Role of anti-apoptotic Mcl-1 gene in human oral cancers and premalignant lesions

By

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CERTIFICATE

I certify that the thesis titled "Role of anti-apoptotic Mcl-1 gene in human oral cancers and premalignant lesions" submitted for the degree of Doctor of Philosophy by Mr. Vinayak C. Palve is a record of the research carried out by him during the period 2007 to 2013 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

Navi Mumbai,

Dr. Tanuja R. Teni

Dedicated to
My Parents

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CONTENTS

SYNOPSIS

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INTRODUCTION

Squamous cell carcinoma of the oral cavity (OSCC) is the most prevalent cancer in males of the Indian subcontinent, predominantly associated with the tobacco-chewing habit [1] and the absolute number of cancer deaths is projected to increase because of population growth and increasing life expectancy [2]. Despite recent advances in surgical treatment and radio/ chemo therapy, the long term survival of oral cancer patients has not changed significantly. Several factors are associated with poor prognosis of OSCC. Firstly, majority of the oral cancer patients are diagnosed at an advanced clinical stage. About 40% of oral cancer patients die from uncontrolled loco-regional disease alone and 24% show metastases to distant sites [3]. Second, the development of multiple primaries has major impact on survival and outcome. Hence, it is important to elucidate the mechanisms involved in the development and progression of oral cancer. Oral carcinogenesis is a multistep process involving functional deregulation of several genes including those involved in cell proliferation and apoptosis and OSCC's have also been repeatedly linked to apoptotic dysregulation [4]. Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with both intrinsic survival advantage and an inherent resistance to therapy [5].

Apoptosis or programmed cell death is controlled by a diverse range of cell signals and effected via two major pathways namely extrinsic and intrinsic. Although their differing modes of initiation are different, both pathways have common downstream events i.e. the activation of caspase cascade leading to apoptosis [6]. The extrinsic pathway is triggered by binding of extracellular ligands, oligomerization of transmembrane receptors and activation of downstream procaspase8 and several adapter molecules to bring above apoptosis. The intrinsic or mitochondrial pathway is triggered by various apoptotic signals like ionizing radiation, chemotherapeutic drugs disrupting the mitochondrial membrane potential with release of cytochrome-c, thereby activating with APAF-1 & procaspase-9 to form 'apoptosome' complex, which leads to disruption of cell.

The Bcl-2 family members are important mitochondrial pathway regulators and are comprised of pro- and anti-apoptotic proteins. The dynamic balance between these opposing members play a critical role in regulating cell survival [7]. Over expression of anti-apoptotic members of the Bcl-2 family like Bcl-2 & Bcl-XL, has been shown to be associated with radioresistance [8, 9]. Bcl-2 members might, therefore, function as indicators of response to radio/chemotherapy. Mcl-1 (Myeloid cell leukemia-1), is an important anti-apoptotic member of the Bcl-2 gene family, essential for development, differentiation and proliferation [10]. Elevated levels of Mcl-1 have been detected in a variety of hematopoietic, lymphoid and solid s including head and neck carcinoma [11, 12]. Overexpression of Mcl-1 has been associated with poor prognosis and resistance to treatment in breast, cervical & gastric cancers [13-15].

Recent studies from our laboratory have demonstrated significant overexpression of Mcl-1 transcripts and protein in oral cancer cell lines, premalignant lesions (OSF) and tumors [16]. Further, we have also demonstrated Mcl-1 to be a prognostic factor in oral cancer patients treated with definitive radiotherapy [17]. Our earlier studies have also demonstrated a five to ten fold higher expression of anti-apoptotic Mcl-1L transcript, versus the pro-apoptotic Mcl-1S in oral tumors [16]. Mcl-1 has been shown to contribute in resistance of cancer cells to chemo/radio therapy [18, 19]. However there are no reports on role of Mcl-1splice variants in radiation induced apoptosis and radioresistance.

Therefore, in the present study we wanted to investigate the association of Mcl-1 isoforms with oral cancer prognosis and with radioresistance and or chemoresistance of oral cancer cells.

Therefore objectives of the project were-

1. To decipher mechanisms of Mcl-1 overexpression in oral cancers

2. To examine the levels of Mcl-1 isoforms in oral cell lines, premalignant lesions & paired oral tumors from different subsites and their correlation with clinico-pathological parameters.

3. To study whether Mcl-1L is a radio-resistance and/or chemo-resistance related factor in oral cancers.

MATERIALS & METHODS:

Oral tissue samples: A total of 130 patients with a diagnosis of oral cancer & 20 OSF were recruited for this study, after approval of the project by the Institutional Review Board (IRB) and with an informed consent of the individual patients. Further, 10 biopsies from patients without any clinically detected lesions undergoing minor surgical procedures like removal of third molar were collected. All the tissues were frozen immediately in liquid nitrogen and stored at -80^{0}C until analysis.

Cell cultures: Eight oral squamous carcinoma cell lines of different origin (Tongue-AW8507, AW13516, SCC40, SCC25, SCC15, QLL1; Buccal mucosa-SCC29B; Alveolar ridge-SCC74) and one each of immortalized Fetal Buccal (FBM) & normal epidermal (HaCaT) & dysplastic oral keratinocyte (DOK) cell lines were used in study. The cells were maintained on DMEM or IMDM supplemented with 10% FBS & 1% standard antibiotic mixture in 5% CO_2 incubator at 37^{0}C.

RNA isolation: RNA from cell pellets and tissues were extracted using TRI reagent (Sigma, USA) according to the manufacturer's protocol. The RNA was dissolved in DEPC-treated water and contaminating DNA was removed by DNaseI treatment. RNA integrity was analyzed by electrophoresis and samples were preserved at −80°C until analysis.

RT-PCR analysis: cDNA was synthesized with 2 µg total RNA, using a First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's instructions. RT-PCR was carried out using primers specific for Mcl-1 isoforms (Mcl-1L, Mcl-1S & Mcl-1ES isoforms) and β-actin.

Real time PCR: qRTPCR was performed using cDNA of cell lines and tissue samples and gene specific Taqman probes (for Mcl-1 isoforms & GAPDH) and universal master mix on ABI-7900HT Real Time PCR System (Applied Biosystems, USA). Comparative C_T method of relative quantification was used for gene expression analysis [20].

DNA isolation & PCR sequencing: The genomic DNA was extracted from cell lines and tissue samples using Blood & Tissue DNA extraction kit, as per manufacturer's instructions (Qiagen, USA). The PCR was performed; gel products were resolved on 2% agarose gel, eluted & purified using QIAquick Gel purification Kit. The products were sequenced in automated sequencing machine and the sequences obtained were aligned with the wild type Mcl-1 gene sequences from NCBI database, using ClustalW, BioEdit, etc software's.

Radiation Treatment: After 48 hrs of plating exponentially growing cells (6×10^3 cells) were treated with IR (D0 dose) using 60Co-γ radiator as described earlier [18]. Cells were incubated up to different time points, harvested and stored in -80°C until use.

Clonogenic Assay: Exponentially growing oral cells were harvested, counted and replated in duplicates. Cells were irradiated, using 60Co-γ radiator along with an untreated control. Cells were then incubated up to 14 days to form colonies which were fixed, stained and counted. The percent plating efficiency and fraction surviving a given radiation dose were calculated based on the survival of non-irradiated cells as described earlier [21].

Western blotting: Cell & tissue lysates were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked and probed with primary antibodies: Bax, Mcl-1, Bcl-xl and ß-actin. Secondary antibodies used were Horseradish peroxidase conjugated IgG. Proteins were visualized with enhanced chemiluminescence kit (GE Healthcare, US).

Densitometry analysis of developed X-ray film was performed using ImageJ software (NIH, Bethesda, MD). β-actin was used as loading control.

Immunofluorescence staining: Cells were grown on glass cover slips and Mcl-1 staining was performed post-IR using an Alexa fluor-488 labeled secondary antibody, as described earlier [16]. The nuclear condensation and apoptosis was analyzed by DAPI staining, cell counting and imaging was done by confocal microscope with LSM Image Browser 4.2 software (Carl Zeiss).

Mcl-1 Knockdown: Transient and regulated knockdown of Mcl-1 was achieved by transfection of Mcl-1L specific siRNA (sc-37007) along with a control siRNA & pTRIPZ mediated lentiviral transduction in oral cell lines. The Mcl-1 knockdown was assessed by western blotting.

Acquired radioresistant sublines: Radioresistant sublines were generated by irradiating AW8507 & AW13516 cells with a fractionated Ionizing radiation strategy as described [22].

MTT assay for cell proliferation: Cells were seeded, in triplicate in a 96-well micro titer plate in 100µl complete medium. Proliferation was studied every 24 hours up to a period of 4 days using MTT assay as described previously [23]. The growth curve was prepared from three independent experiments by plotting O.D. at 540 nm against time.

Drug & inhibitor treatment: In order to determine role of Mcl-1 in chemo-resistance if any, a commonly used drug, Cisplatin was used alone or in combination with Obatoclax (BH3 mimetic small molecule inhibitor) in oral cancer cells. The IC50 doses of both Cisplatin & Obatoclax were calculated by MTT assay as described above.

Statistical analysis: Statistical analysis was performed by using SPSS 16 software. The difference between means was considered statistically significant when $P<0.05$. The data is illustrated as mean ± standard deviation of three independent experiments.

RESULTS:

I. To decipher mechanisms of Mcl-1 overexpression in oral cancers-

1. Genomic alterations:

Controversial reports are available in literature suggesting a role of genomic alterations (6 & 18 bp polymorphic insertions) on Mcl-1 overexpression and disease state. In order to rule out this possibility, all three exons, two introns and promoter of Mcl-1 gene were sequenced in cell lines, healthy volunteers & paired tumor samples.

In cell lines: The sequencing analysis revealed no alterations in exons / introns of Mcl-1 gene amplified from human oral cancer cell lines. Also, sequencing of Mcl-1 isoforms (Mcl-1L & Mcl-1ES) including recently discovered Mcl-1ES, didn't revealed alterations. Further, the promoter region of Mcl-1 was predicted using CHSL (Cold Spring Harbour Laboratory) promoter database and the entire 1.5 kb promoter region was amplified using different pairs of primers. No genomic alterations were observed in promoter region of Mcl-1 in 9 oral cancer cell lines except SCC15 & SCC40 which showed presence of additional 6 bp (GGCCC) repeat.

In healthy volunteers: The PBL's (peripheral blood lymphocytes) were isolated from blood of 25 healthy volunteers, genomic DNA was extracted and PCR sequencing of Mcl-1 gene was carried out. Sequencing and alignment analysis revealed that 8 out of 25 volunteers (32%) showed presence of 18 bp promoter polymorphisms.

In tissue samples: *G*enomic DNA was extracted from 40 paired tumor tissues and sequencing was carried out. The sequencing analysis revealed no differences in presence or absence of polymorphism between adjacent normal vs. tumor tissues. Further, 9 out of 40 tumors (22%) showed presence of 18 bp Mcl-1 promoter polymorphisms. Also, two reported SNP's were observed in oral cancer patient namely C<A-324 & G<C-386.

Effect of promoter polymorphism on Mcl-1 expression:

Real time PCR and western blot analysis of 40 normal & tumors revealed, no significant difference at both mRNA and protein level between patients with or without 18bp Mcl-1 promoter polymorphisms. Also presence or absence polymorphism does not correlate with Mcl-1L/ Mcl-1S isoform levels.

Correlation of Mcl-1 promoter polymorphisms with clinic-pathological parameters:

The χ square test revealed no significant correlation between Mcl-1 promoter polymorphism (18bp) and clinico-pathological parameters (Gender, Age, Habit, Site of tumor, size, Nodal status, Differentiation etc.) of oral cancer patients. Further, Kaplan Meier survival analysis also showed no significant correlation between presence or absence of 18 bp polymorphisms and overall survival of oral cancer patients.

2. Phosphorylation status of Mcl-1:

Western blot and proteosomal inhibitor treatment, revealed phosphorylation of Mcl-1 protein at Ser-159 & Thr-163 residues in AW8507 oral cancer cell line, which is essential for its proper detection by E3 ligase and further proteosomal degradation.

II. <u>Expression of Mcl-1 isoforms and correlation with clinico-pathological parameters</u> -

Expression of Mcl-1 isoform in oral cell lines: qRT-PCR analysis revealed, high expression of anti-apoptotic Mcl-1L isoform over pro-apoptotic Mcl-1S & Mcl-1ES in all the oral cell lines. Elevated expression of Mcl-1L was observed at both mRNA & protein level in majority of oral cancer cell lines (SCC25, SCC29B, SCC40, SCC74, QLL1, AW8507, AW13516) as compared to immortalized normal FBM, HaCaT & DOK cell lines.

Expression of Mcl-1 isoform in oral tissues:

The Immunohistochemistry analysis revealed higher expression of Mcl-1 in OSF tissues versus normal mucosa. Similarly, the qRT-PCR analysis showed an elevated expression of Mcl-1L

mRNA in 64% of oral tumors, in 130 paired samples. However, the relative expression of Mcl-1L isoform was 5 fold higher than Mcl-1S and 10 fold higher to Mcl-1ES isoform in oral tumors. Similarly, western blot analysis has revealed, five to ten fold higher expression of Mcl-1 protein in oral tumors vs. adjacent normal's.

Correlation of Mcl-1 expression with clinico-pathological parameters:

For statistically correlating the expression of Mcl-1 isoforms with clinic-pathological parameters, the data was dichotomized into two groups namely: the Mcl-1 high expressers and low expressers. For comparison the mean expression of Mcl-1 isoforms in healthy normals was used. *Univariate analysis*- revealed significant correlation of high Mcl-1L expression with node positivity ($p = 0.002$) and advanced tumors ($p = 0.001$). However, no significant correlation was observed between expression of Mcl-1 isoforms and gender, age, tobacco/alcohol habits, primary site & differentiation of s in oral cancer patients.

Multivariate analysis- The multivariate analysis revealed that the patients exhibiting a high Mcl-1L exhibited shorter survival as compared to those expressing low Mcl-1L. Our studies indicate that Mcl-1L expression as an independent prognostic factor for oral cancer.

Correlation of Mcl-1 expression with overall survival

The Kaplan–Meier survival curves of low and high expressers of Mcl-1L showed a statistically significant difference ($p = 0.002$). Moreover, patients having high Mcl-1L exhibited poor overall survival. Inversely, patients showing high Mcl-1S significantly better survival ($p = 0.051$) as compared to those having low Mcl-1S. The ratio of Mcl-1L/Mcl-1S, showed a positive correlation with the poor overall survival of oral cancer patients ($p = 0.006$). Univariate analysis also revealed poor overall survival of node positive oral cancer patients ($p = 0.003$). However, the other parameters like age, tobacco/alcohol habits and differentiation did not significantly influence overall survival of patients.

III. Role of Mcl-1L as Radio / Chemo resistance related factor in oral cancers-

In radio-resistance:

To study the role of Mcl-1 isoforms in radiation response of oral squamous carcinoma cells (OSCC), we investigated in the present study, the association of Mcl-1 isoform expression with radiosensitivity of OSCC, using siRNA strategy.

Three to six fold higher expression of anti-apoptotic Mcl-1L versus pro-apoptotic Mcl-1S was observed at mRNA & protein levels in all cell lines, post-irradiation. Sustained high levels of Mcl-1L, downregulation of pro-apoptotic Bax & Bak and a significant ($P < 0.05$) reduction in apoptosis was observed in the more radioresistant AW8507, AW13516 versus FBM cells, post-IR. The ratios of anti to pro-apoptotic proteins were high in AW8507 as compared to FBM. Treatment with Mcl-1L siRNA alone or in combination with IR significantly ($P < 0.01$) increased apoptosis viz. 17.3% (IR), 25.3% (siRNA) and 46.3% (IR plus siRNA) and up regulated pro-apoptotic Bax levels in AW8507 cells. Combination of siRNA & IR treatment significantly ($P < 0.05$) reduced cell proliferation and clonogenic survival of radioresistant AW8507 & AW13516 cells, suggesting a synergistic effect of the Mcl-1L siRNA with IR on radiosensitivity. Interestingly, during the development of radioresistant sublines using FIR, high expression of Mcl-1L was observed.

In chemo-resistance:

To determine role of Mcl-1 as chemo resistance related factor, downregulation of Mcl-1 was achieved via cloning shRNA sequence in pTRIPZ inducible system, transduced in AW8507 cells. Another important member of anti-apoptotic Bcl-2 family, Bcl-xl was also transiently knocked down using siRNA. Western blot analysis confirmed the downregulation of both Mcl-1 and Bcl-xl proteins. Cisplatin, a common drug in treatment of oral cancer was used in the study.

The IC50 values of Cisplatin for oral cell lines by MTT assay showed that majority of oral cells lines were sensitive to Cisplatin treatment. Double knockdown of Mcl-1 & Bcl-xl in combination with Cisplatin significantly reduced cell viability & proliferation as compared to any treatment alone. Interestingly, similar results were obtained using a small molecule anti-Mcl-1 drug, Obatoclax in combination with Cisplatin exhibiting higher induction of cell death and reduction cell proliferation as compared to any treatment alone.

DISCUSSION:

Dysregulation of apoptosis regulating genes may play a key role in the development and progression of many human malignancies. Oral squamous cell carcinomas (OSCCs) have repeatedly been linked to apoptotic dysregulation [16]. Bcl-2 and related pro- and anti-apoptotic proteins are important mitochondrial apoptosis pathway regulators and play a critical role in regulating cell survival [24]. Earlier studies from our lab have shown *Mcl-1,* an anti-apoptotic member of the *Bcl-2* gene family to be overexpressed in oral s [16]. Also, our studies showed together Mcl-1 with PCNA may have potential prognostic value to differentiate patients with poor DFS [17]. We have also for the first time showed the relative levels of Mcl-1 in oral s vs. adjacent normals. Overexpression of Mcl-1 in oral cancer is consistent with an up-regulation of Mcl-1 in hepatocellular carcinomas, cervix cancer, pancreatic cancer, non-small cell lung cancer, and melanomas [11]. Thus, overexpression of Mcl-1 may represent an important mechanism in the development of oral cancer.

In order to elucidate mechanisms of high Mcl-1 expression in oral tumors, we performed the sequencing of Mcl-1 gene which revealed presence of 18bp repeats in 22% of oral s. Interestingly, studies by Moshynska et al, had shown that the presence of 6 and 18 nucleotide insertion in Mcl-1 promoter correlated with increased RNA and protein levels & clinical outcome in CLL patients [25]. The presence of insertions was shown to be associated

with poor overall survival and disease-specific survival, suggesting the potential use of the Mcl-1 promoter insertions as a prognostic factor.

To investigate the clinical significance of the Mcl-1 promoter insertions oral cancer, we analyzed 40 oral cancer patients, 10 oral cell lines and 25 healthy volunteers, utilizing PCR sequencing of Mcl-1 promoter for the presence of these two insertions. We also correlated the sequencing data with clinico-pathological parameters, overall survival & prognosis of OSCC patients. However out data does not support the earlier findings by Moshyanska et al of positive correlation of these insertions Mcl-1 gene expression and outcome. Firstly, we have observed presence of 6/18 bp insertions in both healthy volunteers, as well as s of oral cancer patients. Neither expression of Mcl-1 splice variants nor Mcl-1 protein showed a significant correlation between presence of 6/18 bp insertions in Mcl-1 promoter. The overall survival of patients was also independent of presence or absence of Mcl-1 promoter polymorphisms, indicating that Mcl-1 promoter polymorphism may not be useful to predict outcome/ prognosis of OSCC patients. In support to our findings, several correspondence reports are available [26-29], indicating that the MCL-1 insertions represent hereditary polymorphisms rather than somatic mutations that likely do not predispose to chronic lymphocytic leukemia and were not associated with prognostic markers.

Studies by Reed et al, demonstrated association of between promoter insertion with high MCL-1 mRNA and protein levels, these insertions represent somatic alterations and not hereditary polymorphisms [30]. Thus, the presence of promoter insertions appears be evidently insufficient to reliably drive high levels of MCL-1 expression in CLL. Report from Saxena et al [31], showed that polymorphic insertions were associated with increased promoter activity & Sp1/Sp3 binding sites. However, evidence for the biological effect of MCL-1 promoter polymorphisms on gene expression and the significance of Mcl-1 promoter polymorphism in oral cancer needs to be analyzed in large cohort.

Our studies revealed a high expression of Mcl-1L in both OSF and oral tumors. To evaluate the proportion and contribution of three Mcl-1 isoforms to this overexpression in oral cancer, we analyzed the isoform levels by qRT-PCR in tumor vs. adjacent normal tissues. High levels of anti-apoptotic Mcl-1L isoform were observed in majority of oral cancer cell lines vs. normal immortalized cell lines. Also 64% of oral s showed significantly higher Mcl-1L levels as compared to corresponding adjacent normal tissues. To the best of our knowledge, there are no reports delineating the prognostic significance of Mcl-1 isoforms in human oral cancer. This is the first study demonstrating the correlation of high Mcl-1L levels with poor overall survival and its possible use as an independent prognostic marker for oral cancer patients. However, the only other of Mcl-1 isoforms in CCRCC (clear cell renal carcinoma) by Kempkensteffen et al [32], in contrast showed downregulation of Mcl-1L in CCRCC to be associated with aggressive phenotypes. Our studies revealed a significant correlation of high Mcl-1L expression with node positivity and advanced tumors. The ratio of Mcl-1L / Mcl-1S isoforms also, showed a positive correlation with the poor overall survival, indicating Mcl-1L expression as an independent prognostic factor for oral cancer patients. Our studies are consistent with the previous reports demonstrating high Mcl-1 expression to be associated with poor outcome in breast cancer, cervix cancer, gastric cancer and various hematological malignancies [13, 14, 33].

So far, limited information is available on the role of Mcl-1 in radiation response of cells. To our knowledge, this is the first study to report a time course expression of Mcl-1 isoforms post-IR and effect of Mcl-1L knockdown on radiosentitzation of oral cancer cell lines using siRNA strategy. Our studies demonstrated an inverse correlation of Mcl-1 expression with cellular apoptosis and a synergistic effect of Mcl-1L knockdown along with IR on cell viability and clonogenic survival thereby enhancing the radiosensitivity of OSCC cells. Our studies revealed higher expression of Mcl-1L at both mRNA and protein level in relatively more radioresistant AW8507 & AW13516 cell lines versus FBM, indicating a possible

association of anti-apoptotic Mcl-1L splice variant with radioresistance. Moreover, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [34]. No significant alterations in levels of Mcl-1S & Mcl-1ES were observed post IR, indicating that the predominantly overexpressed Mcl-1L isoform alone may contribute in generation of radioresistance. We assessed the ratios of Mcl-1L/Mcl-1S, Mcl-1L/Bax, Bcl-xl/Bax, wherein radioresistant AW8507& AW13516 showed high ratios as compared to that in FBM indicating predominance of anti-apoptosis which may contribute to radioresistance.

Interestingly, the combination of Mcl-1L siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. However, the expression of pro-apoptotic Bax protein correlated with the increased apoptosis on Mcl-1L knockdown. To address the fact that the induction of apoptosis may not necessarily lead to long-term response to radiotherapy, we performed the clonogenic assay which demonstrated that combination of IR and Mcl-1L downregulation synergistically reduced clonogenic survival as compared to each treatment alone. The high expression of Mcl-1L, in radioresistant sublines developed by fractionated ionizing radiation provides a direct evidence for the role of Mcl-1L in radioresistance of OSCC cells. Therefore, the combination of radiotherapy and Mcl-1L downregulation has the potential to improve the response rate of treatment resistant oral cancer cells. Selective inhibitors like Obatoclax, which specifically overcome Mcl-1 mediated resistance, is already in phase 2 clinical trials [35] and may have important therapeutic implications, when used in combination with radiotherapy in treatment of oral cancer patients.

SUMMARY & CONCLUSION:

Our studies for the first time demonstrate, overexpression of Mcl-1L isoform in oral tumors & its positive correlation with node positivity & advanced tumors. High Mcl-1L significantly correlated with poor overall survival, indicating Mcl-1L to be an independent prognostic factor for oral cancer. Our studies also indicate the association of Mcl-1L isoform with radioresistance/chemo resistance by influencing apoptosis, proliferation and clonogenic survival of oral cancer cells. Thus, Mcl-1L appears to be an important pro-survival and radio/chemo resistance factor, influencing outcome of oral cancer patients. It therefore may be a potential therapeutic target in oral cancers.

REFERENCES:

1. Tandle, A., V. Sanghvi, and D. Saranath, *Determination of p53 genotypes in oral cancer patients from India.* British journal of cancer, 2001. 84(6): p. 739.
2. Dikshit, R., et al., *Cancer mortality in India: a nationally representative survey.* Lancet, 2012. 379(9828): p. 1807-16.
3. Notani, P.N., *Global variation in cancer incidence and mortality.* Curr Sci, 2001. 81(5): p. 465-474.
4. Loro, L., O. Vintermyr, and A. Johannessen, *Cell death regulation in oral squamous cell carcinoma: methodological considerations and clinical significance.* Journal of oral pathology & medicine, 2003. 32(3): p. 125-138.
5. Johnstone, R.W., A.A. Ruefli, and S.W. Lowe, *Apoptosis-A Link between Cancer Genetics and Chemotherapy.* Cell, 2002. 108(2): p. 153-164.
6. Borner, C., *The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions.* Molecular immunology, 2003. 39(11): p. 615-647.
7. Levine, B., S.C. Sinha, and G. Kroemer, *Bcl-2 family members: dual regulators of apoptosis and autophagy.* Autophagy, 2008. 4(5): p. 600-606.
8. Kyprianou, N., et al., *bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells.* International journal of cancer, 1997. 70(3): p. 341-348.
9. Wang, Z.B., et al., *Bcl-xL overexpression restricts [gamma]-radiation-induced apoptosis.* Cell biology international, 2006. 30(1): p. 15-20.
10. Craig, R., *MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis.* Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK, 2002. 16(4): p. 444.
11. Akgul, C., *Mcl-1 is a potential therapeutic target in multiple types of cancer.* Cellular and molecular life sciences, 2009. 66(8): p. 1326-1336.
12. Nagata, M., et al., *Role of myeloid cell leukemia-1 in cell growth of squamous cell carcinoma.* Journal of pharmacological sciences, 2009. 110(3): p. 344-353.

13. Likui, W., et al., *Prognostic role of myeloid cell leukemia-1 protein (Mcl-1) expression in human gastric cancer.* Journal of surgical oncology, 2009. 100(5): p. 396-400.
14. Zhang, T., et al., *The expression of Mcl-1 in human cervical cancer and its clinical significance.* Medical Oncology, 2012: p. 1-7.
15. O'DRISCOLL, L., et al., *Expression and prognostic relevance of Mcl-1 in breast cancer.* Anticancer research, 2004. 24(2A): p. 473-482.
16. Mallick, S., et al., *Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of Mcl-1.* The Journal of pathology, 2009. 217(3): p. 398-407.
17. Mallick, S., et al., *PCNA and anti-apoptotic Mcl-1 proteins predict disease-free survival in oral cancer patients treated with definitive radiotherapy.* Oral oncology, 2010. 46(9): p. 688-693.
18. SKVARA, H., et al., *Mcl-1 blocks radiation-induced apoptosis and inhibits clonogenic cell death.* Anticancer research, 2005. 25(4): p. 2697-2703.
19. Guoan, X., et al., *Adenovirus-mediated siRNA targeting Mcl-1 gene increases radiosensitivity of pancreatic carcinoma cells in vitro and in vivo.* Surgery, 2010. 147(4): p. 553-561.
20. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative CT method.* Nature protocols, 2008. 3(6): p. 1101-1108.
21. Franken, N.A.P., et al., *Clonogenic assay of cells in vitro.* Nature protocols, 2006. 1(5): p. 2315-2319.
22. Fukuda, K., et al., *Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation.* British journal of cancer, 2004. 91(8): p. 1543-1550.
23. Alam, H., et al., *Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells.* Mol Biol Cell, 2011. 22(21): p. 4068-78.
24. Coultas, L. and A. Strasser. *The role of the Bcl-2 protein family in cancer*. in *Seminars in cancer biology*. 2003: Elsevier.
25. Moshynska, O., et al., *Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2004. 96(9): p. 673-682.
26. Tobin, G., et al., *Mcl-1 gene promoter insertions do not correlate with disease outcome, stage or VH gene mutation status in chronic lymphocytic leukaemia.* Leukemia, 2005. 19(5): p. 871-873.
27. Iglesias-Serret, D., et al., *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1090-1091.
28. Dicker, F., et al., *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1092-1093.
29. Coenen, S., et al., *The relevance of sequence insertions in the Mcl-1 promoter in chronic lymphocytic leukemia and in normal cells.* haematologica, 2005. 90(9): p. 1285-1286.
30. Kitada, S. and J.C. Reed, *MCL-1 promoter insertions dial-up aggressiveness of chronic leukemia.* Journal of the National Cancer Institute, 2004. 96(9): p. 642-643.

31. Saxena, A., et al., *Short nucleotide polymorphic insertions in the MCL-1 promoter affect gene expression.* Cancer letters, 2007. 251(1): p. 114-131.
32. Kempkensteffen, C., et al., *Expression of Mcl-1 splicing variants in clear-cell renal cancer and their correlation with histopathological parameters and prognosis.* Tumor Biology, 2009. 30(2): p. 73-79.
33. Ding, Q., et al., *Myeloid cell leukemia-1 inversely correlates with glycogen synthase kinase-3β activity and associates with poor prognosis in human breast cancer.* Cancer research, 2007. 67(10): p. 4564-4571.
34. Bae, J., et al., *MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain.* Journal of Biological Chemistry, 2000. 275(33): p. 25255-25261.
35. Paik, P.K., et al., *A phase II study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in relapsed small cell lung cancer.* Lung Cancer, 2011. 74(3): p. 481-485.

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2. S Mallick, R Patil, R Gyanchandani, S Pawar, **V Palve** et al., '*Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of Mcl-1'*. ***Journal of Pathology****,* 217: 398- 407, 2009.

Signature of Student:

Date: 25/01/2013

LIST OF ABBREVIATIONS:

APS	:	Ammonium persulfate
BME	:	β-Mercapto Ethanol
DAPI	:	4, 6-diamidino-2-phenylindole
DEPC	:	Diethyl pyro carbonate
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTP's	:	deoxyribonucleotide mix
DW	:	Distilled Water
ECL	:	Enhanced chemiluminescence
EDTA	:	Ethylene glycol tetra acetic acid
EtBr	:	Ethidium bromide
FBS	:	Fetal Bovine Serum
FIR	:	Fractionated Ionizing Radiation
FITC	:	Fluorescein isothiocyanate
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GFP	:	Green fluorescent protein
H & E	:	Haematoxylin and eosin
HRP	:	Horse Radish Peroxidase
IHC	:	Immunohistochemistry
LB	:	Luria Broth
MAPK	:	Mitogen activated protein kinase
Mcl-1ES	:	Myeloid cell leukemia-1 Extra Short
Mcl-1L	:	Myeloid cell leukemia-1 full length
Mcl-1S	:	Myeloid cell leukemia-1 Short
nt	:	Nucleotide
OD	:	Optical Density
OSCC	:	Oral Squamous cell carcinoma
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase chain reaction
PI	:	Propidium iodide
PI3K	:	Phosphoinositide 3-kinase
PVDF	:	Polyvinylidene difluoride
qRT-PCR	:	Quantitative Real Time PCR
RNA	:	Ribonucleic Acid
RNAi	:	RNA interference
RNase	:	Ribonuclease
RT	:	Room Temperature
RT-PCR	:	Reverse transcriptase PCR
SDS	:	Sodium dodecyl sulfate
SF	:	Surviving fraction
shRNA	:	Short hairpin RNA
siRNA	:	Small interfering RNA
SOB	:	Super Optimal Broth
TBST	:	Tris buffered saline with tween-20
TEMED	:	N-Tetramethylene diamine
WB	:	Western Blot

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CHAPTER 1

INTRODUCTION

1. Introduction:

Squamous cell carcinoma of the oral cavity (OSCC) is the most prevalent cancer in males of the Indian subcontinent, predominantly associated with the tobacco-chewing habit [1] and the absolute number of cancer deaths is projected to increase because of population growth and increasing life expectancy [2]. Despite recent advances in surgical treatment and radio/ chemo therapy, the long term survival of oral cancer patients has not changed significantly [3]. Several factors are associated with poor prognosis of OSCC. Firstly, majority of the oral cancer patients are diagnosed at an advanced clinical stage, which may be possible due to ignorance or inaccessibility of medical care as knowledge of oral cancer or precancerous lesions and its risk factors among Indian population is limited [4]. Second, the development of multiple primaries has major impact on survival and outcome. About 40% of oral cancer patients die from uncontrolled loco-regional disease alone and 24% show metastases to distant sites [5]. Therefore, it is an urgent need to improve the early detection of oral carcinomas and in depth study of mechanisms involved in the development and progression of oral cancer [6]. Oral carcinogenesis is a multistep process involving functional deregulation of several genes including those involved in cell proliferation and apoptosis and OSCC's have also been repeatedly linked to apoptotic dysregulation [7]. Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with both intrinsic survival advantage and an inherent resistance to therapy [8].

Apoptosis or programmed cell death is controlled by a diverse range of cell signals and effected via two major pathways namely extrinsic and intrinsic [9]. The extrinsic pathway is triggered by binding of extracellular ligands and oligomerization of transmembrane receptors. The intrinsic or mitochondrial pathway is regulated by Bcl-2 family members comprised of pro- and anti-apoptotic proteins. The dynamic balance between these opposing members play a critical role in regulating cell survival [10]. Over expression of anti-apoptotic members of the Bcl-2 family like Bcl-2 & Bcl-XL, has been shown to be associated with radioresistance [11,

12]. Bcl-2 members might, therefore, function as indicators of response to radio/chemotherapy. Mcl-1 (Myeloid cell leukemia-1), is an important anti-apoptotic member of the Bcl-2 gene family, essential for development, differentiation and proliferation [13]. Elevated levels of Mcl-1 have been detected in a variety of hematopoietic, lymphoid and solid s including head and neck carcinoma [14, 15]. Overexpression of Mcl-1 has been associated with poor prognosis and resistance to treatment in breast, cervical & gastric cancers [16-18].

Recent studies from our laboratory have demonstrated significant overexpression of Mcl-1 transcripts and protein in oral cell lines and tumors [19]. Further, we have also demonstrated Mcl-1 to be a prognostic factor in oral cancer patients treated with definitive radiotherapy [20]. Our earlier studies have also demonstrated a five to ten fold higher expression of anti-apoptotic Mcl-1L transcript, versus the pro-apoptotic Mcl-1S in oral tumors [19]. Mcl-1 has been shown to contribute in resistance of cancer cells to chemo/radio therapy [21, 22]. However there are no reports on role of Mcl-1splice variants in radiation induced apoptosis and radioresistance.

Therefore, in the present study we wanted to investigate the association of Mcl-1 isoforms with oral cancer prognosis and with radioresistance and/or chemoresistance of oral cancer cells.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Oral Cancer:

Incidence: Oral cancer is a major problem in the Indian subcontinent where it ranks among the top three types of cancer in the country [23]. Age-adjusted rates of oral cancer in India is high that is, 20 per 100,000 population and accounts for over 30% of all cancers in the country [24]. In 2010, more than 5,56,000 cancer deaths were estimated in India for people of all ages and 71.1% occurred in people aged 30–69 years [2]. Based on these statistics, oral cancer appears to be a major problem in India.

Risk Factors: Epidemiological studies have implicated several factors which are not causal agents but are associated with increased probability or risk of occurrence of oral carcinomas. *Tobacco:* Oral squamous cell carcinoma (OSCC) mainly attributed to the widely used practice of chewing tobacco either alone or along with betel quid and areca nut or slaked lime [25] or in other words the consumption of tobacco is the leading cause of cancers in India, responsible for 65 to 85% cancer incidences in men and women, respectively [26]. *Smoking:* Smoking is a strong factor associated with lung as well as oral cancers in India. The effect of tobacco smoking mainly in form of bidi and cigarette on oral cancer has been studied extensively [27, 28]. However, several studies had proven the carcinogenic nature of bidi components [29, 30]. *Alcohol:* Studies have failed implicate to alcohol as the single important factor for the development of oral cancers [31, 32] however, it has been shown to interact with tobacco and elevate the risk for the development of oral and pharyngeal cancers [33]. Studies suggest that alcohol might act as solvent facilitating the entry of carcinogens across the cell membranes and can activate the carcinogens by enhancing liver metabolism, thereby working as a co-carcinogen [34]. *Virus:* Human papillomavirus (HPV) which is another risk factor associated with benign and malignant oral lesions. Though, HPV positivity is higher in oral cavity (59%) pharynx (43%) and0 larynx (33%) [35], among these only a small fraction of HPV (subtype 16;18) infected lesions rarely proceed to malignant transformation, indicating that the tumorigenic conversion requires the presence of other risk factors [36].

Diet: Several environmental and lifestyle factors including diet and nutritional factors are known to influence occurrence of oral cancer [37]. Fruits and vegetables (high in vitamin A and C) are described as protective in oral cancer and their low intake has been consistently linked with an increase in cancer prevalence/incidence [38]. Many of these studies have shown positive associations between supplementation and reduced risks of oral precancer and cancers in India [39, 40].

2.2 Oral Precancer Lesions:

Oral carcinoma is a multistep disease and it is accepted that most of the oral tumors are preceded by visible change in the oral mucosa accompanied with certain high risk lesions termed as 'precancerous lesions'[6]. A precancerous lesion is defined as any morphological alteration in a tissue which makes it more susceptible to malignant transformation as compared to its adjacent apparently normal tissue [41]. Therefore, activities that are aimed to detect the disease in a precancerous stage would lead to better prognosis and lesser morbidity. The predominant types of oral precancerous lesions include: Leukoplakia, erythroplakia, and submucous fibrosis.

Leukoplakia: Leukoplakia is defined as any white oral lesion that cannot be characterized clinically or pathologically [42]. It is estimated that patients with leukoplakia carry fivefold higher risk of it developing into a cancer as compared to controls. In Western population, leukoplakia occurs predominantly on the tongue whereas in India buccal mucosa is the more common site in tobacco chewers [43]. More often the leukoplakia lesion is seen to develop at the site of placement of smokeless tobacco product and the severity of lesion is also seen to correlate with duration and amount of tobacco used [44].

Erythroplakia: Erythroplakia is an oral precancerous stage which is defined as a red fiery patch that cannot be characterized either clinically or pathologically [45], excluding inflammation induced red patches in the oral cavity. The sites affected by erythroplakia are majorly soft palate, floor of the mouth and buccal mucosa. Since the potential of malignant transformation

of erythroplakia is very high varying from 14 to 50%, which is higher than other precancerous lesions [46], it is necessary to be treated. Tobacco and alcohol are considered to be the major risk factors associated with the development of erythroplakia.

S*ubmucous Fibrosis:* Oral Submucous fibrosis (OSF) is a precancerous condition characterized by burning sensation, blanching and stiffening of oral mucosa, rigidity of lip, palate and tongue leading to difficulty in mastication [47]. This condition is prevalent mostly in the South and South East Asian countries and among the Asian immigrants to UK and Africa. The etiological agents associated with the incidence of OSF are chewing areca nut and betel quid, micronutrient deficiency of iron, zinc etc. The rate of malignant transformation of submucous fibrosis was found to be 7.6% [48].

2.3 Treatment Modalities of Oral Cancer:

Primary OSCC (Oral Squamous Carcinoma) are generally treated by coordination of multidisciplinary approaches to deliver all therapeutic antitumor modalities and appropriate adjunctive services. The generalized treatment includes surgery alone or combined surgery and radiotherapy and/or chemotherapy. However, the use of one treatment over another depends on the tumor location; size (T), nodal status (N) and Metastasis (M) of the primary tumor characterized by TNM classification of UICC (International union against Cancer) and the patient's desires & ability to tolerate treatment. An accurate assessment of the extent of the disease is therefore a prelude to treatment planning of OSCC patients.

Surgery: Being more accessible site, surgery is the most common treatment for H&N cancer. Most salivary gland tumors of the mouth and throat cancers of the tonsil and patients with tumor invasion of the jaw are also treated with surgery initially. Surgical removal of lymph nodes also avoids the tumor spreading. Surgery is used to remove the tumor and some of the healthy tissue around it therefore, surgery also aids in staging and treatment planning of the patient. The type of surgery depends on the type and stage of the cancer. Multiple surgeries

may be necessary to treat the cancer and restore function and appearance. In order to increase the chances of successful outcomes surgery is also followed by chemo or radiotherapy.

Radiation Therapy: Radiation therapy uses high-energy X-rays or ϒ-radiation to kill cancer cells or shrink tumors. In cancer of the H & N, radiation therapy may be used alone to treat small to intermediate-stage tumors or to kill remaining cancerous cells after surgery, or it may be combined with chemotherapy for advanced tumors. Either external or internal radiation therapy, or both, may be used to treat oral cancer, depending on the stage of the cancer. Conventional radiotherapy consists of one fraction daily for three to seven weeks with a total dose varying between 50 -70 Gy. The radiobiological effects of schedules depend on the relation between overall time, total dose and the number of fractions.

Chemotherapy: Chemotherapy uses drugs to kill cancer cells or stop them from growing especially tumors that cannot be surgically removed. The drugs can be given by mouth or injected into a vein or muscle. Chemotherapy may be used to shrink the cancer before surgery or radiation, or it may be combined with radiation to increase the effectiveness of both treatments. Though chemotherapy has a role in organ preservation in oropharyngeal cancers, there are no evidence of increased survival when used alone [49]. Therefore, concurrent administration of chemotherapy with radiotherapy is the current treatment of choice in most cases and several randomized trials has been shown to reduce mortality by 11% [50]. Currently used agents include Cisplatin, Carboplatin, 5-Flourouracil and Taxanes (Paclitaxel and Docetaxel).

Recent advances in treatment have not significantly enhanced survival of patients, which remained less that 50% for past several years which has contributed to its poor outcome [51]. Despite the attainments already achieved concerning OSCC diagnosis and therapy, mortality and morbidity rates are still exceedingly high, challenging the available methods of prognosis assessment and encouraging the search for new and better molecular markers that relate comprehensively with known alterations of tumor progression [52].

2.4 Mechanisms of Cell Death:

Different types of cell death are often defined by morphological criteria and are categorized in to necrotic or apoptotic cell death.

Necrosis: 'Necrotic cell death' or 'necrosis' is morphologically characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents [53]. The cell death via necrosis is an uncontrolled process which leads to lysis of cells and produces inflammatory responses.

Apoptosis: Apoptosis or programmed cell death is highly regulated destruction of a cell, accompanied by rounding-up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing (but maintenance of its integrity) and engulfment by resident phagocytes [54]. A key feature of apoptosis is cleavage of cytoskeletal proteins by aspartate-specific proteases, which thereby collapses sub cellular components [55]. Apoptosis is considered a vital component of various processes including embryonic development, normal cell turnover and functioning of the immune system [56].

Discovery and etymology: The term apoptosis (Greek: *apo* - off, *ptosis* - falling) was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death. Our understanding of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of *C. elegans* [57]. These initial genetic studies in *C.elegans* revealed Ced-3 & Ced-9 which were required for PCDs during the development. Apoptosis has since been recognized and accepted as a distinctive and important mode of "programmed" cell death, which involves the genetically determined elimination of cells [58]. For the discovery of genes and mechanisms involved in controlled destruction of cell Sydney Brenner, Horvitz and John E. Sulston were also awarded the Nobel Prize in Medicine in 2002.

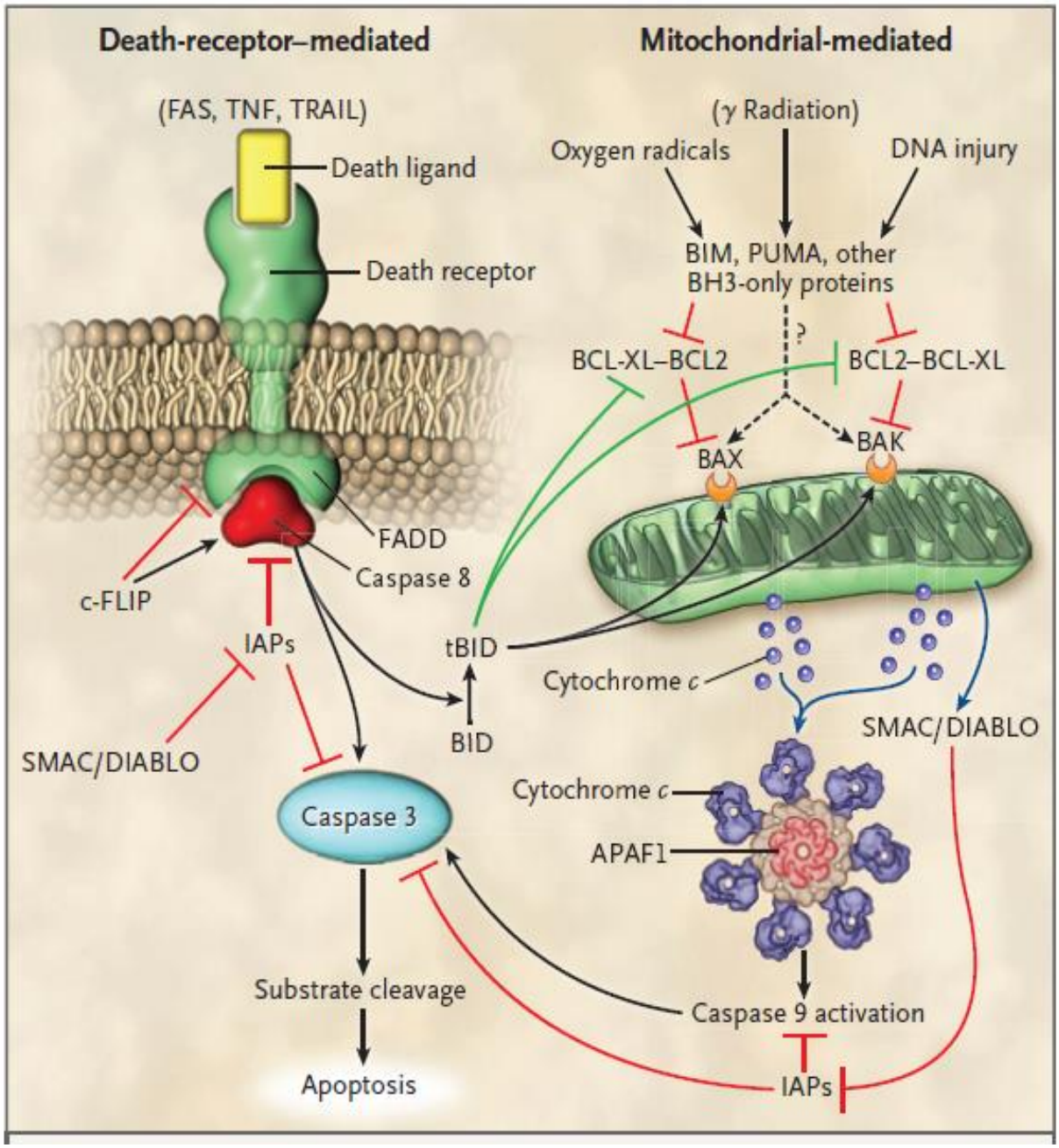
2.5 Apoptotic Pathways:

The process of apoptosis is controlled by a diverse range of cell signals, on the basis of which the apoptotic pathways are characterized as extrinsic or intrinsic pathways-

Extrinsic pathway / Death receptor pathway:

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily [59]. Members of the TNF receptor family share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" [58]. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5 [60]. The sequences of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models.

In these models, there is clustering of receptors and binding with the homologous trimeric ligands. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligands to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP [61]. FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered [62] as depicted in figure1.



(Modified from; Ref-55)

Figure 1: Depicting the two major apoptotic pathways.

Intrinsic pathway/ mitochondrial pathway:

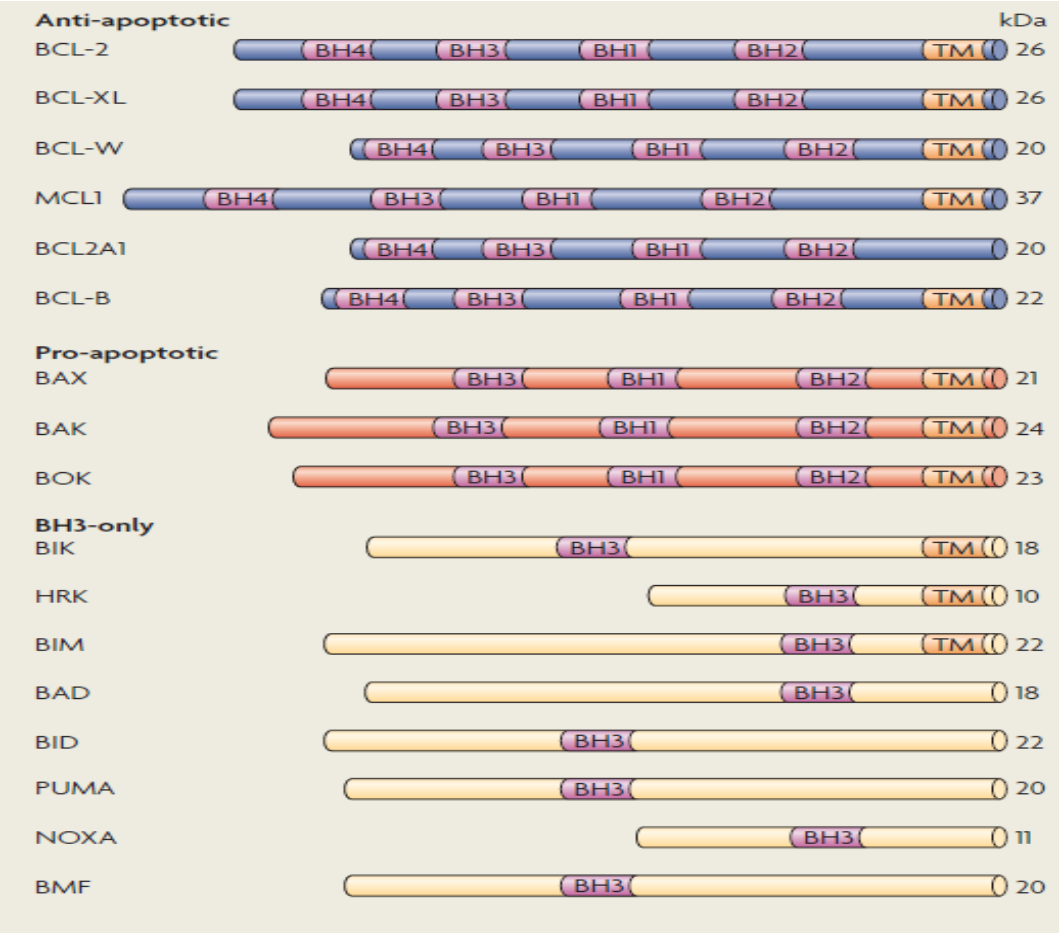
Mitochondria play an important role in the regulation of cell death. Interplay between pro-apoptotic and anti-apoptotic members of the BCL2 family controls the mitochondrial apoptotic pathway [55]. In different cell types, the intrinsic pathway is triggered in response to stress induced by a variety of external stimuli such as DNA damage, hypoxia, and deprivation of growth factors, oncogene deregulation, oxidative damage and microtubule disruption. The BH3 only proteins are the first pro-apoptotic Bcl-2 members to get activated after receiving

damage signals. Activation of BH3 only members occurs through various mechanisms namely increased transcription, protein stabilization and post translational modification [63].

Pro-apoptotic signals redirect these proteins to the mitochondrial membrane where the crucial decision for cell's fate takes place. As depicted in figure1, the activator BH3 only proteins bind to Bax and Bak, induces allosteric change due to which they get inserted into the mitochondrial outer membrane. Here Bax and Bak undergo homo-oligomerization and facilitate the formation of mitochondrial outer membrane pore thereby promoting the release of cytochrome *c* into the cytosol [64]. This is considered a point at which cell is committed to PCD and is associated with activation of caspases. Activated caspase 8 (death receptor pathway) and caspase 9 (mitochondrial pathway) in turn mobilize caspases 3, 6, and 7, proteases that herald demolition of the cell by cleaving numerous proteins and activating DNASES [65].

2.6 Bcl2 Family Proteins:

Bcl-2 family members are the key regulators of the mitochondrial apoptotic pathway, therefore they are also called as "the cellular life or death switch" [66]. The balance between pro-apoptotic and anti-apoptotic BCL2 protein family members controls the mitochondrial apoptotic pathway [67, 68]. *BCL2*, which was originally identified as the gene that is deregulated by the t(14;18) chromosomal translocation in follicular B-cell lymphomas, inhibits apoptosis [69]. Membership in the BCL2 family requires at least one conserved BCL2 homology domain in a protein. This domain allows the protein to regulate apoptosis by joining other proteins through intermolecular forces. The pro-survival members of the family-BCL2, BCL-XL, BCLW, MCL1, A1, have as many as four BCL2 homology regions. These proteins are essential for cell survival and function, specifically in certain cells and on certain stimuli [70]. The pro-apoptotic BCL2 family proteins differ not only in function but also in the number of BCL2 homology domains and their ability to trigger apoptosis [71] as described in figure 2.



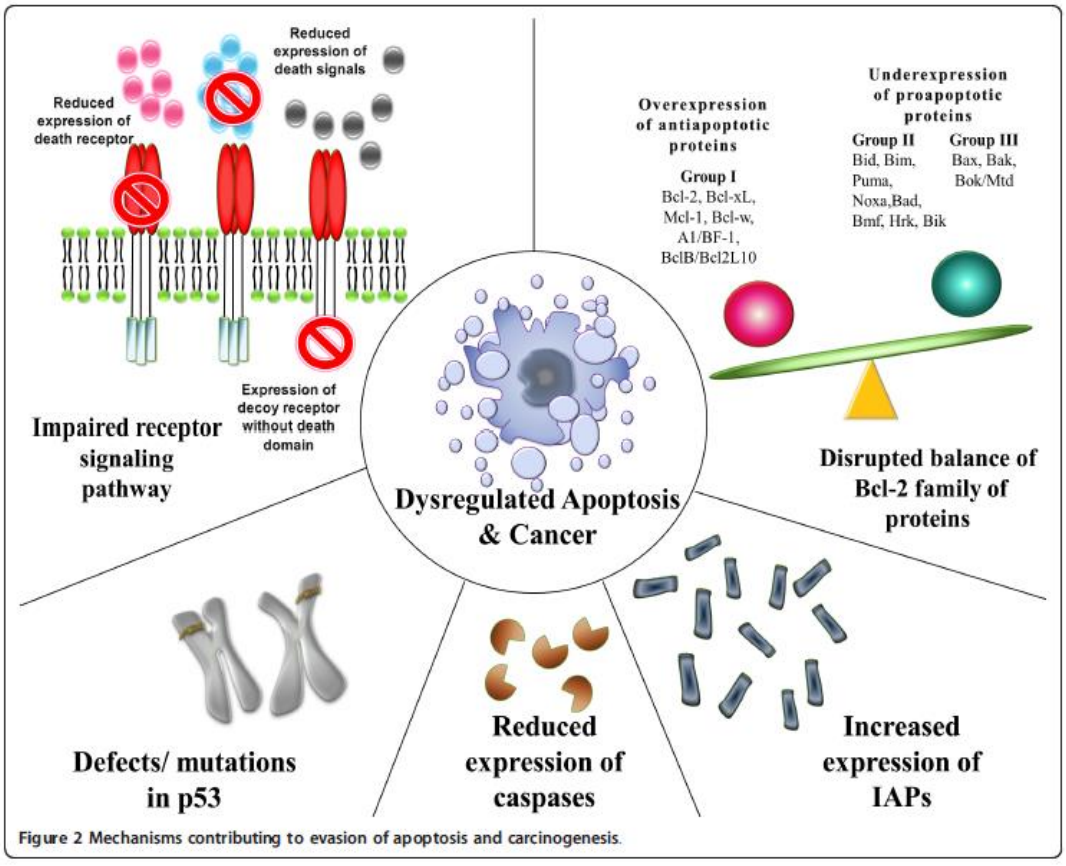
(Source:[62])

Figure 2: The B-cell lymphoma-2 (Bcl-2) family proteins

These "BH3-only" proteins bind to and inhibit anti-apoptotic BCL2 family members, thereby liberating the pro-apoptotic BAX and BAK proteins that cause loss of mitochondrial membrane permeability and subsequent cell death [72]. Three of them (BIM, PUMA, and BID) bind with high affinity to all pro-survival BCL2 family members. Moreover, different apoptotic stimuli preferentially activate certain BH3- only proteins: BIM is essential for apoptosis induced by deprivation of growth factors, whereas PUMA is critical for apoptosis induced by DNA damage [73]. The pro-apoptotic bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins thereby releasing of cytochrome C & activation of downstream caspases cascade. This in turn leads to the formation of the apoptosome and brings out induction of apoptosis (Dash, 2009).

2.7 Apoptosis & Cancer:

A defect in the processes controlling PCD can extend cellular life span contributing to tumor pathogenesis by allowing cell expansion independent of cell division, subverting the need for survival factors and allowing time for accumulating genetic alterations that interfere with proliferation, differentiation, promote angiogenesis, cell motility and invasiveness (Figure3). Defects in the apoptosis regulation are considered to complement the deregulation of oncogenes during the progression of tumorigenesis [74]. Apoptosis defects enables the propagation of clones of genetically unstable cells by overriding DNA repair checkpoint, facilitates metastasis by allowing epithelial cells to grow without attachment, promotes resistance to immune system [75]. Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with both an intrinsic survival advantage and an inherent resistance to chemotherapy and radiotherapy; thereby requiring higher doses for tumor cell kill [76].



(Modified form Ref-[77])

Figure 3: Mechanisms contributing to evasion of apoptosis and carcinogenesis

2.8 Apoptosis & Oral Cancer:

Oral cancer and in particular, squamous cell carcinomas have been repeatedly linked to apoptotic deregulations [78]. In a study done to explore the involvement of apoptosis in oral and oropharyngeal cancers, it was observed that while mitotic index increased from normal to OSCC, the ratio of apoptotic index to mitotic index reaches a peak in carcinoma in situ and then falls indicating block in apoptosis [79]. Alteration in the expression of apoptotic genes have been also found to exhibit variable patterns in both premalignant and malignant oral epithelial lesions. Expression of p53, Bak and Bcl-xl was observed to be an early event and increased expression was associated with increasing histological stages whereas Bax and Bcl-2 demonstrated sporadic expression, indicating that alteration in apoptotic machinery is an early event in oral carcinogenesis and high Bcl-2/Bax ratio as strong independent prognostic marker of OSCC [80, 81]. Further, the expression of Bcl-xL, Bax and p53 increased in lymph node metastatic oral tumors as compared to their corresponding matched primary tumors demonstrating an association of these apoptotic alterations with oral cancer progression and development of metastasis [82]. In another study carried out using immunohistochemistry on normal oral tissues, dysplastic oral lesions and HNSCC biopsy samples, demonstrated that the apoptotic index, expression of bcl-2, bax, bcl-2/bax staining intensity changes drastically in dysplastic lesions and OSCC as compared to normal tissues.

Also the expression of Bcl2 family member proteins was shown to correlate with survival of the oral cancer patients. In this study, high expression of Bax and low expression of Bcl-xL in OSCC was associated with better disease free survival and cancer specific 5 year survival [83]). A multivariate analysis done to evaluate the prognostic significance of Bax and Bcl2 expression in OSCC, revealed that positive Bax expression and negative Bcl-2 expression is associated with better prognosis indicating the significance of apoptotic alteration in the progression of OSCC [84]. Cancer cells can also evade the immune system by altering the expression of death receptors and their respective ligands. A study revealed both OSCC and

oral leukoplakia's exhibit downregulation of Fas and upregulation of Fas L which can be a mechanism to eliminate the infiltrating lymphocytes by triggering cell death [85].

In OSCC, approximately 50% of tumors exhibited dysfunctional p53, resulting in the loss of a check point control. Thus, cells with damaged genomes would not be able to undergo apoptosis, allowing the defective genome to persist and replicate in the offspring cells [86]. Expression of p53 in salivary gland adenoid cystic carcinoma was also associated with poor prognosis as compared to p53 negative tumors [87]. In a study done to explore the involvement of apoptosis in oral and oropharyngeal cancers, it was observed that while mitotic index increased from normal to OSCC, the ratio of apoptotic index to mitotic index reaches a peak in carcinoma in situ and then falls indicating block in apoptosis [79]. A grade wise increased expression of IAP–Survivin was observed in oral squamous cell carcinomas in the Indian subcontinent which might contribute to the resistance of OSCC towards therapy [88]. Survival analysis of OSCC patients with high survivin expression in advanced stages show shorter overall survival than others indicating that an alteration of apoptotic pathway is an important mechanism to evade cell death contributing to the progression of oral cancers [89]. A number of investigations focused specifically on induction of apoptosis in oral cancer cells by synthetic compounds and naturally occurring chemo preventive agents with apoptotic potential [74]. At certain stages in tumor development, the balance between proliferation and apoptosis is interrupted, resulting in deregulated cell proliferation [90].

Study from our lab has also demonstrated an altered expression of Bcl-2 family members and high expression of Mcl-1 suggesting an important role of Mcl-1, early in oral cancer pathogenesis in protecting cells from apoptosis via neutralization of pro-apoptotic members in oral SCC [19]. Emerging knowledge on how the deregulated function of apoptotic signaling networks contribute to the malignant growth of oral squamous cell carcinoma can be exploited to identify novel targets for therapy [91].

2.9 Mcl-1 (*Myeloid Cell Leukemia-1*)

Mcl-1 (*Myeloid cell leukaemia-1*) is an anti-apoptotic member of the Bcl-2 family protein. It was initially identified as an immediate-early gene over expressed during differentiation of ML-1 myeloid leukemia cells [92]. Mcl-1 has been shown to be expressed in multiple cell lineages and has emerged as a key member of apoptosis regulating Bcl-2 family [93].

Mcl-1 Discovery and Characterization: Alignment algorithms identified sequence similarity to Bcl-2 & Bcl-xl, the other pro-survival members of Bcl-2 family. The Bcl-2 family represents a new class of oncogenes that promoted oncogenesis, not through upregulation of proliferation, but by maintaining viability through inhibition of apoptosis[94]. Mcl-1 is rapidly up-regulated & provides short-term enhancement of viability during critical transitions in cell fate. It is normally expressed in a cell type-specific manner in response to specific signals that affect growth, differentiation and viability [13].

Structural and functional properties of Mcl-1:

The human Mcl-1 gene is located on chromosome 1q21 and comprises three exons. Alternative splicing leads to the production of three mRNA species: full length (Mcl-1L), short (Mcl-1S) via splicing of second exon and recently discovered extra short (Mcl-1ES) from splicing within the first coding exon [95, 96]. Excluding exon 2 in Mcl-1S causes exon 3 sequences to be translated in a different reading frame and C-terminal transmembrane (TM) domain, which is a part of Mcl-1L, is not included in Mcl-1S (Mcl-1S/ΔTM).

Bcl-2 family proteins contain BH domains which are short motifs that mediate protein: protein interactions between family proteins and are important for apoptosis regulation. The full length Mcl-1L comprises 350 amino acid residues and has BH1, BH2, BH3, BH4 and C-terminal TM domains, Mcl-1S contains 271 amino acid residues (including the PEST domain) and only a BH3 domain identical to Mcl-1L, but Mcl-1S/ΔTM lacks the BH1, 2 and transmembrane domains [97] as describes in figure 4. Although the significance of this isoform remains to be determined, the structure of Mcl-1S/ΔTM resembles certain pro-apoptotic "BH3

only" proteins and, in marked contrast to Mcl-1L, overexpression of Mcl-1S/ΔTM promotes cell death. However, the deduced sequence of MCL-1ES encodes a protein of 197 amino acids, and the PEST motifs present in MCL-1L are absent. The C-terminal TM domain serves to localize Mcl-1L mainly to the outer mitochondrial membrane [98]. This localization is consistent with a role for Mcl-1 in controlling key mitochondrial events during apoptosis, although localization of Mcl-1 to other intracellular membranes has also been observed. Surprisingly, although Mcl-1L, Mcl-1S and Mcl-1ES are expressed from the same gene via alternative splicing, they have opposing functions, Mcl-1L being anti-apoptotic and Mcl-1S &Mcl-1ES are pro-apoptotic [96].

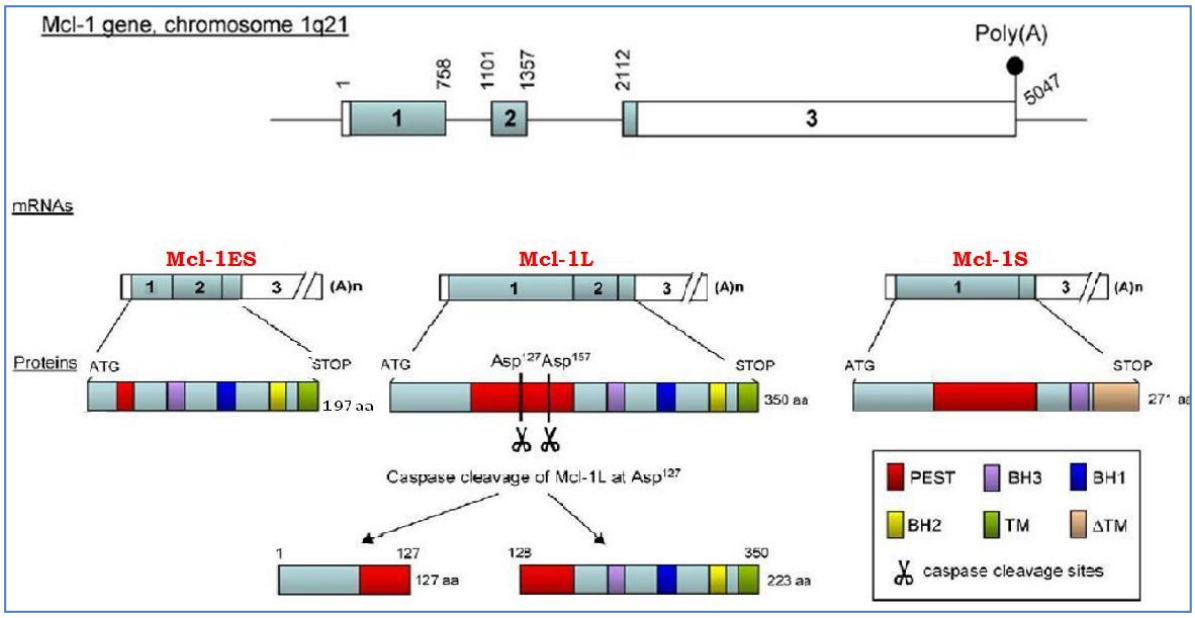


Figure 4: Molecular organization of Mcl-1 (Modified from Source: [93])

Surprisingly, although Mcl-1L, Mcl-1S and Mcl-1ES are expressed from the same gene via alternative splicing and they have opposing functions and Mcl-1L being anti-apoptotic while Mcl-1S & Mcl-1ES are pro-apoptotic [96]. Two PEST sequences located at the N-terminal region of Mcl-1 protein are characteristic sequences found in proteins with rapid turnover and contribute to the short half-life of Mcl-1 protein (one to a few hours) [99, 100]. nterestingly, two residues in the PEST domains (Asp127 and Asp157) have been reported to be critical for caspase cleavage [101] & as the potential sites for phosphorylation (Ser64, Thr92, Ser155, Ser159, Thr163) of Mcl-1 [102].

Mcl-1 (Mcl-1L will be referred to Mcl-1 hereafter) is primarily localized to the outer mitochondrial membrane and promotes cell survival by suppressing cytochrome-c release from mitochondria via hetero dimerization with and neutralization of effector pro-apoptotic Bcl-2 family members including Bak [103]. Mcl-1 also selectively interacts with BH3-only proteins, Bim, tBid, Bik, PUMA and NOXA [104-106]. Mcl-1 may function as an anti-apoptotic factor by sequestering Bak on the outer mitochondrial membrane, preventing Bak oligomerization and cytochrome c release from mitochondria [104-106]. However, when apoptotic signals are received, Bik, NOXA and tBid can selectively disrupt Mcl-1-Bak interaction to displace Bak from Mcl-1, leading to Bak oligomerization and cytochrome c release [104, 105] as described in figure 5.

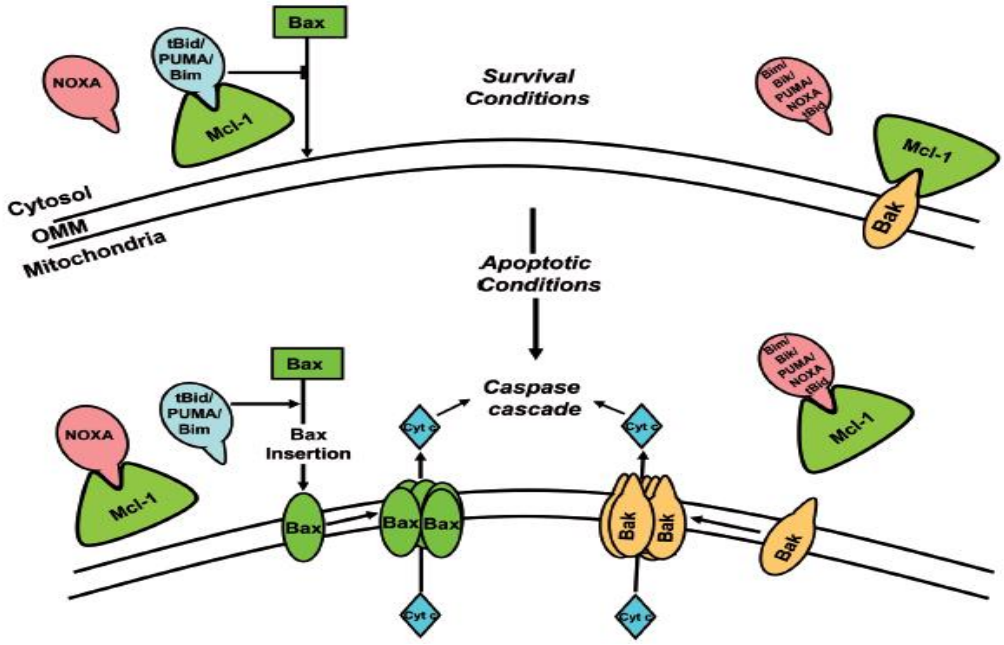


Figure 5: Two main roles of Mcl-1in the cellular apoptosis (Source [94])

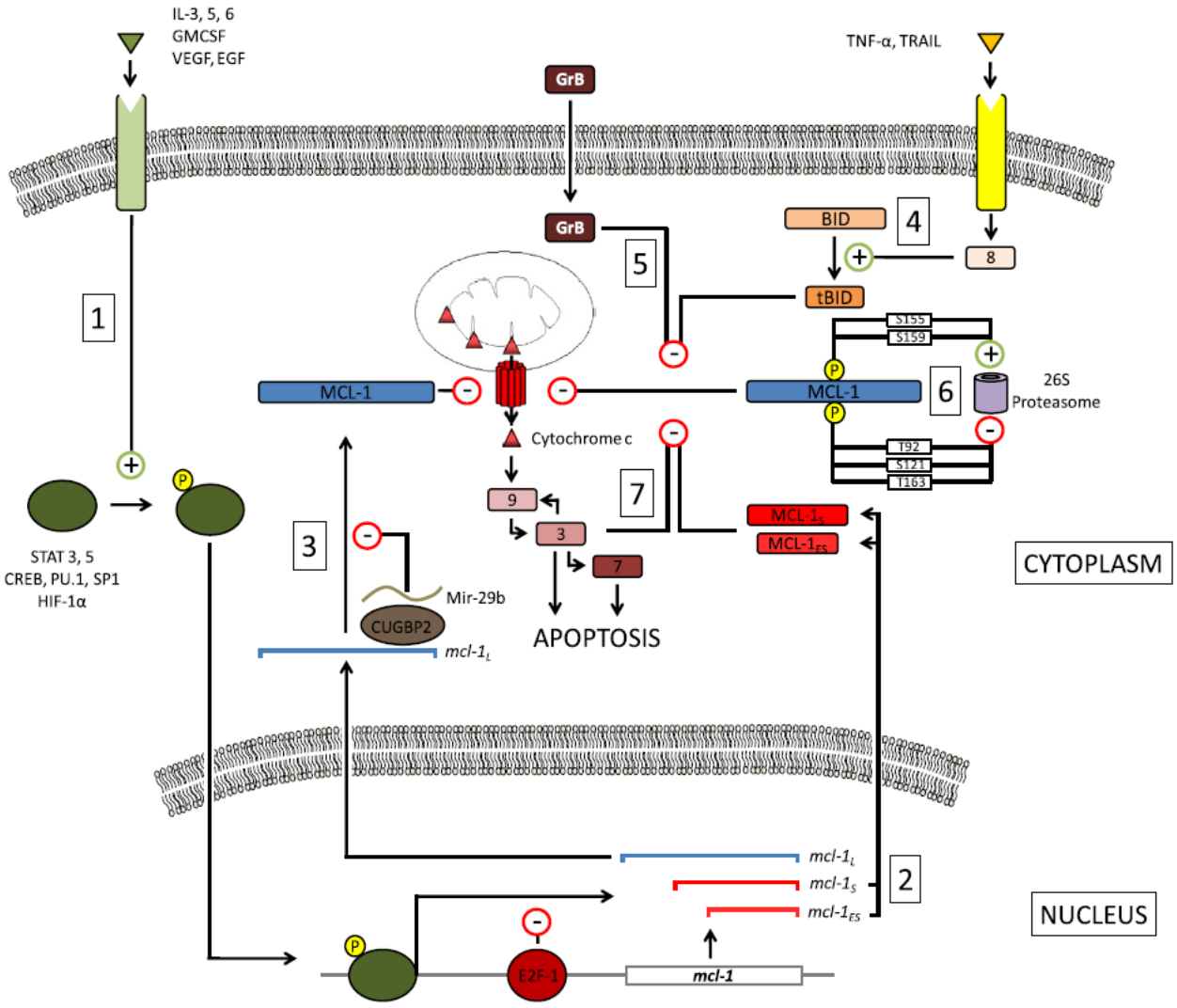
As an alternative mechanism, activator BH3-only proteins (Bim, PUMA, and tBid) bind and activate Bax and/or Bak directly if they are not bound and neutralized by Bcl-2-like proteins including Mcl-1 [67, 107]. However, NOXA can competitively bind to Mcl-1 and prevent it from sequestering activator BH3-only proteins (Fig. 13) [107]. The outer mitochondrial membrane localization of Mcl-1 via its C-terminal TM domain, supports the possibility that Mcl-1 may be primarily acting as a factor sequestering Bak. at the outer mitochondrial membrane in an inactive state [98].

2.10 Regulation of the Mcl-1 expression:

Mcl-1 protein possesses a short half-life and is a highly regulated. Interestingly, the expression of Mcl-1 is induced by survival and differentiation signals, it is also rapidly downregulated during apoptosis in many cell systems. The tight regulation of the Mcl-1 expression suggests that it plays a critical role in apoptosis in response to rapidly changing conditions [14]. Regulation of Mcl-1 expression can occur at multiple levels as summarized in Figure 6.

Transcriptional regulation: Mcl-1 transcription has been known to be regulated by several constitutively activated and/or extracellular signal-activated transcription factors. The signal transducers and activators of transcription (STATs) represent a family of transcription factors that have been shown to be target Mcl-1 promoter. Mcl-1 transcription is upregulated through a STAT3 & STAT5 based activation upon IL-6 treatment and BCR/ABL-dependent upregulation of Mcl-1 transcription [108-110]. IL-3 activates Mcl-1 transcription either by the PI3-K/ Akt-dependent pathway through a transcription factor complex containing CREB [111] or by activation of the PU1 transcription factor through a p38 MAPK-dependent pathway [112]. Mcl-1 transcription can also be upregulated by hypoxia-inducible factor-1 (HIF-1) that protects cells against apoptosis under hypoxia [113]. Mcl-1 gene promoter has also been identified as a target for the ternary complex factor (TCF)-serum response factor (SRF) complex and TCF-SRF-regulated Mcl-1 expression protected cells from apoptosis [114, 115]

Post-transcriptional regulation: Mcl-1 undergoes an alternative splicing giving rise to three distinct mRNA encoding Mcl-1L, Mcl-1S and Mcl-1ES isoform with opposing functions (i.e. anti-apoptotic Mcl-1L and pro-apoptotic Mcl-1S and Mcl-1ES respectively) [96, 97]. It has been recently reported that alternative splicing of Mcl-1 can be regulated in a cell-specific manner. Macrophages up-regulate anti-apoptotic Mcl-1L expression during bacterial infections, and their commitment to apoptosis for the resolution of infection is dynamically regulated by switching Mcl-1 expression from anti-apoptotic Mcl-1L to pro-apoptotic Mcl-1S [98].



(Modified from Source [94])

Figure 6: Overview of the molecular regulation of Mcl-1

Translational regulation: It has very well documented that Mcl-1 mRNA and protein both have very short half-lives and their cellular levels depend on balance between de novo synthesis and degradation [98, 116]. At the translational level, Mcl-1 was shown to be regulated by micro- RNAs through a mir-29b binding in the 3'-UTR of Mcl-1 mRNA [33]. Mir-29b directly inhibits expression of Mcl-1 by binding to its target sequence. Furthermore, mir-29b was found to be overexpressed in non-malignant compared to malignant cholangiocytes, implying a critical role of Mcl-1 protein upregulation in malignant cells [117]. CUGBP2, an RNA binding protein, can also bind to Mcl-1 mRNA 3'- UTR and inhibits its translation, driving the CUGBP2 stably expressing HCT-116 colon cancer cells to apoptosis [118]. mTORC1, the mammalian target of rapamycin complex 1, is a serine/threonine protein kinase and a

downstream target of PI3K/Akt. It has been reported that Mcl-1 is a translationally up-regulated genetic determinant of mTORC1-dependent survival [119].

Post-translational regulation: Multiple modes of post-translational regulation of Mcl-1 have been defined. Mcl-1 possesses many phosphorylation sites, and it is likely that differential phosphorylation of Mcl-1 results in different fates of this protein. Whereas ERK-mediated phosphorylation of Mcl-1 at Thr92 and Thr163 prolongs the Mcl-1 half-life [120], Ser159 phosphorylation by GSK-3b reportedly enhances Mcl-1 ubiquitylation and degradation [121]. On the other hand, a distinct pathway involving phosphorylation at Ser64 enhances its anti-apoptotic function [102]. Furthermore, Mcl-1 is phosphorylated at Ser121/Thr163 and inactivated by JNK in response to oxidative stress [122]. Caspase-mediated and proteasome-dependent degradations are also two main routes responsible for the rapid turnover of Mcl-1. Mcl-1 is subject to rapid turnover through ubiquitin dependent protein degradation by the 26S proteasome.

One of its E3 ligases, MULE/LASU1, is a BH3-only E3-ligase whose BH3 domain interacts with the hydrophobic BH3 binding pocket of Mcl-1, and not with other pro-survival Bcl-2 family members [67, 68]. Such, MULE/LASU1-mediated ubiquitination of Mcl-1 accounts for the constitutive turnover of Mcl-1. The degradation of Mcl-1 can be blocked by proteasome inhibitors, suggesting a role for the ubiquitin proteasome pathway in apoptosis [123, 124]. NOXA is the best-characterized BH3-only protein, and can displace Bak from Mcl-1 to initiate apoptosis. Binding of NOXA to Mcl-1 also induced Mcl-1 degradation via a proteasome-dependent mechanism [104]. On the other hand, specific cleavage of anti-apoptotic Mcl-1 at Asp127 and Asp157 by caspase-3 during the apoptotic process produced a C-terminal domain with a death-promoting activity [125].

2.11 Mcl-1 & Cancer:

Physiological function of Mcl-1:

Mcl-1, is widely expressed but has its own particular tissue distribution and markedly divergent physiological roles from those of other pro-survival Bcl-2 family members [94]. Mcl-1 deletion is peri-implantation lethal in mouse embryogenesis, whereas Bcl-2 deletion is not [126]. Conditional knockout studies have shown that Mcl-1 is essential for both the development and maintenance of B and T lymphocytes [127] and ensuring the homeostasis of early hematopoietic progenitor [128]. Recently, an important role of Mcl-1was observed in regulation of the ovarian primordial follicle reserve of neonatal mouse ovary [17]. It has also been shown to play a critical role in the regulation of macrophage, neutrophil apoptosis and synovial fibroblasts [129, 130] . Mcl-1 also seems crucial anti-apoptotic factor for the liver, contributing to hepatocellular homeostasis and protecting hepatocytes from apoptosis induction [131]. The loss of Mcl-1 expression has shown to promotes apoptosis in human polymorphonuclear leukocytes [132]. In addition to its survival promoting functions, Mcl-1 may also play a positive role in apoptosis. The cell death promoting protein that results from caspase cleavage of Mcl-1 may participate in a positive feedback loop leading to further caspase activation [93].

Depletion of Mcl-1 & Cancer:

Mcl-1 also plays a critical role in the survival of malignant cells since depletion of Mcl-1 via antisense oligodeoxynucleotides are known to trigger apoptosis in cancer cells [133]. Down-Regulation of Mcl-1 by is found to sensitize resistant melanoma cells to Fas-mediated apoptosis and could also sensitize neuroblastoma to cytotoxic chemotherapy and small molecule Bcl2-family antagonists [134, 135]. Also, depleting of Mcl-1 levels could chemo-sensitize human melanoma in a SCID mouse xenotransplantation model [136]. The exact molecular mechanism by which Mcl-1 promotes cell survival is not completely understood but is thought to involve suppression of cytochrome c release from mitochondria, possibly via heterodimerisation with

and neutralization of pro-apoptotic Bcl-2 family proteins, for example, Bim or Bak [93, 127]. The rapid induction and degradation of Mcl-1 suggests it plays an important role in apoptotic control in multiple cell types in response to rapidly changing environmental cues [13]. Interestingly, Mcl-1 may be an apical player in apoptosis control, modulating early events in a cascade leading to cytochrome c release. It is observed from several studies that down-regulation Mcl-1 is able to induce apoptosis in a number of cancer cell types [137], which clearly indicates a crucial role for Mcl-1 for inhibition of cellular apoptosis, further contributing to resistance to various treatments.

Over-expression of Mcl-1 & Cancer:

Mcl-1 overexpression has been reported in several hematopoietic, lymphoid and solid tumors including chronic myeloid leukemia and hepatocellular carcinoma [94]. Mcl-1 expression also appears critical for survival in a subgroup of non-small-cell lung cancer cell lines [138]. Most notably, overexpression of Mcl-1 in transgenic mice results in a high incidence of B-cell lymphoma, demonstrating that Mcl-1 can directly contribute to the development of malignancies [137]. This highly regulated nature indicate that Mcl-1 expression is pivotal to normal animal development, whereas its deregulation may promote neoplastic transformation [139]. Interestingly, MCL-1 over expression has found to be associated with poor prognosis in Gastric, Ovarian & Cervical [16, 17, 140]. Thus, Mcl-1 may play an important role in variety of cancers and it may have potential as a biomarker and therapeutic target.

Mcl-1 as a potential therapeutic target:

Although Mcl-1 is one of the essential anti-apoptotic factor in the development and differentiation of normal cells, deregulation of signaling pathways regulating Mcl-1 expression often results in its overexpression, which contributes to several human diseases including malignancies [14]. Mcl-1 overexpression has been shown in a variety of nonsolid and solid tumors and also appears to be a key factor in the resistance of some cancer types to conventional cancer therapies [141-143]. As described earlier, Mcl-1 down-regulation is often sufficient to

promote apoptosis in cancer cells, suggesting that Mcl- 1 can be a potential therapeutic target in the treatment of several human malignancies. Mcl-1 has also been implicated in the chemoresistance of certain malignancies ([144, 145], notably against the first of a new class of Bcl-2 family targeting compounds, named ABT-737 [146, 147]. Thus Mcl-1 is an attractive and potential therapeutic target in a number of malignancies as MCL-1 has been shown to confer resistance to the BCL-2/BCL-XL/BCL-W selective antagonist ABT-737 and to the proteasome inhibitor bortezomib.

Since ablating Mcl-1 function is sufficient to promote apoptosis in cancer cells, Mcl-1 might be a target for new anti-cancer therapies, for example small molecules interfering with critical BH domains [148]. Small molecule Obatoclax (GX15-070), which is predicted to occupy a hydrophobic pocket within the BH3 binding groove of BCL-2, antagonizes these members and induces apoptosis [141]. Most importantly, Obatoclax overcomes the resistance due to upregulation of Mcl-1[149]. Several ongoing studies are examining the efficacy, potency, and function of several small molecule inhibitor drugs targeted to the Bcl-2 family of proteins and their preclinical progress against different cancers [150].

Hence in the light of above information the specific objectives of the project are-

CHAPTER 3

Aims & Objectives:

The specific objectives of the project-

1. To decipher mechanisms of Mcl-1 overexpression in oral cancers.

2. To examine the levels of Mcl-1 isoforms/protein in oral cell lines, premalignant lesions & paired oral tumors from different subsites and their correlation with clinico-pathological parameters.

3. To study whether Mcl-1L is a radioresistance and/or chemoresistance related factor in oral cancers.

CHAPTER 4

MATERIALS & METHODS

4.1 Source of Reagents:

The following chemicals/reagents/kits were obtained from-

Sigma: Bromophenol Blue, β-mercaptoethanol, Coomassie brilliant blue, Diaminobenzidine, DMSO, DEPC, Ethidium Bromide, ETDA, EGTA, Fast green, Fetal Bovine Serum, 8-Hydroxyquinoline, Oligonucleotides, Proteinase K, Poly-L Lysine, Sulphorhodamine B, Sigmacote, Trypsin, TRIzol, TEMED, βME, Agarose, Acrylamide, Bis acrylamide, Guandine isothiocyanate, Low melting agarose, Sarcosine, Tris base, SDS, Urea, Maxi prep plasmid extraction kit, G-418, MTT reagent, Erythrocin B, Ponceau stain, BSA, DAPI, Hoechst, 3,3'Diamino Benzidine (DAB), Tri-Sodium citrate, Xylene.

Invitrogen/Himedia: DMEM, RPMI 1640, FBS, Opti-MEM, Lipofectamine-2000, TRIzol, Puromycin, Luria agar/broth, Ampicillin.

Roche: dNTPs, Protease inhibitors, Taq polymerase, BM cyclin.

Amersham Lifesciences: Anti-mouse HRP linked secondary antibody, Enhanced Chemiluminescence Kit, PVDF membrane.

MBI Fermentas: DNA ladder (100bp), First strand cDNA kit, 6X loading dye, 25mM $MgCl_2$, Protein ladder, 10X PCR buffer, Protease inhibitors, Taq polymerase, Restriction enzymes (AgeI, BamHI, EcoRI, XhoI), Digestion & Ligation buffers, TA cloning vector.

Santacruz Biotechnology: Anti-rabbit & Anti-mouse HRP linked secondary antibodies, Antibodies for Actin, Mcl-1(polyclonal), Mcl-1L (monoclonal), Bax, Bcl-2, Bxl-xl, and Noxa.

BD Pharmingen: Antibody for Mcl-1, Annexin-V & PI kit.

Cell Signaling: Antibodies for Caspase 8 and Bcl-xL, Bax.

SISCO Research Laboratories: Chloroform, Folin Ciocalteu reagent, Glycine, Isopropanol, Isoamyl alcohol, Methanol, Molecular biology grade ethanol, Phenol, Sodium chloride, Sodium acetate, Trichloro acetic acid, **Thermo scientific:** pTRIPZ vector.

Qualigens: Boric acid, Disodium hydrogen hydrophosphate, MgCl2, NaOH pellets, Sodium dihydrogen orthophosphate, Tri-Sodium citric acid, Glacial acetic acid, Xylene.

Qiagen: Blood & Tissue DNA extraction kit, Gel extraction kit.

Applied Biosysytems: Taqman probes (Mcl-1L, Mcl-1S, Mcl-1ES, GAPDH, 18S RNA), Taqman universal PCR master mix. Tissue culture petridish and flasks were obtained from **Nunc** and **Falcon.** Disposable tips and DNase, RNase free eppendorf tubes were obtained from **Axygen**. The water used for the preparation of all solutions and reagents was obtained from MiliQ water plant (**Millipore**).

4.2 Tissue samples & Reagents used in study:

Tissues samples: A total of 130 patients with a diagnosis of oral were recruited for this study cancer and 20 OSF patient's tissue paraffin blocks were used, after approval of the project by the Institutional Review Board (IRB) and after taking informed consent of the individual patients. During surgery, histologically normal tissue surrounding the tumor were resected and taken as pair-wise normals. Further, 10 oral tissue biopsies were obtained from patients without any clinically detectable lesions but undergoing minor surgical procedures like removal of third molar. All the tissues were frozen immediately in liquid nitrogen and stored at -80^{0}C until analysis.

Characteristics	No. (%)
Gender	
Male	99 (76.2)
Female	31 (23.8)
Age (Range 13-80 years)	
>53	63 (48.5)
<53	67 (51.5)
Habits	
Tobacco chewing	81 (62.3)
Tobacco chewing + Alcohol	15 (11.5)
Smoking	04 (3.1)
Smoking + Alcohol	02 (1.5)
Tobacco chewing + Smoking	13 (10)
Tobacco + Smoking + Alcohol	07 (5.4)
No habits	06 (4.6)
NA	02 (1.6)
Primary site of tumor	
Buccal Mucosa	46 (35.4)
Tongue	45 (34.6)
Alveolus	35 (26.9)
Others	04 (3.1)
Tumor classification	
T1 + T2	42 (32.3)
T3 + T4	88 (67.7)
Lymph Node	
N0	47 (36.2)
N1	32 (24.6)
N2	51 (39.2)
Differentiation	
Well	16 (12.4)
Moderate	82 (63.6)
Poor	31 (24.0)

Table 1: Clinico-pathological characteristics of 130 oral cancer patients-
(Used for Mcl-1 isoform expression studies)

Characteristics	No. (%)
Gender	
Male	33 (76.2)
Female	07 (23.8)
Age (Range 13-80 years)	
>53	23 (48.5)
<53	17 (51.5)
Habits	
Tobacco chewing	26 (65.0)
Tobacco in any form/In combination	13 (32.5)
NA	01 (2.5)
Primary site of tumor	
Buccal Mucosa	19 (47.5)
Tongue	18 (45.0)
Others	03 (7.5)
Tumor classification	
T1 + T2	09 (22.5)
T3 + T4	31 (77.5)
Lymph Node	
Negative	11 (27.5)
Positive	29 (72.5)
Differentiation	
Well	04 (10.0)
Moderate	22 (55.0)
Poor	14 (35.0)

'NA' denotes data not available.

Table 2: Clinico-pathological parameters of 40 oral cancer patients-
(Used for Mcl-1 Polymorphism study)

Mammalian culture:

IMDM *(Iscoves Modified Dulbecco's Medium):* Powdered medium was dissolved in ~800 ml autoclaved D/W, supplemented further with 3.024 gm sodium bicarbonate ($NaHCO_3$) and volume made up to 1 L. The medium was filtered through 0.1 µ sterile filter and stored at 4°C; ***MEM*** *(Minimal Essential medium),* ***DMEM*** *(Dulbecco's Modified Eagle Medium) &* ***RPMI 1640*** (prepared similarly as mentioned above); ***Antibiotic for regular cultures*** (For 10 ml stock): Mixture of 0.2 ml of Amphotericin B (2.5 mg/ml), 2.5 ml of Penicillin (50000 units/ml), 0.4 ml of Streptomycin (100000 units/ml), volume was made up to 10 ml with 6.9 distilled water. The antibiotic solution was stored at 4^0C; ***Antibiotic selection reagent*:** Working concentration of G418 was 400-800 µg/ml and Puromycin 0.5-1 µg/ml; ***Fetal bovine serum***

(FBS)*:** Serum dispensed in 50 ml aliquots/ sterile tube was stored at -20°C; ***Complete medium: IMDM, DMEM or RPMI 1640 supplemented with 10% FBS and 1% of antibiotics; ***Freezing medium*:** FBS was supplemented with 10% DMSO (anti-freeze agent) and stored at -20 °C ; ***Phosphate buffered saline* (PBS)** NaCl-8.0 g, KCl-0.2 g, KH_2PO_4-0.2 g, Na_2HPO_4 -1.5 g, phenol red indicator (0.01%); pH was adjusted to 7.4 and sterilized by autoclaving; ***Phenol Red Solution:*** 0.1 g phenol red powder was dissolved in 100 ml distilled water, autoclaved and stored at 4^oC. ***Trypsin–EDTA*:** 0.25 g Trypsin, 0.58 g $NaHCO_3$, Phenol Red (0.01%), 1.0 g EDTA Sodium salt, 1.0 g D-glucose, 0.4 g KCl, 8.0 g NaCl; ***Erythrocin B*** (0.4 %): 40 mg Erythrocin B in 10 ml PBS, Trypan blue (0.4%).

Protein extraction and quantification- Cell pellets, Proteojet Mammalian cell lysis reagent, Proteoblock protease Inhibitor (Fermentas), Bradford's reagent and BSA 1 mg/ml.

SDS-Polyacrylamide gel electrophoresis (PAGE) - 30 % Acrlyamide solution: 28.8 g Acrylamide and 0.2 g Bis-acrylamide were dissolved in DW on a magnetic stirrer overnight (O/N) at room temperature. The volume was made up to 100 ml and filtered through 0.45 µ filter and stored in a dark bottle at 4^0C; 20% SDS; 10 % Ammonium persulfate (APS); Tetramethylethylenediamine (TEMED), 1.25 M Tris buffer – pH 6.8 & 1.25 M Tris buffer – pH 8.8; 2X & 4X sample loading buffer: 250 mM Tris buffer pH 6.8, 20% glycerol, 8% SDS, 8% β mercapto-ethanol (BME), 0.04 % bromophenol blue; ***electrophoresis buffer-*** 25mM Tris base, 250 mM Glycine and 0.1% SDS; protein molecular weight marker (Fermentas).

Transblotting- Transfer buffer: Tris base-9.0 g, glycine-39.5 g, methanol-600 ml. D/W up to 1 L; PVDF membrane; Whatman filter paper no-3; *Destainer* (100 ml) - 45 ml methanol, 10 ml Glacial acetic acid & 45 ml distilled water. *Coomassie Brilliant Blue-R (100 ml)*- 0.25 gm of CBB powder in 100 ml destainer solution; *Fast green (0.1%)* - 0.1% fast green in 100 ml of destainer; *Ponceau S Staining Solution*- [0.1%(w/v) Ponceau S in 5%(v/v) acetic acid].

Immunoblotting- Tris buffered saline (TBS): 150/500 mM NaCl, 20 mM Tris (pH 7.4); Tris buffered saline with Tween (TBS-T)- TBS + 0.1 % Tween 20; Blocking agent, 3 % BSA or

5% milk in TBS, Primary and HRP labeled secondary antibodies; Detection system: Chemi luminescent substrate (ECL plus & ECL prime) (GE, USA), X-ray films (Kodak) and exposure cassette (Amersham). ***Stripping buffer*** *(100ml)*- 1.63 gm (w/v) of 100 mM beta mercaptoethanol, 10 ml of 20% stock of SDS, 6.23 ml of 1M stock of Tris-Cl (pH:6.7) & make up volume with distilled water.

RNA extraction/c-DNA synthesis - TRIzol reagent, TRI reagent, Chloroform, Isopropanol, ethanol (75%) liquid N_2; *For 10 X Phosphate buffer* (electrode buffer)- 0.2 M Sodium dihydrogen orthophosphate (Monobasic)- 15.6 gms in 500 ml & 0.2 M Disodium hydrogen orthophosphate (dibasic)- 14.2 gms in 500 ml were made separately. For preparing 10 x buffer, 117 ml of 0.2 M of monobasic salt and 183 ml of dibasic salt was added and volume made up to 900ml with DEPC treated water and autoclaved. Revert Aid First strand cDNA synthesis, 5X reaction buffer, 10 mM dNTP mix, random hexamer primer, oligo dT primer, sterile DEPC treated water; routine PCR reagents.

Polymerase Chain Reaction (PCR): Gene specific primers, Taq DNA polymerase buffer with $(NH4)_2SO_4$ (10X), dNTPs, $MgCl_2$ (25 mM), Taq DNA polymerase, Nuclease free water.

Agarose gel electrophoresis: Tris Borate EDTA (TBE) buffer: 0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA (10X buffer stock was made and diluted to 0.5X for use); 6X gel loading dye: 0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol; EtBr: 0.5 μg/ml; agarose, low melt agarose; DNA markers: λ / HindIII, 100 bp and 1 Kb.

Genomic DNA extraction & Gel purification: DNA extraction kit & Gel purification kit.

Quantitative Real time PCR- RNAse free water (0.1% DEPC treated), Taqman probes & Universal master mix, for SYBR chemistry- SYBR green master mix, gene specific primers.

Transfection by CaPO4 method- 2.5 M $CaCl_2$: 3.7 g $CaCl_2.2H_2O$ was dissolved in 10 ml DW, filter sterilized through 0.22 μ filter, 1.0 ml aliquots were made and stored at -20°C; **BES buffer**- 50 mM BES (N, N-bis [2-hydroxy-ethyl]-2-aminoethane sulfonic acid) (1.1 g), 280 mM NaCl (1.6 g), 1.5 mM Na_2HPO_4 (27 mg). The pH was adjusted to 6.95 with 5N NaOH.

The volume was made up to 100 ml with D/W; filter sterilized through 0.22 µ filter 1.0 ml aliquots were made and stored at -20°C; DMEM with 10% FBS and antibiotic.

Lipofectamine method- Control/Nontargeting siRNA, gene specific siRNA, shRNA expressing plasmids, Opti-MEM, IMDM without serum, Lipofectamine-2000 transfection reagent, IMEM with 10% FBS without antibiotic.

Transduction: Polybrene (Hexadimethrine bromide) 2 mg/ml in D/W; sterilized by filtration; Disposable 0.45 µm filtration assembly.

Inhibitors- Obatoclax mesylate ((Z)-2-(5-((3,5-dimethyl-1H-pyrrol-2-yl)methylene)-4-methoxy-5H-pyrrol-2-yl)-1H-indole mesylate); dissolved in 1% DMSO, aliquots were stored in -20^{0}C.

Cytotoxicity assay *(MTT):* 5mg/ml powder dissolved in PBS stored in dark at -20^{0}C, DMSO, 96 well flat bottom plates, Microplate reader with 550 and 600nm wavelength.

Drugs- *Cisplatin* (*cis*-diamminedichloroplatinum); Dissolved in DMSO at 2 mM concentration and diluted further in culture media. Make 10 µM working stock and dilute further in culture media make10 µM working stock.

Immunohistochemistry:

10 mM Sodium citrate Buffer: 500 ml of 10 mM sodium citrate buffer was prepared by dissolving 1.47 gm of Tri-Sodium citrate in 450 ml milli Q water and then adjusting the pH to 6.0 using 1N HCl. The volume was finally adjusted to 500 ml using milli Q water. *1X Phosphate Buffered Saline:* 1000 ml of 1X PBS was prepared by dissolving 8 gms of sodium chloride, 1.6 gms of Disodium hydrogen orthophosphate (anhydrous) and 0.45 gms of sodium dihydrogen orthophosphate anhydrous in 50 ml of milliQ water. The pH was adjusted to 7.4, before adjusting the volume to 1000ml; *3,3'Diamino Benzidine (DAB)* was prepared by dissolving 0.005 gms of DAB powder in 10 ml of 1X PBS and filtering it.

Bacterial culture:

Luria-Bertani medium (LB): For the culture and maintenance of different *E. coli* strains harboring desired plasmids a minimal growth medium was used. Powdered Luria Broth (20 g) was dissolved in 800 ml distilled water (DW) and the volume was adjusted to 1L with DW and sterilized by autoclaving. For making LB-agar plates, 35 g Luria agar powder was dissolved/ L sterilized by autoclaving and poured in 90 mm sterile plates. *For Low salt LB agar*- 10 g Bactotryptone, 5g Yeast extract, 5g NaCl, 20g Agar, 10ml of 1M Tris (pH 7.5) per L was autoclaved and poured in plates, cooled, sealed with paraffin and covered with foil to avoid moisture.

Ultra-competent cells- *E.coli* (DH5α/stable3) strains *;* ***SOB*** (Super optimal broth): Following components were mixed in required volume of DW; 2% Bactotryptone, 0.5%Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, adjust pH between 6.7-7.0

Antibiotics- Ampicillin was used for selection of clones or propagation of plasmids carrying the respective markers. Stock solutions were prepared (50 mg/ml) and stored at -20°C. Antibiotics were added to the media (broth/agar plates) at 50 µg/ml final concentration.

Plasmid Mini-preps by alkaline lysis method*: Resuspension solution* (Solution I)- 50 mM Glucose, 25 mM Tris Cl (pH 8.0), 10 mM EDTA.$2H_2O$; DW to make up the total volume; *Lysis solution* (Solution II): 2M NaOH (1ml), 10% SDS; DW to make up the total volume; *Neutralization solution* (Solution III): 5M Potassium Acetate 60 ml, Glacial acetic acid 11.5 ml, D/W 28.5 ml; 1M Tris (pH 8.0) 1ml, 0.2M EDTA (pH 8.0) 0.5ml, D/W to make up volume to 100 ml. ***Maxi-preps****:* The large scale plasmid extraction was done by following protocol of Maxi prep plasmid extraction kit (Sigma).

Transformation: *Transformation buffer* (TB): The following components were added to 100 ml of D/W; 10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, adjusted pH to 6.7 with 5N KOH, 55 mM $MnCl_2$, filter sterilized through 0.2 µ membrane filter; Ligation reaction (20 µl), Plasmid 5 µl (100 ng), ultra competent *E.coli* cells (DH5α/Stable3); Sterile SOB broth, LB agar (low salt) plates with 50 µg/ml of ampicillin, sterile toothpicks.

METHODS:

4.3 Bacterial Culture:

***Preparation of ultra-competent cells**:*

To recover each plasmid clone higher competency is a prerequisite. Preferably for better cloning efficiency of large size DNA fragments, *E.coli* strains with less/no recombination like DH5α-MCR/stable3 were used for ultra-competent cell preparation and transformation of plasmid vectors. From the glycerol stock, cells were streaked on a freshly made LB agar plate & incubated at 37^0C/overnight. A single colony was inoculated next day in a 5 ml SOB broth and incubated overnight on shaker incubator at 37^0C to prepare starter culture. From the above starter culture 2.5 ml or directly a colony from freshly streaked plate was inoculated in 250 ml SOB in a 1 L flask and incubated at 18^0C while shaking (at 250 rpm) till OD_{600} reached ~0.6. The culture was immediately transferred to pre chilled centrifuge bottles and centrifuged at 3500 rpm at 4^0C for 10 min in a swing out rotor. The cell pellet was resuspended in 80 ml transformation buffer and kept on ice for 10 min. The culture was centrifuged as above. Cell pellet was again resuspended in 20 ml transformation buffer to get a homogeneous suspension, incubated on ice for 10 min and DMSO was added drop-wise to final concentration of 7%. The suspension was mixed and 200 µl aliquots were snap frozen in liquid nitrogen and stored at -80^0C. The competency of cells was checked by transformation of 1pg, 100pg, 1 ng of plasmid.

Transformation:

Competent cells were mixed with either 250-500 ng of plasmid or with ligation mixture, tapped and incubated for 30 min on ice. A heat shock at 42^0C was given for 90 sec and transferred on ice for 2-5 min. 1 ml of LB broth was added and incubated again for 45 min at 37^0C. The mixture was centrifuged for 5 min at 5000 rpm and supernatant was discarded leaving 100 ul behind. The pellet was mixed, spread on LB amp plate and incubated at 37^0C for overnight.

Plasmid mini preparation

(1) By alkaline lysis method: Cultures were aliquoted in 1.5 ml eppendorfs & centrifuged at 14000 rpm for 2 min at 4^0C. The cell pellets were suspended in 100 µl solution l containing RNase A, vortex mixed, 200 µl lysis solution (solution II) was added, inverted for 8 times (do not vortex) and incubated at room temperature for 3 min, 150 µl chilled neutralization solution (solution III) was then added, mixed 10-15 times by inversion and incubated on ice for 10 min. the tubes were centrifuged at 14000 rpm at 4^0C for 10 min. The clear solution was transferred into a fresh tube and the plasmid was precipitated using 1 ml of absolute chilled ethanol at -20^0C for 20 min and centrifuged at 14000 rpm for 20 min. The supernatant was removed and pellet washed with 0.5 ml 70% ethanol, followed by centrifugation for 5 min at 4^0C. The pellet was semi air-dried at RT and dissolved in 25µl DW/ TE buffer. Further, 1 µl of RNase A (10mg/ml) was added, incubated at 37^0C for 1 hr and stored at -20^0.

(2) By GeneJET™ Plasmid Miniprep Kit: In order to sequence the plasmids of the positive clones, the GeneJET™ Plasmid Miniprep Kit was used for plasmid extraction as per manufacture's instruction (Fermentas, USA).

Plasmid maxi preparation:

The large scale plasmid preparation was done by using the protocol of GenElute^{TM} HP Plasmid Maxiprep kit (Sigma, US). Briefly, cells were harvested from, 150 ml culture, resuspended in 12 ml of resuspension/RNase A solution, mixed by pipetting, followed by adding 12 ml of lysis solution & inverted 6-8 times and incubated for 5 min. Further, 12 ml of chilled neutralization solution was added to the mixture gently inverted. Then, 9 mL of binding solution was added and poured into the filter syringe & allowed the lysate to sit for 5 min. Binding column was prepared by passing 12 mL of the column preparation solution. The lysate was filtered and DNA allowed to bind to the column. The column was washed with 12 ml of Wash Solution. The plasmid DNA was eluted in fresh tube by adding 3 ml of Elution solution and stored at -20^0C for further use.

4.4 Mammalian cell culture:

All the cell lines namely immortalized human Fetal Buccal Mucosa (FBM), human epidermal keratinocytes derived normal (HaCaT) and all oral squamous carcinoma cell lines were regularly maintained in different media supplemented with 10% FBS and antibiotics at 37˚ C in 5% CO_2 incubator as described in table below-

Table 3: Culture media used for the different cancer cell lines used in study-

Cell line	Origin	Culture Medium	Reference
FBM	Fetal Buccal Mucosa-Immortalized	IMDM+FBS+ Supplement	Roul *etal.,* Int. J Cancer, 2004
HaCaT	Human Keratinocyte-Immortalized	DMEM +FBS	Fusenig *et al.,* J Invest. Dermato, 1999
DOK	Dysplastic Oral Keratinocyte	DMEM +FBS+HEPES+Hydrocortisone	Chang Int. J. Cancer *et al.,1992*
AW8507	Tongue Squamous Carcinoma	IMDM + FBS	Tatake *et al.,* J Cancer Res Clin, 1990
AW13516	Tongue Squamous Carcinoma	IMDM + FBS	Tatake *et al.,* J Cancer Res Clin, 1990
SCC40	Tongue Squamous Carcinoma	MEM + FBS + HEPES	Gollin *et al.,* Oral Oncol, 2008
SCC25	Tongue Squamous Carcinoma	DMEM + FBS	Price *et al.,* Otol Head Neck Surg, 2010
SCC29B	Buccal mucosa Squamous Carcinoma	MEM + FBS + HEPES	Gollin *et al.,* Oral Oncol, 2008
SCC15	Base of Tongue Squamous Carcinoma	DMEM + FBS+ HEPES	Rheinwald *et al.,*J Cancer research,1999
QLL1	Tongue Squamous Carcinoma	DMEM + FBS+ HEPES	Fusenig *et al.,* J Invest. Dermato, 1999
SCC74	Alveolar ridge Squamous Carcinoma	DMEM + FBS+ HEPES	Gollin *et al.,* Oral Oncol, 2008

Routine maintenance of cell lines-

The spent medium was removed from confluent (80%) cells and one 1 X PBS wash was given to remove the serum adhering to the cells. Trypsin- EDTA solution (1ml) was added per T-25 flask / 60 mm plate for disaggregation and incubated at 37°C for 2 min followed by neutralization of the enzyme by addition of 3 ml of medium to the trypsinized cells. Cells were spun at 1200 rpm/2 min, washed once with medium, counted using hemocytometer and seeded as per requirement. The flasks/plates were incubated at 37˚ C in 5% CO_2.

Cell Count and Viability:

Cell count was taken in Neubauer's hemocytometer using Trypan Blue / Erythrosin B. The cell suspension was diluted in the ratio of 1:10 with PBS. 10 µl of this suspension was mixed with Trypan Blue / Erythrosin B in the ratio of 1:1 and loaded onto the hemocytometer. Live cells will be seen as clear cells whereas the dead cells will take up the dye to appear blue/pink. All the cells in the four WBC chambers were counted to give total count-

Cells per ml = the average count per square x the dilution factor x 10^4

% Viability = viable cells x 100 / Total cells

Freezing and Revival of Cell Cultures:

Freezing: For freezing the cell cultures, 70-80% confluent cultures were trypsinised and harvested as above. Cells were washed twice with 1X PBS to remove the adhering medium. Simultaneously, freezing media was added to the cells to a final concentration of 1 x 10^6 cells / ml. The freezing vials were labeled and suspended in a cylinder with liquid nitrogen vapour and cooled gradually before plunging it into liquid nitrogen.

Reviving Cell cultures: The frozen vial of cells was removed from a liquid Nitrogen container and quickly thawed at 37°C in a water bath & the cell suspension thawed was transferred gently to a tube containing 8 ml of medium. The cell pellet was obtained by spinning the cell suspension at 1000 rpm for 10 min. Subsequently, the cell pellet was washed with 1X PBS twice for complete removal of freezing mixture. The supernatant was discarded and the cell pellet was suspended in 1 ml of complete medium. Cell viability and count was taken as described above. The cell suspension was mixed gently with a Pasteur pipette to remove any clumps and finally plated in a culture plate or flask.

4.5 RNA Extraction:

The total RNA was isolated by TRI reagent as per manufacturer's instructions (Sigma, USA). *Cell lines:* Briefly medium was removed, cells were washed by PBS, either lysed in culture plates using TRI reagent or cell pellets already stored in -80^0C were used for extraction. Cells were lysed completely by repeated pipetting. *Human tissue samples:* 30-50 mg tissue samples stored in -80^0C was finely minced and crushed in mortal and pestle using liquid N_2. The powdered tissues were suspended in 1 ml of TRI Reagent. The suspension from cell lines or tissue samples were transferred into a 1.5 ml tubes and incubated for five min at room temperature for complete dissociation of nucleoprotein. 200 µl of chloroform was added per ml of TRI reagent, vigorously shaken by hand for about 15 seconds, the mixture was kept on the bench top till two phases could be distinguished and then centrifuged at 14,000 rpm for 15 min at 4°C. The clear aqueous phase was carefully transferred to a fresh tube without disturbing

the interphase and the RNA was precipitated by adding 500 µl of chilled isopropanol followed by centrifugation as above. The Isopropanol was gently removed and pellet was washed with 500 µl 75% ethanol; pellet was semi air dried and dissolved in 50 µl DEPC treated water at 55°C; and contaminating DNA was removed by DNaseI treatment. RNA quality and integrity was analyzed by phosphate buffered electrophoresis (1.2%) and samples were preserved at −80°C until analysis.

4.6 *Reverse transcriptase - Polymerase chain reaction (RT-PCR):*

cDNA was synthesized using a First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's instructions. Briefly, 2µg of total RNA and 1ul (0.2 µg/µl) of random hexamer primer in a volume of 12 µl were incubated at 70^0C for 5' and chilled on ice and centrifuged. Following components were added after denaturation of the RNA in the same reaction tube. 5X reaction buffer-4µl, 10mM dNTP mix-2µl, Reverse Transcriptase enzyme-1µl (200 u/µl), RiboLock™ RNase Inhibitor (20 u/µl)-1 µl, DEPC D/W upto 20 µl; the reaction mixture was incubated at 25ºC for 10' followed by 42ºC for 1 hr. The reaction was stopped by heating at 70ºC for 10 min. The efficiency of cDNA synthesis and equal loading were assessed by ß-actin/GAPDH PCR. PCR products were resolved on 2% agarose gel containing ethidium bromide and quantitated using Gel-doc system (UVP, UK). The primer sequences and conditions used to amplify target genes & internal controls and product sizes are shown below.

Sr. No	Gene	Primer Sequence (5'--3')	Conditions (^{0}C – Min/Sec)	Product size (bp)
1	Mcl-1L & Mcl-1S	F- ATCTCTCGGTACCTTCGGGAG R- CCTGATGCCACCTTCTAGGTC	94-1 63-1 72-1 } 35 cycles	444 &196
2	Mcl-1ES	F- AGGGCGACTTTTGGCCACCG R- CCCAGTTTGTTACGCCGTCGCT	94-45S 64-30S 72-45S } 35 cycles	230
3	β-Actin	F- GACTACCTCATGAAGATC R- GATCCACATCTGCTGGAA	94-1 48-1 72-1 } 30 cycles	452
4	GAPDH	F- CAAGGTCATCCATGACAACTTTG R- GTCCACCACCCTGTTGCTGTAG	94-1 52-1 72-1 } 30 cycles	496

F – Forward primer, R – Reverse primer

Table 4: RT-PCR details of target genes (Primer sequence and PCR Condition)

4.7 Quantitative Real time PCR:

A quantitative real-time PCR analysis was performed using either Taqman Universal PCR master mix and gene expression assays or the SYBR Green mix and gene specific primers (Table 3-4). The cDNA was prepared as described above and used as templates for real time PCR. The quantitative RT-PCR was performed with the ABI PRISM7700 sequence detection system. For SYBR green chemistry, the primer & template concentrations were standardized for each primer pair. The threshold cycle (C_T) values were obtained and the comparative C_T method ($2^{\wedge-\Delta\Delta C_T}$) of relative quantification was used to analyze gene expression among samples as described earlier [151]. All amplifications were done in triplicates and expression values were normalized against GAPDH.

S.No	Name	**Accession No**	**Assay ID** *(Applied Biosystems)*
1	Mcl-1L	NM_021960.3	Hs00172036_m1
2	Mcl-1S	NM_182763.1	Hs00766187_m1
3	Mcl-1ES	NM_001197320.1	4331348 (Custom gene expression assay) [F -GGTAATCGGACTCAACCTCTACTGT, R -CTGCCCATTGGCTTTGTGT, FAM Probe -CCGGTGGCCAAAAGTCGCCCT]
4	GAPDH	NM_002046.4	Hs99999905_m1
5	18S RNA	X03205.1	Hs99999901_s1

Table 5: Oligonucleotides sequences for qRT-PCR for different target genes using Taqman assays.

[Mcl-1transcripts: Mcl-1L full Length; Mcl-1S Short; Mcl-1ES Extra Short; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase; 18SRNA- 18S Ribosomal RNA]

S.No	Name	**Sequence**	**Reference**
1	Mcl-1	F -GGGCAGGATTGTGACTCTCATT R -GATGCAGCTTTCTTGGTTTATGG	[152]
2	Mcl-1L	F -AAGAGGCTGGGATGGGTTTG R -CAGCAGCACATTCCTGATGC	*Designed using- NCBI primer tool*
3	Mcl-1S	F -GAGACCTTACGACGGGTTGG R -CCACAAACCCATCCTTGGAA	
4	GAPDH	F -GAAGGTCGGAGTCAACGGATT R -GAGTTAAAAGCAGCCCTGGTG	

Table 6: Primer sequences used in qRT-PCR for different target genes, using SYBR green [F-Forward primer, R-Reverse primer].

4.8 PBL separation from blood: The lymphocytes were separated from peripheral blood using Ficoll-Hypaque density gradient method. Briefly, 3 ml of blood was collected form healthy volunteers with informed consent. The blood was diluted equally in normal saline, layered on to 2.5 ml of sterile Ficoll-Hypaque gradient and centrifuged at 1500 rpm for 20 min at RT. The PBMNC were collected from the intermediate ring. The collected cells were washed twice with normal saline at 1200 rpm for 10 min each and used for DNA extraction.

4.9 Genomic DNA isolation:

The genomic DNA from oral cell lines, tissues samples & peripheral blood lymphocytes was isolated by QiaQuick DNA extraction kit, as per manufacturer's instructions (Qiagen, USA). Briefly, *Animal Tissue:* 25 mg tissue were cut into small pieces and 180 µl Buffer ATL, 20 µl proteinase K was added, mixed by vortexing, and incubated at 55°C on a rocking platform, till the tissue is completely lysed. *Cell lines:* The appropriate numbers of cells (max. 5 x 10^6) were pelleted and resuspended in 200 µl PBS. Further, 20 µl proteinase K and 200 µl Buffer AL was added, vortex and incubated at 70°C for 10 min. To the above, 200µl ethanol (96–100%) was added, vortex & poured in to column & centrifuged at 8000 rpm for 1 min. The column was washed by 500 µl of buffer AW1 & AW2 with intermediate centrifugation and dried by full speed Centrifugation for 3 min. The DNA was eluted by adding 200 µl buffer AE. The quantity and quality of DNA was assessed by measuring the OD_{260}/OD_{280} ratio. The aliquot of DNA was first denatured at 65˚C for 10 min and then snap cooled on an ice bath. The quality of extracted DNA was checked on 1 % agarose gel. The extracted genomic DNA was stored at -20^0C for further use.

4.10 Polymerase Chain Reaction (PCR):

PCR was carried out with either genomic DNA or cDNA synthesized from RNA obtained from cell lines and oral tumor tissues as mentioned above. Amplification of the target gene was carried out in a thermal cycler. PCR amplified for the following target genes: Mcl-1 genomic region (Promoter, 3-Exons & 2- Introns) and expression of Mcl-1 isoforms (Mcl-1L, Mcl-1S

& Mcl-1ES) with β-actin used as housekeeping gene. The concentration of the components of a typical PCR reaction is shown below in table 7.

Reagents	Working Concentrations
PCR buffer (10X)	5 µl (1X)
dNTPs (2 mM)	5 µl (0.2 mM)
MgCl2 (25mM)	3 µl (1.5-3.0 mM)
Forward Primer (1 µg/µl)	1 µl (100 ng) (10-50 pM)
Reverse Primer(1 µg/µl)	1 µl (100 ng) (10-50 pM)
Taq Polymerase Enzyme (5U/ µl)	0.2 µl (1U)
DNA template	1 µl (100-500 ng)
Autoclaved DW	Make up to 50 µl
(DMSO)	5% (For GC rich promoter PCR)

Table 7: Table showing the components of typical PCR reaction

Table 8: Primer sequences & PCR conditions used for Mcl-1 gene and promoter amplification.

PRIMER	SEQUENCE	Ref.	D (^{0}C/M)	A (^{0}C/M)	E (^{0}C/M)	Cycles (^{0}C/M)
Mcl-1 Promoter Pri-1 (-1100 To-700) 373 bp	F- TCACAGCTGTAATCTCAGCACTT R- CTGCTTGCTTCTCCCATCTC	Self-Designed	94 /1	55/1	72/1	30
Mcl-1 Promoter Pri-2 (-700 To -250 bp) 455 bp	F- GGGAGAAGCAAGCAGGGAGGC R- CCGGTCTTCGGAGGCTCTGAGT	Self-Designed	94 /1	67/1	72/1	30
Mcl-1 Promoter Pri-3 (-250 To +300 bp) 586 bp	F- CAGGAGCTCTGGGAGGACCCC R- TCTGGCGTGAGGGTGGACGG	Self-Designed	94/1	69/1	72/1	35
Mcl-1 Promoter Pri-3-1 (-250 To +300 bp) 510 bp	F- AGGTCTCAGGGAAGCACAGA R- GGAAGACCCCGACTCCTTAC	Self-Designed	94/1	64/1	72/1	38
Mcl-1 Promoter Pri-3-2 (-250 To +300 bp) 524 bp	F- CACTTCTCACTTCCGCTTCC R- GTACCCGTCCAGCTCCTCTT	Self-Designed	94/1	59/1	72/1	36
Mcl-1 Promoter Pri-3-3 (-250 To +300 bp) 506 bp	F- AAAAACCTCTGGCGAAAACC R- TTACCGCGTTTCTTTTGAGG	Self-Designed	94/1	57.5/1	72/1	36
MCL1 5'UTR-Exon1 (792 bp)	F- CGACTTTTGGCTACGGAGAA R- TCTTTGAACAAGAGCTGCCA	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 Intron 1 (762 bp)	F- GAGGAGGAGGAGGACGAGTT R- GTCCCGTTTTGTCCTTACGA	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 Exon 2 (757 bp)	F- CAAGTGGGGTCAACCTGAGT R- CCTGCATCAGATCTGGGTTT	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 Intron 2 (748 bp)	F- AGAAAGCTGCATCGAACCAT R- AAGGTTTCCCCCTAAAGCAA	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 Intron 2 (728 bp)	F- AGTCACCAGGGGAGGATAGC R- TCCTCTTGCCACTTGCTTTT	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 Exon3-3'UTR (718 bp)	F- TTGCTTTAGGGGGAAACCTT R- GAGAGGAAAAGCTTCCCTTG	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 3'UTR (780 bp)	F- CCAGTAGCCAGGCAAGTCAT R- AGTATTGCCCAATCAGAGCC	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 3'UTR (681 bp)	F- ACCTGGGATTGAGAGGTTGA R- AGGGAGGGTCACTCAGGTTT	**S**hin et al., 2008	94/1	60/1	72/1	35
Mcl-1 3'UTR (781 bp)	F- GCTGATTGTTCTGCTCCCTC R- GTTTCCACTGGATTTGGCAG	**S**hin et al., 2008	94/1	60/1	72/1	35

Agarose gel electrophoresis

Semisolid agarose was used to resolve nucleic acids on the basis of their negative charge and visualized by intercalating dyes like EtBr. Various sizes of DNA fragments ranging from 200 bp to approximately 50 kb in length can be separated on agarose gels of respective concentrations. Briefly, 2 gm of agarose (2% gel) was melted in 100 ml of 1X TBE buffer. After the melted agarose cooled down, 5 µl of Ethidium Bromide stock (1mg/ml) was added and poured in a casting tray. After it became solid, the tray was shifted to the tank containing 1X TBE buffer enough to submerge the wells of the gel. PCR products were loaded in wells of agarose gel with 6x loading dye. The agarose gel percentage varied (0.7% – 2%) according to the size of the DNA to be resolved. Standard DNA markers of known fragment sizes were run in parallel to the samples in order to have standard reference. The gel was run at a constant voltage not exceeding 10 volts per cm. DNA bands were visualized and documented on a Gel documentation system (UVP, UK).

Gel elution and purification of DNA fragments:

The recovery of restriction digested plasmids or PCR products were purified from agarose gel using QIAquick Gel extraction Kit (Qiagen), according to manufacturer's instructions. Briefly, gel slice with band was excised under UV, weighed and immersed into 3 volumes of the gel solubilizing reagent (QG buffer) & incubated at 50^0C with intermittent vortexing. The above solution was passed through the charged column by centrifugation for 1 min at 14000 rpm followed by 0.5 ml of QG buffer. Further, the column was washed with PE buffer twice and a dry spin was given for 3 min to remove traces of PE buffer. The DNA was eluted by adding 50 µl of EB buffer or DW to the column, allowed to stand for 1 min followed by a quick spin for 1 min at 14000 rpm. Quality of the eluted DNA was checked at A260 and A280 and visualized by loading on 2% agarose gel.

Sequencing of the PCR product:

The PCR product was sequenced in an automated sequencer from Applied Biosystems. The reaction mix for sequencing contained, 2 µl of gel purified PCR product (40-50ng) and 1 µl of primer (1.6 pmoles/µl). The sequencing was done using both forward and reverse primer to confirm any alterations. The sequences obtained were aligned with the standard sequences of gene from NCBI database, using ClustalW, lalign, BioEdit, Chromas lite, etc software's to identify any alterations.

4.11 Western blotting:

Total protein extraction & quantification:

From cultured cells- Cells were harvested either by trypsinization, washed twice with chilled 1X PBS to remove traces of adherent serum proteins or scrapped directly from culture plates with cell lysis reagent & a sterile disposable cell scraper. The cellular proteins were solubilised by ProteoJET mammalian cell lysis reagent containing protease inhibitor (Proteoblock, Fermentas). The lysates were vortex mixed and spun down at 14,000 rpm for 10 min at 4° C. The pellet was discarded and cleared lysate was collected in a fresh tube & total protein was estimated. The lysates were aliquoted & stored in -80 °C for further use.

From tissues samples- 50-100 mg tissues were grounded using liquid nitrogen in mortar & pestle. The powdered tissues were transferred into eppendorf having mammalian cell lysis buffer & protease inhibitor. The solution was mixed by repeated pipetting and vortex. The lysates were centrifuged and the protein content was estimated as described above.

Protein estimation using Bradford's method:

Protein estimation was done using Bradford's reagent as per manufactures instructions. Briefly, BSA standards were made in a 96 well flat bottom plate by serially diluting 1 mg/ml stock. The unknown proteins were diluted approximately in range of standards. 250 µl Bradford reagent was added to the above diluted standards and samples. The plates were mixed gently and incubated in the dark for 20 min at RT. Absorbance was measured at 595 nm, values were

subtracted against the blank (lysis buffer) and concentration of protein was calculated with reference to standards (Table 9).

Concentrations (µg)	BSA (µg/µl)	DW	Bradford Reagent
DW Blank	0.0	5.0	250 µl
1.0	1.0	4.0	250 µl
2.0	2.0	3.0	250 µl
3.0	3.0	3.0	250 µl
4.0	4.0	1.0	250 µl
5.0	5.0	0.0	250 µl
Buffer Blank	0.0	5ul buffer	250 µl
Unknown Protein	(1µl sample)	-	250 µl
Unknown Protein	(2µl sample)	-	250 µl

Table 9: Protein estimation using Bradford's reagent

SDS-Poly acrylamide gel electrophoresis (SDS-PAGE):

Proteins are resolved according to their charge and molecular weight by SDS-PAGE. The samples were diluted in Laemmli buffer according to the amount of protein to be loaded, boiled for 5 min, cooled to RT, loaded on gel and separated on 6-15% SDS PAGE depending on the molecular weight of the proteins being analyzed. Briefly, the resolving gels 12% and 15% were made according to the molecular weight of the proteins to be separated. Pre-stained protein molecular weight marker was loaded for reference. The resolving gel was poured leaving approximately 4 cm of space for the stacking gel. The gel was then allowed to resolve in electrophoresis buffer at 20 mAmp constant current.

*Components of **Stacking** gel (10 ml)*	*5% Gel*	*Components of **Resolving** gel (10 ml)*	*10%*	*12%*	*15%*
Distilled water	6.8	Distilled water	4.0	3.3	2.3
30%Acrylamide Mix	1.7	30%Acrylamide Mix	3.3	4.0	5.0
1.0 M Tris (pH 6.8)	1.25	**1.5** M Tris (pH 8.8)	2.5	2.5	2.5
10% SDS	0.1	10% SDS	0.1	0.1	0.1
10% APS	0.1	10% APS	0.1	0.1	0.1
TEMED	0.01	TEMED	0.004	0.004	0.004

Table 10: Composition of Stacking & resolving gels

Immunoblotting:

This involves detection of target proteins resolved SDS-PAGE and electro-transferred on to a membrane (PVDF) followed by binding of specific antibodies. Briefly, resolving gel was removed from the electrophoresis assembly, rinsed gently in water and immersed in transfer

buffer for 10 min. The PVDF membrane was activated by soaking for 1 min in methanol and immersed in transfer buffer and transfer was set up by placing the gel and membrane in between pieces of filter paper and fiber sheets in the transfer cassette. Wet electro-blotting was carried out of 20 V for overnight or 80V for 4 hrs. The membrane was removed from the sandwich and immersed immediately in transfer buffer or TBS to avoid drying of the membrane. Transfer of proteins was visualized using Ponceau-S and destained by washing with distilled water. The blot was incubated in blocking solution (5% milk or 3% BSA in TBS) for 1 hr at RT on a rocker. After blocking, the blot was incubated with appropriate concentration of primary antibody (diluted in 2.5% milk/1.0 % BSA in TBST), O/N at 4°C or 1hr at RT on a rocking platform. The blot was then washed six times (10 min each) with TBST followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibody for 1 hr at RT on the rocker. The secondary diluted antibody was removed and the blot was washed similarly. Blots were developed using ECL+ chemiluminescence reagent (Amersham, USA) according to the manufacturer's protocol with the use of X-ray films.

Stripping of PVDF membranes:

The membrane was activated with methanol for 1min, if it was stored at -20 °C and equilibrated with 1X TBS at RT with constant agitation. The blot was then incubated with stripping buffer at 60 °C for 45 min with constant agitation in a hybridization chamber. Thorough washes were given with TBST till traces of BME were removed. The efficiency of stripping was checked using ECL for the remnants of antibody used previously. If the blot was clean, then Ponceau-S staining was done to make sure that proteins were present on blot and further reprobed from the blocking step onwards as described earlier.

Coomassie staining:

For Coomassie blue staining, the gel was placed in plastic container containing coomassie staining solution and stained for 2 hr. The background of the gel was removed using destainer and the gels were stored in distilled water. *Drying:* The polyacrylamide gel was placed in 50% methanol containing 1-2% glycerol for 30 min. The gelatin paper was soaked in water for 2

min and covered on glass plate without bubbles. The gel was place on it and covered with another gelatin paper, followed by drying at 40^{o}C in an oven.

Antibody	***Clone***	***Dilution***	***Company***	***Catalogue No.***
Actin (I-19)	Rabbit polyclonal	1:2500	Santa Cruz Biotech	sc-1616
Bak (G-23)	Rabbit polyclonal	1:500	Santa Cruz Biotech	sc-832
Bax (N-20)	Rabbit polyclonal	1:400	Santa Cruz Biotech	sc-493
Bcl-2 (N-19)	Rabbit polyclonal	1:800	Santa Cruz Biotech	sc-492
Bcl-xl (H-5)	Mouse monoclonal	1:500	Santa Cruz Biotech	sc-8392
Bcl-xl (54H6)	Rabbit monoclonal	1:200	Cell Signaling	2764S
BID (C-20)	Goat polyclonal	1:800	Santa Cruz Biotech	sc-6538
Caspase 3	Rabbit monoclonal	1:600	IMGENIX	IMG5700
Caspase 8	Rabbit monoclonal	1:1000	IMGENIX	IMG5703
Caspase 8	Mouse monoclonal	1:500	Cell Signaling	9746
Caspase 8	Mouse monoclonal	1:1000	Cell Signaling	4768
Caspase 9	Mouse monoclonal	1:1000	BD Pharmingen	8086
Mcl-1	Rabbit polyclonal	1:1000	BD Pharmingen	554103
Mcl-1	Mouse monoclonal	1:800	Santa Cruz Biotech	sc-12756
Mcl-1L (K20)	Rabbit polyclonal	1:1000	Santa Cruz Biotech	sc-958
Mcl-1(S19)	Rabbit polyclonal	1:600	Santa Cruz Biotech	sc-819
Mcl-1	Mouse monoclonal	1:700	Chemi-Con	MAB4602
Phospho Mcl-1	Rabbit monoclonal	1:200	Cell Signaling	4579S
Noxa (FL-54)	Rabbit polyclonal	1:1000	Santa Cruz Biotech	sc-30209
Noxa (N15)	Goat polyclonal	1: 1200	Santa Cruz Biotech	sc-26917
PUMA	Rabbit polyclonal	1:500	Abeam	ab-9643
Tubulin-β (9F3)	Rabbit monoclonal	1:1500	Cell Signaling	2128
Anti-Rabbit IgG	Secondary Ab	1:2500 (WB)	Santa Cruz Biotech	sc-2004
Anti-Mouse IgG	Secondary Ab	1:3000 (WB)	Santa Cruz Biotech	sc-2005
Anti-Goat IgG	Secondary Ab	1:2000 (WB)	Santa Cruz Biotech	sc-2020
Alexa Fluor-568	Goat Anti-Rabbit	1:1000 (IF)	Invitrogen	11011
Alexa Fluor-488	Goat Anti-Rabbit	1:1000 (IF)	Invitrogen	11008
Alexa Fluor-488	Goat Anti-Mouse	1:1000 (IF)	Invitrogen	11059

Table 11: Details of primary and secondary antibodies used in the study.

4.12 Antibody array:

In order to simultaneously detect the relative levels of 42 apoptosis related proteins in cell lysates, the RayBio® Human Apoptosis Antibody Array Kit was used as per manufacturer's instructions. Briefly, cell lysates were incubated with the membrane provided with tagged antibodies. Membranes were washed extensively, incubated with a cocktail of HRP-conjugated antibodies, washed and the signals were visualized by ECL kit.

4.13 Clonogenic Assay:

The clonogenic assay was performed as described earlier [153]. Exponentially growing oral cells were harvested, counted thrice using hemocytometer and replated in duplicates in 100 mm petri-dishes. The cell numbers were seeded as increasing numbers (200, 400, 600, 800 and 1000) per plate. After 24 hrs, the cells were treated with different doses of IR (0, 2, 4, 6, 8, 10 Gy) using 60Co-γ radiator along with an untreated control. Medium was changed and cells were then incubated up to 14 days to form colonies. The colonies were fixed and stained with a mixture of Glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) for 20 min at room temperature. Distilled water washes were given to wash off the excess stain and the plates were air dried. The numbers of colonies (≥50 cells) were counted using a microscope. The percent plating efficiency and fraction surviving a given radiation dose were calculated based on the survival of non-irradiated cells as described below-

Calculations:

Plating efficiency is the ratio of the number of colonies to the number of cells seeded:

PE = (No. of colonies in the control plate / No. of cells seeded) x 100%

Surviving fraction is the numbers of colonies arise after treatment, expressed in terms of PE.

SF = No. of colonies formed after IR treatment / No. of cells seeded x PE

The readings of clonogenic survival of each of the cell line were plotted on logarithmic scale.

4.14 MTT assay for cell proliferation:

The cells (1500 cells) were equally seeded per well, in triplicate in a 96-well microtitre plate in 100µl complete medium. Cells were treated with respective drug/inhibitors and proliferation was assessed every 24hrs upto a period of 5 days. At the desired time points (post drug/inhibitor

treatment), 100 µl of the medium was replenished from the designated wells, and 20µl of freshly made MTT solution was added to each well. The plate was incubated at 37°C in a CO_2 incubator for 4 hr to allow MTT to be metabolized and form formazan crystals. The medium was discarded and plates dried on tissue papers. 150 µl of DMSO was added to the plates and mixed at 150rpm for 5 min to mix the formazan crystals in the solvent. OD was measured on an ELISA plate reader at 540 nm against a reference wavelength of 690 nm. Growth curve was plotted from three independent experiments.

4.15 Drug & inhibitor treatment: In order to determine role of Mcl-1 in chemoresistance if any, a commonly used drug, Cisplatin was used alone or in combination with Obatoclax (BH3 mimetic small molecule inhibitor) in oral cancer cells. The IC50 doses of both Cisplatin & Obatoclax were calculated by MTT assay as described above.

4.16 Soft agar colony forming assay:

One milliliter of the basal layer of 1 % agar in complete medium was prepared in 30 mm Petri plates. 1000 cells in complete medium containing 0.4% agar were seeded over the basal layer. Plates were fed with complete medium on alternate days and incubated at 37°C in a 5% CO_2 incubator for 15 days. Observed opaque/dense colonies were counted microscopically on day 15. The assay was done in triplicates.

4.17 Apoptosis detection by Flow cytometry:

The Annexin V-FITC apoptosis detection kit (Santa Cruz Biotechnology, CA) was used for the detection of apoptotic cells in oral cell lines, as per the manufacturer's specifications. Briefly, cells were collected by trypsinization at different time points (Control, 1, 4, 24 & 48 hrs) post-IR treatment. Cells were washed with 1X PBS; treated with 2µg Annexin-V FITC & 10 µl PI; incubated in the dark for 15 min and analyzed on a flow cytometer (FACS Caliber, BD, USA). The flow cytometry was also used to determine effect of Obatoclax on cell death and differentiate between live and dead cell populations. Also flow cytometry helped in sorting the desired cell population having high transgene expression post transfection & transduction.

4.18 Microscopy / Immunofluorescence staining:

Cells were grown on glass cover slips for 48 hrs till they reached 60-70% of confluency, washed twice with PBS. The cells were fixed with chilled methanol or 4% paraformaldehyde for 5 min at -20^{0}C and permeabilised by chilled 100% methanol containing 0.3% Triton-X for 90 sec at RT. Coverslips were washed thrice with PBS & placed in a small humidified chamber and blocking was done using either 5% BSA or preimmune serum and incubated for 1 hr. The BSA was drained and the cells were layered with 50 µl of primary antibody (Mcl-1) diluted in PBS and incubated for 1 hr at RT or overnight at 4^{0}C. Further, the coverslips were washed thrice with 1X PBS for 10 min each followed by incubation with 100µl of anti-mouse (Alexa Fluor 488) or anti-rabbit (Alexa Fluor 568) conjugated secondary antibody for 1 hr and later washed with PBS as above. The nuclei of cells were counterstained by DAPI for 5 min, washed with PBS and coverslips were then mounted using anti-quenching agent and sealed. Confocal images were obtained using a LSM 510 Meta Carl Zeiss Confocal system.

In order to determine the apoptosis induction, Hoechst or DAPI staining was separately done on cells after different treatments and images of condensed nuclei and blebs were taken as described above. The inverted fluorescence microscope (AxioVert 200; Carl Zeiss, Germany) has also helped in selecting the transfected or transduced cells showing high transgene (RFP) expression during screening of shRNA constructs.

4.19 Radiation Treatment

Cells were grown to 60-70% and after 48 hrs of plating; the exponentially growing cells (6 × 10^3 cells) were treated with γ irradiation (D0 dose) using 60Co-γ radiator as described earlier [21]. Cells were incubated in CO_2 incubator at normal conditions up to different time points, harvested and stored at -80°C for further use.

4.20 Trypan blue exclusion assay

Exponential growing cells were harvested; seeded into 24-well plates at a density of 5×10^4 per well and treated with Mcl-1L siRNA and/or irradiation as described above. Post treatment the cells were again incubated for 48 hrs. Further, the cells were trypsinized and trypan blue

staining was performed. The number of viable cells were counted and compared to untreated control using a hemocytometer.

4.21 Acquired radioresistant sublines

The AW8507 & AW13516 cells were irradiated with fractionated doses of 2 Gy each up to 40 Gy earlier in the laboratory. Briefly, the radioresistant sublines of both the oral cell lines were generated using fractionated ionizing radiation (FIR) strategy as described earlier [154]. During development of radioresistant cell lines, cells were collected at different doses (Control, 8, 16, 24, 32, 40 Gy) and lysates from these sublines were loaded on SDS-PAGE to determine Mcl-1L expression by western blotting.

4.22 Transient knockdown of Mcl-1L isoform by siRNA:

Transfection of *siRNA:* Mcl-1L knockdown was achieved using Mcl-1L specific siRNA (sc-43912) along with a control siRNA (sc-37007) from Santa Cruz biotechnology, USA. The siRNA duplexes were transfected using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions [155]. The medium was changed after 16 hrs of transfection and 24 hrs post-transfection the cells were assessed for knockdown by western blotting. The specific silencing of Mcl-1L was confirmed in three independent experiments.

4.23 Inducible knockdown of Mcl-1 by shRNA:

Designing of shRNA constructs: In order to achieve a regulated expression and knockdown of Mcl-1 gene a microRNA-adapted shRNA (shRNAmir) with the pTRIPZ lentiviral inducible vector was used (Thermo Scientific Open Biosystem). These constructs are expressed as human microRNA-30 (miR30) primary transcripts and this design adds a Drosha processing site to the hairpin construct and which has been shown to greatly increase knockdown efficiency [156]. Four oligonucleotides pairs already published (Table-12) were chosen to generate plasmid based short hairpin shRNA constructs, targeting the open reading frame of the Mcl-1 gene. The specificity of oligonucleotides for the target gene was confirmed by BLAST searches. The

restriction sites for EcoRI (CTTAAG) & XhoI (CTCGAG) were added at the end of shRNA cassette in order to clone in to the pTRIPZ plasmid as shown below-

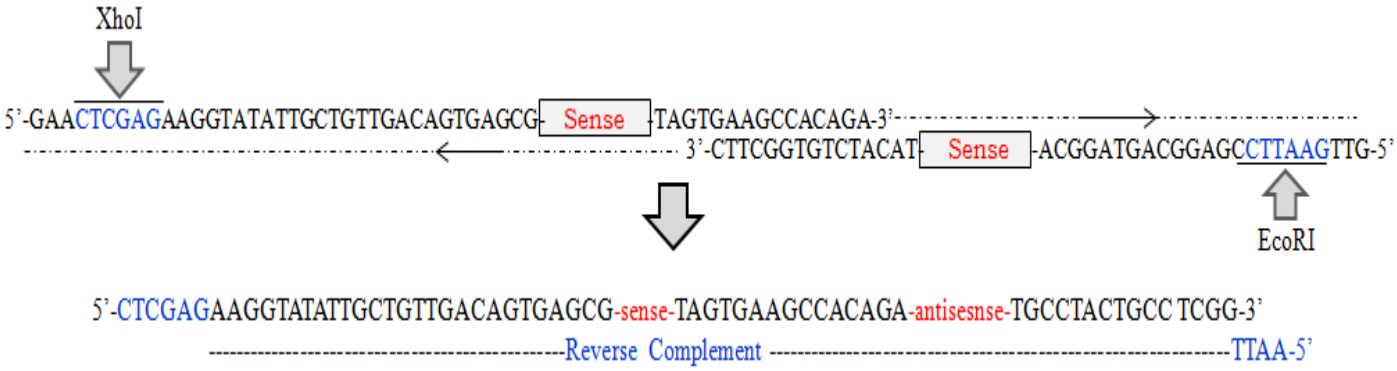


Figure 7: Design of shRNA construct for Mcl-1 knockdown

Sr. No	Target Gene	Target Sequence (5'-3')	Target site	Reference
1	Mcl-1 (Sense) Mcl-1 (Anti-sense)	GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG **GCAAGAGGATTATGGCTAA**TAGTGAAGCCACAGA GTTGAATTCCGAGGCAGTAGGCA**GCAAGAGGATTATGGC TAA**TACATCTGTGGCTTC	1037-10055	[195]
2	Mcl-1L (Sense) Mcl-1L (Anti-sense)	GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG**CGGG ACTGGCTAGTTAAAC**TAGTGAAGCCACAGA GTTGAATTCCGAGGCAGTAGGCA**CGGGACTGGCTAGTTA AAC**TACATCTGTGGCTTC	1312-1330	[146, 196]
3	Mcl-1L (Sense) Mcl-1L (Anti-sense)	GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG**GAAA GTATCACAGACGTTCTCGTAA**TAGTGAAGCCACAGA GTTGAATTCCGAGGCAGTAGGCA**GAAAGTATCACAGACG TTCTCGTAA**TACATCTGTGGCTTC	1004-1028	[197]
4	Mcl-1L (Sense) Mcl-1L (Anti-sense)	GAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG**GCTA AACACTTGAAGACCATA**TAGTGAAGCCACAGA GTTGAATTCCGAGGCAGTAGGCA**GCTAAACACTTGAAGA CCATA**TACATCTGTGGCTTC	953-973	Open Biosystems Clone Id : **TRCN0000005517;** Sigma Clone Id : **TRCN0000005517** & siDESIGN Center Dharmacon.

Table 12: Oligonucleotide sequences of shRNA constructs for Mcl-1 knockdown-

Generation of shRNA cassettes by extension PCR: The extension PCR approach was used which is cost effective, easier and single step technique for generation of shRNA constructs. The PCR products were used for restriction digestion & cloning in pTRIPZ plasmid. Here, the oligonucleotides (Sense and Antisense) were used as primers as well as template for PCR as shown in Table 13 below-

Reagents	Working Concentrations	Conditions
PCR buffer (10X)	5 µl (1X)	94^0C - 5 min
dNTPs (2 mM)	5 µl (0.2 mM)	94^0C - 5 min } 29 cycles
$MgCl_2$ (25mM)	3 µl (1.5 mM)	50^0C - 1.5 min } 29 cycles
shRNA (Sense+ Antisense)	1 µl (100 ng) each	72^0C - 1 min } 29 cycles
Taq Polymerase Enzyme	0.2 µl	72^0C - 20 min
Autoclaved DW	Make up to 50 µl	

Table 13: Requirement & conditions of extension PCR for generation of shRNA constructs.

Restriction Digestion & Ligation: Both the pTRIPZ plasmid and PCR made shRNA constructs were double digested with ECoRI & XhoI enzymes (Fermentas) using 2X Tango buffer, at 37^0C for overnight. The double digested vector backbone and shRNA constructs were loaded on gel, purified by gel elution and then ligated using T4-DNA ligase according to manufacturer's protocol (Fermentas).

Double Digestion Reaction		Ligation Reaction	
Tango Buffer (10X)	10 µl (2X)	Ligase Buffer (10X)	2 µl (1X)
EcoRI enzyme	2 µl	T4 DNA ligase (5U/µl)	2 µl
XhoI enzyme	2 µl	Double Digested pTRIPZ	3 µl (400ng)
pTRIPZ plasmid / shRNA	30 µl	shRNA	12µl (750ng)
DW	4 µl	DW	1 µl
	50 µl		20 µl

Table 14: Digestion and ligation reaction for Mcl-1shRNA cloning in pTRIPZ

Cloning shRNA in pTRIPZ vector: The ligation products were transformed in *E. coli* competent cells and individual colonies were inoculated in broth, grown & plasmid miniprep was done. The screening of positive clones was done by restriction digestion of plasmids (using either AgeI & EcoRI or with BamHI alone) followed by visualization of insert release/loss of restriction site on gel. The cloning & orientation of shRNA was confirmed by sequencing of positive clones. The maxiprep of all four shRNA clones (A, B, C & D) were done and stored at -20^0C for further use.

Co-transfection for preparation of lentiviral particles: The LV was produced by transient co-transfection of HEK-293FT cells using recombinant plasmids carrying transgene/shRNA sequences, helper (packaging) plasmids. Briefly, the HEK293FT cells were grown in complete DMEM. Medium was replaced 4 h before transfection. *Calcium phosphate mediated transfection* was done using desired constructs (pTRIPZ containing shRNA cassette, PMD2G, PAX2) in ratio of 6: 5: 2 (i.e. 12, 10, 4µg up to final volume of 260 µl). Briefly, equal volume

of 0.5µl CaCl2 was added to give a total volume of 520µl, followed by addition of equal volume of 2X BBS to give a total volume of 1040µl. The above solution was mixed and added drop by drop to 40-50% confluent HEK293FT cells with complete medium. Next day, cells were given one wash with plain DMEM and 10ml complete medium was added. After 24 hrs, culture supernatant was collected and centrifuged at 5,000 rpm for 20min at 4^{o}C. The supernatant was divided in aliquots of 1ml and stored at -80^{o}C.

Transduction and generation of stable cell line: Frozen viral particles were thawed & used to transduce ~60-70% confluent target cells (AW9507, AW13516 & SCC29B) along with polybrene (8 µg/ml) to facilitate binding of virus particles and incubated overnight. Next day cultures were washed with PBS, medium changed and incubated further for 48 hr. The transduced cells were selected by using medium containing puromycin (1µg/ml; standardized from puromycin kill curve). The medium was changed every day or alternate days to clear dead cells. About 6-10 isolated colonies of puromycin resistant cells were spot trypsinised and each colony was grown separately in 6 well plates. Post doxycyclin (4µ/ml) induction cells were tested for expression of upstream RFP by microscopy and shRNA mediated knockdown of Mcl-1 by real time PCR and western blot analysis.

4.24 Immunohistochemistry:

The 4 µm paraffin sections of OSF or normal tissues obtained on silane coated slides were deparaffinised by passing them through different grades of xylene and alcohol on shaker in the order given below-

Xylene (100%)	15 Min
Xylene: Alcohol (1:1)	15 Min
Alcohol (100 %)	10 Min
Alcohol (90%)	10 Min
Alcohol (70%)	10 Min

Endogenous peroxidase in the tissue sections was blocked by incubating the slides in 0.3% hydrogen peroxide in 70% methanol in PBS for 20 min on shaker. The slides were washed with 1XPBS on a shaker. Antigen retrieval was done by heating the slides in 10 mM sodium citrate buffer twice for 5 min each in microwave oven at high setting with cooling after first five min.

The slides were then washed twice with 1 X PBS, blocking done by incubating the sections for 45 min at room temperature with 1: 66 diluted goat or horse serum. Primary antibody appropriately diluted in PBS was added to the sections and incubated for 1 hr at 37 °C at room temperature followed by overnight incubation at 4°C. The sections were subsequently washed thrice with 1X PBS containing 0.02 % Triton x-100 (PBST). The sections were then incubated with 1:200 biotinylated secondary diluted in 1:66 blocking serum for 1 hour at room temperature. Sections were again washed with 1 X PBST thrice for 10 min each. Simultaneously 1:50 diluted avidin (A) and horse radish peroxidase conjugated biotin (B) were allowed to stand at room temperature for 30 min. The sections were subsequently incubated with ABC solution for 45 min at room temperature. The sections were briefly washed twice with 1X PBS. The sections were then incubated with DAB containing 10 µl of hydrogen peroxide for 10 min in dark. The slides were then washed in tap water and then in milliQ water. The sections were counterstained with freshly filtered haematoxylin for 30 sec. The slides were rinsed in flowing tap water and dehydrated by passing through grades of xylene and alcohol in reverse order of what was followed in the beginning. The sections were then mounted with DPX and observed for specific staining under the microscope.

4.25 Statistical Analysis:

Statistical analysis was done using licensed version of SPSS software package 15.0 available in the institute. Data of two similar & dissimilar groups were statistically analyzed by Student-t *test &* Mann Whitney test using Graphpad Prism5 software. The correlations between clinico-pathological parameters (gender, age, site, habits, size, node, metastasis, recurrence) were done using Chi Square test (χ^2). Kaplan Meier curves were used for evaluating the overall survival and the difference in the groups was calculated with Log Rank test of significance. The predictive parameters in univariate analysis were incorporated into multivariate analysis using Cox's proportional hazard test to identify the factors that were independent predictors of the survival. The p value < 0.05 was considered statistically significant.

CHAPTER 5

RESULTS

5.1 To decipher mechanisms of Mcl-1 overexpression in oral Cancers-

5.1.1 Genomic alterations in Mcl-1 gene:

Controversial reports are available in literature suggesting a role of genomic alterations (6 & 18-nt polymorphic insertions) on Mcl-1 expression and disease state. In order to check this possibility, all three exons, two introns and promoter of Mcl-1 gene were sequenced in oral cell lines, healthy volunteers & paired tumor samples.

In cell lines: The sequencing analysis revealed no alterations in exons / introns of Mcl-1 gene amplified from human oral cell lines. Also, sequencing of Mcl-1 isoforms (Mcl-1L & Mcl-1S) including recently discovered Mcl-1ES, did not reveal any alterations (Figure 8-10). The promoter region of Mcl-1 has been published in the CHSL (Cold Spring Harbour Laboratory) promoter database. The entire 1.5 kb promoter region of Mcl-1 was amplified using different pairs of primers (listed in appendix-19). No genomic alterations were observed in promoter region of Mcl-1 in 9 oral cancer cell lines analysed, except SCC15 & SCC40 which showed the presence of 6 bp (GGCCC) repeat (6 bp polymorphic insertions as reported earlier).

In healthy volunteers: The Peripheral Blood Lymphocytes (PBL's) were isolated from blood of 25 healthy volunteers, genomic DNA extracted and PCR sequencing of Mcl-1 gene was carried out. Sequencing and alignment analysis revealed that 8/25 volunteers (32%) showed the presence of 18-nt polymorphic insertion in the Mcl-1 promoter.

In tissue samples: *G*enomic DNA was extracted from 40 paired tumor tissues and sequencing analysis was carried out, which has revealed no significant difference in presence or absence of 18-nt polymorphism between adjacent normal versus oral tumor tissues. Further, 9/40 tumors (22%) showed the presence of Mcl-1 promoter polymorphism.

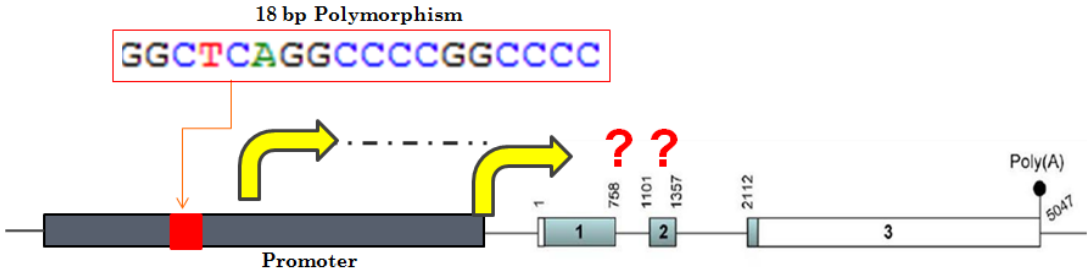


Figure 8: Pictorial representation of 18-nt insertion in Mcl-1 promoter.

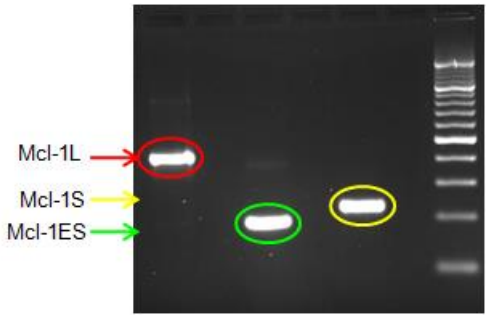


Figure 9: Amplification and sequencing of Mcl-1 isoforms

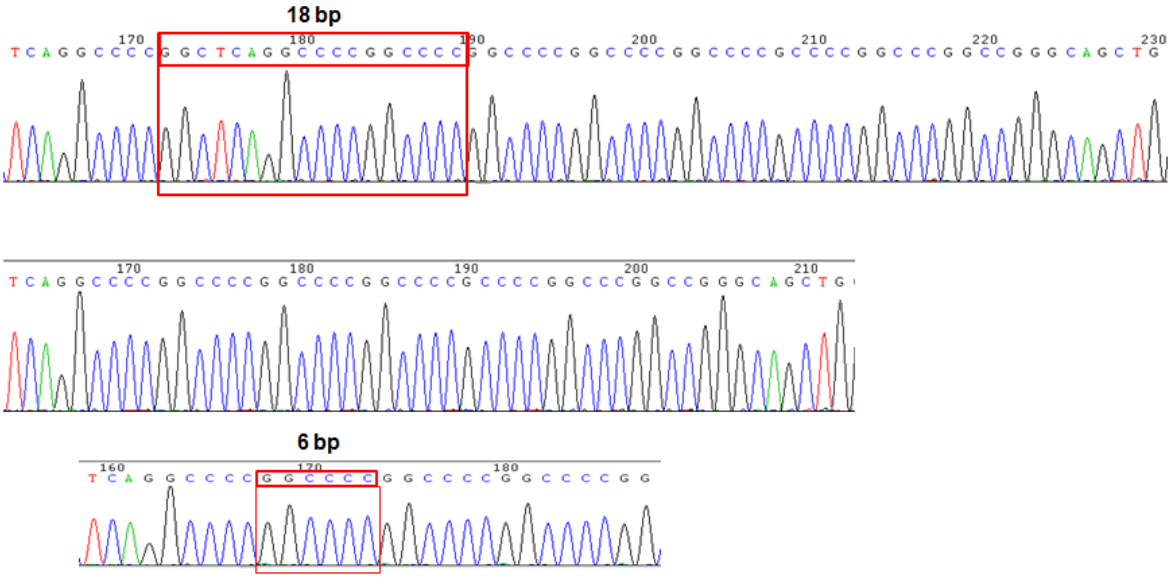


Figure 10: Insertion of 6 & 18 nucleotides Mcl-1 promoter of AW8507 cell line

.***Single Nucleotide Polymorphism:*** The sequencing analysis of Mcl-1 promoter in oral cancer patients also demonstrated the presence of two SNP's namely C<A-324 & G<C-386, earlier reported to be responsible for Mcl-1 overexpression. Interestingly, the TFSEACH analysis has shown that, presence of SNP's G<C-386 & C<A-324 leads to the loss of HSF & CRE-BP binding sites as shown in Figure 11.

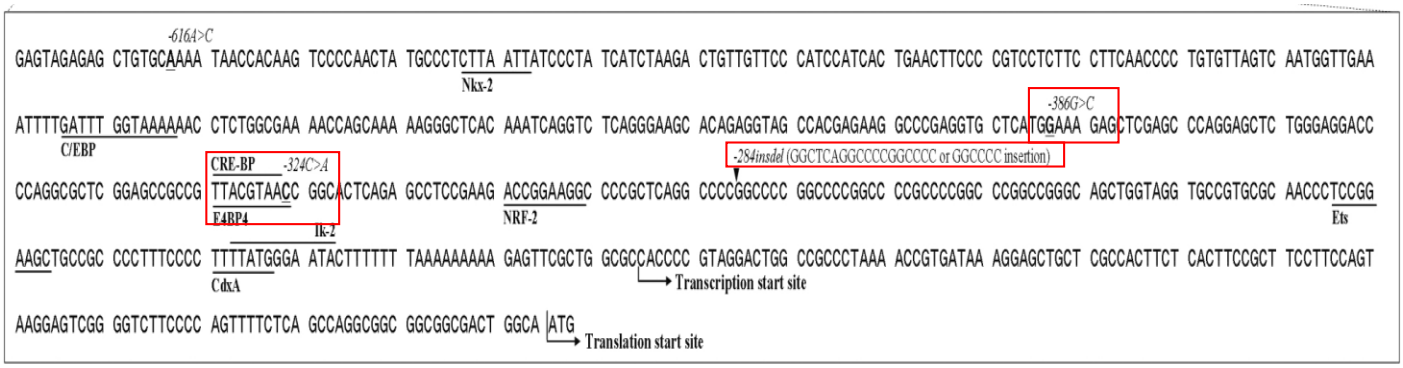


Figure 11: Sites of binding proteins to Mcl-1 promoter region

Additional binding sites: The formation of any additional transcription factor binding sites because of the presence of the polymorphic insertion of 6 & 18-nt were evaluated by TFSEARCH online tool. The results of TFSEARCH analysis of Mcl-1 promoter in oral cell lines and healthy volunteers revealed, formation of additional binding sites for ADR1, ADR2, ADR4, SP1 & CAP proteins, as shown in Table 15.

Sequence	Binding protein	Additional sites	Score
CGGC	ADR1	2	93.8
CGGCC	ADR2	1	93.8
CCGGC	ADR4	1	93.8
CGGCCCCGGC	SP1	1	87.1
CGGC CCCGGCCCC	SP1	1	87.1
TCAGGCCC	CAP	1	86
CTCAGGC	CAP	1	86

Table 15: TFSEARCH analysis of altered binding sites of Mcl-1 promoter

Association of promoter polymorphism and Mcl-1 expression:

Real time PCR and western blot analysis of oral cancer cell lines revealed no correlation between Mcl-1 expression & presence or absence of 6 bp polymorphism. Further, no significant correlation was observed between expression of Mcl-1L/Mcl-1S isoforms in patients (n=40) with or without 18bp polymorphism. Similarly, no such correlation was observed with Mcl-1 protein expression as shown in Figure 12.

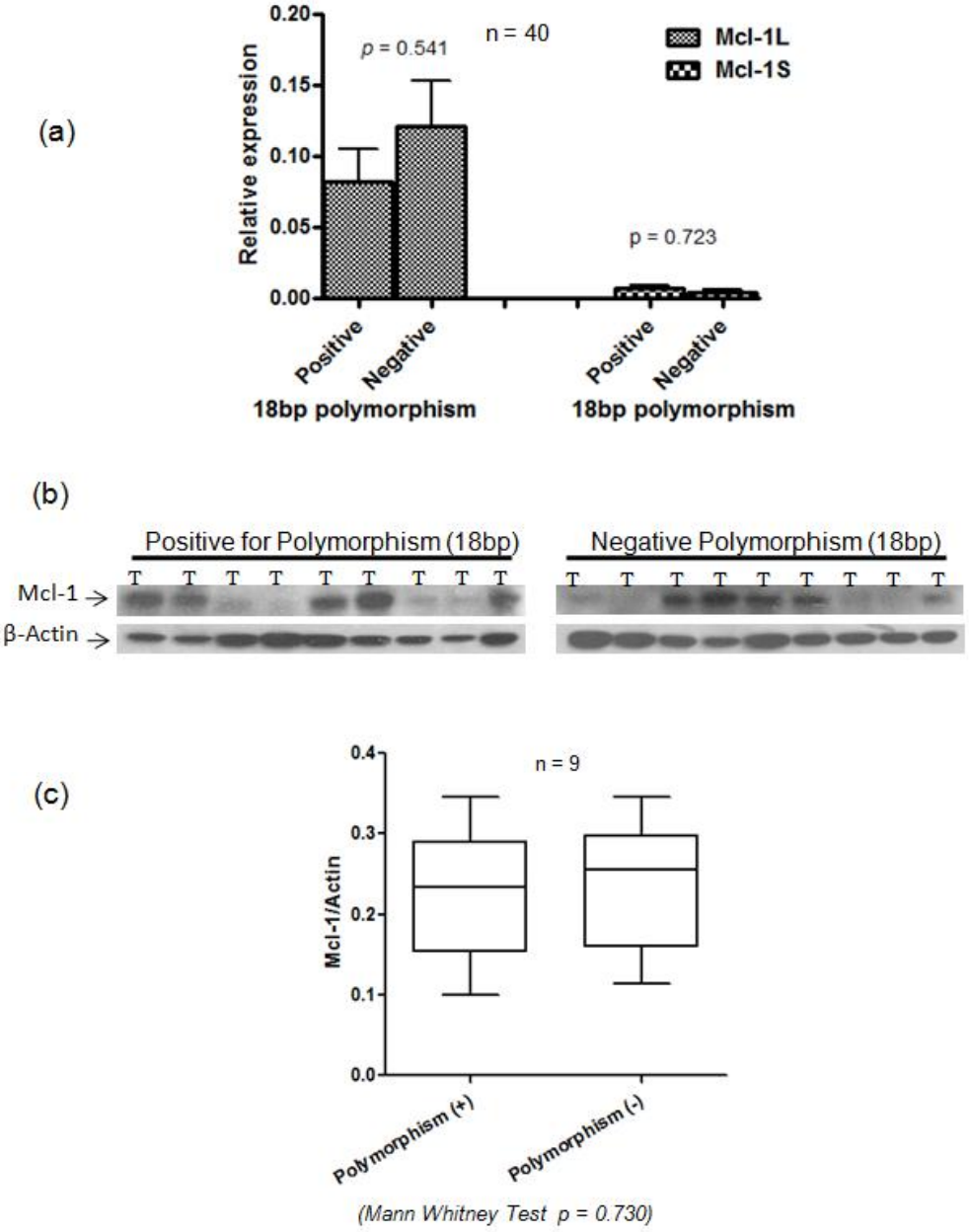


Figure 12: Correlation of presence/absence of 18bp polymorphic insertions with Mcl-1 expression

.

Correlation of Mcl-1 promoter polymorphisms with clinico-pathological parameters:

The χ square test revealed no significant correlation between Mcl-1 promoter polymorphism (18-nt) and clinico-pathological parameters (Gender, Age, Habit, Site of tumor, Size, Nodal status & Differentiation) of oral cancer patients (Table 16). Further, Kaplan Meier survival analysis also showed no significant correlation between presence or absence of 18-nt polymorphisms and overall survival of oral cancer patients (Figure 13).

Parameters	Total	Mcl-1 18bp Polymorphism		P-value
		Presence	Absence	
Gender				
Male	33	9	24	*0.141*
Female	7	0	7	
Age (Years)				
>53	23	5	18	*0.594*
<53	17	4	13	
Habits				
Tobacco chewing	26	5	21	*0.513*
Tobacco in any form/In combination	13	4	9	
NA	1	0	1	
Site of tumor				
Buccal Mucosa	19	4	15	*0.833*
Tongue	18	5	13	
Other	3	0	3	
Tumor size (T)				
T1 + 42	9	2	7	*0.680*
T3 + T4	31	7	24	
Lymph Node (N)				
Negative	11	4	7	*0.874*
Positive	29	5	24	
Differentiation				
Well	4	2	2	*0.057*
Moderate	22	6	16	
Poor	14	1	13	

Table 16: Mcl-1 polymorphism (18bp) & clinico-pathological parameters of oral cancer patients. (n=40) ('NA' denotes data not available; Statistical analysis was performed by the χ^2 test. *P<0.05 was considered significant)

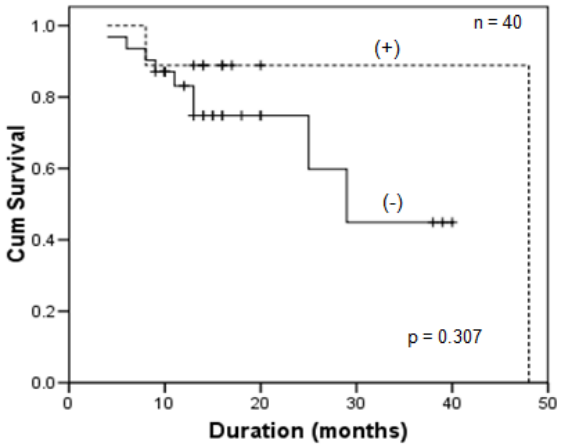


Figure 13: Statistical analysis of survival of patients with and without Mcl-1 promoter polymorphism (By Kaplan–Meier test; *P<0.05 was considered significant)

5.1.2 Phosphorylation status of Mcl-1:

Proteosomal inhibitor (MG132) treatment revealed that Mcl-1 is phosphorylated at Ser-159 & Thr-163 residues in AW8507 cells, which is essential for its proper detection by E3 ligase and further proteosomal degradation (Figure 14). The total Mcl-1 protein level was also found to be increased after inhibition of proteosomal pathway as shown in Figure 15 below-

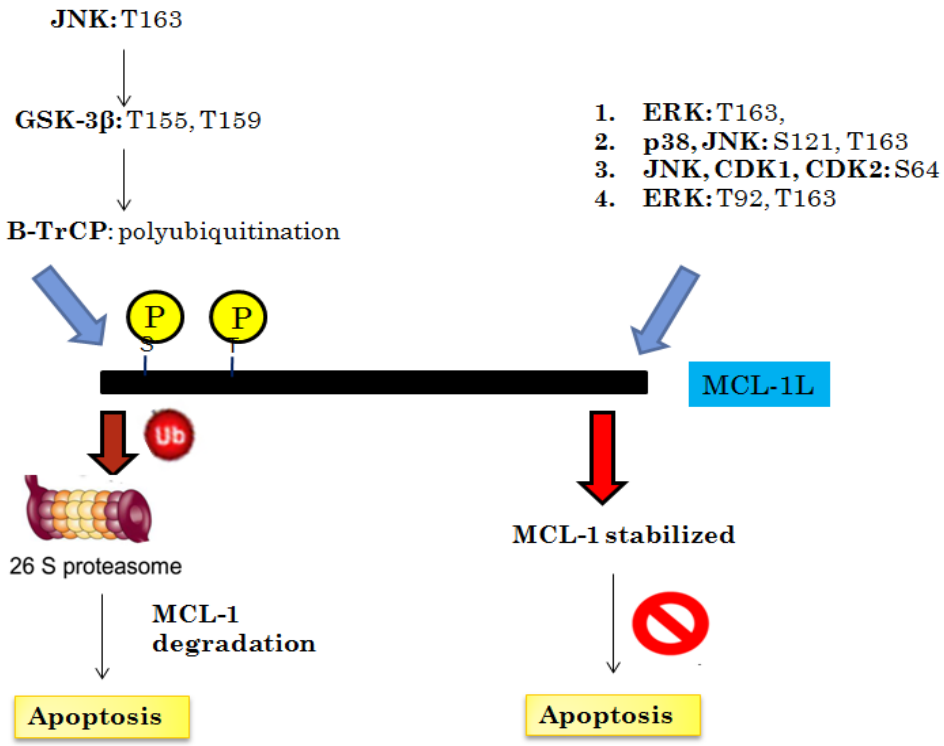


Figure 14: Phosphorylation and degradation of Mcl-1 via proteolytic pathway

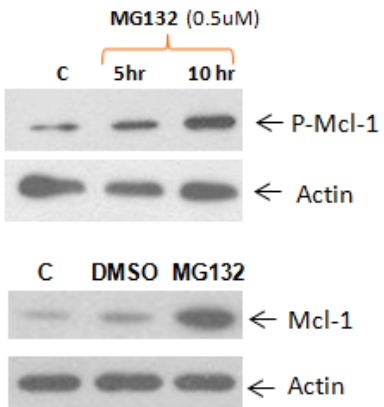


Figure 15: Expression of Phospho & total Mcl-1 after proteosome inhibitor treatment

5.2 Role of Mcl-1 isoforms in pathogenesis of oral cancer:

To examine the levels of Mcl-1 isoforms in oral cell lines, premalignant lesions & paired oral tumors from different subsites and their correlation with clinico-pathological parameters-

5.2.1 Mcl-1 expression in Oral Submucous Fibrosis versus normal mucosa:

In normal oral mucosa:

Out of 10 normal mucosa samples, 4 were from buccal mucosa and 6 from gingiva. The immunohistochemical analysis of tissue sections revealed low expression of Mcl-1 in all layers of epithelia (Figure 16a). The mean Mcl-1 immunoreactivity in normal mucosa was found to be 16.5% (Figure 17).

In Oral Submucous Fibrosis (OSF):

Twenty OSF patients were included, 18 males and 2 females, with mean age of 28 years. The immunohistochemical analysis of 20 OSF samples showed overexpression of Mcl-1 protein. Mcl-1 expression is found to be cytoplasmic, homogenous in all layers of epithelium in most of OSF cases as indicated in Figure 16C&D. The mean Mcl-1 positivity for this group was 51.7% (Figure 17 & Table-17)

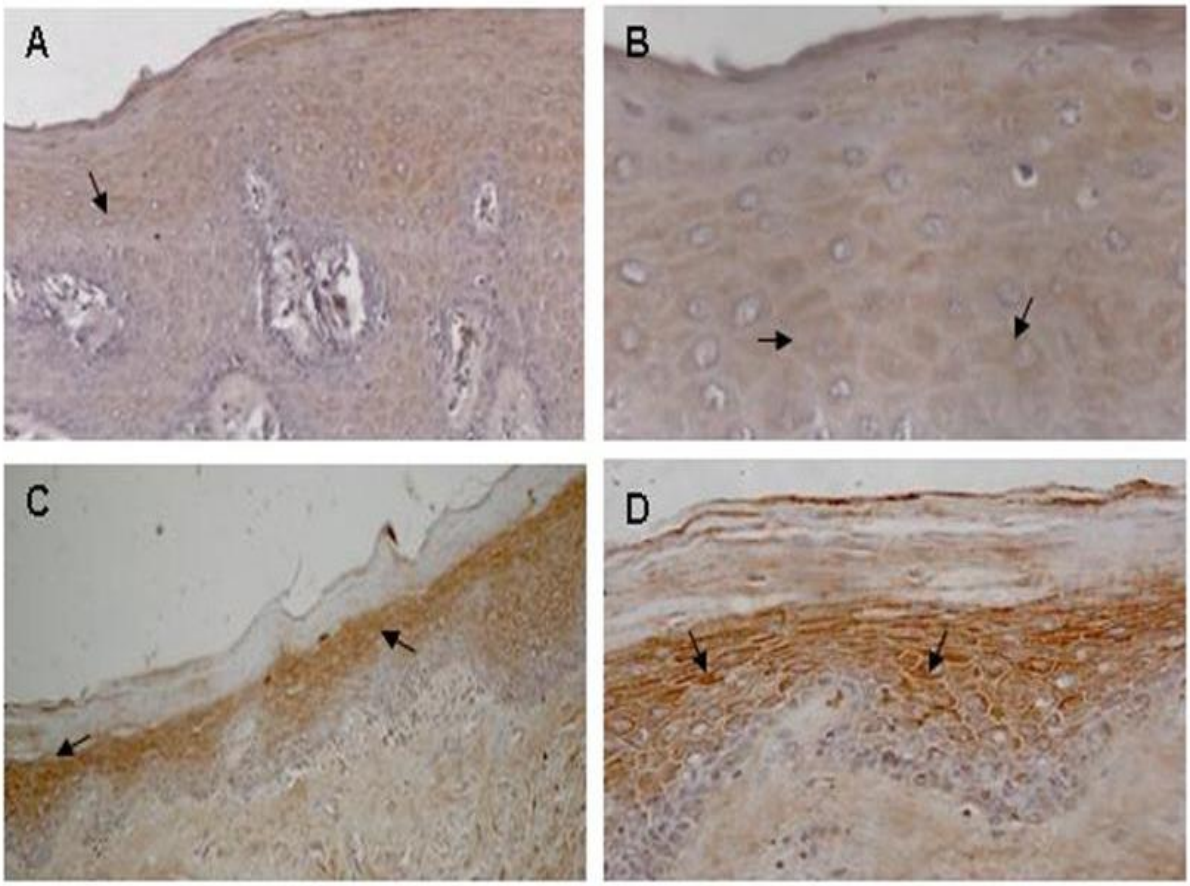


Figure 16: Immunohistochemical staining of Mcl-1 protein in normal mucosa (A & B) versus premalignant OSF tissues (C & D).

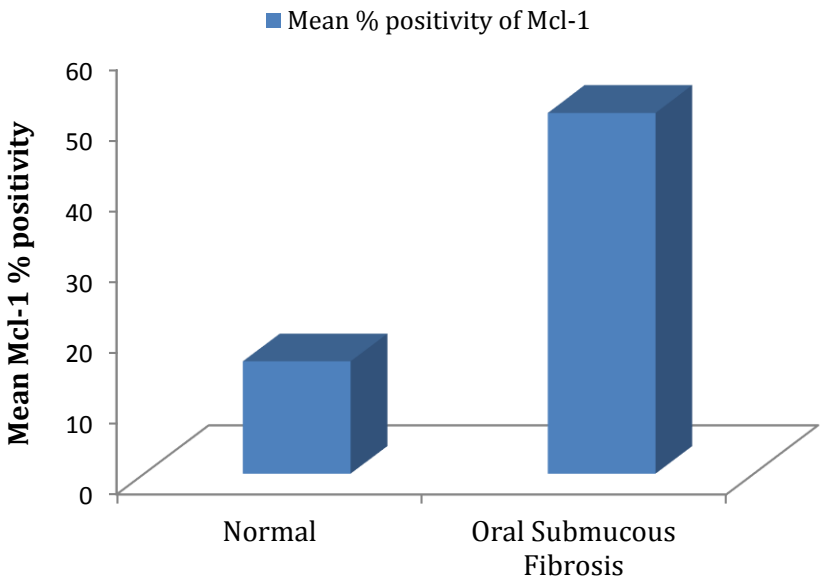


Figure 17: Mean Mcl-1 positivity in different groups of the study (Normal & OSF)

Table 17: Mcl-1 staining and details of patients:

Normal Oral Mucosa			
Sr. No	Site	Mcl-1	
		% Positivity	Grade
1	Buccal Mucosa	20	+
2	Buccal Mucosa	5	Neg.
3	Buccal Mucosa	5	Neg.
4	Buccal Mucosa	20	+
5	Gingiva	10	Neg.
6	Gingiva	20	+
7	Gingiva	10	Neg.
8	Gingiva	40	++
9	Gingiva	5	Neg.
10	Gingiva	15	+

Oral Submucous Fibrosis				
Sr. No	Age (Years)	Gender	Mcl-1	
			% Positivity	Grade
1	21	M	40	++
2	23	M	75	+++
3	25	M	90	+++
4	16	M	78	+++
5	19	M	10	Neg.
6	20	M	45	++
7	23	M	40	++
8	24	F	75	+++
9	25	M	55	+++
10	28	M	45	++
11	31	M	48	++
12	33	M	35	++
13	27	M	85	+++
14	32	M	15	+
15	25	M	45	++
16	26	M	0	Neg.
17	28	M	90	+++
18	24	M	28	+
19	38	M	75	+++
20	34	M	55	+++

5.2.2 To examine the levels of Mcl-1 isoforms in oral cell lines & tumors from different subsites and their correlation with clinico-pathological parameters-

We have examined the expression of Mcl-1 isoforms (Mcl-1L, Mc-1S & Mcl-1ES) in eleven oral cell lines and 130 paired oral tumor samples. The patients tissues used for the study were obtained post-surgery following IRB approval from ACTREC biorepository, with histologically proven oral cancers. The expression of Mcl-1 isoforms was further correlated with patient's clinico-pathological parameters.

Expression of Mcl-1 isoforms in oral cell lines: qRT-PCR analysis revealed, high expression of anti-apoptotic Mcl-1L isoform over pro-apoptotic Mcl-1S & Mcl-1ES in all the oral cell lines. Elevated expression of Mcl-1L was observed at both mRNA & protein level in majority of oral cancer cell lines (SCC25, SCC29B, SCC40, SCC74, QLL1, AW8507 and AW13516) as compared to immortalized normal FBM & DOK cell lines (Figure 18).

Expression of Mcl-1 isoforms in oral tissues:

qRT-PCR analysis showed an elevated expression of Mcl-1L mRNA in 84/130 (64%) oral tumors. The relative expression of Mcl-1L isoform was ~5 fold higher than Mcl-1S and ~10 fold higher to Mcl-1ES isoform in oral tumors. Similarly, western blot analysis has revealed, five to ten fold higher expression of Mcl-1 protein in oral tumors versus adjacent normal's as shown in figure 19.

Correlation of Mcl-1 expression with clinico-pathological parameters:

To statistically correlate the expression of Mcl-1 isoforms with clinico-pathological parameters, the data was dichotomized into two groups namely: the Mcl-1 high expressers and low expressers. For comparison the mean expression of Mcl-1 isoforms in healthy normals was used.

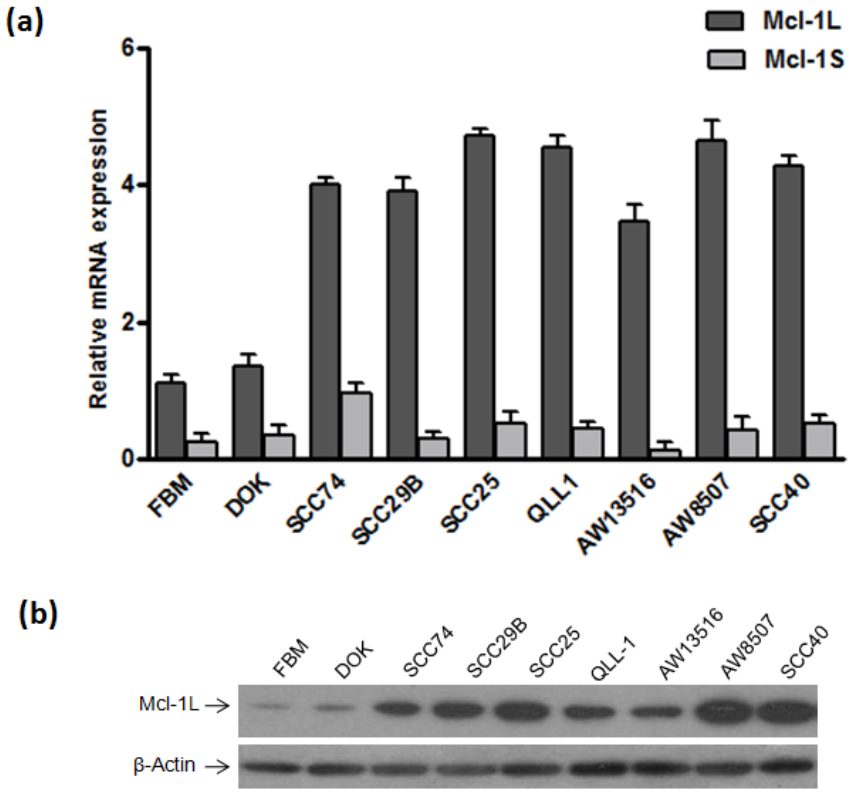


Figure 178: Expression of Mcl-1 isoforms in oral cell lines; (a) indicates expression of Mcl-1L & Mcl-1S mRNA in oral cell lines; (b) indicates Mcl-1L protein expression in oral cell lines.

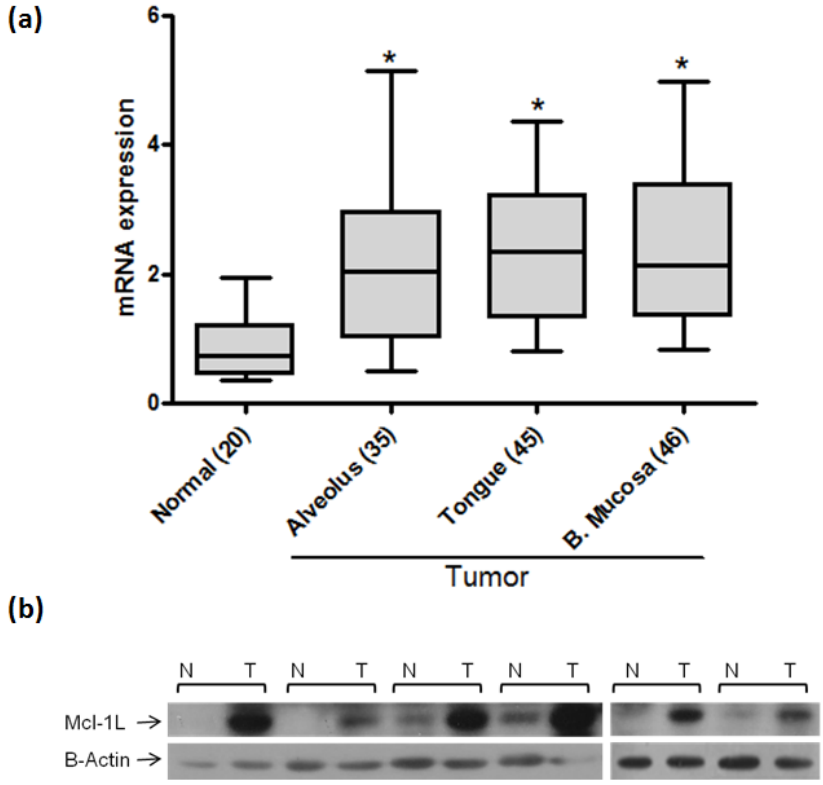


Figure 19: Correlation of Mcl-1L mRNA expression in oral normal vs. tumor tissues; (a) indicates expression of Mcl-1L mRNA in normal vs. oral tumors of different subsites; (b) indicates Mcl-1L protein expression in adjacent normal versus tumors. (* $p < 0.05$)

Univariate analysis- revealed significant correlation of high Mcl-1L expression with node positivity (p = 0.020) and advanced tumors (p = 0.013). However, no significant correlation was observed between expression of Mcl-1 isoforms and gender, age, tobacco/alcohol habits, primary site & differentiation of oral cancer patients (Table 18). Notably, low expression of pro-apoptotic Mcl-1S isoform showed a border line significance with poorly differentiated tumors (p = 0.053).

Multivariate analysis: Among the two isoforms (Mcl-1L & Mcl-1S) analyzed, Mcl-1L expression influenced the overall survival of oral cancer patients. The Mcl-1 variable, which had emerged significant (p = 0.02) in the univariate analysis, was examined using the Cox regression model in the multivariate analysis (Table 19). The patients exhibiting a high expression of Mcl-1L in tumors were at 3.2 time's higher risk of poor survival than patients expressing low Mcl-1L in tumors. The multivariate analysis revealed that the patients exhibiting a high Mcl-1L exhibited shorter survival as compared to those expressing low Mcl-1L. Our studies indicate that Mcl-1L expression is an independent prognostic factor for oral cancer.

Correlation of Mcl-1 expression with overall survival

The Kaplan–Meier survival curves of low and high expressers of Mcl-1L showed a statistically significant difference (p = 0.002). Wherein, patients having high Mcl-1L exhibited poor overall survival as shown in Figure 18. Inversely, patients showing high Mcl-1S exhibited significantly better overall survival (p = 0.051) as compared to those having low Mcl-1S (Figure 20). The ratio of Mcl-1L/Mcl-1S, showed a positive correlation with the poor overall survival of oral cancer patients (p = 0.006). Univariate analysis also revealed poor overall survival in node positive versus node negative oral cancer patients (p = 0.003). However, the other parameters like age, tobacco/alcohol habits and differentiation did not significantly influence overall survival of these patients.

Parameters	Total	**Mcl-1L** expression		P-value	**Mcl-1S** expression		P-value
		Low	High		Low	High	
Gender							
Male	99	35	64	*0.446*	69	30	*0.370*
Female	31	12	19		20	11	
Age (Years)							
>53	63	27	36	*0.087*	47	16	*0.101*
<53	67	20	47		42	25	
Habits							
Tobacco chewing	81	29	52	*0.513*	54	27	*0.323*
Tobacco in any form/In combination	41	15	26		29	12	
No habits	6	3	3		4	2	
NA	2	0	2		2	0	
Site of tumor							
Alveolus	35	10	25	*0.925*	20	15	*0.802*
Buccal Mucosa	46	18	28		33	13	
Tongue	45	17	28		33	12	
Other	4	2	2		3	1	
Tumor size(T)							
T1 + T2	42	37	5	*0.013**	19	14	*0.091*
T3 + T4	88	10	78		70	27	
Lymph Node (N)							
Negative	47	38	9	*0.020**	31	16	*0.785*
Positive	83	9	74		58	25	
Differentiation							
Well	16	7	9	*0.652*	7	9	*0.053*
Moderate	82	29	53		55	27	
Poor	31	11	20		26	5	

Table 18: Mcl-1 polymorphism (18bp) & clinico-pathological parameters of oral cancer patients. [(n=130); ('NA' denotes data not available. Statistical analysis was performed using the χ^2 test. *P<0.05 was considered significant).]

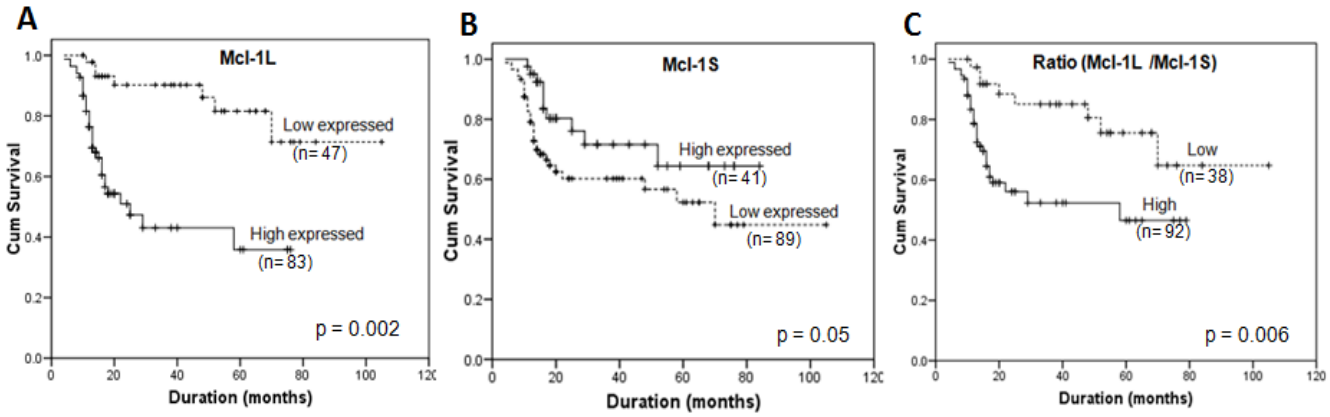


Figure 20: Kaplan–Meier estimates of overall survival of oral cancer patients.
(Expressing the different Mcl-1L & Mcl-1S isoforms)

(a)

Parameter	Category	Hazard Ratio	95% CI	p- value
Node	Negative	1		
	Positive	1.09	0.409-2.941	0.854
T stage	T1+T2	1		
	T3+T4	1.9	0.583-6.195	0.287
Mcl-1L	Low	1		
	High	3.242	1.012-10.383	**0.037***

(b)

Parameter	Category	Hazard Ratio	95% CI	p- value
Node	Negative	1		
	Positive	1.855	0.718-4.791	0.202
T stage	T1+T2	1		
	T3+T4	2.786	0.948-8.187	0.063
Mcl-1S	Low	1		
	High	0.578	0.280-1.193	0.138

Table 19: Multivariate analysis of oral cancer patients.

5.3 Role of Mcl-1L in radioresistance and/or chemoresistance:

This objective aimed to determine whether Mcl-1 plays a role in radiosensitization / chemosensitization of oral cancer cells, using siRNA & shRNA strategies. The effect of Mcl-1 knockdown and/or irradiation/chemotherapeutic drugs on cell death and proliferation was also evaluated using different assays.

5.3.1 Role of Mcl-1 in Radioresistance:

Analysis of clonogenic survival of oral cell lines

Radiosensitivity assessment of the three oral cell lines (FBM, AW13516 & AW8507) used in the study was determined by the clonogenic survival assay (Figure 21a). The D0 values obtained from the surviving fractions of FBM, AW13516 & AW8507 were 2.3, 5.1 & 5.4 Gy respectively, indicating FBM to be most radiosensitive among the three oral cell lines.

Expression of Mcl-1 splice variants in oral cell lines

RT-PCR using a primer pair which amplifies all three isoforms of Mcl-1 showed predominant expression of anti-apoptotic Mcl-1L and low levels of Mcl-1S but undetectable levels of Mcl-1ES in all three cell lines. Separate RT-PCR of the poorly expressed Mcl-1ES isoform confirmed the very low expression of Mcl-1ES as compared to Mcl-1L & Mcl-1S in all the three oral cell lines (Figure 21b). The more radioresistant AW8507 & AW13516 cells showed high expression of Mcl-1L at both mRNA & protein levels as compared to immortalized FBM cells (Figure 21b&c).

Effect of irradiation on expression of Mcl-1 splice variants

The time course expression profiles of Mcl-1 isoforms in the three oral cell lines revealed induction of Mcl-1L protein post-IR in all three cell lines. However, the radiosensitive FBM exhibited a rapid and short induction profile with a peak at 1.5 hrs which declined by 48 hrs.

While the more radioresistant AW8507 exhibited sustained high levels of Mcl-1L up to 48 hrs with a peak observed at 1.5 hrs (Figure 22a). A similar pattern was observed in AW13516 (data not shown). In all cell lines, the expression of short Mcl-1S was elevated at initial time points which later decreased up to 48 hrs while, the short pro-apoptotic Mcl-1ES isoform levels remained unaltered (Figure 22a).

Expression of Bax, Bcl-xl & Bcl-2 protein

Interestingly, AW8507 cell line exhibited a rapid downregulation of pro-apoptotic Bax & Bak proteins, 2hrs post-IR. In contrast the more radiosensitive FBM showed a consistent increase in Bax & Bak levels, 2hrs onwards (Figure 22b). Higher expression of anti-apoptotic Bcl-2 & Bcl-xl protein was observed in AW8507 as compared to FBM. The AW13516 cell line also exhibited similar results.

Ratios of anti to pro-apoptotic members

It is noteworthy that, the more radioresistant AW8507 cell line exhibited higher ratios of anti to pro-apoptotic proteins like Mcl-1L/Mcl-1S, Mcl-1L/Bax & Bcl-xl/Bax as compared to that in FBM post-IR (Figure 23 a-f). A similar pattern was observed in AW13516 (data not shown).

Effect of Mcl-1 expression on apoptosis

As compared to untreated control, post-IR up to 1 hr, a time-dependent increase in apoptotic population was observed in all three oral cell lines. Notably, the number of apoptotic cells in AW13516 & AW8507 significantly ($P < 0.05$ at 24 hrs & $P < 0.01$ at 48 hrs) decreased thereafter as compared to that of FBM viz. from 17.5% to 9% at 24 hrs & 27% to 12% at 48 hrs of post-IR (Figure 24a), coinciding with the high Mcl-1L/Mcl-1S ratio (Figure 23b).

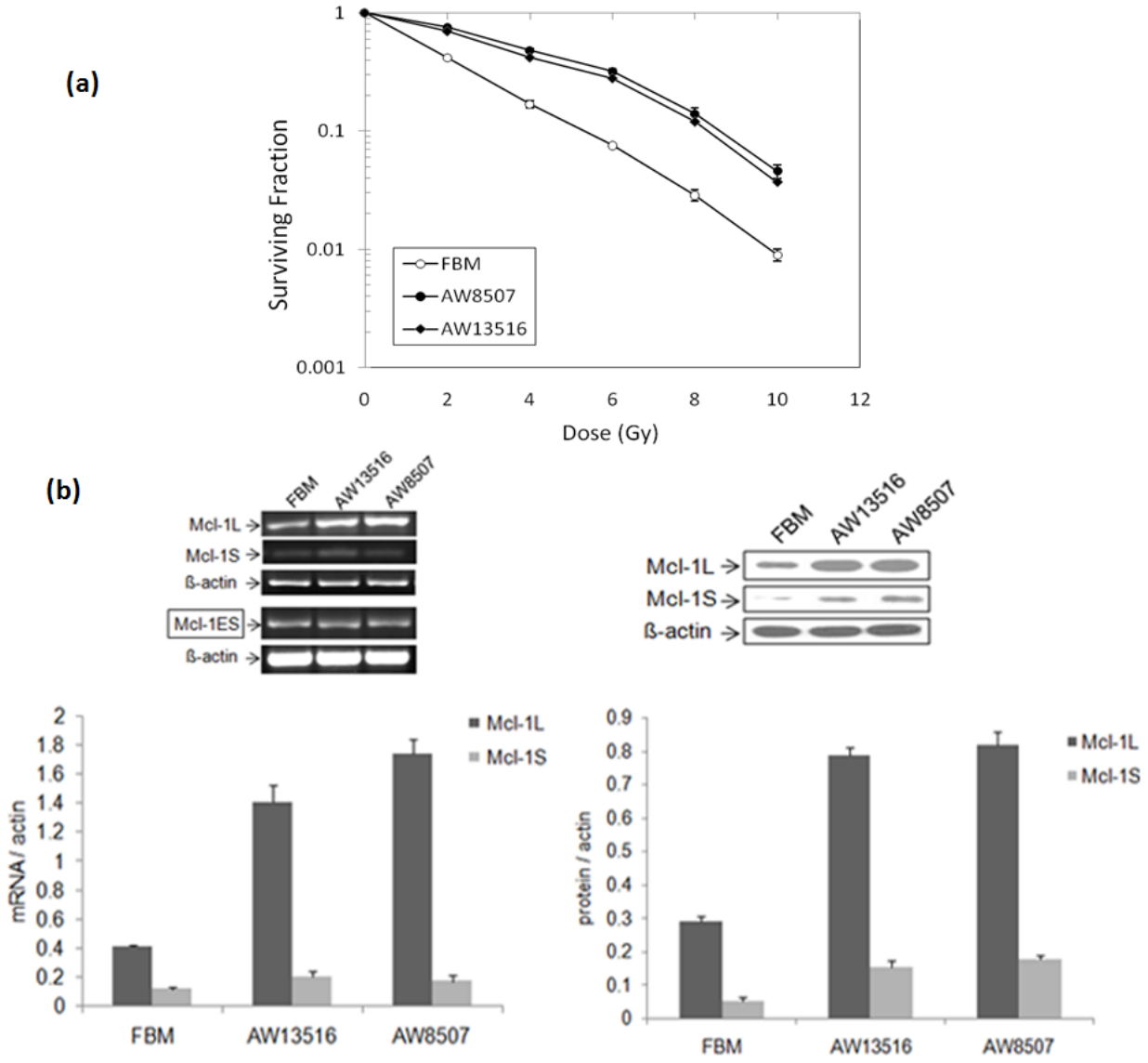


Figure 21: Radiosensitivity assessment and expression of Mcl-1 isoforms in oral cell lines

(a) Clonogenic cell survival assay of FBM, AW8507 & AW13516 oral cell lines. Data given as percentage survival of untreated cell cultures and represent the means (±SD) of three independent colony formation experiments. (b) Expression of Mcl-1 isoforms in oral cell lines. RT-PCR and western blot analysis of Mcl-1 isoforms in the three oral cell lines (FBM, AW8507 & AW13516). A representative blot is shown for three independent experiments. Histogram indicates quantitative expression of Mcl-1 isoforms (Mcl-1L & Mcl-1S) at both mRNA & protein level in oral cell lines.

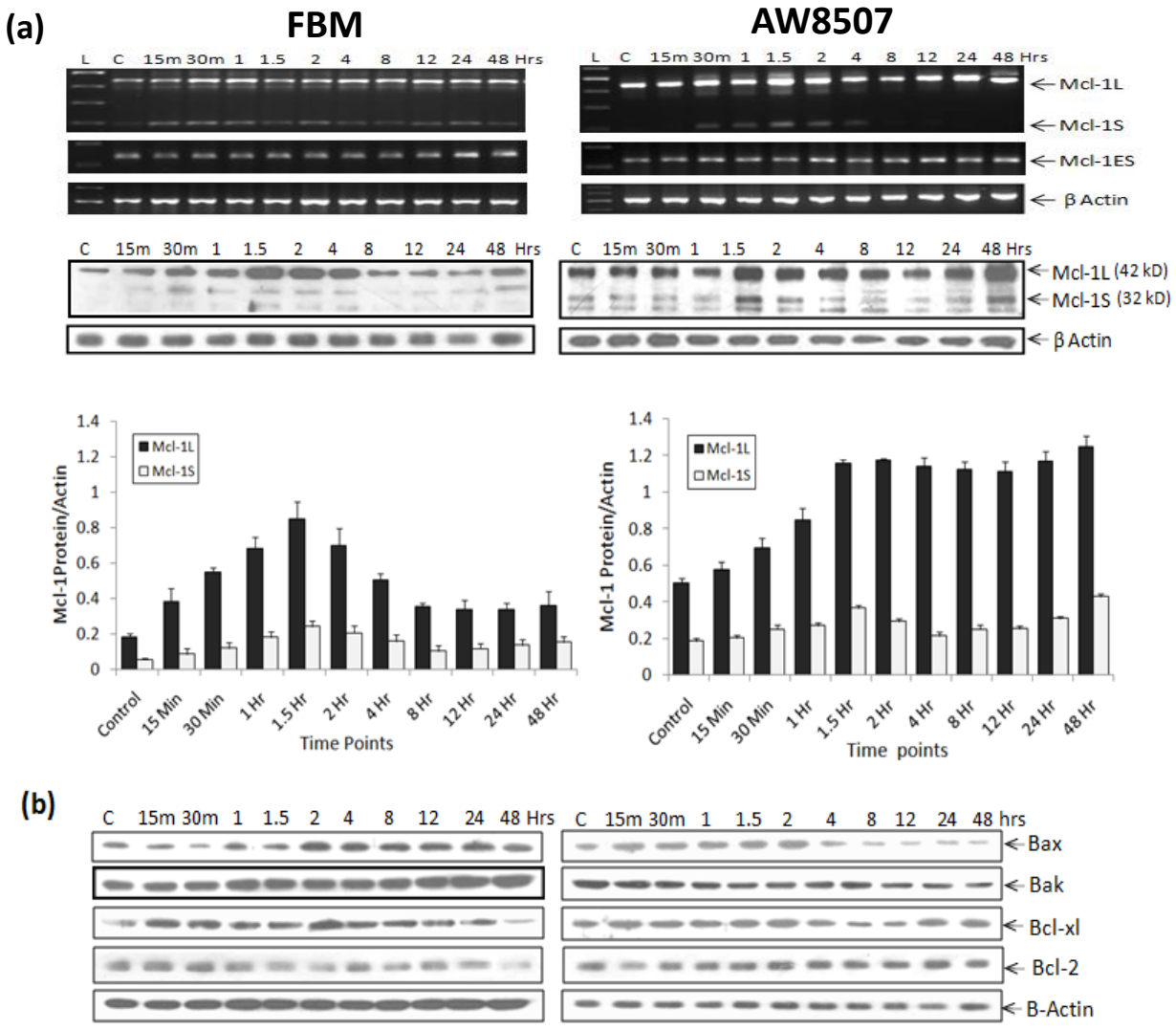


Figure 22: Time course profile of Mcl-1 splice variants & apoptosis related proteins post-IR.

(a) Expression of Mcl-1L, Mcl-1S transcripts and proteins at different time point's post-IR in AW8507 & FBM. Post-IR (D0 dose was used to treat cells, as mentioned in materials & methods) cells harvested at different time points were used for RT-PCR and Western blotting. (b) Western blot illustrates expression of Bax, Bak, Bcl-xl & Bcl-2 proteins at different time points post-IR in oral cell lines, using β-actin as loading control. A representative blot for three independent experiments is shown.

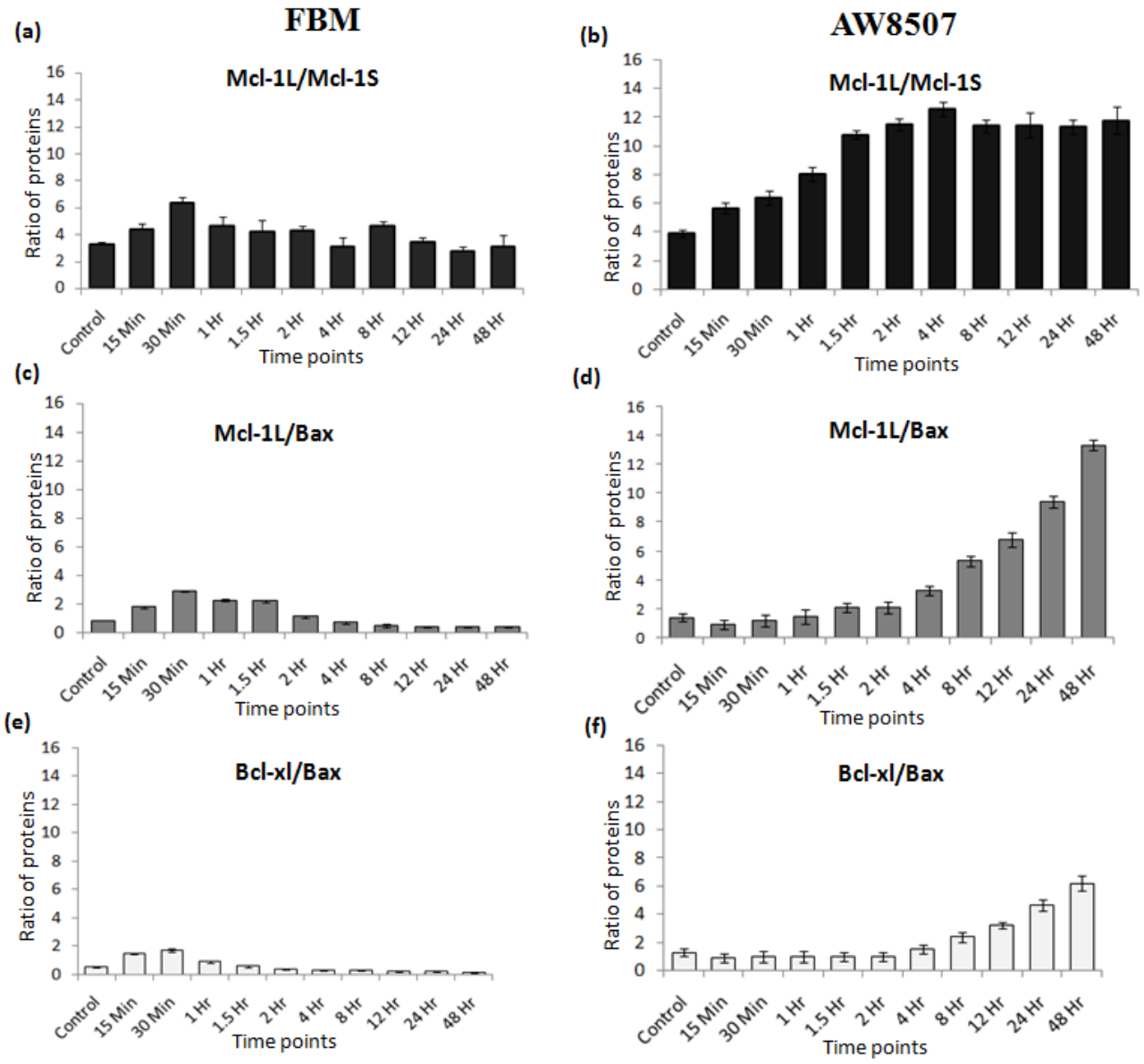


Figure 23: Ratios of anti to pro-apoptotic proteins in AW8507 & FBM cell lines*; Mcl-1L/Mcl-1S (a-b), Mcl-1L/Bax (c-d) and Bcl-xl/Bax (e-f).* The relative ratios of the proteins were obtained by densitometry analysis of blots using ImageJ software (NIH, USA).

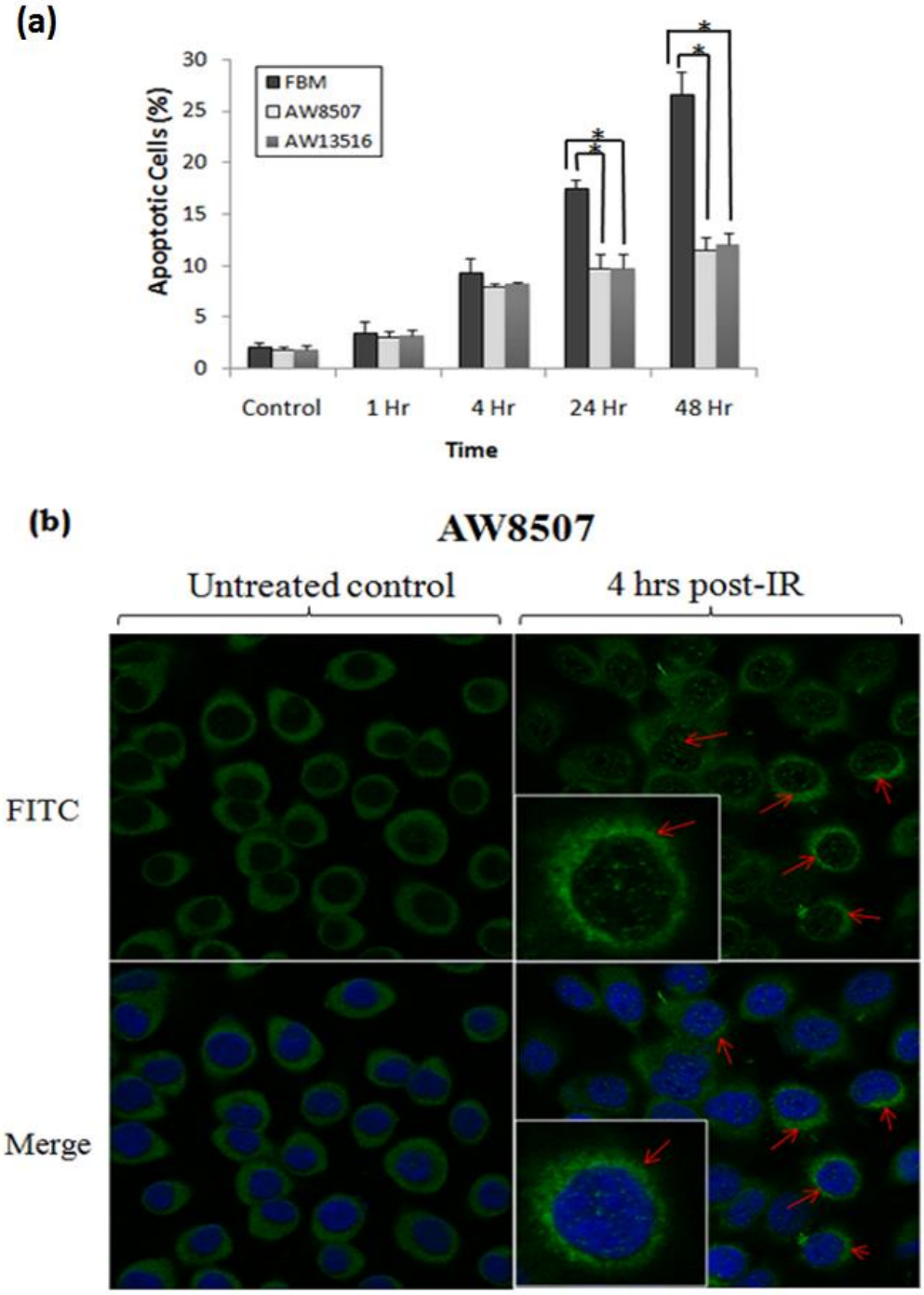


Figure 24: Apoptosis induction and localization of Mcl-1 post-IR. (a) Percentage of apoptosis induction at different time point's post-IR (Do dose) by Annexin-V & PI staining analyzed by FACS. The flow cytometry data shown is representative of three independent experiments (*P < 0.05 & **P < 0.01) (b) Immunofluorescence staining of Mcl-1 protein counterstained with DAPI (blue) shows peri-nuclear accumulation (inset) and additional nuclear localization 4 hrs post-IR, in AW8507 cells

Localization of Mcl-1 protein

Immunofluorescence staining demonstrated that Mcl-1 protein was primarily localized in the cytosolic compartment of untreated AW8507 & FBM cells. Interestingly post-IR a substantial increase in Mcl-1 protein expression with peri-nuclear and nuclear localization at 4 hrs was observed in AW8507 cells (Figure 24b), whereas no significant change in expression and localization was observed in FBM.

siRNA mediated downregulation of Mcl-1L

AW8507 cells exhibited specific downregulation of Mcl-1L levels after transfection with 100nM Mcl-1L siRNA without affecting the Mcl-1S levels (Figure 25a). The effect of Mcl-1L siRNA was maximal between 6 to 72 hrs and the Mcl-1S levels were unaltered. Treatment with siRNA and IR alone or in combination significantly increased expression of pro-apoptotic Bax protein but did not alter Bak & Bcl-xl protein levels (Figure 25b).

Effect Mcl-1L downregulation on cell proliferation and Apoptosis

Trypan blue dye exclusion assay in AW13516 & AW8507 revealed a significant ($P < 0.05$) decrease in viability of cells treated with combination of siRNA plus IR as compared to individual treatments. After 72 hrs, cell viability was reduced to 67% (IR), 42% (siRNA) and 21% (IR plus siRNA) respectively (Figure 26 a&b). Thereby, suggesting a synergistic effect of the combined treatment on cell viability. Immunofluorescence analysis of AW13516 & AW8507 demonstrated an increased nuclear condensation in cells treated with combined Mcl-1L siRNA plus IR as compared to IR or siRNA alone (Figure 26c). The percentage of apoptotic cells in experimental control (EC), UC, IR, siRNA, siRNA plus IR treated AW8507 cells were 2.1% (SD 0.5), 3.2% (SD 0.6), 17.3% (SD 0.5), 25.3% (SD 1.1) and 46.3% (SD 0.6), respectively (Figure 26d). A similar pattern was observed in AW13516. The difference in percentage of apoptosis between IR alone and siRNA plus IR treated cells was highly significant ($P < 0.01$) in both the cell lines.

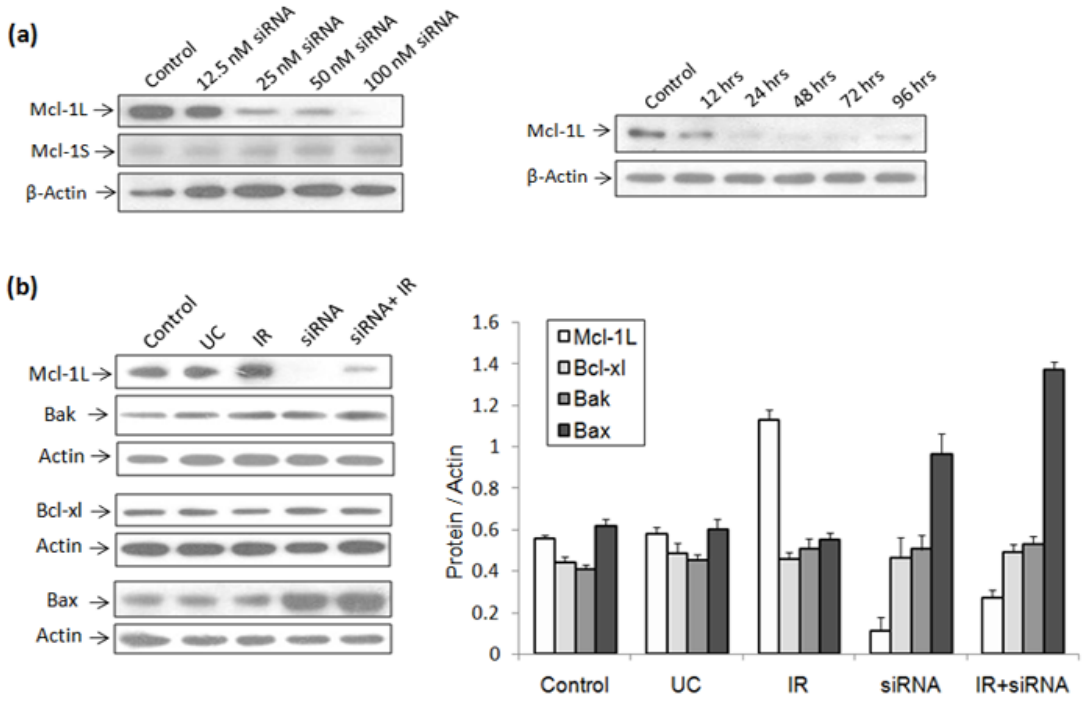


Figure 25: Western blot analysis of Mcl-1L knockdown.

(a) Mcl-1L downregulation using different concentrations of siRNA and unaltered expression of Mcl-1S in AW8507 cells. The effect of Mcl-1L siRNA (100nM) was analyzed upto 96 hrs post transfection. (b) Expression of Mcl-1L, Bak, Bax & Bcl-xl proteins 24 h after transfection of AW8507 cells: [Experimental control without siRNA (EC); universal control siRNA (UC); Mcl-1L siRNA (siRNA); irradiation (IR)]. A representative blot of three independent experiments is shown**.**

Effect of Mcl1L knockdown on clonogenic survival

The effect of Mcl-1L downregulation on long term cell survival was examined by clonogenic assay in AW8507 & AW13516 cells. Interestingly, a reduction in clonogenic survival was observed after treatment of Mcl-1L siRNA (100nM) and increasing doses of IR as compared to the untreated control (Figure 27). The survival of AW8507 post-IR (0, 2, 4, 6 8 and 10Gy) was 78% (SD 2.1), 46% (SD 1.5), 32% (SD 2.3) and 14% (SD 1.8) and 6% (SD 1.9) respectively. However, in presence of Mcl-1 siRNA the survival was further reduced to 42% (SD 2.6), 23% (SD 1.7), 10% (SD 1.0), 4% (SD 2.1) and 2% (SD 1.7), respectively (Figure 27a). Similar reduction in clonogenic survival post Mcl-1L knockdown was observed in

AW13516 cells (Figure 27b). These observations therefore suggest a synergistic effect of the Mcl-1L siRNA with IR on radiosensitivity.

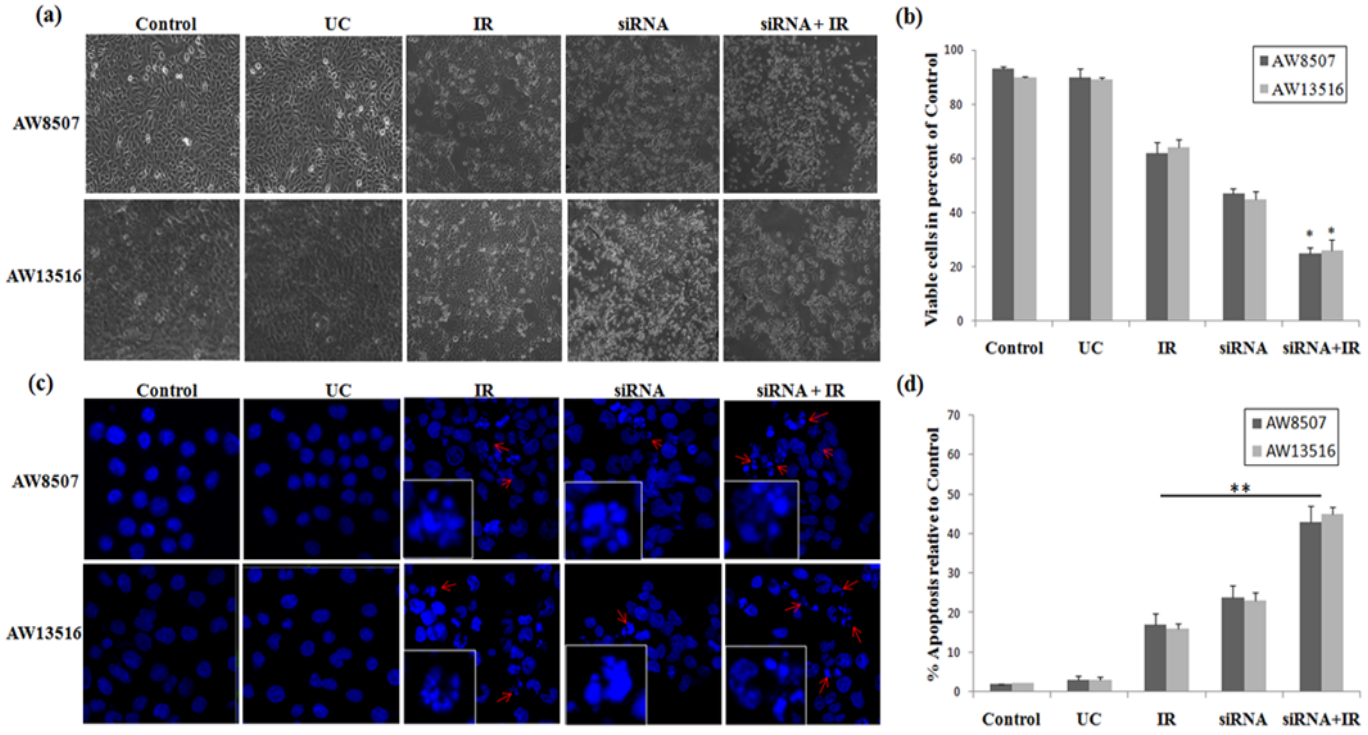


Figure 26: Microscopic analyses of cell proliferation and apoptosis after different treatment combination (a&b) After different treatment combinations as described above, cell growth of AW8507 & AW13516 cells was determined by assessing cell numbers using Trypan blue dye exclusion assay. Cell count is given as percent of untreated controls. Data is mean ± SD of three independent experiments. (c&d) Apoptosis was determined by DAPI staining, the cells were either untreated (Control) or treated with UC siRNA, Mcl-1L siRNA alone or in combination with IR. Apoptotic cells were counted in different fields and shown as percent of apoptosis relative to control. Data is means ± SD of three independent experiments.

[*Indicates $P < 0.05$, ** $P < 0.01$]

Expression of Mcl-1L in radioresistant sublines

To evaluate the association of Mcl-1L with radioresistance, its expression was assessed by western blotting in acquired radioresistant sublines of AW8507 & AW13516 (Using FIR strategy). Figure 28 demonstrates, the high Mcl-1L expression in radioresistant sublines generated by fractionated irradiation as compared to parental untreated cells.

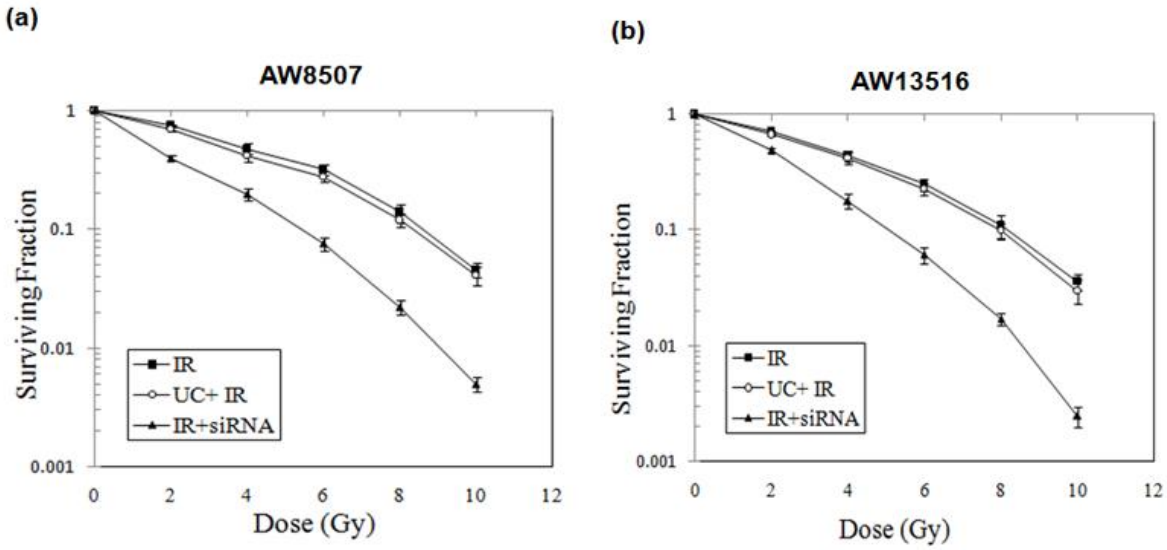


Figure 27: Clonogenic survival analysis post Mcl-1L knockdown and/or irradiation:

(a&b)AW8507 & AW13516 cells were either untreated, or treated with siRNA's. After 24 hrs, cell culture dishes were treated with increasing doses of ionizing irradiation (0, 2, 4, 6, 8 and 10 Gy) as indicated. Survival was assessed by performing clonogenic assay as described in Methods. Data is given as percentage survival of untreated cells and represent the mean (±SD) of three independent colony formation experiments.

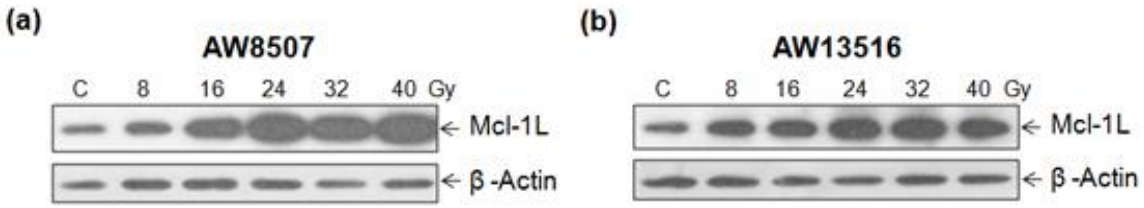


Figure 28: Expression of Mcl-1L in radioresistant sublines: (a&b) AW13516 & AW8507 cells were treated with fractionated irradiation (FIR) and the radioresistant sublines obtained during development of radioreisstant cell lines. The radioresistant sublines were assayed for expression of Mcl-1L. β-actin blot from the same lysates served as control.

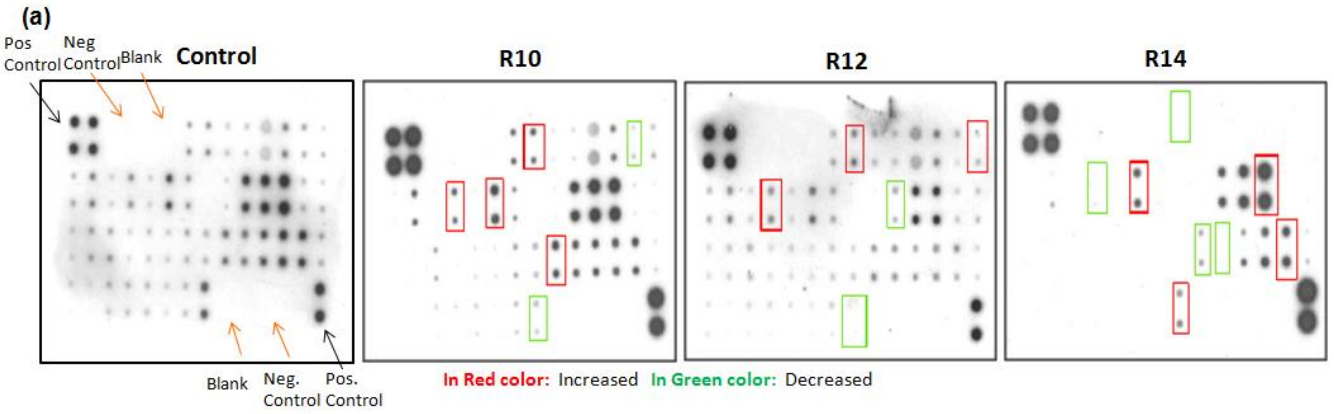
Expression apoptosis related proteins in radioresistant sublines:

The expression profile of 42 different apoptotic proteins (Refer table 20 for array map) was generated using antibody array in radioresistant sublines of AW-13516 as described in methods. The results of antibody array revealed altered expression of several proteins including intrinsic & extrinsic pathways of apoptosis, family of apoptosis inhibitors (IAP), Bcl-2 family, caspases & also the members of HSP family (Heat shock protein), as shown in table 21.

	A	B	B	D	E	Fas	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
2	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
7	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos
8	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos

Table 20: Human Apoptosis Antibody Array Map

(a)



(b)

Sub lines	Up-regulated proteins	Down-regulated Proteins
R-2 (8Gy)	Bad, CytoC, p21, Survivin	Fas, XIAP
R-3 (12Gy)	Bad, p21, CytoC	Fas, XIAP,
R-7 (28 Gy)	P21, CytoC, Fas, Caspase8	XIAP
R-10 (40 Gy)	Bad, Bax, CytoC, Fas, p21	Caspase3, XIAP
R-12 (48 Gy)	Bax, CytoC, Caspase8	HSP27, XIAP
R-14 (56 Gy)	Fas, Survivin, HTRA, SMAC, XIAP	Bax, p21, p27, CytoC,

Table 21: Expression of Apoptosis related proteins in radioresistant sublines of AW8507using Antibody Array.

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5.2.2 Role of Mcl-1 in Chemoresistance:

Effect of Cisplatin treatment to oral cancer cell lines:

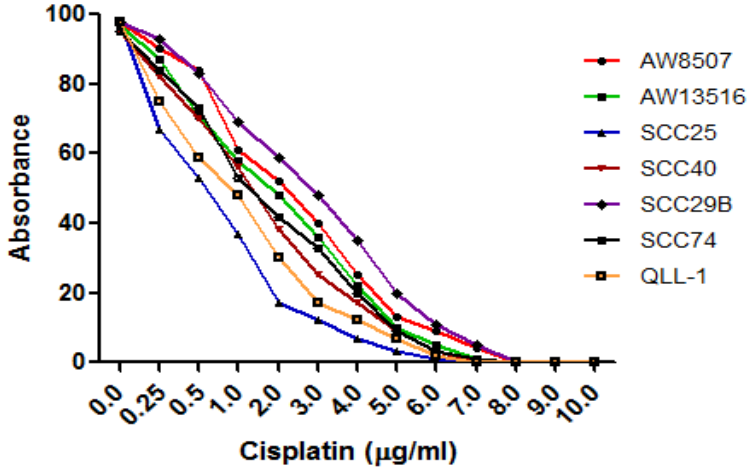
Cisplatin, a common drug in treatment of oral cancer was used in the study. The MTT cell proliferation assay was done for seven oral cancer cell lines (namely AW9507, AW13516, SCC25, SCC40, SCC29B, SCC74 & QLL-1) of different origin, post-Cisplatin treatment. A reduced cell proliferation and induction of cell death was observed post-Cisplatin treatment. The MTT assay revealed that majority of oral cancer cell lines showed the IC50 between 1-3 µg/ml as shown in figure 29.

Effect of Cisplatin on Mcl-1 expression:

The expression of Mcl-1 was analyzed post Cisplatin treatment at different time intervals (6 hrs to 48 hrs). The western blot analysis revealed a reduction in Mcl-1 expression, from 24 hrs post Cisplatin treatment in AW8507 cells (Figure 30a) which indicates that Mcl-1 expression is essential for cell survival post Cisplatin treatment.

Downregulation of Bcl-xl & Mcl-1:

Both predominantly expressed anti-apoptotic proteins, Bcl-xl & Mcl-1 were downregulated using siRNA approach. The western blot analysis revealed a transient effect of knockdown up to 96 hrs post 100 nM Bcl-xl siRNA treatment as shown in figure 30b. Due to rapid turnover of Mcl-1 no significant knockdown was achieved for a longer time (96 hr) post siRNA transfection (Figure 30b). In order to overcome this transient effect, Mcl-1 was downregulated using shRNA cloned in an inducible pTRIPZ vector, further transduced & expressed in AW8507 cells (Figure 31 & 32) as described in methods. The qRT-PCR & immunoblotting indicates downregulation of Mcl-1 at both mRNA & protein level, however the upstream RFP expression by confocal microscopy confirms the expression of Mcl-1 shRNA cassette as shown in Figure 33.



Cell lines	AW8507	AW13516	SCC-25	SCC-40	SCC29B	SCC74	QLL-1
IC50 (µg/ml)	2.0	2.0	0.5	1.5	2.5	2.0	1.0

Figure 29: MTT assay of oral cell lines post Cisplatin treatment

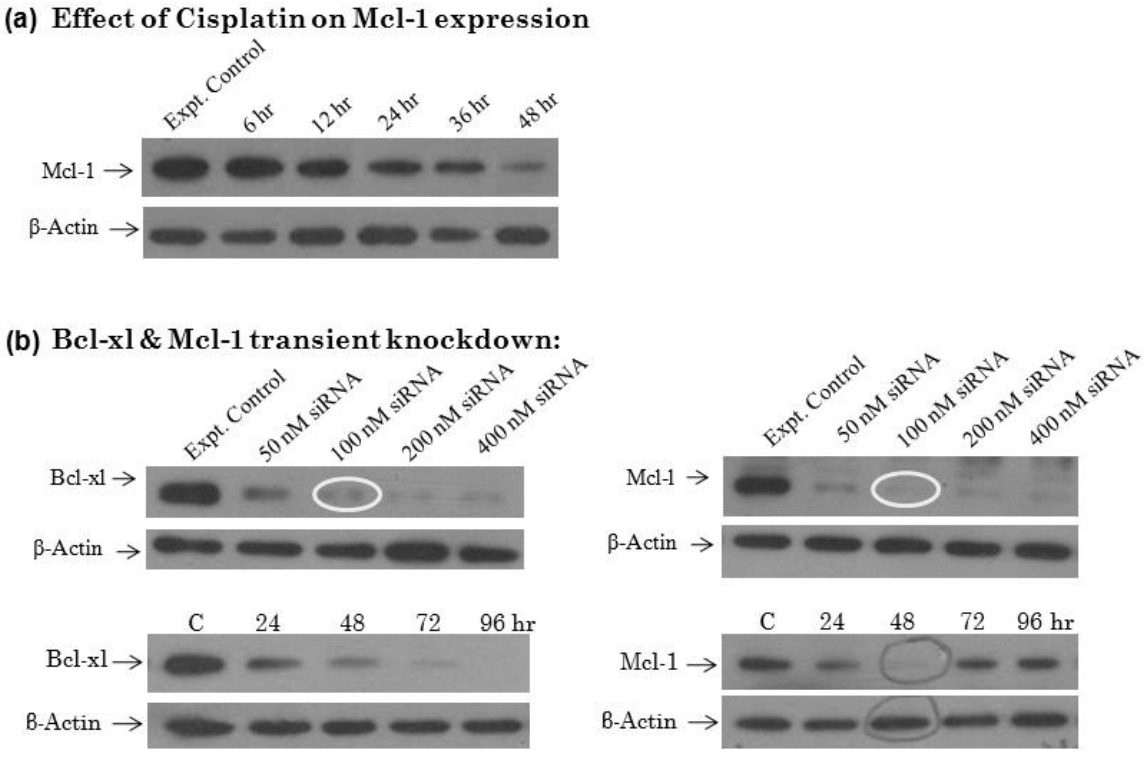


Figure 30: (a) Effect of Cisplatin (2µg/ml) on Mcl-1 expression; (b) Transient knockdown of Mcl-1 & Bcl-xl post siRNA transfection.

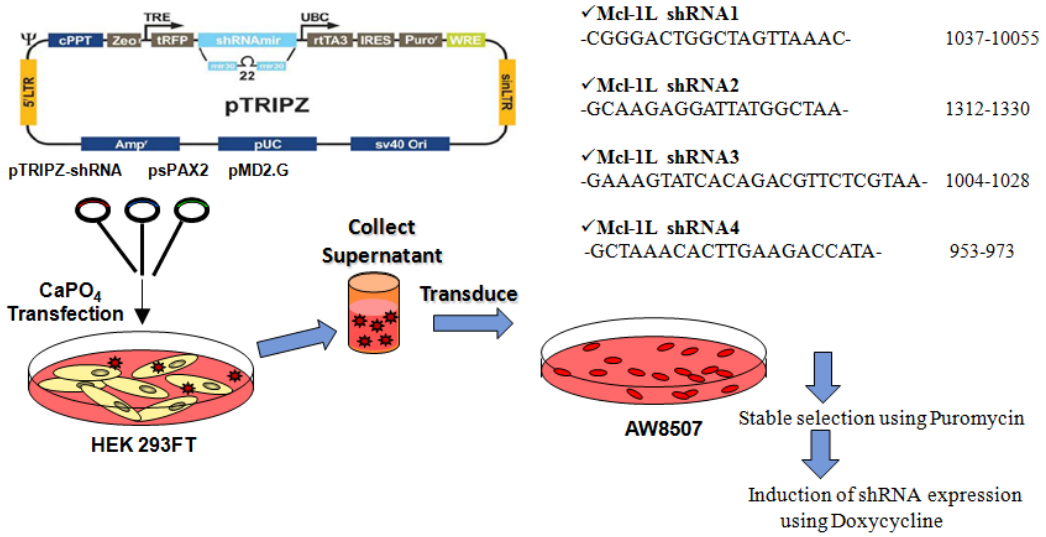


Figure 31: Pictorial representation of shRNA mediated downregulation of Mcl-1

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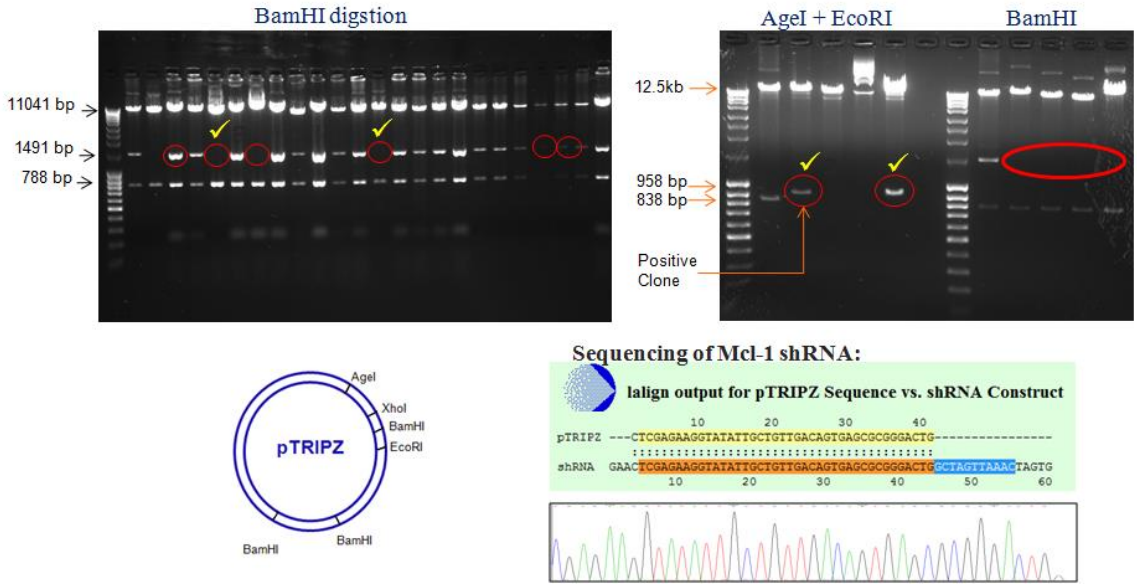


Figure 32: Screening of Mcl-1-shRNA-pTRIPZ clones via restriction digestion & sequencing.

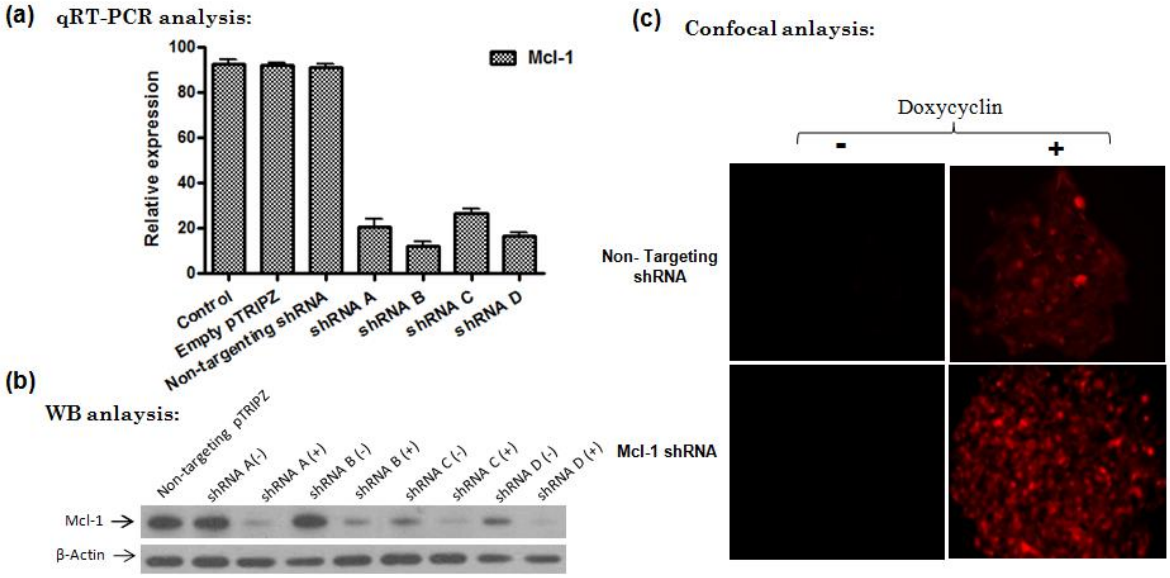


Figure 33: Assessment of Mcl-1 downregulation at both mRNA & protein level

Effect of Mcl-1/ Bcl-xl knockdown and/or Cisplatin on proliferation:

The AW8507 cells were either transfected by shRNA for Mcl-1 & or siRNA for Bcl-xl downregulation and/or treated with Cisplatin. The MTT assay was carried out to evaluate cell proliferation in cells treated with different combinations either individual siRNA mediated knockdown and Cisplatin or combination of both the treatments. Interestingly in AW8507 cells, the double knockdown of Mcl-1 & Bcl-xl in combination with Cisplatin showed a significant reduction in proliferation as compared to any treatment alone (Figure 34).

Effect of Mcl-1/ Bcl-xl knockdown and/or Cisplatin on cell viability:

The AW8507 cells were similarly treated with either shRNA/Cisplatin treatment or combination of both. The trypan blue staining was carried out to determine the cell viability after different treatment combinations. Microscopic & cell viability analysis indicate a higher induction of cell death in cells treated with Cisplatin after depletion of both Mcl-1 & Bcl-xl proteins as compared to individual treatments (Figure 35).

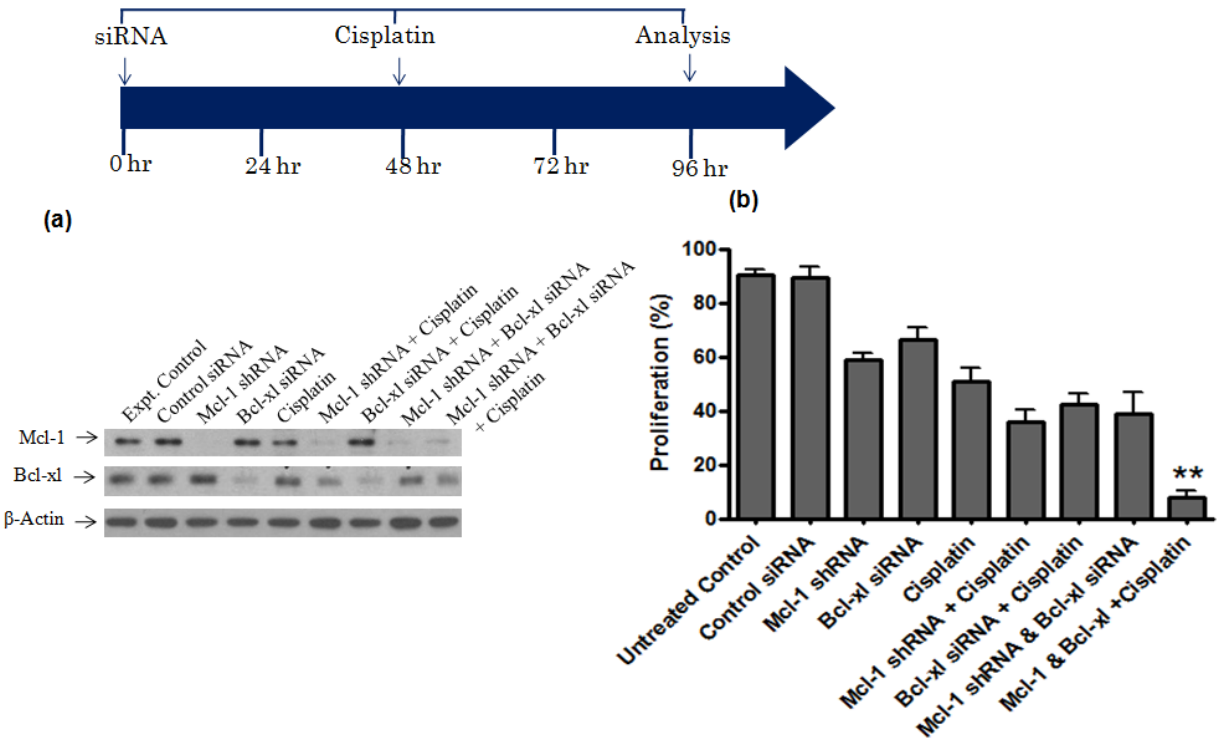
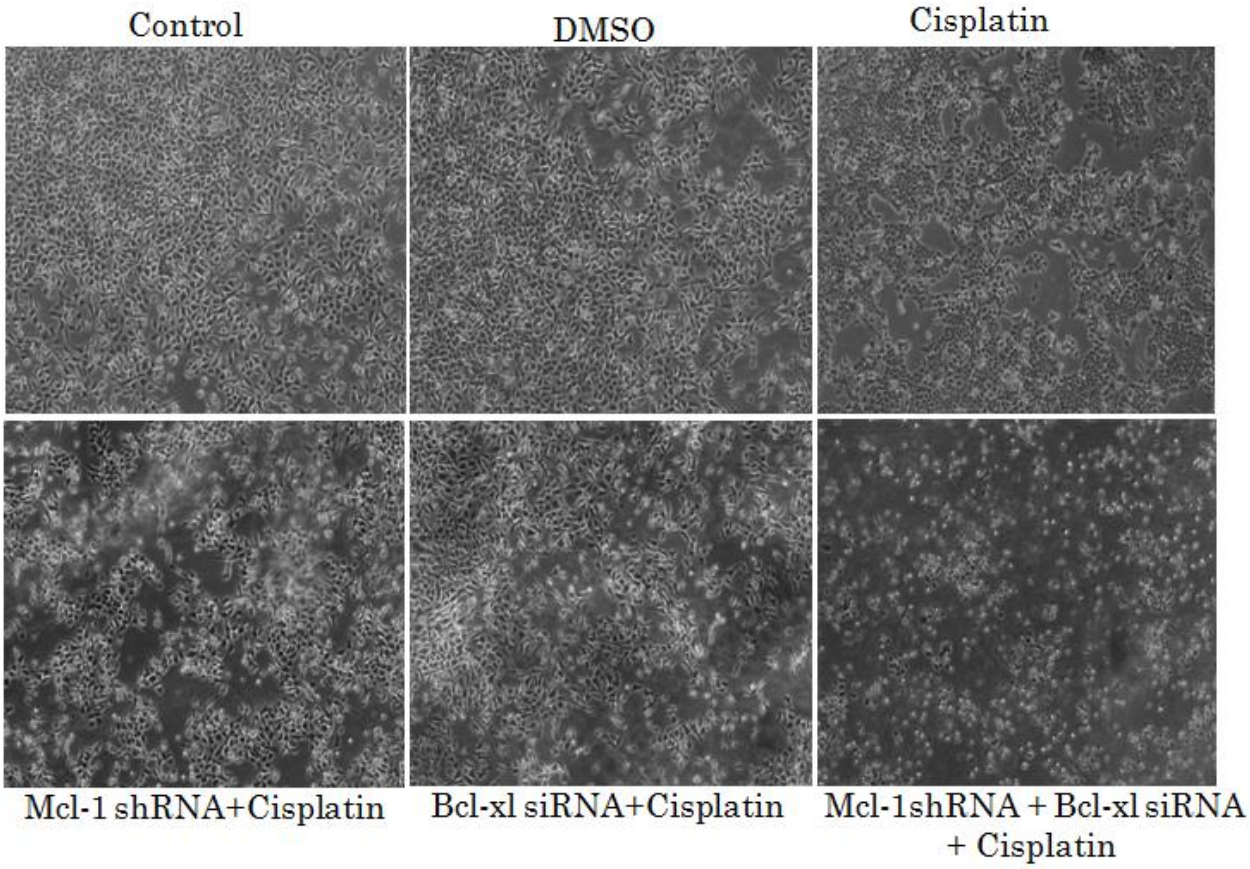


Figure 34: Effect of combination of Mcl-1 & Bcl-xl knockdown and/or Cisplatin on cell proliferation by MTT assay

(a)



(b)

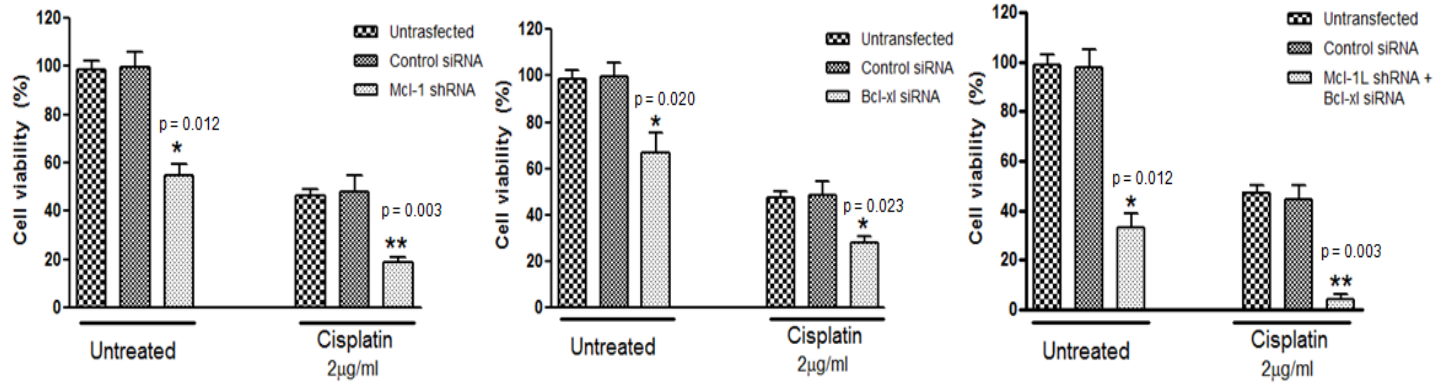


Figure 35: Effect of combination of Mcl-1 & Bcl-xl knockdown and/or Cisplatin on viability of AW8507 cells by trypan blue assay. (The p values determines the difference between cell viability after different treatment combinations).

Effect of Mcl-1 knockdown and/or Cisplatin on cell viability:

The AW8507, SCC40 & SCC29B cells were either downregulated Mcl-1 expression by shRNA and / or treated with Cisplatin. The MTT assay was carried out to evaluate cell viability in cells treated with different combinations either individual siRNA mediated knockdown and Cisplatin or combination of both the treatments. Interestingly in all three coral cancer cell lines (AW8507, SCC40 & SCC29B), the knockdown of Mcl-1 in combination with Cisplatin showed a significant reduction in cell viability as compared to any treatment alone (Figure 35).

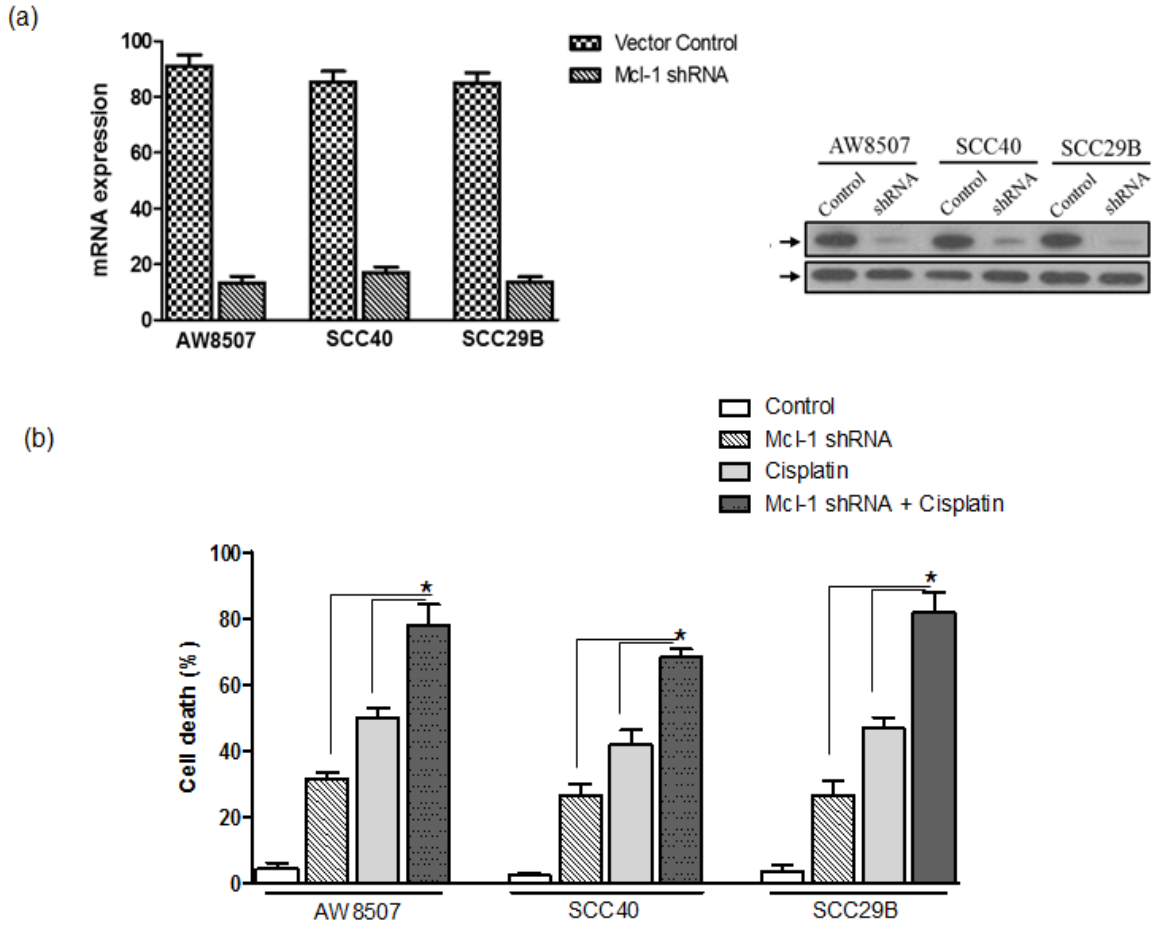


Figure 35i: Effect of combination of Mcl-1 knockdown and/or Cisplatin on viability of oral cacner cells by MTT assay. (The p values determines the difference between cell viability after different treatment combinations; *p<0.05).

Effect of BH3 mimetic Obatoclax and/or Cisplatin treatment:

The IC50 of BH3 mimetic Obatoclax was estimated by PI staining & flowcytometry analysis at different doses, indicated in Table 22. An induction of cell death was observed post Obatoclax treatment in AW98507 cells. The microscopic analysis of cell death, trypan blue staining & MTT assay for cell proliferation indicates that combination of Obatoclax and Cisplatin exhibited significant reduction in cell proliferation & induction of cell death and as compared to any treatment alone (Figure 36).

Table 22: Determination of Obatoclax IC50 by flow cytometry:

Treatment (Obatoclax)	**Control (0.1% DMSO)**	**0.25 uM**	**0.5 uM**	**1 uM**	**2.5 uM**	**5 uM**	**10 uM**	**15 uM**	**25 uM**
Percentage of viable cells	99%	98%	95%	88%	73%	**46%**	11%	3%	1%

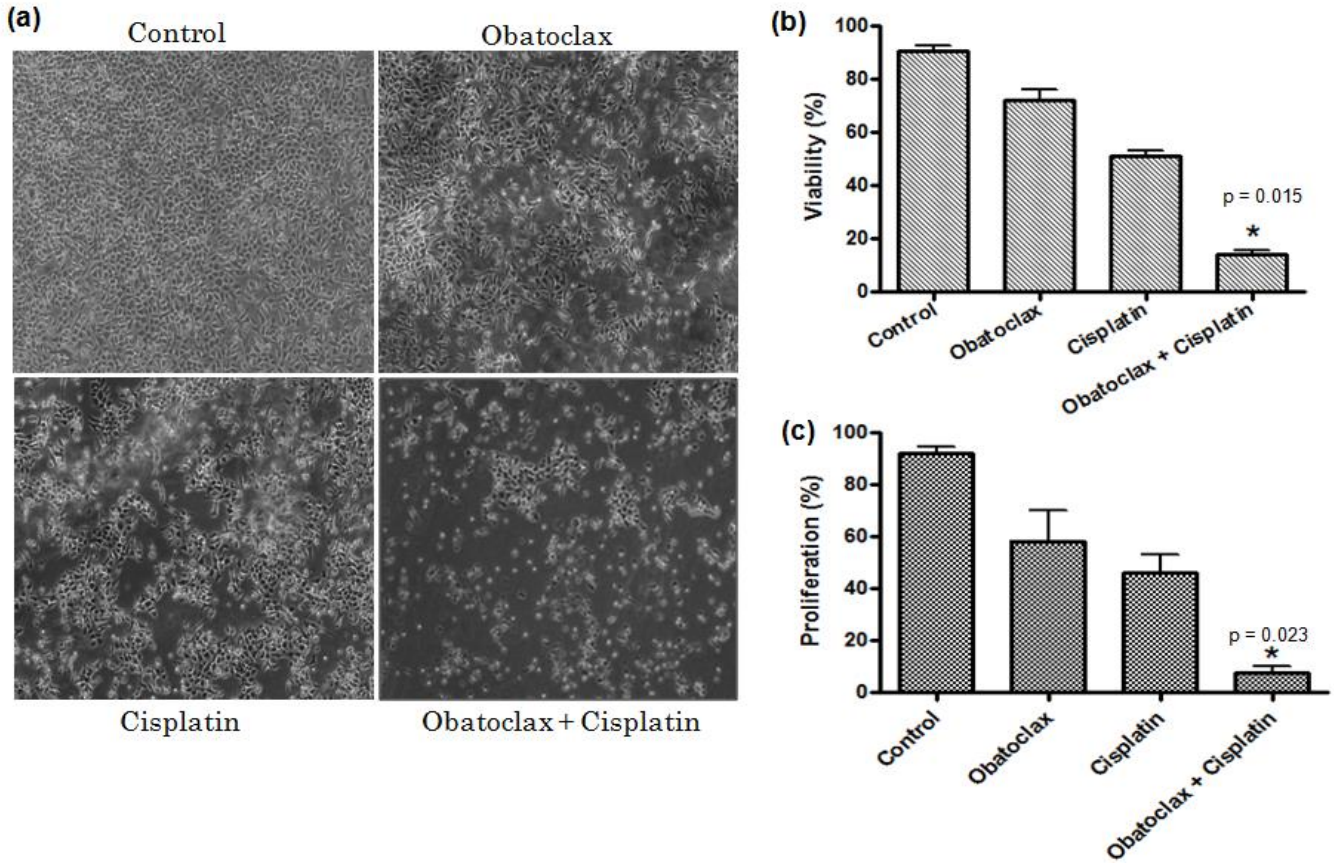


Figure 36: Effect synergistic effect of combination of Obatoclax and Cisplatin on cell viability & proliferation as compared to either treatments alone.

CHAPTER 6

DISCUSSION

DISCUSSION:

6.1 To decipher mechanisms of Mcl-1 overexpression in oral cancers-

Oral cancer is the eighth common cancer worldwide and one of the three most fatal cancers in males of the Indian subcontinent [2]. Despite recent advances in surgical treatment and radio/ chemo therapy, the long term survival of oral cancer patients has not changed significantly [3]. Therefore, it is an urgent need to improve the early detection of oral carcinomas and in depth study to elucidate the mechanisms involved in the development and progression of oral cancer [6]. The treatment outcome depends primarily on early detection therefore; characterization of identifiable molecular markers will help in the early diagnosis and treatment of oral cancer.

Oral squamous cell carcinomas (OSCCs) have repeatedly been linked to apoptotic dysregulation [7]. Bcl-2 and related pro- and anti-apoptotic proteins are important mitochondrial apoptosis pathway regulators and play a critical role in regulating cell survival [68]. Mcl-1 is an anti-apoptotic member of the Bcl-2 gene family have been shown to be overexpressed and involved in progression of variety if solid and nonsolid malignancies [14]. Earlier studies from our lab already have demonstrated over-expression of Mcl-1 in human oral carcinomas and oral cell lines [19, 20].

In the present study we therefore wanted to evaluate the mechanism of Mcl-1 overexpression namely the occurrence of genomic alteration in Mcl-1 gene in oral tumors and correlate the alterations if any with the expression of Mcl-1 isoforms and clinico-pathological parameters of oral cancer patients. Our studies revealed the presence of 18bp polymorphic repeats in the promoter region of 22% oral tumors, which may contribute to the Mcl-1 overexpression in these tumors. Interestingly, a study by Moshynska et al, had demonstrated that the presence of 6 and/or 18 nucleotide insertions in the Mcl-1 promoter correlated with increased RNA and protein levels of Mcl-1 & clinical outcome in CLL patients [157]. The presence of these insertions was also shown to be associated with poor overall survival and

disease-specific survival, suggesting the potential use of the Mcl-1 promoter insertions as a prognostic factor.

Although there are a few reports on the polymorphic insertions in Mcl-1 promoter and their clinical significance, to the best of our knowledge there are no such studies in oral cancer. The present study is the first one evaluating the effect of Mcl-1 promoter alterations on the expression of Mcl-1 isoforms and its clinical significance in oral cancer patients. We analyzed 40 oral cancer patients, 11 oral cell lines and 25 healthy volunteers, utilizing PCR sequencing of Mcl-1 promoter to detect the presence of these two insertions. Neither normal nor oral cancer cell lines showed presence of 18 nucleotide repeats, however only two cancer cell lines (SCC15 & SCC40) showed presence of 6 bp repeats. Interestingly, the 18-nt polymorphic insertions were present in 9/40 tumors (22%) & also in 8/40 healthy volunteers (32%), indicating that these represent hereditary polymorphisms rather than somatic mutations as reported from studies in B-CLL & acute lymphoblastic leukemia (ALL) patients [158, 159]. Our studies did not show any correlation between the presence/ absence of 6/18-nt repeats and Mcl-1 gene expression (neither mRNA nor protein) as reported by Saxena et al [160]. Similar to our findings, several other studies on CML & AML patients and healthy volunteers revealed no significant correlation between 6/18-nt repeats and Mcl-1 expression [161, 162].

Further, we assessed the clinical significance of 6/18-nt repeats i.e with overall survival & prognosis of OSCC patients. Unlike studies from Moshyanska et al and Saxena et al, we did not find any correlation between polymorphic insertions of Mcl-1 & clinical outcome of oral cancer patients. The overall survival of patients was independent of the presence or absence of Mcl-1 promoter polymorphisms, indicating that Mcl-1 promoter polymorphisms may not be useful to predict outcome / prognosis of OSCC patients. In support of our findings, several correspondence letters/reports as Tobin et al, are available [159, 163, 164] indicating that the Mcl-1 insertions represent hereditary polymorphisms rather than somatic mutations that probably do not predispose to CLL and are not associated with prognosis.

In contrast, studies by Reed et al & Moshynska et al, demonstrated association between these promoter insertions with high Mcl-1 mRNA and protein levels. Indicating that these insertions represent somatic alterations and not hereditary polymorphisms [165, 166]. Our studies revealed that the presence or absence of 18bp promoter polymorphism did not affect Mcl-1 expression and was also independent of status of tissue i.e. normal or tumor. Thus, polymorphic insertions in Mcl-1 promoter appear to be the hereditary in nature and not somatic in studies of Indian population. Therefore, the presence of promoter insertions appears be evidently insufficient to reliably drive high levels of Mcl-1 expression in oral cancers. Although, studies by Saxena et al demonstrated that polymorphic insertions were associated with increased promoter activity & Sp1/Sp3 binding sites [160]. Although, our studies found additional binding sites (SP1, CAP, ADR1, ADR2 & ADR4) due to the presence of 18-nt polymorphic repeats in promoter region, no significant effect was observed on Mcl-1 mRNA / protein of oral tumors. Recently, Mcl-1 promoter variants were shown to increase transcriptional activity of Mcl-1 and correlated with reduced risk of lung cancer in nonsmokers, thereby suggesting a dominant anti-proliferative function of Mcl-1 against its anti-apoptosis effect [167]. Thus from our studies indicates, presence or absence of promoter polymorphisms does not appear to influence Mcl-1 expression & therefore may not be the possible mechanism, responsible for Mcl-1 overexpression in oral tumors. However, evidence for the biological effect of Mcl-1 promoter polymorphisms on gene expression and the significance of Mcl-1 promoter polymorphisms in oral cancer needs to be analyzed in a larger cohort.

6.2 Role of Mcl-1 isoforms in pathogenesis of oral cancer:

6.2.1 Expression of Mcl-1 in Oral Submucous Fibrosis versus normal mucosa:

In the present study we studied the expression of Mcl-1 protein in OSF samples versus normal mucosa, to determine whether Mcl-1 expression has a possible role early in oral carcinogenesis. Our study revealed high expression of Mcl-1 protein in OSF as compared to normal, indicating upregulation of Mcl-1 in OSF tissues. The mean Mcl-1 expression was significantly higher in OSF than normal's. The Mcl-1 staining pattern was observed to be homogenous with cytoplasmic distribution.

The molecular mechanism leading to the upregulation of Mcl-1 in OSF is not known. However, Mcl-1 is known to be upregulated through multiple transcriptional and post transcriptional mechanisms. Therefore, alterations in mechanisms of such highly regulated proteins may be responsible of their upregulation. Several genetic alteration have shown to be responsible for Mcl-1 up regulation as discussed earlier [160]. Mcl-1 was originally identified as a gene upregulated early in differentiation of human myeloid cells [92].

The Mcl-1 protein expression may be an important early event in initiation and progression of OSCC. Interestingly, Mcl-1 gene undergo alternative splicing and produces functionally distinct isoforms namely anti-apoptotic Mcl-1L & pro-apoptotic Mcl-1l & Mcl-1ES. Therefore a detailed study, in larger cohort of premalignant samples is needed in order to point out the role of Mcl-1 isoforms in progression of human oral cancers. However, Mcl-1 isoform analysis by qRT-PCR for fresh premalignant samples were not available, hence we compared the relative levels of Mcl-1 isoforms in oral tumors versus the normals.

6.2.2 To examine the levels of Mcl-1 isoforms in oral cell lines & tumors from different subsites and their correlation with clinico-pathological parameters-

In the present study, we assessed the expression Mcl-1L isoforms (Mcl-1L, Mcl-1S & Mcl-1ES) in oral tumors, to determine whether they would be useful as prognostic markers in oral cancer patients. Our studies demonstrate significant high expression of anti-apoptotic Mcl-1L at both mRNA & protein level in majority of oral tumors (64%) versus adjacent normal tissues & in cancer cell lines versus normal cell lines. The observed high expression of Mcl-1 in oral tumors is consistent with reported overexpression of Mcl-1 in hepatocellular carcinomas, cervix cancer, pancreatic cancer, non-small cell lung cancer, testicular germ cell tumors and melanomas [168-171]. The up-regulation of Mcl-1L may be associated with the pathogenesis of oral cancer [149].

Mcl-1 overexpression also appears to be a key factor in the resistance of some cancer types to conventional treatment. An altered expression pattern of Mcl-1 has been reported in association with progression of colorectal cancer [172]. Sieghart et al, have reported overexpression of Mcl-1 protein in human hepatocellular carcinoma tissues and its potential as a molecular drug target in HCC [168]. Moreover, Mcl-1 down-regulation is known to promote apoptosis in cancer cells, suggesting that Mcl-1 can potentially act as a therapeutic target in the treatment of several human malignancies [14].

Dysregulation of apoptosis regulating genes may play a key role in the development and progression of several human malignancies. Overexpression of anti-apoptotic Mcl-1L may represent an important mechanism in the development & progression of oral cancer. Hence, we evaluated the correlation between Mcl-1 isoform expression and clinico-pathological parameters in oral cancer patients. The anti-apoptotic Mcl-1L expression was found to be significantly associated with tumor size ($p = 0.013$) and lymph node positivity ($p = 0.020$)

(table17). A comparison of Kaplan–Meier survival curves of low and high expressers of Mcl-1L mRNA showed that high Mcl-1L expression was significantly associated with poor overall survival ($p = 0.002$). However, the only other study analyzing Mcl-1 isoforms & their clinical significance in clear cell renal carcinoma by Kempkensteffen et al, has shown results in contrast to ours, wherein downregulation of Mcl-1L was associated with aggressive phenotypes in clear cell renal carcinoma [173]. The high expression of Mcl-1 and its association with poor prognosis has also been reported in cervical, ovarian and gastric and breast cancers, however to the best of our knowledge, there are no report delineating the prognostic significance of Mcl-1 isoforms in human oral cancer. This is the first study demonstrating the correlation of high Mcl-1L levels with poor overall survival and its possible use as an independent prognostic marker for oral cancer patients.

Several possible mechanisms can lead to the high Mcl-1 mRNA levels. Mcl-1 is also regulated at the post-transcriptional level by micro RNAs through a mir29 binding in the 3'UTR of Mcl-1 mRNA [117]. Interestingly, the expression of mir29 was also found to be decreased in malignant cholangiocytes, favoring the increased levels of Mcl-1, which might be a possible reason for the observed high levels of Mcl-1 mRNA in oral tumors. Mcl-1 protein has a rapid turnover and possesses a short half-life of about one to 3 hrs. It possess the PEST domain responsible for ubiquitin dependent degradation by the 26S proteosomal machinery [174]. Another reason could be due to loss of S159/T163 phosphorylation sites essential for its detection & degradation, reported to be crucial in nicotine mediated Mcl-1 activation and chemoresistance [175]. Though earlier studies form our lab has shown high Mcl-1protein in OSC versus normals, no statistically significant correlation was found between Mcl-1 protein expression & clinico-pathological parameters of oral cancer patients [19].

Also, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [176]. Interestingly, the anti-apoptotic Mcl-1L was the only predominantly overexpressed variant as compared to both pro-apoptotic Mcl-1S & Mcl-1ES variants indicating that Mcl-1L isoform alone may contribute in the progression of oral cancer. The Mcl-1L not only binds to Mcl-1S but is also known to heterodimerise with pro-apoptotic Bax & Bak etc. preventing the release of cytochrome-c and thereby evading the induction of apoptosis and providing short term survival [94]. Therefore, we looked at the relative ratios of Mcl-1L / Mcl-1S isoforms, which revealed a significant/positive correlation with the poor overall survival of patients, supporting Mcl-1L expression as an independent prognostic factor for oral cancer patients. Our findings are supported by the previous reports in gastric and cervical cancers demonstrating the association between high Mcl-1 expression with tumor size, histological grade, lymph node involvement, metastasis & poor clinical outcome [16, 17]. Also several other studies in breast cancer, ovarian cancer and various hematological malignancies have shown prognostic significance of high Mcl-1 protein expression [140, 177, 178]. However, the studies evaluating the association of the Mcl-1 isoform expression with clinico-pathological parameters are rare.

This is the first study indicating that Mcl-1L expression might be an independent prognostic marker for human oral cancer. Taken together, we have shown that Mcl-1L splice variant was overexpressed in oral cancer cell lines & tumor tissues compared with normal immortalized cell lines and noncancerous tissues. The high Mcl-1L mRNA was correlated with tumor size, nodal involvement & poor overall survival of oral cancer patients. Thus, high Mcl-1L expression was positively correlated with poor prognosis of oral cancer patients. These findings suggest that Mcl-1 may play an important role as a pro-survival factor and could be a potential therapeutic target in oral cancer. However, as cellular expression of Mcl-1 is tightly regulated via multiple mechanisms, further studies are necessary to elucidate the molecular mechanisms and role in the pathogenesis of oral cancer.

6.3 Role of Mcl-1L in radioresistance and/or chemoresistance:

6.3.1 Role of Mcl-1 in Radioresistance:

In the present study we demonstrate the effect of anti-apoptotic Mcl-1L expression on the radiosensitivity of oral cancer cells. So far limited information is available on the role of Mcl-1 in radiation response of tumor cells. To our knowledge this is the first study to report a time course expression of Mcl-1 isoforms post-IR and effect of Mcl-1L knockdown on radiosentitzation of oral cancer cell lines using siRNA strategy. Our studies demonstrated, an inverse correlation of Mcl-1 expression with cellular apoptosis and a synergistic effect of Mcl-1L knockdown along with IR on cell viability and clonogenic survival thereby enhancing the radiosensitivity of OSCC cells.

Various growth factors and cellular stresses like radiation and cytotoxic agents are known to upregulate Mcl-1 levels, thereby enhancing short term viability [174]. Our earlier studies had demonstrated, higher expression of Mcl-1L transcript and its association with poor disease free survival in patients treated with definitive radiotherapy [179, 180]. In the present study two tongue cancer (AW8507 & AW13516) and an immortalized oral (FBM) cell line were used due to their differing radiosensitivities and based on their D_0 values, both AW8507 & AW13516 were demonstrated to be relatively more radioresistant than FBM. Therefore, to evaluate the association of Mcl-1L with radioresistance if any, we evaluated the expression of Mcl-1 isoforms in radioresistant AW8507 & AW13516 as compared to radiosensitive FBM. Our studies revealed higher expression of Mcl-1L at both mRNA and protein level in relatively more radioresistant AW8507 & AW13516 cell line versus FBM, indicating a possible association of anti-apoptotic Mcl-1L splice variant with radioresistance.

Several possible mechanisms can lead to the high Mcl-1 levels in oral cancer cell lines post IR. Mcl-1 is known to be rapidly induced at the transcriptional level and its mRNA has a short half-life [174]. MCL-1 is also regulated at the post-transcriptional level by micro RNAs through a mir29 binding in the 3'UTR of Mcl-1 mRNA [117]. Interestingly, the expression of

mir29 was also found to be decreased in malignant cholangiocytes, favoring the increased levels of Mcl-1, which indicates the possible reason for the observed high levels of Mcl-1 mRNA in our cancer cells lines and also responsible for its immediate degradation within few hours post IR.

Mcl-1, a PEST domain containing protein is also known to undergo ubiquitin-dependent degradation by the 26S proteosome and possesses a short half-life of 1 to 3 hrs & is rapidly downregulated during apoptosis [174]. Notably, the BH3 domains of E3 ligases (MULE/LASU1) specifically interacts with the hydrophobic BH3 binding pocket of Mcl-1 and not with other anti-apoptotic Bcl-2 family members [181] and are responsible for the constitutive turnover of Mcl-1. Such ubiquitin mediated degradation of Mcl-1 has been shown to be essential for the initiation of apoptosis, following UV damage [100]. Hence it was interesting to study the time course expression profile of Mcl-1 isoforms and other bcl-2 family members in the above cell lines post-IR. Also, Mcl-1 protein is known to be phosphorylated by GSK-3β at Ser159, located within the PEST domain, resulting in a significant decrease in the protein half-life and leading to initiation of apoptosis [121]. Such, alterations in the phosphorylation of Mcl-1 protein by GSK3 may also contribute to the elevation of Mcl-1 levels.

Moreover, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [176]. No significant alterations in levels of Mcl-1S & Mcl-1ES were observed post IR, indicating that the predominantly overexpressed Mcl-1L isoform may contribute significantly in the development of radioresistance. Mcl-1L not only binds to Mcl-1S but is also known to heterodimerise with pro-apoptotic Bax, Bak etc. preventing the release of cytochrome-c and subsequent apoptosis [94]. We observed a downregulation of pro-apoptotic Bax & Bak proteins in AW8507 post-IR, coinciding with decreased apoptosis, while in contrast the radiosensitive FBM showed an increase in Bax & Bak protein levels. High expression of anti-apoptotic Bcl-xl was also observed in AW8507 & AW13516 cells. Bcl-xl which has already been shown to be associated with radiosensitivity of

colon cancer cells [182]. Thus, high expression of known radioresistant factors Bcl-xl & Bcl-2 and of Mcl-1L in more radioresistant AW8507 & AW13516 versus FBM may indicate their possible contribution to their radioresistant character.

We assessed the ratios of Mcl-1L/Mcl-1S, Mcl-1L/Bax, Bcl-xl/Bax, wherein radioresistant AW8507& AW13516 showed higher ratios as compared to that in FBM indicating predominance of anti-apoptosis which may contribute to radioresistance. We are the first to elucidate the comparative levels of Mcl-1 isoforms and their association with radioresistance in oral cell lines. The prolonged high expression of Mcl-1L observed in AW8507 & AW13516 could possibly be due to the Mcl-1 protein stabilization via binding with other proteins. Another reason could be its enhanced half-life due to S159/T163 phosphorylation post-IR, reported to be crucial in nicotine mediated Mcl-1 activation and chemoresistance [175].

We observed a significant reduction in apoptosis post-IR which coincided with the high levels of Mcl-1L, indicating a possible association of Mcl-1L expression with radiation response of AW8507 & AW3516 cells. The immunofluorescence staining of Mcl-1 indicated a peri-nuclear accumulation & nuclear localization post IR in more radioresistant AW8507 cell line. Such peri-nuclear accumulation of Mcl-1 has also been observed earlier in polymorphonuclear leukocytes post-etoposide treatment [132]. In AW8507, the observed nuclear and perinuclear accumulation of Mcl-1 may possibly help in cell survival to lower doses of DNA damaging agents. A similar regulatory role for Mcl-1 (snMcl-1), perhaps acting as an adaptor protein in controlling the ATR-mediated regulation of DNA damage checkpoint kinase Chk1 phosphorylation and activation has been reported, placing Mcl-1 at the interface of apoptosis and cell cycle regulation [183]. Mcl-1 has been shown to regulate cell cycle by binding to proteins like CDK1 & PCNA [184] possibly explaining the observed nuclear localization of Mcl-1. High expression of anti-apoptotic Mcl-1L and Bcl-xl proteins and reduced pro-apoptotic proteins like Bak & Bax together may possibly contribute in lowering the sensitivity of AW8507 & AW3516 cells to IR.

The downregulation of Mcl-1L alone leads to the induction of apoptosis, in both AW8507 & AW13516 cells. Interestingly, the combination of Mcl-1L siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. Notably, the expression of closely related Bcl-xl, a known radioresistant factor was not altered. However, the expression of pro-apoptotic Bax protein correlated with the increased apoptosis on Mcl-1L knockdown. This overexpression of Bax, may activate the intrinsic apoptotic pathway resulting in increased cell death. To address the fact that the induction of apoptosis may not necessarily lead to long-term response to radiotherapy we performed the clonogenic assay which demonstrated that combination of IR and Mcl-1L downregulation synergistically reduced clonogenic survival as compared to each treatment alone. Our studies demonstrate that Mcl-1L downregulation potentially enhanced radiosensitivity of AW8507 & AW13516 cells *in vitro*.

Complex interactions occur between Bcl-2 family proteins especially Bak & Bax, where Mcl-1 plays a crucial role in engaging and maintaining pro-apoptotic Bak in an inactive state and accumulates H2AX and ATM proteins to activate DNA repair pathways suggesting that elimination of cellular Mcl-1 may be essential for initiating apoptotic pathways [99]. Overexpression and nuclear accumulation of Mcl-1 in AW8507 may occur due to a protein called IEX-1 which has been shown to interact specifically and timely with Mcl-1 controlling its accumulation and nuclear translocation in response to DNA damage and contribute in the activation of DNA repair pathway by Chk1 activation and G2 checkpoint arrest [185]. The high expression of Mcl-1L in radioresistant sublines developed by fractionated ionizing radiation provides a direct evidence for the role of Mcl-1L in radioresistance of OSCC cells. Thus, the combination of radiotherapy and Mcl-1L downregulation has the potential to improve the response rate of treatment-resistant oral cancer cells.

6.3.2 Role of Mcl-1 in Chemoresistance:

Cisplatin is widely used for chemotherapy of many malignancies, especially of oral squamous cell carcinoma (OSCC). However, the effectiveness of Cisplatin in the treatment of recurrent/metastatic tumors is limited because of acquired or intrinsic resistance [186]. The pro-survival Bcl-2 family members are one of the important factors contributing to the intrinsic resistance to chemotherapy [187]. Interestingly, high Mcl-1 expression has been linked with resistance to Cisplatin in ovarian cancers [188] and high Bcl-xl confers multi drug resistance in several squamous cell carcinoma cell lines [189]. Notably, both Bcl-xL and MCL-1 were known to constitute pertinent targets in ovarian cancers & mesothelioma cells and their concomitant inhibition was sufficient to induce apoptosis [190, 191]. However, to the best of our knowledge no reports are available about the role of both these molecules in oral cancers. Therefore, in present study we investigated the role of anti-apoptotic Bcl-xl and Mcl-1 proteins in chemoresistance of OSCC cells using siRNA mediated knockdown and BH3 mimetic small molecule inhibitor (Obatoclax).

Earlier studies from our lab have demonstrated high expression of both Mcl-1 & Bcl-xl proteins in oral cell lines & tumors. Further, Mcl-1 & Bcl-xl were also shown to be useful as prognostic marker and a predictor of complete tumor response in oral cancer patients respectively [20, 192]. Therefore, it was of interest to study the role of these proteins in chemoresistance of OSCC if any. Our studies indicate, a time dependent depletion of Mcl-1 after Cisplatin treatment in AW8507 cell line, which is consistent with earlier studies in renal tubular epithelial cells where Mcl-1 expression is rapidly declined at the posttranslational level in response to Cisplatin [193]. Further, knockdown of both Bcl-xl & Mcl-1expression using siRNA approach could successfully downregulate Bcl-xl for more than 96 hrs but not Mcl-1. Unlike Bcl-xl, Mcl-1 has a rapid turnover; therefore transient knockdown with siRNA could deplete its levels only upto 48 hrs. In order to overcome this effect a stable system where, Mcl-1 expression was downregulated via an inducible pTRIPZ system transduced in AW8507 cells.

Doxycyclin induced expression of Mcl-1 shRNA has successfully provided a stable & long term knockdown of Mcl-1. Mcl-1 overexpression has been reported in a variety of human hematopoietic cancers, lymphoid cancers including multiple myelomas. Further, it is also suggested that Mcl-1 overexpression could to be a resistance mechanism to conventional cancer therapies in solid tumors [14].

Our results demonstrate induction of cell death post knockdown of both Mcl-1 & Bcl-xl in AW8507 cells. Interestingly, combined depletion of both Mcl-1 & Bcl-xl proteins and Cisplatin treatment significantly reduced cell viability and proliferation compared to any treatment alone, indicating that Mcl-1 & Bcl-xl expression is crucial for survival of oral cancer cells. Bcl-xl and Mcl-1 appear to be important targets in oral squamous carcinomas and using small BH3-mimetics molecules we could induce apoptosis in such cells. In this regard, Obatoclax is a promising BH3-mimetics as it exerts both the conventional BH3-mimetic effect and has the proven ability to overcome resistance generated by Mcl-1 overexpression (unlike other BH3 mimetic like ABT737) [141]. Our results indicate that Obatoclax treatment can successfully sensitize oral cancer cells to Cisplatin treatment by decreasing proliferation and inducing cell death. Thus, BH3-mimetic Obatoclax has the potential use in the treatment of oral cancer. The development of multitargeted therapies directed against Bcl-xl and Mcl-1 constitutes a major challenge for the therapeutic care of chemo resistant oral cancers. However, selective small molecule inhibitors like Obatoclax, which specifically overcome Mcl-1 mediated resistance, is already in phase2 clinical trials [194] and may have important therapeutic implications, when used in combination with chemo/radiotherapy in treatment of oral cancer patients.

CHAPTER 7

SUMMARY & CONCLUSION

SUMMARY & CONCLUSION:

Our studies demonstrate the following-

- ***Mcl-1 isoform expression in oral cancer-***
  - High expression of Mcl-1 transcripts / proteins in oral cancer cell lines, premalignant oral submucous fibrosis & oral tumors as compared to normal mucosa.
  - Five to ten fold higher expression of anti-apoptotic, full length Mcl-1L isoform versus the short & extra short Mcl-1S & Mcl-1ES isoforms in oral tumors & cell lines.
  - High ratio of Mcl-1L/Mcl-1S in oral tumors versus adjacent normals.
  - Significant correlation of high Mcl-1L expression with node positivity, advanced tumors and poor overall survival of oral cancer patients.

  - ***Thus, the predominantly expressed anti-apoptotic Mcl-1L isoform appears to be an independent prognostic factor for oral cancer.***

- ***Mechanism of Mcl-1 over expression-***
  - Absence of genomic alterations in Mcl-1 gene (Promoter, 2 introns & 3 exons) in 10 oral cell lines.
  - Presence of 6-nt polymorphism in Mcl-1 promoter of SCC15 & SCC40 cancer cells & absence of 18-nt polymorphism in 11 Mcl-1 expressing oral cell lines.
  - Presence of 18-nt polymorphism in 7/20 (35%) healthy volunteers and 9/40 (23%) oral cancer patients.
  - Presence of 6/18-nt polymorphism did not correlate with either Mcl-1 gene expression or with clinico-pathological parameters of oral cancer patients.
  - Occurrence of two reported SNP's namely C<A-324 &G<C-386 in Mcl-1 gene promoter of oral cancer patients
  - Phosphorylation of Mcl-1 protein at Ser-159 & Thr-163 residues, which is essential for its detection by E3 ligases and further proteosomal degradation.

  - ***Genomic alterations, 6/18-nt polymorphism in Mcl-1 gene & altered phosphorylation of Mcl-1 protein are possibly not responsible for its overexpression in oral cancers.***

- ***Role of Mcl-1 in Radioresistance and Chemoresistance-***

  - In relatively radioresistant AW8507 versus FBM, post IR-
    - Consistent & prolonged high expression of Mcl-1L mRNA & protein and decrease in percent of apoptosis.
    - Higher anti-apoptotic versus pro-apoptotic proteins ratios (Mcl-1L/Mcl-1S, Mcl-1L/Bax & Bcl-xl/Bax).
    - Higher perinuclear accumulation and nuclear localization of Mcl-1 protein post-radiation treatment.
  - High expression of Mcl-1 in the radioresistant sublines of AW13516 cell line obtained using fractionated irradiation.
  - Combined treatment of Mcl-1L knockdown and IR resulted in increased nuclear condensation, upregulation of Bax & induction of apoptosis as compared to any treatments alone.
  - Double knockdown of Mcl-1 & Bcl-xl in combination with Cisplatin significantly reduced cell viability & proliferation as compared to any treatment alone.
  - Small molecule anti-Mcl-1 drug, Obatoclax in combination with Cisplatin exhibited significantly higher induction of cell death and reduction cell proliferation as compared to any treatment alone.

- ***Mcl-1L downregulation potentially enhanced radiosensitivity and chemosensitivity of oral cancer cells in vitro.***

Thus, our studies demonstrate overexpression of anti-apoptotic Mcl-1L isoform in oral cancers. Further Mcl-1L appears to be an important pro-survival and radioresistance / chemoresistance related factor, influencing outcome of oral cancer patients. It may therefore be a potential therapeutic target in oral cancers.

REFERENCES

References:

1. Tandle, A., V. Sanghvi, and D. Saranath, *Determination of p53 genotypes in oral cancer patients from India.* British journal of cancer, 2001. 84(6): p. 739.

2. Dikshit, R., et al., *Cancer mortality in India: a nationally representative survey.* The Lancet, 2012.

3. Massano, J., et al., *Oral squamous cell carcinoma: review of prognostic and predictive factors.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2006. 102(1): p. 67-76.

4. Elango, J.K., et al., *Factors affecting oral cancer awareness in a high-risk population in India.* Asian Pac J Cancer Prev, 2009. 10(4): p. 627-30.

5. Notani, P.N., *Global variation in cancer incidence and mortality.* Curr Sci, 2001. 81(5): p. 465-474.

6. Mehrotra, R. and S. Yadav, *Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations.* Indian J Cancer, 2006. 43(2): p. 60-6.

7. Loro, L., O. Vintermyr, and A. Johannessen, *Cell death regulation in oral squamous cell carcinoma: methodological considerations and clinical significance.* Journal of oral pathology & medicine, 2003. 32(3): p. 125-138.

8. Johnstone, R.W., A.A. Ruefli, and S.W. Lowe, *Apoptosis-A Link between Cancer Genetics and Chemotherapy.* Cell, 2002. 108(2): p. 153-164.

9. Borner, C., *The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions.* Molecular immunology, 2003. 39(11): p. 615-647.

10. Levine, B., S.C. Sinha, and G. Kroemer, *Bcl-2 family members: dual regulators of apoptosis and autophagy.* Autophagy, 2008. 4(5): p. 600-606.

11. Kyprianou, N., et al., *bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells.* International journal of cancer, 1997. 70(3): p. 341-348.

12. Wang, Z.B., et al., *Bcl-xL overexpression restricts [gamma]-radiation-induced apoptosis.* Cell biology international, 2006. 30(1): p. 15-20.

13. Craig, R., *MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis.* Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK, 2002. 16(4): p. 444.

14. Akgul, C., *Mcl-1 is a potential therapeutic target in multiple types of cancer.* Cellular and molecular life sciences, 2009. 66(8): p. 1326-1336.

15. Nagata, M., et al., *Role of myeloid cell leukemia-1 in cell growth of squamous cell carcinoma.* Journal of pharmacological sciences, 2009. 110(3): p. 344-353.

16. Likui, W., et al., *Prognostic role of myeloid cell leukemia-1 protein (Mcl-1) expression in human gastric cancer.* Journal of surgical oncology, 2009. 100(5): p. 396-400.

17. Zhang, T., et al., *The expression of Mcl-1 in human cervical cancer and its clinical significance.* Medical Oncology, 2012: p. 1-7.

18. O'DRISCOLL, L., et al., *Expression and prognostic relevance of Mcl-1 in breast cancer.* Anticancer research, 2004. 24(2A): p. 473-482.

19. Mallick, S., et al., *Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of Mcl-1.* The Journal of pathology, 2009. 217(3): p. 398-407.

20. Mallick, S., et al., *PCNA and anti-apoptotic Mcl-1 proteins predict disease-free survival in oral cancer patients treated with definitive radiotherapy.* Oral oncology, 2010. 46(9): p. 688-693.

21. SKVARA, H., et al., *Mcl-1 blocks radiation-induced apoptosis and inhibits clonogenic cell death.* Anticancer research, 2005. 25(4): p. 2697-2703.

22. Guoan, X., et al., *Adenovirus-mediated siRNA targeting Mcl-1 gene increases radiosensitivity of pancreatic carcinoma cells in vitro and in vivo.* Surgery, 2010. 147(4): p. 553-561.

23. Elango, J.K., et al., *Trends of head and neck cancers in urban and rural India.* Asian Pacific journal of cancer prevention, 2006. 7(1): p. 108.

24. Sankaranarayanan, R., et al., *Effect of screening on oral cancer mortality in Kerala, India: a cluster-randomised controlled trial.* The Lancet, 2005. 365(9475): p. 1927-1933.

25. Boffetta, P., et al., *Smokeless tobacco and cancer.* The lancet oncology, 2008. 9(7): p. 667.

26. Ali, I., W.A. Wani, and K. Saleem, *Cancer Scenario in India with Future Perspectives.* Cancer Therapy, 2011. 8: p. 56-70.

27. Balaram, P., et al., *Oral cancer in southern India: The influence of smoking, drinking, paan-chewing and oral hygiene.* International journal of cancer, 2002. 98(3): p. 440-445.

28. Muwonge, R., et al., *Role of tobacco smoking, chewing and alcohol drinking in the risk of oral cancer in Trivandrum, India: a nested case-control design using incident cancer cases.* Oral oncology, 2008. 44(5): p. 446.

29. Dikshit, R.P. and S. Kanhere, *Tobacco habits and risk of lung, oropharyngeal and oral cavity cancer: a population-based case-control study in Bhopal, India.* International journal of epidemiology, 2000. 29(4): p. 609-614.

30. Pakhale, S., K. Jayant, and S. Bhide, *Methods of reduction of harmful constituents in bidi smoke.* The Indian journal of chest diseases & allied sciences, 1985. 27(3): p. 148.

31. Notani, P.N., *Role of alcohol in cancers of the upper alimentary tract: use of models in risk assessment.* Journal of epidemiology and community health, 1988. 42(2): p. 187-192.

32. Varshney, P., N. Agrawal, and L. Bariar, *Tobacco and alcohol consumption in relation to oral cancer.* Indian Journal of Otolaryngology and Head & Neck Surgery, 2003. 55(1): p. 25-28.

33. Tuyns, A.J., et al., *Cancer of the larynx/hypopharynx, tobacco and alcohol: Iarc international case-control study in Turin and Varese (Italy), Zaragoza and Navarra (Spain), Geneva (Switzerland) and Calvados (France).* International journal of cancer, 2006. 41(4): p. 483-491.

34. Pöschl, G. and H.K. Seitz, *Alcohol and cancer.* Alcohol and Alcoholism, 2004. 39(3): p. 155-165.

35. Gillison, M.L., et al., *Evidence for a causal association between human papillomavirus and a subset of head and neck cancers.* Journal of the National Cancer Institute, 2000. 92(9): p. 709-720.

36. Scully, C., *Oral squamous cell carcinoma; from an hypothesis about a virus, to concern about possible sexual transmission.* Oral oncology, 2002. 38(3): p. 227-234.

37. Negri, E., et al., *Selected micronutrients and oral and pharyngeal cancer.* International journal of cancer, 2000. 86(1): p. 122-127.

38. Parkin, D., *Cancer incidence in five continents*. Vol. 1. 2006: Iarc.

39. Gupta, P.C., et al., *Influence of dietary factors on oral precancerous lesions in a population-based case–control study in Kerala, India.* Cancer, 2000. 85(9): p. 1885-1893.

40. Zain, R.B., *Cultural and dietary risk factors of oral cancer and precancer—a brief overview.* Oral oncology, 2001. 37(3): p. 205-210.

41. Amagasa, T., M. Yamashiro, and N. Uzawa, *Oral premalignant lesions: from a clinical perspective.* International Journal of Clinical Oncology, 2011. 16(1): p. 5-14.

42. Neville, B.W. and T.A. Day, *Oral cancer and precancerous lesions.* CA: a cancer journal for clinicians, 2009. 52(4): p. 195-215.

43. Haider, S., et al., *Clinical and functional staging of oral submucous fibrosis.* British Journal of Oral and Maxillofacial Surgery, 2000. 38(1): p. 12-15.

44. Warnakulasuriya, K. and R. Ralhan, *Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco–a review.* Journal of oral pathology & medicine, 2007. 36(2): p. 63-77.

45. Warnakulasuriya, S., N.W. Johnson, and I. Van der Waal, *Nomenclature and classification of potentially malignant disorders of the oral mucosa.* Journal of oral pathology & medicine, 2007. 36(10): p. 575-580.

46. Bouquot, J.E. and H. Ephros, *Erythroplakia: the dangerous red mucosa.* Pract Periodontics Aesthet Dent, 1995. 7(6): p. 59-67.

47. Jeng, J., M. Chang, and L. Hahn, *Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives.* Oral oncology, 2001. 37(6): p. 477.

48. Murti, P., et al., *Malignant transformation rate in oral submucous fibrosis over a 17-year period.* Community dentistry and oral epidemiology, 2006. 13(6): p. 340-341.

49. Fu, K.K., *Combined-modality therapy for head and neck cancer.* Oncology-Huntington, 1997. 11(12): p. 1781-1795.

50. Browman, G., et al., *Head and Neck Cancer Disease Site Group. Choosing a concomitant chemotherapy and radiotherapy regimen for squamous cell head and neck cancer: a systematic review of the published literature with subgroup analysis.* Head Neck, 2001. 23(7): p. 579-589.

51. Boring, C.C., et al., *Cancer statistics, 1994.* CA: a cancer journal for clinicians, 2008. 44(1): p. 7-26.

52. Zygogianni, A.G., et al., *Oral squamous cell cancer: early detection and the role of alcohol and smoking.* Head Neck Oncol, 2011. 3(2).

53. Golstein, P. and G. Kroemer, *Cell death by necrosis: towards a molecular definition.* Trends in biochemical sciences, 2007. 32(1): p. 37-43.

54. Kroemer, G., et al., *Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009.* Cell Death & Differentiation, 2008. 16(1): p. 3-11.

55. Hotchkiss, R.S., et al., *Cell death.* New England Journal of Medicine, 2009. 361(16): p. 1570-1583.

56. Dykhuizen, E., *Means to an End: Apoptosis and Other Cell Death Mechanisms. By Douglas R. Green. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press. 79.00(hardcover); 45.00 (paper). xii+ 220 p.; ill.; index. ISBN: 978-0-87969-887-4 (hc); 978-0-87969-888-1 (pb). 2011*, 2012, JSTOR. p. 68-68.

57. Horvitz, H.R., *Genetic control of programmed cell death in the nematode Caenorhabditis elegans.* Cancer research, 1999. 59(7 Supplement): p. 1701s-1706s.

58. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicologic pathology, 2007. 35(4): p. 495-516.

59. Locksley, R.M., N. Killeen, and M.J. Lenardo, *The TNF and TNF receptor superfamilies: integrating mammalian biology.* Cell, 2001. 104(4): p. 487.

60. Suliman, A., et al., *Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and-independent pathways.* Oncogene, 2001. 20(17): p. 2122.

61. Wajant, H., *The Fas signaling pathway: more than a paradigm.* Science Signalling, 2002. 296(5573): p. 1635.

62. Taylor, R.C., S.P. Cullen, and S.J. Martin, *Apoptosis: controlled demolition at the cellular level.* Nature Reviews Molecular Cell Biology, 2008. 9(3): p. 231-241.

63. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science Signalling, 2000. 288(5468): p. 1053.

64. Goldstein, J., et al., *Cytochrome c is released in a single step during apoptosis.* Cell Death & Differentiation, 2005. 12(5): p. 453-462.

65. Strasser, A., *The role of BH3-only proteins in the immune system.* Nature Reviews Immunology, 2005. 5(3): p. 189-200.

66. Cory, S. and J.M. Adams, *The Bcl2 family: regulators of the cellular life-or-death switch.* Nature Reviews Cancer, 2002. 2(9): p. 647-656.

67. Adams, J.M. and S. Cory, *The Bcl-2-regulated apoptosis switch: mechanism and therapeutic potential.* Current opinion in immunology, 2007. 19(5): p. 488.

68. Youle, R.J. and A. Strasser, *The BCL-2 protein family: opposing activities that mediate cell death.* Nature Reviews Molecular Cell Biology, 2008. 9(1): p. 47-59.

69. Marsden, V.S. and A. Strasser, *Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more.* Annual review of immunology, 2003. 21(1): p. 71-105.

70. McKenzie, M.D., et al., *Proapoptotic BH3-only protein Bid is essential for death receptor–induced apoptosis of pancreatic β-cells.* Diabetes, 2008. 57(5): p. 1284-1292.

71. Chipuk, J.E. and D.R. Green, *How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?* Trends in cell biology, 2008. 18(4): p. 157-164.

72. Kuwana, T., et al., *BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly.* Molecular cell, 2005. 17(4): p. 525-536.

73. Jeffers, J.R., et al., *Puma is an essential mediator of p53-dependent and-independent apoptotic pathways.* Cancer cell, 2003. 4(4): p. 321-328.

74. Hsu, S., B. Singh, and G. Schuster, *Induction of apoptosis in oral cancer cells: agents and mechanisms for potential therapy and prevention.* Oral oncology, 2004. 40(5): p. 461-473.

75. Reed, J.C., *Apoptosis-targeted therapies for cancer.* Cancer cell, 2003. 3(1): p. 17.

76. Makin, G. and J.A. Hickman, *Apoptosis and cancer chemotherapy.* Cell and tissue research, 2000. 301(1): p. 143-152.

77. Wong, R., *Apoptosis in cancer: from pathogenesis to treatment.* J Exp Clin Cancer Res, 2011. 30(1): p. 87.

78. Polverini, P. and J. Nor, *Apoptosis and predisposition to oral cancer.* Critical Reviews in Oral Biology & Medicine, 1999. 10(2): p. 139-152.

79. Birchall, M., et al., *Apoptosis in normal epithelium, premalignant and malignant lesions of the oropharynx and oral cavity: a preliminary study.* European Journal of Cancer. Part B: Oral Oncology, 1995. 31(6): p. 380-383.

80. Xie, X., et al., *The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue.* Cancer, 2000. 86(6): p. 913-920.

81. Zhang, M., et al., *Prognostic significance of Bcl-2 and Bax protein expression in the patients with oral squamous cell carcinoma.* Journal of oral pathology & medicine, 2009. 38(3): p. 307-313.

82. BALTAZIAK, M., et al., *Expression of Bcl-xL, Bax, and p53 in Primary Tumors and Lymph Node Metastases in Oral Squamous Cell Carcinoma.* Annals of the New York Academy of Sciences, 2006. 1090(1): p. 18-25.

83. Camisasca, D.R., et al., *Expression of Bcl-2 family proteins and associated clinicopathologic factors predict survival outcome in patients with oral squamous cell carcinoma.* Oral oncology, 2009. 45(3): p. 225-233.

84. Kato, K., et al., *Apoptosis-associated markers and clinical outcome in human oral squamous cell carcinomas.* Journal of oral pathology & medicine, 2008. 37(6): p. 364-371.

85. Kassouf, N. and M.H. Thornhill, *Oral cancer cell lines can use multiple ligands, including Fas-L, TRAIL and TNF-α, to induce apoptosis in Jurkat T cells: possible mechanisms for immune escape by head and neck cancers.* Oral oncology, 2008. 44(7): p. 672-682.

86. Stoll, C., et al., *Prognostic significance of apoptosis and associated factors in oral squamous cell carcinoma.* Virchows Archiv, 2000. 436(2): p. 102-108.

87. Jia, L., et al., *Prognostic value of apoptosis and apoptosis-associated proteins in salivary gland adenoid cystic carcinoma.* Pathology international, 2004. 54(4): p. 217-223.

88. Jane, C., et al., *Increased survivin expression in high-grade oral squamous cell carcinoma: a study in Indian tobacco chewers.* Journal of oral pathology & medicine, 2006. 35(10): p. 595-601.

89. Lin, C.Y., et al., *Survivin expression predicts poorer prognosis in patients with areca quid chewing-related oral squamous cell carcinoma in Taiwan.* Oral oncology, 2005. 41(6): p. 645.

90. Evan, G.I. and K.H. Vousden, *Proliferation, cell cycle and apoptosis in cancer.* Nature, 2001. 411(6835): p. 342-348.

91. O'Connor, O., *Apoptosis: from biology to therapeutic targeting.* Annals of Oncology, 2011. 22(suppl 4): p. iv76-iv79.

92. Kozopas, K.M., et al., *MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2.* Proceedings of the National Academy of Sciences, 1993. 90(8): p. 3516-3520.

93. Michels, J., P.W.M. Johnson, and G. Packham, *Mcl-1*. The international journal of biochemistry & cell biology, 2005. 37(2): p. 267-271.

94. Thomas, L.W., C. Lam, and S.W. Edwards, *Mcl-1; the molecular regulation of protein function.* FEBS letters, 2010. 584(14): p. 2981-2989.

95. Bae, J., et al., *MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain.* Journal of Biological Chemistry, 2000. 275(33): p. 25255-25261.

96. Kim, J.H., et al., *MCL-1ES, a novel variant of MCL-1, associates with MCL-1L and induces mitochondrial cell death.* FEBS letters, 2009. 583(17): p. 2758-2764.

97. Bingle, C.D., et al., *Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death.* Journal of Biological Chemistry, 2000. 275(29): p. 22136-22146.

98. Akgul, C., et al., *In vivo localisation and stability of human Mcl-1 using green fluorescent protein (GFP) fusion proteins.* FEBS letters, 2000. 478(1): p. 72-76.

99. Cuconati, A., et al., *DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells.* Genes & development, 2003. 17(23): p. 2922-2932.

100. Nijhawan, D., et al., *Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation.* Genes & development, 2003. 17(12): p. 1475-1486.

101. Weng, C., et al., *Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells.* Journal of Biological Chemistry, 2005. 280(11): p. 10491-10500.

102. Kobayashi, S., et al., *Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1*. Journal of Biological Chemistry, 2007. 282(25): p. 18407-18417.

103. Shimazu, T., et al., *NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition.* Genes & development, 2007. 21(8): p. 929-941.

104. Willis, S.N., et al., *Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins.* Genes & development, 2005. 19(11): p. 1294-1305.

105. Clohessy, J.G., et al., *Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis.* Journal of Biological Chemistry, 2006. 281(9): p. 5750-5759.

106. Han, J., et al., *Functional linkage between NOXA and Bim in mitochondrial apoptotic events.* Journal of Biological Chemistry, 2007. 282(22): p. 16223-16231.

107. Kim, H., et al., *Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies.* Nature cell biology, 2006. 8(12): p. 1348-1358.

108. Isomoto, H., et al., *Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells.* Hepatology, 2005. 42(6): p. 1329-1338.

109. Aichberger, K.J., et al., *Identification of mcl-1 as a BCR/ABL-dependent target in chronic myeloid leukemia (CML): evidence for cooperative antileukemic effects of imatinib and mcl-1 antisense oligonucleotides.* Blood, 2005. 105(8): p. 3303-3311.

110. Tsutsui, M., et al., *Frequent STAT3 activation is associated with Mcl-1 expression in nasal NK-cell lymphoma.* International journal of laboratory hematology, 2009. 32(4): p. 419-426.

111. Wang, J.M., et al., *The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB.* Molecular and cellular biology, 1999. 19(9): p. 6195-6206.

112. Wang, J.M., M.Z. Lai, and H.F. Yang-Yen, *Interleukin-3 stimulation of mcl-1 gene transcription involves activation of the PU. 1 transcription factor through a p38 mitogen-activated protein kinase-dependent pathway.* Molecular and cellular biology, 2003. 23(6): p. 1896-1909.

113. Piret, J.P., et al., *Hypoxia-inducible factor-1-dependent overexpression of myeloid cell factor-1 protects hypoxic cells against tert-butyl hydroperoxide-induced apoptosis.* Journal of Biological Chemistry, 2005. 280(10): p. 9336-9344.

114. Vickers, E.R., et al., *Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death.* Molecular and cellular biology, 2004. 24(23): p. 10340-10351.

115. Austin, M. and S.J. Cook, *Increased expression of Mcl-1 is required for protection against serum starvation in phosphatase and tensin homologue on chromosome 10 null mouse embryonic fibroblasts, but repression of Bim is favored in human glioblastomas.* Journal of Biological Chemistry, 2005. 280(39): p. 33280-33288.

116. Moulding, D.A., et al., *BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis.* Journal of leukocyte biology, 2001. 70(5): p. 783-792.

117. Mott, J.L., et al., *mir-29 regulates Mcl-1 protein expression and apoptosis.* Oncogene, 2007. 26(42): p. 6133-6140.

118. Subramaniam, D., et al., *Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2008. 294(4): p. G1025-G1032.

119. Mills, J.R., et al., *mTORC1 promotes survival through translational control of Mcl-1.* Proceedings of the National Academy of Sciences, 2008. 105(31): p. 10853-10858.

120. Domina, A.M., et al., *MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol.* Oncogene, 2004. 23(31): p. 5301-5315.

121. Maurer, U., et al., *Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1.* Molecular cell, 2006. 21(6): p. 749-760.

122. Inoshita, S., et al., *Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress.* Journal of Biological Chemistry, 2002. 277(46): p. 43730-43734.

123. Derouet, M., et al., *Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1.* Journal of Biological Chemistry, 2004. 279(26): p. 26915-26921.

124. Opferman, J., *Unraveling MCL-1 degradation.* Cell Death & Differentiation, 2006. 13(8): p. 1260-1262.

125. Michels, J., et al., *Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage.* Oncogene, 2004. 23(28): p. 4818-4827.

126. Rinkenberger, J.L., et al., *Mcl-1 deficiency results in peri-implantation embryonic lethality.* Genes & development, 2000. 14(1): p. 23-27.

127. Opferman, J.T., et al., *Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1.* Nature, 2003. 426(6967): p. 671-676.

128. Opferman, J.T., et al., *Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells.* Science Signalling, 2005. 307(5712): p. 1101.

129. Steimer, D.A., et al., *Selective roles for antiapoptotic MCL-1 during granulocyte development and macrophage effector function.* Blood, 2009. 113(12): p. 2805-2815.

130. Liu, H., et al., *Mcl-1 is essential for the survival of synovial fibroblasts in rheumatoid arthritis.* The Journal of Immunology, 2005. 175(12): p. 8337-8345.

131. Vick, B., et al., *Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes.* Hepatology, 2008. 49(2): p. 627-636.

132. Leuenroth, S.J., et al., *The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis.* Journal of leukocyte biology, 2000. 68(1): p. 158-166.

133. Derenne, S., et al., *Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-xL is an essential survival protein of human myeloma cells.* Blood, 2002. 100(1): p. 194-199.

134. Chetoui, N., et al., *Down-regulation of mcl-1 by small interfering RNA sensitizes resistant melanoma cells to fas-mediated apoptosis.* Molecular Cancer Research, 2008. 6(1): p. 42-52.

135. Lestini, B.J., et al., *Mcl1 downregulation sensitizes neuroblastoma to cytotoxic chemotherapy and small molecule Bcl2-family antagonists.* Cancer biology & therapy, 2009. 8(16): p. 1587-1595.

136. Thallinger, C., et al., *Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model.* Journal of investigative dermatology, 2003. 120(6): p. 1081-1086.

137. Zhou, P., et al., *MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes.* Blood, 2001. 97(12): p. 3902-3909.

138. Zhang, H., et al., *Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines.* Oncogene, 2010. 30(16): p. 1963-1968.

139. Yang-Yen, H.F., *Mcl-1: a highly regulated cell death and survival controller.* Journal of biomedical science, 2006. 13(2): p. 201-204.

140. Shigemasa, K., et al., *Increased MCL–1 Expression Is Associated with Poor Prognosis in Ovarian Carcinomas.* Cancer science, 2002. 93(5): p. 542-550.

141. Nguyen, M., et al., *Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis.* Proceedings of the National Academy of Sciences, 2007. 104(49): p. 19512-19517.

142. Hussain, S.R.A., et al., *Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity.* Clinical Cancer Research, 2007. 13(7): p. 2144-2150.

143. Paoluzzi, L., et al., *Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in in vitro and in vivo models of B-cell lymphoma.* Blood, 2008. 111(11): p. 5350-5358.

144. Fu, J., et al., *The effects of Mcl-1 gene on ATRA-resistant HL-60 cell].* Zhonghua xue ye xue za zhi= Zhonghua xueyexue zazhi, 2005. 26(6): p. 352.

145. Thallinger, C., et al., *Mcl-1 Is a Novel Therapeutic Target for Human Sarcoma Synergistic Inhibition of Human Sarcoma Xenotransplants by a Combination of Mcl-1 Antisense Oligonucleotides with Low-Dose Cyclophosphamide.* Clinical Cancer Research, 2004. 10(12): p. 4185-4191.

146. van Delft, M.F., et al., *The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized.* Cancer cell, 2006. 10(5): p. 389-399.

147. Chen, S., et al., *Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation.* Cancer research, 2007. 67(2): p. 782-791.

148. ME, O.S., et al., *Current strategies to target the anti-apoptotic Bcl-2 protein in cancer cells.* Current medicinal chemistry, 2004. 11(8): p. 1031-1040.

149. Shore, G.C. and M.R. Warr, *Unique biology of Mcl-1: therapeutic opportunities in cancer.* Current molecular medicine, 2008. 8(2): p. 138-147.

150. Masood, A., A.S. Azmi, and R.M. Mohammad, *Small Molecule Inhibitors of Bcl-2 Family Proteins for Pancreatic Cancer Therapy.* Cancers, 2011. 3(2): p. 1527-1549.

151. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative CT method.* Nature protocols, 2008. 3(6): p. 1101-1108.

152. Zhang, C., et al., *Synergistic antitumor activity of gemcitabine and ABT-737 in vitro and in vivo through disrupting the interaction of USP9X and Mcl-1.* Molecular Cancer Therapeutics, 2011. 10(7): p. 1264-1275.

153. Franken, N.A.P., et al., *Clonogenic assay of cells in vitro.* Nature protocols, 2006. 1(5): p. 2315-2319.

154. Fukuda, K., et al., *Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation.* British journal of cancer, 2004. 91(8): p. 1543-1550.

155. Dalby, B., et al., *Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications.* Methods, 2004. 33(2): p. 95-103.

156. Boden, D., et al., *Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins.* Nucleic acids research, 2004. 32(3): p. 1154-1158.

157. Moshynska, O., et al., *Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2004. 96(9): p. 673-682.

158. Nenning, U.C., et al., *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1091-1092.

159. Dicker, F., et al., *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1092-1093.

160. Saxena, A., et al., *Short nucleotide polymorphic insertions in the MCL-1 promoter affect gene expression.* Cancer letters, 2007. 251(1): p. 114-131.

161. Iglesias-Serret, D., et al., *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1090-1091.

162. Vargas, R.L., R.E. Felgar, and P.G. Rothberg, *Re: Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1089-1090.

163. Tobin, G., et al., *Mcl-1 gene promoter insertions do not correlate with disease outcome, stage or VH gene mutation status in chronic lymphocytic leukaemia.* Leukemia, 2005. 19(5): p. 871-873.

164. Coenen, S., et al., *The relevance of sequence insertions in the Mcl-1 promoter in chronic lymphocytic leukemia and in normal cells.* haematologica, 2005. 90(9): p. 1285-1286.

165. Kitada, S. and J.C. Reed, *MCL-1 promoter insertions dial-up aggressiveness of chronic leukemia.* Journal of the National Cancer Institute, 2004. 96(9): p. 642-643.

166. Moshynska, O. and A. Saxena, *RESPONSE: Re: Prognostic Significance of a Short Sequence Insertion in the MCL-1 Promoter in Chronic Lymphocytic Leukemia.* Journal of the National Cancer Institute, 2005. 97(14): p. 1093-1095.

167. Jiang, Y., et al., *Functional regulatory variants of MCL1 contribute to enhanced promoter activity and reduced risk of lung cancer in nonsmokers: Implications for context-dependent phenotype of an antiapoptotic and antiproliferative gene in solid tumor.* Cancer, 2011. 118(8): p. 2085-2095.

168. Sieghart, W., et al., *Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy.* Journal of hepatology, 2006. 44(1): p. 151.

169. Zhuang, L., et al., *Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma.* Modern pathology, 2007. 20(4): p. 416-426.

170. Sano, M., et al., *Overexpression of anti-apoptotic Mcl-1 in testicular germ cell tumours.* Histopathology, 2005. 46(5): p. 532-539.

171. Chung, T., et al., *Expression of apoptotic regulators and their significance in cervical cancer.* Cancer letters, 2002. 180(1): p. 63-68.

172. Schulze-Bergkamen, H., et al., *Bcl-xL and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells.* World journal of gastroenterology: WJG, 2008. 14(24): p. 3829.

173. Kempkensteffen, C., et al., *Expression of Mcl-1 splicing variants in clear-cell renal cancer and their correlation with histopathological parameters and prognosis.* Tumor Biology, 2009. 30(2): p. 73-79.

174. Opferman, J.T., *Unraveling MCL-1 degradation.* Cell Death Differ, 2006. 13(8): p. 1260-2.

175. Zhao, J., et al., *Nicotine enhances the antiapoptotic function of Mcl-1 through phosphorylation.* Molecular Cancer Research, 2009. 7(12): p. 1954-1961.

176. Bae, J., et al., *MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain.* J Biol Chem, 2000. 275(33): p. 25255-61.

177. Pepper, C., et al., *Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers.* Blood, 2008. 112(9): p. 3807-3817.

178. Ding, Q., et al., *Myeloid cell leukemia-1 inversely correlates with glycogen synthase kinase-3β activity and associates with poor prognosis in human breast cancer.* Cancer research, 2007. 67(10): p. 4564-4571.

179. Mallick, S., et al., *Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of Mcl-1.* J Pathol, 2009. 217(3): p. 398-407.

180. Mallick, S., et al., *PCNA and anti-apoptotic Mcl-1 proteins predict disease-free survival in oral cancer patients treated with definitive radiotherapy.* Oral Oncol, 2010. 46(9): p. 688-93.

181. Warr, M.R., et al., *BH3-ligand regulates access of MCL-1 to its E3 ligase.* FEBS letters, 2005. 579(25): p. 5603-5608.

182. Wacheck, V., et al., *Bcl-x(L) antisense oligonucleotides radiosensitise colon cancer cells.* Br J Cancer, 2003. 89(7): p. 1352-7.

183. Jamil, S., et al., *A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1.* Biochemical Journal, 2005. 387(Pt 3): p. 659.

184. Yang-Yen, H.F., *Mcl-1: a highly regulated cell death and survival controller.* J Biomed Sci, 2006. 13(2): p. 201-4.

185. Pawlikowska, P., et al., *ATM-dependent expression of IEX-1 controls nuclear accumulation of Mcl-1 and the DNA damage response.* Cell Death & Differentiation, 2010. 17(11): p. 1739-1750.

186. Hiraishi, Y., et al., *EGFR inhibitor enhances cisplatin sensitivity of oral squamous cell carcinoma cell lines.* Pathology & Oncology Research, 2008. 14(1): p. 39-43.

187. Longley, D. and P. Johnston, *Molecular mechanisms of drug resistance.* The Journal of pathology, 2005. 205(2): p. 275-292.

188. Simonin, K., et al., *Mcl-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin-resistant ovarian carcinoma cells.* Molecular Cancer Therapeutics, 2009. 8(11): p. 3162-3170.

189. Noutomi, T., et al., *Bcl-x< sub> L</sub> confers multi-drug resistance in several squamous cell carcinoma cell lines.* Oral oncology, 2002. 38(1): p. 41-48.

190. Brotin, E., et al., *Bcl-XL and MCL-1 constitute pertinent targets in ovarian carcinoma and their concomitant inhibition is sufficient to induce apoptosis.* International journal of cancer, 2010. 126(4): p. 885-895.

191. Varin, E., et al., *Downregulation of Bcl-xL and Mcl-1 is sufficient to induce cell death in mesothelioma cells highly refractory to conventional chemotherapy.* Carcinogenesis, 2010. 31(6): p. 984-993.

192. Mallick, S., et al., *Bcl-xL protein: Predictor of complete tumor response in patients with oral cancer treated with curative radiotherapy.* Head & neck, 2012.

193. Yang, C., et al., *Mcl-1 is downregulated in cisplatin-induced apoptosis, and proteasome inhibitors restore Mcl-1 and promote survival in renal tubular epithelial cells.* American Journal of Physiology-Renal Physiology, 2007. 292(6): p. F1710-F1717.

194. Paik, P.K., et al., *A phase II study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in relapsed small cell lung cancer*. Lung Cancer, 2011. 74(3): p. 481-485.

195. Taniai, M., et al., *Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells.* Cancer research, 2004. 64(10): p. 3517-3524.

196. Campbell, C.J.V., et al., *The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity.* Blood, 2010. 116(9): p. 1433-1442.

197. Booy, E., E. Henson, and S. Gibson, *Epidermal growth factor regulates Mcl-1 expression through the MAPK-Elk-1 signalling pathway contributing to cell survival in breast cancer.* Oncogene, 2011. 30(20): p. 2367-2378.

PUBLICATIONS

RADIATION ONCOLOGY

RESEARCH Open Access

Association of anti-apoptotic Mcl-1L isoform expression with radioresistance of oral squamous carcinoma cells

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Abstract

Background: Oral cancer is a common cancer and a major health problem in the Indian subcontinent. At our laboratory Mcl-1, an anti-apoptotic member of the Bcl-2 family has been demonstrated to be overexpressed in oral cancers and to predict outcome in oral cancer patients treated with definitive radiotherapy. To study the role of Mcl-1 isoforms in radiation response of oral squamous carcinoma cells (OSCC), we investigated in the present study, the association of Mcl-1 isoform expression with radiosensitivity of OSCC, using siRNA strategy.

Methods: The time course expression of Mcl-1 splice variants (Mcl-1L, Mcl-1S & Mcl-1ES) was studied by RT-PCR, western blotting & immunofluorescence, post-irradiation in oral cell lines [immortalized FBM (radiosensitive) and tongue cancer AW8507 & AW13516 (radioresistant)]of relatively differing radiosensitivities. The effect of Mcl-1L knockdown alone or in combination with ionizing radiation (IR) on cell proliferation, apoptosis & clonogenic survival, was investigated in AW8507 & AW13516 cells. Further the expression of Mcl-1L protein was assessed in radioresistant sublines generated by fractionated ionizing radiation (FIR).

Results: Three to six fold higher expression of anti-apoptotic Mcl-1L versus pro-apoptotic Mcl-1S was observed at mRNA & protein levels in all cell lines, post-irradiation. Sustained high levels of Mcl-1L, downregulation of pro-apoptotic Bax & Bak and a significant ($P < 0.05$) reduction in apoptosis was observed in the more radioresistant AW8507, AW13516 versus FBM cells, post-IR. The ratios of anti to pro-apoptotic proteins were high in AW8507 as compared to FBM. Treatment with Mcl-1L siRNA alone or in combination with IR significantly ($P < 0.01$) increased apoptosis viz. 17.3% (IR), 25.3% (siRNA) and 46.3% (IR plus siRNA) and upregulated pro-apoptotic Bax levels in AW8507 cells. Combination of siRNA & IR treatment significantly ($P < 0.05$) reduced cell proliferation and clonogenic survival of radioresistant AW8507 & AW13516 cells, suggesting a synergistic effect of the Mcl-1L siRNA with IR on radiosensitivity. Interestingly, during the development of radioresistant sublines using FIR, high expression of Mcl-1L was observed.

Conclusion: Our studies suggest that Mcl-1L isoform has an important role in the survival and radioresistance of OSCC and may be a promising therapeutic target in oral cancers.

Introduction

Squamous cell carcinoma of the oral cavity (OSCC) is the most prevalent cancer in males of the Indian subcontinent and is predominantly associated with the tobacco-chewing habit [1]. Radiotherapy is an important treatment modality in oral cancer aiding in tumor size reduction and preservation of oral function [2]. Despite advances in radiotherapy techniques, OSCC patients frequently develop loco-regional recurrence resulting in 5-year survival rates that have remained unchanged for past few decades [3]. Hence for successful radiotherapy, it is crucial to understand the mechanisms involved in the development of radiation resistance in tumor cells.

Anti-apoptotic members of the Bcl-2 family are the key regulators of cellular apoptosis and their over expression has been shown to be associated with radioresistance [4,5]. Mcl-1 (Myeloid cell leukemia-1), an anti-apoptotic member of the Bcl-2 gene family, is essential for development, differentiation, and proliferation

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[6]. The overexpression of Mcl-1 has also been reported in a variety of hematopoietic, lymphoid and solid tumors [7-9]. Our earlier studies demonstrate the overexpression of anti-apoptotic Mcl-1 transcripts & protein in oral tumors and cell lines [10]. Further, we have also demonstrated Mcl-1 to be a prognostic factor in oral cancer patients treated with definitive radiotherapy [11]. Earlier, Mcl-1 has been shown to contribute in resistance of cancer cells to chemotherapeutic agents, however reports on its role in radiation induced apoptosis and radioresistance are rare [12,13]. Further, the situation is complex due to the existence of distinct Mcl-1 isoforms having contrasting functions (anti-apoptotic Mcl-1L, pro-apoptotic Mcl-1S & Mcl-1ES)[14]. Earlier studies from our lab have demonstrated a five to ten fold higher expression of anti-apoptotic Mcl-1L transcript, versus the pro-apoptotic Mcl-1S in oral tumors [10]. Therefore, in the present study we wanted to investigate the association of Mcl-1 isoforms with radioresistance of oral cancer cells using siRNA strategy. To the best of our knowledge, no reports are available on the role of Mcl-1 splice variants in radiation response of OSCC.

The present study was undertaken to compare the time course profile of Mcl-1 splice variants and other Bcl-2 family members, post-radiation (IR) treatment in oral cell lines of differing radiosensitivities. Further, the effect of Mcl-1L knockdown alone or in combination with IR on cell proliferation, apoptosis and radiosensitivity of oral cells was investigated.

Materials and methods

Cell culture

Established AW8507 & AW13516 [10,15] & FBM (fetal buccal mucosa derived immortalized cell line) [16] were selected for the study due to their differing radiosensitivities. The cell lines were cultured in IMDM supplemented with 10% FBS (Gibco, US), 100 units/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and keratinocyte growth supplements only for FBM (Sigma, USA), in 5% CO_2 at 37°C.

Clonogenic Assay

Exponentially growing oral cells were harvested, counted and replated in duplicates. After 24 hrs, the cells were treated with different doses of IR (1, 2, 4, 6, 8, 10 Gy) using ^{60}Co-γ radiator along with an untreated control. Cells were then incubated up to 14 days to form colonies which were fixed and stained with a mixture of glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) and colonies (≥50 cells) were counted using a microscope. The percent plating efficiency and fraction surviving a given radiation dose were calculated based on the survival of non-irradiated cells as described earlier [17].

Radiation Treatment

After 48 hrs of plating exponentially growing cells (6×10^3 cells) were treated with IR (D_0 dose) using ^{60}Co-γ radiator as described earlier [13]. Cells were incubated upto different time points, harvested and stored in -80°C until use.

RNA isolation

Cell pellets were placed in TRI reagent (Sigma, USA) and total cellular RNA was isolated according to the manufacturer's protocol. The RNA was dissolved in DEPC-treated water and contaminating DNA was removed by DNaseI treatment (Sigma, USA). RNA integrity was analyzed by electrophoresis and samples were preserved at −80°C until analysis, as described earlier [10].

Reverse transcriptase-polymerase chain reaction

cDNA was synthesized with 2 μg total RNA, using a First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's instructions. The efficiency of cDNA synthesis and equal loading were assessed by ß-actin PCR. Mcl-1 isoforms (Mcl-1L, Mcl-1S & Mcl-1ES isoforms) were amplified by using primers (forward 5'ACGCGGTAATCGGACTCAACCT3' and reverse 5'GC AGCACATTCCTGATGCCACCT3'), as reported earlier [10]. A separate PCR was performed to determine expression of Mcl-1ES isoform using forward (5'ACGCGGTA ATCGGACTCAACCT3') and reverse (5'GCAGCACATT CCTGATGCCACCT3') primers as shown in Additional file 1. PCR products were resolved on 2% agarose gel containing ethidium bromide and quantitated using Gel-doc system (UVP, UK).

Western blotting

Cell lysates were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, USA). Membranes were blocked with 5% skimmed milk in TBS for 2 hrs. Primary antibodies used were anti-Bax (1:150, Abcam, USA), anti-Mcl-1 (1:1000), anti-Bclxl (1:1000) and anti-ß-actin (1:2000) (Santa Cruz Biotechnology, USA). Secondary antibodies used were Horseradish peroxidase conjugated IgG (1:5000) (Santa Cruz Biotechnology, USA). Proteins were visualized with enhanced chemiluminescence kit (GE Healthcare, US). Densitometry analysis of developed X-ray film was performed using ImageJ software (NIH, Bethesda, MD). β-actin was used as loading control.

Apoptosis detection by flow cytometry

The Annexin V-FITC apoptosis detection kit (Santa Cruz Biotechnology, CA) was used for the detection of apoptotic cells in the three oral cell lines, as per the manufacturer's specifications. Briefly, cells were collected

by trypsinization at different time points (Control, 1, 4, 24 & 48 hrs) post-IR treatment. Cells were washed; 2μg Annexin-V FITC & 10 μl PI were added, incubated in the dark for 15 min and analyzed on a flow cytometer (FACS Caliber, BD, USA).

Immunofluorescence staining

Cells were grown on glass cover slips and Mcl-1 staining was performed at different time points in both FBM & AW8507, post-IR using an Alexa fluor-488 labeled secondary antibody (1:1000, Molecular Probes, USA), as described earlier [10]. The AW8507 cells were treated with siRNA (100nM) and/or exposed to IR as described above. The nuclear condensation and apoptosis was analyzed by DAPI (Sigma-Aldrich, USA) staining, cell counting and imaging was done by confocal microscope with LSM Image Browser 4.2 software (Carl Zeiss).

Knockdown of Mcl-1L isoform

Knockdown was achieved using Mcl-1L specific siRNA (sc-43912) along with a control siRNA (sc-37007) from Santa Cruz biotechnology, USA. The siRNA duplexes were transfected using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions. The medium was changed after 16 hrs of transfection and 24 hrs post-transfection the cells were assessed for knockdown by western blotting. The specific silencing of Mcl-1L was confirmed in three independent experiments.

Trypan blue exclusion assay

Cells were seeded into 24-well plates at a density of 5×10^4 per well and treated with Mcl-1L siRNA and/or IR as described above. Cells were trypsinized and trypan blue staining was performed after 48 hrs of treatment. The number of viable cells were counted and compared to untreated control using a hemocytometer.

Acquired radioresistant sublines

Radioresistant sublines were generated by irradiating AW8507 & AW13516 cells with a fractionated Ionizing radiation (FIR) strategy as described earlier [18]. During development of radioresistant cell lines, cells were collected at different doses (Control, 8, 16, 24, 32, 40 Gy)

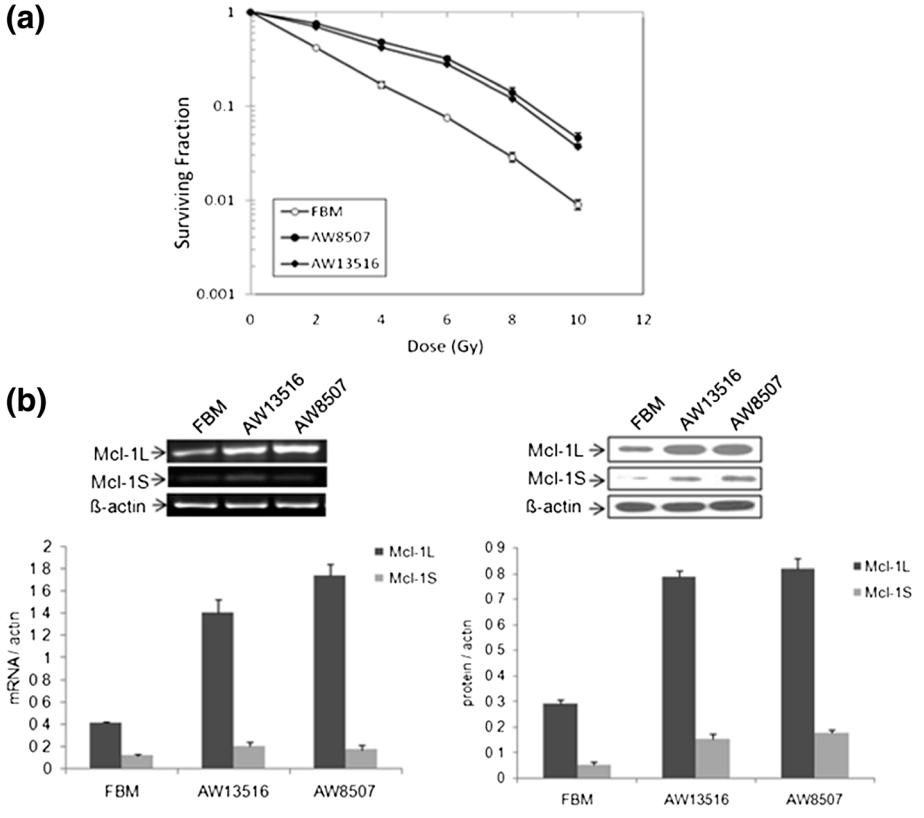

Figure 1 Radiosensitivity assessment and expression of Mcl-1 isoforms in oral cell lines. (**a**) Clonogenic cell survival assay of FBM, AW8507 & AW13516 oral cell lines. Data given as percentage survival of untreated cell cultures and represent the means (±SD) of three independent colony formation experiments. (**b**) Expression of Mcl-1 isoforms in oral cell lines. RT-PCR and western blot analysis of Mcl-1 isoforms in the three oral cell lines (FBM, AW13516 & AW8507). A representative blot was shown for three independent experiments. Histogram indicates quantitative expression of Mcl-1 isoforms (Mcl-1L & Mcl-1S) at both mRNA & protein level in oral cell lines.

and lysates from these sublines were loaded on SDS-PAGE to determine Mcl-1L expression by western blotting.

Statistical analysis

Statistical analysis was performed by using a Student's *t*-test analysis. The difference between means was considered statistically significant when $P < 0.05$. The data is illustrated as mean ± standard deviation of three independent experiments.

Results

Analysis of clonogenic survival of oral cell lines

Radiosensitivity assessment of the three oral cell lines (FBM, AW13516 & AW8507) used in the study was determined by the clonogenic survival assay (Figure 1a). The D_0 values obtained from the surviving fractions of FBM, AW13516 & AW8507 were 2.3, 5.1 & 5.4 Gy respectively, indicating FBM to be most radiosensitive among all three oral cell lines.

Expression of Mcl-1 splice variants in oral cell lines and effect of irradiation

RT-PCR using a single primer which amplifies all three isoforms of Mcl-1 showed predominant expression of anti-apoptotic Mcl-1L and low levels of Mcl-1S but undetectable levels of Mcl-1ES in all three oral cell lines. Separate RT-PCR of poorly expressed Mcl-1ES isoform showed very low levels of Mcl-1ES as compared to Mcl-1L & Mcl-1S in all the three oral cell lines (Additional file 1). The more radioresistant AW8507 & AW13516 cells showed high expression of Mcl-1L at both mRNA & protein levels as compared to immortalized FBM cells (Figure 1b). Post-IR, the time course expression profiles of Mcl-1 isoforms in the three oral cell lines revealed induction of Mcl-1L protein in all three cell lines. However, radiosensitive FBM exhibited a rapid and short induction profile with a peak at 1.5 hrs which declined by 48 hrs. While the more radioresistant AW8507 exhibited sustained high levels of Mcl-1L up to 48 hrs with a peak observed at 1.5 hrs (Figure 2a). A similar pattern was observed in AW13516 (data not shown). In all cell lines, the expression of short Mcl-1S was elevated at initial time points which later decreased up to 48 hrs while, the short pro-apoptotic Mcl-1ES isoform levels remained unaltered (See Additional file 2).

Analysis of Bax, Bcl-xl & Bcl-2 protein expression

Interestingly, AW8507 cell line exhibited a rapid down-regulation of pro-apoptotic Bax & Bak proteins, 2hrs post-IR. In contrast the more radiosensitive FBM showed a consistent increase in Bax & Bak levels, 2hrs onwards (Figure 2b). Higher expression of Bcl-2 & Bcl-xl protein was observed in AW8507 as compared to FBM. The AW13516 cell line also showed similar results.

Ratios of anti to pro-apoptotic members

It is noteworthy that, the more radioresistant AW8507 cell line exhibited higher ratios of anti to pro-apoptotic proteins like Mcl-1L/Mcl-1S, Mcl-1L/Bax & Bcl-xl/Bax as compared to that in FBM post-IR (Figure 3 a-f). A similar pattern was observed in AW13516 (data not shown).

Effect of Mcl-1 expression on apoptosis

As compared to untreated control, post-IR up to 1 hr, a uniform time-dependent increase in apoptotic population was observed in all three oral cell lines. Notably, the number of apoptotic cells in AW13516 & AW8507 significantly ($P < 0.05$ at 24 hrs & $P < 0.01$ at 48 hrs) decreased thereafter as compared to that of FBM viz. from 17.5% to 9% at 24 hrs & 27% to 12% at 48 hrs of post-IR (Figure 4a), coinciding with the high Mcl-1L/Mcl-1S ratio (Figure 3b).

Localization of Mcl-1 protein

Immunofluorescence staining demonstrated that Mcl-1 protein was primarily localized in the cytosolic compartment of untreated AW8507 & FBM cells. Interestingly post-IR a substantial increase in Mcl-1 protein expression with peri-nuclear and nuclear localization at 4 hrs was observed in AW8507 cells, whereas no significant change in expression and localization was observed in FBM (Figure 4b).

siRNA mediated downregulation of Mcl-1L

AW8507 cells exhibited specific downregulation of Mcl-1L levels after transfection with 100nM Mcl-1L siRNA without affecting the Mcl-1S levels (Figure 5a). The effect of Mcl-1L siRNA was maximal between 6 to 72 hrs and the Mcl-1S levels were unaltered. Treatment with siRNA and IR alone or in combination significantly increased expression of pro-apoptotic Bax protein but did not change Bak & Bcl-xl protein levels (Figure 5b).

Effect Mcl-1L downregulation on cell proliferation and apoptosis

Trypan blue dye exclusion assay in AW13516 & AW8507 revealed a significant ($P < 0.05$) decrease in viability of cells treated with combination of siRNA plus IR as compared to individual treatments. After 72 hrs, cell viability was reduced to 67% (IR), 42% (siRNA) and 21% (IR plus siRNA) respectively (Figure 6a&b). Thereby, suggesting a synergistic effect of the combined treatment on cell viability.

Immunofluroscence analysis of AW13516 & AW8507 demonstrated an increased nuclear condensation in cells treated with combined Mcl-1L siRNA plus IR as compared to IR or siRNA alone (Figure 6c). The percentage of apoptotic cells in experimental control (EC), UC, IR,

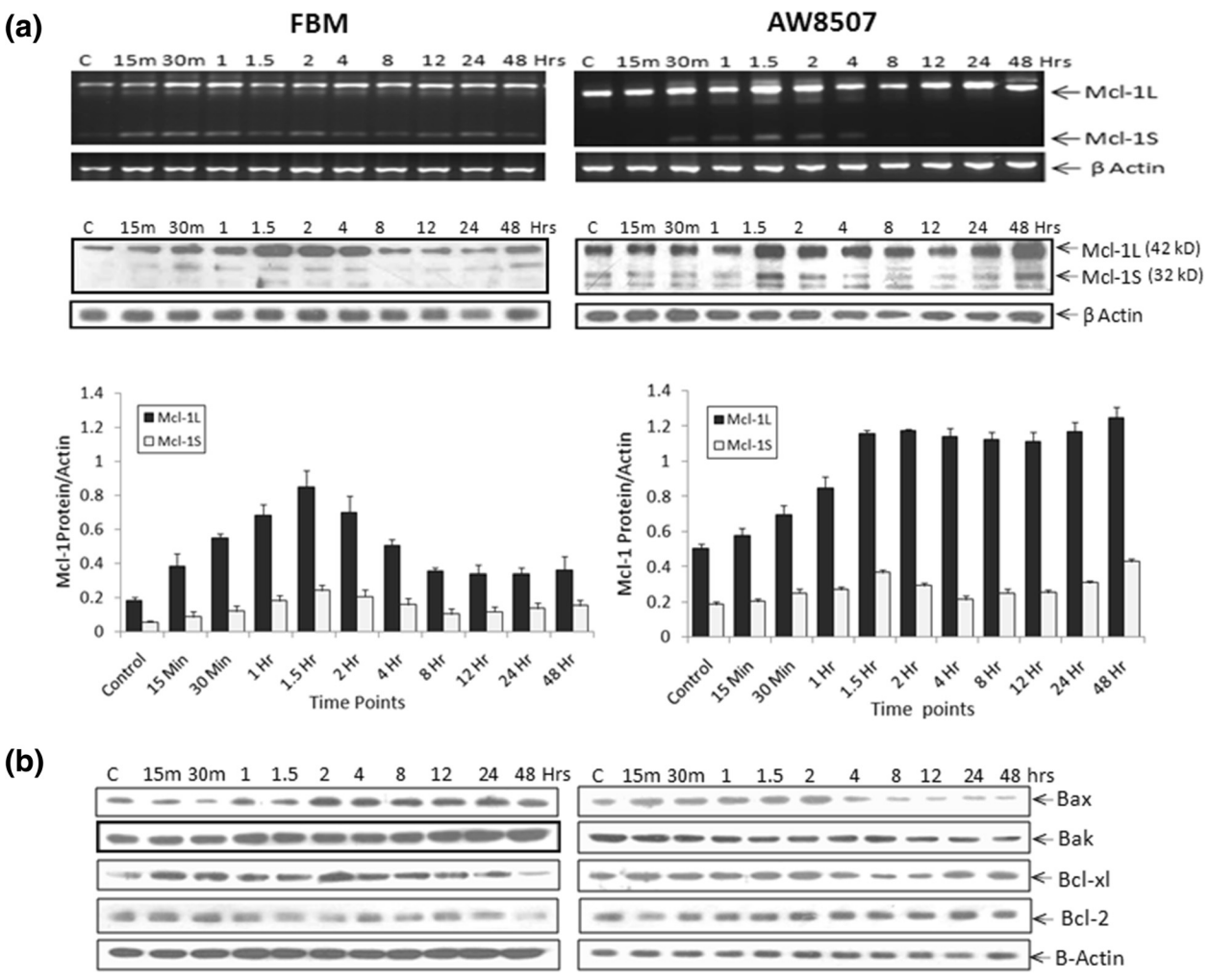

Figure 2 Time course profile of Mcl-1 splice variants & apoptosis related proteins post-IR. (**a**) Expression of Mcl-1L, Mcl-1S transcripts and proteins at different time points post-IR in FBM & AW8507. Post-IR cells were harvested at different time points, used for RT-PCR and Western blotting. (**b**) Western blot illustrates expression of Bax, Bak, Bcl-xl & Bcl-2 proteins at different time points post-IR in oral cell lines, using β-actin as loading control. A representative blot for three independent experiments is shown.

siRNA, siRNA plus IR treated AW8507 cells were 2.1% (SD 0.5), 3.2% (SD 0.6), 17.3% (SD 0.5), 25.3% (SD 1.1) and 46.3% (SD 0.6), respectively (Figure 6d). A similar pattern was observed in AW13516. The difference in percentage of apoptosis between IR alone and siRNA plus IR treated cells was highly significant ($P < 0.01$) in both the cell lines.

Effect of Mcl1L knockdown on clonogenic survival

The effect of Mcl-1L downregulation on long term cell survival was examined by clonogenic assay in AW8507 & AW13516 cells. Interestingly, a reduction in clonogenic survival was observed after treatment of Mcl-1L siRNA (100nM) and increasing doses of IR as compared to the untreated control (Figure 7). The survival of AW8507 post-IR (0, 2, 4, 6 8 and 10Gy) was 78% (SD 2.1), 46% (SD 1.5), 32% (SD 2.3) and 14% (SD 1.8) and 6% (SD 1.9) respectively. However, in presence of Mcl-1 siRNA the survival was further reduced to 42% (SD 2.6), 23% (SD 1.7), 10% (SD 1.0), 4% (SD 2.1) and 2% (SD 1.7), respectively (Figure 7a). Similar reduction in clonogenic survival post Mcl-1L knockdown was observed in AW13516 cells (Figure 7b). These observations therefore suggest a synergistic effect of the Mcl-1L siRNA with IR on radiosensitivity.

Expression of Mcl-1L in radioresistant sublines

To evaluate the association of Mcl-1L with radioresistance, its expression was assessed by western blotting in acquired radioresistant sublines of AW8507 & AW13516. Figure 8 demonstrates the high Mcl-1L expression in

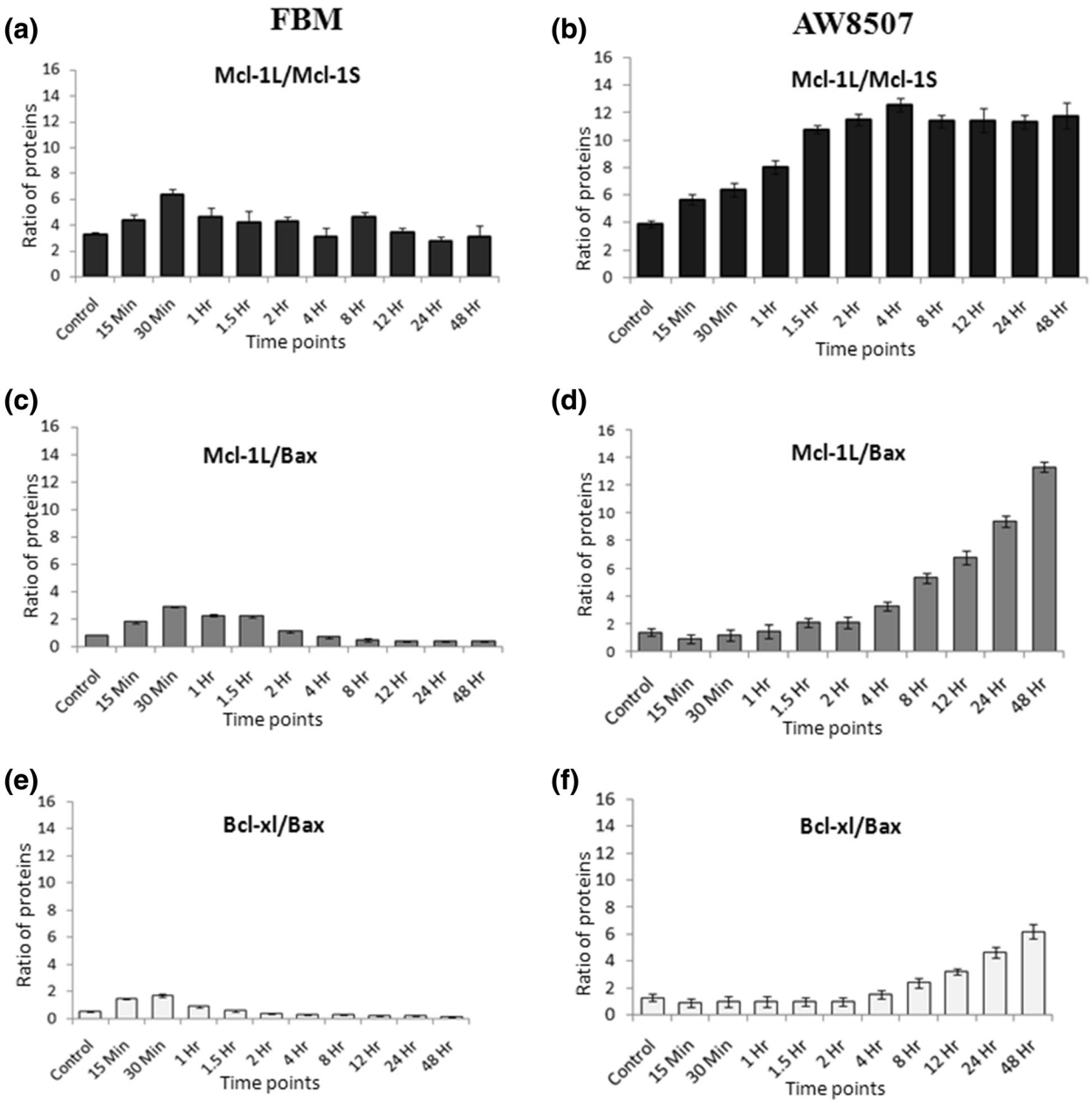

Figure 3 Ratios of anti to pro-apoptotic proteins; Mcl-1L/Mcl-1S (a-b), Mcl-1L/Bax (c-d) and Bcl-xl/Bax (e-f) in AW8507 & FBM cell lines. The relative ratios of expression of proteins were obtained by densitometry of blots using ImageJ software (NIH, USA).

radio-resistant sublines generated by fractionated irradiation as compared to parental untreated cells.

Discussion

In the present study, we demonstrate the effect of anti-apoptotic Mcl-1L expression on radiosensitivity of oral cancer cells. So far, limited information is available on the role of Mcl-1 in radiation response of tumor cells. To our knowledge, this is the first study to report a time course expression of Mcl-1 isoforms post-IR and effect of Mcl-1L knockdown on radiosentitzation of oral cancer cell lines using siRNA strategy. Our studies demonstrated an inverse correlation of Mcl-1 expression with cellular apoptosis and a synergistic effect of Mcl-1L knockdown along with IR on cell viability and clonogenic survival thereby enhancing the radiosensitivity of OSCC cells.

Various growth factors and cellular stresses like radiation and cytotoxic agents are known to upregulate Mcl-1 levels, thereby enhancing short term viability [19]. Our

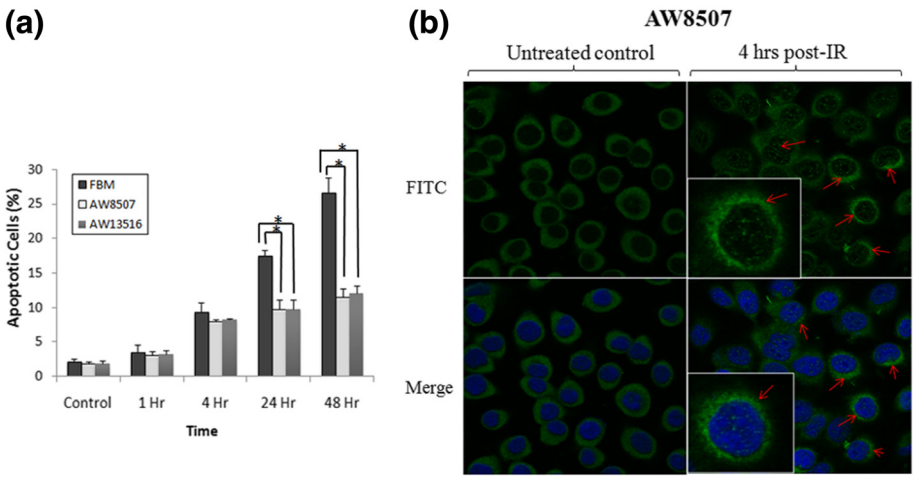

Figure 4 Apoptosis induction and localization of Mcl-1 post-IR. (**a**) Percentage of apoptosis induction at different time points post-IR by Annexin-V & PI staining analyzed by FACS. The flow cytometry data shown is representative of three independent experiments (*$P < 0.05$ & **$P < 0.01$) (**b**) Immunofluorescence staining of Mcl-1 protein counterstained with DAPI (blue) shows peri-nuclear accumulation (inset) and additional nuclear localization 4 hrs post-IR in AW8507 cells.

earlier studies had demonstrated higher expression of Mcl-1L transcript and its association with poor disease free survival in patients treated with definitive radiotherapy [10,11]. In the present study, two tongue cancer (AW8507 & AW13516) and an immortalized oral (FBM) cell line were used due to their differing radiosensitivities and based on their D_0 values, both AW8507 & AW13516 were relatively more radioresistant than FBM. Therefore, to evaluate the association of Mcl-1L with radioresistance if any, we evaluated the expression of Mcl-1 isoforms in radioresistant AW8507 & AW13516 as compared to radiosensitive FBM. Our studies revealed higher expression of Mcl-1L at both mRNA and protein level in relatively more radioresistant AW8507 & AW13516 cell lines versus FBM, indicating a possible association of anti-apoptotic Mcl-1L splice variant with radioresistance.

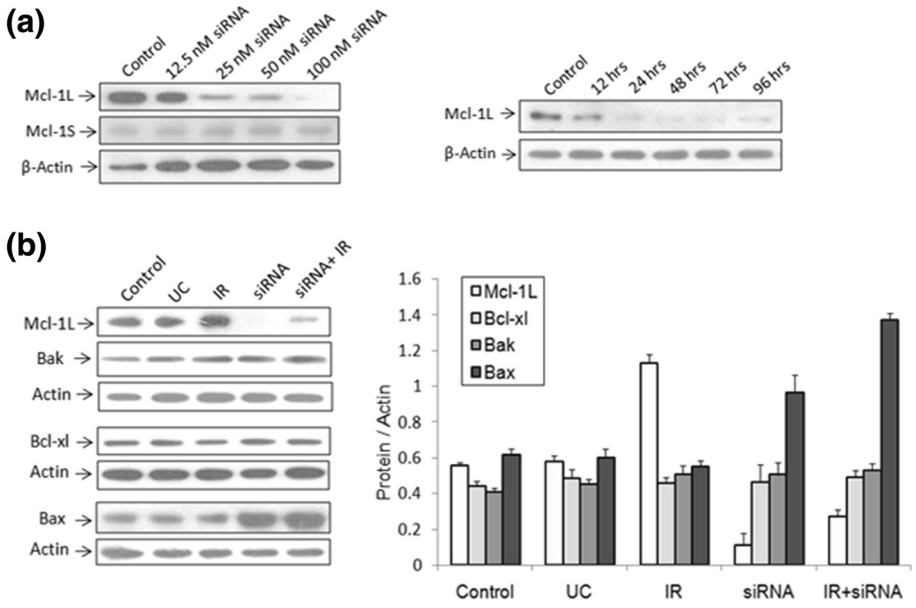

Figure 5 Western blot analysis of Mcl-1L knockdown. (**a**) Mcl-1L downregulation using different concentrations of siRNA and unaltered expression of Mcl-1S in AW8507 cells. The effect of Mcl-1L siRNA was analyzed upto 96 hrs post transfection. (**b**) Expression of Mcl-1L, Bak, Bax & Bcl-xl proteins 24 hrs after transfection of AW8507 cells: [Experimental control without siRNA (EC); universal control siRNA (UC); Mcl-1L siRNA (siRNA); irradiation (IR)]. A representative blot of three independently performed experiments is shown.

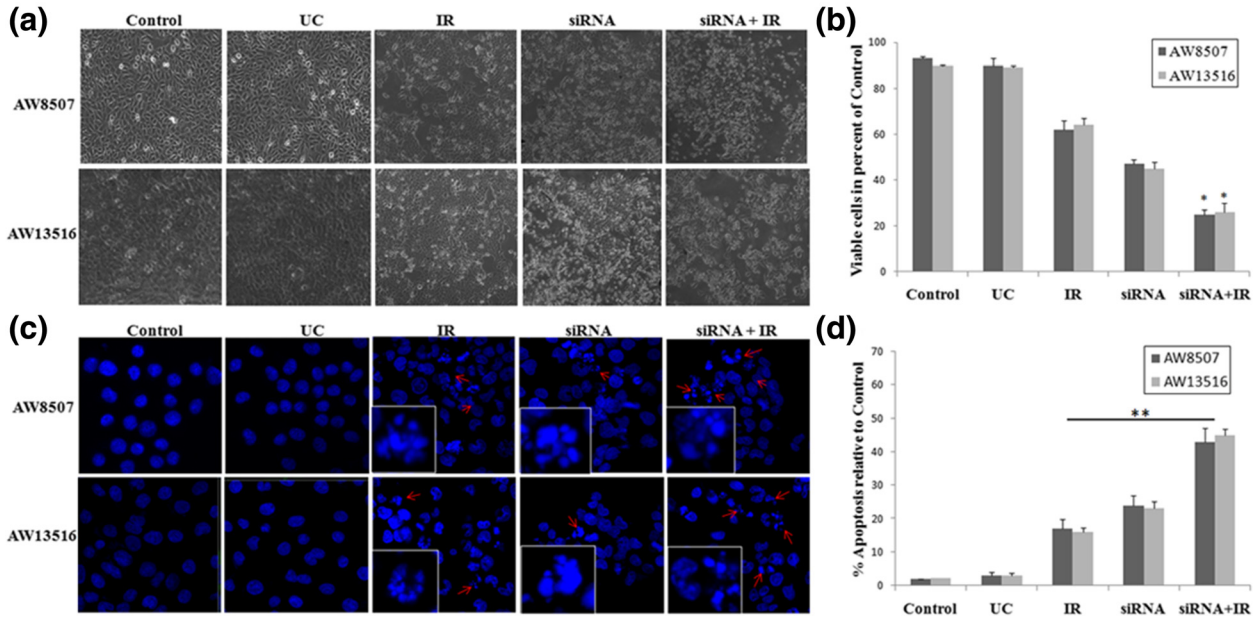

Figure 6 Microscopic analyses of cell proliferation and apoptosis after different treatment combinations. (**a & b**) After different treatment combinations as described, cell growth of AW8507 & AW13516 cells were determined by counting cell numbers using Trypan blue dye exclusion assay. Cell numbers are given in percent of untreated controls. Data are means ± SD of three independent experiments. (**c & d**) Apoptosis was determined by DAPI staining, cells either untreated (Control) or treated with UC siRNA, Mcl-1L siRNA alone or in combination with IR. Apoptotic cells were counted in different fields and shown as percent of apoptosis relative to control. Data are means ± SD of three independent experiments. *indicates $P < 0.05$, ** $P < 0.01$.

Several possible mechanisms can lead to the high Mcl-1 levels. Mcl-1 is known to be rapidly induced at the transcriptional level and its mRNA has a short half life [19]. Mcl-1 is also regulated at the post-transcriptional level by micro RNAs through a mir29 binding in the 3'UTR of Mcl-1 mRNA [20]. Interestingly, the expression of mir29 was also found to be decreased in malignant cholangiocytes, favoring the increased levels of Mcl-1, which indicates the possible reason for the observed high levels of Mcl-1 mRNA in our cancer cells lines and also responsible for its immediate degradation within few hours post IR.

Mcl-1, a PEST domain containing protein is also known to undergo ubiquitin dependent degradation by the 26S proteosome and possesses a short half life of one to 3 hrs is rapidly downregulated during apoptosis

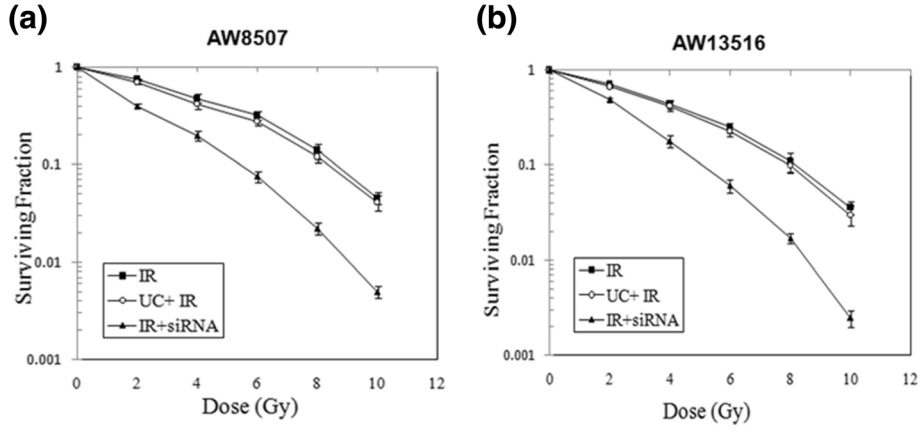

Figure 7 Clonogenic survival analysis after Mcl-1L knockdown and/or irradiation. (**a & b**) AW8507 & AW13516 cells were either untreated, or treated with siRNA. After 24 hrs, cell culture dishes were treated with increasing doses of ionizing irradiation (0, 2, 4, 6 8 and 10 Gy) as indicated. Survival was assessed by performing clonogenic assay as described in Methods. Data are given as percentage survival of untreated cells and represent the means (±SD) of three independent colony formation experiments.

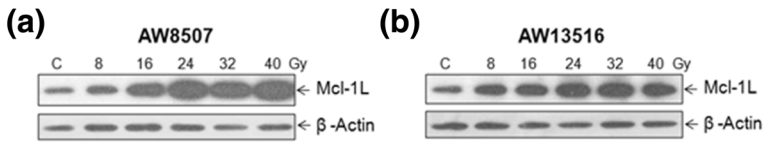

Figure 8 Expression of Mcl-1L in radio resistant sublines. (**a & b**) AW8507 & AW13516 cells were treated with fractionated irradiation and the radio resistant sublines were assayed for expression of Mcl-1L. β-actin blot from the same lysates serve as loading control.

[19]. Notably, the BH3 domains of MULE/LASU1 E3 ligase specifically interact with the hydrophobic BH3 binding pocket of Mcl-1 and not with other anti-apoptotic Bcl-2 family members [21] and are responsible for the constitutive turnover of Mcl-1. Such ubiquitin mediated degradation of Mcl-1 has been shown to be essential for the initiation of apoptosis, following UV damage [22]. Hence, it was interesting to study the time course expression profile of Mcl-1 isoforms and other bcl-2 family members in the above cell lines post-IR. Also, Mcl-1 protein is known to be phosphorylated by GSK-3β at Ser159, located within the PEST domain, resulting in a significant decrease in the protein half-life and leading to initiation of apoptosis [23]. Such, alterations in the phosphorylation of Mcl-1 protein by GSK3 may also contribute to the elevation of Mcl-1 levels.

Moreover, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [24]. No significant alterations in levels of Mcl-1S & Mcl-1ESwere observed post IR, indicating that the predominantly overexpressed Mcl-1L isoform alone may contribute in generation of radioresistance. Mcl-1L not only binds to Mcl-1S, but is also known to heterodimerise with pro-apoptotic Bax & Bak etc. preventing the release of cytochrome-c and subsequent apoptosis [25]. We observed a downregulation of pro-apoptotic Bax & Bak proteins in AW8507 post-IR, coinciding with decreased apoptosis, while in contrast, FBM showed an increase in Bax & Bak protein levels. We observed high expression of anti-apoptotic Bcl-xl which has already been shown to be associated with radiosensitization of colon cancer cells [26]. Thus, high expression of Bcl-xl & Bcl-2, known radioresistant factors and Mcl-1L in more radioresistant AW8507 & AW13516 than FBM may indicate their possible role in radioresistance.

We assessed the ratios of Mcl-1L/Mcl-1S, Mcl-1L/Bax, Bcl-xl/Bax, wherein radioresistant AW8507& AW13516 showed high ratios as compared to that in FBM indicating predominance of anti-apoptosis which may contribute to radioresistance. We are the first to elucidate the comparative levels of Mcl-1 isoforms and their association with radiation response in oral cell lines. The high and prolonged expression of Mcl-1L observed in AW8507 & AW13516 could possibly be due to Mcl-1 protein stabilization via binding with other proteins. Another reason could be its enhanced half life due to S159/T163 phosphorylation post-IR, reported to be crucial in nicotine mediated Mcl-1 activation and chemoresistance [27].

We observed a significant reduction in apoptosis post-IR which coincided with the high levels of Mcl-1L, indicating a possible association of Mcl-1L expression with radiation response of AW8507 & AW3516 cells. The immunofluorescence staining of Mcl-1 indicated a perinuclear accumulation & nuclear localization post IR in more radioresistant AW8507 cell line. Such, peri-nuclear accumulation of Mcl-1 has also been observed earlier in polymorphonuclear leukocytes post-etoposide treatment [28]. In AW8507, the observed nuclear and perinuclear accumulation of Mcl-1 may possibly help in cell survival to lower doses of DNA damaging agents. A similar regulatory role for Mcl-1 (snMcl-1), perhaps acting as an adaptor protein in controlling the ATR-mediated regulation of DNA damage checkpoint kinase Chk1 phosphorylation and activation has been reported, placing Mcl-1 at the interface of apoptosis and cell cycle regulation [29]. Mcl-1 has been shown to regulate cell cycle by binding to proteins like CDK1 & PCNA [30], possibly explaining the observed nuclear localization of Mcl-1. High expression of anti-apoptotic Mcl-1L and Bcl-xl proteins and reduced pro-apoptotic proteins like Bak & Bax together may possibly contribute in lowering the sensitivity of AW8507 & AW3516 cells to IR.

The downregulation of Mcl-1L alone was efficient in induction of apoptosis in both AW8507 & AW13516 cells. Interestingly, the combination of Mcl-1L siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. Notably, the expression of closely related Bcl-xl, a known radioresistant factor was not altered. However, the expression of pro-apoptotic Bax protein correlated with the increased apoptosis on Mcl-1L knockdown. This overexpression of Bax, a downstream pro-apoptotic member, may execute the intrinsic apoptotic pathway resulting in increased cell death. To address the fact that the induction of apoptosis may not necessarily lead to long-term response to radiotherapy, we performed the clonogenic assay which demonstrated that combination of IR and Mcl-1L downregulation synergistically reduced

clonogenic survival as compared to each treatment alone. Our studies demonstrate that Mcl-1L downregulation potentially enhanced radiosensitivity of AW8507 & AW13516 cells *in vitro*. Complex interactions occur between Bcl-2 family proteins especially, Bak & Bax, where Mcl-1 plays a crucial role in engaging and maintaining pro-apoptotic Bak in an inactive state and accumulates H2AX and ATM proteins to activate DNA repair pathways, suggesting that elimination of cellular Mcl-1 is crucial to initiate apoptotic pathway [31]. Overexpression and nuclear accumulation of Mcl-1 in AW8507 may occur due to a protein called IEX-1 which has been shown to interact specifically and timely with Mcl-1 controlling its accumulation and nuclear translocation in response to DNA damage and contribute in the activation of DNA repair pathway by Chk1 activation and G2 checkpoint arrest [32].

The high expression of Mcl-1L, in radioresistant sublines developed by fractionated ionizing radiation provides a direct evidence for the role of Mcl-1L in radioresistance of OSCC cells. Therefore, the combination of radiotherapy and Mcl-1L downregulation has the potential to improve the response rate of treatment-resistant oral cancer cells. Selective inhibitors like Obatoclax, which specifically overcome Mcl-1 mediated resistance, is already in phase 2 clinical trials [33] and may have important therapeutic implications, when used in combination with radiotherapy in treatment of oral cancer patients.

Conclusion

Our studies indicate the association of Mcl-1L isoform expression with radioresistance by influencing apoptosis, proliferation and clonogenic survival of OSCC. Thus, Mcl-1L appears to be a promising molecular target for improving outcome of radiotherapy in oral cancer patients.

Additional files

Additional file 1: Figure S1. Indicating expression of Mcl-1ES isoform in three oral cell lines done by separate RTPCR.

Additional file 2: Figure S2. Time course profile of Mcl-1ES splice variant (done by separate PCR) along with another two (Mcl-1L & Mcl-1S in single PCR) isoforms, at different time point's post-IR in AW8507 & FBM cell lines.

Abbreviations

Post-IR: Post Irradiation; FIR: Fractionated Ionizing Radiation; IR: Ionizing Radiation; IMDM: Iscove's Modified Dulbecco's Media; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; PVDF: Polyvinyldene fluoride; DAPI: 4'-6-Diamidino-2-phenylindole; SD: Standard deviation; SDS-PAGE: Sodium dodecyle sulfate polyacrylamide gel electrophoresis.

Competing interests

Both authors declare that they have no competing interests.

Authors' contributions

VP aided in study design, performed experimental procedures, analyzed the data and drafted manuscript. TT designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

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References

1. Tandle AT, Sanghvi V, Saranath D: **Determination of p53 genotypes in oral cancer patients from India.** *Br J Cancer* 2001, **84:**739–742.
2. Burri RJ, Lee NY: **Concurrent chemotherapy and radiotherapy for head and neck cancer.** *Expert Rev Anticancer Ther* 2009, **9:**293–302.
3. Mathew Iype E, Pandey M, Mathew A, Thomas G, Krishnan Nair M: **Squamous cell cancer of the buccal mucosa in young adults.** *Br J Oral Maxillofac Surg* 2004, **42:**185–189.
4. Kyprianou N, King E, Bradbury D, Rhee J: **bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells.** *Int J Cancer* 1997, **70:**341–348.
5. Wang ZB, Zhang Y, Liu YQ, Guo Y, Xu H, Dong B, Cui YF: **Bcl-xL overexpression restricts gamma-radiation-induced apoptosis.** *Cell Biol Int* 2006, **30:**15–20.
6. Craig R: **MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis.** *Leukemia* 2002, **16:**444–454.
7. Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, Bodingbauer M, Crevenna R, Monia BP, Peck-Radosavljevic M, Wacheck V: **Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy.** *J Hepatol* 2006, **44:**151–157.
8. Derenne S, Monia B, Dean N, Taylor J, Rapp M, Harousseau J, Bataille R, Amiot M: **Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-xL is an essential survival protein of human myeloma cells.** *Blood* 2002, **100:**194.
9. Cho-Vega JH, Rassidakis GZ, Admirand JH, Oyarzo M, Ramalingam P, Paraguya A, McDonnell TJ, Amin HM, Medeiros LJ: **MCL-1 expression in B-cell non-Hodgkin's lymphomas.** *Hum Pathol* 2004, **35:**1095–1100.
10. Mallick S, Patil R, Gyanchandani R, Pawar S, Palve V, Kannan S, Pathak KA, Choudhary M, Teni TR: **Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of Mcl-1.** *J Pathol* 2009, **217:**398–407.
11. Mallick S, Agarwal J, Kannan S, Pawar S, Kane S, Teni T: **PCNA and anti-apoptotic Mcl-1 proteins predict disease-free survival in oral cancer patients treated with definitive radiotherapy.** *Oral Oncol* 2010, **46:**688–693.
12. Guoan X, Hanning W, Kaiyun C, Hao L: **Adenovirus-mediated siRNA targeting Mcl-1 gene increases radiosensitivity of pancreatic carcinoma cells *in vitro* and in vivo.** *Surgery* 2010, **147:**553–561.
13. Skvara H, Thallinger C, Wacheck V, Monia BP, Pehamberger H, Jansen B, Selzer E: **Mcl-1 blocks radiation-induced apoptosis and inhibits clonogenic cell death.** *Anticancer Res* 2005, **25:**2697–2703.
14. Kim JH, Sim SH, Ha HJ, Ko JJ, Lee K, Bae J: **MCL-1ES, a novel variant of MCL-1, associates with MCL-1L and induces mitochondrial cell death.** *FEBS Lett* 2009, **583:**2758–2764.
15. Tatake RJ, Rajaram N, Damle RN, Balsara B, Bhisey AN, Gangal SG: **Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors.** *J Cancer Res Clin Oncol* 1990, **116:**179–186.
16. Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M: **Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype.** *Int J Cancer* 2004, **111:**662–668.
17. Franken N, Rodermond H, Stap J, Haveman J, van Bree C: **Clonogenic assay of cells *in vitro*.** *Nat Protoc* 2006, **1:**2315–2319.
18. Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y, Yamagishi H: **Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation.** *Br J Cancer* 2004, **91:**1543–1550.
19. Opferman JT: **Unraveling MCL-1 degradation.** *Cell Death Differ* 2006, **13:**1260–1262.
20. Mott JL, Kobayashi S, Bronk SF, Gores GJ: **mir-29 regulates Mcl-1 protein expression and apoptosis.** *Oncogene* 2007, **26:**6133–6140.

21. Warr MR, Acoca S, Liu Z, Germain M, Watson M, Blanchette M, Wing SS, Shore GC: **BH3-ligand regulates access of MCL-1 to its E3 ligase.** *FEBS Lett* 2005, **579:**5603–5608.
22. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X: **Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation.** *Genes Dev* 2003, **17:**1475.
23. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR: **Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1.** *Mol Cell* 2006, **21:**749–760.
24. Bae J, Leo CP, Hsu SY, Hsueh AJ: **MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain.** *J Biol Chem* 2000, **275:**25255–25261.
25. Thomas LW, Lam C, Edwards SW: **Mcl-1; the molecular regulation of protein function.** *FEBS Lett* 2010, **584:**2981–2989.
26. Wacheck V, Selzer E, Gunsberg P, Lucas T, Meyer H, Thallinger C, Monia BP, Jansen B: **Bcl-x(L) antisense oligonucleotides radiosensitise colon cancer cells.** *Br J Cancer* 2003, **89:**1352–1357.
27. Zhao J, Xin M, Wang T, Zhang Y, Deng X: **Nicotine enhances the antiapoptotic function of Mcl-1 through phosphorylation.** *Mol Cancer Res* 2009, **7:**1954–1961.
28. Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH: **The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis.** *J Leukoc Biol* 2000, **68:**158.
29. Jamil S, Sobouti R, Hojabrpour P, Raj M, Kast J, Duronio V: **A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1.** *Biochem J* 2005, **387:**659.
30. Yang-Yen HF: **Mcl-1: a highly regulated cell death and survival controller.** *J Biomed Sci* 2006, **13:**201–204.
31. Cuconati A, Mukherjee C, Perez D, White E: **DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells.** *Genes Dev* 2003, **17:**2922–2932.
32. Pawlikowska P, Leray I, de Laval B, Guihard S, Kumar R, Rosselli F, Porteu F: **ATM-dependent expression of IEX-1 controls nuclear accumulation of Mcl-1 and the DNA damage response.** *Cell Death Differ* 2010, **17:**1739–1750.
33. Paik PK, Rudin CM, Pietanza MC, Brown A, Rizvi NA, Takebe N, Travis W, James L, Ginsberg MS, Juergens R: *A phase II study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in relapsed small cell lung cancer.* Amsterdam, Netherlands: Lung cancer; 2011:74–481.

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Original Paper

Human oral cancers have altered expression of Bcl-2 family members and increased expression of the anti-apoptotic splice variant of *Mcl-1*

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Abstract

Expression of Bcl-2 family proteins in tumours can modulate apoptosis, influencing tumour behaviour and treatment. To investigate their role in oral tumourigenesis, nine *Bcl-2* family transcripts were examined in three oral cell lines and 25 oral tumours, using ribonuclease protection assay. Since *Mcl-1* mRNA was elevated in these samples, *Mcl-1* splice variants were assessed by RT–PCR and Mcl-1 protein was studied in normal, premalignant and malignant oral tissues and cell lines, by immunohistochemistry and/or immunoblotting. The cell lines exhibited significantly higher levels of 7/9 *Bcl-2* family transcripts as compared to those in normal tongue, and significantly higher ($p = 0.030$, $p = 0.004$) anti-apoptotic versus pro-apoptotic transcripts. Elevated *Mcl-1* mRNA was observed in 11/25 (44%) tumours as compared to normal tissues with a five- to ten-fold higher expression of full-length anti-apoptotic *Mcl-1* transcript versus the pro-apoptotic short isoform. Strong cytoplasmic Mcl-1 immunoreactivity was detected predominantly in differentiated epithelia in 27/33 (82%) oral tumours, 18/20 (90%) leukoplakia, 25/30 (83%) submucous fibrosis and 3/3 oral cell lines, with weak staining in 8/15 (53%) normal mucosa samples. Mcl-1 positivity in malignant and premalignant tissues was comparable but significantly higher ($p < 0.01$) than that in normal mucosa. The expression of *bcl-2* family genes, including Mcl-1 in tumours, did not correlate significantly with clinicopathological parameters. This is the first report delineating the *in vivo* expression patterns of Mcl-1 protein and *Mcl-1* transcripts in oral cancers and premalignant lesions. The observed imbalance between expression of anti-apoptotic and pro-apoptotic *Bcl-2* family genes may promote survival in the oral cell lines. Since the majority of oral tumours associated with tobacco-chewing evolve from premalignant lesions, the sustained expression of full-length anti-apoptotic Mcl-1 protein in these tissues suggests an important role for Mcl-1, early in oral cancer pathogenesis in protecting cells from apoptosis via neutralization of pro-apoptotic members and could be a potential therapeutic target for oral cancers.

Keywords: Mcl-1; Bcl-2 family members; apoptosis; oral cancer; ribonuclease protection assay; oral lesions; oral cavity; oral cell lines

Introduction

Oral cancer comprises 30–40% of the total malignancies in India and is generally associated with tobacco habits (mainly chewing with/without smoking/alcohol). It is usually preceded by premalignant lesions, most often a persistent leukoplakia or oral submucous fibrosis (OSMF) [1]. The majority of the oral cancer patients are diagnosed at an advanced clinical stage and the 5-year survival rate has not changed, despite advances in surgical treatment and radiochemotherapy. About 40% of oral cancer patients die from uncontrolled loco-regional disease alone and 24% show metastases to distant sites [2]. It is therefore important to elucidate the mechanisms involved in the development and progression of oral cancer.

Oral squamous cell carcinomas (OSCCs) have repeatedly been linked to apoptotic dysregulation [3–5]. Bcl-2 and related pro- and anti-apoptotic proteins are important mitochondrial apoptosis pathway regulators and play a critical role in regulating cell survival [6]. *Mcl-1* (myeloid cell leukemia-1), an anti-apoptotic member of the *Bcl-2* gene family was originally identified in 1993 during a screen for genes

that increase in expression early in the differentiation of ML-1 human myeloblastic leukemia cells [6]. Gene products that influence cell viability as a major effect, such as Mcl-1, Bcl-2 and other family members, can act as key determinants in cell proliferation, differentiation and tumourigenesis [7].

Cellular expression of Mcl-1 is tightly regulated through multiple transcriptional and post-transcriptional mechanisms. It is induced in response to specific signals that affect growth, differentiation and viability, such as a number of growth factors or cytokines, including IL-3, IL-5, IL-6, GM-CSF, VEGF, α-interferon and EGF [8]. In addition, Mcl-1 can be rapidly up-regulated in response to diverse cellular stresses, e.g. radiation and cytotoxic agents [9]. Mcl-1 can produce moderate short-term viability enhancement in a broad range of cell types, including haematopoietic cells of various lineages [10]. Enforced expression of Mcl-1 in a susceptible cell type and conductive environment can enhance viability over the long term and thereby open the window for immortalization and additional genetic changes that lead to tumourigenesis [10]. The exact molecular mechanism by which Mcl-1 promotes cell survival is not completely understood but it is thought to involve suppression of cytochrome *c* release from the mitochondria, possibly via neutralization of pro-apoptotic Bcl-2 family proteins [11]. The potential role of *Mcl-1* as an oncogene in lymphoid malignancies was confirmed in transgenic mouse models in which animals with deregulated Mcl-1 expression eventually developed widely disseminated B cell lymphoma [10,12]. Although the *Mcl-1* gene has been studied extensively in multiple myeloma and leukaemia, there are rare reports on *Mcl-1* analysis in head and neck cancers [13].

Our previous studies indicate that the transition from oral lesions to oral cancer is accompanied by inhibition of cell death, enhancement of proliferation and frequent overexpression of *Bcl-2* and altered p53 [14,15]. The mechanisms leading to inhibition of apoptosis in oral cancers is poorly understood. Therefore, in the present study we carried out analysis of *Bcl-2* family gene expression in oral cancers and oral cell lines, using ribonuclease protection assay (RPA) and focused on *Mcl-1* expression.

Methods

Patients

Oral tissue specimens were obtained from the Tata Memorial Hospital (TMH), Parel, Mumbai, and Sharad Pawar Dental College and Hospital (SPDCH), Wardha, under respective Institutional Review Board approval and after patient consent.

Twenty-five primary oral tumour specimens and biopsies of histologically normal tissue surrounding the tumour, taken as pairwise normal, were obtained during surgery from patients admitted to TMH. Their clinicopathological features are listed in Table 1. Only tissues with $\geq$80% tumour cells as observed by histological examination were included in the study. The tissues were collected in RNA later (Sigma, USA) to prepare RNA for RPA and RT–PCR analysis. Informed consent was obtained from each subject according to the institutional guidelines.

Table 1. Clinical characteristics of oral cancer patients

Patient characteristics	
Cases	25
Age (years)	
<50	15
>50	10
Sex	
Male	18
Female	7
Site	
Buccal mucosa	8
Tongue	8
Alveolus	9
Size	
T1	2
T2	9
T3	4
T4	10
N Status	
N0	10
N1	8
N2b	4
N2c	3
Histological differentiation	
Well	5
Moderate	13
Poor	7

Thirty-three OSCC, 30 OSMF, 20 leukoplakia and 15 normal mucosa samples were obtained, formalin-fixed and paraffin-embedded, at SPDCH, Wardha. The clinicopathological features of OSCC, leukoplakia and OSMF patients are listed in Tables 2–4, respectively. Normal oral mucosa samples were collected from patients who did not have clinically detectable lesions and were undergoing minor surgical procedures, such as surgical removal of impacted third molar, alveoloplasty and mucogingival surgery, and major surgical procedures, such as reconstruction surgery for cleft lip and cleft palate and open reduction of mid-facial and mandibular fractures.

Cell culture

Established human tongue cancer cell lines AW 8507, AW 13 516 [16], FBM (a fetal buccal mucosa derived immortalized cell line) [17] and HeLa were used in the study. These cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine [and keratinocyte growth supplements (Sigma, USA), only for FBM], in 5% CO_2 at 37 °C.

Table 2. Clinicopathological features of OSCC patients and the correlation with Mcl-1 protein expression

	Degree of Mcl-1 staining in OSCC				
	0–10% Negative	11–30% +	31–50% ++	>50% +++	*p*
Cases	6	3	12	12	
Age (years)					
<50	0	1	2	1	0.477
>50	6	2	10	11	
Sex					
Male	2	1	6	4	0.828
Female	4	2	6	8	
Location					
Buccal mucosa	2	2	6	7	0.674
Tongue	0	0	1	2	
Other	4	1	5	3	
Staging					
I	0	0	0	0	0.352
II	0	0	0	0	
III	0	0	1	3	
IV	6	3	11	9	
Size					
T1	0	0	0	0	0.344
T2	0	0	1	0	
T3	0	0	3	5	
T4	6	3	8	7	
N status					
N0	0	0	1	0	0.09
N1	1	0	3	4	
N2b	2	1	6	5	
N2c	3	2	2	3	
Histological differentiation					
Well	0	0	3	11	<0.001
Moderate	3	1	9	1	
Poor	3	2	0	0	

RNA isolation

Oral tissue samples/cell lines were homogenized/placed in Tri-reagent (Sigma, USA) and total cellular RNA was isolated according to the manufacturer's protocol. The precipitated total RNA was dissolved in DEPC-treated water and contaminating DNA was removed by DNase I treatment (Sigma, USA). RNA integrity was analysed by electrophoresis, using a reference RNA (Stratagene, USA), and RNA samples were preserved at −80 °C until analysis.

Ribonuclease protection assay

Levels of mRNA for *Bcl-2* family members were examined in 25 oral tissues (Table 1), three oral cell lines and commercially procured normal adult tongue RNA (Biochain, USA), using a RPA. The multi-probe *Bcl-2* family template set, hAPO-2 RiboQuant (BD Biosciences, USA), with housekeeping genes *L32* and GAPDH as internal controls was used. Total RNA from oral tissues and cell lines was isolated, RNA probes generated and RPA performed according to the manufacturer's instructions (BD Biosciences). The dried gels were exposed to X-ray films for different time intervals to obtain non-saturating levels of GAPDH/*L32*. The radioactive bands were quantified and normalized to GAPDH/L32 levels using Quantity1 software (Biorad, USA) or a Molecular Dynamics Phosphorimager.

Reverse transcriptase-polymerase chain reaction analyses

Splice variants of *Mcl-1* were assessed in 25 oral tumours and three oral cell lines by RT–PCR. cDNA was synthesized with 2 μg total RNA, using a First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer's instructions. The efficiency of cDNA synthesis was estimated using β-actin. *Mcl-1*/β-*actin* primers (Sigma, USA), as reported earlier [18], were amplified in a PCR reaction using annealing temperatures of 60 °C for *Mcl-1* and 48 °C for β-*actin*. PCR products were separated on 2% agarose gels containing ethidium bromide, and amount of each PCR product was determined using a Gel-doc system (Amersham, UK).

Western blot analyses

Western blot analysis for Mcl-1 and β-actin was performed on the three oral cell lines, 25 oral tumours and corresponding adjacent normal mucosa, as described earlier [19]. A specific Mcl-1 antibody (1 : 1000, rabbit polyclonal, Pharmingen, USA) was used, which recognizes amino acids 121–139 (PEST region) of human Mcl-1 protein and can detect both the full-length and short isoforms of Mcl-1. A β-actin antibody (1 : 2000, mouse monoclonal; Sigma, USA) was used as control.

Immunohistochemical analyses

Levels of Mcl-1 protein *in situ* were estimated in 15 normal, 20 leukoplakias, 30 OSMFs and 33 OSCCs using an Avidin-Biotinylated-Peroxidase Complex kit (Vector Labs, USA). Paraffin wax sections were cut (4 μm), mounted on amino-propyltriethoxysilane-coated slides and stained using an anti-Mcl-1 rabbit polyclonal antibody (1 : 200; Pharmingen), using a protocol described earlier [14]. Immunostaining was also performed on cover-slip cultures of oral cell lines using an FITC-labelled secondary antibody (1 : 1000, Molecular Probes, USA), as described earlier [20]. The percentage of positive tumour cells was scored as follows: a minimum of 1500 cells were counted at ×400 magnification, using a conventional light microscope (Nikon, Japan) in 10–15 randomly selected different fields. The number of positively-stained cells were expressed as a percentage of the total number of cells and graded semi-quantitatively: 11–30% positive cells, grade (+); 31–50% positive cells, grade (++); >50% positive cells, grade (+++); and no positive cells or ≤10% positive tumour cells, grade negative.

Table 3. Correlation of Mcl-1 expression and clinicopathological features of oral leukoplakia.patients

	Degree of Mcl-1 staining in leukoplakia				
	0–10% Negative	11–30% +	31–50% ++	>50% +++	*p*
Cases	2	0	10	8	
Age (years)					
<40	0	0	5	3	0.413
>40	2	0	5	5	
Sex					
Male	0	0	1	1	0.870
Female	2	0	9	7	
Site of the lesion					
All oral sites except FOM, tongue	2	0	10	8	No statistics can be computed
FOM, tongue	0	0	0	0	
Clinical aspect					
Homogeneous	0	0	6	7	0.061
Non-homogeneous	2	0	4	1	
Histopathology					
No dysplasia	1	0	7	6	0.783
Mild	1	0	2	2	
Moderate	0	0	1	0	
Severe	0	0	0	0	
Stage grouping					
I	1	0	6	7	
II	1	0	3	1	
III	0	0	0	0	
IV	0	0	1	0	0.608

Statistical analyses

The SPSS v. 11 software package (SPSS Inc., USA) was used for all statistical evaluation of correlation with various clinicopathological features in OSCC, leukoplakia and OSMF cross-tabulation. Pearson's χ^2 test was carried out to assess the significance of the association. Fisher's exact test was performed when the expected cell frequency was <5. Variation among group means was analysed by one-way analysis of variance (ANOVA) test. Multiple comparison testing was carried out using the Tukey HSD procedure. Variables associated with the *Bcl-2* family genes and RPA were examined using the Mann–Whitney U-test. Anti- and pro-apoptotic transcripts were compared by *t*-test. The data are reported as mean ± SD. The difference between means was considered statistically significant when $p < 0.05$.

Table 4. Clinicopathological features of OSMF patients and the correlation with Mcl-1 protein expression

	Degree of Mcl-1 staining in OSMF				
	0–10% Negative	11–30% +	31–50% ++	>50% +++	*p*
Cases	4	4	7	15	
Age (years)					
<20	0	1	1	1	0.348
20–30	2	3	6	9	
30–40	2	0	0	5	
Sex					
Male	1	0	0	2	0.492
Female	3	4	7	13	
Groups					
I	0	0	0	0	0.581
II	1	2	1	5	
III	3	2	5	10	
IV	0	0	1	0	

Hierarchical cluster analysis

The normalized values for each gene in tumours by RPA, was analysed using Genesis software release 1.6.0 Beta 1 (Institute for Genomics and Bioinformatics, Graz University of Technology, Austria). The resulting expression map was visualized with Tree View in the same software package. The increased and decreased expression of genes in tumour specimens was indicated by red and green colours, respectively.

Results

Expression of *Bcl-2* family transcripts in oral cell lines and tissues by RPA

The mRNA expression of *Bcl-2* family genes in oral cell lines, tumour tissues, normal adjacent mucosa and commercial normal tongue RNA was evaluated using RPA. Figure 1a illustrates the mRNA expression of nine *Bcl-2* family members comprising of the anti- and pro-apoptotic genes and two housekeeping genes in oral cell lines AW 8507, AW 13 516, FBM, HeLa

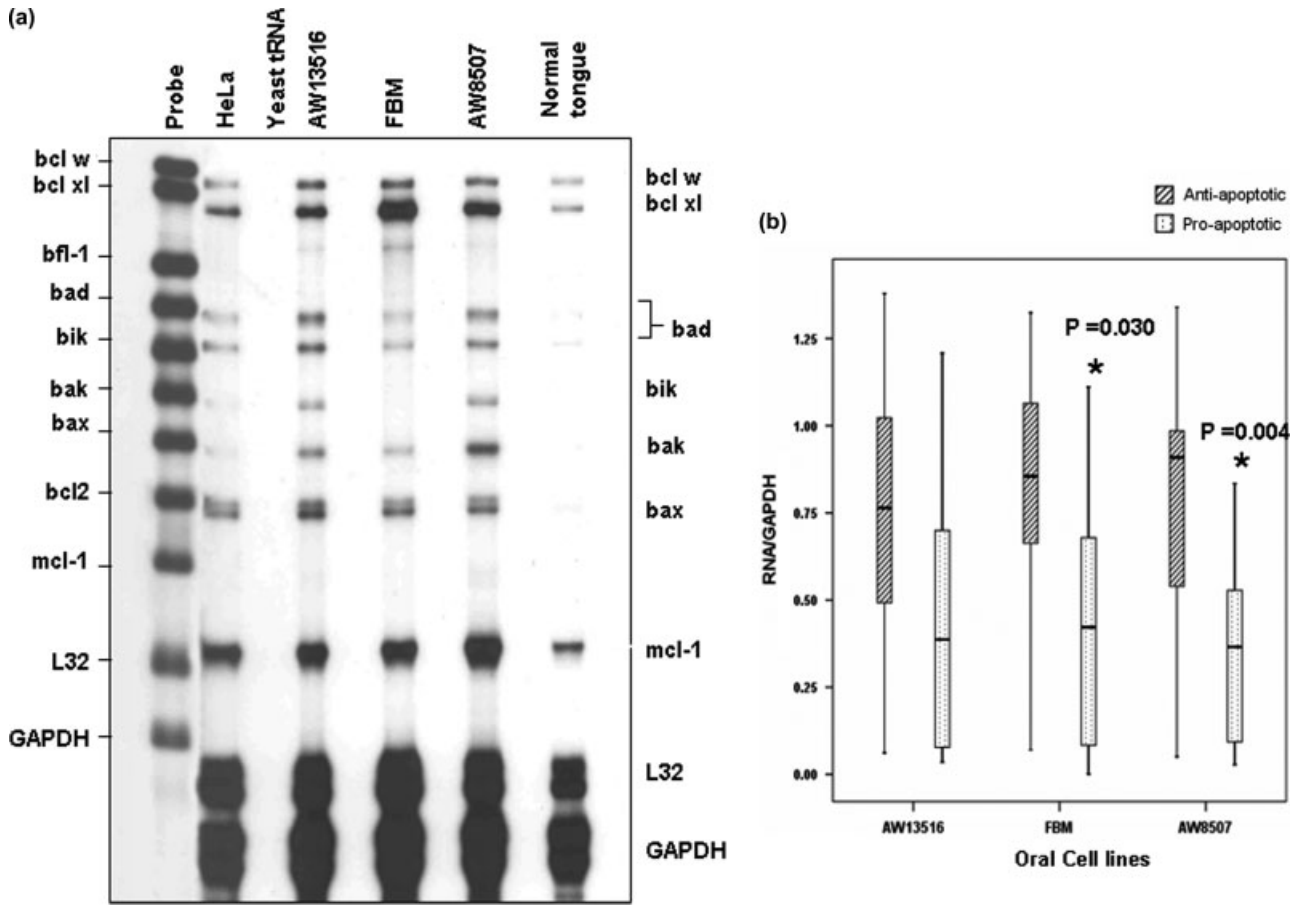

Figure 1. RPA image illustrating the expression of nine BCL-2 family transcripts in oral cell lines and normal tongue. (a) Anti- and pro-apoptotic *Bcl-2* family members in oral cell lines, AW13 516, AW8507 and FBM, and HeLa cell lines and commercially procured normal tongue RNA. (b) Box plot showing comparison between anti- and pro-apoptotic members in each oral cell line, using *t*-test

and commercially procured human adult tongue RNA. Further, the expression of each *Bcl-2* family member in all the three oral cell lines was statistically compared to that in normal tongue, using ANOVA test. Seven of the nine members of the *Bcl-2* family were expressed in the three oral cell lines and at significantly higher levels than in normal tongue. The significance values of *Mcl-1* gene expression alone is indicated in Figure 2a. Analysis by RPA was repeated three times in oral and HeLa cell lines and a summarized profile is illustrated in Figure 2a, demonstrating the levels of anti-apoptotic and pro-apoptotic members in the cell lines studied. The combined gene expression values of pro-apoptotic members versus the anti-apoptotic members in each cell line was analysed using t-test. As shown in Figure 1b, comparison between anti-apoptotic and pro-apoptotic members in each of the three oral cancer cell line demonstrated significantly higher expression of anti-apoptotic members in FBM and AW 8507 ($p = 0.03$, $p = 0.004$, respectively). In AW13 516, although a similar trend was seen the difference was not statistically significant ($p = 0.139$). Figure 3d is a histogram illustrating up-regulated normalized *Mcl-1* mRNA levels in the three oral and HeLa cell line as compared to that of normal tongue RNA.

To determine the *Bcl-2* family expression profile in tumour tissues, RPA was performed on 25 oral tumours and their corresponding normal adjacent mucosa. The normalized transcript levels in 25 oral tissues are illustrated as a scatter plot in Figure 2b. Eleven of 25 (44%) tumours exhibited *Mcl-1* transcript abundance greater than the corresponding normal adjacent mucosa. No statistically significant correlation was found between *Mcl-1* expression and clinicopathological parameters of oral cancer patients. Clustering of the *Bcl-2* family gene expression data using Tree View analysis in these 25 oral cancer patients (Figure 2c) identified a cluster of five oral tumours exhibiting a high *Bcl-2* family gene expression profile.

Mcl-1 splice variants in oral cell lines and tumours

Mcl-1 splice variants were assessed in 25 oral tumuors (T) and paired normal tissue (N) and the three oral cell lines by RT–PCR. Confirming the RPA analysis, 44% of the tumours and all three oral cell lines demonstrated overexpression of *Mcl-1* transcripts as compared to normal tissue. Figure 3a shows a representative gel illustrating the PCR amplified full-length and short isoforms of *Mcl-1* in the oral cell lines (AW8507,

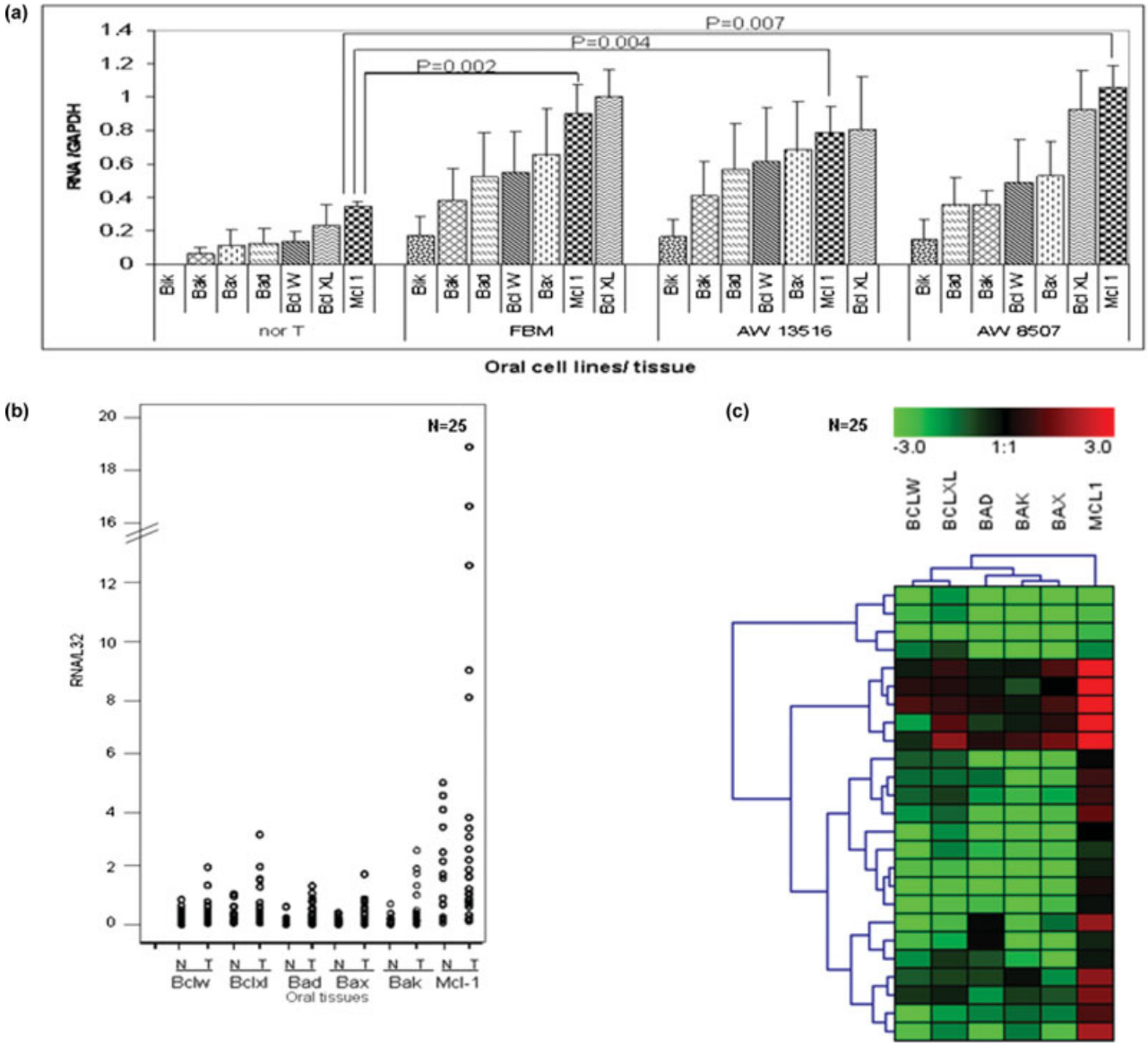

Figure 2. Normalized *Bcl-2* gene family expression profiles in oral cell lines and tumours. (a) Histogram indicating the profile in the oral cell lines and in normal tongue. (b) Scatter plot showing the *Bcl-2* gene expression in 25 oral tumours as compared to normal adjacent mucosa. (c) A tree view analysis of the gene expression data in 25 oral cancer patients is shown. One of the clusters identified consists of five tumours exhibiting a high gene expression profile

AW13 516 and FBM) and in oral tumours (T) and normal adjacent mucosa (N). A 5–10-fold higher expression of *Mcl-1* (L) full-length, anti-apoptotic transcript (446 bp product) as compared to the *Mcl-1* (S) pro-apoptotic mRNA (198 bp product) was observed in the above samples.

Mcl-1 protein expression in oral cell lines and tissues

Western blotting analysis (Figure 3b) demonstrated abundant expression of Mcl-1(L) (42 kDa) protein in the oral cell lines AW8507, AW13 516 and FBM and oral tumours (T) as compared to undetectable levels in adjacent normal (N), human tongue and buccal mucosa tissues. The pro-apoptotic 30 kDa protein was not detected in these samples. The cytoplasmic localization of Mcl-1 protein is demonstrated in the AW8507 oral cancer cell line in Figure 3c. Similar Mcl-1 staining was also observed in AW13 516 and FBM cell lines (data not shown).

Mcl-1 protein expression in normal oral mucosa, premalignant lesions and OSCC

In normal oral mucosa, weak cytoplasmic expression of Mcl-1 protein was observed predominantly in the differentiated squamous epithelial cells (Figure 4a). Figure 4b illustrates homogeneous expression of Mcl-1 in squamous epithelial cells of leukoplakic tissue. Intense cytoplasmic Mcl-1 immunoreactivity in differentiated squamous cells of the spinous epithelial layer and low or absent expression in the parabasal and basal cell layers was observed in most OSMF cases (Figure 4c). In OSCC, the Mcl-1 staining pattern

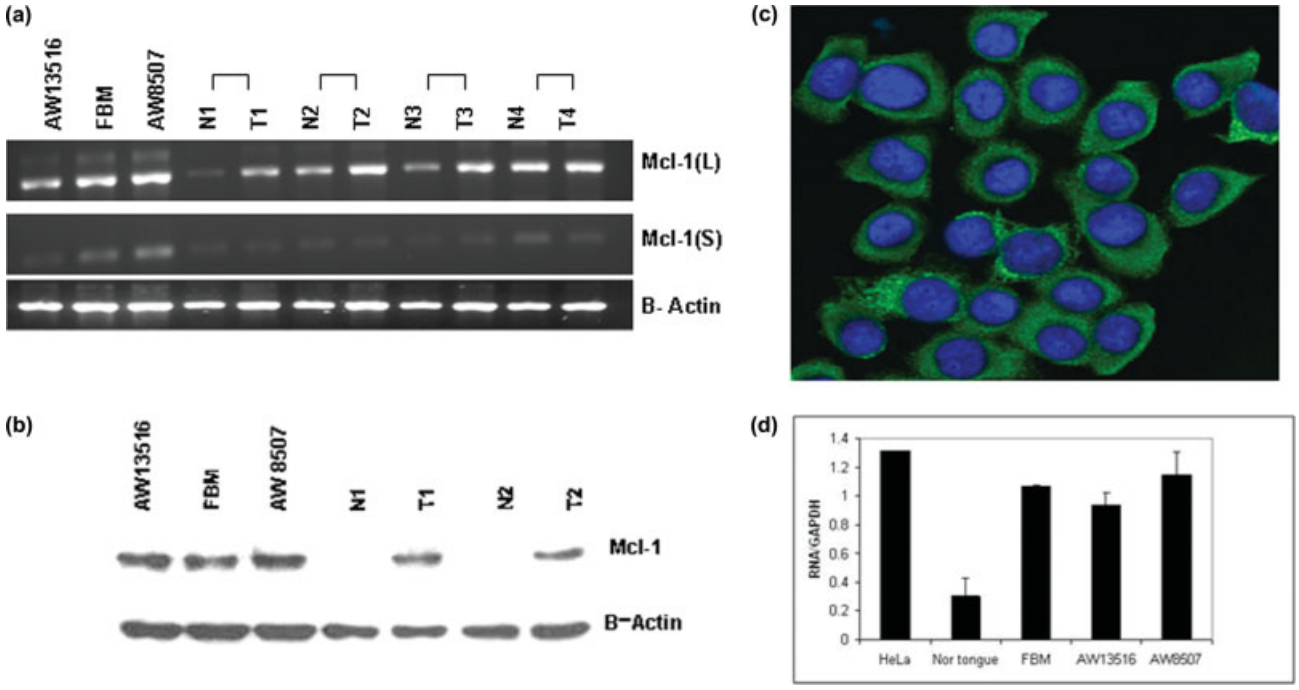

Figure 3. Expression of Mcl-1 protein and transcripts in oral cell lines and tumours. (a) RT–PCR reveals predominant expression of *Mcl-1* (L) versus low expression of *Mcl-1* (S) form in all the samples and β-actin mRNA is used as internal control. (b) Western blot indicates high expression of the 42 kDa Mcl-1 (L) form in the oral cell lines and oral tumours, using β-actin as the loading control. (c) Immunohistochemistry in AW13516 cell line illustrates cytoplasmic (green) localization of Mcl-1 protein, counterstained with DAPI (blue). (d) Histogram illustrating the normalized *Mcl-1* transcript levels in the oral cell lines, AW13516, AW8507 and FBM, as compared to commercial normal tongue RNA and HeLa cell line RNA

was homogeneous, with a more generalized cytoplasmic distribution (Figure 4d). In epithelial islands of OSCC, Mcl-1 expression was low in peripheral basal and parabasal cells. In poor and moderately differentiated OSCC, Mcl-1 staining was more prominent in cohesive epithelial areas than in isolated malignant cells which form a major part of the tissue.

Mcl-1 expression was detectable in 8/15 (53%) normal tissues, 18/20 (90%) leukoplakias, 25/30 (83%) OSMF and 27/33 (82%) OSCCs. The mean proportion of cells with Mcl-1 immunoreactivity was 19% for normal tissue, and significantly increased ($p < 0.01$) to 49% for leukoplakias, 45% for OSMF and 45% for OSCC (Figure 5a). The number of cases showing Mcl-1 immunoreactivity in the different staining grades in the above oral tissue groups is illustrated in Figure 5b. No statistically significant correlation was found between Mcl-1 expression and clinicopathological parameters of leukoplakia, OSMF and OSCC. Mcl-1 expression differed depending on whether the tumours were well-differentiated (14 cases), moderately differentiated (14 cases) or poorly differentiated (five cases). Expression of the Mcl-1 protein decreased from well- to moderately to poorly differentiated OSCC (Table 2), although this difference is not statistically significant. No Mcl-1 expression was detectable in three of five poorly-differentiated OSCC samples. The sample distribution and expression of Mcl-1 across the stages of OSCC, leukoplakia and OSMF are illustrated in Tables 2, 3 and 4, respectively.

Discussion

Signalling pathways that regulate apoptosis can directly modify Bcl-2 family proteins, as well as alter the expression of Bcl-2 family members at both the transcriptional and translational levels. In the present study, sensitive RPA was used to detect and quantify nine *Bcl-2* family members in oral cell lines and tumours. Our study demonstrated the predominance of anti-apoptotic (*Mcl-1, Bclx*(L) and *Bclw*) over pro-apoptotic (*Bax, Bad* and *Bak*) gene expression in oral cell lines. The possibility of a common intrinsic pathway regulation resulting in the over-expression of anti-apoptotic members which promote cell survival via neutralization of pro-apoptotic member activity is indicated by the overlapping *Bcl-2* family expression profile seen in all the cell lines studied.

Further, the RPA revealed up-regulation of *Mcl-1* transcripts in oral tumours as compared to normal mucosa. Mcl-1 is reported to play a critical role in the survival of malignant cells, since depletion of Mcl-1 via antisense oligonucleotides triggers apoptosis in cancer cells [21]. The factors leading to up-regulation of Mcl-1 in oral cancers is unclear. Several signalling pathways, including MAP kinase, PI3k/Akt and Jak/Stat, have been implicated in stimulation of *Mcl-1* transcription, acting via specific transcription factor response elements in the *Mcl-1* promoter [11]. Previous studies from our laboratory have demonstrated alterations in the MAP kinase signalling

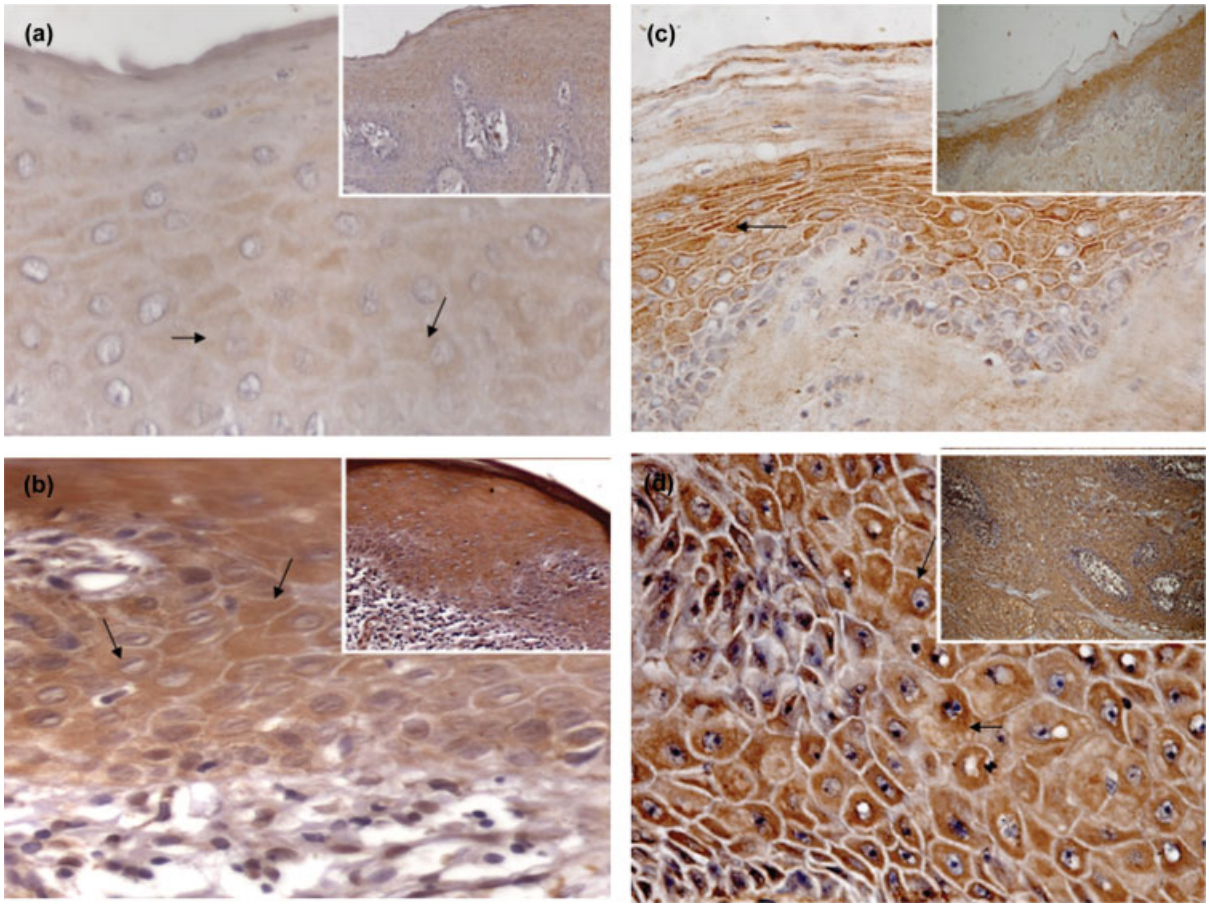

Figure 4. Immunohistochemical staining patterns of Mcl-1 protein in normal oral and leukoplakia, OSMF and OSCC tissues, with lower magnification as inset. (a) Normal oral tissue showing weak cytoplasmic expression of Mcl-1 protein in differentiated epithelium. (b) Homogeneous expression of Mcl-1 in leukoplakia. (c) OSMF epithelium, showing expression of Mcl-1 in the spinous layer, with low or no expression in the basal cells. (d) Well-differentiated oral squamous cell carcinoma, illustrating cytoplasmic expression of Mcl-1 protein

pathway components in oral cancers [22,23]. Mcl-1 function can also be regulated through alternate splicing [11]. Our study demonstrates a 5–10-fold higher expression of *Mcl-1*(L) transcripts as compared to the weakly expressed *Mcl-1*(S) transcript in oral cells indicating the predominance of the anti-apoptotic isoform.

Genetic changes in the *Mcl-1* gene may also be directly responsible for altered expression of *Mcl-1* transcripts and protein. In a report by Saxena *et al* [24], polymorphic insertions of 6 or 18 nucleotides in the promoter region of the *Mcl-1* gene were demonstrated to affect gene expression.

Mcl-1 protein has a short half-life and is rapidly down-regulated during apoptosis. Studies have shown that Mcl-1 contains PEST domains and is targeted for proteasome-mediated degradation by the E3 ubiquitin ligase MULE and rapidly degraded with an estimated short half-life of 30 min to 3 h [25]. Our studies demonstrate abundant expression of 42 kDa anti-apoptotic Mcl-1(L) protein in AW8507, AW13 516 and FBM oral cell lines and undetectable levels in normal oral tissues by western blotting. The 30 kDa Mcl-1(S) pro-apoptotic form was not detected, probably due to the low levels of its transcripts observed by RT–PCR in these samples. Cytoplasmic localization of Mcl-1 protein was demonstrated in all the three oral cell lines. Besides its predominant localization at the mitochondrial membranes, Mcl-1 has also been demonstrated in the nucleus and cytoplasm [19,26]. The amino terminus of anti-apoptotic Mcl-1 has been reported to regulate its localization and function [11].

There are few reports of Mcl-1 analysis in head and neck cancers. A study in locally-advanced head and neck cancer revealed expression of Bcl-2 in 15%, Mcl-1 in 92%, Bax in 85% and Bak in 92% cases evaluated [13]. Abundant expression of anti-apoptotic Bclx and Mcl-1 proteins were also illustrated in normal mucosa and SCC of larynx [27]. Frequent expression of Mcl-1 was also reported in 39 cases of thyroid carcinomas [28]. To our knowledge this is the first report on Mcl-1 expression in oral premalignant lesions and oral cancers.

The mean Mcl-1 expression levels observed in leukoplakia, OSMF and OSCC were similar but significantly ($p < 0.01$) higher than in normal mucosa. The reason for this up-regulation is unclear and the possible reasons have been discussed earlier in this section. No significant association was found between Mcl-1 expression and clinicopathological parameters.

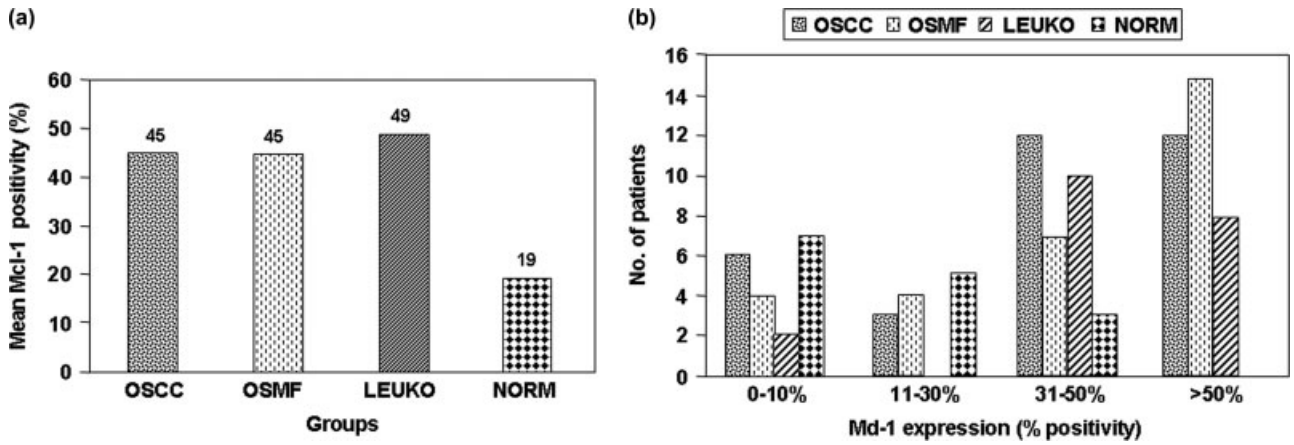

Figure 5. Graphical representation of percentage positivity of Mcl-1 expression in the different study groups. (a) Histogram indicating the mean percentage immunoreactivity in the four groups. (b) Histogram indicating the number of patients showing the different percentage positivity in the four groups

Mcl-1 is normally expressed in a cell type specific manner, with tissue- and differentiation-specific variations in expression levels. High levels of Mcl-1 are reported in the more differentiated apical layers of epithelia, whereas Bcl-2 expression tends to be higher in the basal cell layer [29]. In oral tissues we observed homogeneous, cytoplasmic distribution, predominantly in the differentiated squamous epithelial cells, whereas the basal, parabasal and keratinized upper layers expressed low or no Mcl-1. The expression of Mcl-1 protein in the differentiated cells supports its role in differentiation apart from its anti-apoptotic activity. Mcl-1 protein expression tended to decrease from well to moderately to poorly differentiated oral tumours. This may be related to the tendency of more differentiated cells to express this protein. *Mcl-1* was originally identified as a gene up-regulated early in the differentiation of a human myeloid leukaemia cell line and increased expression was discovered during cell commitment to differentiation [6]. The association of Mcl-1 expression may therefore be an indirect indication of cells that are more differentiated, although not obvious by simple morphological criteria.

Studies involving larger cohorts of patients with follow-up data are needed to explore any prognostic significance of Mcl-1 in OSCC. As chewing tobacco-associated oral cancers evolve in a series of distinct steps, wherein they are preceded by oral premalignant lesions, these precancerous lesions are therefore considered an early stage in oral carcinogenesis. The observed high expression of anti-apoptotic Mcl-1 protein in leukoplakia, OSMF and OSCC, as compared to normal mucosa, suggests that Mcl-1 protein expression may be an important early event in initiation and progression of OSCC, possibly via attenuation of pro-apoptotic bcl-2 members and leading to evasion of apoptosis. Further, the reported role of Mcl-1 as a chemoresistance factor in cancer [30] indicates it to be a potential therapeutic target in oral cancers.

Acknowledgements

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References

1. Notani PN. Epidemiology and prevention of head and neck cancer: a global view. In *Contemporary Issues in Oral Cancer*, Saranath D (ed.). Oxford University Press: New Delhi, 2000; 1–29.
2. Notani PN. Global variation in cancer incidence and mortality. *Current Sci* 2001;**81:**465–474.
3. Loro LL, Vintermyr OK, Johannessen AC. Cell death regulation in oral squamous cell carcinoma: methodological considerations and clinical significance. *J Oral Pathol Med* 2003;**32:**125–138.
4. Polverini PJ, Nor JE. Apoptosis and predisposition to oral cancer. *Crit Rev Oral Biol Med* 1999;**10:**139–152.
5. Kamer AR, Krebs L, Hoghooghi SA, Liebow C. Proliferative and apoptotic responses in cancers with special reference to oral cancer. *Crit Rev Oral Biol Med* 1999;**10:**58–78.
6. Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. *MCL-1*, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to *BCL-2*. *Proc Natl Acad Sci USA* 1993;**90:**3516–3520.
7. Craig RW. MCL-1 provides a window on the role of the BCL-2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* 2002;**16:**444–454.
8. Jourdan M, De Vos J, Mechti N, Klein B. Regulation of Bcl-2 family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-α and insulin-like growth factor 1. *Cell Death Differ* 2000;**7:**1244–1252.
9. Zhan Q, Bieszczad CK, Bae I, Fornace AJ Jr, Craig RW. Induction of BCL-2 family member MCL-1 as an early response to DNA damage. *Oncogene* 1997;**14:**1031–1039.
10. Zhou P, Qian L, Bieszczad CK, Noelle R, Binder M, Levy NB, *et al*. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. *Blood* 1998;**92:**3226–3239.
11. Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005;**37:**267–271.
12. Zhou P, Levy NB, Xie H, Qian L, Lee Chi-Yu G, Gascoyne RD, *et al*. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. *Blood* 2001;**97:**3902–3909.
13. Hotz MA, Bosq J, Zbaeren P, Reed J, Schwab G, Krajewski S, *et al*. Spontaneous apoptosis and the expression of p53 and Bcl-2

family proteins in locally advanced head and neck cancer. *Arch Otolaryngol Head Neck Surg.* 1999;**125:**417–422.
14. Saranath D, Tandle AT, Teni TR, Dedhia PM, Borges AM, Sanghavi V, *et al*. p53 activation in chewing tobacco-induced oral cancers and leukoplakias from India. *Oral Oncol* 1999;**35:**242–250.
15. Teni T, Pawar S, Sanghvi V, Saranath D. Expression of bcl2 and bax in chewing tobacco-induced oral cancer and oral lesions from India. *Pathol Oncol Res* 2002;**8:**109–114.
16. Tatake RJ, Rajaram N, Damle RN, Balsara B, Bhisey AN, Gangal SG. Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors. *J Cancer Res Clin Oncol* 1990;**116:**179–186.
17. Raul U, Sawant S, Dange P, Kalrayia R, Ingle AD, Vaidya M. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: Induction of transformed phenotype. *Int J Cancer* 2004;**111:**662–668.
18. Hartley PS, Bayne RA, Robinson LL, Fulton N, Anderson RA. Development changes in expression of myeloid cell leukemia-1 in human germ cells during oogenesis and early folliculogenesis. *J Clin Endocrinol Metab* 2002;**87:**3417–3427.
19. Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J Leukocyte Biol* 2000;**68:**158–166.
20. Vaidya MM, Sawant SS, Borges AM, Naresh NK, Purandare MC, Bhisey AN. Cytokeratin expression in human fetal tongue and buccal mucosa. *J Biosci* 2000;**25:**235–242.
21. Derenne S, Monia B, Dean NM, Rapp MJ, Harousseau JL, Taylor JK, *et al*. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x (L) is an essential survival protein of human myeloma cells. *Blood* 2002;**100:**194–199.
22. Saranath D, Bhoite LT, Deo MG. Molecular lesions in human oral cancer: the Indian scene. *Eur J Cancer B Oral Oncol* 1993;**29B:**107–112.
23. Rai R, Mahale A, Saranath D. Molecular cloning, isolation and characterisation of ERK3 gene from chewing-tobacco induced oral squamous cell carcinoma. *Oral Oncol* 2004;**40:**705–712.
24. Saxena A, Moshynska OV, Moshynskyy ID, Neuls ED, Qureshi T, Bosch M, *et al*. Short nucleotide polymorphic insertions in the MCL-1 promoter affect gene expression. *Cancer Lett* 2007;**251:**114–131.
25. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 2005;**121:**1085–1095.
26. Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* 1995;**128:**1173–1184.
27. Whisler LC, Wood NB, Caldarelli DD, Hutchinson JC, Panje WR, Friedman M, *et al*. Regulators of proliferation and apoptosis in carcinoma of the larynx. *Laryngoscope* 1998;**108:**630–638.
28. Branet F, Brousset P, Krajewski S, Schlaifer D, Selves J, Reed JC, *et al*. Expression of the cell death-inducing gene *bax* in carcinomas developed from the follicular cells of the thyroid gland. *J Clin Endocrinol Metab* 1996;**81:**2726–2730.
29. Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K, *et al*. Immunohistochemical analysis of Mcl-1 protein in human tissues. *Am J Pathol* 1995;**146:**1309–1319.
30. Thallinger C, Wolschek MF, Wacheck V, Maierhofer H, Gunsberg P, Polterauer P, *et al*. Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model. *J Invest Dermatol* 2003;**120:**1081–1086.