Role of Epstein-Barr Virus in Epithelial Cell Cancer

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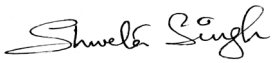

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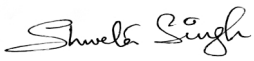

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List of Publications

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3. **Shweta Singh** and Subrata Banerjee. Downregulation of HLA-ABC expression along with MIC-A/B surface expression in LMP2A-positive epithelial carcinoma cell lines. *SINP School and Symposium on ADVANCES IN BIOMEDICAL MASS SPECTROMETRY 2019 & Metabolomics Workshop;* November 11-17, 2019, SINP, Kolkata, India.

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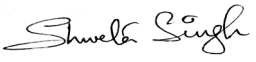

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DEDICATIONS

Dedicated to my grandparents……

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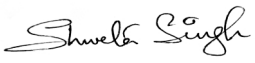

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Chapter-1

Introduction

Cancer

Cancer is a disease where cells of a specific tissue undergo abnormal proliferation and differentiation, finally resulting in accumulation of clonal cells referred to as 'tumour formation' (1). Increased tumour cell number and burden results in adverse effects on the host. A tumour may be characterized as benign or malignant in terms of behaviour and response to the treatment (Figure 1.1). Benign tumour endures to its original location, and is incapable of invading surrounding normal tissue or spreading to obscured body sites. However, malignant tumour has the ability to invade surrounding normal tissue and transmitting to distant body sites through the circulatory or lymphatic systems by a process referred to as 'metastasis' (2). Benign tumours can be treated through surgery in the affected area, whereas malignant tumours due to their metastasizing property fails to respond to localized treatments. As a result, in most of the cases malignant tumours becomes life threatening.

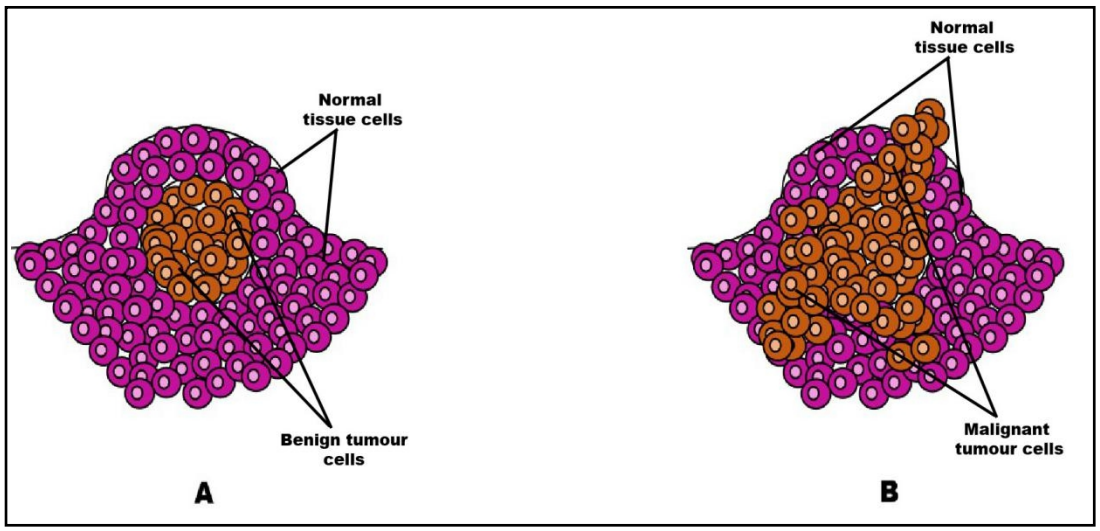

Figure 1.1: Tumour characterization in terms of localization. *(A) Benign Tumour- Growth of tumour cells is localized and does not spread through invasion or*

metastasis. (B) Malignant Tumour- Invade neighbouring tissues and metastasize to different sites.

Benign and malignant tumours are further grouped on the basis of cell type from which they ensued. Most of the tumuors reside to one of the four major groups: carcinomas, lymphomas, sarcomas, and leukemias . Carcinomas, are malignancies of epithelial cells, approximately 90% of human cancers worldwide fall under this category. Sarcomas, are solid tumours of connective tissues, such as bone, cartilage, muscle and fibrous tissue, they are rarely found in humans. Leukemias, are cancers of blood-forming cells. Lymphomas are tumours associated with cells of the immune system. Leukemias and Lymphomas account for approximately 8% of human cancers throughout the world. Tumours are also classified according to tissue from which it originated (e.g., lung or breast carcinomas) and the cell type involved. At the cellular level, cancer development is marked as a multistage process which involves mutation and selection of cells possessing successively intensified scope for proliferation, survival, invasion, and metastasis (3). The first step in the development process is tumour initiation, the consequence of genetic alteration resulting in uncontrolled and abnormal cell proliferation. Abnormal cell proliferation drives the outgrowth of a clonal cell population acquiring tumourogenic properties (4,5). Tumour progression pursues as an effect of additional mutations occurring within the tumour cell population. Some of these mutations allocates various selective leverage to the tumourogenic cell, such as, promotion of rapid growth, and the scions of a cell carrying such mutations will therefore turn into dominant ones (6). The process is referred to as clonal selection, leading to derivation of a new clone of tumour cells on the basis of heightened proliferation rate along with additional

properties, like survival, metastasis and invasion, which provides a selective advantage. Clonal selection exists throughout the process of tumour development, as a result tumour constantly become more fast-growing and highly malignant (7). In 2000, Hanahan and Weinberg in a review proposed six major hallmarks of cancer which together are capable of enabling tumour growth and metastatic circulation. The hallmarks constitute a logical framework for understanding the biology of cancer cells. They include insensitivity to anti-growth signals, self-sufficiency in growth signals, uncontrolled replicative potential, defying programmed cell death, sustained angiogenesis, and stimulating tissue invasion and metastasis (8). Fundamental to these hallmarks includes genome instability, which bring about the genetic diversity that facilitates their procurement, and inflammation, which promotes different hallmark functions. In the last decade two emerging hallmarks of potential generality was included in this list on the basis of conceptual progress—reprogramming of energy metabolism and evading immune destruction (Figure 1.2) (9). In addition to cancer cells, tumours manifest another magnitude of complexity by creating a ‘tumour micro-environment’ which contains a repertoire of recruited, evidently normal cells to accomplish the acquisition of hallmark traits (10,11).

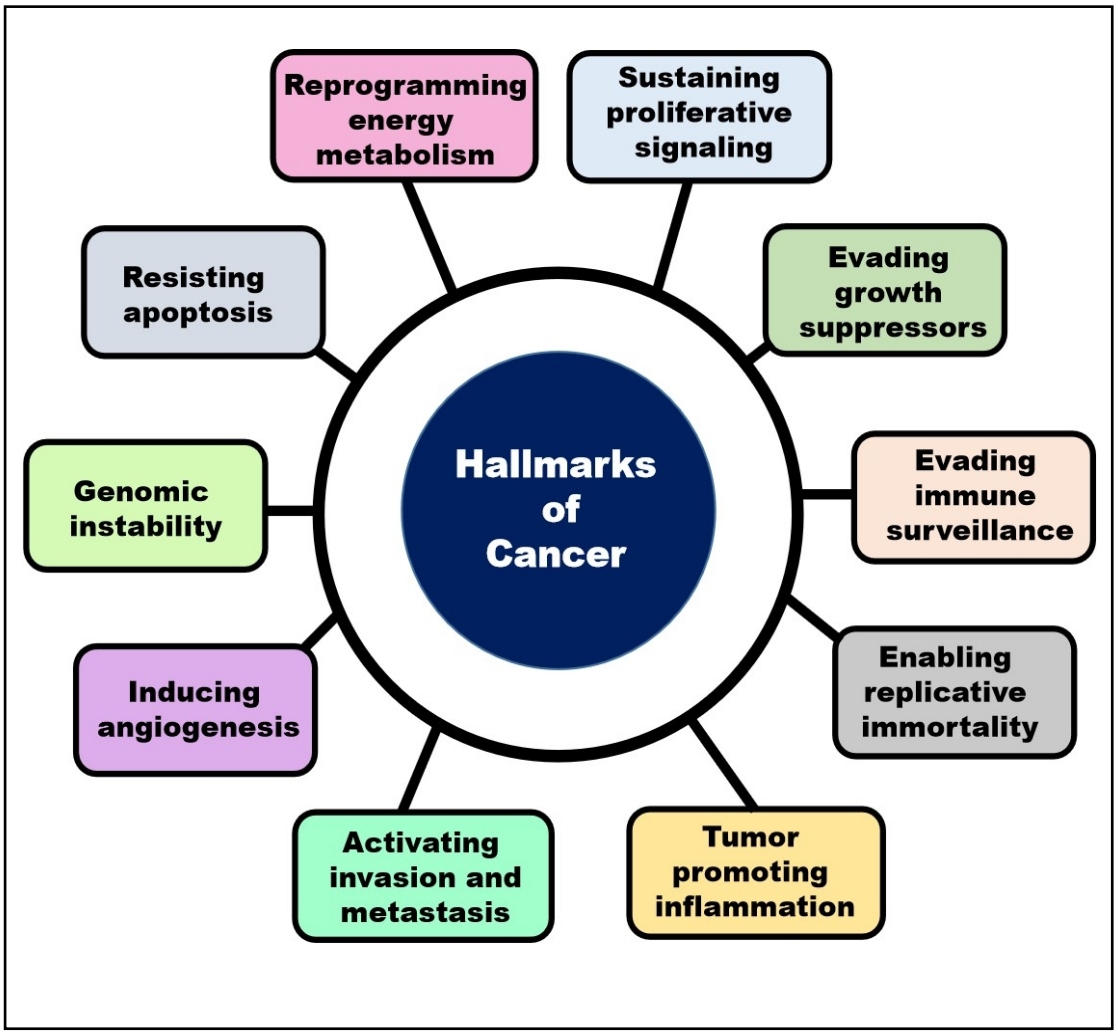

Figure 1.2: Major hallmarks of Cancer (Adapted and modified from Hanahan and Weinberg, 2011). *Schematic representation of characteristic hallmarks of Cancer*.

Cancer Causing Agents

Agents that cause cancer, are referred to as carcinogens, have been determined through studies in experimental animals along with an epidemiological analysis of cancer prevalence in human populations (e.g., cigarette smokers highly prone to lung cancers) (12). Tumour development is a perplexed multi-step procedure, various factors may influence the probability for the development of cancer and to become

malignant and invasive. The International Agency for Research on Cancer (IARC) has till date considered the potential of almost 1000 different agents to cause cancer through evaluation of scientific literature. Nevertheless, a number of agents, including radiation, chemicals, and infectious pathogens, have been reported to play important role in inducing cancer in both experimental animals and humans. Radiation and a number of chemical carcinogens induce mutations through DNA damages and are generally referred to as initiating agents. Initiating agents (such as ultraviolet radiation, tobacco smoke and aflatoxin) are those substances which are capable of inducing mutations in key target genes and crucial for the initiation of cancer development (13-15). Many mutations (Gain of function mutations for oncogenes) cause increased cell proliferation and thereby inducing cancer. They are referred as tumour promoters, hormones are classic examples for tumour promoter (16). Apart from chemicals and radiation, some infectious pathogens including many viruses and bacteria (specifically Helicobacter pylori) are known to cause cancer. Above mentioned agents can directly or indirectly lead to cancer and are referred to as direct or indirect carcinogens, respectively. Direct carcinogens are viruses such as Epstein Barr virus (EBV), Human Papillomaviruses (HPV), Hepatitis B virus (HBV), Kaposi Sarcoma associated Herpesvirus/ Human Herpesvirus 8 (KSHV), Hepatitis C virus (HCV), Human T Lymphotropic virus (HTLV-1) along with recently identified Merkel Cell Polyomavirus (MCV) (17-21) (Table 1.1). Indirect carcinogens include, bacteria, such as, Helicobacter pylori (22).

Virus	Genetic material	Associated cancers	Year first identified
Epstein-Barr virus (EBV also known as human herpesvirus-4,HHV-4)	Double-stranded DNA	Burkitt's lymphoma, Hodgkin's and non-Hodgkin's lymphoma, lymphoproliferative disorders, nasopharyngeal carcinoma and gastric carcinoma.	1964
Hepatitis B virus (HBV)	Partially double-stranded circular DNA	Hepatocellular carcinoma	1965
Human T-lymphotropic virus-1 (HTLV-1)	Positive, single-stranded RNA	Adult T cell leukaemia	1980
Human papillomaviruses	Double-stranded DNA	Cervical cancer and penile cancers, anogenital cancers, head and neck cancers	1983-1984
Hepatitis C virus	Positive, single-stranded RNA	Hepatocellular carcinoma and lymphomas	1989
Kaposi's sarcoma associated herpesvirus (KSHV; also known as human herpesvirus 8, HHV-8)	Double-stranded DNA	Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's di	1994
Merkel cell polyomavirus (MCV)	Double-stranded DNA	Merkel cell carcinoma	2008

Table 1.1: Viruses known to cause human cancers are discussed along with salient features (Adapted and modified from Moore and Chang, 2010).

Epstein Barr Virus

Viruses are known to be an important class of microorganisms capable of establishing successful infection of the host. The International Agency for Research on Cancer (IARC) evaluates that 20% of the cancer cases reported worldwide is associated with viral infection. Tumour viruses play crucial role in elucidating the molecular events liable for the development of cancers (Figure 1.4) activated by both viral and non-viral carcinogens (23-25).

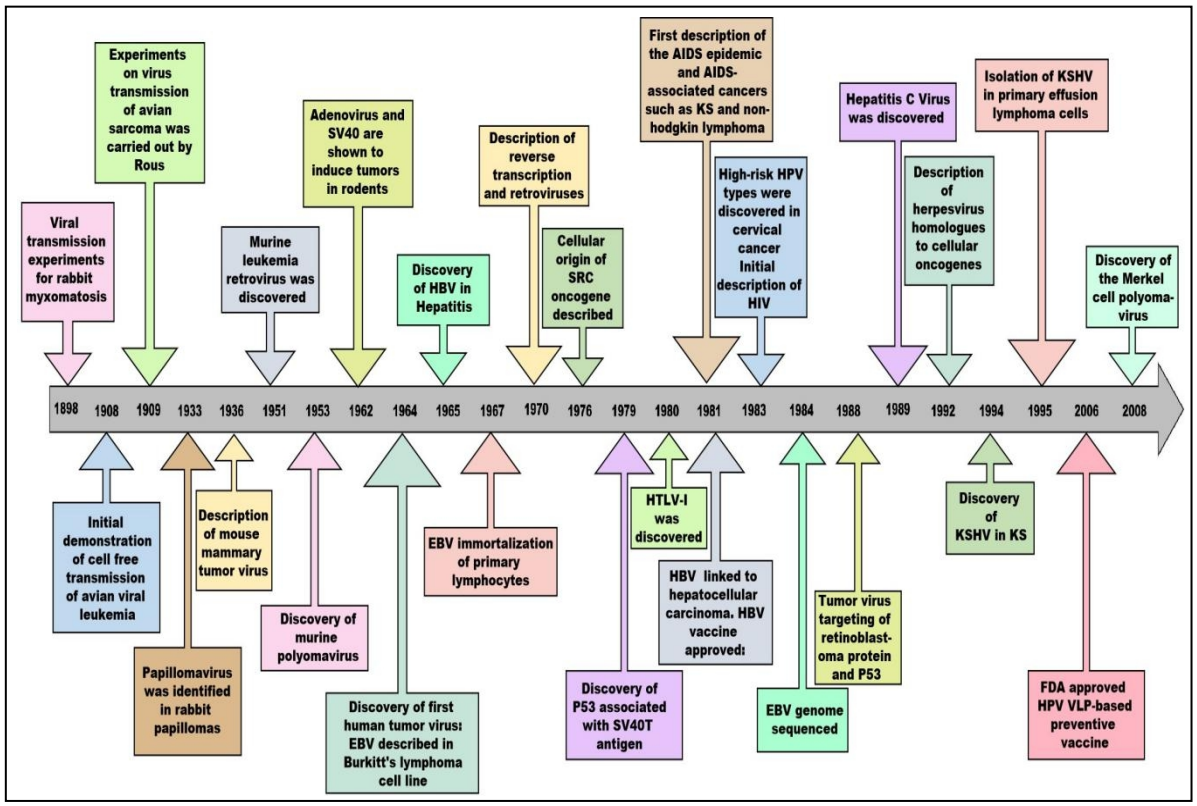

Figure 1.3: Major milestones and events in the area of viruses and human cancer during the first century (Adapted and modified from Moore and Chang, 2010). *In this Timeline an overview of the major milestones in the research areas on viruses and human cancer are highlighted.*

Epstein-Barr virus (EBV also known as human herpesvirus 4) was the first human tumour virus to be identified from patient with Burkitt's Lymphoma in the year 1964 (26,27). EBV is known to account for more than 200,000 cancer cases per year and is estimated to cause 1.8% of all cancer deaths (28). EBV establishes most prevalent and persistent virus infection in humans, with approximately more than 90% of the world's population being an asymptomatic carrier of the virus throughout lifetime. EBV infection has been classified as a Group I carcinogen by IARC (IARC monograph,1997) (18), indicating the carcinogenicity of the virus in blood cell and epithelial cell malignancies. Immunocompromised hosts are susceptible to EBV infection, where the virus can cause opportunistic lymphomas. EBV infection is also prevalent in several other malignancies, such as Burkitt's lymphoma, Hodgkin Lymphoma, peripheral natural killer/ T-cell lymphoma, nasopharyngeal carcinoma, smooth muscle tumours, and gastric cancers (29-32).

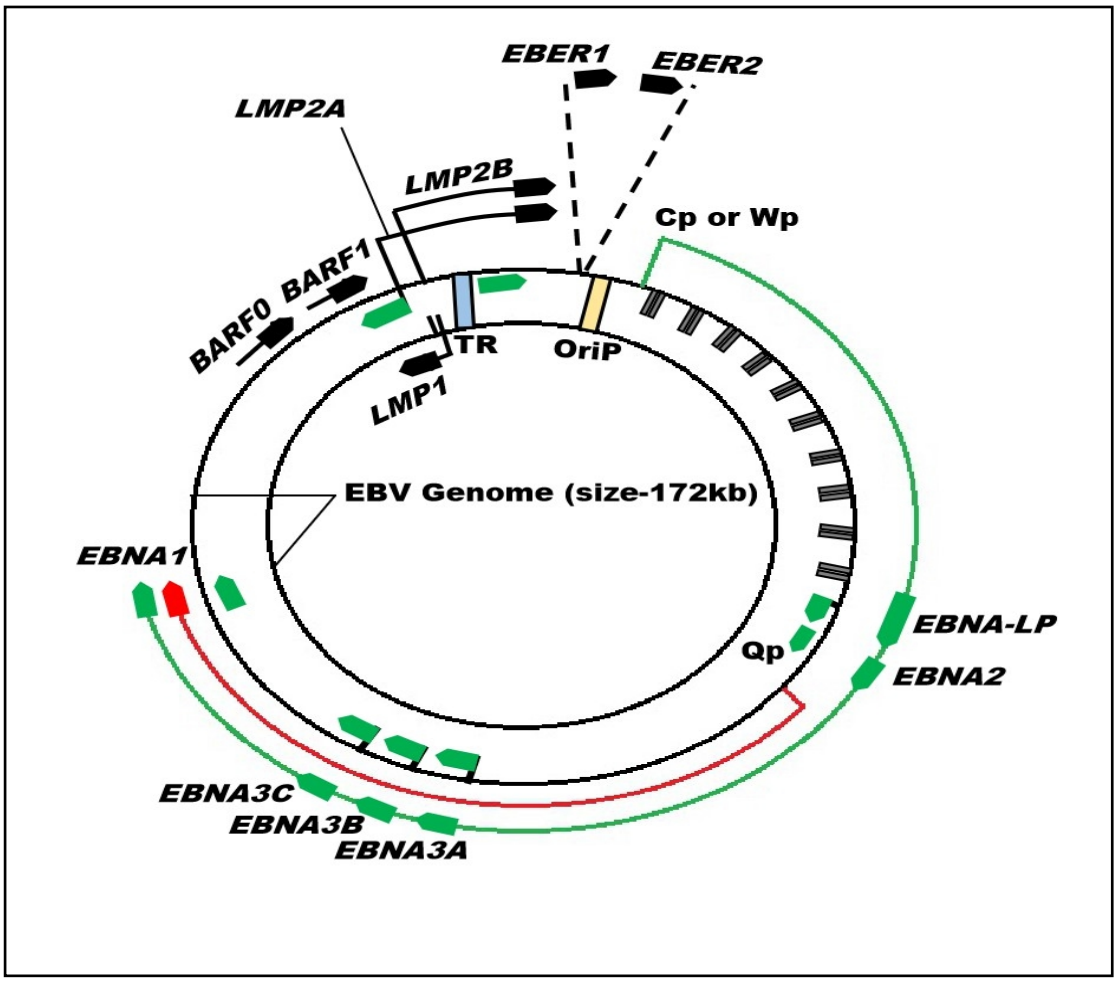

Figure 1.4: EBV double-stranded DNA episome (Adapted and modified from Young and Rickinson, 2004). *EBV circular genome: latent genes.*

EBV has a relatively large, 172 kb double-stranded DNA genome (Figure 1.5). The EBV genome is known to express about 80 proteins along with 46 functional small untranslated RNAs. Some of the proteins play role in replication of the viral genome and production of new viral particles during the lytic phase. However, herpesviruses are well-known for another mode of gene expression which allows them to establish a persistent long-term infection within the host without the production of infectious

viral particles during the latent phase. The normal life cycle of EBV comprises of infection of B lymphocytes and some epithelial cells (33-40).

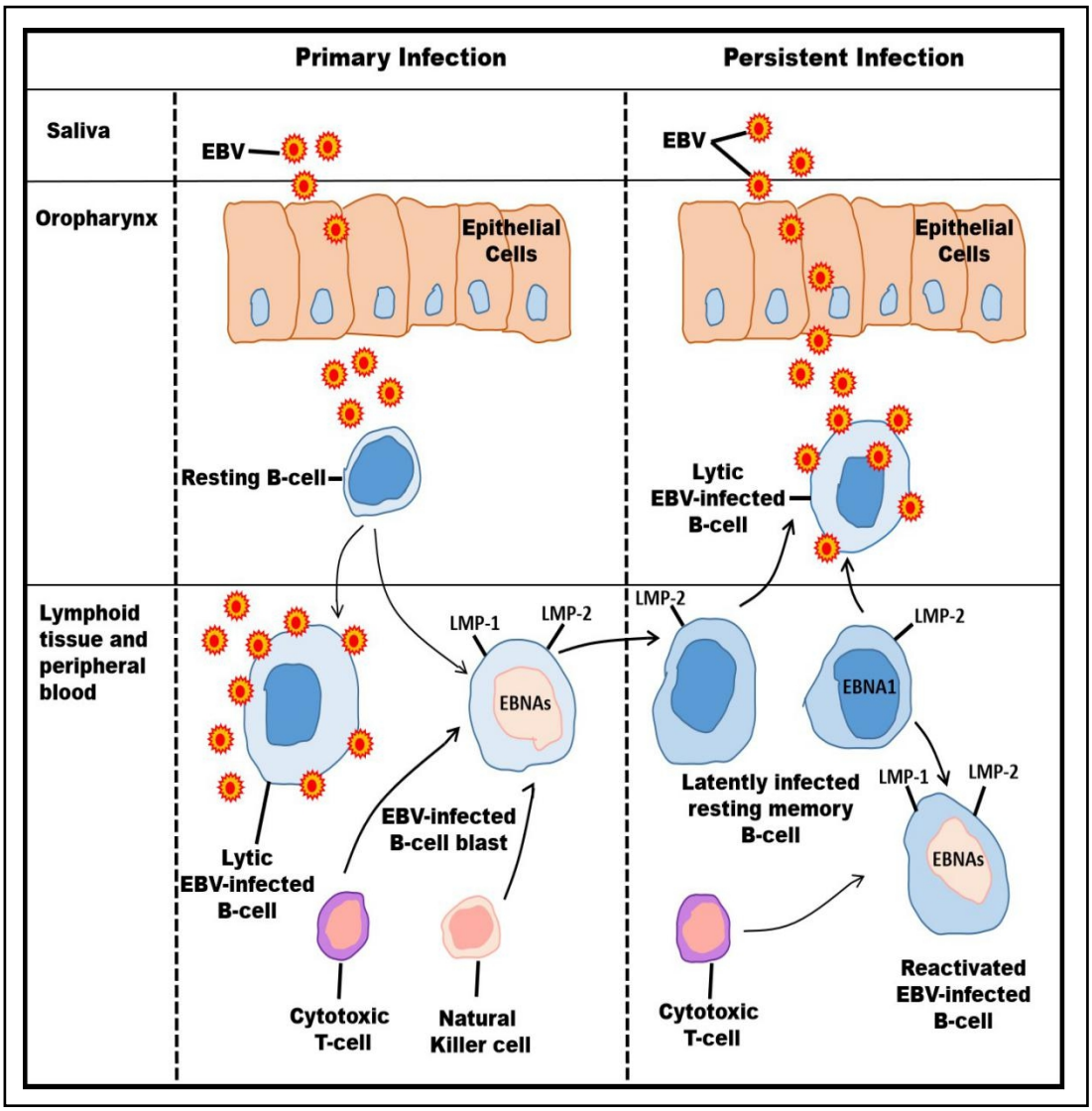

Figure 1.5: Model for establishment of primary and persistent infection by EBV in humans (Adapted and modified from Cohen, 2000). *In the oropharynx, EBV instantaneously infects resting B cells or epithelial cells, which further infect the resident B cells. During the course of primary infection, B cells infected with EBV endures lytic infection resulting in cultivation of infectious virus or express the full*

complement of viral latent proteins. The later cells are further monitored by CTLs and NK cells. After convalescence, the peripheral blood harbors EBV in latently infected memory B cells where the virus expresses latent membrane protein 2 (LMP2) along with EBV nuclear antigen1 (EBNA1). EBV reactivation may take place in the latter cells and express other viral latent proteins, leading to their identification and lysis by CTLs. In the oropharynx, few cells having latent infection undergo lytic replication, leading to production of infectious virus and shedding of virus through saliva or infection of other epithelial cells.

EBV is usually transmitted in saliva through shedding, and primary infection is initiated when EBV crosses the oropharynx epithelium, further infecting the naive B cell population existing in the Waldeyer's tonsillar ring which circumscribes the access to the nasopharynx and oropharynx. A number of viral latency transcription events takes place, which drives the EBV-infected B cells into resting memory B cells and establishes a life-long infection (Figure 1.6). The differentiation of memory B cells into plasma cells bring about lytic EBV infection leading to release of infectious virus particles that further establishes infection of the oropharyngeal epithelial cells for frequent replication and transmission of the virus (41). Persistent life-long EBV infection is sustained through a check-and-balance of the host immune system to phase out infected cells.

EBV is known to readily infect B lymphocytes by means of it major viral envelope protein, gp350, which binds to the CD21 receptor, expressed on the surface of the B-cells. Viral glycoprotein, gp42 binds with the human leukocyte antigen (HLA) class II protein on the surface of B-cell and turns on the core fusion machinery of EBV,

which consists of the envelope proteins, gB and gHgL (42,43). Infection of epithelial cells is ineffective; as epithelial cells fail to express the two membrane proteins, HLA class II protein and CD21. A recent report showed that viral protein complex gHgL interacts with the host integrin complex, αvβ6 and αvβ8. The integrin complex is localized on the surface of epithelial cells and expedites the fusion of the host cell membrane with the viral envelope (43). Gp42 cripples the entry of EBV into epithelial cells by blocking the binding of gHgL complex to its cognate receptor. Interestingly, EBV particles originating from epithelial cells are rich in gp42, efficiently infects B-cells but fails to infect epithelial cells. Whereas, those released from B cells better infects epithelial cells due to lack of gp42 (Figure 1.7). This dual cell tropism helps the virus to shuttle from epithelial cells to B cells and then back to epithelial cells during its infection period (44).

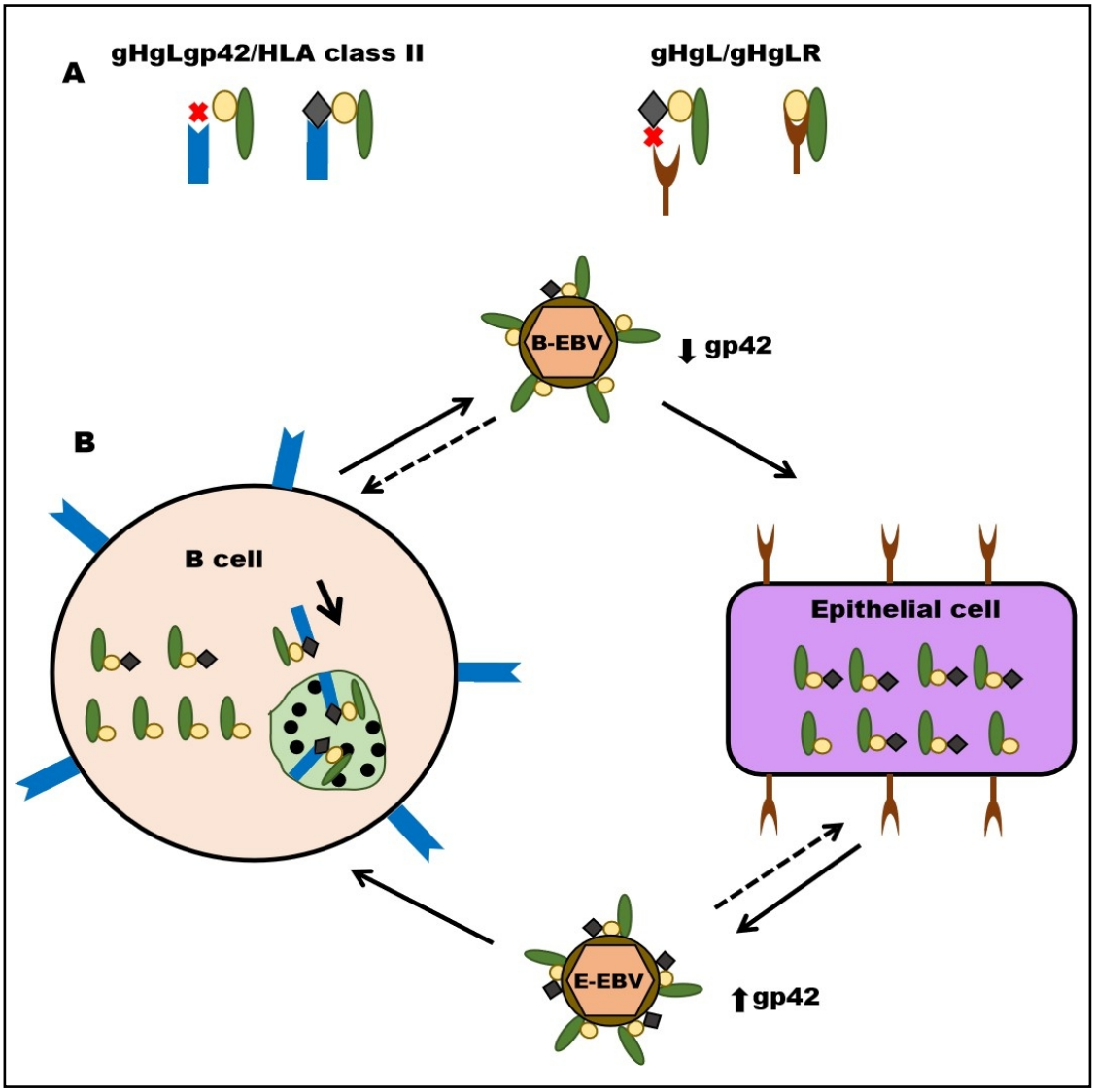

Figure 1.6: Glycoproteins (gp) 42 involved in the entry of EBV into B cells and epithelial cells (Adapted and modified from Hutt-Fletcher, 2007). *(A) B-cell infection involves interaction between gHgLgp42 and HLA class II surface molecules, whereas epithelial cell infection involves interaction between gHgL and gHgLR. Complexes deficit of gp42 fails to bind HLA class II, and complexes having gp42 fails to bind gHgLR. (B) gHgLgp42 complexes can bound to HLA class II molecules in the endoplasmic reticulum of infected B cell. They may then be destined to endosomal vesicles rich in proteases, site for generation of antigenic peptides from incoming*

proteins for loading onto the peptide binding groove of HLA class II molecule before localization on to the cell surface. This results in loss of gHgLgp42 complexes due to degradation and a relative lower gHgLgp42 complexes in viruses thus produced from B cells. gHgL complexes in epithelial cells are not lost through this degradative pathway, and viruses generated from epithelial cells are in turn consist of higher levels of gp42. Epithelial cells with high levels of gp42 are most effective in infecting B cells, and B cells with low levels of gp42 are more effective in infecting epithelial cells.

EBV-associated epithelial cancers has gained importance in the past two decades that represents 80% of all EBV-associated malignancies worldwide (45). Nasopharyngeal carcinoma (NPC) and EBV-associated gastric cancers (EBVaGCs) are the most common among the EBV-associated malignancies (Figure 1.8) (45). Clonal EBV genome and the expression of a group of viral latent genes are frequently reported in these type of cancers (46,47).

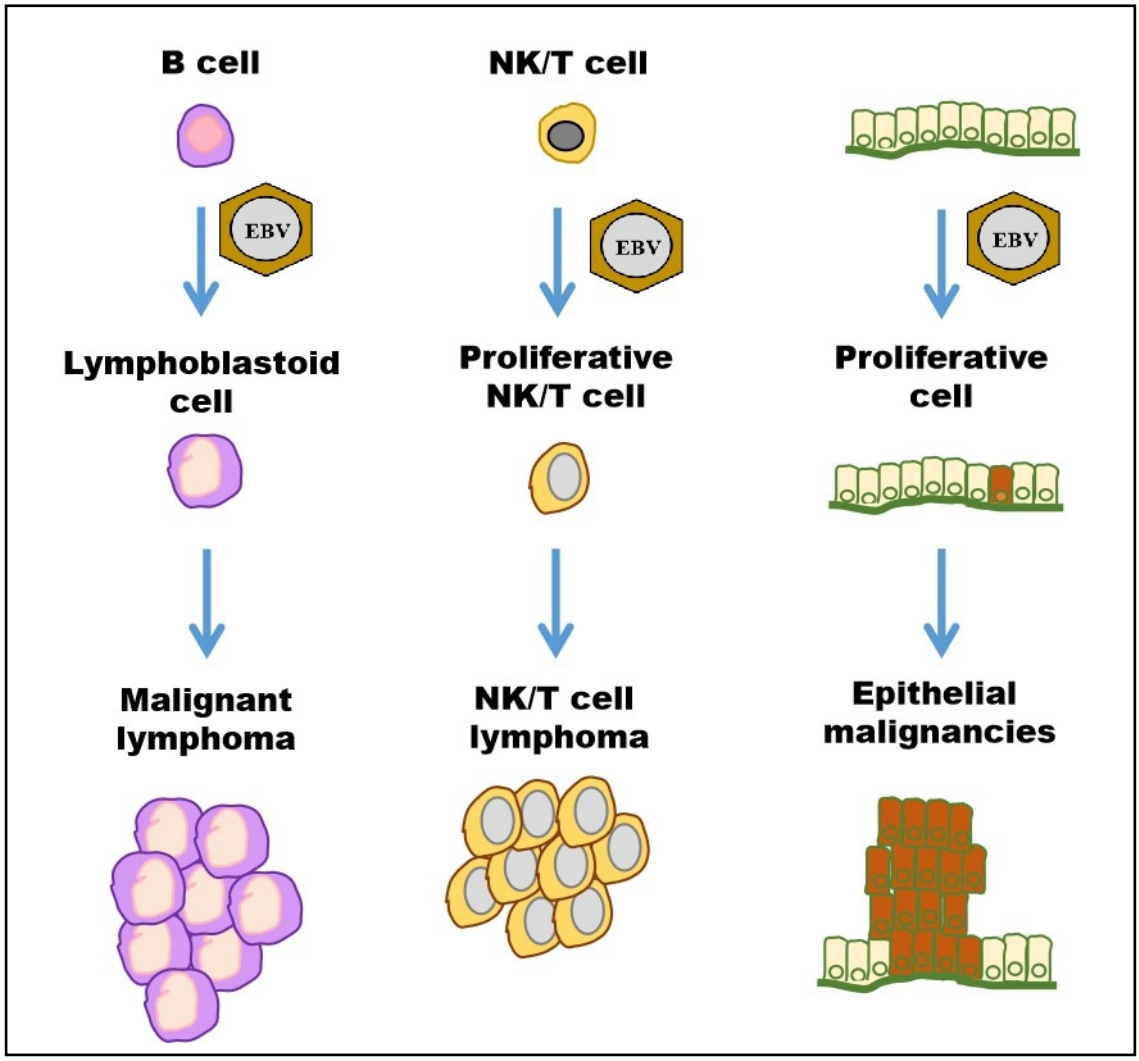

Figure 1.7: EBV infection is associated with a number of neoplasms (Adapted and modified from Yin et al. 2019). *EBV is commonly known to infect B lymphocytes, making them malignant, and results in the formation of a malignant lymphoma, such as BL. EBV is also known to infect epithelial cells and transform into epithelial malignancies, such as EBVaGC, NPC. Recent reports show that EBV can also establish infection of NK/T cells leading to malignancy, referred to as natural killer/T cell lymphoma (NK/T cell lymphoma).*

Chapter 2

Aims and Objectives

The International Agency for Research on Cancer (IARC) evaluates that one-fifth of the cancer cases reported worldwide is due to viral infection.

Epstein-Barr virus (EBV) is a ubiquitous tumorigenic virus, known to be associated with both lymphoid as well as epithelial cancers. The role of EBV specifically in epithelial cancers remains unclear to a great extent. Among epithelial cancers, nasopharyngeal carcinoma (NPC) and EBV-associated gastric cancers (EBVaGCs) are the most prevalent. The expression of viral latent genes (type II latency) is restricted and is thought to result in malignant transformation of epithelial cells by regulating several vital cellular pathways. EBV has developed a number of strategies to evade host immune responses in order to ensure its survival and maintenance within the host. EBV-encoded latent membrane protein, LMP2A is already reported to have immune-evasive properties in EBV associated malignancies. Previous studies showed downregulation of HLA-ABC expression in EBV associated gastric carcinomas through viral latent protein, LMP2A (48,49). However, the detailed mechanism that leads to this downregulation remains unclear. The overall aim of this work was to examine the regulation of host immune system in epithelial cell cancers by EBV.

Major Objectives:

1. To determine the role of epigenetic regulators in regulating the expression of HLA-A, HLA-B and HLA-C gene in response to viral oncoprotein, LMP2A.
2. To evaluate the methylation status of HLA-ABC promoter region using MSP in stable LMP2A-expressing AGS and HEK293 cells. Additionally, in this context, methylation mediated regulation of HLA-ABC expression was

further investigated in response to treatment with 5'-Aza-2′-deoxycytidine (demethylating agent).

3. To investigate the role played by the ITAM region of LMP2A in regulating the expression of HLA-A, HLA-B and HLA-C by means of promoter hypermethylation in AGS and HEK293 cells.
4. To evaluate the expression of NKG2D ligands (MIC-A and MIC-B) in response to viral oncoprotein, LMP2A in epithelial cell carcinomas.
5. To study the role played by the ITAM region of LMP2A in regulating the expression of NKG2D ligands, MIC-A and MIC-B in LMP2A expressing epithelial cells.
6. To investigate whether ER stress regulates NKG2D ligand expression in response to viral and mutated LMP2A in epithelial carcinomas.
7. To evaluate the role of Protein Disulfide Isomerase (PDI) in the regulation of NKG2D ligands (MIC-A/B) expression in response to viral gene, LMP2A.
8. To study whether PI3k-Akt or Shh pathways had an altered expression of MIC-A/B in AGS-LMP2A and SNU-719 cells.

Chapter 3

Scientific Background

Host immune response plays vital role in tumour progression and development. Viruses stimulate carcinogenesis in human cancers through different mechanisms that have not been fully exemplified but consist of promoting host immune evasion. Immune evasion acts as a hallmark for viral persistence. For the already known human tumor viruses in order to establish lifelong infection within the hosts, they must successfully regulate the host response to them. Viral inhibition of host immune responses occurs at several stages. Over 90% of the world's population is reported to be infected with EBV (50). The percentage is lower in developed parts of the world. Most individuals encounter primary infection before attainment of adolescence in an asymptomatic manner. However, if primary infection continues until adolescence or beyond, it may result in acute Infectious Mononucleosis (IM) (51,52). Persistent infection is accompanied with several malignancies, which includes Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma and Hodgkin's lymphoma (53-56). The increased frequency and severity of EBV infection in immunocompromised individuals feature the importance of the immune system in restraining the infection. The lower incidence of EBV-associated diseases in healthy individuals proves the effectiveness of the EBV-specific immune response reinforced by the majority of people. Evidence upholds the idea that both the innate and adaptive immune responses play crucial role in providing protective immunity against EBV.

EBV life-cycle

EBV has a biphasic life cycle which comprises of two stages: the lytic cycle, EBV produces infectious virions which infect new cells, whereas the latency cycle, allows viral persistence within infected cells. Latency phase is further divided into four types (Table 3.1) in which the gene expression pattern is restricted to avoid immune surveillance.

Latency type	Features	Gene expression profile
Type 0	Naturally EBV-infected B cells within some healthy individuals	EBERs, LMP2A
Type I	Memory B cells, EBV+BL and BL-derived cell lines	BARTs, EBERs, EBNA1
Type II	EBV+NPC, EBV+GC, HL and T cell lymphoma	BARTs, EBERs, EBNA1, LMP2A, LMP1
Type III	LCLs, B-cell tumors in immunosuppressed individuals, such as, PTLD, AIDS-related immunoblastic lymphomas.	BARTs, BHRF1, EBERs, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B

Table 3.1: Latency profiles of EBV-infected cells along with there gene expression repertoire (Adapted and modified from Young et al.2007).

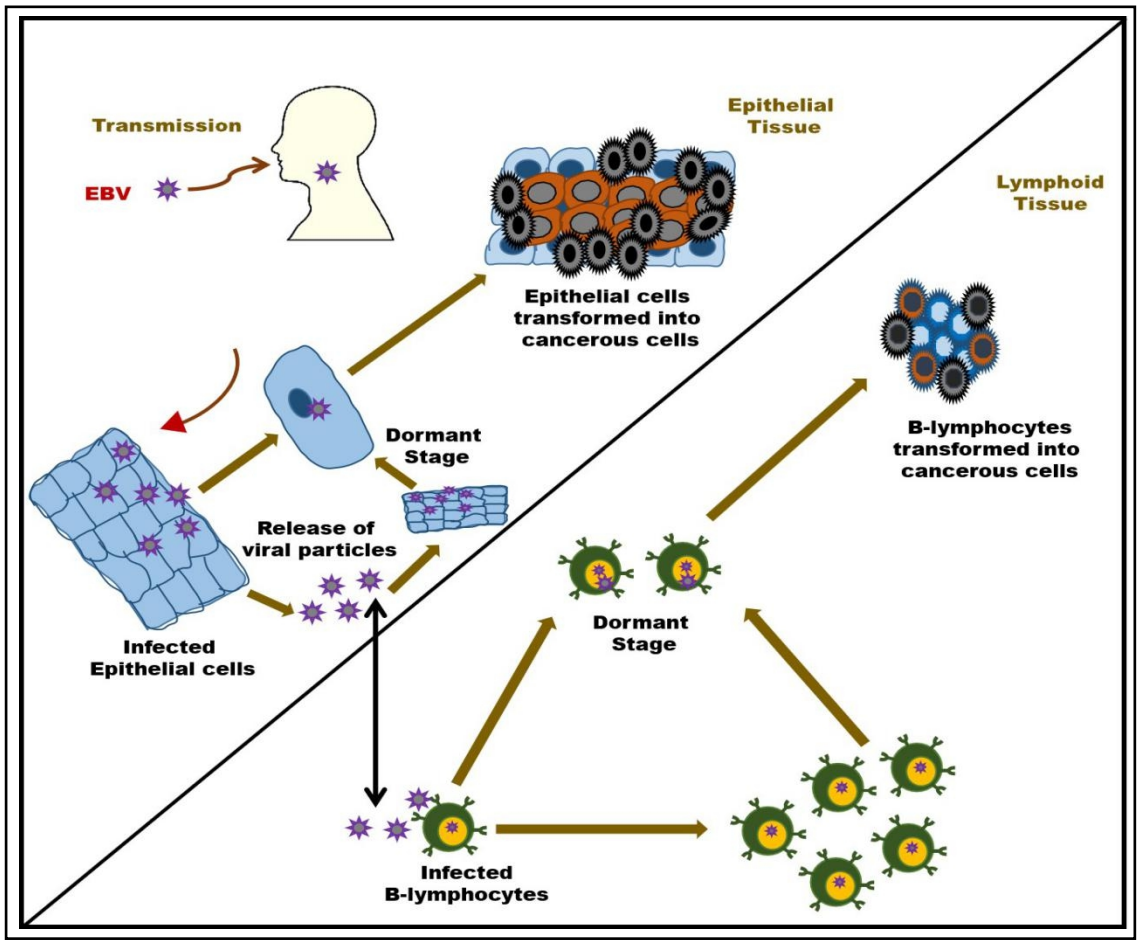

Figure 3.1: EBV mediated transformation of B lymphocytes and Epithelial cells into malignant cells (Adapted and modified from Ayee et al. 2020). *Cells of the epithelium and B lymphocytes are transformed into malignant cells by EBV as a consequence of expression of viral latent genes.*

Viral gene expression varies when EBV enters the lytic phase (57,58). Epithelial cells exhibit gene expression programme distinct from that of B cells during EBV infection (Figure 3.1). Primary B cells upon infection with EBV, expresses type III latency genes which includes six nuclear proteins (EBNA1, EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C), non-coding RNAs (EBERs) and three membrane

proteins (LMP1, LMP2A and LMP2B) (59). EBV nuclear antigen proteins, EBNA-LP, EBNA2 and EBNA3C, which play an essential role in cell cycle progression and B cell immortalization, are absent in virus-infected epithelial cells. During infection of epithelial cells, number of latent genes expressed is restricted to a greater extent (type II latency), which includes EBNA1, EBERs, LMP1 and LMP2A (60-63). Noticeably, increased expression of BamHI A rightward transcripts (BARTs) in epithelial malignancies, NPC and EBVaGC, suggests its involvement in the transformation of epithelial malignancies (63,64). An overview of the EBV encoded latent genes with their established roles is given in Table 3.2.

Latent Genes	Roles
EBNA-1	Essential for episomal maintenance of EBV genome through replication and segregation during mitosis, enhances p53 degradation, inhibition of MHC class I.
EBNA-2	Transcriptional co-activator that upregulates expression of host and viral proteins (especially c-myc). Essential for EBV mediated B-cell immortalization.
EBNA-3A	Essential for virus-mediated cell immortalization, interacts with Cp promoter binding factor 1 (CBF1), repression of cell cycle arrest pathways, suppression of pro-apoptotic signals
EBNA-3B	Suppresses the detrimental effects of EBNA3A and EBNA3C mediated oncogenic functions
EBNA-3C	Essential for cell immortalization, overcomes retinoblastoma protein (pRB) mediated cell cycle checkpoint, repression of

	cell cycle arrest pathways, suppression of pro-apoptotic signals, interacts with CBF1, induces LMP1 production
EBNA-LP	Interacts with EBNA-2 and inactivate p53 and Rb, contributes to EBV mediated immortalization of B-cell.
LMP-1	Mimics CD40 ligand binding signal, elevate levels of antiapoptotic proteins and NF-kB and acts as a constitutively active receptor.
LMP-2A and -2B	B-cell survival, restricts EBV-infected B-cells in the latent phase, activates growth and cell survival promoting signaling pathways, modulates antigen presentation and recognition by immune cells
EBER-1 and -2	Binds to L22 and PKR, activates cellular growth factors like IGF-1 and interleukin (IL-10) in gastric cancers, modulates innate immune signaling contributing to EBV mediated oncogenesis, leads to induction of type-I IFNs and inflammatory cytokines
BARTs	Suppress antigen presentation and activation of immune cells, inhibit the destruction of B cell and epithelia cell derived cancers by down-regulating expression of tumor suppressor genes, targets MAPK signaling pathway

Table 3.2: EBV encoded latent genes and their roles (Adapted and modified from Thompson and Kurzrock, 2004).

The oncogenic potential of the EBV latent proteins which contribute to development of malignancies are subject to intensive study. A detailed study of the regulation of host immune response by EBV latent protein, LMP2A is addressed in this thesis. In brief, LMP2A is a latent trans-membrane protein expressed in several types of EBV-associated malignancies, and in latently infected cells in healthy individuals. Latent membrane protein 2A (LMP2A) aids in B cell activation and proliferation. It is still unknown and not well studied, how epithelial cell cancer latently infected by the virus escape recognition by immune cells. It is obscure whether LMP2A can regulate the recognition and interaction of infected cells by host immune cells.

EBV associated Malignancies

Hodgkin's Lymphoma: Hodgkin lymphoma (HL) is an uncommon malignant hematopoietic neoplasm characterized by cancerous Hodgkin and Reed-Sternberg (HRS) cells surrounded by an inflammatory milieu. The inflammatory milieu comprises of lymphocytes, eosinophils, neutrophils, plasma cells and histiocyte. In most of the cases, the HRS cells are derived from mature B-cells (65). In about 40-60% of patients with HL in the, HRS cells are latently infected by EBV (66). EBV monoclonal genome, suggests that EBV infection is an early event in HL pathogenesis (66-68). EBV exhibits Latency II program in infected HRS cells where the virus expresses EBNA1, LMP1 and LMP2A. LMP1 constitutively activate the CD40 pathway, by replacing the signal which is generally produced by cognate T cells during selection of memory cells. LMP2A mimics signaling from surface immunoglobulins, which substitutes the frequent prerequisite for high-affinity binding to the cognate antigen. Immunosuppression in a variety of medical conditions

increases the risk of HL. Increased incidence of HL is reported in the human immunodeficiency virus (HIV)-infected population than in the normal individuals (68).

Burkitt's Lymphoma: Burkitt's lymphoma (BL) is a rare and aggressive lymphoma of rapidly growing B-cells in the germinal centre. It is manifested in three clinical subtypes which include endemic, sporadic, and immunodeficiency-associated forms. BL is marked by expression of MYC gene through translocations of chromosomes 8 and 14, leading to increased expression of c-myc protein transcription further resulting in upregulated cell proliferation, differentiation, and apoptosis (69). The endemic form is prevalent in Africa is associated with EBV in 90% of the cases (70,71). Sporadic Burkitt's lymphoma, extant in United States and Europe, is EBV DNA positive in only one-fifth of the cases. HIV infected individuals are reported to have increased risk for Burkitt's lymphoma (72). Clinically, BL is known to affect most of the organ system, mainly the abdomen and pelvis in the sporadic form.

T-cell non-Hodgkin's lymphoma: Apart from being a B-lymphotropic virus EBV may also lead to infection of T-cells and NK-cells. Till today, three major categories of T-cell non-Hodgkin's lymphomas (NHL) is known to be associated with EBV infection. The above mentioned categories include, 1. virus-associated hemophagocytosis associated T-cell lymphocytosis/lymphoma, 2. peripheral T-NHL, and 3. nasal T-NHL. It has been hypothesized that during the process of B-cell destruction, EBV may enter the T-cell through an activated cytotoxic T-cell (73). Since T-cells are known to exhibit highly restricted expression of CD21, the virus may enter the T-cell through CD21, or through a different receptor or mechanism (74-76).

Intensive chemotherapy is inadequate to provide an effect during EBV related T-cell NHL. Notably, fulminant T-cell NHL ensued at the site of chronic active infection has a bleak effect (77).

Nasopharyngeal Carcinoma: Nasopharyngeal carcinoma (NPC) is commonly widespread in southern China, Alaskan Eskimos and northern Africa (78). NPC has been classified by World Health Organization (WHO) into two broad categories-keratinizing (type I) and non-keratinizing (type II and III) squamous cell carcinoma-on the basis of tumor cells morphology under the light microscope. The non-keratinizing squamous cell carcinoma is also subdivided on the basis of differentiation into differentiated (type II) and undifferentiated (type III) carcinoma. The non-keratinizing squamous cell carcinoma are mostly EBV-positive. However, the association of EBV with type I form of NPC has been specifically found in the geographical area with an increased occurrence of undifferentiated NPC (79,80). EBV exhibits latency programme in NPC, particularly in the transformed epithelial cells and not present in the neighbouring lymphoid infiltrate. However, the synergy between the eminent lymphoid stroma of the undifferentiated NPC and adjoining tumour cells emerges out to be pivotal for the sustained growth of malignant NPC cells (81,82). Expression of EBV latent genes is principally restricted to EBNA1, LMP2A and LMP2B along with BamHI-A transcripts. Approximately 20% of tumours also reported to express the viral LMP1 protein. EBV genome exists in the malignant epithelial cells but is nonexistent in the tumourogenic lymphocytes (83,84).

Gastric Carcinoma: Approximately 10% of gastric carcinoma (GC) cases throughout the world are associated with EBV infection (85,86). Histologically, EBV associated GC (EBVaGC) is categorized into two types; lymphoepithelioma-like carcinoma-type and conventional-type adenocarcinoma, a morphological continuum between these two types always exist. Lymphoepithelioma-like carcinoma is characterized as undifferentiated carcinoma with dense infiltration of lymphocytes, which is similar to that of NPC (87). Conventional-type adenocarcinoma histology exhibits well to moderately differentiated adenocarcinoma with irregular load of infiltrating lymphocytes. EBV manifests latent form of infection in GC. EBVaGC belongs to latency type I or II, in which the gene expression pattern is restricted to EBERs, EBNA-1, BARTs, BART miRNAs along with LMP2A (88). EBV-positive gastric carcinomas shows phenotype and clinical features distinct from EBV-negative tumours, which include loss of expression of p16 and enhanced patient prognosis. Pre-malignant gastric lesions lacking the EBV infection, assists the idea that viral infection is a comparatively late event in the process of gastric carcinogenesis.

Biochemical properties of EBV-encoded gene products

EBV-encoded nuclear antigens (EBNAs)

EBNA-1 is expressed in all forms of latent EBV infection. It is encoded by the ORF BKRF1, protein of highly variable size ranging from 60–100 kDa, due to glycine–alanine repeats (59). The glycine-alanine repetitive sequence of EBNA-1 prevents its processing by the ubiquitin–proteasome system and further inhibits MHC-class 1 association of the derived viral peptides, a prerequisite for recognition by the CD8+CTLs (59). EBNA-1 is a DNA-binding protein, is capable of binding

three different specific palindromic target sites on the viral DNA, each of which occurs several times in the viral genome. EBNA-1 is involved in the episomal maintenance of viral genome, DNA replication, and viral gene expression during latency.

EBNA-2 is one of the earliest viral protein expressed in freshly infected B cells. EBNA-2 is a phosphoprotein and and acts as a potent transactivator of viral and cellular genes. The EBNA-2 does not bind directly to DNA but instead interacts with viral (LP) and cellular factors (RBPJ transcriptional repressor and ZNF143) for transcriptional activation. The EBNA-2 protein complexes are reported to induce chromatin remodelling. EBNA-2 responsiveness elements have been found in the EBV-Cp, LMP-1, LPMP-2, and CD23 promoters (59,62). EBNA-2 is plays important role in the transformation of B cells into immunoblasts, and derivation of lymphoblastoid cell lines.

EBNA-LP is a nuclear phosphoprotein and is also known as EBNA-5. Like EBNA-2, EBNA-LP is the earliest viral protein expressed in newly infected B cells. EBNA-LP expression along with EBNA-2, cooperatively activates EBNA-2 mediated transcriptional activation of genes (59,62). EBNA-LP is tightly associated with the nuclear matrix, and often accumulates in the nuclear promyelocytic leukaemia bodies. EBNA-LP is also necessary for immortalization (59).

EBNA-3 family consists of EBNA-3A, EBNA-3B and EBNA-3C. EBNA-3 comprises of three large stable nuclear phosphoproteins that accumulate in intranuclear clumps, sparing the nucleolus (59,62). EBNA-3 family proteins share a limited homology in a region near the *N* terminus, and this conserved domain facilitates the binding to CBF1/RBP-Jk. All EBNA-3 families are coactivators of EBNA-2. EBNA-3C

functions as a coactivator and corepressor. EBNA-3A and EBNA-3C, but not EBNA-3B, are necessary for *in vitro* immortalization (59).

EBV-encoded RNAs

EBV-encoded RNAs, EBERs (EBER-1 and EBER-2) are transcribed by host RNA polymerase III as small non coding nonpolyadenylated RNAs. The role of EBERs in EBV-induced B-lymphocyte transformation is not well understood. EBERs binds to La, PKR, ribosomal protein L22 (also called as EAP), pattern-recognition receptors, retinoic acid-inducible gene I (RIG-I), melanoma differentiationassociated gene-5 and AU-rich element binding factor 1. EBER-mediated RIG-I activation likely contributes to EBV oncogenesis (59). Interaction of EBERs with RIG-I, AU-rich element binding factor 1 and pattern-recognition receptors could activate host innate immune responses. EBER double-stranded RNA structures also activate RIGmediated NF-κB and IRF-3 signaling and subsequently type I IFN induction (64).

EBV-encoded miRNAs

EBV genomes express many miRNAs from two regions of EBV's genome: BART and BHRF1 (BamHI fragment H rightward open reading frame 1). The EBV genome transcribes at least 25 pre-miRNAs that encode 40 short single-stranded RNAs. The miR-BART2-5p targets a stress-induced natural killer cell ligand, MICB, allowing EBV-infected cells to escape recognition and subsequent elimination (59). Most EBV miRNAs have the ability to sustain latently infected cells. BHRF1 miRNA facilitates progressive growth, in vitro transformation of infected cells and acute systemic EBV infection but not the overall oncogenic potential of EBV in vivo (63). In addition,

BHRF1 and BART miRNAs prevent primary B cells or BLs, respectively, from apoptosis.

Latent membrane proteins

EBV expresses three latent membrane proteins (LMPs) during latency II and III in immunoblasts as well as in derived tumours and cell lines: LMP-1, LMP-2A, and LMP-2B. All three proteins are also detected in epithelial tumours of the nasopharynx, and during the early stages of oral hairy leukoplakia (59). LMP-2A transcripts can also be expressed in resting virus-carrying B lymphocytes in healthy individuals – the reservoir of persistently latent EBV (59). LMP-2A together with LMP-1 are necessary for continued lymphoma cell survival via TRAF2 regulation of NF-κB. LMP-1 as an integral membrane protein acts like a constitutively activated receptor. It almost completely mimics the CD40-mediated signalling, and is thus functionally homologous to the TNF-receptor (TNFR)-family of proteins in B lymphocytes and epithelial cells (59,68). The three LMP proteins are highly multifunctional and interact with several cellular signalling pathways. They are expressed at the cell surface membrane as well as in intracellular membranes of the Golgi and endoplasmic reticulum.

EBV-LMP2A

EBV establishes life-long latent infection in most of the epithelial malignancies and expresses latent protein, Latent Membrane Protein 2A (LMP2A) besides several other EBV-encoded genes (53). LMP2A plays significant role in the sustenance of latency programme and is also known to be associated with several vital cellular processes,

such as, anchorage, motility, differentiation and transformation in epithelial cells (89). LMP-2A/B are constitutively expressed primarily in the plasma membrane, and also in cytoplasmic location, in all EBV-infected cells. LMP-2 associates with and is a substrate for a B-lymphocyte tyrosine kinase, Lyn and Syk protein tyrosine kinases through the first 167 of the LMP-2A 497 amino acid, co-localizes with the cellular tyrosine-phosphorylated proteins on the plasma membrane and is also serine and threonine phosphorylated. Although in B cells LMP-2 is tyrosine phosphorylated by the Src family kinase (Lyn, Syk), in epithelial cells it is mediated by the C-terminal Src kinase, which is triggered by epithelial cell adhesion to extracellular matrix proteins. The immunoreceptor tyrosine-based activation (ITAM) motif contributes to LMP-2A phosphorylation and participates in signal transduction events in epithelial cells. The BCR block by LMP-2A is bypassed by raising intracellular-free Ca^{2+} levels with an ionophore or by activating protein kinase C with phorbol 12-myristate 13-acetate. LMP-2A is secreted through exosomes similarly to LMP-1. Cholesterol depletion from the plasma membrane increases LMP-2A abundance and LMP-2A exosome secretion and also blocks endocytosis, phosphorylation and ubiquitylation of LMP-2A, indicating that cholesteroldependent LMP-2A traffificking determines the fate of LMP-2A. We previously reported role of LMP2A in increased cellular migration by means of altered mitochondrial dynamics (90). LMP2A is an integral membrane-embedded protein, includes a protracted tyrosine rich 119 amino acid cytoplasmic tail in the N terminus, along with 12 membrane-spanning domains and a terse 27 amino acid C-terminal cytoplasmic tail. LMP2A-ITAM in the cytoplasmic N-terminus contains eight tyrosine residues as well as proline and tyrosine rich motif (PY) along with tyrosine, glutamic acid, glutamic acid and alanine motif (YEEA)

(Figure 3.2) (91,92). LMP2A is proclaimed to constitutively turn on PI3-kinase (PI3-k) and Akt signaling cascade (93). Earlier studies show defects in the classical HLA Class I mediated antigen processing and presentation of viral peptide amid EBV infection in Burkitt's lymphoma and nasal NK/T-cell lymphoma (94,95).

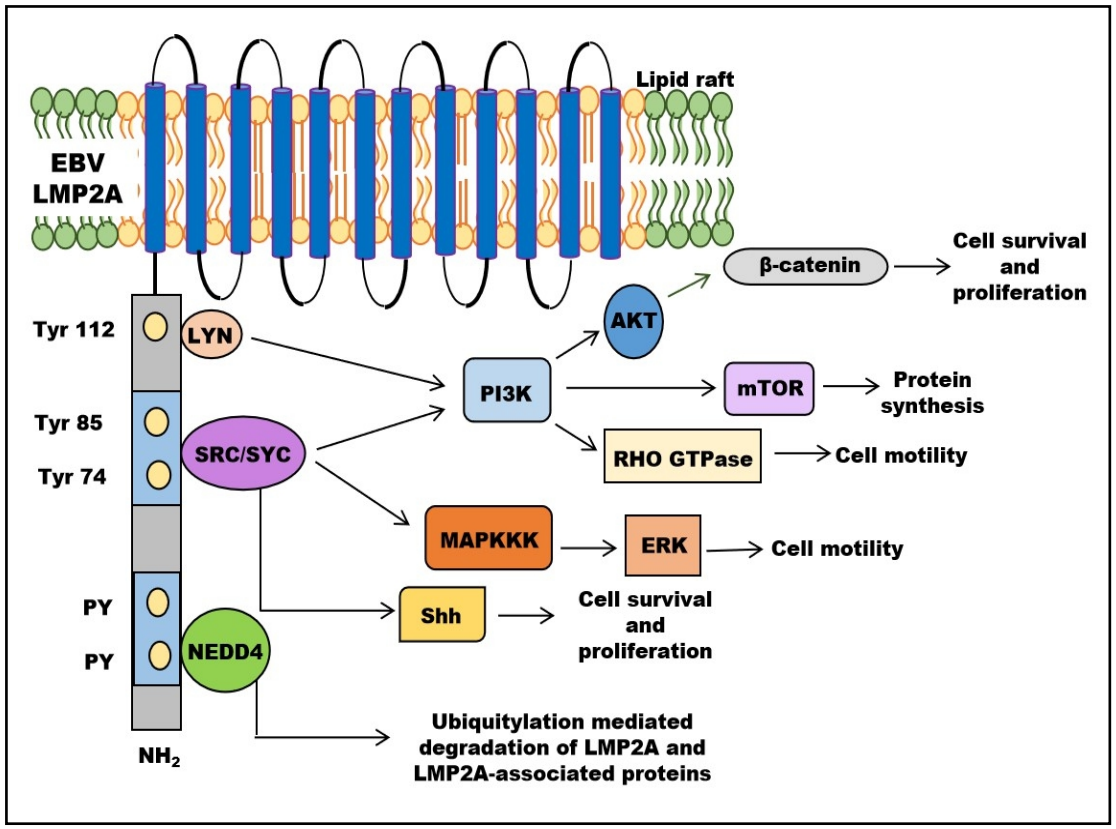

Figure 3.2: Structure and Function of EBV-LMP2A (Adapted and modified from Young and Rickinson, 2004). *The structure of the Epstein–Barr virus (EBV) latent membrane protein, LMP2A; it consist of 12 transmembrane domains along with a short 27-amino-acid cytoplasmic C-terminal domain. EBV-LMP2A also contains a long 119-amino-acid cytoplasmic N-terminal domain that consists of eight tyrosine residues, Tyr74 and Tyr85 in the N-terminal region encompasses the immunoreceptor tyrosine-based activation motif (ITAM). The phosphorylated tyrosine residues in the*

ITAM recruits members of the SRC and the SYK tyrosine kinase and regulates their function. A membrane-proximal tyrosine residue, Tyr112 is known to bind the LYN tyrosine kinase and constitutively phosphorylates different tyrosine residues in LMP2A. ITAM impedes signaling from the B-cell receptor (BCR) through sequestering of the tyrosine kinases and further obstructing the translocation of BCR into lipid rafts. LMP2A-PY motifs binds to NEDD4-like ubiquitin protein ligases and promotes degradation of LYN tyrosine kinase and LMP2A through a ubiquitin-dependent mechanism.

Regulation of CTL response by EBV

Cytotoxic T lymphocytes (CTLs) constitutes of a specific sub-population of lymphocytes, and are activated by a number of distinct stimulation, which includes protein antigens, major histocompatibility antigens and pathogenic microbes (viruses, parasites and bacteria). CTLs identify antigenic peptides displayed by major histocompatibility complex (MHC) class I molecules, which further results in CTL activation and proliferation. Activated CTLs secrete the essential cytotoxic factors, such as, perforin, granzyme and trigger apoptosis in target cells (tumor cells, virus-infected cells, etc.). CTLs have specialized surface proteins, called T cell receptors (TCRs) which facilitates the recognition of virus-infected cells. The TCR specifically recognizes a particular antigenic peptide bound to MHC on the surface of infected cells. If the T cell receptor encounters antigenic peptide from a virus, it leads to release of cytolytic mediators by the CTL to destroy the virus-infected cell (Figure 3.3). Moreover, activated CTLs secrete several cytokines, such as, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), resulting in enhanced

antigen presentation. The regulation of CTLs activation and functions is essential for designing effective strategies in providing protection against various viruses and tumors.

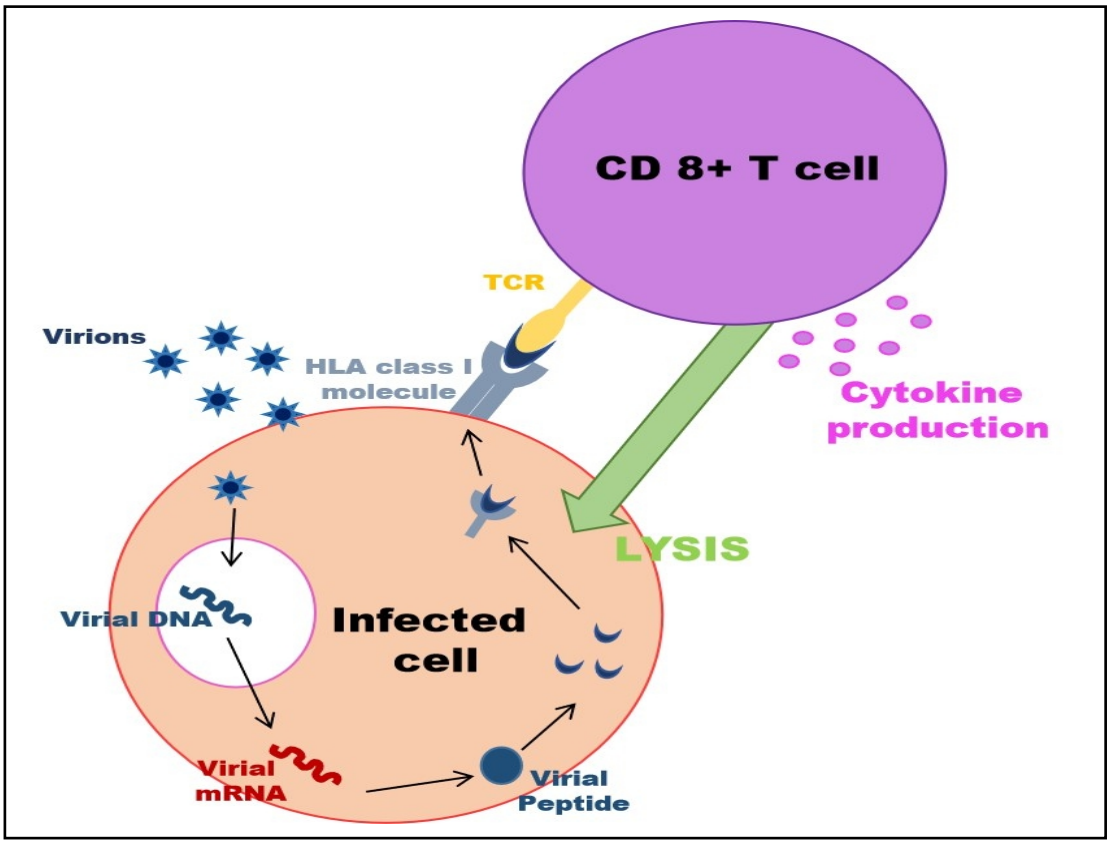

Figure 3.3: CD8+ T cells recognizes and kills virally infected cells through T cell receptor (TCR) (Adapted and modified from Elemans et al. 2012). *The TCR binds HLA class I molecules displaying viral antigenic peptides at the surface of infected cells. Upon recognition of infected cell by CD8+ T, the CD8+ T cell effector functions are triggered. CD8+ effector functions can either be lytic resulting in death of infected cell or non-lytic during which cytokines are secreted resulting in reduced cell infection and viral production.*

The human leukocyte antigen (HLA, also referred to as MHC), class I antigens, HLA-A, HLA-B and HLA-C constitutes the MHC class I complex in humans, play significant role in identification of virus-infected cell or transformed cell by CTLs. HLA class I antigen processing pathway downmodulation (96,97) along with proteasome subunits downmodulation serve as methods exploited by the viruses to surmount host immune response. Viral infection also targets transporter associated with antigen presentation, HLA class-I heavy chains and β-microglobulin (98). Earlier reports (99,100) have disclosed HLA deformity during processing and presentation of antienic peptides by HLA Class Ia molecules in EBV-associated, nasal Natural Killer (NK) cell/T-cell carcinoma and Burkitt′s lymphoma. Moreover, several reports (101-104) have shown a number of EBV-infected carcinomas including Nasopharyngeal Carcinoma and Hodgkin′s Lymphoma to exhibit locus-specific downregulation of the HLA Class I molecule. Furthermore, previous reports from our group have also shown HLA Class I downregulation in EBV associated gastric cancer (48,105). Furthermore, our group also reported EBV latency I gene, LMP2A to bring about decrease HLA Class Ia expression in EBV associated gastric cancer cells (49). Nevertheless, the procedure by which HLA-ABC gene is downregulated besides decreased surface expression in EBV associated epithelial cell cancer needs to be studied.

Loss of expression of HLA class I antigen at the cell surface can occur due to regulation at genetic, transcriptional and post-transcriptional levels. A direct association between lack of cell surface protein expression and a decrease in mRNA levels has been observed for HLA class I genes in EBV associated gastric carcinomas, suggesting transcriptional regulation. Molecular research has provided information for

regulation of gene expression through DNA methylation, especially 5'-CpG methylation (Figure 3.4). DNA methylation may precisely interfere with the basal transcriptional machinery through alteration in the DNA secondary structure. DNA methylation can also bring about chromosome remodelling through histone deacetylation, leading to transcriptional repression. Aberrant DNA methylation is one of the characteristics of cancer cells (106-108) DNA methylation-mediated regulation of gene expression is executed by DNA methyltransferases (DNMTs) (109). DNMTs are essential for mammalian development, as the transfer of methyl group yakes place between the universal methyl donor, S-adenosyl-L-methionine (SAM) and cytosine residues in DNA (96). DNMTs play significant role in genomic integrity, disruption of which may lead to chromosome instability and tumor progression (110). The DNMT family mainly consist of three members, which include DNMT1, DNMT3A and DNMT3B. DNMT1 functions as maintenance methyltransferase, whereas DNMT3A and DNMT3B functions as de novo methyltransferases (111,112), needed for the establishment as well as maintenance of the genomic methylation. Although maintenance vs de novo division has been favourable, there is definite evidence for utilitarian overlap between maintenance and de novo methyltransferases (113,114).

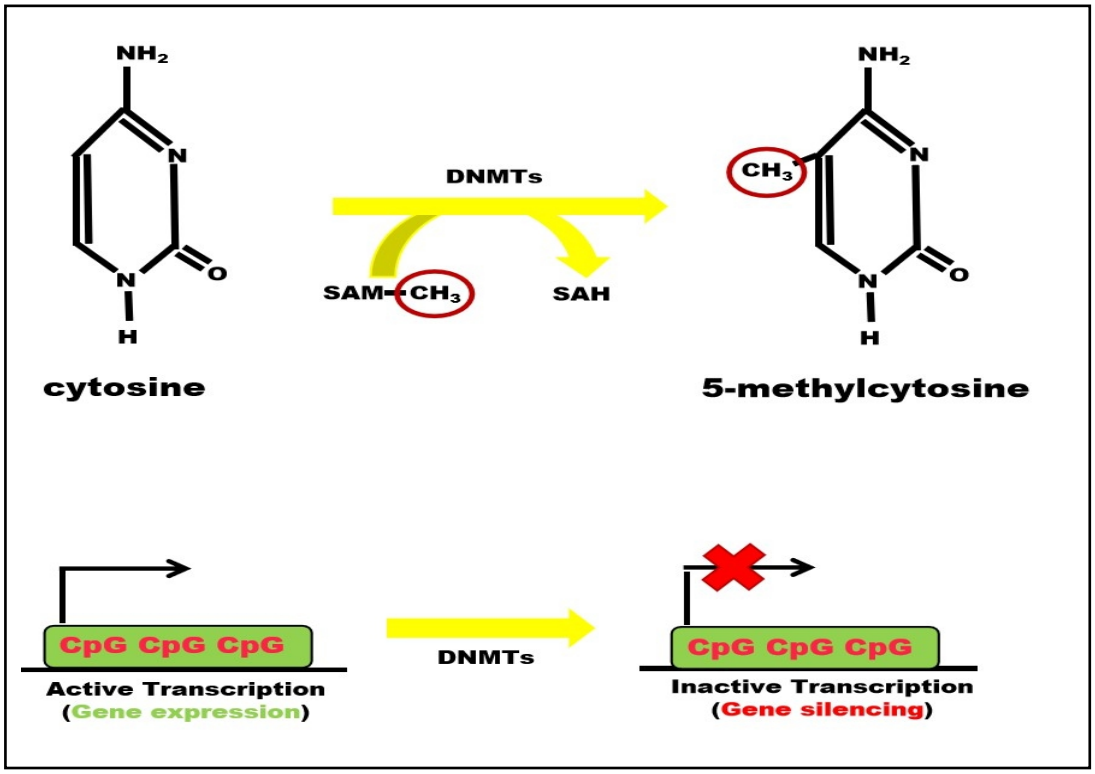

Figure 3.4: Characteristic feature of DNMTs (Adapted and modified from Cui et al 2016). *DNA methyltransferases (DNMTs) facilitate the conversion of cytosine to 5'-methylcytosine. Cytosine is the DNA nucleotide which is typically methylated in the context of CpG islands, and the methylation of CpG islands in the promoter region results in gene silencing. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.*

Ubiquitin-like with PHD and Ring Finger Domain 1 (UHRF1) is a multi-domain nuclear protein that efficiently regulates epigenetic modifications (115). Inheritance of DNA methylation during DNA replication is one of the major functions performed by Uhrf1. Uhrf1 is reported to bind hemi-methylated DNA through its Set- and RING-Associated (SRA) domain and assists DNMT1 loading on to the newly synthesized DNA strand during the process of cell division (116). Here, we

investigated the role of DNMTs and Uhrf1 in methylation-mediated regulation of HLA-ABC expression in response to the viral latent gene LMP2A. The expression of HLA-A, HLA-B and HLA-C was reported to be down-regulated in EBV associated gastric cancers. Promoter hypermethylation acts as one of the major epigenetic modifications responsible for gene inactivation, playing an important role in carcinogenesis. However, HLA-A, HLA-B and HLA-C gene promoter methylation in EBV-infected epithelial cell cancer was never been studied previously. The current study evaluates the expression and alteration of HLA-A, HLA-B and HLA-C in LMP2A expressing epithelial cell carcinomas by using quantitative real-time polymerase chain reaction (qRT-PCR), MSP and fluorescence activated cell sorting (FACS) analysis. Further validation of methylation mediated decreased HLA-ABC expression was executed by carrying out demethylation study using 5'-azacytidine in epithelial cell carcinomas expressing the viral gene, LMP2A.

Regulation of NK cell response by EBV

The importance of natural killer (NK) cells in response to viral infections that is complementary to CTL has recently been documented (117,118). Human NK cells are a key component of the early innate immune defense. NK cells can cause lysis of virus-infected and malignant cells, through release of perforins. They are also known to augment the antigen-specific immune response through immunoregulator cytokine secretions (119,200). NK cells express a series of inhibitory and activating receptors that act in accordance to control NK cell activation (121). A number of inhibitory receptors are known to recognize HLA class 1, and expression of HLA class 1 on healthy cells impedes NK cell activation, further causing self-destruction (Figure 3.5).

Alterations in HLA class 1 expression in both virus-infected and tumour cells hinders signaling through inhibitory receptors, and thus activating signals may dictate and bring about NK cell activation (122).

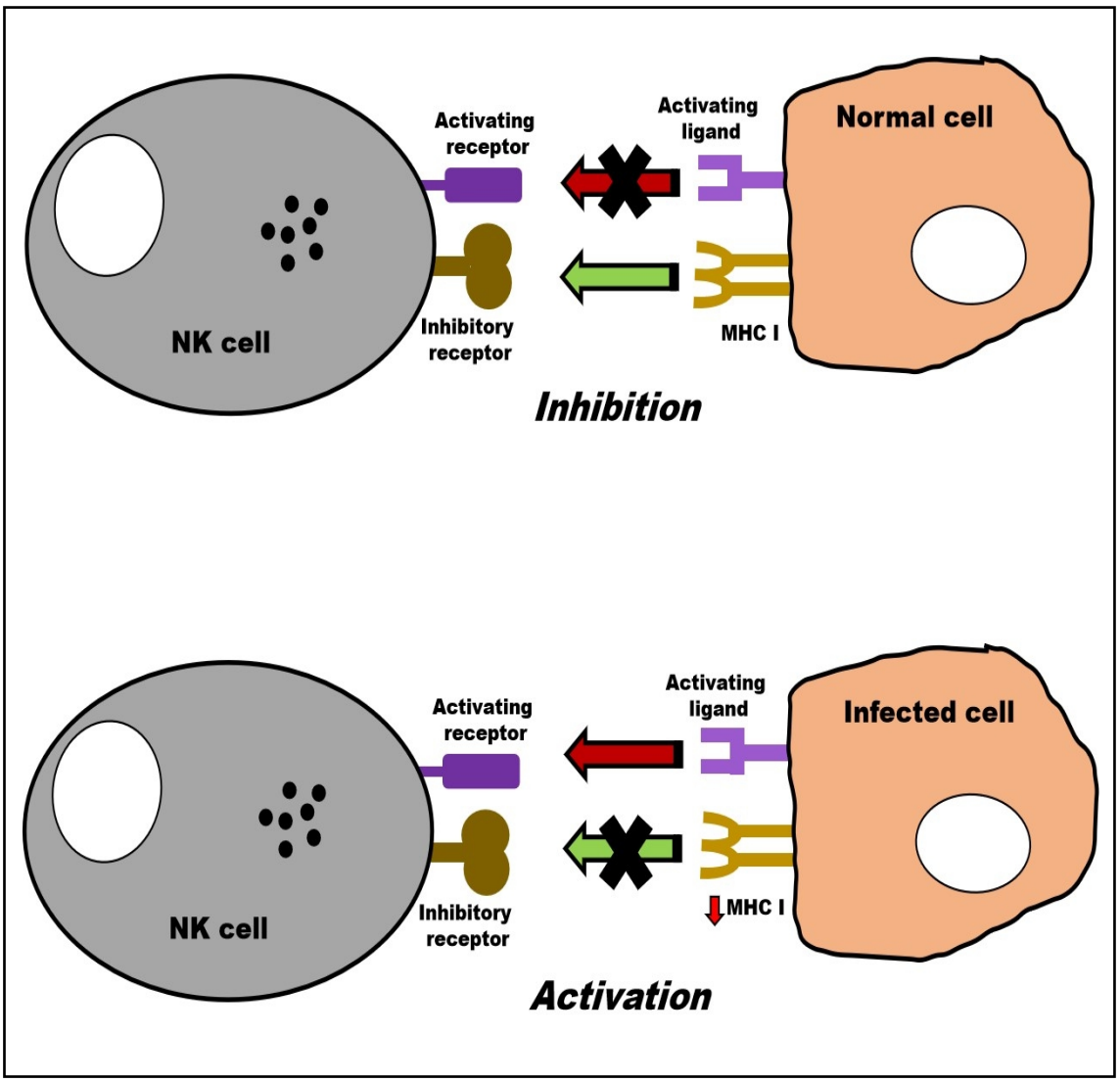

Figure 3.5: Regulation of NK cell activation according to 'missing-self' hypothesis (Adapted and modified from Wilensky et al. 2000). *Natural killer (NK) cells avoid normal healthy cells expressing MHC class I molecules on their surface and facilitate their recognition by inhibitory receptors on the surface of NK cells leading to*

inhibition of NK cell mediated cytotoxicity. In response to viral infection and tumor transformation the expression of MHC class I molecules is restricted and, accordingly, restrains the fail-safe inhibitory process. As a result, the activating receptors are triggered, leading NK cell mediated death of the target cells.

Virus-infected cells undergo NK cell-mediated lysis through the expression of the activating receptor, NKG2D (natural killer group2, member D) on NK cells, NKT cells, and some CTLs. Eight tumour-related ligands are recognized for NKG2D activating receptors in humans (Figure 3.6) which consists of, MHC class I chain-related proteins A and B (MIC-A and MIC-B), along with six UL-16 binding proteins (ULBP1-6) also referred as retinoic acid early transcript-1 proteins (RAET-1) (123,124).

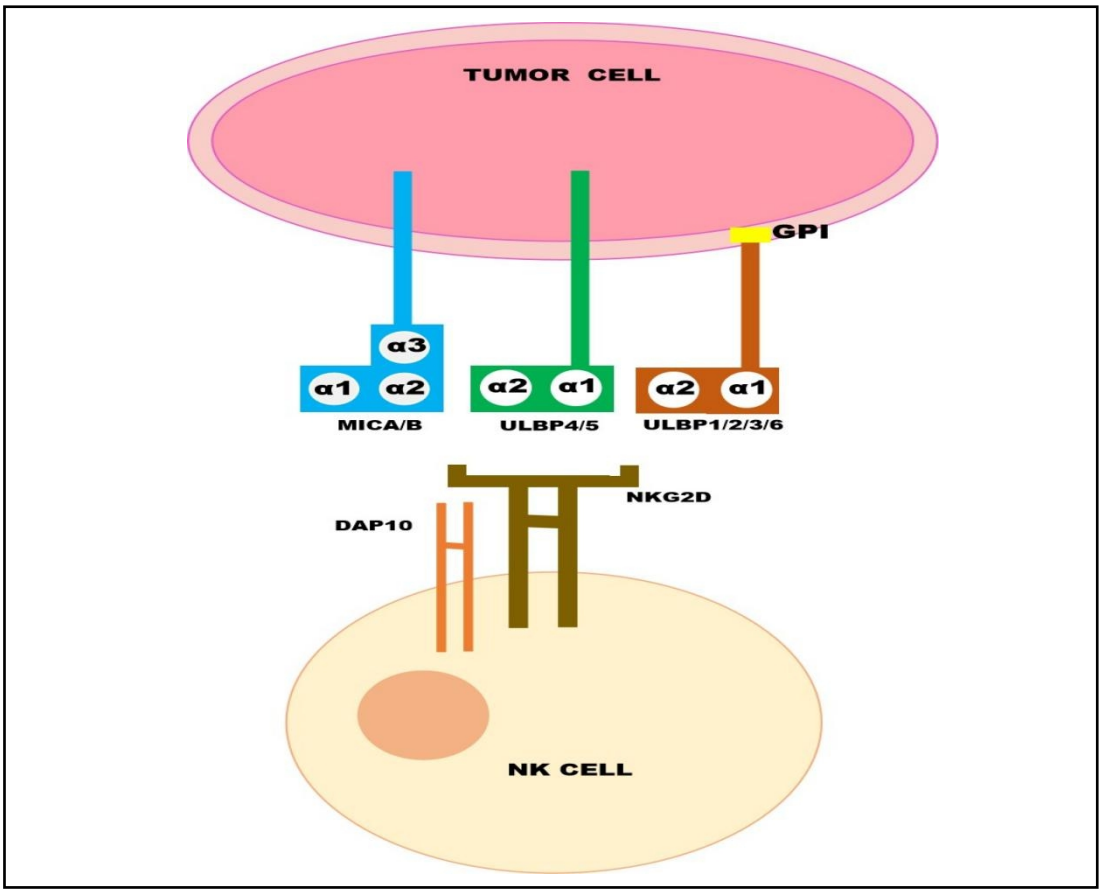

Figure 3.6: NKG2D receptor and its ligands expression in response to tumour (Adapted from Duan et al. 2019). *NKG2D receptor is a homodimer, type II transmembrane glycoprotein that tethers to DAP10 non-covalently in the transmembrane domain. NKG2D ligands, MICA\B consists of α1–3 domains, a membrane-embedded structure, and a cytoplasmic tail. NKG2D ligands, ULBP-1/2/3/4/5/6 consist of only the α1 and α2 domains. ULBP-1, -2, -3, and -6 also have a GPI-anchoring structure, whereas, ULBP-4 and - 5 contains a transmembrane structure and a cytoplasmic tail.*

NKG2D ligand expression is regulated at different levels which includes transcriptional, post-transcriptional, and post-translational mechanisms. Among

post-translational mechanisms, the release of membrane-bound activating ligands through proteolytic cleavage in the extracellular milieu or by extracellular vesicle secretion are mechanisms utilized for controlling surface expression levels of NKG2D ligand. In particular, the shedding of membrane-bound NKG2D ligands by proteases represents an immune evasion strategy employed by cancer cells as it causes lower levels of NKG2D ligands on the cell-surface, thus rendering cancer cell unseen to NKG2D-mediated surveillance (125,126). During NKG2D-mediated recognition of malignant cells, lower levels of membrane-bound NKG2D ligands offers an important strategy by which malignant cells ensues inhibition of NK cell mediated recognition (127,128).

EBV-infected cells upon transition from latent to productive infection is associated with sensitization to NK cell killing through elevated levels of NKG2D ligand, ULBP1 (129). EBV has recently been reported to avoid NK cell activation by suppressing MIC-B translation with the help of viral miRNA miR-BART2-5p (130). Remarkably, till date, immune evasion mechanisms involving regulation of NKG2D ligands expression during latent EBV infection of epithelial cell carcinomas remains unexplored. We investigated the expression of NKG2D ligands', primarily MIC-A and MIC-B. Expression of NKG2D ligands' is documented to be governed by cellular stresses (131).

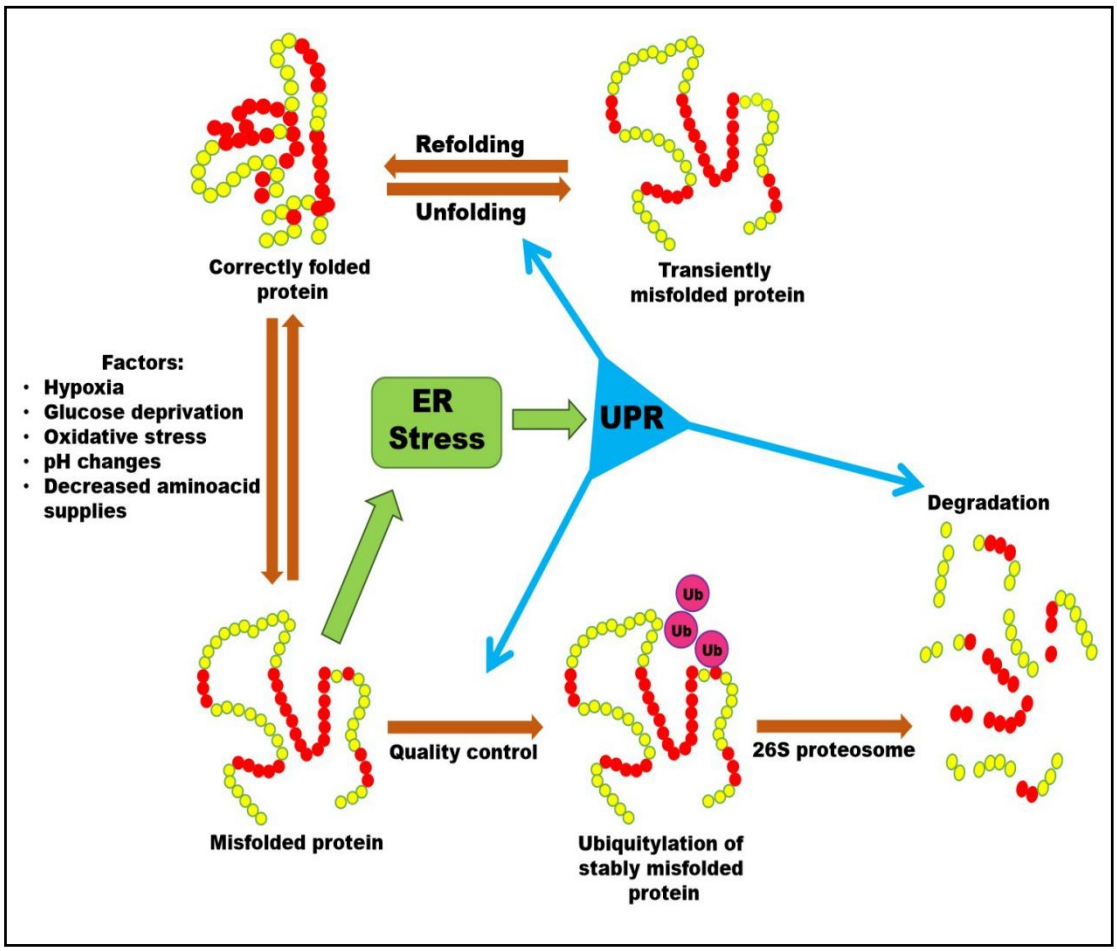

Figure 3.7: The anomaly of Unfolded Protein Response (UPR) in Cancer (Adapted and modified from Vandewynckel et al. 2013). *Cellular stress results in protein misfolding. Endoplasmic Reticulum (ER) consists of rigid quality control systems that judiciously exports properly-folded proteins, whereas, culls terminally-misfolded proteins for ubiquitination mediated proteolytic degradation, a procedure referred as ER-associated protein degradation (ERAD). Molecular chaperones play important role by stabilizing and folding newly synthesized proteins. During tumour formation, continuous ER stress eventually causes damages that the molecular chaperones fails to correct as a result the damaged proteins are destructed by ubiquitin-proteasome*

system (UPS). However, if incomplete degradation take place leading to accumulation of misfolded proteins, resulting in activation of unfolded protein response (UPR). In response to continuous ER stress chaperones are transcriptionally induced resulting in UPR.

Accumulation of unfolded and mis-folded proteins in the ER leads to induction of UPR (Figure 3.7). Binding Immunoglobulin protein (BIP), C/EBP homologous protein (CHOP), Inositol-requiring transmembrane kinase/endoribonuclease 1α (IRE1-α), Protein Disulfide Isomerase (PDI) as well as other proteins play significant role in maintenance of UPR (132,133). The UPR acts as a protective mechanism for tumours by contributing to the growth, progression, and chemo-resistance of cancer. UPR proteins are reported to be frequently detected in EBV-infected Nasopharyngeal Carcinoma (NPC) leading to increased expression of EBV oncoprotein, Latent Membrane Protein1 (LMP1) (134). Disulfide isomerases are recently studied to promote shedding of membrane bound NKG2D ligands, thereby inducing immune evasion (135). However, the correlation between UPR and MIC-A/B surface expression patterns in EBV-associated epithelial carcinomas has not been studied well. In this study, we investigated the role of UPR proteins in regulating the surface expression of MIC-A/B in response to viral gene, LMP2A.

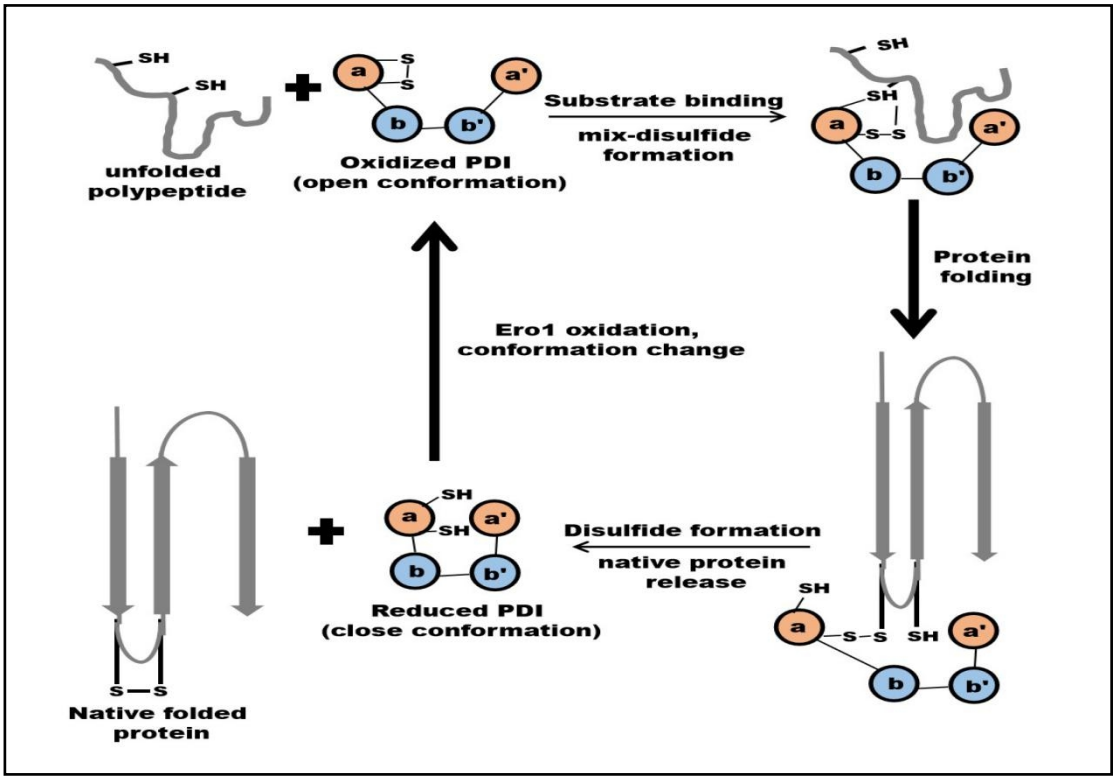

Figure 3.8: Formation of dislufide bond by PDI (Adapted and modified from Lu and Holmgren, 2014). *Oxidized PDI undergoes reduction by transferring the oxidizing equivalent to its substrate and becomes reduced.*

PDI is an UPR protein having dithiol-disulfide oxidoreductase activity by which it catalyzes disulfide bonds formation and isomerization (Figure 3.8) (136,137). PDI is reported to shuttle between the cell cytoplasm and the cell outer-membrane in certain malignancies (138). Membrane-bound PDI aids disulfide bonds reduction in cell surface proteins (139). Therefore, we tried to investigate the role of PDI in surface expression of MIC-A/B in response to EBV-LMP2A. This comprehensive study of cell surface proteins in EBV infected epithelial cell cancers, provides insights into detailed molecular mechanisms by which the viral protein, LMP2A regulates immune response.

Chapter 4

Materials and Methods

Materials.

Thapsigargin, 5'-Aza-2′-deoxycytidine, Forskolin, along with LY294002 were purchased from Sigma Aldrich and dissolved in DMSO for experimental treatment, while G418 was purchased from Gibco and solubilized in PBS for the selection of clones stably expressing pcDNA-3.1 along with the gene of interest. The HLA-ABC and MIC-A/B antibody was procured from BD Biosciences. Antibodies against DNMT1, DNMT3B, UHRF1, MIC-A, MIC-B, LMP2A, and β-ACTIN were purchased from Abcam and BIP, CHOP, IRE1α and PDI primary antibodies, along with anti-rabbit, anti-mouse, anti-rat horseradish peroxidase (HRP)-conjugated secondary antibodies were procured from Cell Signaling Technology. Antibodies' information is provided in Table 4.1.

ANTIBODY	**MANUFACTURER**
HLA-ABC (FACS)	BD Biosciences
MIC-A/B (FACS)	BD Biosciences
EBV-LMP2A (Western Blot)	Abcam
DNMT1 (Western Blot)	Abcam
DNMT3B (Western Blot)	Abcam
UHRF1 (Western Blot)	CST
IRE1α (Western Blot)	CST
BIP (Western Blot)	CST
PDI (Western Blot)	CST
CHOP (Western Blot)	CST
β-ACTIN (Western Blot)	Abcam
MIC-A (Western Blot)	Abcam
MIC-B (Western Blot)	Abcam

Table 4.1: List of antibodies used in this study.

Cell Culture.

AGS, EBV negative human gastric adenocarcinoma cell line and SNU-719, EBV positive gastric carcinoma cell line were cultured in RPMI1640 (Gibco, California, USA) with 10% FBS (Gibco, CA, USA). HEK 293, EBV negative human embryonic kidney cells 293 and HepG2, EBV negative human liver cancer cell line were maintained in DMEM (Gibco, CA, USA) supplemented with 10% FBS (Gibco, CA, USA). SNU5, EBV negative human gastric cancer cell line was cultured in DMEM (Gibco) supplemented with 20% FBS. All the cell lines mentioned above were maintained in 5% CO_2 humidified atmosphere at 37ºC.

DNA constructs and transfection.

Latent membrane protein 2A cDNA and ITAM mutants cDNA (generous gift from Prof. R. Longnecker, Northwestern University, Chicago). LMP2A cDNA and LMP2A ITAM mutant (PY1/PY2 and Y74/85F) cDNA were cloned into the Eco R1 site of mammalian vector, pcDNA3.1 (Invitrogen) to produce plasmids, LMP2A/pcDNA, LMP2A (PY1/PY2)/pcDNA and LMP2A (Y74/85F)/pcDNA, respectively. The plasmids generated were transfected using lipofectamine 2000 (Invitrogen) into the EBV-negative cell line, AGS. AGS cells stably expressing the pcDNA vector, LMP2A cDNA, and ITAM mutant LMP2A cDNA were selected in medium containing G418 (Gibco) at a concentration 800 ug/mL. Further experiments were carried out in EBV negative cell lines, HEK293, HepG2 and SNU5 by transfecting the plasmids for LMP2A and mutant versions of LMP2A using lipofectamine 2000.

RT-PCR and quantitative real-time PCR.

Total RNA was extracted from the cells through Tripure isolation reagent (TRIZOL, Roche). Quality and quantity of RNA was assayed through spectroscopy (Eppendorf BioPhotometer). 2µg of the total RNA was reverse transcribed 42 ºC for 90 min utilizing M-MuLV reverse transcriptase and oligo dT primers (Fermentas) according to manufacturer's protocol. Quantitation of gene expression was performed using quantitative real-time PCR (qRT-PCR) and SYBR Green core PCR reagents (Thermo Scientific, Massachusetts, USA). HPRT was used as the endogenous control (Primer sequences for quantitation of genes are provided in Table 4.2). The qRT-PCR reactions and analysis were performed on 7500 Sequence Detection System (Applied Biosystems).

Gene	***Primer Sequence***
DNMT1 (Fw)	5'-ATGCTTACAACCGGGAAGTG-3'
DNMT1 (Rv)	5'-TGAACGCTTAGCCTCTCCAT-3'
DNMT 3A (Fw)	5'-ATCTCCAAGTCCCCATCCAT-3'
DNMT 3A (Rv)	5'-CAGCCATTTTCCACTGCTCT-3'
DNMT 3B (Fw)	5'-AGATCAAGCTCGCGACTCTC- 3'
DNMT 3B (Rv)	5'-GGCTTTCTGAACGAGTCCTG-3'
HLA-A (Fw)	5'-CCTGGGCAGTCGCACTGC-3'
HLA-A (Rv)	5'-GATTCTCCCCAGACGCCGAG-3'
HLA-B (Fw)	5'-GGACAGCCAGACCAGCAACA-3'

HLA-B (Rv)	5’-GATTCTCCCCAGACGCCGAG-3’
HLA-C (Fw)	5’-TCAGAGCCCTGGGCACTGTT-3’
HLA-C (Rv)	5’-GATTCTCCCCAGACGCCGAG-3’
HLA-E (Fw)	5’-CCACCATGGTAGATGGAACCCTC-3’
HLA-E (Rv)	5’-GCGCTTTACAAGCTGTGAGACTC-3’
HLA-F (Fw)	5’-ATCGTTGCTGGCCTTGTTGTCCTT-3’
HLA-F (Rv)	5’-GGCACAAGTGGAATTCTGCTAC-3’
HLA-G (Fw)	5’-GGAAGAGGAGACACGGAACA-3’
HLA-G (Rv)	5’-TGAGACAGAGACGGAGACAT-3’
HPRT (Fw)	5’-GACACTGGCAAAACAATGCAGAC-3’
HPRT (Rv)	5’-TGGCTTATATCCAACACTTCGTGG-3’
MIC-A (Fw)	5’-CCTTGGCCATGAACGTCAGG-3’
MIC-A (Rv)	5’-CCTCTGAGGCCTCGCTGCG-3’
MIC-B (Fw)	5’-ACCTTGGCTATGAACGTCACA-3’
MIC-B (Rv)	5’-CCCTCT GAGACCTCGCTGCA-3’
UHRF1 (Fw)	5’-TGAATGACACCATCCAGCTC-3’
UHRF1 (Rv)	5’-TCTCATCCCACATGTCCTCA-3’
ULBP1 (Fw)	5’-CAAGTGGAGAATTTAATACCCATTGAG-3’
ULBP1 (Rv)	5’-TGTTGTTTGAGTCAAAGAGGA-3’

ULBP2 (Fw)	5’-TTACTTCTCAATGGGAGACTGT-3’
ULBP2 (Rv)	5’-TGTGCCTGAGGACATGGCGA-3’
ULBP3 (Fw)	5’-CCTGATGCACAGGAAGAAGAG-3’
ULBP3 (Rv)	5’-TATGGCTTTGGGTTGAGCTAAG-3’
ULBP4 (Fw)	5’-CCTCAGGATGCTCCTTTGTGA-3’
ULBP4 (Rv)	5’-CGACTTGCAGAGTGGAAGGATC-3’
ULBP5 (Fw)	5’-TGGCCGACCCTCACTCTCT-3’
ULBP5 (Rv)	5’-CCGTGGTCCAGGTCTGAACT-3’
ULBP6 (Fw)	5’-AATCTCTTGTCCCCAGCCCT-3’
ULBP6 (Rv)	5’-GTGAGGGTCGTCTCGCCTA-3’

Table 4.2: Primer sequences used during qRT-PCR.

Bisulfite Modification and Methylation Specific PCR (MSP).

1×10^5 cells were gathered and dissolved in 5% PBS, were used for further experiments. Bisulfite modification of genomic DNA was carried out using EpiTect Fast LyseAll Bisulfite Kit (Qiagen) as per the manufacturer's protocol. Modified genomic DNA samples was then used as a template for PCR. The methylation status of HLA-ABC promoter in the cell lines was analysed using methylation-specific PCR (MSP) (Primer sequences used for MSP study are provided in Table). PCR reaction was carried out in a volume of 50 µl buffer (high GC) containing 100 ng or less genomic DNA along with TaKaRa EpiTaq HS (TaKaRa). Denaturation was carried

out at 98ºC for 10 s for 35 cycles followed by annealing at 60ºC for 30 s and extension at 72ºC. The amplified product was analyzed using DNA gel electrophoresis and photographed through Gel Doc chemiluminescence detector (BioRad).

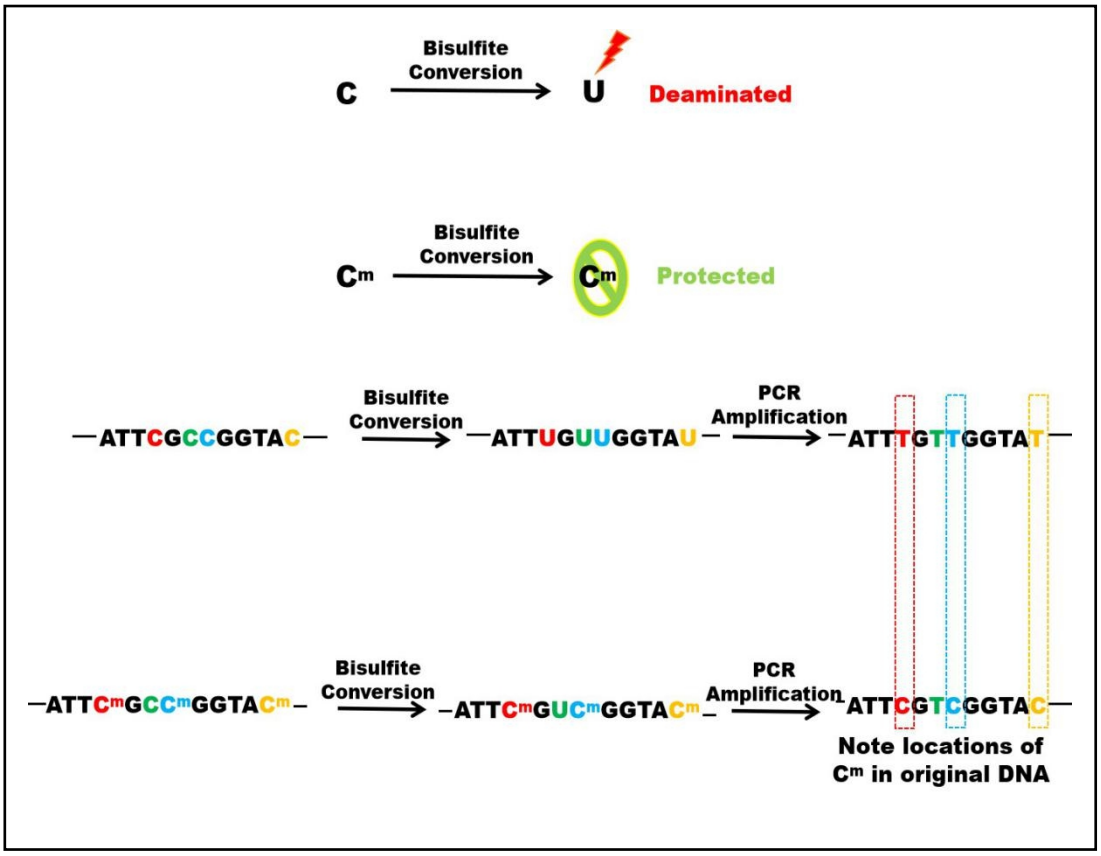

Figure 4.1: Bisulfite conversion and MSP (Adapted and modified from Li et al. 2019). *Bisulfite treatment leads to deamination of unmethylated cytosine and converts it to uracil. DNA polymerase then subsequently substitutes uracil for thymidines during a PCR reaction. However, methylated cytosines are resistant to deamination and will not undergo conversion.*

The qRT-PCR reactions were carried out by using SYBR Green core PCR reagents and genomic DNA template (bisulfite-modified) for the quantitation of methylated

products of HLA-ABC promoter region in cell lines. GAPDH was used as the endogenous control for qRT-PCR analysis. Similar primer sequences (Table 4.3) were used for PCR and qRT-PCR analysis upon bisulfite-modification to amplify product size of 167 kb approximately.

Gene	***Primer***	***Sequence***	***Size***
HLA-AM	Forward	5'-TAATTTCGATATTTTGGGAGGTC-3'	173 bp
	Reverse	5'-CCAAATAACTAAAACTACAAACGC-3'	
HLA-BM	Forward	5'-TTTTTTTTAATTTGTGTCGG-3'	165bp
	Reverse	5'-ACTTTAAAACTAAAACCGCGAC-3'	
HLA-CM	Forward	5'-TTTTTTGAATATTTATGACGCGT-3'	163bp
	Reverse	5'-CCTCTAAAAAAAATATAAATCCGA-3'	
GAPDH (Methylated)	Forward	5'-TTTTTTTATGTTGGGGTTTATATG-3'	142bp
	Reverse	5'-AAAACCTAAACTACATACACCCAT-3'	

Table 4.3: Sequences used for amplification of methylated products upon bisulfite modification using qRT-PCR.

Western blot analysis.

Cells were harvested and lysed in Nonidet P-40-lysis buffer [Tris-HCl (20 mM, pH 8.0), NaCL (137mM), glycerol (10% v/v), Nonidet P-40 (1% v/v), EDTA (2mM), Na_3VO_4 (200mM), phenylmethylsulfonyl fluoride (100mM), phosphatase inhibitor and protease inhibitor cocktail (Roche, Mannheim, Germany)]. Whole cell protein lysates were obtained by freeze-thaw method and centrifuged for 5 min at 16,000 g ice cold, supernatant was collected for further experiments. Quantitation of the protein

concentration was determined through Bradford protein assay, equal amounts of protein (25-50 µg) from different samples were separated on the basis of molecular weight by using 10%-12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (GE Healthcare). Nitrocellulose membrane was then subjected to blocking using BSA (5% v/v) in TBS containing Tween 20 (0.1% v/v) (Sigma Chemical Co.) at room temperature for an hour and then washed with TBST. Primary antibodies (1:1000 dilution in TBS-T) (Table 4.1) were incubated with blotted nitrocellulose membrane overnight at 4 ºC. Membranes were then subjected to washing with TBS-T, probed with horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology) at room temperature for an hour (1:3000 dilution in TBS-T), and washed with TBS-T to remove excess secondary antibody. The protein bands specific to primary antibody were visualized using enhanced chemiluminescent reagent (Amersham Biosciences), photographed using Versa Doc chemiluminescence detector and quantitation of bands was executed using Quantity ONE software (BioRad).

5'-Aza-2'-deoxycytidine treatment.

The cell lines were seeded at low density and incubated with different doses of 5'-Aza-2′-deoxycytidine (2uM and 4uM) for a period of 6 days, independent sets were replenished with fresh 5'-Aza-2′-deoxycytidine after a time-period of 24 hr. Cells were harvested after treatment for FACS analysis of HLA-ABC surface.

RNA interference.

The siRNA sequence used in the study for inhibiting LMP2A mRNA expression was 5'-AACUCCCAAUAUCCAUCUGCU-3', identical to the one which was previously studied (140). The siRNA sequence for inhibiting PDI mRNA expression was 5'-GGACCAUGAGAACAUCGUC-3', was similar as previously reported (141). A control scrambled siRNA sequence was used as Mock (Sigma Aldrich, St. Louis, MO). The transfection of siRNAs was performed by utilizing Lipofectamine 2000 (Invitrogen).

Cell surface analysis of HLA-ABC surface expression.

1×10^5 cells were harvested and dissolved in PBS (5%) and incubated with allophycocyanin (APC) conjugated anti-HLA-ABC monoclonal antibody at 4ºC for 30 min in dark. The concentration of antibody used for the purpose of staining was as per the manufacturer's instructions. Stained samples were washed with PBS to remove excess unbound antibody, further resuspended in PBS and subjected to Fluorescence-Activated Cell Sorting (FACS) Analysis on FACS Calibur (Becton Dickinson). The results were analyzed and quantitated using Cell Quest Pro software (B.D. Biosciences).

Cell surface analysis of MIC-A/B surface expression.

1×10^5 cells were harvested and dissolved in PBS (5%) and incubated with phycoerythrin (PE) conjugated anti-MIC-A/B monoclonal antibody at 4ºC for 30 min in dark. The concentration of antibody used for the purpose of staining was as per the manufacturer's instructions. Stained samples were washed with PBS to remove excess

unbound antibody, further resuspended in PBS and subjected to Fluorescence-Activated Cell Sorting (FACS) Analysis on FACS Calibur (Becton Dickinson). The results were analyzed and quantitated using Cell Quest Pro software (B.D. Biosciences).

Thapsigargin (Tg) treatment.

The cell lines were seeded at low density, incubated with different doses of thapsigargin (1uM and 2uM) for a time period of 6hr. Treated and untreated cells were harvested for determination of protein level measurement of PDI and FACS analysis for MIC-A/B surface expression.

Statistical analysis.

Results acquired from the experiments (n=3) were represented as mean ± s.e.m. The significance of the data analyzed was calculated using student's two-tailed t-test. Only P-values < 0.05 were considered statistically significant.

Chapter 5

Results

LMP2A expression in epithelial cell carcinomas.

Epstein-Barr virus (EBV) is reported to transform human B cells and epithelial cells, resulting in cancer. EBV latent protein, LMP2A promotes proliferation and activation of EBV-infected epithelial cells and is expressed in several epithelial cell carcinomas. It is still unclear, how latent EBV infection of epithelial cell cancers escape host immune response, and whether the viral protein, LMP2A plays any significant role in influencing the interaction and recognition of EBV-infected epithelial cells by the host immune cells. The LMP2A cDNA was cloned within the EcoR1 site of the mammalian expression vector, pcDNA 3.1 containing the G418 resistance gene to allow selection of transfected cells. EBV-negative epithelial cells, AGS and HEK-293 were transfected with plasmid containing LMP2A cDNA. Stable clones were procured using G418 selection treatment. Protein level expression of LMP2A was determined in AGS and AGS-LMP2A cells [Figure 5.1(A & B)]. Protein level expression of LMP2A was also determined in HEK293 and HEK293-LMP2A cells [Figure 5.1(C & D)]. Clones which showed similar expression levels for LMP2A compared to EBV-positive cell, SNU-719 (marked with orange box; Clone2 and Clone4 for both AGS-LMP2A and HEK-293 cells) were used for further studies [Figure 5.1(E & F)].

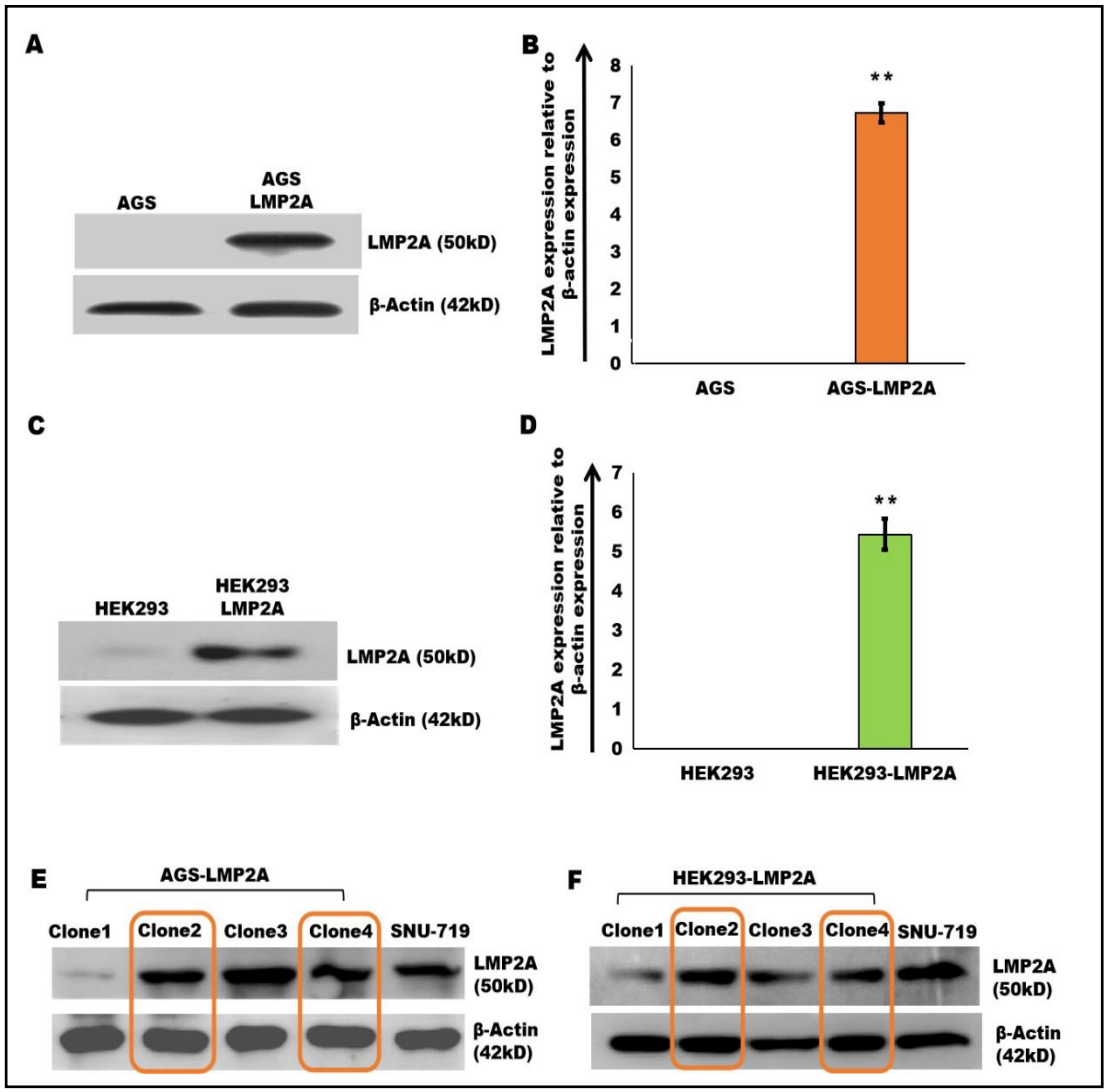

Figure 5.1: LMP2A expression in epithelial cell carcinomas. *(A & B) Immunoblotting analysis of LMP2A in AGS and AGS-LMP2A cells. Bar graph illustrates the densitometry quantification of LMP2A expression in AGS and AGS-LMP2A cells compared to respective β-Actin expression. (C & D) Immunoblotting analysis of LMP2A in HEK293 and HEK293-LMP2A cells. Bar graph depicts the densitometry quantification of relative expression of LMP2A in AGS and AGS-LMP2A cells compared to respective β-Actin expression.(E) Immunoblotting analysis of stable LMP2A expression in AGS cells. (F) Immunoblotting analysis of stable LMP2A*

*expression in HEK293 cells. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A regulates HLA-ABC expression.

LMP2A is latent protein of EBV that may alter identification of infected cells by CTLs, especially by regulating the expression of HLA-ABC through Sonic hedgehog (Shh) pathway in gastric cancer cells (49). However, detailed mechanism which results in HLA-ABC downregulatiom still remains unclear. We found that expression of HLA-A, HLA-B and HLA-C at the transcript-level was somewhat reduced in LMP2A expressing cells. This argued against a possible role of LMP2A in contributing to decreased recognition of LMP2A cells through downregulated expression of HLA-ABC. Transcript-level expression of HLA-A, HLA-B and HLA-C was measured through qRT-PCR in epithelial cells, AGS [Figure 5.2(A)] and HEK293 [Figure 5.2(B)] expressing LMP2A gene. A decreased expression of HLA-A, HLA-B and HLA-C transcripts was observed in response to LMP2A gene. Next, we investigated whether siRNA mediated silencing of LMP2A [Figure 5.2(D)] modulated the expression of HLA-A, HLA-B and HLA-C transcripts in EBV-positive, SNU-719 cells [Figure 5.2(C)] . Increased transcript level expression of HLA-A, HLA-B and HLA-C was observed in response to siRNA mediated knockdown of LMP2A in SNU-719 cells.

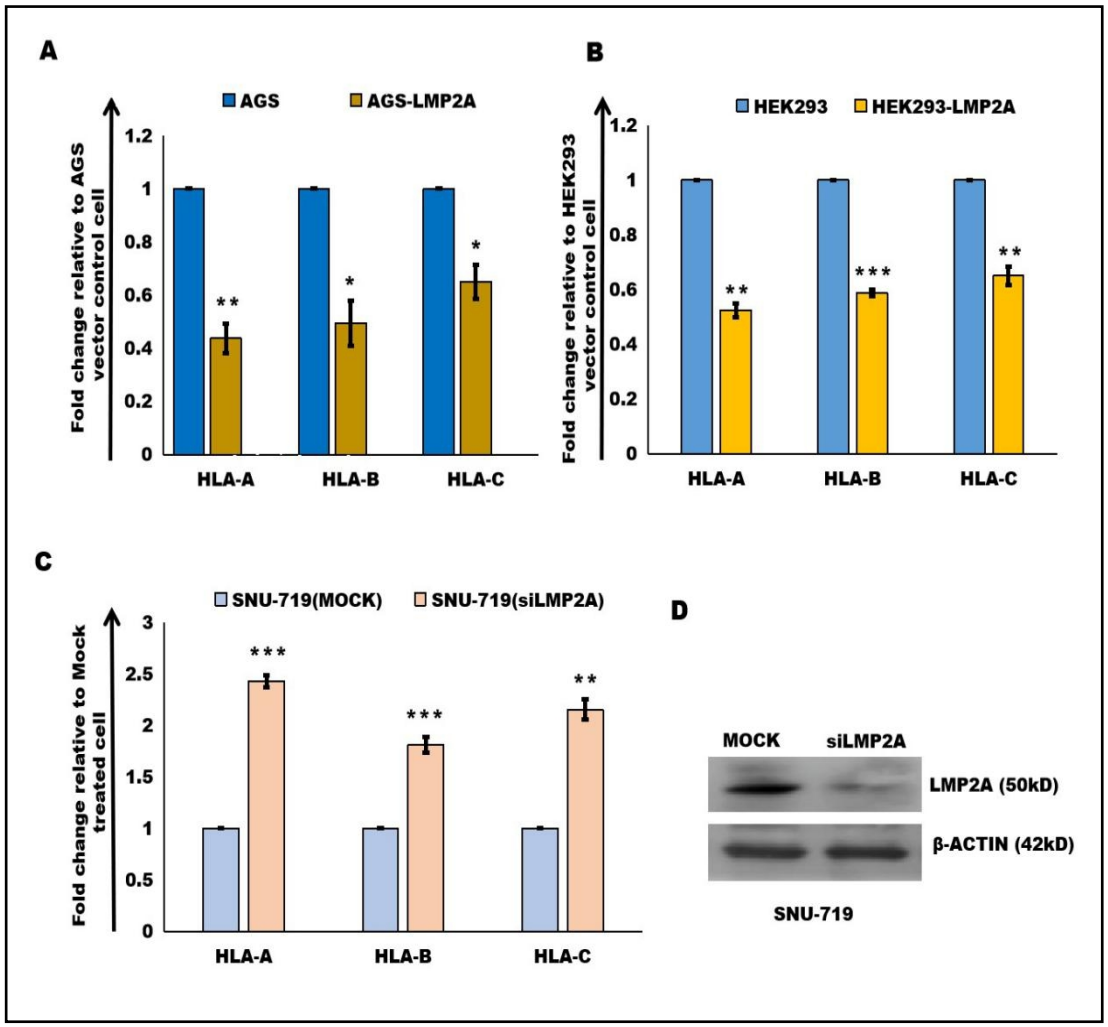

Figure 5.2: LMP2A regulates HLA-ABC expression. *(A) Quantitative RT-PCR of HLA-A, HLA-B, and HLA-C genes in AGS-LMP2A with respect to AGS vector control. (B) qRT-PCR of HLA-A, HLA-B, and HLA-C genes in HEK293-LMP2A with respect to HEK293 vector control. (C) qRT-PCR of HLA-A, HLA-B, and HLA-C genes in SNU-719 upon siRNA mediated knockdown of LMP2A expression after 72 hr compared to Mock treatment. (D) Immunoblotting experiments to determine LMP2A levels in SNU-719 upon siRNA mediated silencing of LMP2A expression after 72 hr compared to Mock treatment. Results are represented as mean ±s.e.m. of triplicate*

*experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

EBV-LMPA induces expression of epigenetic regulators.

EBV has been reported to stably regulate gene expression by means of aberrant DNA methylation in EBV-associated epithelial cell cancers (142). Epigenetics play crucial role in the regulation of gene expression; thus, we studied expression of epigenetic regulators in context of LMP2A expression. First, we recognized the epigenetic regulators with increased expressions in, AGS [Figure 5.3(A)] and HEK293 [Figure 5.3(B)] cells expressing LMP2A by quantitation of mRNA levels with respect to vector control cells through qRT-PCR. An increased expression of epigenetic regulators, DNMT1, DNMT3B, and UHRF1 at transcript level was detected upon analysis in response to LMP2A expression in epithelial cells. Further validation was done through immunoblotting experiments in AGS [Figure 5.3(C & D)] and HEK293 [Figure 5.3(E & F)] cells expressing the viral protein, LMP2A.

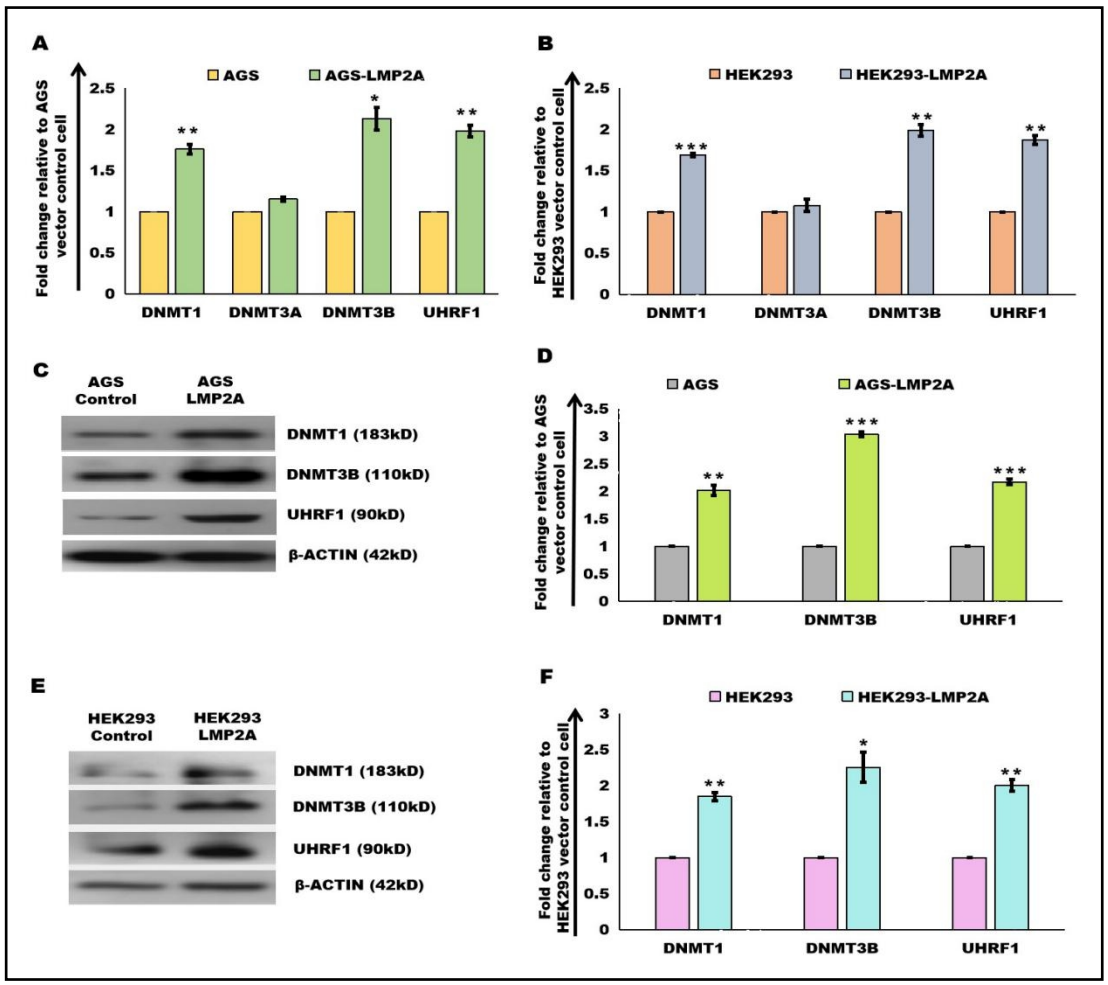

Figure 5.3: EBV-LMPA induces expression of epigenetic regulators. *(A) qRT-PCR of epigenetic regulators (DNMTs and UHRF1) in AGS-LMP2A cells with respect to AGS vector control. (B) qRT-PCR of epigenetic regulators (DNMTs and UHRF1) in HEK293-LMP2A cells relative to HEK293 vector control. (C & D) Protein level measurement of DNMT1, DNMT3B, and UHRF1 in AGS vector control and AGS-LMP2A. (E & F) Protein level measurement of DNMT1, DNMT3B, and UHRF1 in HEK293 vector control and HEK293-LMP2A. Bar graph depicts the densitometry quantification of relative expression of DNMT1, DNMT3B, and UHRF1. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001, **P ≤ 0.01 and *P < 0.05.*

LMP2A silencing resulted in decreased expression of epigenetic regulators.

To further validate the role of viral protein, LMP2A on gene regulation through DNA methylation, the expression of epigenetic regulators was examined upon siRNA mediated silencing of LMP2A gene in epithelial cells AGS-LMP2A and SNU-719. Immunoblotting experiments were performed for AGS-LMP2A [Figure 5.4(A & B) and SNU-719 [Figure 5.4(C & D) cells at 48 hr and 72 hr post-knockdown of LMP2A. Expression of DNMT1, DNMT3B and UHRF1 was observed to be downregulated in a time-dependent approach in response to siRNA mediated silencing of the viral protein, LMP2A in AGS-LMP2A and SNU-719 cells.

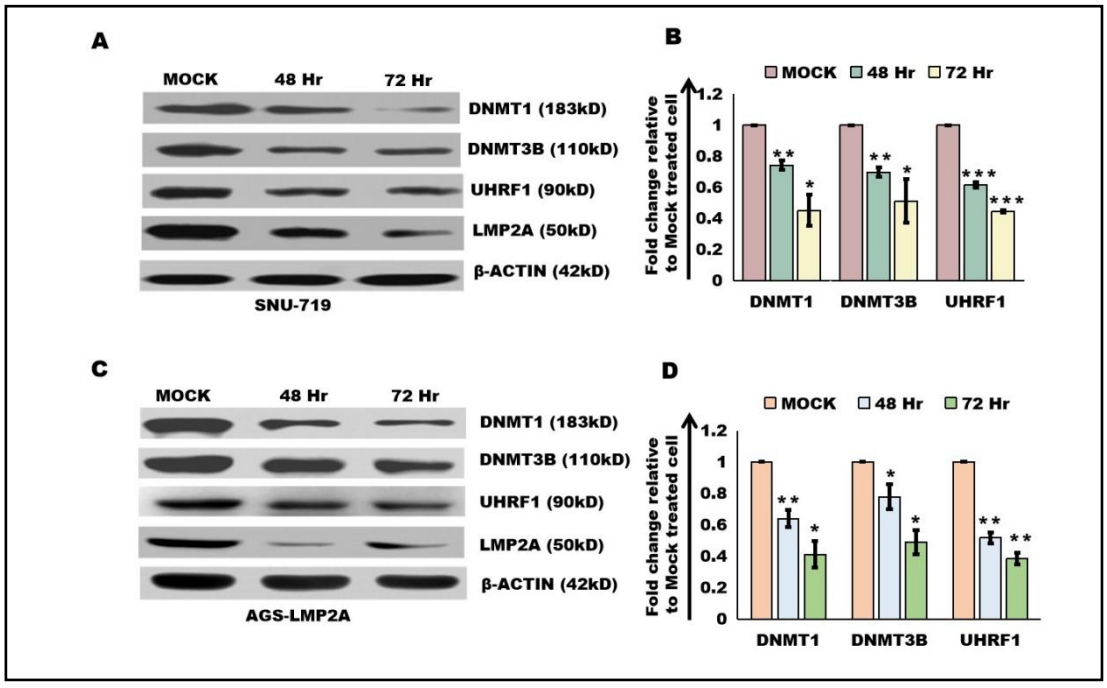

Figure 5.4: LMP2A silencing resulted in decreased expression of epigenetic regulators. *(A & B) Protein level measurement of DNMT1, DNMT3B, UHRF1 and LMP2A levels in response to siRNA mediated targeting of LMP2A expression in AGS-LMP2A at 48 and 72 hr with respect to Mock treatment. (C & D) Immunoblotting experiments to determine DNMT1, DNMT3B, UHRF1 and LMP2A levels in SNU-719 upon siRNA*

*mediated silencing of LMP2A expression at 48 and 72 hr with respect to Mock treatment. Bar graph depicts the densitometry quantification of relative expression of DNMT1, DNMT3B, UHRF1 and LMP2A. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A promotes methylation of HLA-ABC gene promoter region.

Loss of HLA-ABC expression can take place at genetic, transcriptional and post-transcriptional stages. DNA methylation, especially methylation of 5′-CpG islands, acts an important technique in silencing the gene expression. Aberrant DNA methylation has been reported in several tumour suppressor genes, leading to their decreased expression in tumors. The methylation status of promoter region of HLA-ABC gene was studied in SNU-719, AGS-LMP2A [Figure 5.5(A & B)] and HEK293-LMP2A [Figure 5.5(C & D)] together with their respective vector control cells using mehylation-specific PCR (143,144). Hypermethylation of HLA-A, HLA-B and HLA-C gene promoter region was detected in EBV-positive gastric cancer cell, SNU-719 as well as in EBV-negative epithelial cell expressing viral latent gene LMP2A, AGS-LMP2A and HEK293-LMP2A.

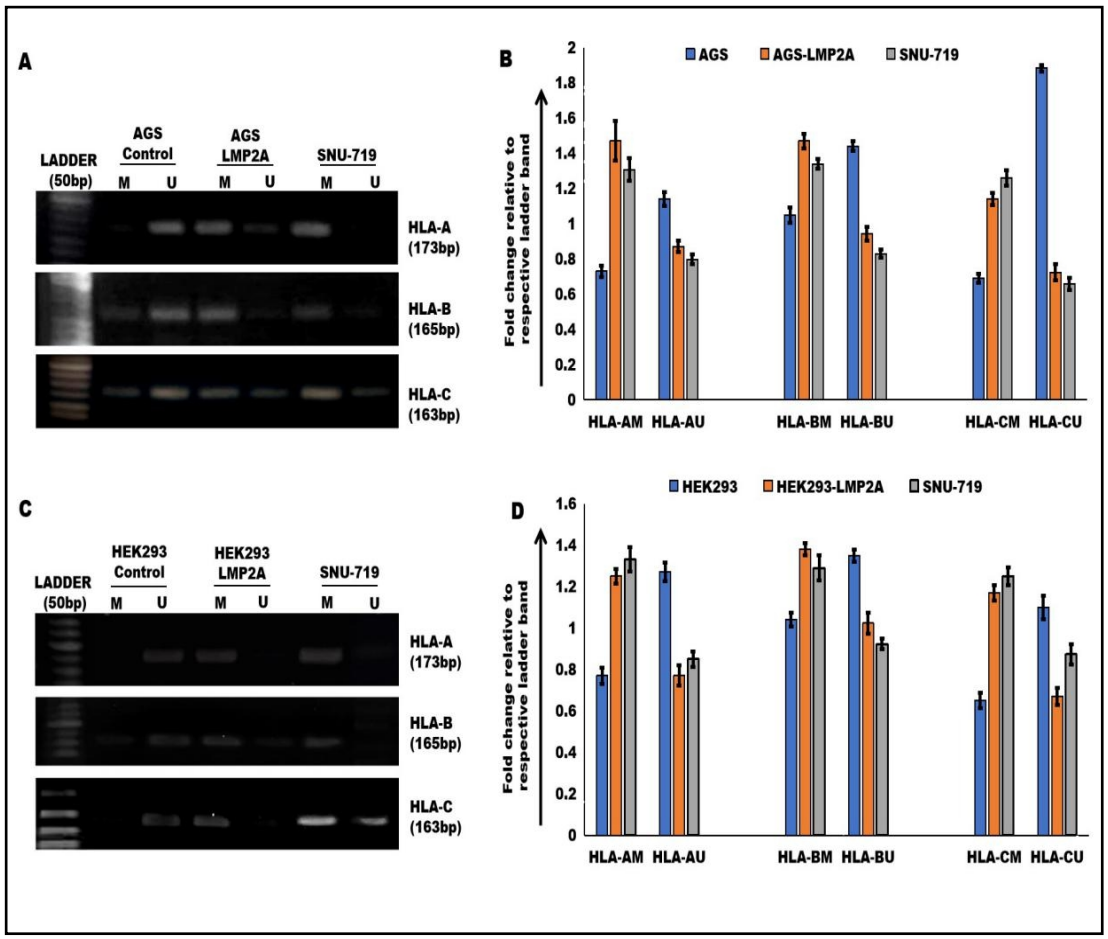

Figure 5.5: LMP2A promotes methylation of HLA-ABC gene promoter region. *(A & B) MSP analysis of the human leukocyte antigen (HLA) class Ia; HLA-A, HLA-B and HLA-C gene promoter in AGS, AGS-LMP2A, and SNU-719 cells. ImajeJ software was used for the quantitation of results by measuring the median values. (C & D) MSP analysis of the human leukocyte antigen (HLA) class Ia; HLA-A, HLA-B and HLA-C gene promoter region in HEK293, HEK293-LMP2A, and SNU-719 cells. ImajeJ software was used for the quantitation of results by measuring the median values. Genomic DNA was subjected to sodium bisulfite treatment and amplification through specific primers for methylated (M) and unmethylated (U) PCR products. Results are represented as mean ±s.e.m. of triplicate experiments.*

LMP2A mutants expression in AGS cells.

LMP2A acts as a latent viral trans-membrane protein and plays significant role in the maintenance of latent infection. LMP2A comprises of an ITAM region identical to BCR, contains eight tyrosine residues. Two such mutants were generated in the ITAM region; 1) PY1/PY2 and 2) Y74/85F using 'Site-directed mutagenesis' [Figure 5.6(A)]. LMP2A mutants were referred as pcDNA3.1/LMP2A-PY1/PY2 [LMP2A(PY1/PY2)] and pcDNA3.1/LMP2A-Y74/85F [LMP2A(Y74/85F)], they were stably transfected into AGS and screened through G418 treatment. The expression of mutant LMP2A genes at the transcript level was quantitated in AGS-LMP2A(PY1/PY2) [Figure 5.6(B)] and AGS-LMP2A(Y74/85F) [Figure 5.6(C)] compared to AGS cells expressing pcDNA3.1 (AGS). Clones which showed similar expression pattern as that of AGS-LMP2A were selected for further study.

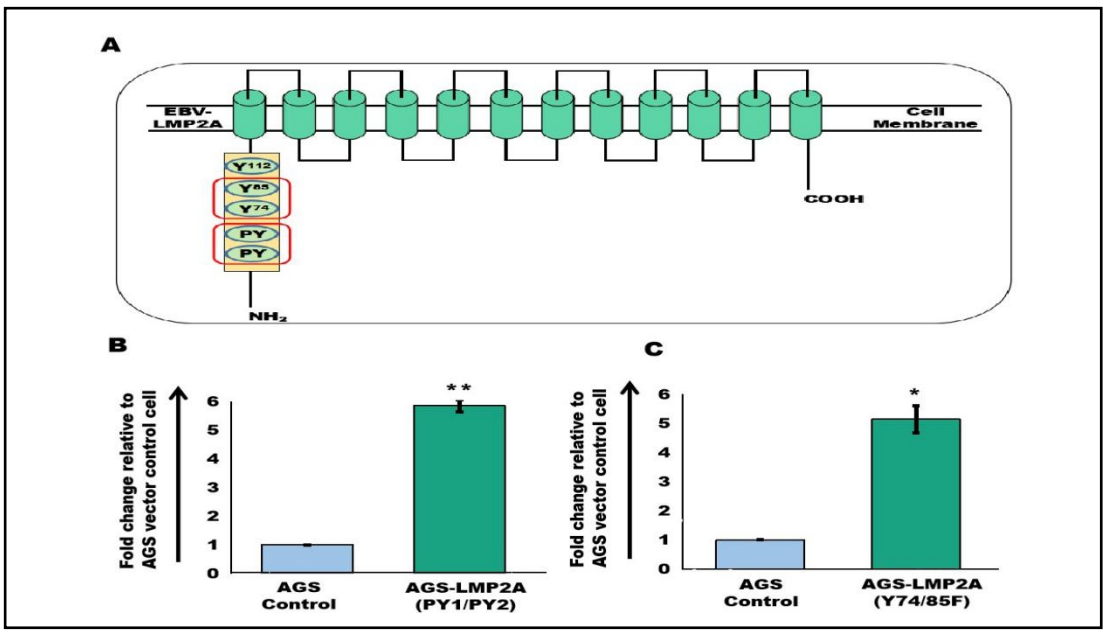

Figure 5.6: LMP2A mutants expression in AGS cells. *(A) Schematic representation of EBV-LMP2A. Red boxes indicate the mutated regions in the ITAM. (B) qRT-PCR of*

*LMP2A(PY1/PY2) in AGS cells stably expressing the mutated gene, AGS-LMP2A(PY1/PY2) relative to AGS vector control cell. (C) qRT-PCR of LMP2A(Y74/85F) in AGS cells stably expressing the mutated gene, AGS-LMP2A(Y74/85F) relative to AGS vector control cell. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A mutants were screened for HLA-ABC surface expression.

HLA-ABC expression at surface-level was investigated in AGS-LMP2A(PY1/PY2) [Figure 5.7(A)] and AGS-LMP2A(Y74/85F) [Figure 5.7(B)] with respect to AGS cells. HLA-ABC surface-level expression was also studied in HEK293 cells transiently transfected to express viral LMP2A as well as LMP2A mutants [Figure 5.7(C and D)]. HLA-ABC was observed to be downregulated in AGS and HEK293 cells expressing viral LMP2A and mutated LMP2A(PY1/PY2) genes. However, EBV negative epithelial cell, AGS and HEK293 cells expressing mutated LMP2A(Y74/85F) displayed increased HLA-ABC surface expression similar to the vector control cells.

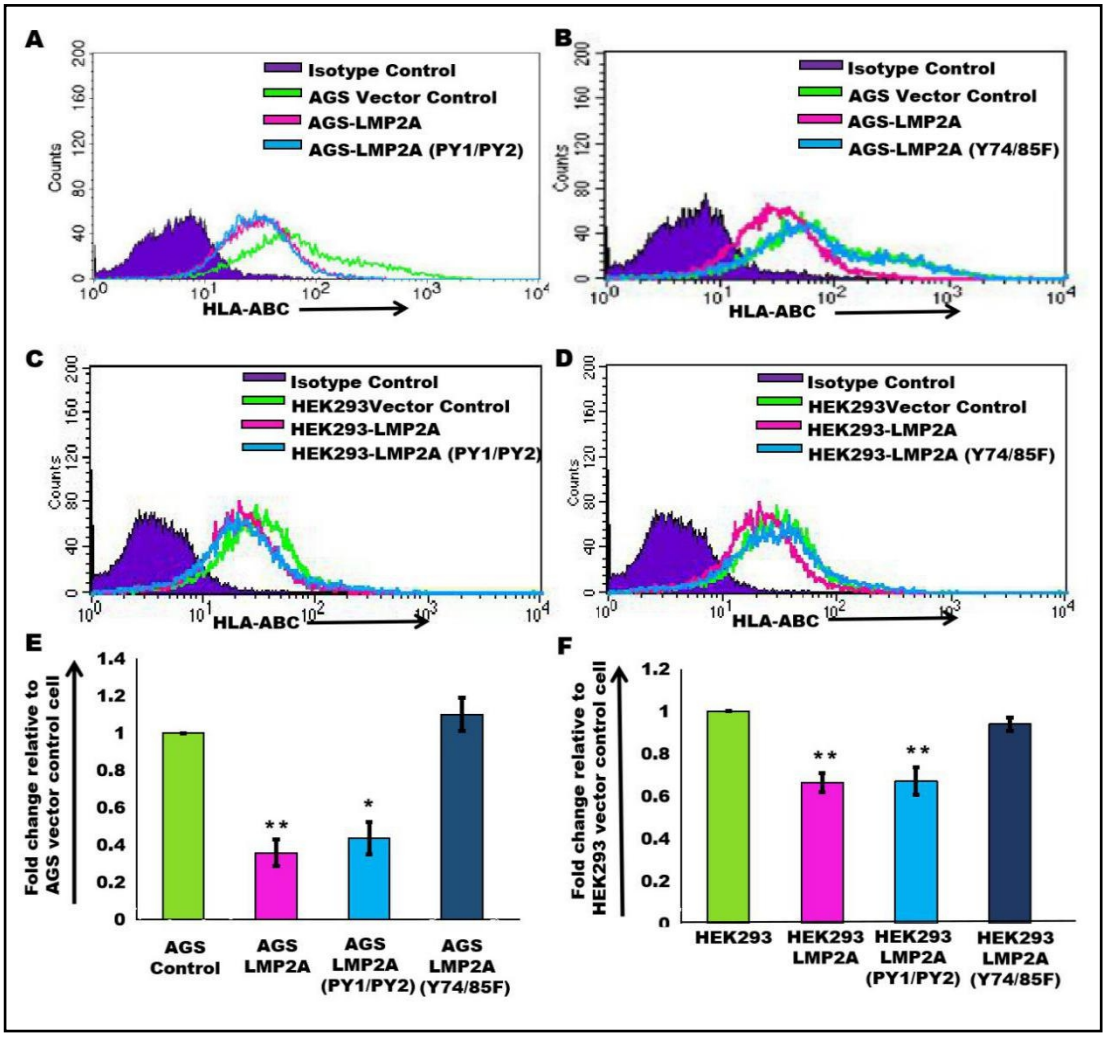

Figure 5.7: LMP2A mutants were screened for HLA-ABC surface expression. *(A) Surface expression of HLA-ABC was analyzed through FACS analysis, in response to expression of mutant LMP2A (PY1/PY2) in AGS cells compared to vector control. (B) Surface expression of HLA-ABC was analyzed through FACS analysis, in response to expression of mutant LMP2A (Y74/85F) in AGS cells compared to vector control. (C) Surface expression of HLA-ABC was analyzed through FACS analysis, in response to expression of mutant LMP2A (PY1/PY2) in HEK293 cells compared to vector control. (D) Surface expression of HLA-ABC was analyzed through FACS analysis, in response to expression of mutant LMP2A (Y74/85F) in HEK293 cells compared to*

*vector control. (E and F) Cell Quest Pro software was used for the quantitation of results by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A mutants were screened for expression of epigenetic regulators and HLA-ABC promoter hyper-methylation.

LMP2A mutants were further screened for the expression of epigenetic regulators, DNMT1, DNMT3B, and UHRF1. Decreased protein-level expression of DNMT1, DNMT3B, and UHRF1 was verified in AGS-LMP2A(Y74/85F) cells. AGS-LMP2A(PY1/PY2) cells manifested similar expression levels as AGS-LMP2A cells [Figure 5.8(A)]. Immunoblotting analysis exhibited that tyrosine residues (Y74 and Y85) in ITAM region is responsible for increased expression of the above-mentioned epigenetic regulators. HEK293 cell transiently expressing LMP2A and LMP2A (PY1/PY2) exhibits increased protein levels of DNMT1, DNMT3B and UHRF1 [Figure 5.8(B)] along with HLA-ABC gene promoter hyper-methylation [Figure 5.8(D)]. Increased methylated amplicons of HLA-A, HLA-B, and HLA-C gene promoter regions were screened in AGS [Figure 5.8(C)] and HEK293 [Figure 5.8(D)] cells stably expressing LMP2A and LMP2A(PY1/PY2) genes by qRT-PCR.

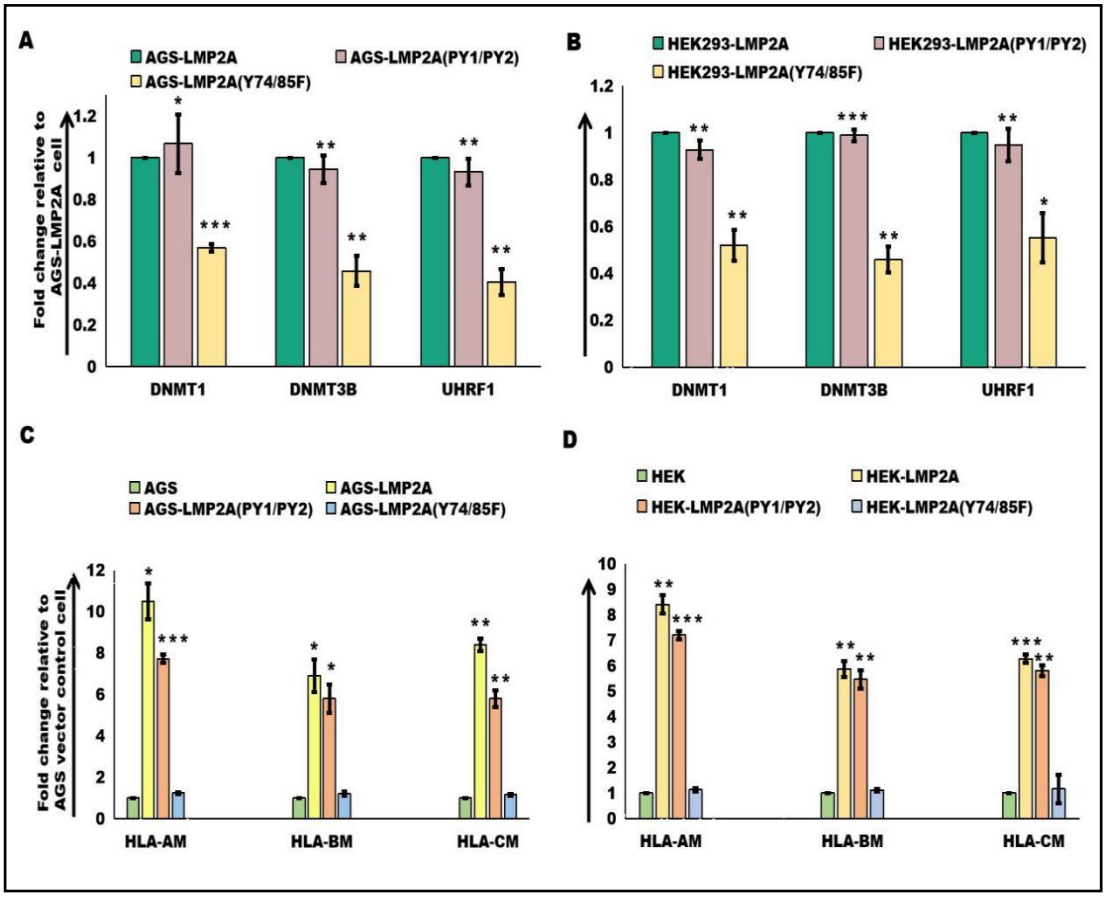

Figure 5.8: LMP2A mutants were screened for expression of epigenetic regulators and HLA-ABC promoter hyper-methylation. *(A) Protein level measurement of DNMT1, DNMT3B and UHRF1 levels in AGS LMP2A (PY1/PY2) and AGSLMP2A (Y74/85F) relative to AGS-LMP2A cells. (B) Determination of protein levels of DNMT1, DNMT3B and UHRF1 in HEK293, HEK293-LMP2A, HEK293-LMP2A(PY1/PY2) as well as HEK293-LMP2A(Y74/85F) cells compared to HEK293 control cells. (C) Bisulfite modified genomic DNA template was used for qRT-PCR mediated quantitaion of methylation of HLA-A, HLA-B and HLA-C promoter using MSP specific primers in AGS-LMP2A, AGS-LMP2A(PY1/PY2) and AGS-LMP2A(Y74/85F) with respect to AGS control cell. (D) Bisulfite modified genomic DNA template was used for qRT-PCR mediated quantitaion of methylation of HLA-A, HLA-B and HLA-C*

*promoter using MSP specific primers in HEK293, HEK293-LMP2A, HEK293-LMP2A(PY1/PY2) and HEK293-LMP2A(Y74/85F) cells. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

Restoration of HLA-ABC expression upon treatment with 5'-Aza-2'-deoxycytidine.

Methylation mediated regulation of HLA-BC surface expression was further verified, LMP2A expressing epithelial cell gastric carcinomas, SNU-719 and AGS-LMP2A were subjected to 5'-Aza-2'-deoxycytidine (demethylating agent) (145-147) treatment. 5'-Aza-2'-deoxycytidine treatment ensued restoration of HLA-ABC surface levels in AGS-LMP2A and SNU-719 cells in a dose-dependent manner [Figure 5.9(A, B, C)]. AGS-LMP2A(PY1/PY2) cells treated with 5'-Aza-2'-deoxycytidine treatment exhibited increased HLA-ABC surface levels in a dose-dependent manner [Figure 5.9(D, F)]. On the contrary, AGS-LMP2A(Y74/85F) cells treated with 5'-Aza-2'-deoxycytidine failed to show any noticeable change in HLA-ABC surface levels in response to dose-dependent treatment [Figure 5.9(E, F)]. Thus, further validating the role of tyrosine residues (Y74 and Y85) within the ITAM motif in regulation of surface expression of HLA-ABC by promoter hypermethylation.

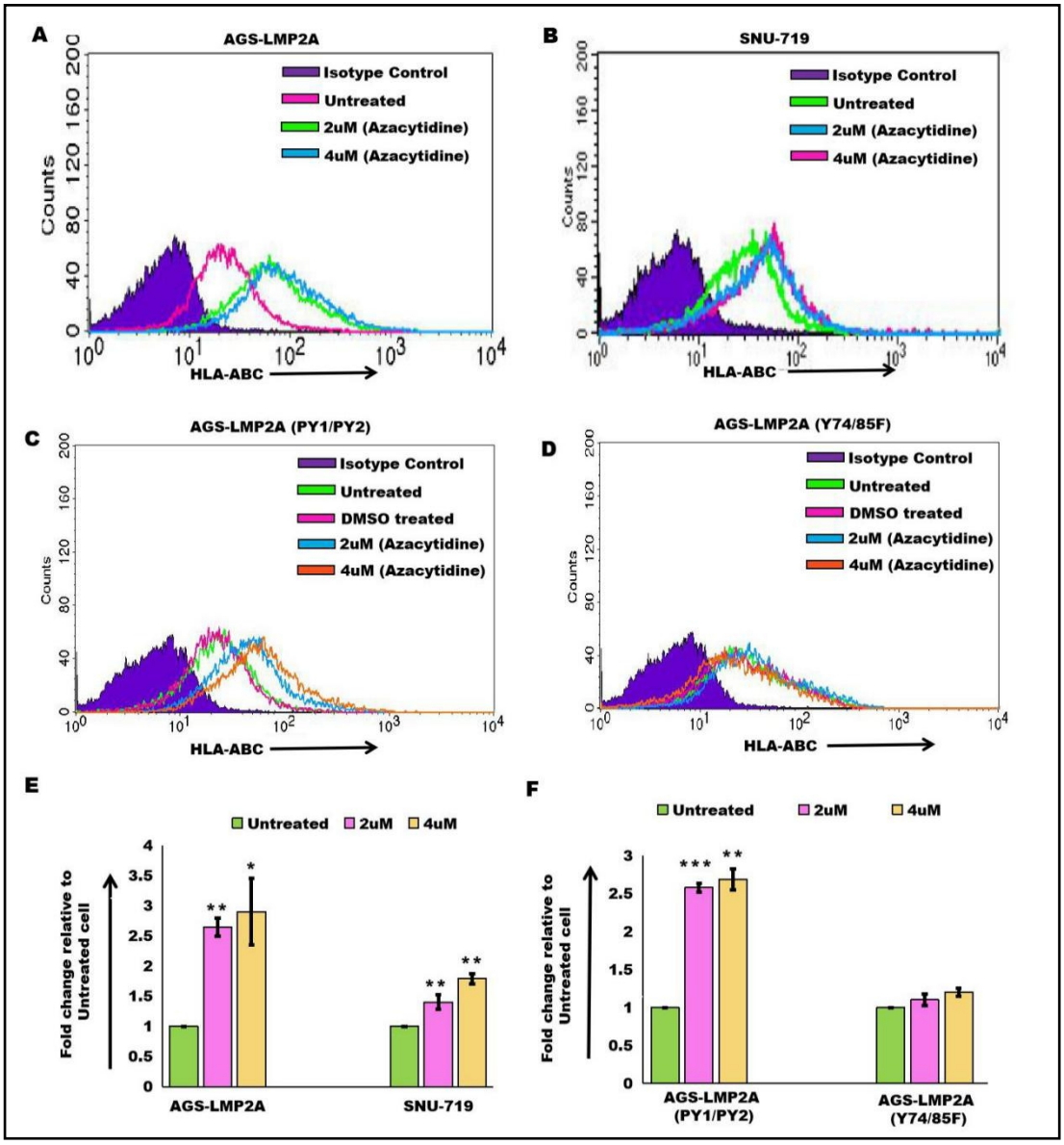

Figure 5.9: Restoration of HLA-ABC expression upon treatment with 5'-Aza-2'-deoxycytidine. *(A, B, C) Surface expression of HLA-ABC was analyzed using FACS analysis in response to treatment with 5'-Aza-2'-deoxycytidine in AGS-LMP2A and SNU-719 cells with respect to untreated cells in a dose (2uM and 4uM) dependent manner. (D, E, F) Surface expression of HLA-ABC was analyzed using FACS analysis in response to treatment with 5'-Aza-2'-deoxycytidine in AGS-LMP2A (PY1/PY2) and AGS-LMP2A (Y74/85F)cells with respect to untreated*

*cells in a dose (2uM and 4uM) dependent manner. Cell Quest Pro software was used for the quantitation of results by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

EBV-LMP2A induces MIC-A and MIC-B expression.

Major histocompatibility homologs, MIC-A and MIC-B are polymorphic proteins that are induced in response to stress, damage or transformation of cells, acts as key regulators of NK cell mediated cytotoxicity through the natural-killer group 2, member D receptor. Expression of MIC-A/B has been reported for most tumour types, however there expression in normal healthy cells is somewhat deprived (148). To determine whether viral latent protein, LMP2A could regulate MIC-A and MIC-B at transcriptional level, we examined the change of MIC-A and MIC-B expression in the LMP2A expressing epithelial cell lines. The expression levels of MIC-A [Figure 5.10(A)] and MIC-B [Figure 5.10(B)] was significantly increased in AGS-LMP2A and AGS-LMP2A (PY1/PY2). Increased transcript-level expression of MIC-A [Figure 5.10(C)] and MIC-B [Figure 5.10(D)] was further detected in HepG2 cells expressing LMP2A and LMP2A(PY1/PY2) gene.

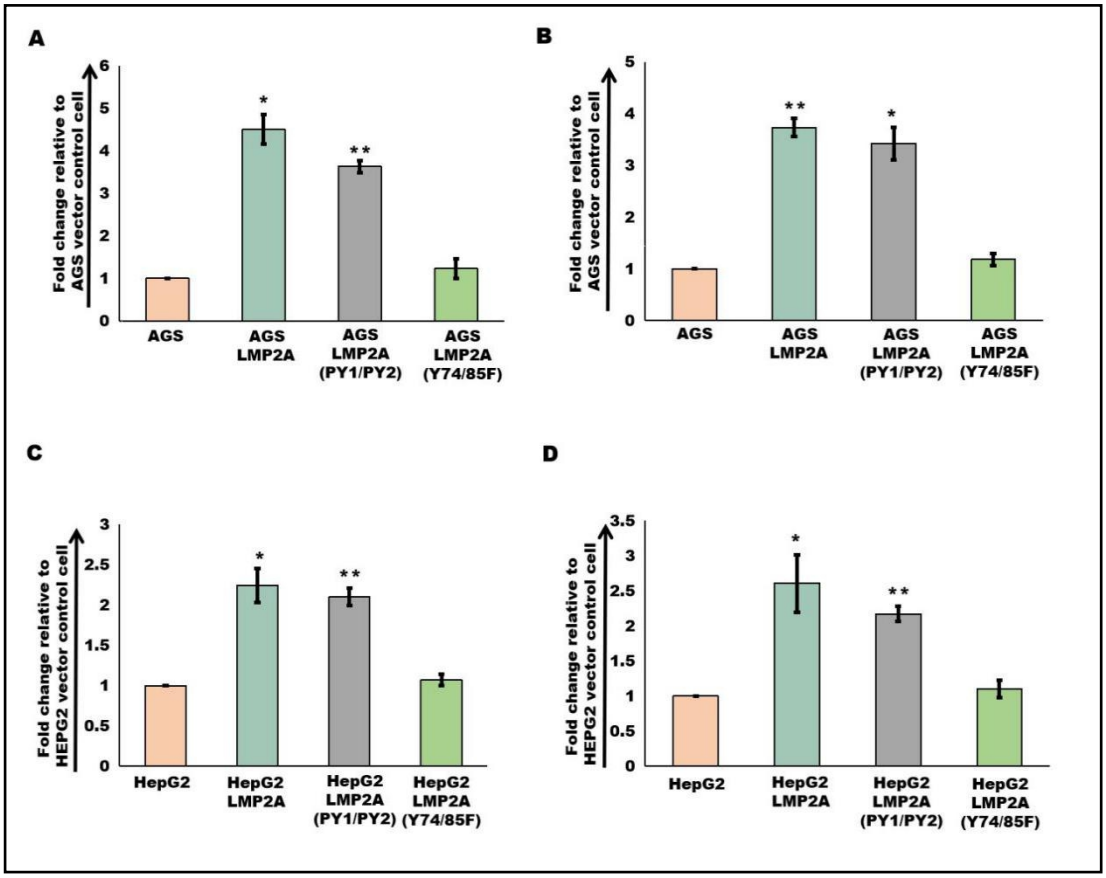

Figure 5.10: EBV-LMP2A induces MIC-A and MIC-B expression. *(A) Transcript level expression of MIC-A was determined in AGS-LMP2A, AGS-LMP2A(PY1/PY2) and AGS-LMP2A(Y74/85F) with respect to AGS control cell using qRT-PCR analyis. (B) Transcript level expression of MIC-B was determined in AGS-LMP2A, AGS-LMP2A(PY1/PY2) and AGS-LMP2A(Y74/85F) with respect to AGS control cell using qRT-PCR analyis. (C) qRT-PCR to analyze MIC-A transcript level expression in HepG2-LMP2A, HepG2-LMP2A(PY1/PY2) and HepG2-LMP2A(Y74/85F) cells relative to HepG2 vector control cells. (D) qRT-PCR to analyze MIC-B transcript level expression in HepG2-LMP2A, HepG2-LMP2A(PY1/PY2) and HepG2-LMP2A(Y74/85F) cells relative to HepG2 vector control cells. Results are*

*represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

Expression of ULBPs in response to LMP2A.

Apart from MIC-A/B, unique long 16 (UL16) binding proteins (ULBPs) are also known to bind NKG2D receptors and play important role in NK cell mediated cytotoxicity. Expression of ULBP-1, ULBP-2, ULBP-3, ULBP-4, ULBP-5 and ULBP-6 was studied at the transcript-level in association with LMP2A expression. ULBP-1 showed decreased expression, whereas all other ULBPs showed increased expression in LMP2A [Figure 5.11(A)] and LMP2A(PY1/PY2) [Figure 5.11(B)] expressing AGS cells.

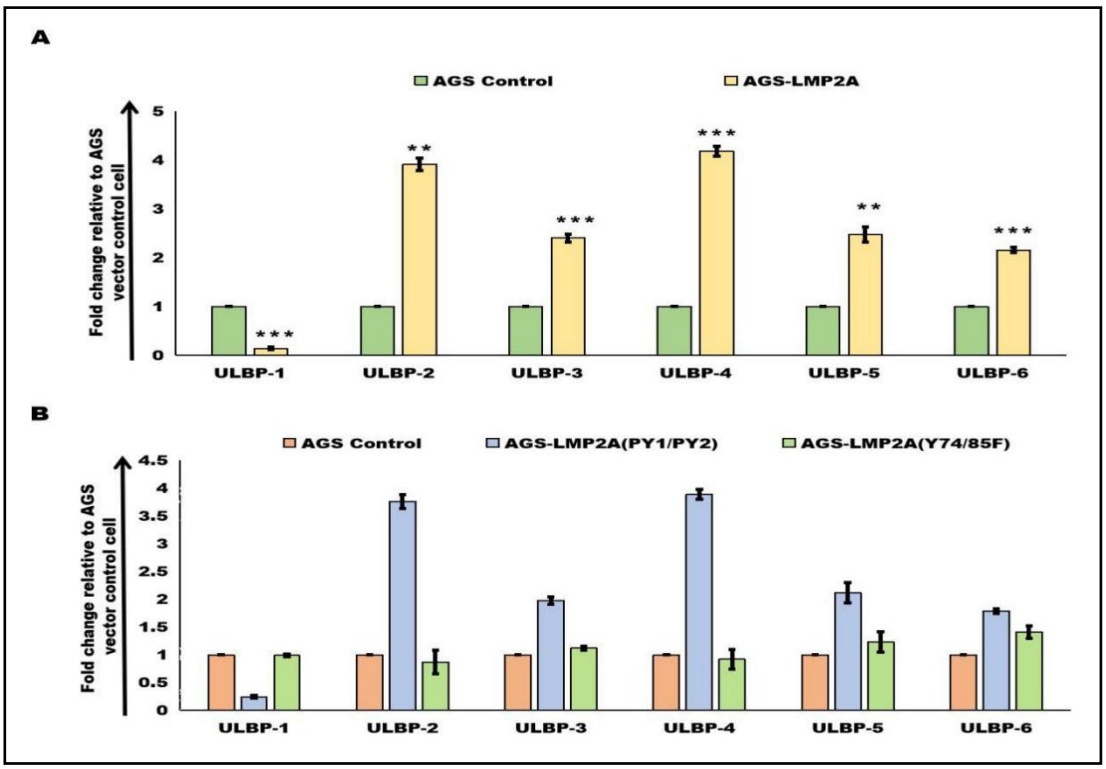

Figure 5.11: Expression of ULBPs in response to LMP2A. *(A) qRT-PCR to analyze ULBP (1-6) transcript level expression in AGS-LMP2A cells relative to AGS vector*

*control cell. (B) qRT-PCR to analyze ULBP (1-6) transcript level expression in AGS-LMP2A (PY1/PY2) and AGS-LMP2A(Y74/85F) cells relative to AGS control cell. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

Expression of HLA-EFG in response to LMP2A.

HLA class-I molecules consist of extremely polymorphic classical HLA class-I molecules denoted as HLA class-Ia, which includes, HLA-A, HLA-B, and HLA-C alleles along with the minimal polymorphic non-classical HLA class-I molecules denoted as HLA class-Ib, which includes, HLA-E, HLA-F and HLA-G. Studies suggest the role of HLA-E as the specific ligand for the NKG2 inhibitory receptors. Therefore, we investigated the mRNA levels of HLA-E, HLA-F, and HLA-G in AGS-LMP2A [Figure 5.12(A)], AGS-LMP2A(PY1/PY2) [Figure 5.12(B)] and AGS-LMP2A(Y74/85F) [Figure 5.12(B)]. Increased expression of NKG2D inhibitory ligand, HLA-E was detected in association with LMP2A and LMP2A(PY1/PY2) expression.

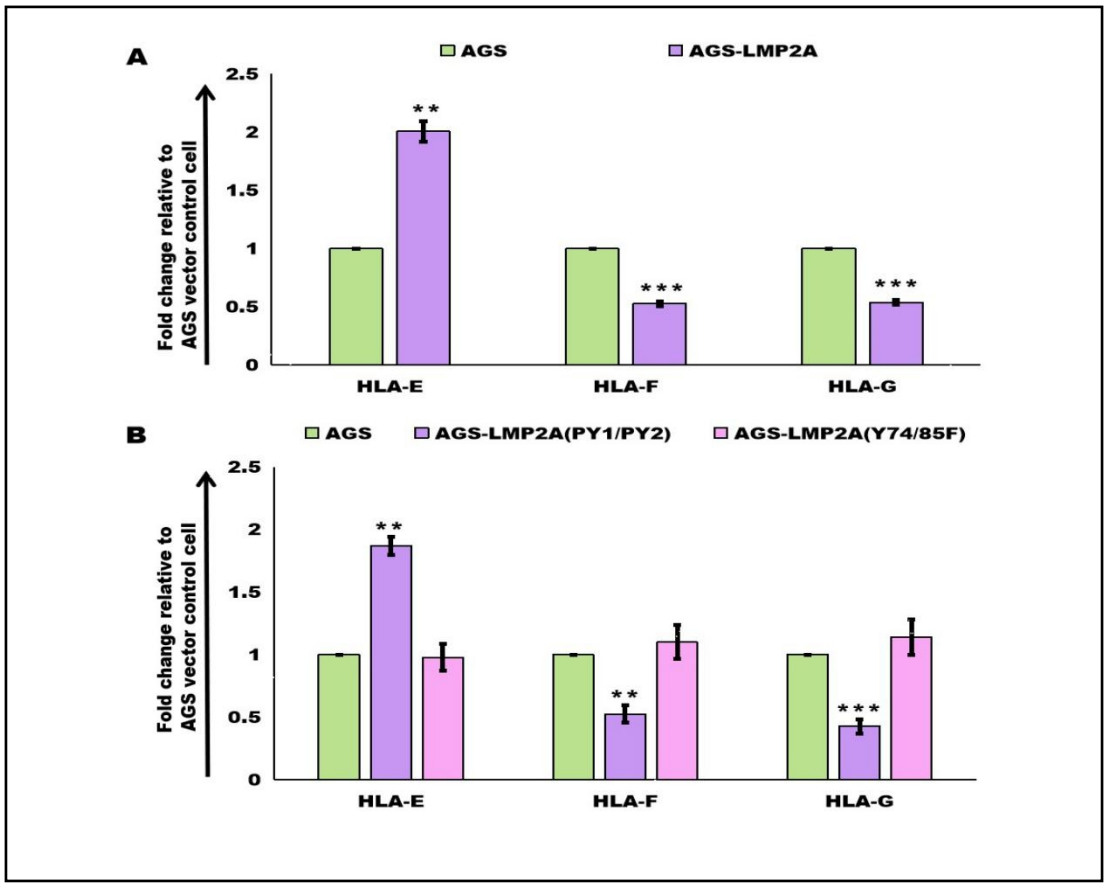

Figure 5.12: Expression of HLA-EFG in response to LMP2A. *(A) qRT-PCR to analyze HLA-E, HLA-F and HLA-G transcript level expression in AGS-LMP2A cells relative to AGS vector control cell. (B) qRT-PCR to analyze HLA-E, HLA-F and HLA-G transcript level expression in AGS-LMP2A (PY1/PY2) and AGS-LMP2A(Y74/85F) cells relative to AGS control cell. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A regulates MIC-A/B expression in AGS cells.

To determine the expression of MICs at protein level in response to LMP2A expression western blotting experiments were performed using AGS cells expressing the viral LMP2A. Despite increased mRNA expression of MIC-A and MIC-B NKG2D ligands, reduced expression pattern at translational level was detected in response to viral LMP2A in AGS cells [Figure 5.13(A & B)]. Decreased protein-level expression in response to mutated LMP2A (PY1/PY2) was further encountered in AGS-LMP2A(PY1/PY2) cells [Figure 5.13(C & D)]. Consistent with the protein level measurements, MIC-A/B surface expression was also recognized to be reduced in AGS-LMP2A and AGS-LMP2A(PY1/PY2) cells [Figure 5.13(E & F)].

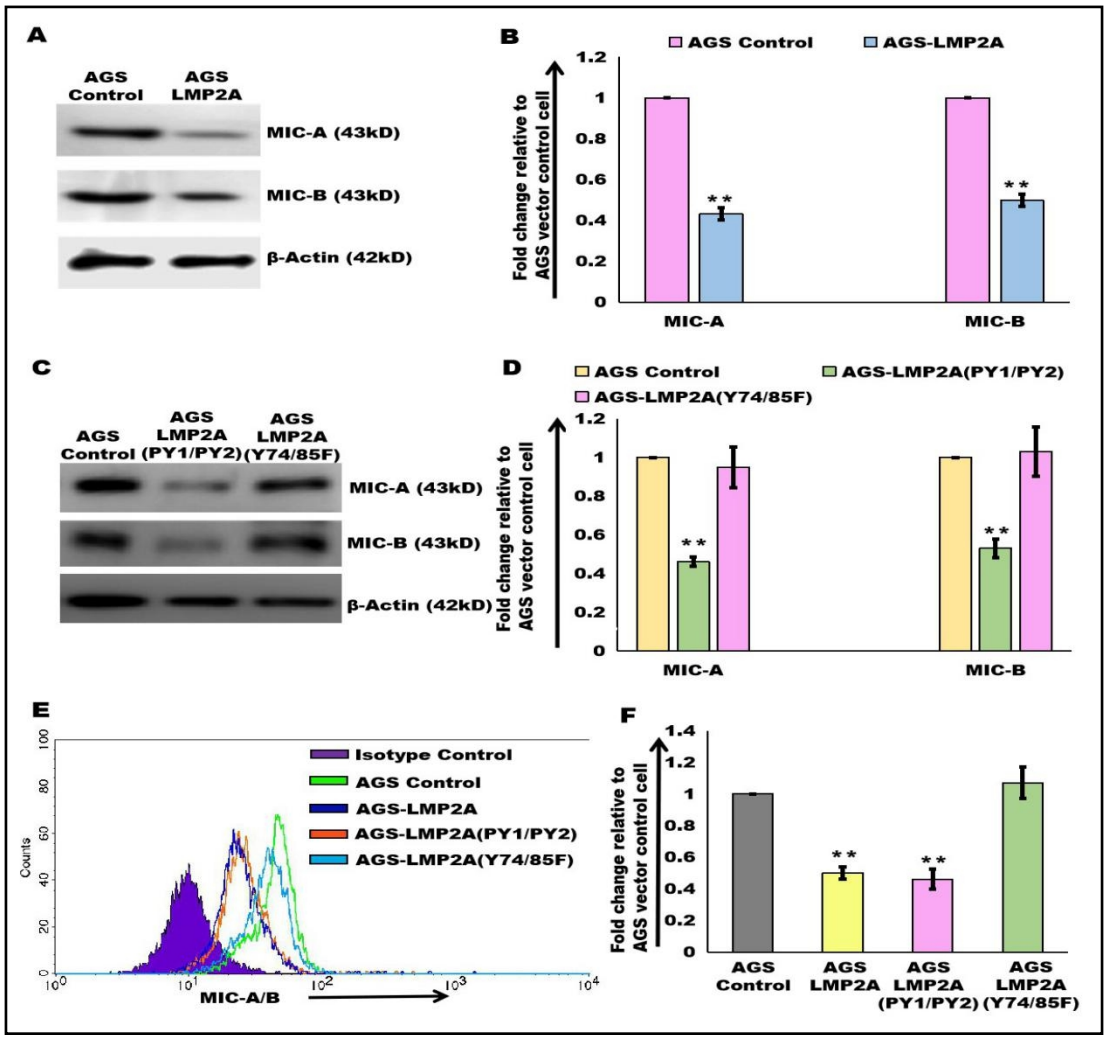

Figure 5.13: LMP2A regulates MIC-A/B expression in AGS cells. *(A & B) Protein level determination of MIC-A and MIC-B in AGS control and AGS-LMP2A cells. (C & D) Immunoblotting experiments to determine MIC-A and MIC-B levels in AGS-LMP2A (PY1/PY2) and AGS-LMP2A (Y74/85F) cells with respect to AGS control cell. Bar graph illustrates the densitometry quantification of MIC-A and MIC-B expression levels. (E & F) Surface expression of MIC-A/B was determined using FACS analysis in AGS-LMP2A, AGS-LMP2A (PY1/PY2) as well as AGS-LMP2A(Y74/85F) with respect to AGS control cell. Cell Quest Pro software was used for the quantitation of results by measuring the median values. Results are*

*represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001, **P ≤ 0.01 and *P < 0.05.*

LMP2A regulates MIC-A/B surface expression in epithelial cells.

MIC-A/B surface expression was further checked in other epithelial cells, HEK293 [Figure 5.14(A & B)] and HepG2 cells [Figure 5.14(C & D)] expressing the viral latent gene LMP2A. A down-regulated expression pattern similar to that of AGS cells was observed in HEK293 and HepG2 cells expressing the LMP2A and LMP2A(PY1/PY2) genes.

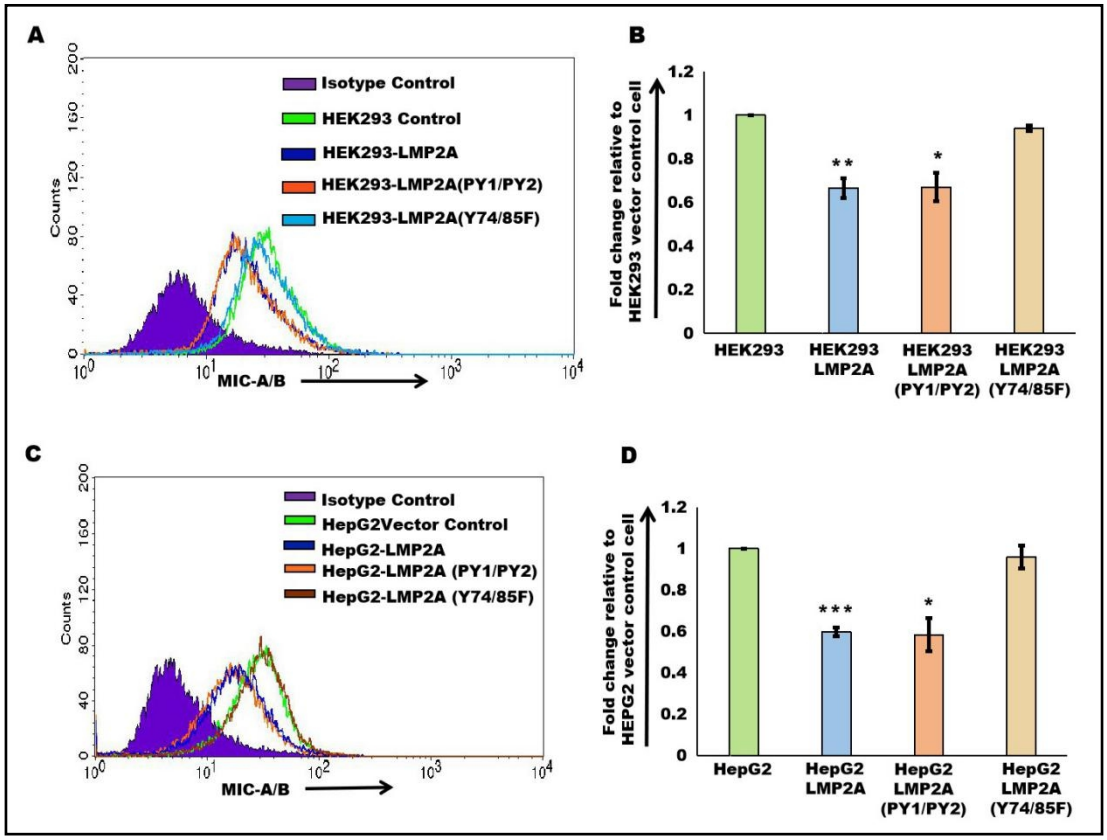

Figure 5.14: LMP2A regulates MIC-A/B surface expression in epithelial cells. *(A & B) Surface expression of MIC-A/B was determined using FACS analysis in HEK293-LMP2A, HEK293-LMP2A (PY1/PY2) as well as HEK293-LMP2A(Y74/85F)*

*with respect to HEK293 control cell. (C & D) Surface expression of MIC-A/B was determined using FACS analysis in HepG2-LMP2A, HepG2-LMP2A (PY1/PY2) and HepG2-LMP2A(Y74/85F) with respect to HepG2 control cell. Cell Quest Pro software was used for the quantitation of results by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

EBV-LMP2A regulates MIC-A/B expression in SNU cell lines.

MIC-A and MIC-B protein levels were further measured in SNU-5 cells, EBV-negative Korean cell line. Decreased protein-level expression of MIC-A and MIC-B NKG2D ligands was further encountered in SNU5-LMP2A and SNU5-LMP2A(PY1/PY2) [Figure 5.15(A)]. Consistent with the protein level measurements, MIC-A/B surface expression was also recognized to be down-regulated in SNU5-LMP2A and SNU5-LMP2A(PY1/PY2) [Figure 5.15(B & C)]. SNU-719, which is an EBV-positive Korean cell line when checked for surface expression showed decreased MIC-A/B surface expression [Figure 5.15(D)].

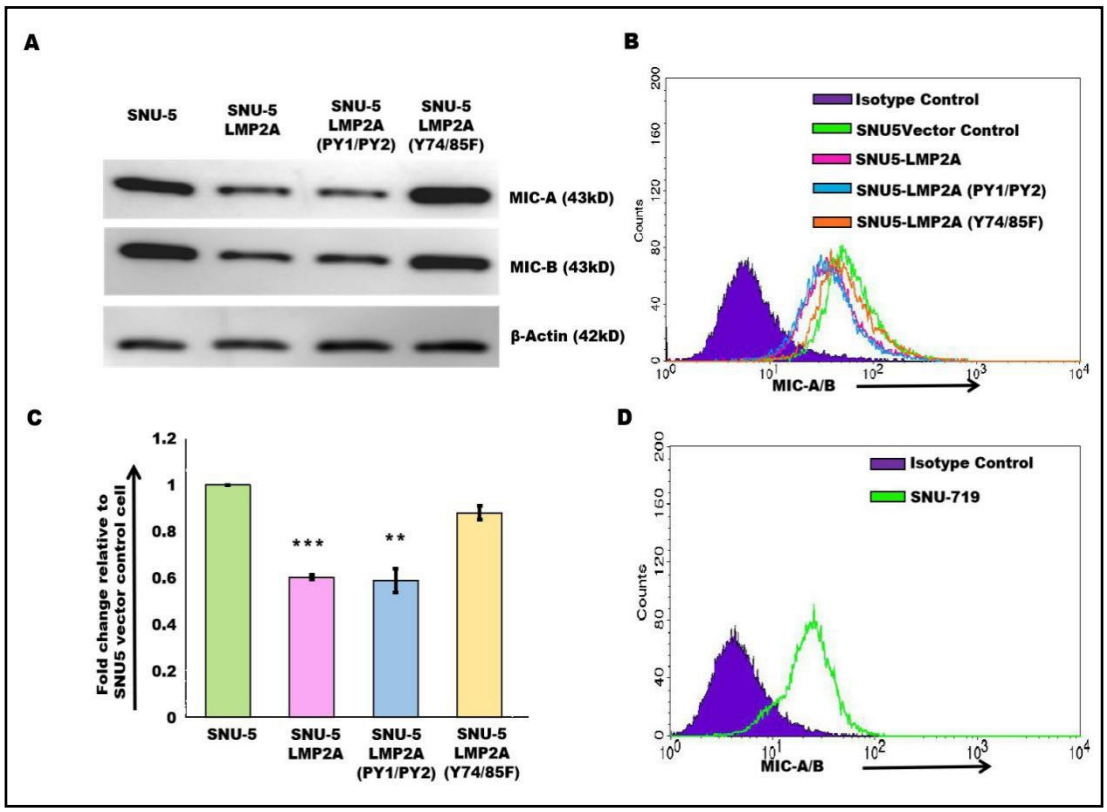

Figure 5.15: EBV-LMP2A regulates MIC-A/B expression in SNU cell lines. *(A) Protein level measurement of MIC-A and MIC-B in SNU5 control, SNU5-LMP2A cells, SNU5-LMP2A (PY1/PY2) and SNU5-LMP2A (Y74/85F) cells. (B & C) Surface expression of MIC-A/B was determined using FACS analysis in SNU5-LMP2A, SNU5-LMP2A (PY1/PY2) and SNU5-LMP2A(Y74/85F) with respect to SNU5 vector control cell. (D) Surface expression of MIC-A/B was further studied using FACS analysis in SNU-719 cells. Cell Quest Pro software was used for the quantitation of results by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A induces expression of UPR protein markers.

NKG2D ligands expression is reported to be controlled by a number of cellular stresses, thus supervising NK cell activation (149-155). UPR has recently been recognized to modulate NKG2D ligands expression in certain cases (156). Gastric cancer cell AGS, expressing viral LMP2A (AGS-LMP2A) was studied for expression of UPR proteins, such as, BIP, CHOP, IRE1-α and PDI. Immunoblotting analysis showed enhanced UPR protein expression in response to viral LMP2A expression [Figure 5.16(A, B)]. Furthermore, decreased protein levels of UPR proteins was detected in AGS-LMP2A(Y74/85F), whereas AGS-LMP2A(PY1/PY2) displayed expression pattern similar to that of AGS-LMP2A cells [Figure 5.16(C, D)]. Increased expression of UPR proteins was further verified in response to LMP2A and LMP2A(PY1/PY2) in HEK293 cells [Figure 5.16(E)].

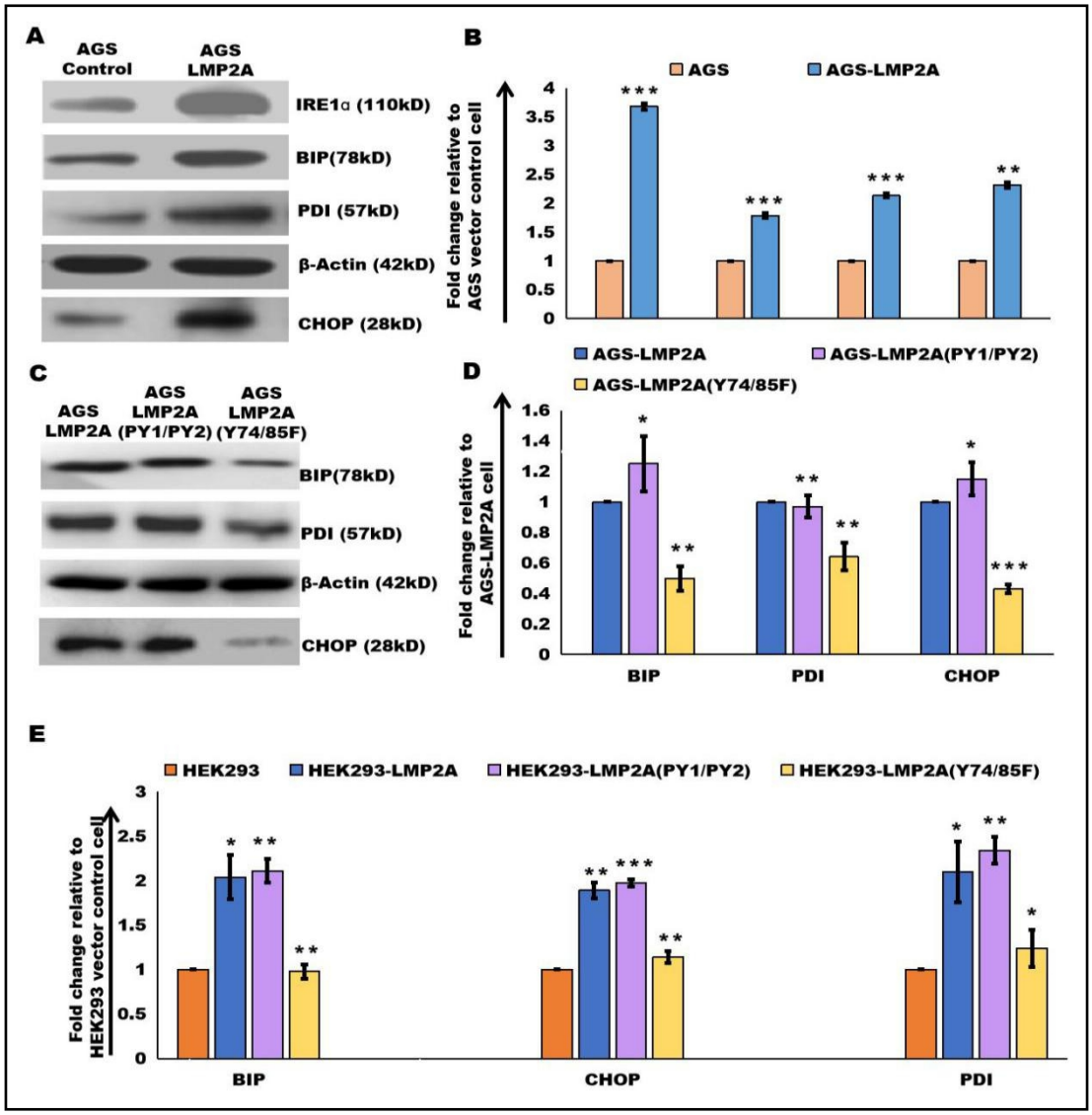

Figure 5.16: LMP2A induces expression of UPR protein markers. *(A, B) Protein level measurement of UPR protein markers; IRE1-a, BIP, PDI and CHOP in AGS control and AGS-LMP2A cells. Bar graph illustrates the densitometry quantification of IRE1-a, BIP, PDI, and CHOP levels. (C, D) Protein level measurement of UPR protein markers; BIP, PDI and CHOP in AGS-LMP2A (PY1/PY2) and AGS-LMP2A (Y74/85F) with respect to AGS-LMP2A cells. (E) Protein level measurement by means of immunoblotting experiments for BIP, CHOP and PDI levels in HEK293 transiently expressing LMP2A, LMP2A(PY1/PY2) and LMP2A(Y74/85F) with respect to HEK293*

*control cell. Bar graph illustrates the densitometry quantification of BIP, PDI and CHOP levels. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A knockdown resulted in decreased expression of UPR proteins.

To further confirm the role of viral latent protein, LMP2A in the regulation of UPR proteins expression the viral gene was targeted by means of siRNA in epithelial cells, AGS-LMP2A and SNU-719. Immunoblotting experiments showed decreased expression of UPR proteins upon siRNA mediated silencing of viral protein, LMP2A in AGS-LMP2A [Figure 5.17(A & B)] and SNU-719 [Figure 5.17(C & D)].

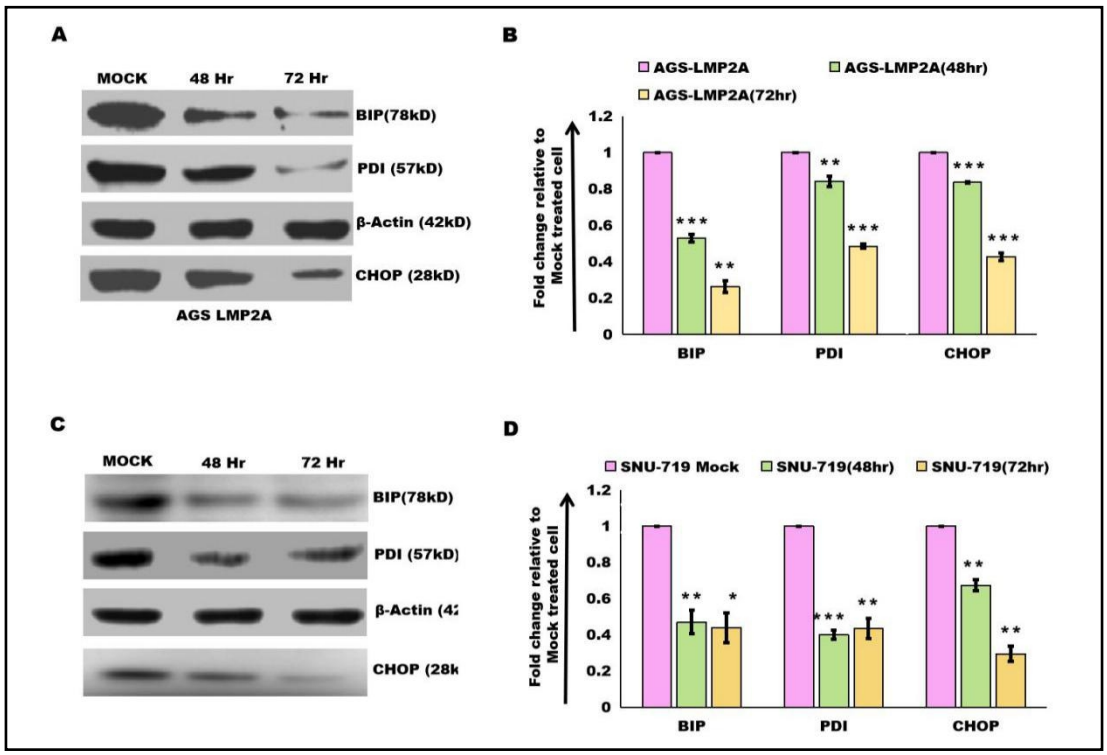

Figure 5.17: LMP2A knockdown resulted in decreased expression of UPR proteins. *(A & B) Protein level measurement by means of immunoblotting experiments to*

*determine expression of BIP, PDI and CHOP in response to siRNA mediated silencing of viral LMP2A in AGS-LMP2A cells at 48 hr and 72 hr with respect to Mock-treatment. (C & D) Protein level measurement by means of immunoblotting experiments to determine expression of BIP, PDI and CHOP in response to siRNA mediated silencing of viral LMP2A in SNU-719 cells at 48 hr and 72 hr with respect to Mock-treatment. Bar graph illustrates the densitometry quantification of BIP, PDI and CHOP levels. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

LMP2A silencing results in restoration of MIC-A/B surface expression.

We sought to further validate the role of viral LMP2A in regulation of surface level expression of MIC-A/B. LMP2A-expressing epithelial cell, AGS-LMP2A and SNU-719 were treated with siRNA against the viral gene LMP2A and studied for the expression of MIC-A/B on the surface of targeted cells. An increased surface level expression of MIC-A/B was detected in AGS-LMP2A [Figure 5.18(A & C)] and SNU-719 [Figure 5.18(B & C)] cells upon siRNA-mediated silencing of LMP2A expression.

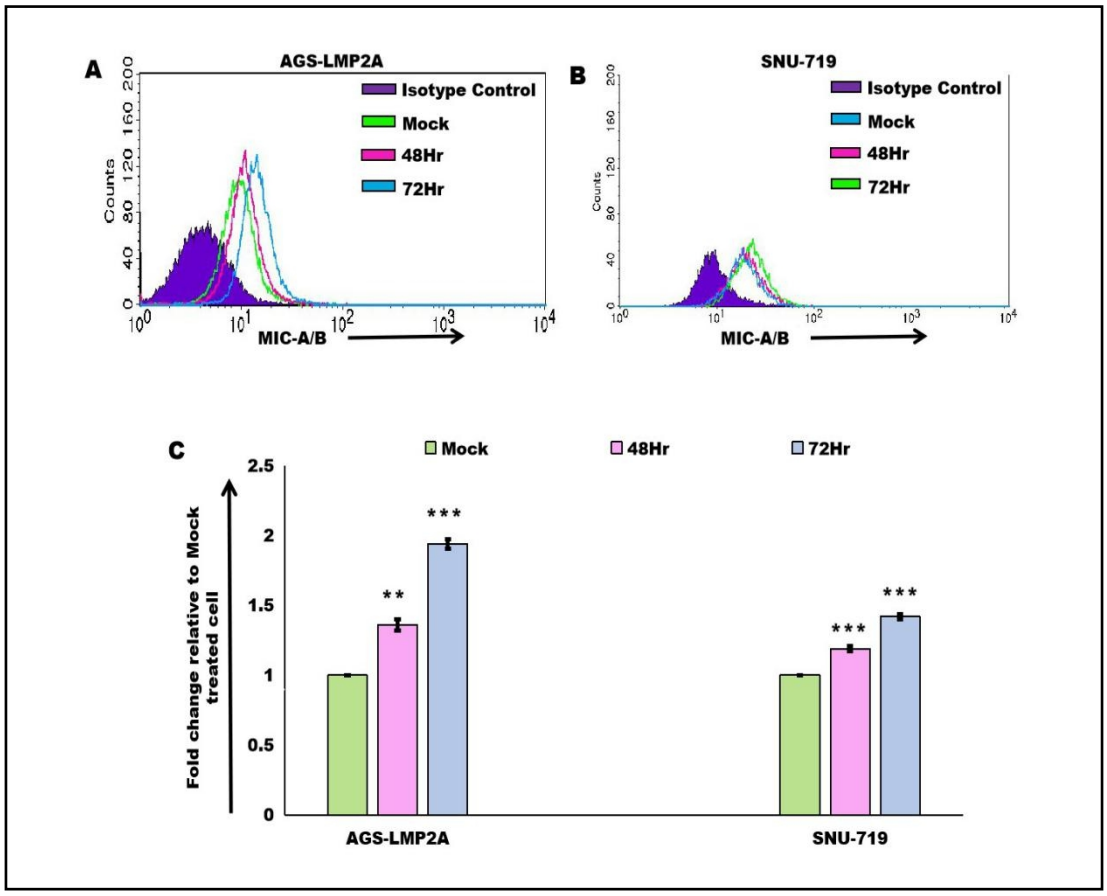

Figure 5.18: LMP2A silencing results in restoration of MIC-A/B surface expression. *(A) Surface expression of MIC-A/B was determined using FACS analysis in response to siRNA mediated targeting of viral LMP2A in AGS-LMP2A cells with respect to Mock treatment. (B) Surface expression of MIC-A/B was determined using FACS analysis in response to siRNA mediated targeting of viral LMP2A in SNU-719 cells with respect to Mock treatment. (C) Cell Quest Pro software was used for the quantitation of MIC-A/B surface expression in AGS-LMP2A and SNU-719 cells by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

PDI inhibits MIC-A/B expression in AGS-LMP2A cells.

Disulfide isomerases are reported to regulate tumour migration by modulating tumour invasive properties (157,158). PDI is a distinguished UPR protein known to catalyse proteolytic shedding of membrane-bound proteins (159-161). We therefore investigated the role of PDI in LMP2A-expressing epithelial cell carcinoma was further verified through siRNA mediated knockdown of PDI. MIC-A/B protein [Figure 5.19(A & B)] and surface-levels [Figure 5.19(C & D)] were restored in AGS-LMP2A cells upon siRNA mediated targeting of PDI. Knockdown analysis verified the role of PDI in the surface expression of MIC-A/B in response to viral LMP2A.

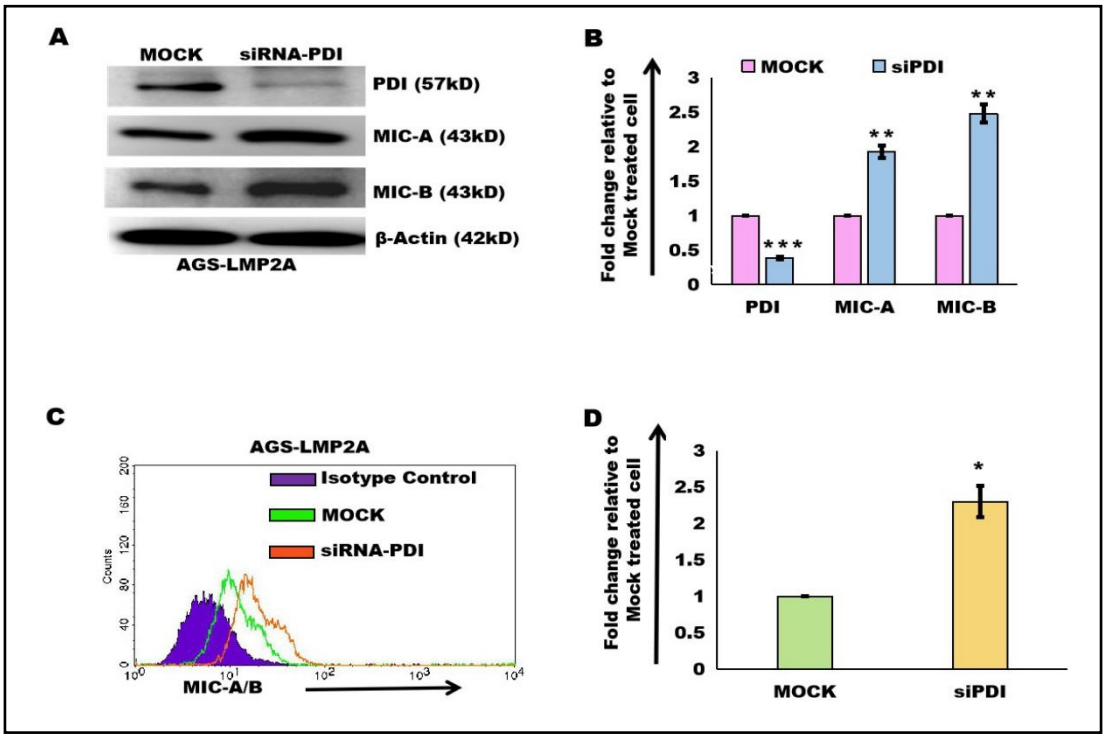

Figure 5.19: PDI inhibits MIC-A/B expression in AGS-LMP2A cells. *(A & B) Immunoblotting experiments to determine the expression of PDI, MIC-A and MIC-B in response to siRNA mediated knockdown of PDI in AGS-LMP2A cells with respect*

*to Mock treatment. (C & D) Surface expression of MIC-A/B was determined using FACS analysis in response to siRNA mediated silencing of PDI in AGS-LMP2A cells with respect to Mock treatment. Cell Quest Pro software was used for the quantitation by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

PDI inhibits MIC-A/B expression in SNU-719 cells.

The role of PDI in regulating the surface expression of MIC-A/B was further investigated in SNU-719 cells in response to siRNA mediated knockdown of PDI. MIC-A/B protein [Figure 5.20(A & B)] and surface-levels [Figure 5.20(C & D)] were restored in SNU-719 cells [Figure 5.20(A & B)] upon siRNA mediated targeting of PDI. Knockdown analysis further pinpointed the role of PDI in down-regulation of MIC-A/B surface expression by means of proteolytic shedding.

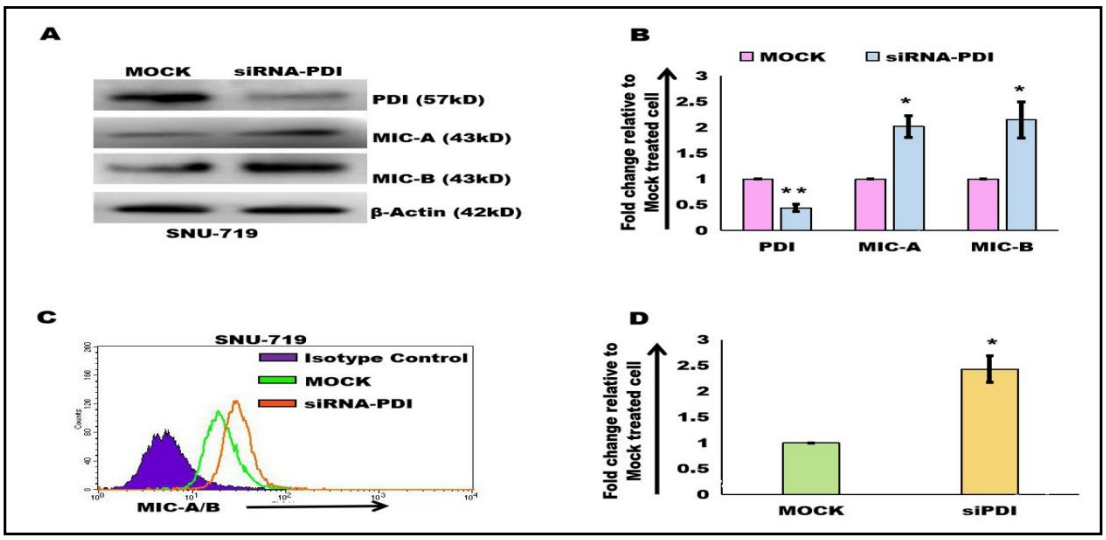

Figure 5.20: PDI inhibits MIC-A/B expression in SNU-719 cells. *(A & B) Immunoblotting experiments to determine the expression of PDI, MIC-A and MIC-B*

*in response to siRNA mediated knockdown of PDI in SNU-719 cells with respect to Mock treatment. (C & D) Surface expression of MIC-A/B was determined using FACS analysis in response to siRNA mediated silencing of PDI in SNU-719 cells with respect to Mock treatment. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

UPR mediated down-regulation of MIC-A/B surface expression in AGS cells.

We further analysed the role of UPR protein in the regulation of MIC-A/B surface expression by thapsigargin (induces UPR by increasing cytosolic calcium ion concentration) (162) treatment (162) . AGS cells upon treatment with thapsigargin in a dose (1uM and 2uM) dependent manner for a period of 6 hr, displayed increased PDI [Figure 5.21(A & B)] expression along with decreased MIC-A/B [Figure 5.21(C & D)] surface expression. Our results thus indicated regulation of MIC-A/B surface expression through UPR protein, PDI in AGS cells.

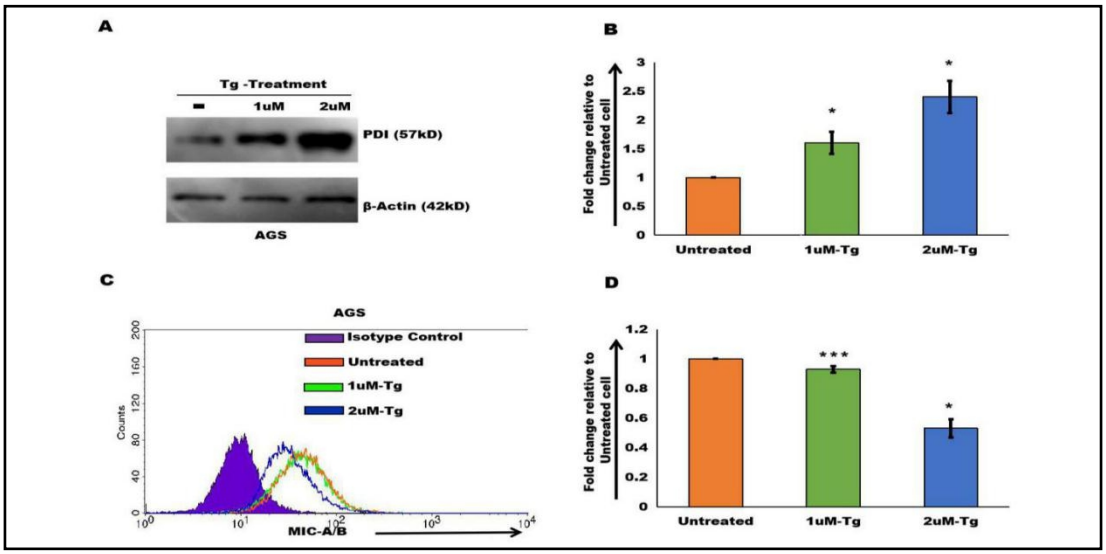

Figure 5.21: UPR mediated down-regulation of MIC-A/B surface expression in AGS cells. *(A & B) Protein level measurement of UPR protein, PDI in response to thapsigargin (Tg) treatment in a dose (1uM and 2uM) dependent manner in AGS cells compared to the untreated cells. (C & D) Surface expression of MIC-A/B was determined using FACS analysis in response to thapsigargin (Tg) treatment in a dose (1uM and 2uM) dependent manner in AGS cells compared to untreated cells. Cell Quest Pro software was used for the quantitation by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001, **P ≤ 0.01 and *P < 0.05.*

UPR mediated down-regulation of MIC-A/B surface expression in SNU5 cells.

The role of UPR protein was further verified in MIC-A/B surface expression through thapsigargin treatment. SNU5 cells upon treatment with thapsigargin in a dose (1uM and 2uM) dependent manner for a period of 6 hr, showed increased PDI [Figure 5.22(A & B)] expression along with decreased MIC-A/B [Figure 5.22(C & D)] surface-level expression. Our results thus indicated down-modulation of MIC-A/B surface expression through UPR protein, PDI in SNU5 cells.

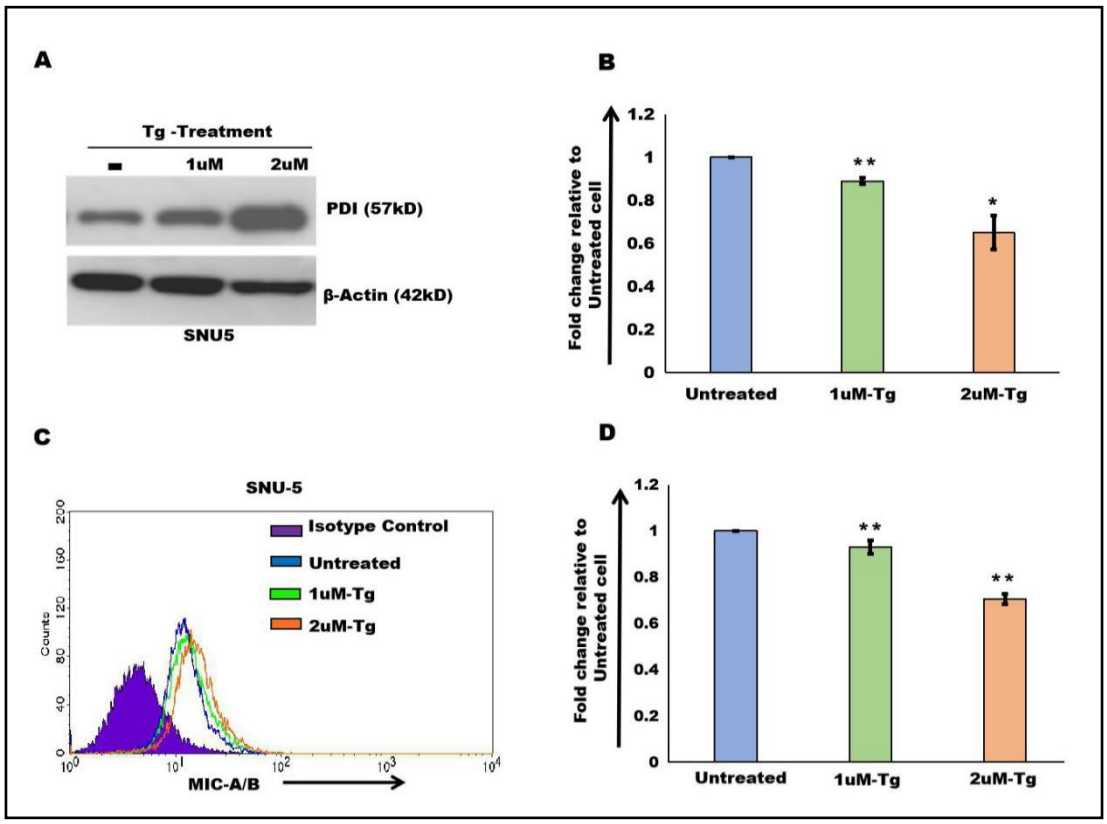

Figure 5.22: UPR mediated down-regulation of MIC-A/B surface expression in SNU5 cells. *(A & B) Protein level measurement of UPR protein, PDI in response to thapsigargin (Tg) treatment in a dose (1uM and 2uM) dependent manner in SNU5 cells compared to the untreated cells. (C & D) Surface expression of MIC-A/B was determined using FACS analysis in response to thapsigargin (Tg) treatment in a dose (1uM and 2uM) dependent manner in SNU5 cells compared to untreated cells. Cell Quest Pro software was used for the quantitation by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

Role of signaling pathways in regulation of MIC-A/B expression in AGS-LMP2A cells.

LMP2A ITAM mutant (Y74/85F) failed to manifest similar phenotype as viral LMP2A. Previous studies show activation of signaling pathways, PI3k-Akt pathway (163,164) as well as Sonic Hedgehog (Shh) pathway (164,49) by the viral LMP2A. In order to study the role played by the above-mentioned signaling pathways in the regulation of MIC-A/B surface expression, cells were subjected to treatment with Forskolin (PI3k/Akt inhibitor) and LY294002 (Shh inhibitor) in a dose-dependent manner. MIC-A/B surface expression was significantly enhanced in response to Forskolin treatment in AGS-LMP2A cell [Figure 5.23(A & B)], indicating the role of Shh pathway in regulation of MIC-A/B surface expression in response to viral LMP2A.

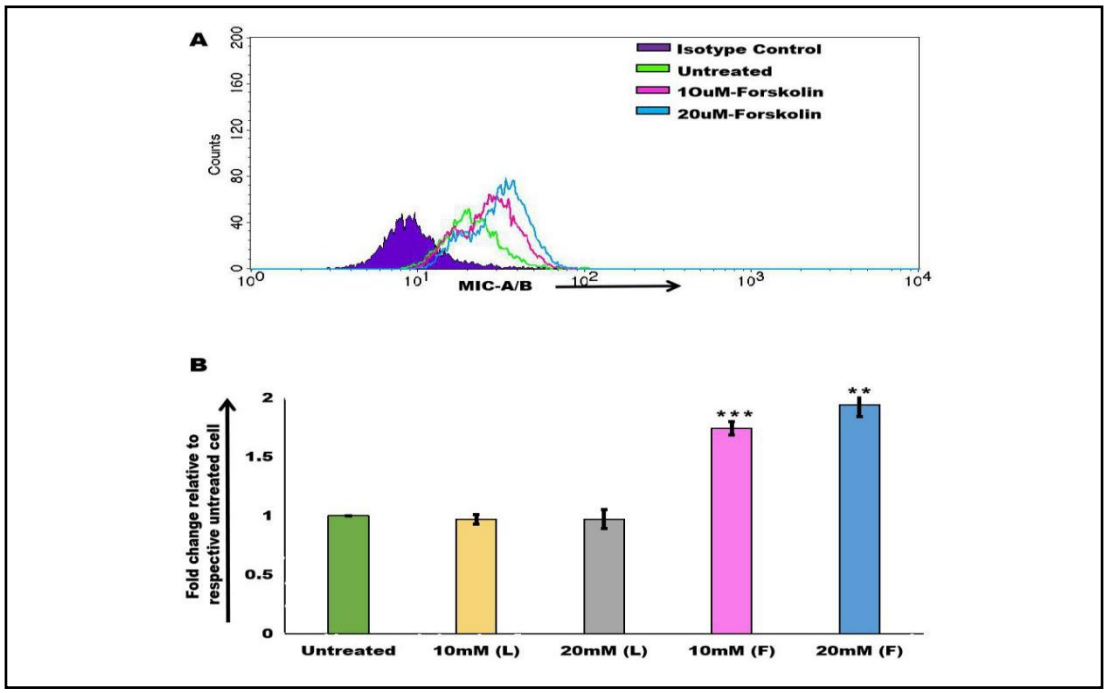

Figure 5.23: Role of signaling pathways in regulation of MIC-A/B expression in AGS-LMP2A cells. *(A & B) MIC-A/B surface expression determination by using*

*FACS analysis in response to Forskolin 'F' and LY294002 'L' treatment in a dose dependent manner in AGS-LMP2A cells with respect to untreated cells. Cell Quest Pro software was used for the quantitation by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001, **P ≤ 0.01 and *P < 0.05.*

Role of signaling pathways in regulation of MIC-A/B expression in SNU-719 cells.

The role of PI3k-Akt and Shh signaling pathways in the regulation of MIC-A/B surface expression was determined in SNU-719 cells through a dose-dependent treatment with Forskolin (PI3k/Akt inhibitor) and LY294002 (Shh inhibitor). MIC-A/B surface expression was observed to be up-regulated in response to Forskolin treatment in SNU-719 cell [Figure 5.24(A & B)], indicating the role of Shh pathway in MIC-A/B surface expression in response to viral latent gene, LMP2A.

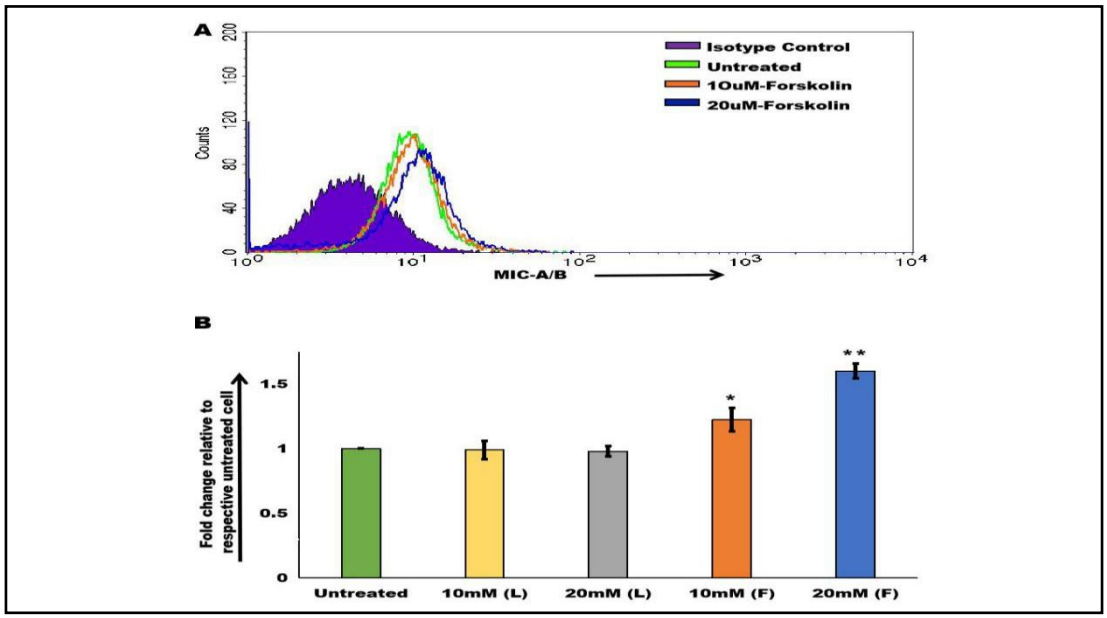

Figure 5.24: Role of signaling pathways in regulation of MIC-A/B expression in SNU-719 cells. *(A & B) MIC-A/B surface expression determination by using FACS*

*analysis in response to Forskolin 'F' and LY294002 'L' treatment in a dose dependent manner in SNU-719 cells with respect to untreated cells. Cell Quest Pro software was used for the quantitation by measuring the median values. Results are represented as mean ±s.e.m. of triplicate experiments. Data depicts an average of n=3 individual experiments. ***P ≤ 0.001,**P ≤ 0.01 and *P < 0.05.*

Chapter 6

Discussion

EBV was the first human neoplastic virus to be identified more than 50 years ago and remains the most prevalent asymptomatic virus in humans. The persistent life-long relationship between EBV and its host immune system is an outcome of the ability of the virus to prevail in healthy individuals (165). The significance of EBV infection on the development of epithelial carcinomas may be an effect of the aberrant establishment of latency programme in epithelial cells that have previously sustained premalignant changes- a pathogenic process that is different from EBV-induced lymphomagenesis in which the virus emerges to be the initiator (166). EBV infection grants immune evasion and survival benefits to infected malignant cells (167,168). EBV has been reported to develop strategies to avoid recognition by CTLs and imperiling host's ingenuity to overcome EBV infection in EBV-infected epithelial cells.

LMP2A is frequently expressed in EBV-associated malignancies (169-174) and thus may have significant role in EBV-infected tumorigenesis. Previous investigations have shown the capability of LMP2A to transform cells, alter epithelial cell motility as well as inhibit epithelial cell differentiation (175,176). The current study focuses on the role played by viral LMP2A in regulation of host immune response by means of epigenetic regulators and UPR proteins in response to LMP2A expression in epithelial cell carcinomas. At first we analyzed the role of epigenetic modification in the decreased expression of HLA-ABC in response to viral LMP2A. Furthermore, since modifications in epigenetic marks have been linked with regulated expression of HLA class I gene (177-179). As a result we continued to explore the role of viral LMP2A in regulating the expression of HLA-ABC gene by means of epigenetic regulators. The promising facet of epigenetic regulators in tumour progression has gained importance

in several carcinomas (180-183). Our investigation provide information for LMP2A mediated increased methylation of HLA-A, HLA-B and HLA-C gene promoter regions and increased expression of epigenetic regulators; DNMT1, DNMT3B, and UHRF-1. Thus, it is noteworthy that methylation mediated modification of HLA-A, HLA-B and HLA-C promoter region is used as a strategy by the virus to target the surface availability of HLA-ABC in response to viral LMP2A expression (Figure 6.1). Methylation mediated decreased HLA-ABC expression was further justified by 5'-aza-2'-deoxycytidine (demethylating agent) treatment. An increased HLA-ABC surface expression was detected in response to demethylation treatment in LMP2A-expressing gastric cancer cell, AGS and SNU-719. As already discussed before LMP2A, consists of a long cytoplasmic tail at the N-terminus, 12 trans-membrane domains, along with a short cytoplasmic tail at the C-terminus. The N-terminal cytoplasmic tail of LMP2A consists of a stretch of eight constitutively phosphorylated tyrosine residues along with several proline-rich regions that play important role in interactions with other cellular proteins. The intracellular N-terminal tail of LMP2A contains an immunoreceptor tyrosine-based activation motif (ITAM) similar to that present in BCR (184). LMP2A ITAM is reported to associate with Src and Syk family protein tyrosine kinases, which is known to form part of the BCR signaling complex (185). Furthermore, LMP2A contains two polyproline motifs that have been shown to be involved in the recruitment of Nedd4 ubiquitin ligases (186). Very little information is available in relation to these viral trans-membrane ITAM-containing proteins and host immune evasion, as well as the role played by LMP2A ITAM in immune evasion during infection of epithelial cell carcinomas. The purpose of the current study is to investigate the importance of LMP2A ITAM in

LMP2A-mediated regulation of immune signaling. This study is the first report which provides direct link between ITAM (Tyr74 and Tyr85) and viral LMP2A mediated regulation of immune evasion in epithelial malignancies.

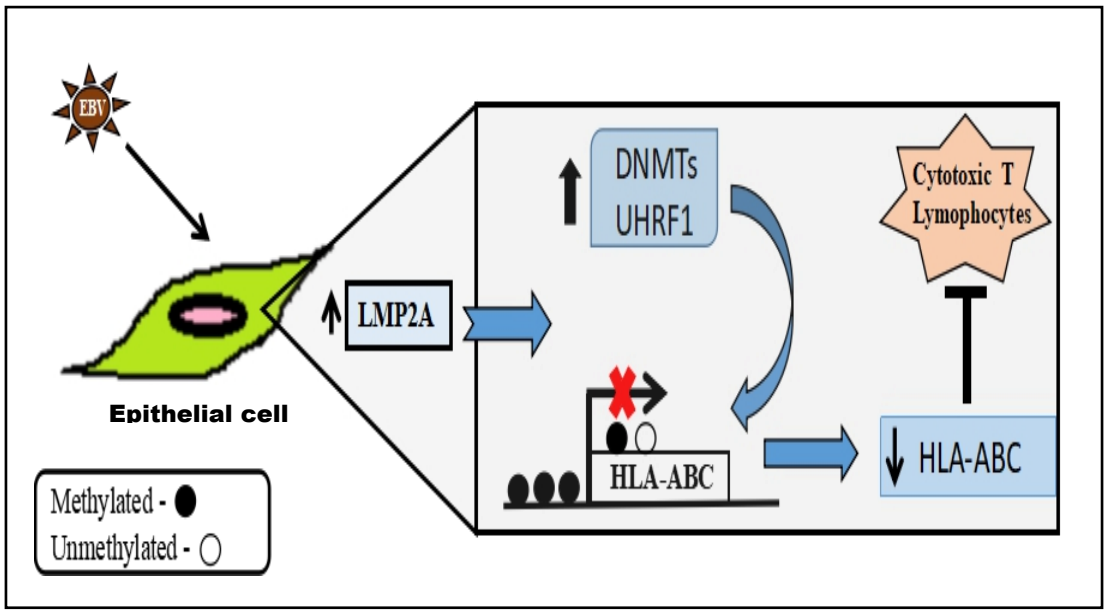

Figure 6.1: EBV-LMP2A inhibits activation of CTLs. *LMP2A, the viral latent gene is consistently detected in several epithelial carcinomas. LMP2A dramatically affects HLA-ABC expression by means of promoter hypermethylation which is mediated by increased expression of epigenetic regualtors. As a result, the infected cells escape recognition and attack by CTLs.*

Apart from CTLs, NK cells play pivotal role in providing protection against viral infection (187). NKG2D ligands expression on the surface of infected cells may prompt an immune response by binding to NKG2D activating receptor, localized on the surface of NK cells as well as a subset of T cells. Therefore, selective outgrowth of tumours that fails to express these NKG2D ligands on the surface of infected cells may act as a mechanism of tumor immune escape. On the contrary, NKG2D ligands

overexpression could result in overstimulation leading to insensibility of host immune cells, further resulting in immune evasion by tumours overexpressing NKG2D ligands. In addition to this hypothesis, it has been studied that the availability of membrane-bound NKG2D ligands on the surface of transformed cells may be monitored by production of cleaved soluble NKG2D ligand molecules. This proteolytic-shedding of membrane-bound NKG2D ligands could consistently regulate the expression of NKG2D receptor and thereby lead to impaired NK and T cells mediated anti-tumour reactivity. To conclude, the strategies through which NKG2D ligands mediate immune function or dysfunction may vary depending upon tumour type and circumstances(188-194). The interaction of the activating receptor NKG2D with MIC-A and MIC-B on the surface of tumours has been extensively reported in several epithelial tumors. Previous studies report downregulated expression of NKG2D ligand, MIC-B expression through BARTs in EBV-infected nasal natural killer / T - cell lymphoma. LMP2A-deficient EBV-infected lymphoblastoid cell lines (LCLs) exhibit increased susceptibility to CTLs (195,196). Our current findings were in good agreement with viral LMP2A mediated loss of availability of membrane-bound MIC-A/B. HLA-E expression also plays significant role in desensitization of NK cells (197-200). Surprisingly in this study, we also found increased transcript-levels of HLA-E in association with LMP2A expression. NKG2D ligands expression is transcriptionally regulated by a number of cellular stresses in response to viral infection. The stress signals that alerts immune cells includes oxidative stress (149), DNA damage response (151,152), and UPR (155,57) during tumour progression. UPR proteins acts as an important regulator for viral persistence and function. Meanwhile expression of UPR proteins was found to be regulated in

response to viral LMP2A in epithelial cell cancers. It might be suggested that these alteration in expression of UPR proteins might regulate the expression of MIC-A/B. PDI is reported to be localized on the surface of plasma membrane as well as inside the cytoplasm, where it facilitates proteolytic cleavage of membrane-bound proteins (139). Therefore, we focused to examine the role of PDI in regulation of MIC-A/B expression in response to viral LMP2A. An increased MIC-A/B surface expression was detected in response to siRNA mediated targeting of PDI in LMP2A-expressing gastric cancer cell, AGS and SNU-719. Thus, the current findings validates the significant role played by PDI in the surface expression of MIC-A/B. PDI could therefore used as a drug target to enhance recognition of infected cells by NK cells. This study indeed verified the importance of ITAM tyrosines (Tyr74 and Tyr85) in the surface-expression of MIC-A/B. Replacement of viral LMP2A with LMP2A ITAM mutant (Y74/85F) results in successful release of the effect of viral LMP2A on the MIC-A/B surface availability in epithelial cell carcinomas. Notably, ITAM region tyrosine residues (Tyr74 and Tyr85) are reported to activate various signaling cascades, which includes PI3k-Akt and Shh pathways. Therefore, in this study we targeted the two pathways using LY294002 and Forskolin, respectively and demonstrated their role in MIC-A/B surface expression. Restoration of MIC-A/B surface expression was detected in response to treatment with Shh pathway inhibitor (Forskolin), indicating the role of Shh pathway in regulation of MIC-A/B surface expression. The current work highlights, regulation of CTL response by promoter hypermethylation mediated decreased expression of HLA-A, HLA-B and HLA-C gene along with modulation of NK cell response through decreased MIC-A/B surface expression in association with viral latent gene, LMP2A (Figure 6.2).

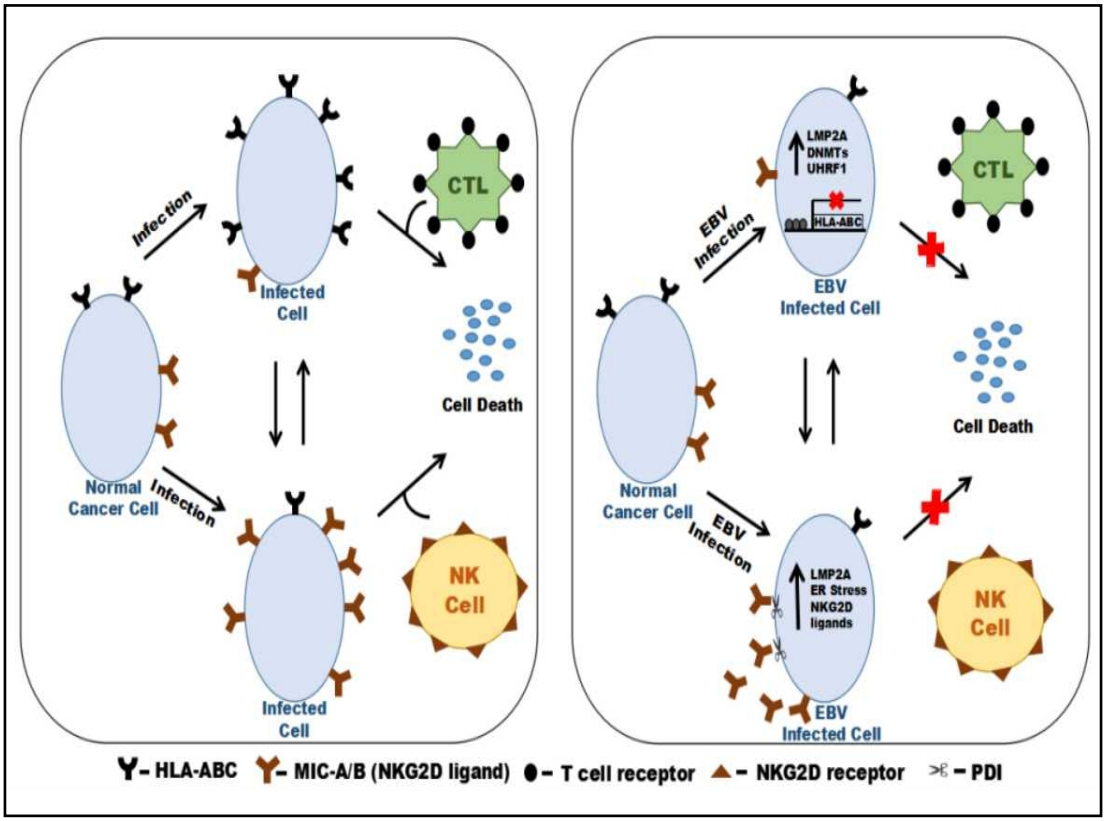

Figure 6.2: Schematic representation of the current findings. *EBV-LMP2A plays crucial role in evasion of immune attack by CTLs and NK cells.*

In conclusion, reduced expression of HLA-ABC on the cell surface is associated with the establishment of life-long EBV infection in epithelial cell carcinomas. Impaired HLA-ABC expression is common during viral infection. MIC-A and MIC-B belongs to the MHC class I family and can specifically bind to surface NKG2D receptor on NK cells and activates their immune functions. Therefore, reduced MIC-A and MIC-B expression protects the EBV-infected epithelial cancer cells from immune attack. EBV-LMP2A acts as a potent regulator of host immune response by removing recognition signatures from the surface of infected cells. Immune evasion acts as a marker for poor prognosis in cancer patients, particularly in epithelial malignancies.

Taking all together, regulation of immune response through different mechanisms, such as, promoter hypermethylation mediated gene inactivation and proteolytic shedding of membrane proteins, provides better understanding about the survival strategies utilised by EBV to establish persistent life-long infection.

SYNOPSIS

Epstein Barr Virus (EBV), ubiquitous γ-herpesvirus, is known to be establish persistent life-long infection in humans. EBV infection is categorized as Group I carcinogen by the International Agency for Research on Cancer (IARC monograph,1997), indicating its carcinogenicity in lymphoid and epithelial cell malignancies. EBV exhibits latency programme in most of the EBV-infected epithelial cell cancer. EBV expresses viral oncoprotein, Latent Membrane Protein 2A (LMP2A) along with several other genes during latency. We previously showed that LMP2A downregulates HLA-ABC expression in EBV-infected gastric carcinomas. However, the detailed mechanism that results in HLA-ABC downregulation remain unclear.

Transcriptional regulation through promoter methylation has recently gained importance in tumour progression. In this thesis, we therefore analysed methylation-mediated regulation of HLA-ABC expression by the viral protein, LMP2A. Our results indicated LMP2A mediated hypermethylation of the HLA-ABC promoter region through increased expression of epigenetic regulators; DNMT-1, DNMT-3B, and UHRF-1. Methylation-mediated regulation of HLA-ABC expression was further justified by 5'-azacytidine (demethylating agent) treatment, which resulted in restoration of HLA-ABC surface expression. Hence the current study signifies the role of methylation in downregulated expression of HLA-ABC in response to the viral gene, LMP2A. Decreased HLA-ABC surface expression resulted in ablation of cytotoxic T Lymphocytes (CTLs) response as the infected cells could no longer display the viral antigenic peptides to the respective CTLs. Fascinatingly, according to the ‘missing self’ hypothesis, when there is loss of HLA-ABC surface expression,

there should be an increased expression of NKG2D ligands' on the surface of infected cells to facilitate killing by Natural Killer (NK) cells. We therefore analyzed NKG2D ligands' (MIC-A/B) expression and reported downregulation of MIC-A/B surface expression in response to viral gene, LMP2A. Several cellular stresses are known to transcriptionally regulate the expression of NKG2D ligands' in response to viral infection. The stress signals that alerts the host immune cells of the infection includes DNA damage response, oxidative stress, and Unfolded Protein Response (UPR). Furthermore, we investigated the role of UPR proteins in the regulation of MIC-A/B surface expression in epithelial cells stably expressing viral protein, LMP2A. Membrane-bound Protein Disulfide Isomerase (PDI), UPR protein reported to promote reduction of disulfide bonds in cell surface protein through dithiol-disulfide oxidoreductase activity leading to loss of membrane-bound proteins. Knockdown study of PDI through siRNA resulted in increased MIC-A/B surface expression in LMP2A expressing epithelial cells, thus further pinpointing on the role of PDI in regulation of MIC-A/B surface expression. UPR-mediated regulation of MIC-A/B surface expression was further validated through thapsigargin (ER stress inducer drug) treatment, which resulted in increased surface expression of NKG2D ligands' (MIC-A/B). Therefore, the current research signifies the role of UPR protein, PDI in the downregulated expression of membrane-bound MIC-A/B in response to the viral gene, LMP2A. The current research highlights, decreased HLA-ABC expression due to hyper-methylation of the promoter region leading to regulation of CTL response, along with down modulation of MIC-A/B surface expression by means of stress protein, PDI resulting in regulation of NK cell mediated cytotoxicity in LMP2A expressing epithelial cell carcinomas.

List of Abbreviations

AKT	Protein Kinase B (PKB)
APC	Allophycocyanin
BARTs	Bam HI A rightward transcripts
BIP	Binding Immunoglobulin Protein
BL	Burkitt's lymphoma
CD	Cluster of Differentiation
CHOP	CCAAT/enhancer-binding protein (C/EBP) homologous protein
CIITA	Class II major histocompatibility complex Transactivator
CTL	Cytotoxic T Lymphocyte
DNMTs	DNA methyltransferases
EBV	Epstein Barr Virus
EBER	EBV encoded small RNAs
EBNA	Epstein Barr Nuclear Antigen
ER	Endoplasmic Reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gastric carcinoma
Gp	Glycoprotein
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HPRT	Hypoxanthine phosphoribosyl transferase
HRS	Hodgkin and Reed-Sternberg cells
IM	Infectious Mononucleosis
IRE1α	Inositol-requiring transmembrane kinase/endoribonuclease 1 alpha
ITAM	Immunoreceptor tyrosine-based activation motif

LMP	Latent membrane protein
MHC	Major Histocompatibility Complex
MIC	MHC class I chain-related proteins
MSP	Methylation specific PCR
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin's lymphomas
NK cell	Natural Killer cell
NKG2D	Natural killer group2, member D
NPC	Nasopharyngeal Carcinoma
PDI	Protein disulfide isomerase
PE	Phycoerythrin
PI3K	Phosphoinositide-3-Kinase
RFX5	Regulatory factor X 5
SAM	S-adenosyl-L-methionine
Shh	Sonic Hedgehog
TCR	T cell receptor
TF	Transcription Factor
Tg	Thapsigargin
UHRF1	Ubiquitin-like PHD and RING finger domain 1
ULBP	UL-16 binding protein (Retinoic acid early transcript-1 proteins)
UPR	Unfolded Protein Response

Thesis Highlight

Name of the Student: ***Shweta Singh***

Name of the CI/OCC: ***Saha Institute of Nuclear Physics*** **Enrolment No.:** ***LIFE05201504007***

Thesis Title: ***Role of Epstein-Barr Virus in Epithelial Cell Cancer***

Discipline: ***Life Sciences*** **Sub-Area of Discipline:** ***Cancer Immunology***

Epstein Barr Virus (EBV) is an ubiquitous γ -herpesvirus, known to be establish persistent life-long infection in humans. EBV infection has been classified as a Group I carcinogen (IARC monograph,1997), indicating its carcinogenicity in blood cell and epithelial cell cancer. In most of the EBV-associated epithelial malignancies, the virus has developed number of strategies to evade host immune response in order to establish latent infection. During latency, EBV expresses viral latent gene, Latent Membrane Protein 2A (LMP2A) along with few other genes. LMP2A plays crucial role in maintenance of latency during infection. As a result, it is reported to alter a number of cellular signaling pathways. In this thesis, the role of viral protein, LMP2A in regulation of host immune response is investigated. Previous studies report LMP2A causes downregulation of HLA-ABC surface expression in EBV associated gastric carcinoma. Cytotoxic T Lymphocytes (CTLs) identify viral antigenic peptides displayed by Human Leukocyte Antigen (HLA) class I molecules (HLA-ABC) on the surface of infected cell. However, the detailed mechanism that leads to this downregulation remain unclear.

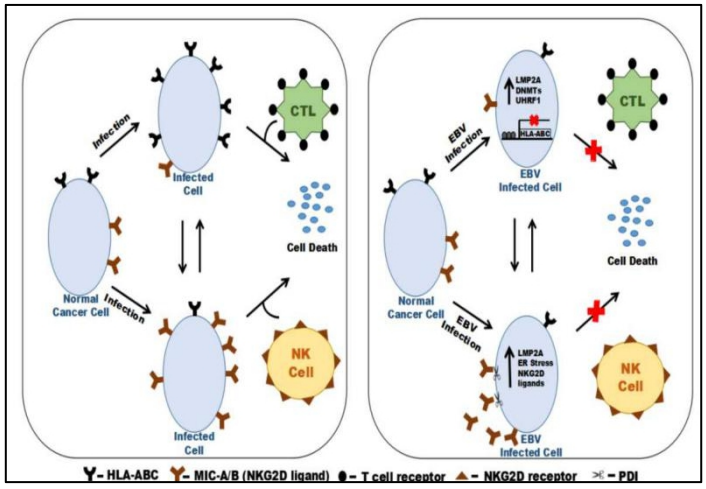

Figure. Schematic description of the current findings

Here, in this study it is shown that LMP2A exhibits hypermethylation of the HLA-ABC promoter region through increased expression of epigenetic regulators; DNMT-1, DNMT-3B, and UHRF-1 in epithelial cell cancer. Decreased HLA-ABC surface expression resulted in ablation of CTL response as the infected cells could no longer display the viral antigenic peptides to the cognate CTLs. Interestingly, according to the 'missing self' hypothesis, loss of HLA-ABC expression, should result in increased NKG2D ligands expression on the surface of infected cells to facilitate Natural Killer (NK) cell mediated cytotoxicity. Expression of NKG2D ligands (MIC-A/B) on the surface of infected cell plays important role in recognition of the infected cell by the NK cells. Interestingly, MIC-A/B surface expression in response to viral gene, LMP2A was observed to be downregulated in epithelial cell cancer. Expression of NKG2D ligands is transcriptionally regulated by several cellular stresses associated during viral infection. The stress signals that alerts the host immune cells of the infection includes DNA damage response, oxidative stress, and Unfolded Protein Response (UPR). Further, UPR proteins were studied for their role in the regulation of MIC-A/B surface expression. The current study signifies the role of UPR protein, PDI in the downregulated expression of membrane-bound MIC-A/B in response to the viral gene, LMP2A. In summary, the regulation of HLA-ABC expression through epigenetic regulation resulted in ablation of CTL response along with UPR mediated regulation of MIC-A/B surface expression helps in regulating NK cell mediated cytotoxicity of the virus-infected cell (Figure).