# Association of MMTV-like Sequences with Human

# Breast Cancer in Pakistani Population.


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# Association of MMTV-like Sequences with Human Breast Cancer in Pakistani Population.

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# List of abbereviations

| | |
|---|---|
| AIDS | Acquired Immune Deficiency syndrome |
| ASR | Age Standerdized Rates |
| ATL | Adult T-Cell Leukemia |
| BLV | Bovine Leukemia Virus |
| dATP | Deoxy Adenosine Triphosphate |
| dCTP | Deoxy Cytosine Triphosphate |
| dGTP | Deoxy Guanosine Triphosphate |
| DNA | Deoxyribo Nucleic Acid |
| dNTP | Deoxyribo Nucleotide triphosphate |
| dsDNA | Double Stranded DNA |
| dTTP | Deoxy Thymidine triphosphate |
| DVM | Doctor of Veterinary Medicine |
| E.coli | *Escherichia coli* |
| EBV | Epstein Barr Virus |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme Linked Immuno Sorbent Assay |

| | |
|---|---|
| FFPE | Formalin Fixed Paraffin Embedded Tissue |
| GFP | Green Fluorescent Protein |
| HBV | Hepatitis B Virus |
| HCC | Hepatocellular Carcinoma |
| HCV | Hepatitis C Virus |
| HERV | Human Endogenous Retrovirus |
| HHV4 | Human Herpes Virus 4 |
| HPV | Human Papilloma Virus |
| Htlv1 | Human T-Cell Leukemia Virus |
| IARC | International Agency for Research on Cancer |
| Ig | Immunoglobulin |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| IRF | Interferon Regulatory Factor |
| KSHV | Kaposi's Sarcoma associated Herpesvirus |
| LANA1 | Latent Nuclear Antigen 1 |
| LANA2 | Latent Nuclear Antigen 2 |
| LB | Luria Bertani |

| | |
|---|---|
| LTR | Long Terminal Repeat |
| MCV | Merkel Cell polyomavirus |
| MHC-II | Major Histocompatibility Class 2 |
| MMTV | Mouse Mammary Tumor virus |
| NCBI | National Center for Biotechnology Information |
| NF | Nuclease Free |
| NORI | Nuclear Medicine, Oncology and Radiotherapy Institute |
| OD | Optical Density |
| PCR | Polymerase chain reaction |
| Rb1 | Retinoblastoma 1 |
| RBC | Red Blood Cells |
| SDS | Sodium Dodecyl Sulphate |
| ssRNA | Single Stranded Ribo Nucleic Acid |
| TAE | Tris- Acetate- EDTA |
| UV | Ultra Violet |
| WHO | World Health Organization |

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# ABSTRACT

Breast cancer is the most common cancer in females and leading cause of cancer related deaths in females worldwide. Breast cancer is more common in developed countries as compared to developing countries. Even though Pakistan lies in the region of low prevalence of breast cancer there is substantially higher rate reported as compared to its neighbors. The known risk factors of breast cancer include mutations in P53 gene BRCA1 and BRCA2 genes, hormonal factors and lifestyle based factors. As reported for other cancers viral etiology of breast cancer is also suspected. Mammary tumors in mice are also caused by virus known as Mouse Mammary Tumor Virus (MMTV).

The search for human homolog of MMTV gained direction when highly specific MMTV DNA sequences were shown to be present in human breast cancers. Different levels of association are reported from different regions of the world however there is no consensus on whether they present any risk to humans in regard with breast cancer or not. We hypothesized that among other risk factors viral infection may also contribute to development of breast cancer. In order to study the prevalence of MMTV-like sequences we collected blood and formalin fixed paraffin embedded breast cancer samples from Pakistan. We extracted the DNA and samples confirmed for DNA integrity by amplification of β-globin gene fragment. At the same time we also obtained tissue and blood sample from mouse to compare the two samples. MMTV sequences amplified from mouse were cloned into TA vector and sent for sequencing. While human breast cancer samples still need to be screened for prevalence of MMTV sequences.

# 1 INTRODUCTION

Breast cancer is the most common cancer in the women worldwide with an estimated 1.38 million new cases every year. It is also the leading cause of death in women with approximately 69% of them in the developing countries (GLOBOCAN 2008, WHO Global burden of disease 2004).

Pakistan has the highest incidence of breast cancer in Asia after Jews in Israel (Bhurgri,*et al*. 2000). It has also unusually higher rate than the neighboring countries (Denic,*et al* 2005). One in every nine women in Pakistan, develop breast cancer at some stage in life (Sohail S.,*et al*.). The major risk factors associated with breast cancer are genetic disorders, early menarche, late menopause, endogenous estrogen, late first child birth, alcohol, obesity and physical inactivity (IARC, 2008). The most common genetic aberration is mutation in P53 gene which is associated with Breast Cancer followed by mutations in BRCA1 and BRCA2 genes.

However, since the discovery of Mouse Mammary Tumor Virus (MMTV) as a cause of Breast cancer in Mice (Bittner *et al*., 1936), the viral etiology of Human breast cancer has also been suspected. Moreover, the discovery of Human Papilloma virus as a cause of cervical cancer further strengthened the argument. Earlier methods used for the detection of human analogue of MMTV were immunohistochemistry based. The results became controversial because of the presence of Human endogenous retroviruses (HERVs) that were shown to be able translate envelope proteins. However with the advent of PCR more specific MMTV-related sequences could be targeted (Cato, 1987). In 1995 for the first time highly specific primers differentiating MMTV from HERV-K10

were developed and the prevalence of this sequence was shown to be 38% in Human Breast Cancer (Wang *et al*., 1995). The conserved sequences from the *env* gene were used to screen for MMTV-like virus in Human. The full length proviral genome has also been sequenced from Human Breast cancer sample with 95% homology to MMTV.

MMTV is a Beta-retrovirus and causes tumors by random insertions into the genome. Its LTRs contain hormone responsive elements that respond to glucocorticoid hormones, this is why its expression increases incredibly during lactation in mice. Similar is the case in humans where Johal *et al*. (2010) reported that it is also associated with other hormone induced cancers like prostate cancer, endometrial cancer and ovarian cancer.

Another important feature of MMTV is the Super Antigen (SAg), encoded by an unusual open reading frame in 3'LTR. This SAg is responsible for malignant transformation in mice. In humans this SAg has also been discovered. Its ability to grow in various human cell types has also been demonstrated (Wang *et al.,* 2004).

Its association with Breast Cancer differs in the different regions of the world. As Stewart *et al*. (2000) also linked the incidence of Human Breast Cancer to the population of *Musdomesticus*, the zoonotic origin of some cases of breast cancer has also been suspected. However, data must be provided from different regions of the world about its prevalence in Breast cancer patients and its association with other risk factors.

Reasons for the high rate of Breast and Ovarian Cancer in Pakistan have not been justified although theories like negative heterosis (Denic *et al.,2005)* have been presented. Both of these cancers belong to hormone sensitive parts of the body. Among the established risk factors, genetic factors and reproductive/hormone associated factors

are most common and both these factors are also responsible for MMTV induced cancers in Mice.Among the other known risk factors data on the MMTV in Pakistan is not available. In order to establish a strong argument for the role of MMTV-like sequences in high incidence of Breast Cancer in Pakistani population, a study must be conducted for the prevalence of MMTV in Human Breast and Ovarian Cancer in Pakistan. Our study will be investigating the presence of MMTV in Breast Cancer only.

## 1.1 Proposed Methodology

1. Cloning and sequencing of *env* gene fragments from mouse tumors using primers reported by Wang *et al.* (1995).
2. Infected and healthy FFPE and blood sample collection from various labs from Pakistan.
3. DNA Extraction using rapid salt extraction method
4. Beta Globin PCR for the quality of sample.
5. Nested PCR for MMTV envelope protein using primers reported by Wang *et al*. (1995)
6. Gel electrophoresis.
7. Statistical analysis.

# 2 REVIEW OF LITERATURE

Breast cancer is the most common cancer in women and accounts for the 23% of the total cancer cases. Breast cancer has Age Standardized Rate (ASR) of 66.4 per 100,000. It is also the leading cause of cancer deaths in women accounting for 14% of the total cancer deaths. According to GLOBOCAN statistics, every year approximately 1.38 million new cases are reported and around 458,000 die from breast cancer. Other common cancers among the female are of Lung and Bronchi, Colon and Rectum and importantly (because of the steroid hormone sensitivity of these regions like breast) Cervix uteri (ASR 9.0 per 100.000), Corpus uteri (ASR 12.9 per 100,000) and Ovary (ASR 9.4 per 100,000). According to the GLOBOCAN cancer statistics (2008), Pakistan is included in the South-Eastern Asian region which has medium to low incidence of the breast cancer (ASR 31.0 per 100,000) and breast cancer associated mortality rate of 13.4 per 100,000. (GLOBOCAN cancer Statistics, 2008)

In the South Asian countries, Pakistan has the highest incidences of the breast and ovarian cancer. These are also the highest in Asia (excluding the Jews). The ASR for breast cancer in Pakistan is 51.7 per 100,000 (Bhurgri*et al.*, 2006). This rate is significantly higher than in the neighboring countries- Iran, Afghanistan, India, China and Tajikistan. Similar is the case with the ovarian cancer with ASR 10.7 per 100,000 being significantly higher than in the neighboring countries (Denic *et al.*, 2005).

## 2.1 Risk Factors

In contrast to other diseases and disorders, cancers are not defined by a single cause, there are several risk factors playing important roles in the development and

progression of cancers (the illusion of certainty: Health benefits and risks chapter 2: p16.). The risk factors considered by WHO (Breast cancer: prevention and control, http://www.who.int/cancer/detection/breastcancer/en/index.html) for breast cancers include

i. Genetic Factors
ii. Reproductive or Hormone associated factors
iii. Lifestyle (smoking, alcohol, obesity, physical inactivity etc.)
iv. Breast feeding.

However another hypothesis also proposes the role of Viruses in breast cancer.

### 2.1.1 Genetic Factors

Family History of breast cancer is known to increase the risk of breast cancer in females. Different genes associated with breast cancer include P53, BRCA1 and BRCA2. Mutations in P53 have been found to be associated with 20% of all breast cancers in the meta-analysis by Pharoah *et al.*, (1999).

BRCA1 and BRCA2 mutations have been specifically known for breast and ovarian cancer. Approximately 10% of breast cancer cases are due to mutations in BRCA1 and BRCA2 (Narod *et al.*, 2000). In Pakistani population Alexander Liede *et al.*, (2002) reported 6.7 % association of these mutations with MMTV. However, they did not cover the complete region of BRCA1 and BRCA2 for mutational analysis, so in a another family based and well organized study, Rashid *et al.*, (2006) reported the association of breast cancer to mutations in these two genes to be around 11.9% in Pakistani population.

### 2.1.2 Reproductive/Hormonal Factors

There is an established role of factors like Parity, age at Menarche and Menopause on the incidence of breast cancer. Similarly the use of exogenous hormones in the form of contraceptive pills and hormone replacement therapy also affect the development of breast cancer. To put it in simple words "higher the number of ovarian cycles, greater is the risk of breast cancer". The risk of breast cancer decreases by 15% with each year delay in menarche age and increases by 3% by each year delay in menopause. (Colditz, Cancer epidemiology and prevention, 2006). Similarly, breast cancer risk is decreased by 4.3% for each year of lactation (breast cancer and breast Feeding. Lancet, 2002).

Pregnancies in humans have beneficial effects against breast cancer. Higher level of prolactin and lower level of sex-hormone binding prolactin in parous as compared to nulliparous women gives the reason for the protective role of early age of 1$^{st}$ pregnancy (IARC, 2008). The users of oral contraceptives are at 15-25% higher risk of developing breast cancer (Breast cancer and hormonal contraceptives: collaborative analysis of individual data on 53,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies. Collaborative group on hormonal factors in breast cancer. Lancet, 1996.). According to Chlebowski *et al.*, (2003) estradiol concentrations are linked directly to breast cancer risk in post-menopausal women.

Other Factors like alcohol, obesity, physical inactivity also contribute significantly to the development of breast cancer. (Lacey *et al.*, 2009).

### 2.1.3 Viral Infection

Viral infections have already been established as a risk factor for a number of cancers. Most notable are HPV, associated with cervical cancer; EBV (Epstein Barr Virus), associated with Burkitt's B-cell Lymphoma; HCV (Hepatitis C Virus) and HBV (Hepatitis B Virus) with hepatocellular carcinoma and several infectious agents in animals like, MMTV with Mouse Mammary Tumors. Lawson and Heng, (2010) have reviewed the studies associating HPV, EBV, MMTV and BLV (Bovine Leukemia Virus) with Human breast cancer. They presented a good case by giving strong arguments for these infectious agents to be involved in the etiology of breast cancer, the strongest evidence was found for MMTV which has been known as a causative agent in murine mammary tumors (Bittner *et al.*, 1936).

### 2.1.4 Suggested Reasons for High Incidence of breast cancer in Pakistan

As stated earlier Pakistan has the highest incidence of breast and ovarian cancer in Asia (excluding Jews) (Bhurgri*et al.*, 2006). High prevalence of BRCA1 and BRCA2 mutations in Pakistan which are 42-50% involved in familial breast and ovarian cancers. For single cases of breast cancer at young age (≤ 30 years) the association is about 11.9% (Rashid *et al.*, 2006).

Another proposed reason is cancer by Negative Heterosis (Denic *et al.*, 2005). Since, the population of Pakistan is derived from previously inbred population from Iran, India, Afghanistan and the locals of this Region, so the present population, a hybrid, has higher incidence as compared to the parent populations. The explanation to this phenomenon is that the individual is a heterozygote with both parents having normal homozygous alleles, but the two allele association is susceptible to a third factor. It was proposed by Denic *et al.*, (2005) that the heterozygote will have the gender (or other

factor)-directed inactivation of a tumor suppressor gene that crosses between previously inbred subpopulations and generate more different heterozygotes thus resulting in increased chances of cancer by negative heterosis. However, this hypothesis has not, yet, been tested.

### 2.1.5 Viruses in cancer

The first valid proof that an infectious agent may be involved in cancer was demonstrated by Oluf Bang and VilhelmEllermen's experiments in 1908 i.e. the avian leucosis virus could be transmitted after cell-free filteration to the new chickens, causing leukemia.

In 1911, Peyton Rous demonstrated the transmission of sarcoma by only using the cell-free filterate from the sarcoma marking the discovery of Rous Sarcoma Virus (Peyton Rous Biography).

Richard Edwin Shope reported Cottontail rabbit papillomavirus, the first mammalian oncovirus, in 1934. Further tumor causing ability of the virus was confirmed by Rous (Rous *et al.,* 1935). Bittner *et al.,* (1936) reported an extra-chromosomal agent secreted in mouse milk responsible for mouse mammary tumors.

The trend of the discovery of viruses involved in cancers and their specific associations continued to support the viral causes of cancers. The first virus discovered to be involved in Human cancers was EBV (HHV4) (Epstein *et al.,* 1964). It was shown to be associated with Burkitt's lymphoma. The milestone in the search of oncoviruses was the discovery of HPV being responsible for 70% of the cervical cancers. (WHO, viral cancers)

Seven Viruses with established role in human cancers include EBV, HBV, HPV, HCV, HLTV, KSHV and Merkel cell polyomavirus (MCV), (Moore and Chang, 2010).

### 2.1.6 Human Oncoviruses

All cancerous cells have almost same characteristics of self-sustained growth, evasion to apoptosis, resistant to immune responses, etc. These cellular changes can be genetic or acquired. Viruses are ubiquitous obligate parasites that are the major cause of persistent infections. About 20% of human cancers reported each year are attributed to viral infection (Farral, 2002). First evidence of viruses as a reason for carcinogenesis were provided by Ellerman and Bang (1908) when they found experimentally that cell filtrate can transfer Leukemia from one chicken to another. This was later confirmed by Peyton *et al.*, (1911). Epstein Bar virus (EBV: also known as HHV4) was the first human tumor virus isolated from Burkitt's Lymphoma cells from an African patient (Epstein *et al.*, 1964). EBV is a dsDNA virus of 172 Kbps (Baer *et al.*, 1984). More than 90% of world population is infected with EBV. Mostly EBV causes asymptomatic infections but in rare cases it can cause carcinomas or lymphomas. EBV causes translocation of c-MYC gene under ubiquitous promoter of Ig heavy chain.

Hepatitis viruses are the major cause of hepatocellular carcinoma (HCC), worldwide. HBV and HCV cause persistent infection in 50-80% individuals. The causal relation of HBV and HCC was established in a large scale epidemiological study where incidence of HBV infection was strikingly similar with HCC. HBV increases risk of HCV 200 fold as compared to non-carriers (Beasly, 1988; Szmuness, 1978). Alone HCV caused a death toll of 308000 in year 2004 (WHO report, 2004). Exact mechanism of

virus induced HCC has not been established, yet, it is proposed to work by modulating immune system and cellular machinery.

Human Papilloma virus is dsDNA virus of 8 kbps. HPV was first discovered in 1932 in cottontail rabbits (Shope *et al.*, 1932). There are more the then 130 strains associated with HPV. HPV as a cause of cancer was established in 1980s when strain 16 and 18 were isolated from cervical cancer patients (Boshart*et al.*, 1984, Durst *et al.*, 1983). HPV is the second leading cause of cancer in women (Parkin, D.M., 2006). Habner and Laimins, (2006) reviewed the pathogenesis of HPV in cervical cancer and reported more than 95% prevalence of HPV in cervical cancers. HPV causes carcinogenesis by inactivation of check points in cell cycle. HPV E7 oncoprotein down regulates Rb1 mediated inhibition of cell proliferation. Simultaneously E6 eliminates premature apoptosis via ubiquitin mediated degradation of p53 (Habner and Laimins, 2006).

Human T cell leukemia virus 1(HTLV1) is a ssRNA virus from retrovirus family. HTLV1 is the only human tumor causing retrovirus reported till time. It was discovered as causative agent of T cell lymphomas (Poiesz*et al.*, 1980). About 10-20 million people are infected with HTLV1 worldwide (Pagano *et al.*, 2002). HTLV is a causative agent of Adult T cell leukemia (ATL) and is found integrated as provirus in most of the ATL cases. HTLV increases the risk of ATL, 1 - 4.5 times as compared to non-carriers (Telzak*et al.*, 1998).

Kaposi Sarcoma associated herpes virus (KSHV) also known as human herpes virus 8 (HHV8) is dsDNA virus of 162 kbps, during persistent infection KSHV exist as

an episome inside the nucleus. Jarrett (2001) reported an uneven distribution of KSHV among European, Mediterranean and African population. KSHV infections are usually asymptomatic; however, co-infection with HIV increases the risk of Kaposi Sarcoma. Kaposi Sarcoma is the leading cause of AIDS associated mortality and morbidity (Ganem, 2006). KSHV encodes several proteins that have striking homology with human cytokines, e.g. KHSV encodes four IRF homology proteins, including vIRF1 which behaves like IRF2 in inhibiting interferon signaling and initiates cell cycle. Latent nuclear antigens (LANA1 and LANA2) have a well-established role in regulation of innate immune system.

## 2.2 MMTV

Mouse Mammary Tumor Virus first described by Bittner *et al.*, in 1936 as an extra-chromosomal agent secreted in milk, has since been a known cause of mammary tumors in certain strains of mice and it has now been established that MMTV causes cancers in laboratory mice (Callahan, 1996). By 1945, Andorvent went on to describe this milk factor as a virus (Gross, 1970), and gradually, this became widely known as the 'Bittner Virus'. Today MMTV also includes closely related strains in addition to the Bittner Virus.

MMTV is a retrovirus and thus exists in proviral form inserted into the DNA, for its replication it must, therefore, insert itself into the host genome. In order to replicate it requires the DNA and cell division however the neoplastic transformation occurs only in mammary epithelium. This specific association is suggestive of special mechanisms involved in MMTV induced transformation. It can be considered as a heritable somatic mutagen whose target range is limited to mammary epithelium (Callahan, 2000).

The basic mechanism of transformation by MMTV is by insertional mutagenesis. These mutations are the cause of pre-malignant lesions and leading to malignant tumors (Nusse, R. and Varmus, H.E., 1982)

Callahan and Smith (2000) reviewed the commonly mutated genes and eight different genes were found to be genetically altered in multiple mammary tumors due to MMTV. These genes include Wnt1, Wnt3, Wnt10b, fgf3, fgf4, fgf8, Int3 and Int6.

### 2.2.1 Infectious Pathway

It transfers from viremic mother to the Gastro-intestinal tract of the pup. There it infects lymphoid tissues like peyer's patch. Its U3 region encodes super antigen SAg that is presented on MHC-II molecules. This results in the proliferation of cognate T-cells that further stimulate bystander B-cell proliferation. It results in a reservoir of infection-competent cells that spread the virus to other lymphocytes. The infection spreads in this way and infects other epithelial tissues along with mammary epithelium as well. However mammary epithelium is malignantly transformed in contrast to other cells. (Callahan and Smith, 2000). They also proposed that the cancerous cell population is monoclonal and derived from a single infected stem cell.

### 2.2.2 MMTV Genome

MMTV is a beta-retrovirus. It has and 8.8 kb ssRNA genome length comprising 7 genes (NCBI Genome MMTV). In contrast to many other retroviruses it has an open reading frame in the LTR region encoding SAg protein (Webster and Granoff, 1994). The genome structure of MMTV is shown the figure below (NCBI Genome).

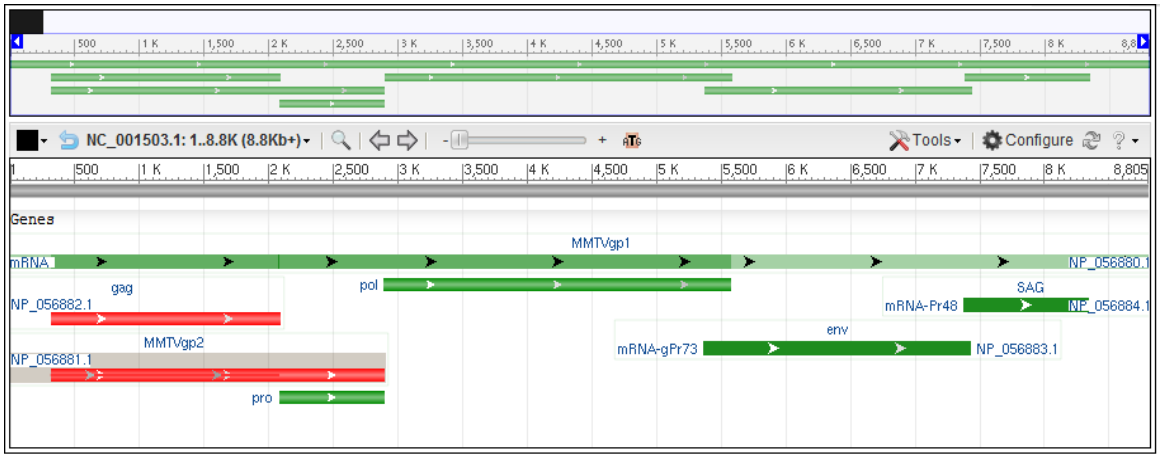


Figure 2.1. The MMTV genome

### 2.2.3 MMTV in Human cancer

Once it has been established that viruses cause cancers and in the one of the best known animal models it can cause breast cancer, the search started for its human analog. Initially immunohistochemistry based study by Mesa-Tejada *et al.* (1978) found 39% of breast cancer tissues positive for MMTV-Envelope protein. These techniques faced controversies when human endogenous viruses (HERVs) were discovered and few were shown to encode envelope proteins. The landmark event was the use of PCR technique for the detection. Wang *et al.* (1995) used highly specific primers that could easily distinguish the MMTV envelope protein from HERV-encoded proteins. The 660 bp homologous sequence was found in 38% of the breast cancer samples and a 250 bp homologous sequence in about 40% of the analyzed cancer. Wang *et al.*, (1998) again showed the expression of 660 bp that was 98% homologous to MMTV. Etkind *et al.* (2000) analyzed 250 bp sequence and found 37% of 73 breast cancer samples positive for the MMTV 250 bp envelope sequence. Importantly they analyzed 19 samples of Non-Hodgkin's lymphomas and found 3 of them Positive for MMTV viral sequence.

After establishment of some level of association of MMTV-envelope sequences with human breast cancers, Melana *et al.* tried to establish them as an exogenous source. They examined both normal and cancer tissues of the patient. The paired samples had 30% of the cancerous tissue samples positive for MMTV and only 1 positive in normal tissue of 106 paired samples (Melana *et al.*, 2001). Similarly other groups have worked on the association of these sequences with human cancers, even the full proviral genome (9.9 kb) with 95% homology to MMTV was reported by Bingren Liu *et al.* (2001).

Indik *et al.* used GFP labeled MMTV to show the infection of a number of human cell types in culture (Indik *et al.*, 2005). Similarly it was shown to be present in Lung cancer (Trejo-aliva*et al.*, 2011), Biliary liver cirrhosis (Indik*et al.*, 2009) and also in lymphoma (Etkind *et al.* 2000) and intestine lymphoid tissue of breast cancer patients (Lushnikova*et al.*, 2004).

In the same way different researchers from different geographical regions found different levels of association between MMTV and Human breast cancer. Hachana*et al.*, (2008) reported 74% of breast cancers positive for MMTV from Tunisia. Ford *et al.*, (2003) reported 42.2% of 45 Caucasian-Australian women positive for MMTV, while only 1 of the 120 Vietnamese-Australian and Vietnamese women was positive for MMTV. Argentina had 31% (Melana *et al.*, 2002), Italy 37.7% (Pogo *et al.*, 1999) and US 36 % (Levine *et al.*, 2004).

In Pakistan MMTV has not yet been studied at any level even though the high prevalence of breast cancer in Pakistan is suggestive of other risk factors associated with

breast cancer. Our study is to examine the prevalence of MMTV in breast cancer in Pakistani population.

# 3 MATERIALS AND METHODS

## 3.1 Sample Collection

Blood samples were collected from different hospitals of Islamabad (Holy family hospital, Benazir Bhutto Hospital and NORI) and Lahore region during February-May, 2012. These samples were already confirmed positive for breast cancer by using tissue biopsy. The tissue samples were collected in formalin fixed paraffin embedded (FFPE) form while the blood was collected in sterile vacutainers containing EDTA as anticoagulant. The tissue samples were stored at room temperature while blood samples were stored at 4ºC until further processing.

### 3.1.1 Extraction ofDNA from Tissue

DNA form formalin fixed paraffin embedded (FFPE) tissues was extracted by following protocol. 10-15 sections of 5-10 um size was cut from the tissue and placed these in microfuge tube. Paraffin was removed by adding 1 mL of xylene followed by shaking for 30 minute at room temperature. Dissolved paraffin was removed by decanting the supernatant after centrifugation at 14000 rpm for 10 minute at room temperature. The whole procedure from addition of xylene was repeated for the complete removal of paraffin.

### 3.1.2 DNA extraction from FFPE tissues

The xylene was removed by washing the sections with 100% ethanol and then ethanol was removed by centrifugation at 14000 for 10 minute at room temperature. Tissue pellets were then air dried briefly. To each tube, 500 µL of tissue digesting buffer

was added and incubated at 50°C for overnight. Digestion buffer stock of 100 mL was prepared. The ingredients are 1M Tris-HCl (4 mL), 0.5M EDTA (0.2 mL), 10% SDS solution (10 mL), distilled water (80.8 mL) and proteinase K (5 mL).

On the second day, Proteinase K was inactivated by heating at 95 °C for 10 minute. After that, 120 µL of NaCl (6M) was added in each tube and to pellet down the protein, tubes were centrifuged at 14000 rpm for 10 minute. Supernatant was transferred to another tube, 3M sodium acetate was added and vortexed. DNA was pelleted down by adding 600 µL isopropyl alcohol and incubated at -20 °C for overnight.

To isolate the DNA, tubes were centrifuged at 14000 rpm for 20 minute at -4 °C. Supernatant was discarded and the pellet was washed with 70% ethanol. After centrifugation, ethanol was discarded and pellet was air dried. Finally, DNA was dissolved in 40 µL of Nuclease Free water (NF $H_2O$).

### 3.1.3 Extraction of DNA from Blood

Genomic DNA was extracted from whole blood by using kit method (GentraPuregene, Hilden, Germany). Firstly, 900 µL RBC lysis solution was addedto the 1.5 mL centrifuge tube containing 300 µL blood.The mixture was incubated for 1 minute at room temperature and inverted gentlyfor 10 times during the incubation. It was centrifuged at 13,000 x g for 20 seconds. The supernatant was removed completely leaving behind the visible white cell pellet and about 10-20 µL of the residual liquid.

The tube was vortexed vigorously using vortex mixer to resuspend the cells in the residual liquid so that white cell pellet was not visible any more. Later on 300 µL cell lysis solution was added to the resuspended cells and pipetted up and down to lyse the cells. Afterwards 1.5mL RNase A solution was added to the cell lysate and tube was

inverted for 25times, followed by incubation at 37°C for 15 minutes in incubator. The samples were placed on ice for 1 minute to be cooled quickly. Protein precipitation solution (100 µL) was added to the cell lysate.

The mixture was vortexed vigorously at high speed using vortex mixer for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate and was centrifuged at 13,000 x g for 1 minute. A tight dark brown pellet of the precipitated proteins was formed. The supernatant containing the DNA was poured into a clean 1.5 mL microfuge tube containing 300 µL isopropanol (100 per cent) while the precipitated protein pellet was discarded.

The supernatant was mixed by inverting the tubes gently for about 50 times until the white threads of DNA were seen. It was centrifuged at 13,000 x g for 2 minutes to sediment the DNA as a white pellet. The supernatant was poured off and tubes were dried on clean absorbent paper. Then 300 µL of ethanol (70 per cent) was added in the tubes containing DNA and tubes were inverted several times to wash the DNA pellet.

The tubes were centrifuged at 13,000 x g for 1 minute and then the ethanol was poured off carefully. The tubes were inverted and drained on clean absorbent paper and allowed to air dry for 1 hour. The DNA hydration solution (50-100 µL) was added and vortexed for 5seconds to mix thoroughly using vortex mixer.The samples were incubated overnight at room temperature and stored at -20°C.

### 3.1.4 Determination of DNA Concentration

Concentration of viral DNA was estimated by spectrophotometer. Optical density was measured at 260 and 280 nm. The ratio of absorbance at 260/280 nm was used to

determine the quality of DNA. Samples having the ratio below 1.7 were treated with phenol-chloroform to remove contaminating proteins.

### 3.1.5 Analysis of DNA through Agarose Gel Electrophoresis

To analyze the integrity of genomic DNA isolated from blood, 0.8 % agarose gel was prepared in 1X Tris borate EDTA (TBE) and was run in the same buffer composition. 10X TBE buffer (pH 8.3) was prepared as stock solution by dissolving 7.5 g EDTA, 55 g of boric acid and 108 g Tris base in 800 mL of distilled water and then the volume was raised to 1 liter with distilled water. For gel preparation, working solution of TBE was prepared by diluting the stock solution by 1:10 with distilled water. For making gel, 0.5 g of agarose was dissolved in 40 mL of 1X TBE and heated in microwave oven to dissolve agarose. The gel mixture was cooled to ~60 °C and upon cooling, 5 µL of ethidium bromide (10 mg/mL) was added to stain the gel. To analyze, 2 µL of DNA from each sample was run on the gel along with 1X loading dye. This gel was run at a constant current of 60 mA for 30-40 minutes. Gel was visualized under ultra-violet light and photographed by gel documentation system (Wealtec, Sparks, USA). The DNA was stored at -20 °C until further use.

## 3.2 Animals Handling

Balb/C mice strain gifted by AMSON Pakistan were maintained and bred in ASAB animal house at NUST. A few females of the first progeny of mice developed mammary tumors. The incidence of the mammary tumors increased as mice population was continuously inbred. The pattern of inheritance and lactation was observed.

Moreover the tumors developed generally during pregnancy. The pattern suggested the infection of MMTV. Mice which developed cancers were maintained in separate cages. The average tumor mass was around 1 cm. The tumors reaching size more than 1cm generally became invasive and mouse died in a week after development of tumors.

## 3.3 Collection of Samples from Murine

One female mouse (52 gram weight) of around 6 months age, with unusually large tumors was selected, food was provided in surplus amount and water was changed on daily basis 2 days before sample collection.

### 3.3.1 Tissue Biopsy

Mouse was killed by cervical dislocation and dissected. The skin was carefully removed from the tumor under the supervision of a qualified DVM Dr. Abdul Ghaffar. The tumor was excised from its base and washed with Phosphate Buffer Saline (PBS). The tumor was weighed to be 2.2 g.

### 3.3.2 Blood

After slaughtering the mouse, aortic blood (500 µL) was extracted in an EDTA containing vacutainer tube.

### 3.3.3 DNA Extraction

Both the samples, blood and tissue, were immediately processed. Genomic DNA from tissue was extracted by rapid salt extraction method (Aljanabi*et al.*, 1997) while blood was extracted using GentraPuregene DNA extraction kit by method stated above (section 3.1.2).

## 3.4 Optimization of Polymerase Chain Reaction (PCR) Conditions

### 3.4.1 ß-Globin PCR

The DNA integrity was confirmed using PCR for human β-globin gene as used by Daniel *et al.*, (2011). The 50 µL PCR reaction mixture consisted of Taq polymerase buffer (1x), $MgCl_2$ (2 mM), 2 mM of dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20 pmole each), thermostable*Taq* polymerase (1 U) and 20 ng ofmouse DNA as template. The nuclease free water was added to make the volume up to 50 µL. The primers used to amplify the β-globin gene are given in table 3.1.

Table 3.1. Sequence of primers for amplification of β-globin amplification

| S# | Primer Code | Sequence (5′---3′) | Tm |
|---|---|---|---|
| 1 | β-globin –F | ACACAACTGTGTTCACTAGC | 58.4°C |
| 2 | β-globin –R | CAACTTCATCCACGTTCACC | 60.4°C |

PCR mixture was placed in thermocycler and run on following profile.

| | | |
|---|---|---|
| Initial denaturation (95°C) | 5 min | |
| Denaturation (95 °C) | 30 sec | 35 cycles |
| Annealing (55 °C) | 30 sec | |
| Extension (72 °C) | 30 sec | |

Final extension (72 °C) 10 min

Hold at 4°C

### 3.4.2 3.2.5 Analysis of PCR Product by Gel Electrophoresis

To analyze the PCR products, 1.2% agarose gel was prepared in 1X TBE and 3 µL of PCR product was run on the gel along with 1X loading dye. This gel was run at a constant current of 60 mA for 30-40 minutes. Gel was stained with ethidium bromide and visualized under ultra-violet light.

### 3.4.3 PCR Amplification of MMTV ENV gene

Primers described by Wang *et al.*, (1995) were used for the examination of MMTV envelope sequences. Primer 5L and 3N product was used as the outer 600 bp region and primer set 2N and 3L for inner 191 bp region.The sequences for forward and reverse primer sets used for amplification of *env* gene are given in the Table 3.2

Table 3.2: Primers Sequences used for Amplification of MMTV-env gene

| S# | Primer Code | Sequence (5′---3′) | Tm |
|---|---|---|---|
| 1 | 5L | CCAGATCGCCTTTAAGAAGG | 53°C |
| 2 | 3N | ATCTGTGGCATACCTAAAGG | 52°C |
| 3 | 2N | CCTACATCTGCCTGTGTTAC | 53°C |

| | | | |
|---|---|---|---|
| 4 | 3L | TACAGGTAGCAGCACGTATG | 52°C |

To amplify the *env* gene, the extracted mouse genomic DNA was subjected to PCR amplification, using primers for partial MMTV *env* gene. The 50 µL PCR reaction mixture consisted of Taq polymerase buffer (10X), $MgCl_2$ (2mM), 2 mM dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20 pmole each), thermostable *Taq* polymerase (1 U) and 20 ng of DNA as template. The nuclease free water was added to make the volume up to 50 µL. PCR mixture was placed in thermocycler and run on following profile.

| | | |
|---|---|---|
| Initial denaturation (95°C) | 5 min | |
| Denaturation (95 °C) | 30 sec | 35 cycles |
| Annealing (50 °C) | 40 sec | |
| Extension (72°C) | 40 sec | |
| Final extension (72 °C) | 10 min | |
| Hold at 4°C | | |

The sequences for forward and reverse primer sets used for amplification of *env* gene are given in the Table 3.2 and the sites where these primers bind on DNA are diagrammatically shown in Figure 3.1.

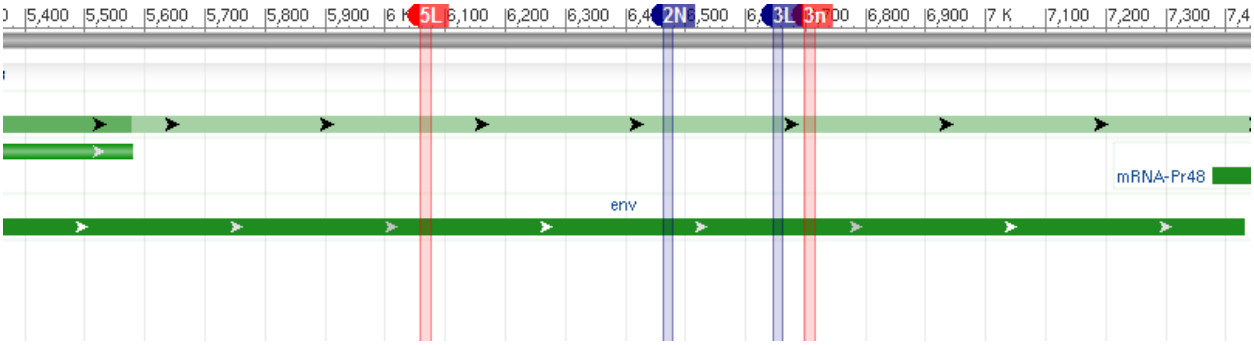


Figure 3.1: Labels show the site of primers binding on gene. Red tags show outer primers and blue tags show inner primers

## 3.5 Analysis of PCR Product by Gel Electrophoresis

To analyze the PCR products, 1.5% agarose gel was prepared in 1X TBE and was run in the same buffer composition. For making gel, 0.6 g of agarose was dissolved in 40 mL of 1X TBE and heated in microwave oven to dissolve agarose. The gel mixture was cooled to ~60 °C and upon cooling, 5 µL of ethidium bromide was added to stain the gel. To analyze, 5 µL of PCR product was run on the gel along with 1X loading dye. This gel was run at a constant current of 60 mA for 30-40 minutes. Gel was visualized under ultra-violet light and photographed by gel documentation system. The PCR product was stored at -20 °C until further use.

## 3.6 Cloning of *env*-gene sequences in TA Vector

The purified PCR product was ligated in a T/A cloning vector pCR® II (Invitrogen). The vector contains two marker genes, lac promoter which allows bacterial expression of the lacZα fragment for α-complementation (blue-white screening); lacZα fragment encodes the first 146 amino acids of β-galactosidase. It also contains antibiotic resistant genes, ampicillin and kanamycin, allowing selection and maintenance of

*Escherichia coli* (*E.coli*) in antibiotic containing media. The pUC origin maintains high copy number of plasmid in *E. coli*. T7 promoter and priming site allows *in vivo* or *in vitro* transcription of antisense RNA and also provide a site for sequencing. The map of TA vector is given in Figure 3.2.

Beta-galactosidase, which is encoded by the bacterial lacZ gene, is the first reporter protein and still is very popular. In bacteria, β-galactosidase cleaves the disaccharide lactose (sugar found in milk) into glucose and galactose. In transformation procedure, β-galactosidase cleaves the colorless substrate X-gal (5-bromo-4-chloro-3-indolyl-β -galactopyranoside) into galactose and a blue insoluble product of the cleavage that turns the colony into blue (i.e. blue/white selection).Since the complete lacZ gene is too large to be carried by a plasmid, cloning vectors contain only the alpha region of the lacZ gene. Expression of the alpha region of lacZ yields an inactive protein. However, the truncated protein encoded by the alpha region can combine into active β-gal when complemented with a protein from lacZ lacking the alpha region. Such "intragenic complementation" can be achieved if a plasmid carrying the alpha region of lacZ is inserted into a bacterial cell containing a mutated alpha region of the lacZ gene. Such cells will turn β-gal positive if the plasmid is present.

DNA cloning into lacZ vectors occurs such that the fragment is likely to be inserted into the alpha lacZ sequence of the plasmid which renders the alpha-region dysfunctional. In this case intragenic complementation no longer occurs. Hence, cells carrying plasmids with transgene will remain β-gal negative. β -gal negative cells can readily be isolated from positive ones by incubation of bacteria on chromogenic culture

media containing X-gal and isopropyl β-D-1-thiogalactopyranoside (IPTG) an inducer of lacZ promoter.

### 3.6.1 Gel Extraction

Gel elution kit of PureLink™ Invitrogen was used to elute the PCR Product inorder to continue with the cloning. Standard procedure was followed to extract the product from the agarose gel which involved the cutting of the gel slice containing the desired fragment corresponding to the Product size as compared to known DNAladder. Then added Gel solubilizing buffer and incubated the gel at 55º C for approximately 25minutes until the gel was completely dissolved. 100 µL chilled Isopropanol was then added. Subsequently the product was passed through the Gel elution column and the flow through was discarded. Next the column was washed with Wash buffer W1 which was 70% high purity ethanol. Finally the product was eluted in 25 µL of Elution Buffer and run on a1% TAE gel to confirm successful gel elution procedure and visualized by means ofUV transilluminator.

### 3.6.2 Ligation

For ligation in T/A vector, 2 µL of vector, 5 µL of purified product, 1 µL of 10X ligase buffer and 1 U of T4 ligase along with 1 µL of NF water was used and made total volume 10µL. The ligation mixture was incubated at 16 °C for overnight. Top 10 strain of *E. coli* were transformed with the ligation mixture.

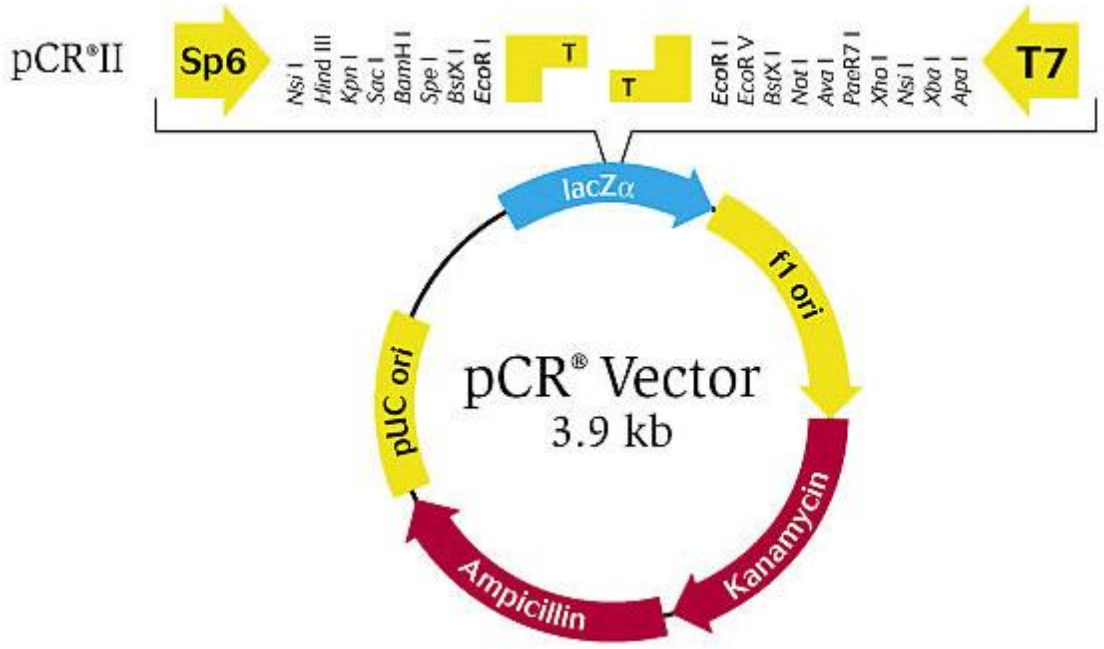


Figure 3.2. Map of TA vector showing site of cloning, lacZ-α, ampicillin and kanamycin resistant gene and origin of replication.

### 3.6.3 Preparation of*E. coli* Competent Cells

A single colony of Top-10 cells was grown in Luria-Bertani (LB) medium. The medium was prepared by using 1% trypton, 0.5 % yeast extract and 0.5%NaCl in 1L of distilled water. The pH was adjusted at 7.5 using NaOH and sterilized by autoclaving. The medium was incubated at 37 °C at 125 rpm with constant shaking for 16-24 hours. Next morning, 1 mL of overnight grown culture was inoculated in 50 mL LB medium. This culture was incubated at 37 °C with constant shaking. The concentration of cells was checked by spectrophotometer after every hour. When the optical density ($OD_{600}$) of the cells reached 0.6, cells were harvested in a 50 mL centrifuge tube by centrifuging at 4000 rpm at 4 °C (Eppendorf, Germany). Supernatant was discarded and the pellet was dissolved in 10 mL of ice cold solution A (100mM $CaCl_2$, 50mM $MgCl_2$) and placed on ice for half an hour. The cells were then harvested by centrifuging at 4000 rpm for 10

minutes at 4 °C. The supernatant was discarded and cells were resuspended in 2 mL of Solution B (50mM $MgCl_2$, 25% glycerol). 100 µL of this cell suspension was dispensed in microfuge tubes for single transformation reaction. Competent cells were stored at -80 °C.

### 3.6.4 Transformation

Transformation was carried out using Heat Shock Method (Woodcock *et al.*, 1989). Five µL of ligation mixture was mixed with 100µL of competent cells and the mixture was incubated on ice for half an hour. Heat shock was given to the cells at 42 °C for 2 minutes and snaps cooled on ice for 2-3 minutes. Transformants were allowed to grow in 1 mL LB media, without antibiotic, for 1 hour at 37 °C for the expression of antibiotic resistance gene. Transformants were harvested by centrifugation at 14,000 rpm for 2 minutes and were plated on LB agar plate containing ampicillin (200 µg/ mL). Using spread plate method, IPTG (40 µL of 100mM) and X-Gal (40 µL) were spread on the plates prior to spreading transformants for the induction of *lacZ* gene and blue/white selection. Plates were incubated at 37 °C for overnight.

### 3.6.5 Screening and Selection of Clones

From each plate, 8 different white colonies were selected and each colony was inoculated in 3mL LB media containing ampicillin (200 µg/mL) and the cells were allowed to grow at 37 °C with continuous shaking. After 16 hours of growth, plasmids were isolated by Alkaline Lysis Method (Sambrook and Russell, 2001). For plasmid preparation, 1.5 mL of overnight grown culture was taken in microfuge tube and spun for 1 minute at 14,000 rpm. Supernatant was aspirated and the cells were resuspended in 100

µL solution I (25 mMTris-HCl, pH 8.0, 10 mM EDTA). To each tube, added 200 µL solution II (0.2 N sodium hydroxide (NaOH), 1.0 % sodiumdodecylsulfate (SDS) and mixed gently by inversion. Added 150 µL solution III (60 mL 3 M potassium acetate (KOAc), pH 4.8, 11.5 mL acetic acid, 28.5 mL $H_2O$), vortexed briefly to mix, and was spun for 5 minutes at 14,000 rpm. Supernatant was transferred to fresh tube containing 500 µL phenol:chloroform:isoamyl alcohol (25:25:1), vortexed and centrifuged for 5 minutes at 14,000 rpm. Aqueous layer was transferred to fresh tubes containing 1 mL ethanol, mixed by inversion, and spun for 5 minutes at 14,000 rpm. Supernatant was discarded and the pellet was washed with 100 µL of 75 % ethanol and spun for 10 minutes at 14,000 rpm. Ethanol was removed and the pellet was air-dried by leaving on bench with the lid open for 5 minutes. DNA was resuspended in 40 µL $dH_2O$ containing 20 µg/mL RNaseA. The extracted plasmid DNA was resolved on 1 % agarose gel, stained with ethidium bromide and visualized under UV trans-illuminator to check the quality of isolated plasmid.

### 3.6.6 Confirmation of clones

#### *3.6.6.1 Colony PCR*

Insert was first confirmed by colony PCR. For colony PCR, ten different colonies were picked. These were dissolved in 15 µL of NF water and vortexed vigorously. Five µL of this solution was used as template for PCR amplification. PCR mixture consisted of Taq buffer (1X), dNTPs (2 mM), $MgCl_2$(2 mM), forward and reverse primers (20 pmole each), and 1 U of Taq polymerase in a final volume of 25 µL. Thermal profile was the same as for the amplification of the gene except initial denaturation was of 12

minutes. After run over, PCR product was resolved on 1.2 % agarose gel under the constant current of 60 mA and visualized under UV transilluminator.

#### *3.6.6.2 Restriction digestion*

Insert was confirmed by digestion with of *Eco*R1 (Fermentas, USA) because the plasmid contained the restriction sites of *Eco*R1 on both side of insert. Five µL of plasmid was treated with 5U of enzyme in a mixture of 20 µL having 1X buffer for*Eco*R1. This mixture was incubated at 37 °C for 2-3 hours and the confirmation was made by resolving the digested product on the 1.5 % agarose gel. The gel was stained with ethidium bromide and visualized under UV transilluminator.

# 4 RESULTS

## 4.1 DNA integrity

The extracted DNA was confirmed for adequacy of the DNA by PCR for detection β-globin gene. The presence of 121-bp segment confirmed the presence of DNA. The results are shown in Figure 4.1.

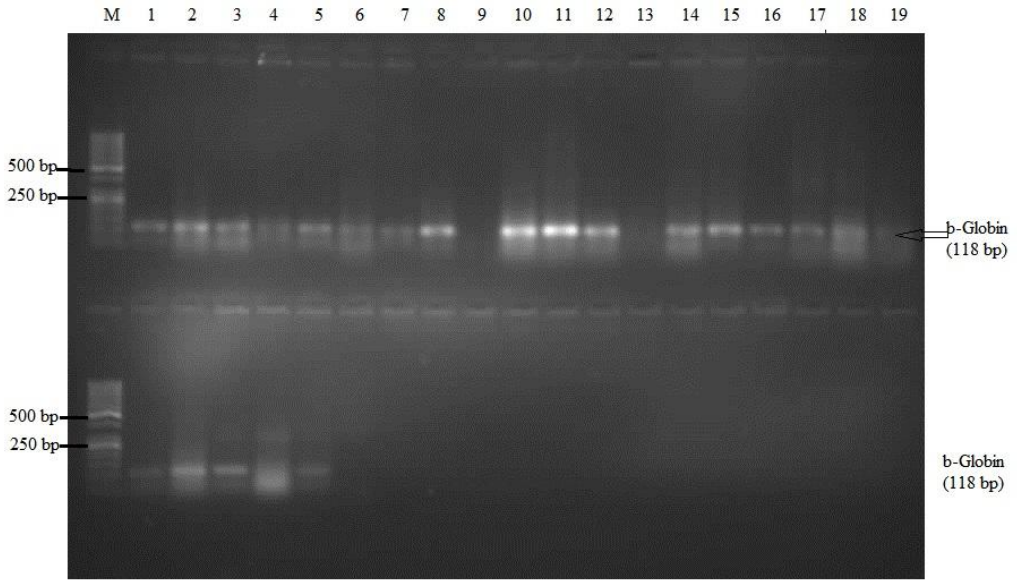


Figure 4.1. 1.5 % agarose gel showing PCR amplification of β-globin gene. (Lane M shows 50bp ladder marker. Lane 1 contains positive control using DNA extracted from healthy individual's blood.

## 4.2 PCR for *env*-gene sequences

PCR results for gene sequences obtained from infected mouse blood are shown below in the figure 4.2 and 4.3. both reactions were carried out separately, not as a nested PCR.

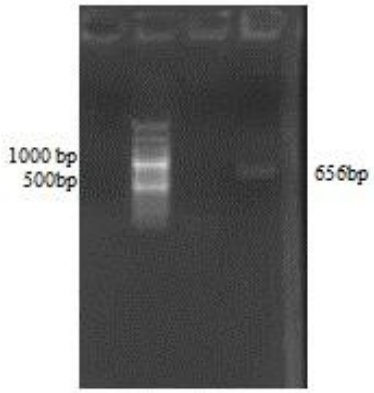


Figure 4.2. 1Kb ladder followed by negative control and 5L-3N PCR to the left 656 bp product was observed at the conditions mentioned above from Tumor extracted DNA of Mouse.

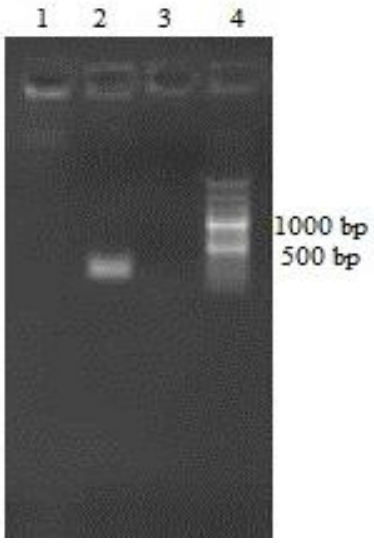


Figure 4.3. 1.5 % agarose gel showing the PCR amplification using internal primers 2N and 3L. (Lane 1: Negative control, Lane 2: 191 bp PCR amplicon of env region, Lane 4: 100 bp DNA Ladder.)

## 4.3 Cloning of genes in T/A Vector

PCR products from both regular and nested sets of primers were cloned in TA vector. The clones were confirmed using *Eco*RI. The fragments generated after restriction were analyzed on 1.5 % agarose gel as shown in figure 4.4 and 4.4.

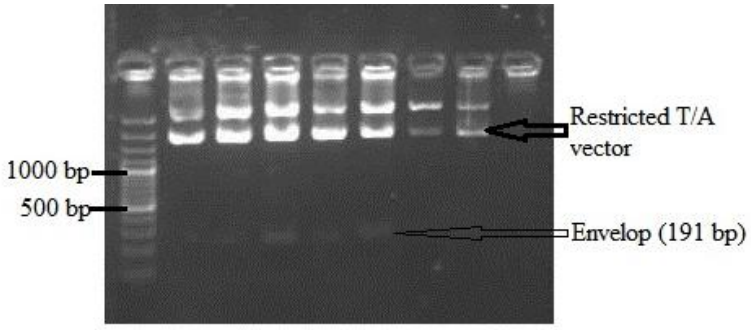


Figure 4.4. Restriction digestion of plasmids isolated from transformed colonies. Lane M: 100 bp DNA Ladder, Lane 1: Undigested Vector, Lane 2-7: Restriction digestion of plasmids with EcoRI.

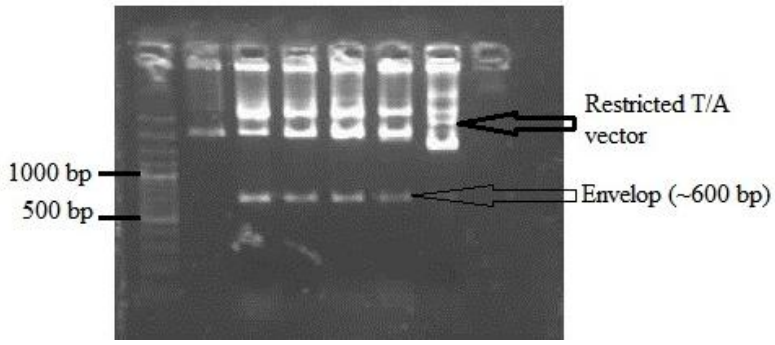


Figure 4.5. Restriction digestion of plasmids isolated from transformed colonies. Lane M: 100 bp DNA Ladder, Lane 1: Undigested Vector, Lane 2-7: Restriction digestion of plasmids with EcoRI.

## 4.4 Screening of samples for MMTV *env* gene

Total 28 samples were included in the study. Of all the tissue samples of 21 patients two were fibroadenomas, one benign hyperplasia and the rest were invasive ductal carcinomas. The average age was 44 years, ranging from 25 to 77 years. DNA from all these patients was positive for β-globin gene. Screening of samples for MMTV *env* gene is in process.

# 5 DISCUSSION

Breast cancer being the most common cancer and the leading cause of cancer deaths in women worldwide requires more attention to be paid. Although it is a point of interest for a lot of researchers worldwide but still a lot more is needed to be known in order to get rid of breast cancer. People have identified many risk factors, so that being careful one can avoid breast cancer as much as possible yet there is an increasing incidence of breast cancer. According to IARC report 2008, the incidence of breast cancer is higher in developed countries but breast cancer related deaths are more common in developing countries. The main reason is unavailability of treatment and resources in developing countries.

Pakistan is also one of the developing countries with an incidence of breast cancer similar to those of developed world. The risk factors that contribute to higher incidence in developed world are the results of urbanization i.e. pollution, unhealthy food, physical inactivity, alcohol abuse etc. (lacey *et al*,. 2009). Pakistan's situation is much similar for these risk factors in urban areas.

To date at least 7 viruses have been confirmed to contribute significantly to the human cancers (Patrick S. Moore, 2010). For two of these viruses vaccines are used as a preventive measure. The virus under study (MMTV) has also been strongly linked to breast cancer because of two main reasons,

1. Being established as a causative agent in most well-known animal model i.e. mouse.

2. Most of the studies on the association of MMTV-like sequences and Human breast cancers have found a strong correlation with very few studies for null association.

Although such data is not enough to establish the causal role of this virus but at least 3 different studies have tried to establish MMTV as an exogenous source. Firstly, Melana *et al.* (2001) used paired samples and found MMTV in 30 % of the cancerous but only in one of the 106 normal tissue samples from the same patients. Secondly, Etkind *et al.* found MMTV-homologous sequences in father, mother and daughter living together for years and all developed breast cancers. And thirdly, Stewart *et al.* 1999 associated breast cancer prevalence to the population density of *Mus musculus.* The above mentioned three researches the variation in prevalence in different region, the variation of results in different populations living in the same region as reported by Caroline *et al.* 2003, the establishment of the fact that MMTV can infect human cells in culture (Stanislav*et al.* 2005), and isolation of the same sequences in from biliary liver cirrhosis and lung cancer are all suggestive of human susceptibility to MMTV. These facts also point towards the zoonotic link to MMTV as we have seen in case of HIV and HTLV.

In contrast, other scientists criticize these results to be insufficient and suggest that there are missing links as some have found no sequences in human cancers. Those in favor of viral-association argue the sensitivity of the detection method (Vickie brower, 2009).

Here in this study we have tried to find out the presence of MMTV sequences in Pakistani population. We started with unselected FFPE tissue samples and unrelated blood samples. The obtained DNA was examined for its integrity by β-globin PCR. At

the same time we obtained blood and tumor sample from MMTV infected mice. Since proviral-MMTV could have been present in low copy number in human cells (if present), we decided to use nested PCR. Primer sets reported by Wang *et al.* (1995) were used. At first we optimized the reaction conditions for mice and successfully amplified 600 bp and 191 bp fragments from both tissue and blood of the mouse. The tissue was sent to histopathology lab for fixation and histology analysis. The amplified fragments were cloned in the TA vector and sent for sequencing. The positive colonies will serve as a continuous stock for further studies on MMTV envelope gene. By now, we have DNA from breast cancer patients, clones of MMTV and optimized reaction conditions. However, the sample size is small (only 30 tissue samples) and we wish to obtain a few more samples.

The purpose of using Blood is that during infection in mice MMTV creates a reservoir of memory B-cells and also infect T-cells in order to reach the mammary epithelium. We suppose that a similar mechanism may be present in humans and low copy number of virus may still be detected by nested PCR.

The obtained data from Pakistani population will serve as another proof to either of the hypothesis that whether MMTV is a risk factor for cancer or it just grows in transformed mammary epithelium. Moreover it will also provide a link in geographical distribution of MMTV-like sequences.

In future based on the level of risk associated with MMTV infection, preventive measures could be defined.

## 5.1 Conclusion

Presences of MMTV like sequences in human breast cancer is quite evident, however a causal role of MMTV cannot be established yet, because of lack of data available on MMTV in humans. Our study has contributed in part to understand the role of MMTV as a risk factor involved in higher incidence of breast cancer in Pakistan.

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