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Significant correlation of MMTV (Mouse mammary tumor virus) LTR gene with hormone receptor status in peripheral blood samples of breast cancer patients from North Pakistan

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Abstract

Mouse Mammary Tumor Virus (MMTV); a Beta retrovirus is the causative agent of mammary tumors in mice. Studies have suggested role of MMTV, as the contrasting factor that could cause Breast cancer in humans however, the exact mechanism of action is still to be explored. In this study, our aim was to investigate the association of MMTV-Ltr gene prevalence with hormone receptor status in breast cancer patients. A total of 50 peripheral blood samples of breast cancer patients were collected for this study. Detection of MMTV-Ltr sequences was done by end time PCR. Statistical analysis was carried out using SPSS 16.0 software. Molecular analysis of blood samples revealed the prevalence of MMTV-Ltr gene sequences in 19 of 50 (38%). In the current study we report the presence of MMTV LTR sequences in blood samples of breast cancer patients and their significant correlation with hormone receptor status of the breast cancer disease. A significant ($p \leq 0.05$) correlation was found between the Estrogen receptor, her2/neu status and metastasis of the breast cancer patients with presence of MMTV-Ltr gene. No significant correlation was found in case of progesterone receptors and MMTV-Ltr gene presence in breast cancer samples. Presence of MMTV-Ltr and its correlation with hormones receptor status revealed the etiological involvement of MMTV in breast cancer patients. These results strengthen the role of MMTV as a hormone responsive virus and its role in causing Hormone positive breast cancer.

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Introduction

Breast cancer stands out as one of the most widespread malignancy in women worldwide, but the etiological factors involved are still being discovered (Lawson *et al.,* 2006; Ringold, 1979). Studies in the past few decades have shown that Estrogens and growth hormones are well-established risk factors for breast cancer in females (Lawson *et al.,* 2006). Consequently, breast cancer research on mouse models and cell lines, suspected that hormone-responsive viruses may act as etiological agents for human breast cancer.

Mouse Mammary Tumor Virus (MMTV) a beta retrovirus, is best known for its causative role for mammary tumors in mice but no conclusive evidence for MMTV pathogenesis in human breast cancer, is not yet available. There is considerable indirect and limited direct evidence that MMTV with additional cofactors such as diet and hormones, may influence human breast carcinogenesis (Wang *et al.,* 1998). MMTV is related to oncogenic expressions in all organs but specifically in epithelial cells of mammary gland and this expression of MMTV in mammary tissue high lightened the relevance of MMTV with the hormones receptors in women. MMTV expression requires cofactors glucocorticoids and progesterone, to be active at molecular level (Groner, 1983).

Glucocorticoid hormones act rapidly and specifically to stimulate the synthesis of mouse mammary tumor virus RNA in a variety of mouse mammary tumor cells and infected heterologous cells (Ringold, 1979) and viral gene expression is also increased by the addition of dexamethasone (Buetti & Diggelmann, 1981). Studies have shown that the MMTV-HRE (hormone regulatory element) is responsive to progesterone and estrogen receptors (Golovkina *et al.,* 1992). Glucocorticoid hormones enhance the transcription of mouse mammary tumor virus DNA by mechanisms involving a direct interaction of the hormone receptor with four binding sites in a hormone regulatory element (HRE) located between the main transcription initiation site within the proviral long terminal repeat region (Ltr) (Scheidereit & Beato, 1984).

Findings with gene transfer experiments discovered a super antigen (Sag) encoded in the open reading frame within Ltr of MMTV, which is important for viral replication and hence infectivity (Pucillo *et al.*,1995; Scheidereit & Beato, 1984).

Since, viral infection is dependent upon several cofactors including hormones; and also breast cancer depends on estrogens and other hormones, the influence of these hormones and hormone receptors on MMTV is of paramount interest. Around the world there are contradictory reports regarding prevalence and etiological role of MMTV in breast cancer. Prevalence rates vary from 73.7% in Tunisia to 4.2% in Mexico to none in Japan (Fukuoka *et al.*, 2008; Levine *et al.*, 2004). We have previously reported the presence of MMTV like sequences in 22% of breast cancer tissue biopsies from Pakistani population (Naushad *et al.*, 2014).

As the research in the past few decades, displayed association between the hormones receptor status and metastasis with the retroviral sequences specifically the super antigen (sAg) in LTR gene of MMTV. The current study, aimed to identify MMTV Ltr sequences and their association with hormone receptor status in peripheral blood samples of Breast cancer patients from north Pakistan. Our data supplements to the ongoing research for MMTV expression and tumorigenesis in breast cancer disease development and aggressiveness that leads to metastasis.

Materials and methods

Patient group

The patient group comprised of peripheral blood samples of clinically diagnosed female breast cancer patients from different hospitals of North Pakistan. The study adhered to national and institutional regulations. Consent was obtained directly from patients. All patients included in the study were females and mean age of the study group was 50 years (30-70 years). Respective clinical details like ER, PR, her2/neu, metastasis and number of lymph nodes of all the cancer patients under study was obtained.

DNA isolation and PCR amplification of Ltr gene

DNA isolation was performed manually using phenol-chloroform method. To determine the quality of the isolated DNA, a 119bp β-globin gene was amplified using single round PCR with specific primer set BGF/BGR (Table 1).

The amplification of β-globin gene confirmed the quality of DNA for subsequent analysis.

Genomic DNA concentration was estimated using a spectrophotometer by measuring the optical density of DNA at 260 and 280 nm and ratio of absorbance at 260/280 nm was used to assess the purity of DNA.

A ratio of ~1.8 is generally accepted as "pure" for DNA; so the samples having the ratio below 1.7 were treated with phenol-chloroform to remove the contaminating proteins.

Table 1. Primers utilized for amplification of β-globin and MMTV-Ltr gene.

Primers		Primer Sequence (5'-3)	Annealing temperature	Gene amplified (bp)	Reference
β-globin	Forward	ACACAACTGTGTTCACTAGC	55°C	β-globin (119bp)	(Saiki *et al.*, 1988)
	Reverse	CAACTTCATCCACGTTCACC			
LTR	Forward	GGTGGCAACCAGGGACTTAT	50°C	MMTV-Ltr (630bp)	(Naushad *et al.*, 2014)
	Reverse	CGTGTGTTTGTGTCTGTTCG			

To screen a 630-bp region within the MMTV-Ltr open reading frame (ORF) that codes for the MMTV super-antigen (*sag*) gene, previously reported conditions and primer sequences for MMTV-Ltr gene amplification were used (Liu *et al.*, 2001). A reaction without template DNA was routinely tested to detect possible contamination of master mix components.

Reactions with isolated DNA from normal controls were done to rule out contamination of samples. For positive control a 6-month old female mouse weighing 52gm with unusually large sized mammary tumor was slaughtered and tumor and blood were removed for DNA extraction and subsequent analysis. The amplified PCR product was analyzed by electrophoresis in 2% agarose gels. Ladder 100bp (Fermentas) was used as a marker to identify the size of the PCR products.

Statistical Analysis

Pearson correlation was performed, in order to determine the association between MMTV-Ltr prevalence and metastasis. To compare the MMTV-Ltr prevalence in hormone receptors positive and negative population of breast cancer patients, paired T-test was performed. A p value of less than 0.05 was taken as statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences, version 16.0 (SPSS Inc, Chicago, IL, USA).

Results

Prevalence of MMTV-like Ltr sequences in breast cancer

Normal women and breast cancer patients' blood samples were tested for the presence of MMTV-Ltr gene sequences. MMTV Ltr gene sequences (630-bp) were detected using a positive control of MMTV-Ltr gene cloned from mice. Fig. 1a represents the ethidium bromide-stained agarose gel electrophoresis of an MMTV-Ltr primed PCR from different patients' blood samples. No amplification was observed in negative controls and normal women breast tissue samples. A total of 19 out of 50 (38%) samples were positive for presence of MMTV-Ltr gene. Fig. 1b, represents the amplified 119bp β-globin gene.

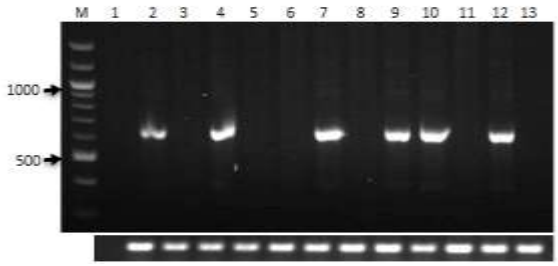

Fig. 1. Screening of 630bp MMTV-Ltr gene from blood samples of breast cancer patients. Lane M 100bp ladder (Fermantas, US), lane 1 negative control, lanes 2 positive control, lanes 3–12 blood samples screened for the presence of 630-bp MMTV-Ltr gene. Lower gel shows119 bpβ- globin gene amplification, as a DNA quantity control.

Correlation of MMTV-Ltr gene prevalence with Hormone receptor status, metastasis and presence of lymph nodes

Table 2 shows the statistical correlation of MMTV-Ltr gene prevalence with Hormone receptor status and a significant correlation (*p= 0.016*, r=0.36*) was observed between Estrogen receptor and MMTV-Ltr gene prevalence in breast cancer patients.

In case of progesterone receptor similar positive association was seen with MMTV-Ltr prevalence (*p= 0.041*, r=0.28*).

A significant correlation (*p= 0.033*, r=0.302*) was observed between metastasis and MMTV-Ltr gene prevalence in breast cancer patients (Table 3). No significant association was found between Her2/neu receptor, lymph nodes and MMTV-Ltr prevalence.

Table 2. Statistical analysis of MMTV-Ltr prevalence with Hormone receptor status of Breast cancer patients.

Association of MMTV-Ltr prevalence with Hormone receptor status			
	Ltr positive (n = 19)	Ltr negative (n= 31)	*p*-value
Estrogen receptor (n= 50)			
Positive (n=26)	14	12	0.000*
Negative (n=24)	5	19	
Progesterone receptor (n= 50)			
Positive (n=17)	8	9	0.000*
Negative (n=33)	11	22	
Her2/neu receptor (n= 50)			
Positive (n=25)	12	13	0.151
Negative (n=25)	7	18	

Table 3. Statistical analysis of MMTV-Ltr prevalance with metastatic and non-mestastatic Breast cancer.

Association of MMTV-Ltr prevalence with Breast cancer metastasis			
	Ltr positive (n = 19)	Ltr negative (n= 31)	P-value
Metastasis (n=50)			
Present (n=22)	12	10	0.033*
Absent (n=28)	7	21	

Discussion

Breast cancer, being a multistep disease, viral infection is considered as a key player in one or more steps of pathogenesis (Labrecque *et al.*, 1995). Role of MMTV, a beta retrovirus as the contributing agent of mammary tumors in mice has previously been proven (Choi *et al.,* 1991; Mant *et al.,* 2004). Various studies have also revealed that direct infection with MMTV can infect human cells (Ford *et al.,* 2003; Melana *et al.*, 2007). We have previously reported MMTV like sequences in the Pakistani breast cancer patients. In the current study, we have found the prevalence of MMTV-Ltr gene and its significant correlation with hormone receptor status of breast cancer patients.

For over 50 years, MMTV-like virus has been suspected to cause human breast cancer because of the rational that MMTV is the well-established etiological agent causing mammary tumors in field and experimental mice.

Furthermore, MMTV like sequences have been detected in breast cancer patients (Salmons & Gunzburg, 1987). Despite the considerable evidence that MMTV-like virus may be linked with breast cancer cause and tumor genesis, the exact mechanism and relationship of MMTV and breast cancer in humans is still a subject of research (Lawson *et al.*, 2006). The frequency of detection of MMTV-like sequences greatly varies from 0 to 100% depending on the specimen, the country where the study is conducted, and the methods used (Taneja *et al.*, 2009).

It has been established that replication of MMTV is enhanced by sex and growth hormones (Lawson *et al.*, 2006). MMTV expression is hormone responsive, resulting in increased amount of exogenous MMTV being produced during pregnancy and lactation (Ross, 2000; Salmons & Gunzburg, 1987). Hormone stimulation occurs via HRE (Gunzburg & Salmons, 1992).

For ER and PR HRE is located in the Ltr of MMTV genome(Lawson *et al.,* 2006). Hormonal response elements present in the MMTV-like LTR may play a role in promoting cell growth as they do in the mouse system (Wang *et al*., 2003).

Studies on MMTV-Ltr and sAg showed that there is a direct or indirect influence of hormone receptors on the infectivity of MMTV and breast tumor genesis. Our data shows a positive association of estrogen and progesterone receptor status with MMTV-Ltr gene (p = 0.01). No correlation between her2/neu and MMTV Ltr sequences was found. The positive association between MMTV-like DNA sequences and progesterone receptor indicates steroid hormones and MMTV may play a role in human breast cancer (Faedo *et al.,* 2004; Langerod *et al*., 2007).

During lactation, MMTV expression markedly increases under the influence of steroid hormones. Estrogen receptors (ER) and progesterone receptors (PR) make MMTV transcription ally active by binding to HRE located in LTR (Truss *et al*., 1995). Curtis *et al*., 2012 also showed that ER and PR are necessary for LTR to make MMTV transcription ally active by the help of nuclear factor 1 and DNA set as viral chromatin gets changed mechanically.

The positive association between MMTV-like DNA sequences and progesterone receptor indicates steroid hormones and MMTV may play a role in human breast cancer. In line with these finding our current study also confirmed positive correlation of MMTV Ltrwith ER and PR status implying the importance of the receptor status in etiology of breast cancer. Her2/neu over expression is reported in invasive ductal carcinoma serving as a prognostic marker (Craig *et al.,* 1992; Park, Neve *et al.,* 2008). But our findings did not show any correlation of her2/neu with presence of MMTV-Ltr sequences in breast cancer patients.

We concluded from current study that MMTV LTR gene is significantly correlated with ER/PR status of Breast cancer patients, though our data only correspond to the blood samples of Breast cancer patients.

Our findings also suggest that presence of MMTV Ltr gene in the blood samples of breast cancer patients is significantly correlated with the metastatic breast cancer. Since ER/PR status is important in the prognosis of breast cancer, presence of MMTV Ltr gene may hinder the prognosis as well as may progress breast cancer towards metastasis.

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Conflict of interest

No conflict of interest is declared by the authors.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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