Molecular Studies on env Gene of Mouse Mammary Tumor Virus in Human Breast Cancer

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Introduction

Breast cancer is the foremost cancer amongst women worldwide as well as in India. With about 1.68 million cases, breast cancer accounts for a quarter (25.2%) of all female cancers reported in 2012. For the same year, with about more than half a million deaths it accounts for 14.7% of cancer related mortality worldwide. The situation in India is no different, with respect to overall incidence of cancer; breast cancer (27%) has overtaken cancer of cervix and uteri (22.9%) to become number one female malignancy in the country. It accounts for 21.5% of cancer mortality in females in India. However when India’s share with respect to of global breast cancer incidence and mortality are computed, the results are startling and alarming at the same time. This calls for studies related to various facets of breast cancer to be carried out specifically keeping population of Indian subcontinent in mind. This thereby would assist in better understanding and management of the disease at population level.

The research undertaken on various facets of breast cancer over the decades has still not leaded us in finding its definitive causal or etiological factor. However studies so far have been successful in shortlisting the most probable risk factors which enhances ones chance of developing breast cancer. Though these factors like family history, personal history, lifestyle, area of residence, ethnicity, etc. help is assessing the risk of developing breast cancer, but most of them fail to pinpoint the actual commencement of the disease. For the better management of breast cancer in terms of prevention, screening and treatment it is quint essential to know exactly what triggers the onset of the disease.
The thought of involvement of infective agents like versus in the etiology of human breast cancer was first perceived when; MMTV was shown to be the etiological agent that causes breast cancer in mice. The research in this regard has come a long way spanning close to five decades. It’s a laborious and tedious task to establish a causal link between MMTV like agent and human breast cancer.

The first Electron microscopy based studies that reported detection of B-type virus particles in human breast cancer specimens set the stage for the quest to explore the association of MMTV and human breast cancer. Viral particles morphologically resembling MMTV have been reported in human milk, MCF-7 and TD-4 cell lines and human breast cancer tissue. Further antigenic reactivity against envelop protein (env) of MMTV was reported in human breast cancer tissue, human breast cancer cell lines, human milk, sera of patients, cyst fluid and particles produced by a human breast carcinoma cell line. Targeting env protein gp52 of MMTV using antibodies was however discounted because epitopes of human proteins displayed antigenicity towards these antibodies.

Genetic evidence to support MMTV as causative agent of human breast cancer was challenging to attain, principally because human genome has endogenous retroviruses known as HERV. HERV-K10 in particular exhibits very high nucleotide homology with MMTV. Low stringency hybridization of DNA from human breast cancer tumor and MMTV showed a degree of homology between the two sequences. Further on the lines of similar technique, reports suggest the detection of 1) MMTV related RNA in malignant cells of breast cancer in humans 2) lymphocytes of breast cancer patients exhibited sequences related to MMTV, and 3) reverse transcriptase activity in monocytes. However these results have been challenging to decipher because the
detected sequences were thought to be associated with the sequences of HERVs, and MMTV-like sequences (MMTV-LS) were found to be repeats of DNA arranged tandemly in the genome of MCF-7 cells.

The probable role of MMTV in breast cancer of humans was revived in the scientific community by a study that reported detection of the MMTV-LS by PCR based method. The sequences detected using this approach had minimal similarity to any of the viral or human sequences including even that of HERVs. Soon many studies exploring the possible role of MMTV in breast cancer of humans were reported using sophisticated, sensitive and specific molecular techniques like PCR, Real Time-PCR (RT-PCR), Fluorescence-PCR (FN-PCR), Reverse Transcriptase In situ PCR (RT-IS-PCR), In situ PCR (IS-PCR) and Microarray. Different rates of detection of MMTV-LS in human breast cancer have been noted in various populations. Other than variation in the methodology used for viral amplification, genetic and environmental factors may be causing variation of the geographical prevalence of the virus. In the primary human breast cancer tumor cultures, MMTV-like viral env DNA has been detected by PCR and RNA by RT-PCR. Furthermore, a 2.7kb env- LTR sequence as well fragments representing the full length proviral structure was reported in two breast carcinomas. The MMTV-like-virus proviral DNA identified via nested extra-long PCR using different primer sets was found to be 95% homologous to MMTV. Further these sequences had a low similarity of only 57% to HERV-K10 in a 3.5kb region of the pol and protease (PR) genes. Most of the human breast cancer derived cell lines are susceptible to infection by MMTV in in vitro conditions. MMTV has also been shown to infect human embryonic kidney epithelial cells, further supporting the ability of MMTV to infect human cells.
Extensive research has been carried out employing various tools to associate MMTV infection with human breast cancer. Since 1990’s many reports have emerged regarding the link of MMTV and human breast cancer based on sophisticated and sensitive molecular biology techniques. However these reports are diametrical in nature, while some suggesting a positive association and the remaining giving a converse link. Further no study has been reported from the Indian subcontinent regarding the role of MMTV in human breast cancer. Hence the current study was carried out in an attempt to explore the link between MMTV and human breast cancer in the Indian sub-context.

The objectives of this research are as follows;

1. Identification of MMTV like gene sequences in human breast cancer.

2. To estimate the frequency of breast cancer linked to MMTV infection.

**Material and Methods**

Institutional Ethical Committee Clearance was obtained from KCTRI and Ethics Committee (Humans) KLE University, Belgaum prior to commencement of the work. Further to facilitate smooth/hassle-free sampling and to understand the trends in breast cancer profiles, a decade long (2001-2010) retrospective case report analysis was conducted at KCTRI. Clinically and histopathologically diagnosed cases of breast cancer scheduled to undergo surgery at KCTRI and Dr. R. B. Patil Cancer Hospital, Hubballi were considered for the study. Breast tissue samples comprising of both malignant tumor and adjacent normal tissue were provided by onco-surgeon from discarded surgical material of consenting patients. 2-5 mm3 of tissue sample (breast tumors and adjacent normal breast tissue) was collected in individual autoclaved,
nuclease free 5 ml vails containing 2 ml of Ambion's RNAlater®, a storage reagent that prevents the degradation of cellular DNA and RNA. The vails were colour and number coded. After each collection, the vails containing the samples were transported in ice cold conditions to the laboratory. Once in the laboratory the vails were kept in freezers at -20°C. Samples comprising of, breast tumor (BC) and adjacent normal breast tissue (NB) was collected from 132 patients who underwent surgery. Along with the sample collection the details of patients’ clinicopathological and demographic details viz. age, family history of cancer, residence (urban or rural), occupation, income, etc. were collected in a predesigned data collection sheet from the case records. The detection of MMTV sequences in the clinical samples was first carried out by employing PCR amplification based detection. Genomic DNA was isolated from the clinical samples and MCF-7 breast cancer cells using DNeasy® Blood & Tissue Kit (Cat. no. 69504, Qiagen). Agarose gel (0.8%) electrophoresis was done for visual inspection of the isolated genomic DNA of both the clinical samples and MCF-7 breast cancer cell pallet. The quantification was done on a Micro-volume spectrophotometer (Quawell Technology). The sequence of MMTV that was selected for PCR based detection should be highly specific and should not have homology to any of the human or other viral sequences. Hence in the current study a region comprising of env gene and SAG LTR (6056 to 8617) was targeted for PCR based detection of MMTV. PCR assays were conducted with utmost care to rule out any possibility of cross contamination resulting in false results. PCR mixture preparation of the isolated genomic DNA from the samples was carried out in a separate laboratory. The components of PCR mixture were checked on regular bases. All the consumables used in these assays were sterile and nuclease free. The PCR amplicons were analysed by agarose gel electrophoresis (Bio-Rad Laboratories, Inc.). Agarose
gel (4% w/v) in 1X TAE, with ethidium bromide (0.5 μg/mL) was prepared. The gel was casted in a gel casting tray with comb and allowed to solidify. 10μL of PCR product mixed with 2μL of 6X loading dye was loaded into the well. Along with this DNA ladder of 100 bp was also electrophoresed. Electrophoresis was carried out at 100 Volts/cm, for 1 hr. Following electrophoresis the gel profile was visualized under UV trans-illuminator at 254nm and photographed in a gel documentation system (Vilber Lourmat).

Microarray based gene expression profiling for the detection of MMTV was also done at Genotypic Technology Pvt Ltd, Bangalore. Microarray based human gene expression profiling on a 8x15K Array encompassing 14,992 genes which included the env and gag genes of MMTV was done for 13 cancer (6 tissue samples of breast cancer with family history, 7 tissues of without any history of familial breast cancer i.e. sporadic breast) and 3 normal breast tissue samples. Total RNA was isolated from all the tissue samples as per instructions using the Qiagen RNA Easy minikit (Cat.No.74104). Using RNA 6000 Nano Lab chip the integrity of the extracted RNA was analysed on the 2100 Bioanalyser as per protocol. The purity of the extracted RNA analysed by UV-VIS Biophotometer from Eppendorf. Total RNA with OD260/OD280>1.8 and OD260/OD230>1.3 was fit for further micro array analysis. The RNA was used if the ratios of rRNA 28s/18s was more than or equal to 1.0 with the rRNA proportion being at least 30% and the RNA Integrity Number (RIN) to be >7.0. Agilent's One-Color Microarray-based Gene Expression Analysis employs cyanine 3-labelled targets to measure gene expression in FBC, SBC and NB tissue samples. For this experiment Agilent's Low Input Quick Amp Labeling Kit (Agilent p/n 5190-2305) which produces fluorescent cRNA (complimentary RNA) with 10 ng and 200 ng of total RNA or at list 5 ng of poly A+ RNA for one-color processing was
used. T7 RNA polymerase is used in this method, which concurrently amplifies target material and incorporates cyanine 3-labelled CTP.

**Results**

The average age of the patients was 48.12 years, ranging from 25-72 years. Most of the patients (61.36%) were younger than 50 years of age. Of the 132 patients 4 were in 21-30 years, 35 were in 31-40 years, 42 were in 41-50 years, 37 were in 51-60 years, 8 were in 61-70 years and 6 were in 71-80 years. The rural representation was more with 84 (63.63%) patients compared to urban area with 48 (36.37%). Most of the patients belonged to the majority community of Hindus (n=118; 89.39%) followed by Muslims (n=12; 9.09%) and Christians (n=2; 1.52%). With respect to pathology of the tumors apart from one Phyllodes tumor the remaining all were IDC. Left and right breast tumors were 68 and 63 patients respectively and bilateral tumor in 1 patient. Family history of breast cancer was reported by 6 patients and 4 patients reported a history of other cancers.

There was no amplification with any of the primers when MCF-7 cell line DNA was used. This indicates that the MMTV-LS were absent in the of MCF-7 cell line DNA. However appropriate amplification was obtained for all the primer sets when 2.7 kb DNA encompassing env and SAG LTR regions of MMTV (GenBank AF243039) was as template. After standardization of the protocol, all the PCR based detection of MMTV sequences in the clinical specimens was carried out were in the positive control was 2.7 kb DNA fragment. The PCR based detection of MMTV sequences in the clinical samples did not produce any amplification in MMTV env gene using primers 5L-3N and 2N-3N. Similarly, no amplification of SAG LTR region using primers LTR5-LTR3. Thereby, indicating the absence of these sequences.
The filter probesets by expression and flag call reports indicate that there were no detectable signals for MMTV specific probes i.e. GT_EA531_env_V_4 and GT_EA531_gag_V_3. Thereby indicating the absence of these sequences in the current study specimens.

This result of non-detection of MMTV like sequences in the study population/geographical area is in agreement with previous reports that used PCR based detection. Results of negative association have been also reported using RT-PCR, based techniques as well. Further Microarray based gene expression profiling for the detection of MMTV was also done using Human 8x15K Array covering 14,992 genes which included the env and gag genes of MMTV. Total of 16 samples comprising of, 13 cancer (6 familial breast cancer tissue samples, 7 sporadic breast) and 3 normal breast tissue samples were assayed. In this analysis too no detection of MMTV sequences was reported. This result is in concurrence with an earlier report based on Microarray. Different rates of MMTV-LV in human breast cancer have been noted in various populations. Other than variation in the methodology used for viral amplification, environmental and genetic factors may be involved in the variation of the geographical prevalence of the virus.

**Discussion and Conclusion**

Therefore considering the younger age of onset of breast cancer and the rural background combined with poor socioeconomic status of majority patients, there is a need for the evaluation of screening efficacy in Indian settings for determining the best screening strategy in different Indian sub-populations. Similarly the breast cancer management strategies should be standardized to fit specific sub-populations.
Considering the results of MMTV-LS detection in genomic DNA of MCF-7 cells and the previous diametrical reports, it can be stated that MCF-7 cells are not an appropriate referral template for the detection of MMTV-LS using PCR based techniques. Further it is suggested that plasmids contain the cloned genes or sequences of MMTV be used as positive control for detection of MMTV-LS for PCR based assays. Choice of proper positive and negative control to substantiate a viral etiology in human breast cancer is very important. This would further lead to the development of novel anti-oncoviral therapies and prophylactic strategies to combat this ever increasing trend of breast cancer.

Results of PCR and Microarray based investigation indicate that MMTV like viral sequences are absent in the clinical specimens analysed. The comparison of these results with other studies lead to the conclusion that significant differences in MMTV-like virus prevalence in various countries might be a reflection of regional viral epidemiology.

If viral etiology were substantiated, it could lead to the development of novel anti-oncoviral therapies and prophylactic management strategies to combat with this dreaded cancer. For the first time detection of MMTV sequences in human breast cancer samples was done by simultaneously targeting env and SAG genes. This is also the first study conducted in India to assess involvement of MMTV in human breast cancer using modern molecular tools like PCR and Microarray. This study also suggests that MCF-7 cells are not an appropriate referral template for the detection of MMTV-LS using PCR based techniques. Samples from patients reporting to major tertiary cancer hospitals in Hubballi, were assayed for presence of MMTV like sequence. However, in order to authoritatively accept or reject the involvement of
viral role in the development of breast cancer, such investigation encompassing a larger geographical area comprising of more subjects should be carried out. Indian population comprises of heterogeneous group of subpopulations having a large sociodemographic difference. In future multicentre based study can be undertaken spanning a larger geographical area and population. More candidate infectious agents can be examined for their role in breast cancer development. Such studies can aid in better understanding of the involvement of infectious agents in the etiology of breast cancer.

This investigation has certainly set a platform to undertake research on infectious etiology of breast cancer, and also for development of better management strategies to curtail the burden of this disease.