RESEARCH ARTICLE

Lack of Detection of the Mouse Mammary Tumor-like Virus (MMTV) Env Gene in Iranian Women Breast Cancer using Real Time PCR

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Abstract

Background: Mouse mammary tumor virus (MMTV) is the major cause of mammary tumors in mice. There is limited controversial evidence about the probable etiologic role of MMTV- like virus in human breast cancer. Materials and Methods: A total of 40 Formalin fixed paraffin embedded samples with diagnosis of breast cancer were collected in a period of 3 years from cancer institute of Iran. We selected both pre-menopausal and post-menopausal patients with different histologic grades and different ethnic groups. We evaluated presence of MMTV-like virus env gene through real time PCR method. Results: Forty patients (20 pre and 20 post-menopausal women) were evaluated with the mean age of 49.67. The average tumor size was 39 mm. None of the studied samples were positive for MMTV-like virus env gene target sequences. Conclusions: We found no evidence on the potential role of MMTV-like virus in the carcinogenicity of breast cancer among Iranian women.

Keywords: Mouse mammary tumor virus - human breast cancer - PCR method - Iran

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Introduction

Breast cancer is the most common neoplasms among women worldwide (Grayson, 2012). Incidence is about 200 per 100000 women in the United State of America (Anderson et al., 2011). Incidence rate of breast cancer is rising almost everywhere including the middle income countries.

Family history of breast cancer, early menarche, late menopause, late first pregnancy, physical activity and body fatness are the main risk factors for breast cancer (Fund et al., 2007).

Moreover viruses are implicated in the development of various cancers including breast cancer (Glenn et al., 2012). Human Papilloma Virus (Glenn et al., 2012), Epstein-Barr virus (Huo et al., 2012), and Mouse mammary tumor virus (MMTV) (Mok et al., 2008) are viral agents which have been linked to breast cancer.

The geographic distribution of mouse species which are primarily infected by this virus can considerably affect human breast cancer.

Mouse mammary tumor virus (MMTV) is the major cause of mammary tumors in mice(Bittner, 1936), which could be transmitted as an endogenous provirus or like an exogenous infectious agent (Amarante et al., 2009). A virus called MMTV–like virus has about 95% sequence homology to MMTV (Lawson et al., 2006). There is controversial evidence on the probable etiologic role of MMTV-like virus on the development of human breast cancer. Although the role of MMTV as an etiologic factor for mammary tumor development in mice is confirmed, it is still unclear whether MMTV-like virus is a risk factor for breast cancer in human.

A highly variable reports are available on the present of MMTV- like virus in female breast tumor ranging from 78.8% in Australia (Ford et al., 2004) to no virus (0%) in Japan (Fukuoka et al., 2008) and Germany (Frank et al., 2008). this results implies that geographic and ethnic variations may play an important role in MMTV-like virus infection and its role on breast cancer development.

Accordingly, in this study we examined the presence of MMTV- like env gene in Iranian women breast cancer patient.

Materials and Methods

Breast cancer tumors

We used 40 Formalin fixed paraffin embedded samples with the diagnosis of invasive ductal carcinoma diagnosed between 2007 and 2009 at the pathology department of

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Cancer Institute of Iran. The specimens were obtained from archived breast cancer samples, besides that, we have ethical committee approval.

Genotyping

Nucleic acid was extracted form deparafinized sections of each paraffin block using Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) as instructed by the manufacturer. Subsequently, internal control amplification was performed for a 119 base-pair region of Homo sapiens hydroxymethylbilane synthase (HMBS) gene, as previously described, with some modifications (Moberg et al., 2003; Shahsiah et al., 2011).

The PCR for the target sequence was performed in 20 μL reaction containing 0.5 μM of each forwardand reverse primers, and 20 μL SYBR Premix Ex TaqII (Takara Bio, Ostu, and Shiga, Japan). PCR conditions were as follows: initial denaturation at 94°C for 30 seconds, and then 45 cyclesof 95°C for 5 s, 55°C for 20 s and 72°C for 20 s; the real-time acquisition of SYBR green fluorescence was performed during the annealing phase on green channel. Finally, a 5 min terminal extension followed by melting curve construction by heating up the PCR product from 60°C to 95°C at a rate of 1°C/s with acquisition on green channelwas performed.

A 104 base pair oligonucleotide was ordered based on the gene bank MMTV-like virus envelope protein (ACCESSION #: GU109516) and was used as a positive control.

Positive and negative controls were included in each run. Reactions with Ct of less than 35 and the melting peak of less than 0.5°C differences from positive control were considered positive. Table 1 reveals the target sequence and size of the studied genes.

Results

Clinical and pathological data of the patients are summarized in Table 2. Half of the patients were pre-menopausal and half were post-menopausal women. There various ethnic group among patients including Persian (32.5%), Gilak (22.5%), Lur (5.0%), Kurd (12.5%) and Azari (27.5%). In addition, there was almost equal frequency of tumor grads, i.e 30% were well differentiated (grade 1), 35% poorly differentiated (grade II) and 35% were undifferentiated (grade III). The mean age was 49.7 (SD 13.4) years. Estrogen and Progesterone receptor status varied among the selected patients.

None of the examined samples was positive for MMTV-like target sequences and no virus particles were found by real time PCR. The qPCRdata, including positive controls standard curve and melting curve, are presented in Figure 1.

Table 1. Target Sequence and Gene Size

Target		Sequence	Product Size
HMBS	Forward	GCCTGCAGTTTGAAATCAGTG	119
	Reverse	CGGGACGGGCTTTAGCTA	
MMTV	Forward	GCTCTAGTTCCCCATACAGA	104
	Reverse	GCAGATGTAGGAATCATCTCATG	
PC	GCTCTAGTTCCCCATACAGAATTGTTTCGCTTAGTT GCAGCCTCAAGACATCTTATTCTCAAAAAGCCAGG ATTTCAAGAACATGAGATGATTCCTACATCTGC		

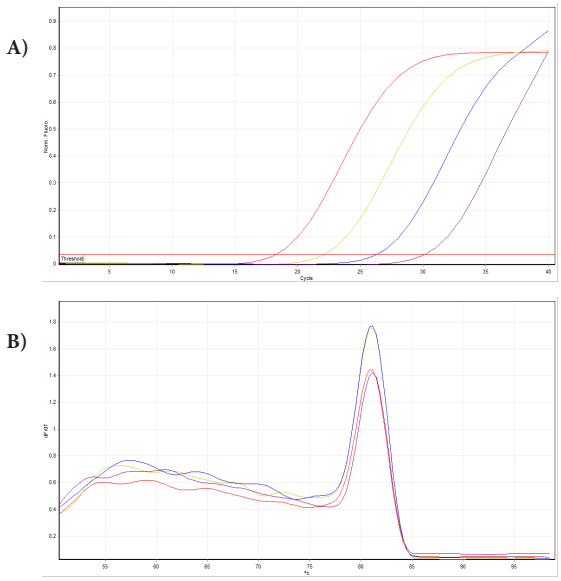

Figure 1. A) Positive Controls Standard Curve, B) Melting Curve

Table 2. Clinicopathological Characteristics of Iranian Patients with Invasive Breast Ductal Carcinoma (N=40)

Variable		Number	(%)
Menopause Status	Pre-menopause	20	(50)
	Post-menopause	20	(50)
Ethnicity	Persian	13	(32.5)
	Gilak	9	(22.5)
	Lur	2	(5.0)
	Kurd	5	(12.5)
	Azari	11	(27.5)
Histologic grade	Grade I	12	(30.0)
(Nattingham score)	Grade II	14	(35.0)
	Grade III	14	(35.0)
Estrogen receptor (ER)	Positive ER	25	(62.5)
	Negative ER	15.0	(37.5)
Progesterone receptor (PR)	Positive PR)	11	(27.5)
	Negative (PR)	29	(72.5)
Average age in year (SD)		49.7	(13.4)
Average tumor size in millimeter (SD)		39	(24.8)

Discussion

It is extremely difficult to reveal that malignant transformation of human breast epithelium is induced by exogenous MMTV- like virus infection. Needless to say, this process requires mutagenetic and oncogenetic analysis. In addition, cancer development is usually multifactorial and the only presence of a specific virus could not confirm the casual role for tumorogenesis.

On the other hand, gathering biological and clinical evidence along with the identification of specific viral genome as MMTV-like env gene in cancer or precancerous cells (including breast tumor cells) could complete a part of criteria as a proof of viral oncogenesis.

Our study which evaluates the possible existence

of MMTV-like genome in invasive ductal carcinoma, demonstrated that despite the high sensitivity of real time PCR method, the prevalence of this nucleotide sequence in Iranian breast cancer patients was nil and could not trace the MMTV-like genome among Iranian patients. In our study we select the patients from a multi ethnic population with a variable clinic-pathologic status.

In 2012, motamedifar etal studied about MMTV-like sequences in 50 Iranian breast cancer tissues in the city of Shiraz, located in southwest Iran. Two regions of MMTV, 660 bp and 250 bp, were evaluated by nested PCR. They also found no virus in their specimens (Motamedifar et al., 2012).

In 2008, Fukuoka et al evaluated the presence of MMTV- like virus in invasive breast cancer of 46 Japanese women by PCR and southern blot hybridization and similar to our study they didnot detect any virus particle in the tissue specimen tested (Fukuoka et al., 2008). Findings of Brindra et al. (2007) in Swedish women and Frank et al. (2008) in German women also support our results. However, Ford CE et al detected very low ratio of MMTV DNA in Vietnamese and Vietnamese-Australian women (0.8% and 0% respectively) using PCR method (Ford et al., 2003).

In contrast, there are many studies that indicate the high prevalence of this genome in invasive breast carcinoma. To our knowledge, the highest ratio is reported from Australia (78.8%) (Ford et al., 2004) and Tunisia (73.7%) (Levine et al., 2004).

In US, the reported results range from 38.5% to 62% (Wang et al., 1995; 2003). Lawson et al. (2006) reported present of MMTV-like virus env DNA in37.3% of 59 human breast cancer patients. They conclude that some breast cancers not only are positive for MMTV–like env DNA but also have histological similarities to MMTV–associated mammary tumor of mice.

In 2011 Mazzanti et al. (2011) analyzed human breast cancer (HBC) preinvasivelesions for the presence of MMTVels.MMTV env-like exogenous sequences were found in 19% of normal epithelial cells collateral to ductal carcinomas in situ (DCISs) or infiltrating ductal carcinomas (IDCs), 27% of atypical ductal hyperplasias, 82% of DCISs, and 35% of IDCs.

The comparison of our 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 virus epidemiology. In spite of variable ethnicity of the patients selected in this study and also varieties in the age, age, tumor size, histologic grades estrogen and progesterone receptor status in this study, it seems that MMTV-like virus does not play an important role in development of breast cancer among Iranian population.

Molecular method of the virus detection may also influences the rate of virus detection in the tumor samples. For instance, Zammarchi et al. (2006) evaluated the presence of MMTV-like virus env gene sequence in Italian women by fluorescence nested PCR method which was positive in 33% of human breast cancer cases. They rely ontheir more accurate method, i.e. a combined use of frozen material and FNPCR which was able to identify very low copies of retroviral gene (Zammarchi et al., 2006). Although we could not use fluorescence nested PCR method in this study, however, by applying positive and negative controls during each run of real time PCR along with double checking of all specimens, we tried to increase sensitivity of the detection rate.

Although qPCR is a very sensitive method to detect the presence of HMTV, there is a chance that it may produce false negative results if the sequences of the target and the designed primers do not match well enough. It is possible that the sequence of MMTV in Iranian population may be different than that provided by the gene bank.

Limitation of sample size still remains a concern to make firm conclusion, though.

In conclusion, while negative results of our study could not completely rule out the potential role of MMTV-like virusas risk factor for developing breast cancer among Iranian women. Our null results imply that, if anything, it would be a remote etiologic agent.

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