Lack of Association of Mouse Mammary Tumor Virus-Like Sequences in Iranian Breast Cancer Patients

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Key Words

Mouse mammary tumor virus • Breast cancer patients • Shiraz • Iran

Abstract

Objective: The present study aimed to detect mouse mammary tumor virus (MMTV)-like sequences in Iranian breast cancer patients in the city of Shiraz, located in southwest Iran. **Subjects and Methods:** We searched for two MMTV genetic regions in the peripheral blood leukocytes of 300 women with breast cancer, 300 age-matched healthy control subjects, and 50 breast tumor tissues. Two regions of MMTV, 660 bp and 250 bp, were searched by nested polymerase chain reactions. **Results:** None of the above two regions were detected. There were no differences between the control group and the breast cancer group. **Conclusion:** Our findings did not show any association of MMTV-like sequences with breast cancer development in Iranian patients in Shiraz.

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Introduction

Among known risk factors for development of breast cancer, it has been suggested that an infectious agent may be one [1]. A great deal of effort has been invested in searching for a tumor virus associated with human breast cancer, yet the existence of such a virus, which has been postulated for many years, has not yet been proven.

The mouse mammary tumor virus (MMTV) is undoubtedly the major etiological agent of breast cancer in laboratory mice [1, 2] and, as such, has been the retrovirus most extensively searched for in human breast tumor development. MMTV, a non-acute transforming type B retrovirus, was first identified in the milk of breast-feeding mice by Bittner [3]. A mandatory step in the replication of MMTV is the integration of a DNA copy of its proviral genome into the host cellular genome, an event that is potentially mutagenic for the host cell. Although a large body of evidence for MMTV involvement in human breast tumors has been reported [4–11], such reports have often lacked verification from other laboratories and thus have led to much controversy in this field. In addition, the

presence of human endogenous retrovirus (HERV) sequences in the human genome, which are homologous to MMTV [12, 13], has made it difficult to distinguish endogenous from exogenous MMTV-like sequences. Wang et al. [14] compared the sequences of the MMTV *env* gene with the sequences of the HERV-k10 *env* gene and localized a region of 660 bp of low homology (16%) between MMTV *env* nucleotides 976–1640. They then searched for sequences homologous to it using polymerase chain reaction and found that an MMTV *env* gene-like sequence that is 90–98% homologous to the *env* gene of the MMTV was present in 38% of breast cancer samples but not in normal tissues [14]. Researchers [15] therefore suggested that a related virus, called human mammary tumor virus (HMTV) with 85–95% homology to MMTV, may be involved in human breast cancer [15]. Intrigued by this suggestion of MMTV involvement in human breast cancer, we attempted to detect these MMTV-like sequences in Iranian breast cancer patients.

Subjects and Methods

Specimens and Samples

In this cross-sectional study, DNA samples of peripheral blood of 300 women with breast cancer (mean age: 49.1 ± 11.5 years), and 300 age-matched healthy controls, extracted by the salting-out method, were taken at the Shiraz Institute for Cancer Research for detection of MMTV-like *env* gene sequences in this study. All patients were recruited during the period from 2005 to 2008 at the Breast Clinic, Shiraz Medical School, Shiraz, Iran. The breast cancer cases included 241 invasive ductal carcinoma (IDC), 19 medullary carcinoma (MC) and 40 non-well-differentiated breast cancers. All patients and controls originated from Shiraz, southwest of Iran. Breast cancer was proved from surgical and pathological reports. Controls did not have any evidence of malignancy or autoimmunity.

Also, 50 paraffin-embedded sections of breast tumor specimens from the above-mentioned patients operated at the Faghihi Hospital, Shiraz Iran, were collected from the Department of Pathology, Shiraz Medical School, Shiraz, Iran. DNA samples of the peripheral blood of the same patients were obtained as well. The protocol was approved by the Ethics Committee of the Shiraz University of Medical Sciences.

DNA Extraction and PCR

DNA of paraffin-embedded sections was isolated using DNA extraction system I (Vienna Lab, Labordiagnostika gmbH, Vienna, Austria). Briefly, nuclei were lysed with SDS and proteinase k, proteins were precipitated by addition of saturated NaCl solution and DNA was recovered by ethanol precipitation. High molecular weight DNA was spooled on plastic inoculation loops, dissolved in TE buffer (10 mM Tris-HCl (pH 7.4), 1 mM Na₂ EDTA) and stored at -80°C .

PCR was performed in 25 μl reactions containing 1 μl DNA plus 0.6 μl of each MMTV 660 bp *env* gene-like sequence primers 1N (5'-CCTCACTGCCAGATCGCCT-3'; nucleotides 976–993 of the MMTV *env* gene; Wang et al. [15]) and 3N (5'-ATCTGTGGCATACTAAAGG-3'; nucleotides 1640–1621 of the MMTV *env* gene; Wang et al. [15]), 1 μl of β globin primers (BF: 5'-ACACAACTGTGTTCCTACTAGC-3' and BR: 5'-CAACTTCATC-CACGTTCCACC-3') as the internal control [16], 0.7 μl dNTPs, 2.5 μl 10-fold buffer, 2 μl Taq polymerase, 0.65 μl MgCl₂, and 4.95 μl distilled water (Aqua dest, GmbH, Germany). To detect possible contamination of the master mix components, a control lacking DNA was routinely included. Thermocycling was performed by denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

The product of the PCR was analyzed by electrophoresis in a 2% agarose gel with ethidium bromide staining. A sequence of the cloned *env* gene that was obtained from the American Type Culture Collection (ATCC No. 45006, p 203) was used as a positive probe to determine optimal Mg² primer concentrations and requirements for the different cycling temperatures. To detect possible contamination of the master mix components, a control lacking DNA was routinely included. A pUC19/Mspl DNA ladder was used to identify the size of the PCR products.

Detection of MMTV 250 bp *env* gene-like sequence in DNA of paraffin-embedded sections and peripheral blood used nested PCR. In our study, to maximize the sensitivity and specificity, we analyzed human breast tumors for the presence of the 250 bp sequence fragment after nested PCR reactions. Screening of this 250 bp fragment was performed with two sets of primers: MMTV2 (5'-TACATCTGCCTGTGTAC-3'; nucleotides 1388–1405 of the MMTV *env* gene 917) and MMTV3 (5'-ATCTGTGGCATACT-3'; nucleotides 1640–1626 of the MMTV *env* gene [17]).

For the nested PCR round, 1 μl of the product of the first PCR was added to a reaction volume of 25 μl containing 0.6 μl of each MMTV 250 bp *env* gene-like sequence primers, 1 μl of β globin primers as internal control, 0.7 μl dNTPs, 2.5 μl 10-fold buffer, 2 μl Taq polymerase, 0.75 μl MgCl₂ and 14.85 μl aqua dest. Thermocycling was performed by denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, and the product of the PCR was analyzed as mentioned above.

Results

Only the probe that was used as positive control revealed the presence of the 660 bp sequence, but not in the DNA of breast tissues of patients (fig. 1). Similarly, figure 2 reveals the presence of 250 bp sequence was revealed in the ATCC probe only, not in breast tissues (fig. 2).

The stained agarose gels containing the products of PCR of peripheral blood samples are shown in figure 3 and 4. Again, the positive template was 660 and 250 bp sequence-positive, whereas none of the peripheral blood samples were positive for these sequences. Similar results were shown in the normal control group.

Fig. 1. Image of the ethidium bromide stained gel of the 660 MMTV-like sequence in breast tissues PCR products. CP = Control positive; MW = molecular weight ladder. 100 bp bands are β globin gene fragments as the internal control.

Fig. 3. Image of the ethidium bromide stained gel of the 660 MMTV-like sequence in peripheral blood PCR products. CP = Control positive; MW = molecular weight ladder. 100 bp bands are β globin gene fragments as the internal control.

Fig. 2. Image of the ethidium bromide stained gel of the 250 MMTV-like sequence in breast tissues PCR products. CP = Control positive; MW = molecular weight ladder. 100 bp bands are β globin gene fragments as the internal control.

Fig. 4. Image of the ethidium bromide stained gel of the 250 MMTV-like sequence in peripheral blood PCR products. CP = Control positive; MW = molecular weight ladder. 100 bp bands are β globin gene fragments as the internal control.

Discussion

As the etiology of breast cancers remains unknown, it is important to verify observations of diverse breast cancer populations worldwide. Previous studies have purported to disclose an association between MMTV and human breast cancer. Recent reported associations between MMTV or HMTV and human breast cancer have shed new light on the role of MMTV in breast cancer [18].

Our results indicate that 660 bp and 250 bp DNA sequences that are homologous to MMTV *env* gene sequences are not present in the studied Iranian patients breast tumors and their analyzed blood samples and also in the normal control group. Both the ethnic background and age range of normal populations and breast cancer patients were quite similar.

Many previous studies have shown that MMTV-like sequences are detectable in mammary tissues of women

with breast cancer [15, 18]. An investigation by Zapata et al. [19], showed the presence of MMTV-like sequences in Mexican women. In that study, using specific primers for MMTV, 3 breast cancer cell lines and 119 breast cancer samples from Mexican women were tested. MMTV-like gene sequences were amplified in 5 of 119 (4.2%) breast cancer biopsy tissues but not in cell lines that had been used as a positive breast control in other reports. Evidence from paraffin embedded samples produced supporting data for 37% of breast cancers from Italy [20, 21]. These data were then highlighted in a study of the prevalence of MMTV DNA PCR positivity in breast cancer patients from Vietnam with 1% positivity and Australia with 42% positivity [22]. MMTV-like *env* sequences have also been detected in human breast samples from women in Australia, Argentina, China, Tunisia and United States [23].

Several of the most recent papers have, however, suggested significantly different interpretations for such discordant experimental findings. For example, Yin et al. [24] were only able to detect MMTV-like virus by PCR in one (~2%) of 60 breast cancers and, in a careful quantitative PCR study of 17 ductal carcinomas, tested in 7 different MMTV PCRs, none produced a positive PCR reaction [25]. Similarly, a laboratory was able to detect PCR amplicons of the expected size in 16% of 44 breast cancer patients but, upon DNA sequencing, all 110 DNA sequences turned out to be false positive, comprising host genomic DNA [26]. In a study in Sweden using a sensitive real-time PCR method, none of the blood leukocyte samples, cancer samples and controls were reported to indicate 1–10 copies of MMTV target DNA, and it was concluded that previous reports on MMTV in human breast cancers may have been due to unspecific (e.g. due to plasmid or amplicon contamination) PCR amplification [27]. Also in a recent report, no MMTV sequences were found in 42 specimens in tumor-enriched DNA using a sensitive test based on strong β globin nested PCR [28]. However Pogo et al. criticized that report, as the methodology was unable to amplify low copies of MMTV sequences. They claimed that their methods were based on a report by Wang et al. [14], which could show that one copy of plasmid containing 660 bp of MMTV-like *env* sequences was more sensitive [29].

In light of our finding, one might conclude that the methodology and sample sizes herein were not capable of detecting MMTV-like *env* gene sequences with low homology to HERVs in the 50 samples of Iranian breast cancer DNA. By using a sensitive PCR [29], we were unable to detect the MMTV-like sequences in blood DNA sam-

ples of 300 Iranian breast cancer patients. Therefore, the discrepancy between previous studies and the present study may arise from differing geographic distribution of etiologic agents, vectors, or clustering of populations with different infection of cancer susceptibilities, or may result from different MMTV diagnostic methods. The prevalence of MMTV in both feral mice and human breast cancer varies greatly between populations. Stewart et al. [30] proposed a zoonotic theory for MMTV as a viral cause of human breast cancer and suggested that the highest incidence of such cancers occurs in regions where *Mus domesticus* is the prevalent mouse species. However, some studies of Australian, Japanese and Chinese women revealed no association between MMTV or MMTV-related retrovirus and breast carcinogenesis [28, 29]. The absence of MMTV DNA in both breast cancer samples and controls indicates either that the concentration of putative MMTV or HMTV DNA in the breast cancers was too low for detection or that it did not exist there. It also suggests that MMTV-like viral expression in humans may be influenced by hormones [15, 23].

Conclusion

MMTV-like sequences were not detected in Iranian patients in Shiraz with breast cancer. We therefore recommend that probable association between MMTV-like sequences with development of breast cancer be interpreted with caution.

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