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Research article

Molecular Detection of Mouse Mammary Tumor-like Virus (MMTV-Like) in Breast Carcinoma for Iraqi Female Patients

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Abstract

Background: The mouse mammary tumor virus (MMTV)-like env gene have previously been reported to be present in some human breast cancers, however, their role in developing that cancers is still unclear.

Aim: This study aims to investigate the presence of MMTV-like env gene among Iraqi women who have breast cancers.

Methods: The prevalence of MMTV-like env gene and beta-globulin gene in formalin-fixed paraffin-embedded (FFPE) breast tissue specimens of 88 Iraqi women with breast cancers was detected using conventional PCR and Real- Time techniques. Eight benign breast tumors' specimens were also used as controls. Fisher's exact test was used to analyse data.

Results: MMTV-like env gene were detected in 16 (18.8%) specimens of those with breast malignancies, while they were not identified in benign breast tumors specimens. However, no significant difference between two groups was noted.

Conclusion: MMTV-like env and beta-globin genes were detected in Iraqi women with breast cancer, and further research is needed to study the role and association of these env gene with cancer development.

Keywords: Breast cancer; MMTV-like; Iraq.

1. Introduction

Breast cancer is the most often diagnosed cancer and the one cause of death from cancer in women worldwide (1). Mouse mammary tumor virus (MMTV) is a milk-transmitted retrovirus, belonging to the Betaretrovirus family, MMTV was formerly referred to as the Bittner virus, and previously called milk factor, relating to the extra-chromosomal vertical spread through adoptive nursing of murine breast cancer, shown by John Joseph Bittner while worked at the Jackson Laboratory in Bar Harbor, Maine, in 1936. Bittner developed the hypothesis that a milk factor, or cancer agent, Cancerous mothers might be spread to young mice by a virus in their mother's milk(2). MMTV causes breast cancer and lymphoma in mice(3). Also there is a mutation and overexpression in murine breast cancers associated with MMTV(4).In mice, MMTV expression is up regulated during pregnancy with the influence of steroid hormones(5)(6). env gene like to Mouse Mammary Tumor Virus (MMTV) were found in human breast cancer in multiple experiments from various geographical region, Many studies have been it was done in various countries of the world, and their results confirmed that there is a link between MMTV virus which infects mice and the virus similar to its with the sequence that was found in human samples. Former studies have demonstrated that MMTV-like env gene, which share at least 95% identity with MMTV, are highly expressed in human breast cancer(7)(8). The similarity of the Mouse Mammary Tumor Virus (MMTV) has been linked with the occurrence of breast cancer in humans with a range from 4.2% to 8.7% in Breast Cancers cases in several countries around the world, including: Mexico, Austria, Egypt and Saudi Arabia(9)(10)(11)(12), and other countries a range from 32.2% to 57.14% in Iran, Sudan, USA and Morocco(13)(14)(15)(16). All of these studies confirm the true prevalence of the MMTV-like env gene In many geographic regions of the world. In this study, we sought to discover and Determination positive results of MMTV-LIKE in Iraqi cities where breast cancer is common among Iraqi women, and to link this virus to the disease.

2. Materials and methods

2.1. Samples

Formalin-fixed paraffin-embedded (FFPE) tissue specimens of breast cancer patients were obtained from oncology laboratories at three Iraqi cities: Basra (n=22), Amara (n=44), and Najaf (n=22), during the period extended between May to November, 2020. In addition, eight FFPE tissue specimens of benign breast tumors were included as controls. Duration of storage of these samples ranged from 1 to 5 years at sample obtaining and patients' ages ranged from 22 to 59 years at sample collection. Research ethics committees of general health directorates at Basra, Maysan, and Najaf have all approved the current study and its protocols. As well, Çankırı Karatekin Üniversitesi has approved the project.

2.2. DNA and RNA isolation

In order to test our hypothesis, both DNA and RNA were isolated for further investigations. For DNA isolation, the commercial kit (G-spinTM, Korea) was used and its instructions were followed to extract DNA from thin tissue slices trimmed by microtome. To confirm DNA isolation, gel electrophoresis for each sample was performed. RNA was also isolated from all tumor, benign and malignant, samples using Trizol protocol (AccuZolTM, Korea).

2.3. Quantitation of extracted DNA

After extracting the DNA, concentration and purity of each sample was measured. DNA concentration and purity were quantified using spectrophotometer (BIORAD SpecPlus, USA). Then, all samples were kept at −20°C until the day of processing.

2.4. Detection of MMTV-like env gene by PCR

At the same time by using conventional PCR was performed to detect the beta-globin genes using of following primers Beta-globin-F5(5'-TAA GGT GAA GGC TCA TGG CAA-3'),Beta-globin-R5(5'-GCA GCT CAC TCA GTC TGG CA-3') and MMTV-like using DNA extracted with a fragment of following primers MMTV-F5 (5'-ATG GGT AGA ACC TAC WTG GTT CTG-3') and MMTV-R5 (5'-ATA AGG

RTA AGT AAC ACA GGC AGA-3'). table(2.1). all Primers for MMTV-like env gene and beta-globin gene were designed according to (Al Dossary *et al*, 2018). These primers amplify a fragment of 187bp of MMTV-like env gene highly conserved in the gene encoding the viral coat protein, Amplification reaction was performed in a total volume of 20µl, The amplification mixture contained 1µl of each primer, one-step master mix 3µl, and 4µl of DNA. For every reaction, a negative control in which DNA template was omitted from the amplification mixture, and a positive control MMTV DNA were included. The thermal cycler was programmed for 35 cycles with an initial denaturation at 94°C for 4min. Each cycle was performed with a denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 24 sec. At the end of the last cycle, the mixtures were incubated at 35°C for 3min. The amplified products were submitted to electrophoresis on a 2% agarose gel in TBE buffer at pH of (8.6) ,The gel was stained with Ethidium Bromide of 10 mg/ml 5µl in 100mL (TBE and D.W.). and the 187bp amplified bands were visualized on an ultraviolet transilluminator (UVP BioDoc-it Imaging System, GERMANY) to check for DNA amplification.

Table 1: List of PCR amplification and DNA primers.

Gene	Primer		Size of fragment resulted	Reference
MMTV	F	5'-ATGGGTAGAACCTACWTGGTTCTG-3'	24	(Al Dossary et al., 2018)
	R	5'-ATAAGGRTAAGTAACACAGGCAGA-3'	24	
β-globin	F	5'-TAAGGTGAAGGCTCATGGCAA-3'	21	
	R	5'-GCAGCTCACTCAGTCTGGCA-3'	20	

2.5. Detection of MMTV-like env gene by PCR (RT-qPCR)

Reverse transcription begins by reverse transcribing the desired RNA transcript into cDNA with the RTase, The cDNA that is first created is single stranded and is consecutively used as the template for PCR, The RT-qPCR technique is very useful because of its high sensitivity to low input RNA quantities, There are various types of primers that can be used for this process – oligo(dT) primers, random primers or gene specific primers, The primers that are used may target a known mRNA transcript that will lead to the detection of a desired transcript or simply may transcribe the entire RNA profile (Bachman 2013; Simpson and Brown 1995). The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA), Reverse transcriptases use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, That can be used directly as the Polymerase Chain Reaction template. This combination of reverse transcription and PCR(RT-qPCR) It facilitates the identification and development of the corresponding cDNA of low abundance RNAs in a sample, Enabling the cloning of reduced-copy genes.

In our project Reverse transcription was performed in (RT-qPCR) with fluorescent dye eva green probs, that bind to amplified gene product and give us a Ct value for the MMTV-like env gene. In pcr tubes a total volume of (20 µL), all containing (1µL of F primer,1µL of R primer, DNase 0.5 µL, EV 0.5 µL , RNA-free H2O 4.5 µL and RNA 3 µL), according the protocol (One-Step qRT-PCR, withG471One-Step BrightGreen qRT-PCR MasterMix.abm's ExCellenCT Lysis Kit). cDNA generate with the primers MMTV-F5(5'-ATG GGT AGA ACC TAC WTG GTT CTG-3') and MMTV-R5 (5'-ATA AGG RTA AGT AAC ACA GGC AGA-3'), Primers for MMTV env gene amplification were designed according to (Al Dossary et al, 2018).

2.6. Data analysis

As percentages, numerical data were presented. In addition, Fisher's exact test (GraphPad Prism 8.0.1, GraphPad, US) was used to assess the importance of of differences between groups' findings.

4 Result

4.1 Results of extracted DNA

To make sure the accuracy and quality of extracting DNA of all samples are checked by detection of Beta-globin gene, where as the result of this gene confirms that presence of DNA in our positive samples (fig.1) shows samples of Beta-globin genes from 27-42 malignant cancer and N represent for negative control without sample. Either result of MMTV-like env gene were as follows: in (fig.2) show positive results for s2 and s5 other samples of malignant and benign show negative results. (fig.3) show positive results for s13,s15,s16,s17,s18,s19,s21,s23,s24,s25 other samples malignant show negative results and N represent benign samples show negative results. (fig.4) show positive results for s31,s33,s38,s39 other samples malignant show negative results and N represent benign samples show negative results. Thus the total samples of a positive 16 of 88 cancer samples (18.18%), the PCR reactions was repeated to confirm the results.

4.2 Results of extracted RNA

cDNA from RNA produced by Real time PCR with used eva green probs, the RNA extraction and real time amplification was applied only to the (16) samples that were positive in the previous phase of DNA, also the existence of the Beta-globin gene was for the purpose of confirming the purity and accuracy of extraction and they were positive for all 16 samples, MMTV-like env gene has been attended by Real time PCR and (fig.5) represents the results of amplifications of DNA, where the result was also positive for all samples under the test compared with negative control and samples control .

Fig.(4.1) shows results of electrophoresis for samples of Beta-globin genes from 27-42 malignant cancer and N represent for negative control without sample .

Fig.(4.2) shows results of electrophoresis for samples of MMTV-like env gene s2 and s5 malignant cancer, and N represent for negative control without sample .

Fig.(4.3) shows results of electrophoresis for samples of MMTV-like env gene from s13,s15,s16,s17,s18,s19,s21,s23,s24,s25 malignant cancer and N represent benign samples show negative results.

Fig.(4.4) shows results of electrophoresis for samples of MMTV-like env gene s31, s33, s38, s39 malignant cancer and N represent benign samples show negative results.

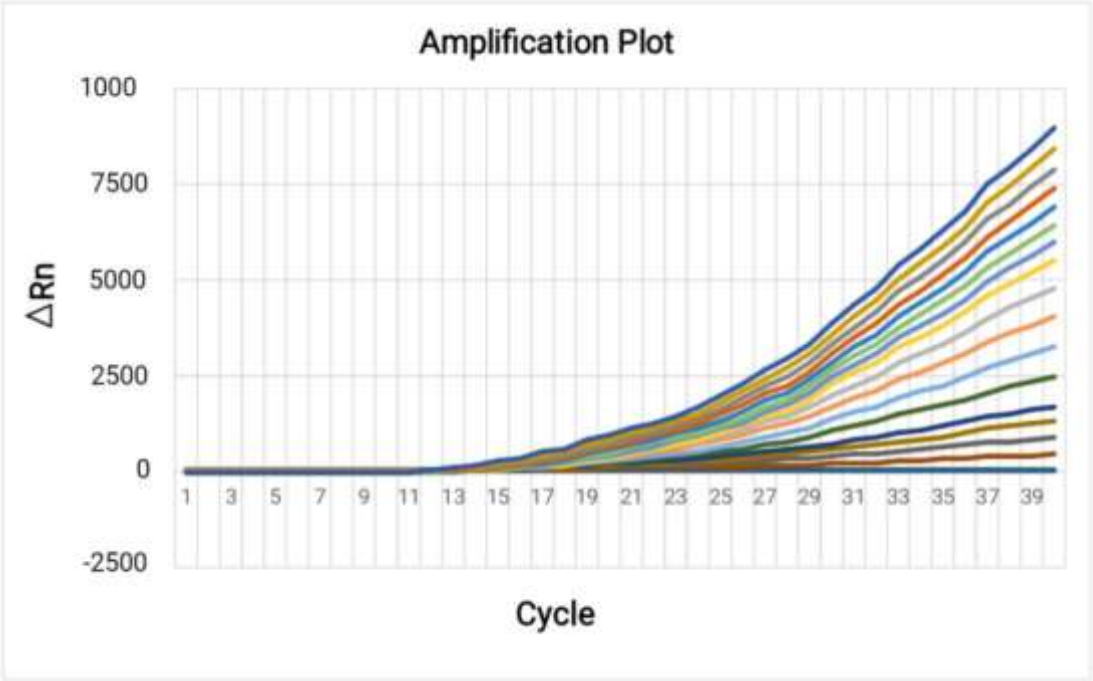

Fig.(4.5) This figure shows quantitative PCR result were plot represent amplification acquired from eva green probes and the test was applied to the DNA samples that were used in conventional PCR and which were positive, each of the lines in the curve represents a positive pathology sample (malignant cancer). Negative pathology sample (benign cancer) and negative control (without sample).

DISCUSSION

The association between MMTV infection and breast cancer, and the differential regional prevalence of breast cancer that is well in line with the prevalence of the mouse population, led some researchers to use these interrelationships to support the idea that MMTV may be considered a significant pathogen implicated in the growth of breast cancer in areas with a higher mouse population. high homology between human and mouse in view of identical responses of humans and mice to immunosuppression It is hypothesized that humans receive MMTV from rodents, and the analogous trend of human BC occurrence and mouse ranges. If followed by more scientific data, this zoonotic hypothesis for the mouse-viral origin of human BC may be of significant significance in the prevention of breast cancer, at least in some cases(17). detection of MMTV env gene is variable in different regions of the world. In addition to the importance played by this virus in breast cancer, the reason for that geographic variation and the type of transmission are not well known. However, data suggesting the interaction of this virus with breast cancer risk is accumulating. In reality, conducted a comprehensive search of several datasets to investigate this relationship and discovered that MMTV like virus was present, In western countries, the virus is associated with an elevated risk of developing breast cancer and is slightly higher than in Asian countries (18). In addition, in breast biopsies of Australian women before and after breast cancer, MMTV-like env gene were identified, a criterion that satisfies a potential causal association between the virus and breast cancer (18). In olden times , MMTV appears to have leapt into the human population by Zoonotic transmission from mice and retained itself by utilizing those transmission paths , two routes of transmission, saliva and milk, have been reported, In breast cancer patients, healthy adults and infants, but not in newborns, MMTV DNA was found in saliva , In the same study, the discovery of MMTV RNA in saliva was documented in patients with breast cancer and their salivary glands, indicating saliva as a human transmission route (19). In the milk of healthy lactating women (20)(21). and lactating women at high risk of developing breast cancer (21). MMTV have also been found , indicating another path of MMTV transmission among humans. The results that appeared in our project are higher than the results of some countries such as Saudi Arabia, Mexico, Australia and Egypt, While the results of our research compared to other countries of the same study showed that they are lower than That

countries, as in the United States of America, Morocco, Iran and Sudan, as follows: Saudi Arabia 8.7% (12). Mexico 4.2% (9). Australia 5% (10). Egypt 6.7%(11). USA 45% (15). Morocco 57% (16). Iran 32% (13). Sudan 36% (14). All these results are evidence that this env gene is actually widespread in most countries of the world. like-Although the results we obtained indicate the prevalence (18.18%) of the MMTV env genein our samples, now we do not hide that some of the samples taken had old dates, which led to difficulty in isolating and extracting the nuclear material, on the her hand, the lack of some modern devices in Our laboratories, these reasons may ot have led to the emergence of some negative results in our study.

Conclusions

For the first time in Iraq the MMTV-like env gene was confirmed in three Iraqi cities, Basra, Maysan and Najaf for women with breast cancer. Although there were no statistically significant correlations between virus infection and BC, the high prevalence for the positive results where that16 samples from 88 tested (18.18%) of MMTV-like env gene in our samples indicated that MMTV infection may be a contributing factor in the development of breast cancer in Iraq. Thus, more detailed studies are needed at the country level by examining larger carcinogenic samples, as well as the use of fresh samples in order to avoid the nuclear material from preservatives such as formalin and paraffin wax. Methods for extracting DNA and RNA must be far from pollutants and be done with high accuracy, and it must be Make sure of the sample taken from the sources that you have breast cancer with certainty to investigate the extent of the spread of this virus in other areas of the country in order to be more able to accurately assess its relationship with breast cancer in Iraq.

Authors' contribution

Author HA was mainly involved in planning, studying and designing the project, obtaining funds, writing and publishing research as well as providing patient samples and data. Co-author ME participated in the design of the practical part, testing of

samples, and validation of results. Co-author MF has been involved in planning, studying, designing the project and verifying results. All authors reviewed and approved the study.

Conflict of interest

Authors have no conflict of interest.

Funding resources

Nil.

Abbreviations

MMTV-like env gene: Mouse Mammary Tumor Virus like envelope gene; FFPE: Formalin fixed paraffin embedded; MMTV : Mouse Mammary Tumor Virus; HMTV: Human Mammary Tumor Virus; PCR: polymerase chain reaction; RT-qPCR: quantitative real time polymerase chain reaction.

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Ethical approval

All mentioned institutions have approved the practical part of the current study and protocols used.

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