http://doi.org/10.36582/j.Alkuno.2023.06.02
Al-Kunooze University College
Journal homepage: http://journals.kunoozu.edu.iq/1/archive &
http:// www.iasj.net

Frequency of Human Mammary Tumor Virus in Breast Tumor Patients from Basrah , South Iraq

Hanaa Ali Naeem[a], Wijdan Nazar Ibraheim[b], Saad Abdulbaqi Abdullah Alomar[c]

College of Medicine, University of Basrah , Iraq [a,b,c]

Corresponding Email: hanaaalinaeem@gmail.com

Abstract

Breast cancer is one of the most frequent malignancies in women globally, but its causes is still obscure, and it remains a major public health problem. Human mammary tumor virus(HMTV) is the name given to sequences that have been identified in multiple investigations from various geographical regions in human breast cancer. However its role in the development of breast cancer is not well known. The aim of this study is to detect the frequency of HMTV envelope gene in malignant and benign breast tumor tissues in Basrah women. For Detection of HMTV envelope proviral sequences, two techniques were used the real-time PCR and Nested PCR using primers.

A total of 80 samples of formalin fixed paraffin embedded tissues(FFPET)were collected from females aged ranged from(17 to 77) years comprising as (60) malignant tumors and (20) benign tumors, were examined by qPCR technique. The results showed that 23 (28.8%) out of the (80)samples were positive for HMTV. Out of the (60) malignant tumors, 15 (25%) were found positive for HMTV, and 8 (40%) from benign tumors gave positive results for HMTV env. gene. While all samples re-examined by nested PCR , only two samples gave positive results for HMTV envelope gene . No significant associations were found between viral positivity and each of the age, grade, and molecular subtypes of breast cancer. Conclusion:

* *Corresponding author*. Tel.: +0-000-000-0000 .
E-mail address: author@institute.xxx

HMTV env genes were detected in Basrah women with breast malignant and benign tumors, this indicates that infection with HMTV may have a role in breast cancer development.

Keywords
Human mammary tumor virus, breast tumor, Real-time PCR.

1. Introduction

Breast cancer is one of the most frequent malignancies in women globally, but its causes is still obscure, and it remains a major public health concern as the main cause of cancer mortality in women. In the past three decades , breast carcinoma remains the most prevalent malignant tumor nationwide , every year about (2.09 million) females are diagnosed with breast cancer and 627 000 die from this disease (1).

Breast cancer starts, progresses, and develops as a result of internal and external influences. Viruses like the Mouse Mammary Tumor Virus(MMTV), and other environmental factors, in particular, can have a significant influence on the development of human breast cancer . Several investigations have also shown that virus identical to MMTV, known as (MMTV-like virus), also known as Human Mammary Tumor Virus (HMTV), could be a risk factor for development of human breast cancer(2).

In 1936, a viral infectious pathogen known as a "milk factor," discovered by John Bittner, could be passed via milk from mouse mothers suffering mammary tumors to their pups, who eventually developing breast cancer as adults (3). HMTV is 90% to 95% homologous to Mouse Mammary Tumor Virus, the etiological agent of mammary tumors in mice. Several reports indicate that HMTV sequences is associated with breast carcinoma and is not present in the normal breast tissues, these Retroviruses sequences were detected in about 39% of human breast cancer and subsequently, entire proviral structure with 95% homology to MMTV were amplified from two separated human breast carcinomas, while these sequences are not detected in normal mammary tissue specimens from the same breast that contained the tumor(4). For MMTV-like (HMTV) gene sequences identification in

breast cancers several methods have been used such as hybridization techniques, in situ PCR, standard liquid PCR , microdissected PCR and whole genome sequencing ,and using of various techniques for the HMTV detection in human breast cancer is essential as this eliminates concern about contamination and false positive outcomes (5).

HMTV is a Beta RNA Retrovirus that is closely linked to the Mouse Mammary Tumor Virus (MMTV). In, 2001 , Liu. et al amplified the entire proviral containing a typical structure of a Retrovirus long terminal repeat (LTR), group specific antigen (gag), capsid region, polymerase (pol), reverse transcription region, and envelope (env) from two(env) positive human breast carcinomas and they succeeded in amplification of overlapping env- LTR, LTR-gag, gag-pol, and pol-env segments, and this provirus with molecular weight of 9.9-kb has 95% homology to MMTV but only 57% of both gag and pol genes(3.5 kb) are identical to the human endogenous retrovirus K10 (6).

provirus LTRs comprise all of the enhancer and promoter components characteristic of a virus capable of replication, in addition to the open reading frame coding for the super-antigen and the glucocorticoid- responsive element. HMTV(MMTV-like) promote Breast cancer by the insertional mutagenesis mechanism which leads to uncontrolled cellular proliferation and then to cancer beginning and progression (6). The most reasonable way for MMTV to spread among humans is through human saliva. Among women with a higher risk of breast cancer than normal ,the prevalence of MMTV in human milk is significantly higher . This shows that human breast milk is a possible means of MMTV transmission(7). While human to human saliva is the most likely means of MMTV-like transmission, but in many countries additional ways of transmission are also possible, such as consumption of uncooked cereals and other foods which contaminated with mouse or rat fecal materials(8) . MMTV-like gene sequence has also been detected in breast tumors in dogs and cats. Women living with companion dogs have twice the risk of BC as expected, indicating that MMTV can be transmitted to humans in dog saliva(9). In this study, we investigated the presence of the HMTV env gene among Basrah Breast Cancer patients and correlated between HMTV gene and

clinicopathological parameters of BC.

2. Methods

2.1-Sample collection and DNA extraction

This retrospective case-control study included paraffin embedded blocks of tumor samples from 60 Iraqi women patients diagnosed with primary breast cancer and 20 cases of benign tumors of breast. All of them had diagnosed by oncologist (physician) and operated upon. The FFPT of the patients were collected from histopathology unit in Al-Sadder teaching hospital, and from privet histopathology laboratory in Basrah city during the period between October 2020 to October 2021 Ages of patients range from 25 to 77 years with a mean of (52.4) and the ages of control group ranges from 17 to 45 years with mean of (26.2). Information included the patients name, age , histological types of the breast cancer, molecular types of BC and grades were obtained from patients reports. All Samples were cut into10 um thick sections and the blade cleaned between sections to avoid contamination, and the slices were put in to expand off tubes for DNA extraction and stored at deep freeze until work done.

The genomic DNA was extracted from formalin-fixed paraffin-embedded tissue by using the QIAamp DNA FFPE Tissue Extraction Kit (catalog no. 56404) manufactured by Qaigen company and arranged by guideline of manufacturer. When the DNA was extracted, verified its presence and concentration by utilizing the QuantiFluor dsDNA System (Cat.# E2670)and using Quantus Fluorometer (Cat.# E6150 / promega/ USA)),which estimated DNA concentration (ng/µl) .

2.2- Real Time PCR analysis

For the identification of the specimens positive for the envelope (env)gene of Human Mammary Tumor virus (MMTV like) , primers and probe were designed based on previously published sequences from the entire C3H MMTV (GenBank AF033807) and human genomes (AF346816,) and the envelope gene of the C3H strain (GenBank AF228552) was utilized as a positive control. and free nuclease water as negative control (Cedro et al. 2014) were used.

Table 2.1 – Primers and probe

	Sequence (5'-3')	**Location**
Foreword	5'-AAGGGTGATAAAAGGCGTATGTG-3'	5943–5964
Reverse	5'- TTTTGTATTGGCCCCTGAGTTC-3'	5990–6011
Probe	5'-FAM-AACTTTGGTTGACTACCTT-MGB-3'	5969–5986

The PCR run was set up by utilizing Go Taq probe qPCR Master Mix with(Cat# A6100) and this master mix done by organization guidelines as following procedure:

PCR master mix	**Volume**
DNA Template	4µl
Foreword primer(10µm)	1µl
Reverse primer (10µm)	1µl
Probe (10µm)	1µl
Nuclease free water	3µl
Master mix	10µl
Total volume	20µl

The tubes were transferred into a thermal cycler pre-warmed. The accompanying table display cycling conditions:

Table (2.2) –Real Time PCR program

PCR step	**Temp.**	**Time**	**Repeat**
Initial denaturation	95 C◦	10min.	1
Denaturation	95C◦	15 second	50cycles
Annealing and extension	58C◦ 60C◦	20 second 30 second	

2.3- Nested PCR for HMTV env gene detection

All 80 samples examined by nested PCR using following primers were synthesized according to the reference (Cedro, et al ,2014) .

Table 2.3- Nested PCR primers

Primer Round	**Primer**	**Sequence (5'-3')**	**Product**
First Round	Foreword	CCTCACTGCCAGATC	660bp
	Reverse	GAATCGCTTGGCTCG	
Second Round	Forward	TACATCTGCCTGTGTTAC	250bp
	Reverse	ATCTGTGGCATACCT	

A fragment from HMTV env gene of purified genomic DNA was amplified over two rounds of nested PCR using outer and inner primers (Table 2.3) .The products was amplified according to PCR reaction mixture which is consist of 12.5 µl of master mix and 1µl of each of foreword and reverse primer and 5 µl DNA template and 5.5 µl of nuclease free water .following table show program was used for amplification. Then reaction products are analyzed by agarose gel electrophoresis in a 1% agarose gel.

2.4- Nested PCR conditions

Steps	**Temperature**	**Time**	**Cycles**
Denaturation	94	3 min.	1
Denaturation Annealing Extension	94 52 72	1.5 min. 2 min. 3 min.	50

3. Results

3.1- Age Distribution of the study population.

The age distribution of the (60) females with primary breast cancer and (20) with benign breast tumor was summarized in table (3.1).

Table 3.1-Age distribution among study population

Age	Breast cancer		Benign tumor		Total
	No.	%	No.	%	
35 ≥	8	13.33%	16	80%	24
36-55	24	40%	4	20%	28
≤ 56	28	46.6%	0	0%	28
Total	60	100%	20	100%	80

3.2- HMTV detection by real time PCR

A total of 80 cases of breast tumors from females with age ranged from(17 to 77) years with a mean of (46.03) and Std. Deviation 16.43 , comprising as: (60) malignant tumors and (20) benign tumors, were examined by qPCR technique. The results showed that 23 (28.8%) out of the (80)samples were positive for HMTV .out of the (60) malignant tumors, 15 (25%) were found positive for HMTV, and 8 (40%) from benign tumors gave positive results for HMTV env. gene (Table 3.2).

Table (3.2) – Frequency of HMTV among study population.

Parameter		Group		Total	P value
		Patient	Control		
HMTV	Positive PCR	15	8	23	0.199
		25.0%	40.0%	28.8%	
	Negative PCR	45	12	57	
		75.0%	60.0%	71.3%	
Total		60	20	80	
		100.0%	100.0%	100.0%	

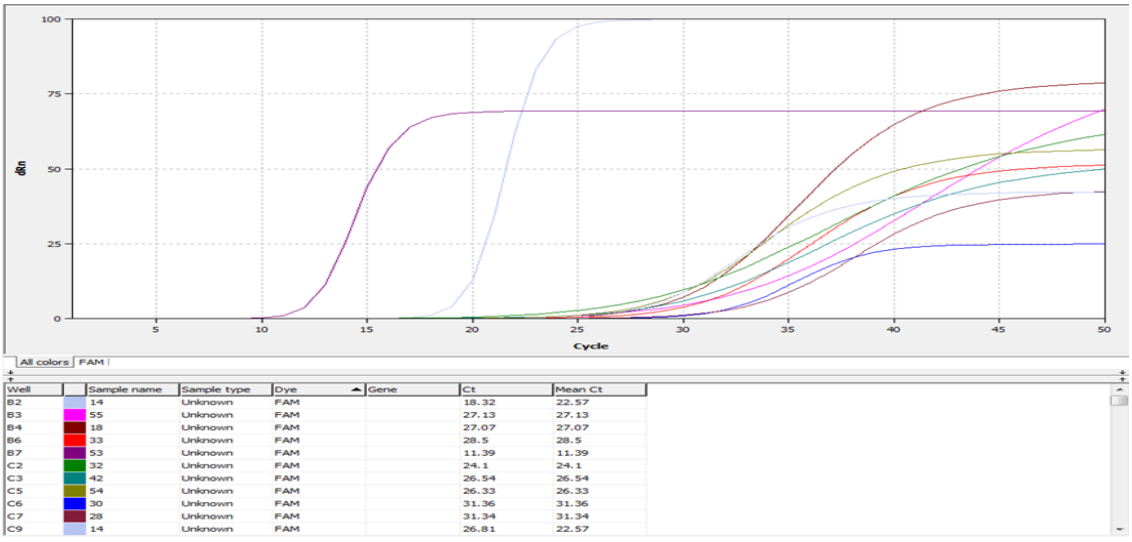

Figure 3.1- This plot illustrate amplification achieved using Taq Man probe and positive control and test DNA samples.

3.3- HMTV detection by nested PCR

The HMTV env gene sequence was screened in the genomic DNA from 60 breast cancer specimens, 20 breast benign tumors specimens using Nested PCR method . In all 80 specimens, the PCR products of expected size(250 bp) were amplified(weak band) from only Two specimens as shown in (fig. 3.2)

Figure 3.2- a 250 bp PCR product

Table (3.3)- Distribution of HMTV positive Result according to age groups.

Group	Age groups	HMTV		Total	P value
		Positive	Negative		
Patient	≥ 35 years	2	6	8	1.000*
		13.3%	13.3%	13.3%	
	36 - 55 years	6	18	24	
		40.0%	40.0%	40.0%	
	≤ 56	7	21	28	
		46.7%	46.7%	46.7%	
	Total	15	45	60	
		100.0%	100.0%	100.0%	
Control	Thirty five years or younger	6	10	16	1.000**
		75.0%	83.3%	80.0%	
	From 36 to 55 years	2	2	4	
		25.0%	16.7%	20.0%	
	Total	8	12	20	
		100.0%	100.0%	100.0%	

* Chi-Square / ** Fisher's Exact Test

Table (3.4) –Association of HMTV with molecular subtypes

Molecular subtypes	HMTV		Total	P value
	Positive PCR	Negative PCR		

Luminal A	4	20	24	0.319
	26.7%	44.4%	40.0%	
Luminal B1	3	7	10	
	20.0%	15.6%	16.7%	
Luminal B2	2	2	4	
	13.3%	4.4%	6.7%	
Her2 over expression	2	1	3	
	13.3%	2.2%	5.0%	
Basal like	3	9	12	
	20.0%	20.0%	20.0%	
None	1	6	7	
	6.7%	13.3%	11.7%	
Total	15	45	60	
	100.0%	100.0%	100.0%	

* Fisher's Exact Test

Table3.5- Association of HMTV positive with hormone receptors status and Grade

Variables		**HMTV**		**Total**	**Sig.***
		Positive	Negative		
Estrogen receptor	Positive	10(66.7%)	32(71.1%)	42(70%)	0.754
	Negative	5(33.3%)	13(28.9%)	18(30%)	
	Total	15(100%)	45(100%)	60(100%)	
Progesterone	Positive	10(66.7%)	31(68.9%)	41(68.3%)	1.000
	Negative	5(33.3%)	14(31.1%)	19(31.7%)	

	Total	15(100%)	45(100%)	60(100%)	
Her2	Positive	5(33.3%)	9(20%)	14(23.3%)	0.309
	Negative	10(66.7%)	36(80%)	46(76.7%)	
	Total	15(100%)	45(100%)	60(100%)	
Histological grade	Grade1	0	1	1	0.708
	Grade 2	15	45	58	
	Grade 3	0	1	1	

* Fisher's Exact Test

4. **Discussion**

Breast cancer is complicated disease more likely caused by the interaction of genetic susceptibility , environmental exposures, hormone levels, as well as health behaviors, the contribution of viral infection to development and progression of mammary carcinogenesis remains unclear. MMTV has been involved in development of mammary cancer in mice (10), although its contribution in the pathogenesis of human breast carcinoma has long been suppose but has never been confirmed. Furthermore , several studies propose that the incidence of human breast cancer is geographically associate with the natural ranges of different species of mice (11; 12). The prevalence rate of HMTV sequences in human breast carcinoma ranges from(6 - 78) percent, with commonly prevalence being about 40 percent, in comparison to low percent in human breast normal and benign tissues (5). The current study showed that (28.8%) of total number of breast tumor cases gave

positive results to HMTV divided as (25%) malignant tumors and (40%) benign tumors which comparable to results carried out by Hasan *et al* (13) in 1raq which detected HMTV env gene in (18.8%)specimens of Iraqi women with breast carcinoma. In agreement with study performed in Australia in 2017 which detected HMTV sequences in 24% of benign breast tumors and 36% of BC samples and of these Six HMTV positive benign tumor samples, five eventually developed HMTV positive cancer(14). The virus env gen was detected in benign specimens as well as malignant cases in the current study, which could be attributed to the small sample size of the control group (benign) and the low concentration of viral DNA in tissue specimens. It is especially difficult to obtain consistent results from PCR analyses when retroviruses are present in extremely low viral concentrations. The fact that the MMTV-like(HMTV) viral load increases as breast cancer advances but decreases in late stage of cancer is another reason for higher detection of virus in benign in more than breast cancer. The decrease in viral load appears to be the result of a breakdown in cell physiology(15).

The presence of HMTV in benign and malignant tumors indicates that virus infection contributes to the formation of breast tumors and its presence in benign breast tumors indicate an increase in risk of breast carcinoma over time when combined with other carcinogenic factors such as genetic possibility, weakened immunity due to disease or aging, and other carcinogenic exposures, as demonstrated by Lawson and Glenn, 2017 and Nartey et al, 2017(14) , studies that found HMTV (MMTV-like) in human breast normal tissues and benign tumors 1 to 11 years prior the development of HMTV positive BC in the same patient women. Many investigations, such as those conducted in Japan and Iran countries, have been unable to demonstrate the prevalence of HMTV sequences in cancer tissue specimens (16,17). This disparity could be due to differences in detection methodologies or tissue heterogeneity.

In the current investigation, real-time PCR and nested PCR were utilized as two molecular approaches to identify HMTV in breast tumor tissues. When comparing the findings of both methods, we observed that 23 samples had positive real-time PCR results for HMTV. Only two cases, though, tested positive for HMTV using nested

PCR, and those two cases also tested positive using real-time PCR. In general, our findings show the high sensitivity of the Taq Man-based PCR test utilizing specific primers for HMTV diagnosis. This assay is quick and easy to perform, as compared to nested PCR and it has fewer risk of contamination.

As regards correlations between HMTV env positive specimens and clinicopathologic characteristics. Wang et al in 2014 ,found no correlation between HMTV env and age, hormonal status (ER, PR, HER2, TNBC), or tumor grade in their meta-analysis. Agree with these researchers, this study did not found association between HMTV env in tumor samples and clinicopathological characteristics in the general study population, similar findings have been described in Egypt (18). Current study showed there is no statistical significant difference was noted in the prevalence of HMTV between molecular subtypes of BC. This finding disagree with a study done in china which found that the presence of HMTV env sequence was significantly correlated with HER-2 expression in breast cancer tissues (19).

In conclusion HMTV env genes were detected in Basrah women with breast malignant and benign tumors , this finding indicate that infection of HMTV may increase risk for breast cancer development. and Real time PCR is more reliable and sensitive than nested PCR for proviral detection in human genomic DNA because low viral concentration in FFPT samples.

Acknowledgments

The authors would like to thank Miss Haneen and Dr. Hussam for their technical assistance.

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