The prevalence of Human Mammary Tumor (HMTV) sequences in Breast carcinoma tissues and its association with the *P53* expression in Basrah Province

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Abstract

Background Although it has previously been revealed that a virus related to Mouse Mammary Tumor Virus (MMTV) is found in some human breast tumors, their causal role in oncogenesis is still unclear. **Aim** The purpose of present study is to detect HMTV (MMTV-like) in breast tumor tissues from women in Basrah province who have primary malignant and benign breast tumors, as well as to analyze the rate of concordance translational expression of the *P53* with HMTV in breast tissues. **Methodology** The presence of env gene of HMTV in breast tumors tissue (FFPT) samples of 60 women with primary breast cancers was identified using a TaqMan probe by Real time PCR method. Twenty specimens from benign breast tumors' were used as control group. The expression of *P53* was examined by immunohistochemical staining technology. From the patients' reports, information such as their name, age, histological and molecular types of BC, and tumor grades were recorded. Data was analyzed using the Chi square and Fisher's exact test. **Results** The Human Mammary Tumor provirus was found in 15 (25 %) of breast carcinoma samples and 8 (40 %) of benign breast tumor samples. But, there was no statistical significant difference between the two groups ($p > 0.05$). In a total of 80 samples of malignant and benign tumors, the expression level of *p53* was 55 % and 15%, respectively. Reduced *P53* expression was statistically associated with the prevalence of HMTV infection, with a significant difference (P value =0.031). **Conclusion** HMTV provirus were found in Basrah women with breast primary malignant and benign tumors and significantly associate with loss expression of *p53*. Further studies of large numbers of cases is required to investigate the etiology and association of this virus infection with carcinogenic development.

Keywords: HMTV, Basrah, p53. Breast cancer

1. Introduction

Breast cancer is one of the most frequent diagnosed cancers in women around the world, although the causes of this disease remains controversy and it remains a public health problem as the major cause of death in women. Breast cancer will be diagnosed in 2.3 million women in 2020, accounting for 11.7 percent of all cancer cases and 685,000 fatalities, accounting for 6.9% of all cancer deaths in women [1]. The role of virus infection in the beginning and progression of breast carcinogenesis is unknown [2].

Breast cancer is the most common cancer type among adult females in Basrah, and it occurs at least a decade younger in women from southern Iraq than in the western world, with over 60% of cases occurring in the 40-59 year age group [3]. Poor survival is significant associated with advanced stage and grade of the cancer, in Basrah the 3-year survival average of females with BC was similar to that in several developing countries. Despite being poorer than that in developed countries [4]. Mouse Mammary Tumor viruses (MMTV) derived from genus *Beta-retrovirus* have been linked to mammary cancers in both feral and experimental mice [5]. John Bittner was the first who discovered a pathogenic factor and he named its as "milk factor" that could be passed in milk from infected mice mothers to their pups, they eventually developed mammary cancers as adults [6, 7].

These virus molecules were later specified as RNA *beta-retrovirus* which named as Mouse Mammary Tumor Virus. Many experimental studies from different geographical areas have found sequences similar to the MMTV envelope gene in human breast tumor, and their findings have proven that there is a link between the MMTV virus that infects mice and the virus that is similar to it was detected in human tumor specimens [8]. Retrovirus sequences with 90–95 percent similarity to MMTV were discovered in 39 percent of human breast tumors in 1955. Then the complete structure of provirus in two samples from human breast tumors was amplified in the United States whereas these sequences were not detected in normal breast tissue in the same breast that harbor tumor. Recently the retrovirus with MMTV-like sequence was named (HMTV) Human Mammary Tumor Virus [9]. Until now, the evidence of causal role of HMTV in the development of BC is controversial [10]. HMTV sequences were also found in other human biological samples like milk, saliva, and lymphocytes, indicating that the transmission of virus in humans is similar to that observed in mice, and it infect the lymphocytes of mucosa before reach the mammary tissue [11].

For HMTV sequences detection in BC, several methods

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have been used such as hybridization techniques, in situ PCR, standard liquid PCR, microdissected PCR and genome sequencing, the use of a variety of approaches for detecting HMTV in human breast cancer is essential since it reduces problems regarding contamination and false positive results [12]. *P53* was the first gene to be recognized as a tumor suppressor, and first described in 1979, initially thought to be an oncogene. *P53* acts to eliminate and suppress abnormal cell proliferation, hence preventing neoplastic growth. In mammalian cells, the *p53* protein serves an important function in maintaining genetic integrity, and the *p53* gene is inactivated in human malignancies [13]. More than half of all human malignancies have *p53* mutations, and mutant *p53* is predictive marker in numerous cancers. The mutations of *P53* have been connected to poor prognosis and disease-free survival in BC and have also been linked to anticancer therapy resistance [14, 15]. Other known mechanisms affect *p53* function in a large percentage of tumors without mutations. This may occur via interference with virally encoded proteins in virus-associated cancers, leading to sequestration or enhanced *p53* degradation [16]. Onco-suppressor protein mutations are uncommon in viral-related malignancies. Moreover, carcinogenic viruses interfere with activity of *p53* in these malignancies through a variety of direct and indirect mechanisms, including binding of viral oncoproteins to *p53*dirctly, p53 phosphorylation due to viral kinases, activation of MDM2,which is a negative regulator of *p53 [17].*

Oncogenic viruses produce many onco-proteins have the capacity to inactivate the *p53*, causing dysregulation of many regulated genes of *p53*, such as those contributed in apoptosis, DNA integrity, and cell growth. The BZLF1,E6,NS5 oncoproteins from the EBV, HPV and the hepatitis C virus respectively, all these have been found to bind to and cause degradation of *p53*. Other viral oncoproteins like HBx and Tax from hepatitis B virus and HTLV-1 cause *p53* activity inhibition by modulating nuclear factors, whereas other oncogenic viruses weaken *P53* resulting reduction in its levels in infected cell such as Kaposi's sarcoma herpes virus (HHV8) and Merkel cell polyomavirus (MCPyV) [18].

2. Methods

Patients and Tissue samples

This retrospective case control study included (FFPET)paraffin embedded blocks of tumor samples from 60 Basarh women diagnosed with primary breast carcinoma and 20 cases of breast benign tumors. All of them had diagnosed by oncologist (physician) and operated upon. The FFPET samples of the patients were collected from the histopathology unit in the Al-Sadder teaching hospital, and from the privet histopathology laboratory in the Basrah city during the period between (October 2020 to October 2021). All of the histological data included in the analysis was taken directly from patients' original pathology reports. The information included the patients name, age, histological types of breast cancer, molecular types of BC and grades. About 10 um thick sections of all Samples were cut and slices were put in to (1.5) eppendroff tubes for DNA extraction. For immunohistochemistry staining, four micrometer sections were cut on Apex bond IHC charged slides and stored until work done.

DNA extraction and estimation of DNA concentration

The human genomic DNA was extracted from FFEP tissue specimens using the Extraction Kit (QIAamp, catalog no. 56404) from Qaigen company and arranged according to the manufacturer's instructions. When the DNA was extracted, its presence and concentration was confirmed by using The QuantiFluor dsDNA System (Cat. # E2670) and Quantus Fluorometer (Cat. # E6150 / promega/ USA)),which estimated DNA concentration (ng/µl).Then the extracted DNA samples was stored at deep freeze(-35) until PCR work done.

Real Time PCR analysis

In this study Primers and probe were designed according to [19] based on the reported sequences of the whole C3H MMTV and human genomes (GenBank AF033807 and AF346816, respectively) for identification of positive cases for the Human Mammary Tumor virus envelope gene and we used the MMTV env gene from the C3H strain (GenBank AF228552) as a positive control,while free nuclease water was employed as a negative control [20] as following: Foreword 5'-AAGGGTGATAAAAGGCGTATGTG-3'loction (5943–5964) Reverse 5'- TTTTGTATTGGCCCCTGAGTTC-3' location(5990–6011). Probe 5'-FAM-AACTTTGGTTGACTACCTT-MGB-3' location (5969–5986). The total volume of PCR reaction mix contained a (10 µl of qPCR Master Mix with (Cat# A6100); 4µl of DNA template; 1 µl of each primer and probe, and 3 µl nuclease free water. The thermocycler conditions for PCR are shown in Table (2.1). The examination of a threshold cycle number (Ct value) that displayed positive amplification in Real-Time PCR cycle number was used to analyze Real-Time data.

Table (2.1) –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	

Immunohistochemistry

Immunohistochemical labeling was carried out in accordance with the manufacturer's guidelines on serial 4µm-thick sections from the FFPT blocks mounted on charged slides and subjected to IHC staining using Bond Max (Leica Microsystems, Germany). In brief, 4µm FFPT sections were dewaxed with BOND Dewax Solution (AR9222. Leica, Germany). Antigen retrieval was carried out for 20 minutes using the BOND Epitope Retrieval Solution ER2 (Leica Microsystems). By using streptavidin-biotin-peroxidase complex method (Bond Polymer Refine Detection kit with cat # DS9800, Leica Microsystems), the sections were treated for 15 minutes

with anti-human *p53* mouse monoclonal antibody p53 (DO-7; PA0057, Leica, Germany). The nuclei were then counterstained with hematoxylin in a BOND-MAX automatic slide stainer.

3. Results

Molecular detection of HMTV

A total of 80 cases of breast tumors from females with age ranging from(17 to 77) years with a mean of (46.03) and Standard deviation 16.43[figure 2.1], including 60 malignant tumors and 20 benign tumors, were examined by real time PCR technique. The results showed that 23 (28.8%) samples divided as: 15 (25%) from malignant tumors, and 8 (40%) from benign tumors gave positive results for HMTV *env* gene [Table 3.1].

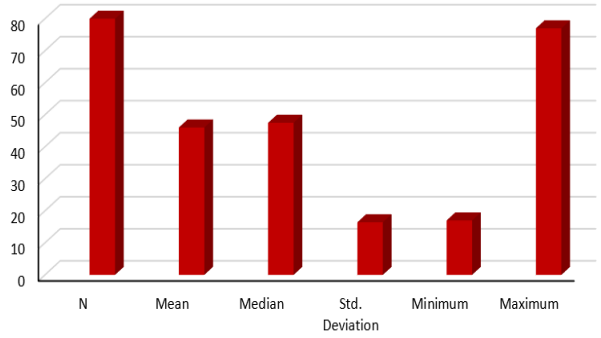
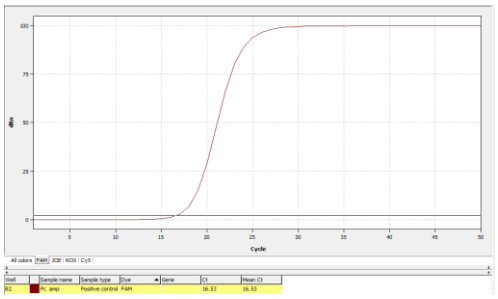


Figure (3.1)-Age distribution in study population

Table (3.1) – Frequency of HMTV among the study population.

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

A



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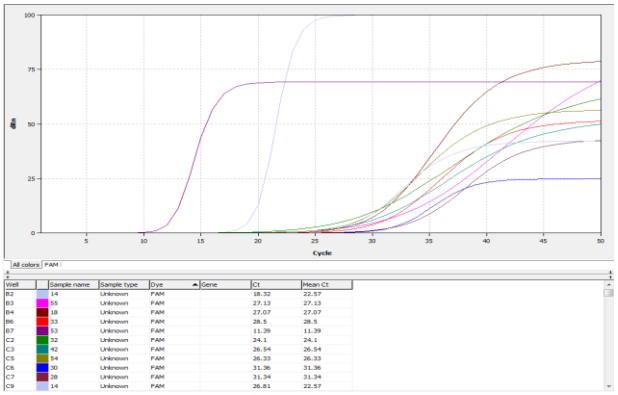

Figure (3.2) - The amplification achieved using TaqMan probes and a positive control is shown in plot A. The PCR cycles are plotted on the x-axis, and the Rn values (indicating the fluorescence units) are plotted on the Y axis. The plot B show amplification obtained using Taq Man probe and test DNA samples.

Table (3.2) - Association between HMTV sequences in Breast cancer with clinicopathological features in the study population.

Parameter	HMTV			(p-Value)
		Positive (n=15)	Negative (n=45)	
Age	≤ 35 years 36 - 55 years ≥ 56 years	2(13.3%) 6(40%) 7(46.7)	6(13.3%) 18(40%) 21(46.7)	1.000
Grade	Grade1 Grade 2 Grade 3	0 15 0	1 45 1	0.708
ER status	Positive Negative	10(66.7%) 5(33.3%)	32(71.1%) 13(28,9%)	0.754
PR status	Positive Negative	10(66.7% 5 (33.3%)	31(68.9%) 14(31.1%)	1.000
Her2	Positive Negative	5(33.3%) 10(66.7%0	9(20%) 36(80)	0.309
TNBC	TNBC Non TNBC	4(26.7%) 11(73.3%)	8(17.8%) 37(82.2%)	0.472

Table (3.3)- The prevalence of HMTV among 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%	

Expression of *P53*

A total of 80 FFPT samples of breast malignant and benign tumors were IHC automatically stained, using the automated protocol of the Leica Bond Max Auto Stainer. The positive expression of *P53* was considered as positive staining for P53 if there was brown nuclear staining of tumor cells with *P53* antibody in at least 5% of the tumor cells (fig. 3.3).

Table 3.4 - Frequency of the P53 expression in the study population.

P53	Group		Total	P
	Patient	Control		
Positive	33	3	36	0.002
	55.0%	15.0%	45.0%	
Negative	27	17	44	
	45.0%	85.0%	55.0%	
Total	60	20	80	
	100.0%	100.0%	100.0%	

Table (3.5)- Association of the P53 expression and HMTV.

P53	HMTV		Total	P
	Positive PCR	Negative PCR		

Positive	6	30	36	0.031
	26.1%	52.6%	45.0%	
Negative	17	27	44	
	73.9%	47.4%	55.0%	
Total	23	57	80	
	100.0%	100.0%	100.0%	

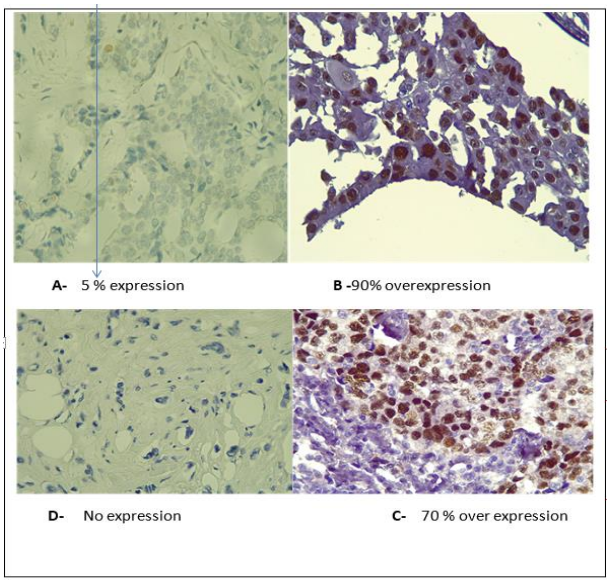

Figure 3.3- IHC staining for P53 expression in breast cancer samples. A (Invasive Ductal carcinoma); B (Ductal carcinoma); and C (Ductal carcinoma) showed nuclear overexpression of P53, D showed no expression (IHC,40x).

Table(3.6)- Association between *P53* expression in Breast cancer with other clinicopathological features of the study population.

Parameter	P53			(p-Value)
		Positive (n=36)	Negative (n=44)	
Age	≤ 35 years 36 - 55 years ≥ 56 years	7(19.4%) 13(36.1%) 16(44.4%)	17(38.4%) 15(34.1) 12(27.3%)	0.127
Grade	Grade1 Grade 2 Grade 3	0 32 1	1 26 0	0.710
Histological type	Ductal carcinoma Invasive ductal carcinoma Benign tumor	30(83.3%) 3(8.3%) 3(8.3%)	20(45.5%) 7(15.9%) 17(38.6%)	0.002
Her2	Positive Negative	8(24.2%) 25(75.8%)	6(22.2%) 21(77.8%)	0.854
TNBC	TNBC Non TNBC	7(21.2%) 26(78.8%81.5%	5(18.5%) 22(81.5%)	0.795

4. Discussion

Breast cancer is the most prevalent type of malignancies and the second greatest cause of mortality in women worldwide. Where the number of cases of breast cancer has risen sharply. Between 2008 and 2012, the incidence of BC grew by even more than 20%, whereas death rates increased by 14% [21] On over the years,the search for a viral causes of human breast cancer has created great controversy [22] Mouse Mammary Tumor Virus (MMTV) has been involved in the development of mammary cancer in mice [7], although the role of the MMTV in the pathogenesis of human breast carcinoma has long been assumed, but never proven. By using a hybridization test and a PCR method, HMTV sequences were identified in human tissues of BC and some human biologic specimens like sera, saliva, and milk. Furthermore, several studies have proposed that the incidence rate of human BC is linked to the natural ranges of certain mouse species [20, 23].According to Amarante *et al* novel review [24], the HMTV env gene was detected in varying amounts in human breast tumor tissue around the world, ranged from (0% - 74%) of samples. In the current study, we detect HMTV env gene in 25 % of breast malignant tumor tissues which comparable to result of study carried out by Hasan *et al* in1raq which detected HMTV env gene in 18.8% specimens of Iraqi women with breast carcinoma [25] Also the prevalence of HMTV in China and Pakistan was high as (17.65 and 20.0%, respectively) [26, 27]. Otherwise, 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 [28, 29]. According to the findings of this case control study,the prevalence of HMTV in BC tissue (25%) was lower than in breast benign tissues, (4o %).There was no statistically significant difference between groups (p value > 0.05). Because breast cancer is a multifactorial heterogeneous illness, this finding suggests that HMTV infection may be a risk factor for breast tumor development and may play an essential role in breast carcinogenesis when combined with other carcinogenic factors. In 2017, a recent study by Nartey *et al* proved that HMTV sequences were found in benign tumor tissue of breast from women who acquired HMTV positive breast cancer several years later. These findings contribute significantly to the understanding of HMTV's role in human breast carcinoma and achieve a key standard for a probable causation for the HMTV virus in human breast cancer [5]. The discovery of categorical evidence linking HMTV to human breast cancer will lead to a new age of treatment and prevention. As regards correlations between HMTV env positive specimens and clinicopathologic characteristics Wang et al. [26]. 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, we did not found association between HMTV env in tumor samples and clinicopathological characteristics in the general study population (table 3.2). There is no significant difference was noted in the prevalence of HMTV among molecular subtypes of breast cancer. We disagree with a study done in China which found that the detection of HMTV sequences was significantly associated with Her2 expression in BC tissues [26].

The *P53* gene, also named as the "guardian of the genome," has been shown to be mutated in about half of all human malignancies. Enhanced tumor cell invasion and metastasis are linked to mutations in the tumor suppressor *P53*[29]. By targeting *p53* and other suppressor genes, oncoproteins from oncogenic viruses can interrupt the cell cycle, stimulate transformation and tumorigenesis, then finally lead to tumor formation. Interference with or destruction of *p53* and other suppressor genes, which play essential roles in cell cycle and genomic repair, ultimately lead to excessive cellular proliferation and, as a result, cancer [30]

Immunohistochemistry (IHC) has proven to be quite

useful in analyzing cancer prognostic and predictive markers. The predictive value of immunohistochemistry stained *p53* protein in Triple Negative Breast Cancer has remained a point of contention until now [31]. Current study showed the ratio of *P53* expression in 80 samples of malignant and benign tumors was 55%,15 % respectively. There was statistical significant difference between *p53* positivity and *p53* negativity in study groups. However, the expression of *P53*was elevated in the cancer group as P value reach (0.002). Also, the *P53* expression levels was compared between HMTV positive and HMTV negative cases diagnosed with breast malignant and benign tumors and it was revealed that these levels were significantly reduced or absent in HMTV-positives cases as compared to HMTV-negatives cases (p value= 0.031). In disagreement with previous studies found that the nuclear expression of p53 was considerably higher in HMTV positive cases compared to HMTV negative cases.

In the analysis of the association between irregular expression of *P53* and clinicopathological parameters, this study found that no significant association appeared between *p53* expression and age, histologic tumor grade, TNBC, and Her2 status. This contrasted prior research findings [32], and disagree with study done in India [33] which is reported that the *p53* overexpression associate with high histologic tumor grade and Her2/neu expression. Regarding the histological types we observed significant association between *P53* expression and histological types of breast cancer (p= 0.002), as over expression of *p53* was associate with Ductal carcinoma in stiu with 83.3% versus 8.3% in invasive ductal carcinoma. Larger sample sizes may be required to confirm the findings and evaluate whether there are any correlations that were overlooked in smaller studies.

5. conclusion

In the present study HMTV *env* sequences genes were found in Basrah women with breast malignant and benign tumors. This finding indicate that infection of HMTV may be a risk factor for breast cancer development. This study showed that levels of *p53* expression were significantly reduced or absent in HMTV-positives breast tumors cases compared to HMTV-negatives breast tumors cases. Despite the fact that these findings do not prove causation, they are consistent with HMTV viruses playing a role in the development of certain human breast cancers. More large-scale studies on virus transmission, infectivity, and pathogenesis are required to study the role and relation of HMTV in human breast cancer development.

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