Detection of Mouse Mammary Tumor Virus MMTV Antigens in Different Pathological Tissue forms of Iraqi Women- Breast Samples using Immunohistochemistry Staining

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Abbreviations: (mouse mammary tumor virus , MMTV) (formalin fixed paraffin-embedded tissues, FFPT) (IHC, immunohistochemistry ) (BC, breast cancer), (F, fibroadenoma), (M, mastitis), (D, ductectesia), (Wingless--related integration site, Wnt) (Fibroblast growth factor, Fgf) (HMTV) human mammary tumor virus.

Abstract
Mouse mammary tumor virus (MMTV) is a β-retrovirus that cosider as the most common cause of breast cancer in mice. However its role in human breast cancer is very controversial yet. Iraq having increasing rate of breast cancer and high prevalence in comparison to other cancer types among Iraqi people, Iraqi women suffering from a very aggressive fast-growing breast cancer, revealing causes and predisposing factors for such cases may help in saving lives of such women. Accordingly, the current study worked on detection of MMTV antigens (envelope glycoprotein 52(gp52) and capsid protein 27 (p27)) within Iraqi women breast cancer tissue samples and other comparative groups, cases were chosen from area highly endemic with house mouse. Immunohistochemical staining was performed using 97 samples of Formalin-Fixed Paraffin–Embedded (FFPE) Breast Tissues using specific antibodies targeting gp52 & p27 antigens. Although, results showed non significant differences for percentage of MMTV antigens positive results within Breast Cancer samples 7.31% (3 of 41), Fibroadenoma samples 5.26% (1 of 19), Mastitis samples 5.55% (1 of 18) and Ductectesia samples 5.26% (1 of 5.26). Despite of this low positive percentage, the presence of MMTV in human breast tissues is significant because MMTV is an animal virus so, the results may support the possibility of viral transmission from mice to human by direct or indirect contact and act as a co-factor with other oncogenes to induce breast cancer.

Keywords: MMTV, Iraqi, Breast cancer.

1. Introduction
Mouse mammary tumor virus (MMTV) belongs to Retroviridae family, Orthoretrovirinae subfamily, Betaretroviruses genus. It is responsible for over 95% of breast cancer in mice (1). MMTV consider as an animal model for the study of human breast cancer. It can be transmitted longitudenally and vertically through milk from mother to offspring. After ingestion, the viruses spread through the gastrointestinal tract to the Peyers’ patches where they infect the new host’s macrophages, dendritic cells, and lymphocytes. This establishes a reservoir of viral infection within the new host. The infected lymphocytes carry the virus to the mammary gland as part of the mucosa-associated lymphoid tissue (2 ). MMTV integrates into the DNA of the host mammary epithelial cells. Although the integration of MMTV proviral DNA is thought to be essentially random, integration of an MMTV provirus in the vicinity of a number of host oncogenes, particularly near the Wingless – related integration site (Wnt) and Fibroblast growth factor (Fgf) family genes, results in inappropriate oncogenes expression and clonal outgrowth of the infected cell (3, 4, 5).

The mature MMTV virion is an enveloped structure around 100nm in diameter. Envelope proteins are composed of a transmembrane glycoprotein protein and a surface glycoprotein, which are located on the surface of the viral lipid envelope. Beneath the lipid envelope there are three structural proteins, namely, the matrix, capsid, and nucleocapsid proteins (2). The matrix protein is located directly beneath the lipid bilayer, whereas the capsid and nucleocapsid proteins comprise the core of the virion, which contains three additional proteins, ( protease, the reverse transcriptase, and the integrase) (6). MMTV envelope protein consisting of two chains generated by processing a precursor polyprotein, a cell surface (SU) domain of 52 kilodaltons and a transmembrane domain of 36 kilodaltons . It is the cell surface protein that binds the cellular receptor for the virus, since antecell surface antibody blocks MMTV infection of cultured cells (7). Forty years ago, electron microscopic images of MMTV-like particles were identified in milk from 5% of healthy lactating women. These observations, however, have not been confirmed by modern methods (8). The current work investigated the presence of MMTV antigens within certain abnormal human mammary tissues through Immunohistochemical staining using specific antibodies targeting gp52 and p27 antigens.

2. Materials and Methods
2.1. Patients
After mastectomy, patient’s FFPT were collected at Al-Sader Teaching Hospital cancer unit in AL-Najaf
Governorate. Patient’s description data were collected through patient's history sheets (Table 1 and 2).

Table (1) Total Patients Number

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>41</td>
<td>42.26</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>19</td>
<td>19.58</td>
</tr>
<tr>
<td>Mastitis</td>
<td>18</td>
<td>18.55</td>
</tr>
<tr>
<td>Ductectesia</td>
<td>19</td>
<td>19.58</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>100%</td>
</tr>
</tbody>
</table>

(Table 2) Clinic-pathological Characteristics of Breast Carcinoma Samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number of cases and percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≤35</td>
<td>2 (4.87)</td>
</tr>
<tr>
<td>35–50</td>
<td>22 (53.65)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>17 (41.46)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>33 (80.48)</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>4 (9.75)</td>
</tr>
<tr>
<td>Modularly carcinoma</td>
<td>1 (2.43)</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>1 (2.43)</td>
</tr>
<tr>
<td>Intraductal papillary carcinoma</td>
<td>1 (2.43)</td>
</tr>
<tr>
<td>Intraductal carcinoma Insitu</td>
<td>1 (2.43)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>1 (2.43)</td>
</tr>
<tr>
<td>III</td>
<td>40 (97.56)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
<tr>
<td>Staging</td>
<td></td>
</tr>
<tr>
<td>T2N2MX</td>
<td>15 (36.58)</td>
</tr>
<tr>
<td>T3N0MX</td>
<td>3 (7.31)</td>
</tr>
<tr>
<td>T3N2MX</td>
<td>12 (29.26)</td>
</tr>
<tr>
<td>T2N1MX</td>
<td>7 (17.07)</td>
</tr>
<tr>
<td>T3N3MX</td>
<td>4 (9.75)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>38 (92.68)</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (7.31)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
</tr>
</tbody>
</table>

2.2. Samples Preparation

The current study was conducted at the University of Iowa/Carver College of Medicine/ United states of America.

Two slides were prepared for each FFPT sample, the tissues (4 µm thickness) were laid over a Fisherbrand™ Superfrost™ Plus Microscope Slides, tissues were cut using sterile microtome blades. To confirm histopathological diagnosis, One slide was stained with Hematoxylin and Eosin stain, and the second slide was immunohistochemically stained using Alkaline phosphatase universal AK-5200 (Vectastain® ABC kit).

2.3. Antibodies

Antibodies used in the current study were envelope gp52 antibody (gp52)= 52,000-dalton exterior envelope glycoprotein and Capsid antibody P27 (p27)= 27,000-dalton nucleoid protein (9,10) (a gift provided from Dr.Chioma Okeoma /Microbiology Department /Carver college of medicine/Iowa University/USA). Control slide was prepared and tested using mouse monoclonal IgG1(Abcam) for detection of nonspecific binding.
2.4. Immunohistochemistry staining

Immunohistochemistry Procedure was performed using vector laboratory Manufacturer instructions as follow:

1. Deparaffinization and rehydration were done using Dacko auto-staining machine.
2. Antigen Epitope retrieval performed by cooking the slides for 15 minutes in Sodium-citrate buffer (10 mM, pH 6.5) in Pelco Biowave microwave.
3. Blocking of endogenous peroxidase activity was done with 1% H$_2$O$_2$ (1.5 ml of 30% stock Sigma H-1009 in 50 ml PBS) for 20 min, then slides were soaked in phosphate-buffered saline for 5 minutes.
4. Blocking of non-specific antigens reaction was done by incubating of slides in vector blocking serum (one drop of horse serum in 5 ml phosphate-buffered saline) for 20 minutes, then excess serum was removed from sections.
5. The primary antibodies gp52 & p27 were diluted (1:50), slides were incubated with both antibodies on tissue section, slides were incubated for one hour, then soaked in phosphate-buffered saline, for 5 minutes at room temperature.
6. Control slide was incubated with mouse monoclonal nonspecific IgG1 antibody for checking of false positive results or nonspecific binding.
7. The conjugated Secondary antibody was incubated for 30 minutes at room temperature, then soaked in phosphate-buffered saline, for 5 minutes.
8. ABC reagent (two drops of reagents A and B in 10ml of phosphate-buffered saline), was incubated for 30 min at room temperature, soaked in phosphate-buffered saline, for 5 minutes, then rinsed under cold tap water for 5 minutes.
9. The slides were dipped in counter stain Hematoxylin (Fisher CS401-D) for 20 seconds and rinsed in tap water until water came out clear.
10. Slides were rehydrated through alcohol gradient started at 30% ethanol up to 100% ethanol (2 minutes each), then soaked in xylene 3times for 5 minutes.
11. Slides were mounted with premount (Fisher SP15-100) and pressed with coverslip.
12. Slides were examined and imaged with Olympus BX51 microscope.
13. Note all incubation steps were performed using humidity chamber.

3. Results

For Histological diagnosis slides were stained with Hematoxylin and Eosin stain, Figure (1).

Figure (1) Hematoxylin and Eosin stain of breast tissue forms A. Invasive Ductal Carcinoma X40. B. Fibroadenoma X10. C. Mastitis X10. D. Ductectesia X40

3.1. Immunohistochemical staining results

Ninety seven samples were tested using immunohistochemistry method with MMTV monoclonal gp52 and p27 antibodies (1:50). The results revealed positive reaction for both antibodies as follows: breast cancer 3 of 41 (7.31%), fibroadenoma 1 of 19 (5.26%), mastitis 1 of 18 (5.55%) and ductectesia 1 of 19 (5.26%).
Immunohistochemistry positive slides and control slide shown in Table (3) and Figure (2 and 3).

Figure (2) positive results of MMTV Ags by IHC  A. Breast carcinoma, B. Fibroadenoma, C. Negative control (mouse IgG1), photos were taken using Olympus BX51 microscope

Figure (3) positive results of MMTV Ags by IHC  A. Mastitis, B. Ductectia, C. Negative control (mouse IgG1), photos were taken using Olympus BX51 microscope
Table (3) Results of MMTV positive by immunohistochemistry technique (No significant differences among the results)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Immunohistochemistry results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>3</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>1</td>
</tr>
<tr>
<td>Mastitis</td>
<td>1</td>
</tr>
<tr>
<td>Ductectesia</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>

\[ \text{ChiSq} = 2.64097 \times (E-33) \]

4. Discussion

Iraq having increasing rate of breast cancer and high prevalence in comparison to other cancer types among Iraqi people (11). Ruddy mentioned that Iraqi women suffering from a very aggressive fast-growing breast cancer and that human mammary tumor virus (HMTV or MMTV-like) may play a role as a potential co-factor of Iraqi and Kuwaiti women (12).

In Arabian regions, although there were no studies worked on MMTV, few studies worked on MMTV-like (HMTV), some studies confirmed absence of this virus in breast cancer samples among Iraqi women (13), and Iran (14, 15). While in Tunis, Hachanaannd colleagues found MMTV-like sequences with a very low percentage comparing to previous studies among Tunisian women (16). However, HMTV is still a virtual agent of breast carcinoma, while, MMTV is a proven cause of mouse mammary carcinoma but infection with this virus, has not been investigated among Iraqi women suffering from breast carcinoma or other non-neoplastic conditions till now.

Strong evidence for humans becoming infected with MMTV comes from a study of laboratory personnel working with MMTV-infected mice, they were found to develop a specific serologic response to MMTV when compared to age and gender matched controls, most strongly to gp55, a surface glycoprotein of MMTV (17).

However, the use of this antibody against the MMTV envelope protein has been disputed and places an uncertainty on the validity of these results (18). In addition, viral particles morphologically similar to MMTV were found in 60% of milk specimens from patients with history of breast cancer, whereas only 5% of milk samples from normal women contained these particles (19,20,21). These findings were not reproducible in other laboratories (22). It was also demonstrated that human genome may normally have endogenous retroviral sequences with 50% homology to MMTV(23). These findings further challenged the concept of an MMTV-like exogenous infection as a cause of breast cancer. Some observers even questioned the specificity of the antibodies used in the identification of viral antigens(24). In human earlier immunohistochemistry studies that worked on detection of MMTV antibodies in human breast cancer, they suggested presence of antigenic crossreactivity with the MMTV envelope glycoprotein gp52, and that the same reaction was found in human milk and in breast cystic fluid (25). Same results were found by Tsubura and Morii in 1985 who used gp52 (envelope protein) and p27 (core protein) for detection of viral antigens in sweat and sebaceous glands of mice (26).

Immunohistochemical results in the current study showed detection of MMTV antigens (gp52 and p27) in low percentage of patients (7.31% of BC, 5.26% of fibroadenoma, 5.55% of mastitis, and 5.26% of ductectesia samplese), so the theory of crossreactivity mentioned by numerous immunological studies (27) might be unapplicable in our results, because their study showed high percentage of positive results, in addition we used mouse monoclonal IgG1 antibody to exclude non-specific reaction.

The MMTV-positive results of malignant and non-malignant breast tissues may enhance an idea, that MMTV may act as a co-factor with other carcinogens in the pathogenesis of human breast tissues. The interest of this paper is very high because we are talking about an oncogenic animal virus present in human cancer tissues, MMTV use transferrin receptor 1 (TIR1) for mouse-cells entrance, in human the virus used a different entry mechanism other than TIR1 in mammary epithelial cells (28, 29). The presence of MMTV antigens consider a significant signal because:

1. MMTV is an oncogenic animal virus.
2. It’s proved to be oncogenes and cause breast cancer in the mice only.
3. Mice have a coexistence behavior with human life, like food and water, so the possibility of its transmission among Iraqi patients might be related to dry cereal food contaminations with mice fecal debris and via oral route infection.

In addition, the positivity of MMTV Ags can be explained by the wide distribution of house mice in different Iraqi areas, and theoretically, it means that there is a possible route for transmission of exogenous form of MMTV particles from contaminated food, water and house materials with mice's products (saliva, milk, and fecal materials) to human.
The geographical endemic of the *Mouse musculus* ranges from central Europe east to China and Japan, *Mouse castaneus* occurs from southern China to central Iran, and *Mousetdomesticus* lives from western Iran to western Europe. *M. domesticus* mice also expanded their range to North and South America, Australia, New Zealand and Hawaii via ships sailing from western European ports. Inbred laboratory mice have mostly *Mouse domesticus* genes (30), MMTV has been isolated from human breast cancer samples North America, Australia and Tunisia (31,32,33 and 34). While Regions of Vietnam, Sweden and Austria, did not isolate the virus(35, 36 and 37). In addition, according to the international union for conservation of nature and natural rescores (IUCN), Iraq recorded high prevalence of house mouse (38).

An earlier studies used an antisera against the mouse mammary tumor virus (MMTV) structural proteins to detect MMTV cell surface antigens on several mouse models. Indik and colleagues have demonstrated infection and spread of MMTV in human cells *in vitro* (39,40).

The current study concluded that the MMTV appeared positive in different Iraqi breast tissues (malignant and non malignant), even if it was low percentage but it can be consider as a critical evidence for transmission of virus from animal to human. For further confirmation future studies need molecular work in addition to cell culture testing for any neoplastic changes due to tissue challenging with MMTV.

5. **Acknowledgment**

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