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Original Article

The Impact of Human Mammary Tumor Virus (HMTV) on the Expression of Epidermal Growth Factor Receptor (EGFR) and Death–Domain Associated Protein (DAXX) in Breast Carcinoma Tissues

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Abstract

The presence and characteristics of HMTV in Iraqi breast cancer women are still unknown. Furthermore, the identification of HMTV in human breast carcinoma tissue of patients differs by country, and the factors that influence it are still unknown. In many epithelial tumor types, the EGFR and its signaling pathways outcomes are necessary for the behavior of cells and regulating their proliferation, and it has been discovered that DAXX has strong carcinogenic characteristics and could be a new treatment target. This case-control retrospective study investigated the presence of HMTV in paraffin-embedded blocks (FFPT) of tumor samples from 60 Iraqi women patients diagnosed with primary breast cancer and 20 cases of benign tumors as a control group. *HMTV env* sequences were identified by Real-time PCR. EGFR and DAXX expression were immun0-detected by the immuno-histochemistry technique. HMTV sequences were detected in 15 (25%) samples of malignant breast tumors and 8 (40%) samples of benign breast tumors. There was no statistically significant association between the detection of env sequences of HMTV and age, grade, hormone receptors, EGFR, or DAXX expression compared to clinicopathological characteristics. However, statistically, the data showed a highly significant difference in the Expression of EGFR between study groups, age, and histological types (P=0.0001), and a significant negative association was observed between EGFR and both Her2 and TNBC. There was a statistically significant difference between DAXX (+) and DAXX (−) in study groups (P=0.002), and it was significantly associated with age and histological types of breast cancer (P=0.031 and 0.007, respectively). No significant association was found between DAXX and EGFR, grade, Her2. TNBC of breast cancer. The current study found HMTV *env* sequences in breast tumors of Iraqi women, suggesting that a larger sample size is needed to illustrate the potential causative role of HMTV in the development of human breast malignancy. Moreover, a negative association was found between HMTV and DAXX and EGFR Expression.

Keywords: Breast cancer, HMTV, EGFR, DAXX

1. Introduction

Despite the fact that prevention can reduce risk, breast carcinoma remains the most prevalent type of cancer in women in both developed and developing nations and the major cause of cancer mortality in women globally (1). BC is the most common type of malignancy in Iraq's whole population. It accounts for nearly one-third of all reported female malignancies, with an age-standardized incidence rate of (34.9/1000) females (2).

The etiology of BC disease is linked to a complex set of factors, including demographics, reproductive factors, lifestyle choices, and other environmental factors, advanced age, a positive family history, socioeconomic level, diet, endogenous or exogenous

hormones, atypical breast illnesses, benign tumors, oncogenic viruses, and carcinogenic exposure, all these factors have been linked to an elevated risk (3). Accumulating data indicated that viral infection might contribute to the initiation and development of BC. Oncogenic viruses contribute to various stages of the carcinogenic process, and their relationship with specific cancer can range from 15% to 100%; also, human oncoviruses contribute to tumorigenesis by containing viral oncogenes or by insertional mutagenesis in genetic materials to activate proto-oncogenes (4). Several oncogenic viruses have been determined, including Mouse Mammary Tumor virus (MMTV), the leading cause of malignant mammary tumors in mice, and the milk-transmitted retrovirus previously named Bittner virus (5). Sequences similar to MMTV have been identified in human BC tissue, and an entire proviral sequence with more than 95% homology to MMTV, named MMTV-like virus and also known as HMTV, has been sequenced outside of human breast carcinoma tissue, including a proper integration into the genome of human cell (6).

An association of the HMTV with human breast cancer development remains inconclusive. Recently, HMTV sequences were found in tissues of benign breast tumors before the evolution of HMTV-positive breast carcinoma, suggesting a probable causative agent in breast cancer development (7). In the study and treatment of tumors, discovering new genetic predictors, prognostic indicators, and treatment response is a field of intensive investigation. Also, progression in this field will undoubtedly alternate cancer medication treatment from non-targeted to targeted anticancer therapeutics in patients chosen based on the molecular biology of tumor cells.

The most crucial cancer molecular targets discovered recently are members of the ErbB family, also called the epidermal growth factor receptor (EGFR) family, which consists of (HER1/EGFR), HER2, HER3, HER4, which are also known as ErB1, ErB2, ErB3, ErB4 respectively (8, 9). Although overexpression of EGFR can be identified in all forms of BC, it is most prevalent in triple-negative (TNBC) and inflammatory types of breast cancer. It is related to large tumor size and poor clinical consequences (9, 10).

DAXX (Death Domain-Associated Protein) is a nuclear and cytoplasmic protein have multifunction; it involved in various cellular processes. It can shuttle between the two sites in response to different cellular stresses. It was previously thought to be a cytoplasmic protein that interacts with the Fas death domain to facilitate the activation of Fas-mediated apoptosis thru JNK signaling independent of FADD, hence the protein's name. Other cell death processes involving DAXX include TGF-induced apoptosis, necrosis, and autophagy (11). Furthermore, DAXX regulates transcription, programmed cell death (apoptosis), tumorgenesis, and antiviral response (12). The purpose of the present study was to search the presence and prevalence of the HMTV sequences in Breast carcinoma tissues in Basrah females and investigate the association of the virus with irregular Expression of EGFR and DAXX (tumor markers) and with some clinicopathological characteristics.

2. Materials and Methods

2.1. Sample Collection

Specimens of FFPT blocks from 60 females diagnosed with primary breast cancer were obtained from the histopathology unit in Al-Sadder teaching hospital and a privet histopathology laboratory in Basrah city during the period between September 2020 to October 2021. A control group of 20 FFPE tissue samples from benign breast tumors was also included. At the time of sample collection, the patients' ages ranged from 17 to 77.

2.2. DNA Extraction

Human genomic DNA was extracted for the additional analysis required to demonstrate our hypothesis. The commercial kit (QIAamp DNA FFPE Tissue Extraction, catalog No. 56404) was used to extract DNA from thin tissue sections cut by microtome, n accordance with the manufacturer's instructions. The extracted DNA concentrations of

samples were measured by The QuantiFluor dsDNA System (Cat.# E2670) and Quantus Fluorometer (Cat.# E6150 / Promega/ USA). Then, all samples were kept at deep freeze at -35°C until the work of PCR was done.

2.3. Real-Time PCR Analysis

The HMTV *env* positive specimens were identified using DNA extracted with a fragment of following primers (foreword 5'-AAGGGTGATAAAAGGCGTATGTG-3') and (Reverse 5'- TTTTGTATTGGCCCCTGAGTTC-3'), and Probe (5'-FAM-AACTTTGGTTGACTACCTT-MGB-3'). The sequences of the MMTV *env* gene from the C3H strain (GenBank AF228552) were used as a positive control. Free nuclease water was used as a negative control, and all primers and probes for HMTV env were designed according to previously described methods (13). The amplification reaction mixture was performed utilizing Go Taq probe qPCR Master Mix with(Cat# A6100) with a total volume of (20µl); the reaction mixture included 1µl of each primer and probe; 10 µl of Master Mix, nuclease-free water, 3 µl, and 4 µl of template DNA. Positive and negative control (the amplification mixture without DNA template) were included for every reaction. The thermocycler conditions were set up for one cycle; initial denaturation was at 95°C for 1 minute, followed by 50 cycles at 95°C for 15 seconds, 58°C for 20 seconds, and 60°C for 30 seconds. Real-Time PCR data was analyzed by investigating the *Ct value* representing a threshold cycle number that exhibited the positive amplification in the Real-Time PCR cycle number.

2.4. Immunohistochemistry

For immunohistochemical staining, the following primary antibodies are anti-EGFR (Anti-human EGFR clone E 30 with (cat# M7239), Dako, Denmark) and anti-DAXX antibody (Rabbit anti Human, Mouse polyclonal IgG antibody, Mybiosource, USA) were used. FFPT blocks were cut in series at 4 µm using a sterile microtome. After introducing it in the stainer (Lieca Bond MAX autostainer, Germany), the sections were automatically deparaffinized, utilizing Bond dewax solution (AR9222. Leica, Germany). Automatically, the reactive has been added after the specimens were processed with EDTA buffer (pH 9.0) and antigen retrieval solution (ER2) from (Leica Microsystems) and treated for 20 minutes. Then the slides were incubated for 15 min with primary antibodies specific to the EGF receptor at a dilution of (1: 25) and anti-DAXX antibodies. The slides were subsequently stained with chromogen (DAB) and counterstained with hematoxylin using the streptavidin-biotin-peroxidase complex procedure, "Bond Polymer Refine Detection kit with cat # DS9800, Leica".

2.5. Statistical Analysis

The results were analyzed using available statistical packages for social science-version 24 (SPSS) to assess the significance of differences in group findings. Fisher's exact test and chi-square were used. A difference was considered significant if (*P*-value<0.05)

3. Results

3.1. Detection of HMTV in the Study Population

Eighty samples of malignant and benign breast tumors were included in this work. HMTV *env* gene was detected in 28.8% (23/80) of tumor tissues analyzed by qPCR, divided as 25% (15/60) from malignant tumors and 40% (8/20) from benign tumors (Table 1). Statistically, the difference was not significant (*P*> 0.05) (Table 2 and Figure 1).

Table 1. Prevalence of HMTV among the study population

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

Table 2. Association between HMTV-env in Breast cancer for other clinicopathological features study population

Parameter		HMTV		(*P*-value)
		Positive (n=15)	Negative (n=45)	
Age	≤ 35 years	2(13.3%)	6(13.3%)	1.000
	36 - 55 years	6(40%)	18(40%)	
	≥ 56 years	7(46.7)	21(46.7)	
Grade	Grade1	0	1	0.708
	Grade 2	15	45	
	Grade 3	0	1	
Estrogen	Positive	10(66.7%)	32(71.1%)	0.754
	Negative	5(33.3%)	13(28,9%)	
Progesterone	positive	10(66.7%)	31(68.9%)	1.000
	negative	5 (33.3%)	14(31.1%)	
HER2	positive	5(33.3%)	9(20%)	0.309
	negative	10(66.7%0	36(80)	
TNBC	TNBC	4(26.7%)	8(17.8%)	0.472
	Non-TNBC	11(73.3%)	37(82.2%)	

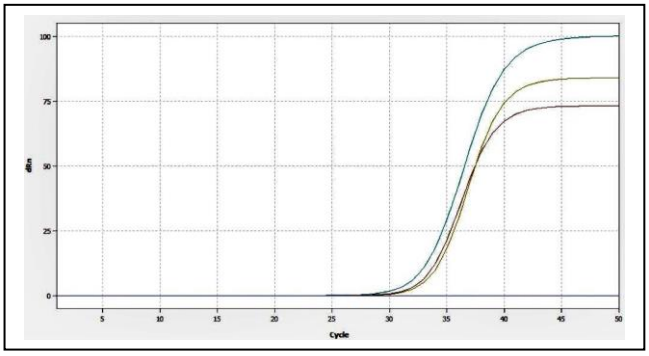

Figure 1. The figure above illustrates the results of real-time PCR, where the plot represents amplification obtained from Taq Man probes and DNA samples. Each line in the graph shows a positive cancer sample and negative control (without a DNA template)

3.2. Epidermal growth factor receptor (EGFR) detection

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 EGFR was indicated by the intensity of brown membranous staining of tumor cells. As shown in (Table 3), the EGFR transmembrane proteins were IHC detected in the malignant cells in 25 (41.7%) versus 2 (10%) in benign tumors. Nuclear expression was detected in 14 (70%) benign tumors versus (0%) malignant cases. Statistically, the data showed a highly significant difference in the EGFR expression between cancer and benign groups (*P*=0.0001).

Out of 23 samples positive for HMTV, only Four were EGFR overexpressed. The data of this table 4 demonstrated that the association between Egfr expression and the presence of the virus in studied samples is not significant (Table 5 and Figure 2).

Table 3. Frequency of EGFR expression in the study population

EGFR	Group		Total	P
	Patient	Control		
Positive	25 41.7%	2 10.0%	27 33.8%	0.0001
Negative	35 58.3%	4 20.0%	39 48.8%	
Nuclear Positive	0 0.0%	14 70.0%	14 17.5%	
Total	60 100.0%	20 100.0%	80 100.0%	

Table 4. An association between EGFR expression and HMTV

EGFR	HMTV		Total	P
	Positive	Negative		
Positive	4 (17.4)	23 (40.4)	27 (33.8)	0.145
Negative	14 (60.9%)	25 (43.9%)	39 (48.8%)	
Nuclear Positive	5 (21.7%)	9 (15.8%)	14 (17.5%)	
Total	23 (100%)	57(100%)	80(100%)	

Table 5. Association between EGFR expression for other clinicopathological features in Breast cancer

Parameter	EGFR				(*P*-value)
	Group	Positive (n=27)	Negative (n=39)	Nuclear N=14	
Age	≤ 35 years	3(11.1%)	11(28.2%)	10(71.4%)	0.0001
	36 - 55 years	8(29.6%)	16(41%)	4(28.6%)	
	≥ 56 years	16(59.3%)	12(30.8%)	0(0%)	
Grade	Grade1	0(0%)	1(2.9%)		0.671
	Grade 2	24(96%)	34(97.1%)		
	Grade 3	1(4%)	0(0%)		
Histological type	DC	23(85.2%)	27(69.2%)	0(0.0%)	0.0001
	IDC	2 (7.4%)	8(20.5%)	0(0.0%)	
	Benign	2(7.4%)	4(10.3%)	14(100%)	
Her2	Positive	5(20%)	9(25.7%)		0.606
	Negative	20(80%)	26(74.3%		
TNBC	TNBC	5(20%)	7(20)		1.000
	Non-TNBC	20(80%)	28(80)		

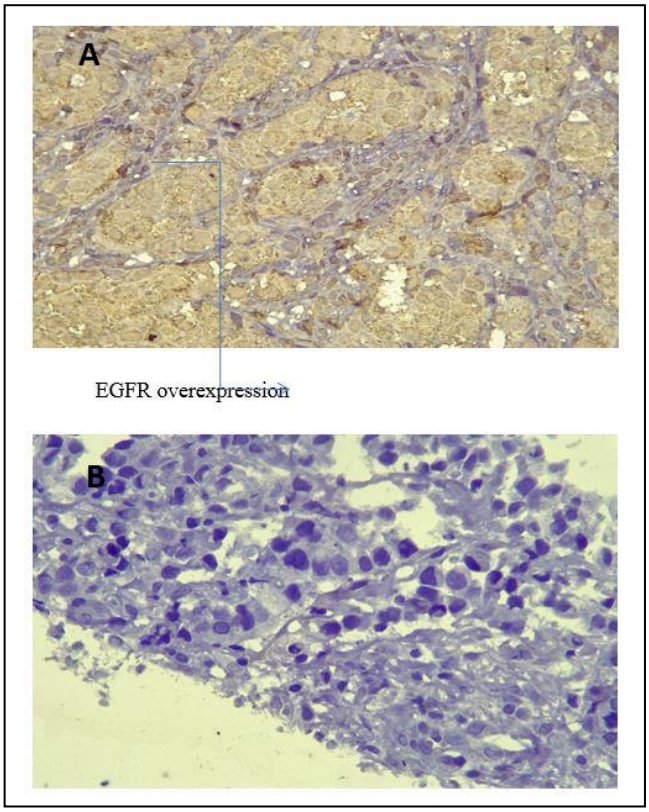

Figure 2. IHC staining for EGFR in breast cancer samples, **A** (Ductal carcinoma) showed overexpression of EGFR, and **B** showed no expression (IHC, 40X)

3.3. Death-Domain Associated Protein (DAXX) Detection

DAXX expression was evaluated using IHC staining in all breast cancer and benign tumor samples. A strong and weak nuclear expression with or without cytoplasmic staining of DAXX positivity identified in the nuclei of tumor cells is based on the intensity of Brown pigment in tumor cells (Figure 3). DAXX was over-expressed in (16.7%) out of 60 specimens of BC, whereas it was overexpressed in (50%) out of 20 benign tumors. Weak expression was noted in 28.3% of BC vs. 35% in benign tumors, and loss of expression was found in 55% of BC versus 15% in benign tumors (Table 6). A significant difference between DAXX (+) and DAXX (−) in study groups was observed as (*P*=0.002) (Tables 7 and 8).

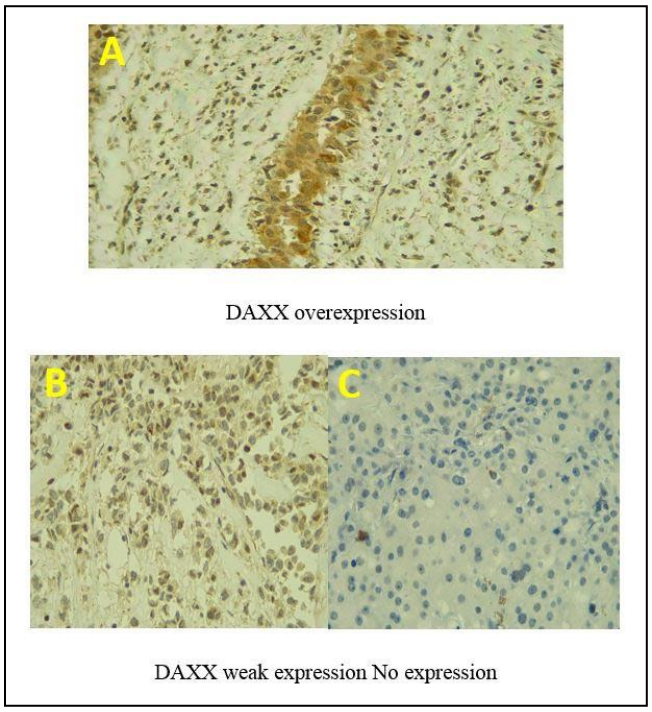

Figure 3. DAXX expression in Breast Tumor cells, **A** (positive); **B** (weak positive); and **C** (negative). (IHC, 40 X)

Table 6. Prevalence of DAXX expression in the study population

Daxx	Group		Total	P
	Patient	Control		
Positive	10 16.7%	10 50.0%	20 25.0%	0.002
Negative	33 55.0%	3 15.0%	36 45.0%	
Weak positive	17 28.3%	7 35.0%	24 30.0%	
Total	60 100.0%	20 100.0%	80 100.0%	

Table 7. Association between DAXX expression and HMTV

HMTV	Daxx			Total	P
	Positive	Negative	Weak positive		
Positive	5 25.0%	10 27.8%	8 33.3%	23 28.8%	0.819
Negative	15 75.0%	26 72.2%	16 66.7%	57 71.3%	
Total	20 100.0%	36 100.0%	24 100.0%	80 100.0%	

Table 8. Association between DAXX Expression and other clinicopathological features in Breast cancer

Parameter	DAXX				(*P*-value)
	Group	Positive (n=)	Negative (n=)	Weak positive	
Age	≤ 35 years	11(55.0%)	7(19.4%)	6(25%)	0.031
	36 - 55 years	7(35%)	13(36.1%)	6(33.3%)	
	≥ 56 years	2(10%)	16(44.4)	10(41.7%)	
Grade	Grade1	0(0%)	0(0%)	1(5.9%)	0.300
	Grade 2	10(100)	32(96.7%)	16(94.1%)	
	Grade 3	0(0%)	1(3.3%)	0(0%)	
Histological type	DC	8(40%0	29(80.6%)	13(54.2%)	0.007
	IDC	2(10%)	4(11.1%)	4(16.7%)	
	Benign	10(50%)	3(8.3%)	7(29.2%)	
Her2	Positive	2(20.0%)	6(18.2%)	6(35.3%)	0.421
	Negative	8(80%)	27(81.8%)	11(64.7%)	
TNBC	TNBC	2(20%)	8(24.2%)	2(11.8%)	0.616
	Non-TNBC	8(80%)	25(75.8%)	15(88.2%)	

4. Discussion

Breast cancer is a complicated disease more likely caused by genetic susceptibility, environmental exposures, hormone levels, and health behaviors; the contribution of viral infection to the development and progression of mammary carcinogenesis remains unclear. In Western countries, the frequency of HMTV (MMTV-like) sequences is higher (30-40%) of IBC (invasive breast cancer) than in Asian countries (10-20%). The disparities could be attributed to regional differences in MMTV prevalence in mice, which could affect the occurrence of MMTV-like in humans (14). The prevalence rate of HMTV sequences in human breast carcinoma ranges from 6 - 78 percent, with a common prevalence being about 40 percent, compared to a low percent in human breast normal and benign tissues (15). The current study detected the HMTV env gene in 25% of malignant tumor tissues and 40% in benign samples. In agreement with a study performed in Australia, which detected HMTV sequences in 24% of benign breast tumors and 36% of BC samples. Five SixHMTV positive, benign tumor samples developed HMTV-positive cancer (7). In the present study, no significant difference was observed between HMTV sequences detected in cases and controls specimens; this may be due to the small sample size of the control group and the low concentration of viral DNA in tissue specimens. Other investigations, however, have never been able to find HMTV sequences. This disparity could be due to differences in detection methodologies or tissue heterogeneity (16-18). This study showed no association between the presence of virus and clinicopathological features such as age, tumor grade; ER; PR, Her2, and TNBC. We could not find any correlation between the presence of HMTV and any of

the clinicohistopathological features of Breast Cancer like age, hormone receptors, triple-negative breast cancer, or tumor grade; similar findings have been described in Egypt (19).

EGFR (epidermal growth factor receptor) and Her2/neu are proteins belonging to the ErbB family of receptor tyrosine kinases. Their increased expression in breast cancer patients indicates a bad prognosis. Her2/neu has been verified as a therapeutic target after being found to be upregulated in 20–25 percent of breast cancer patients. Furthermore, EGFR is typically overexpressed in TNBC, suggesting that it could be utilized as an antitumor therapeutic target (10, 20, 21). Immunohistochemistry (IHC) is being used to investigate EGFR expression in breast carcinoma, and the incidence rate of EGFR overexpression in malignancy varies greatly, ranging between 7-43 percent (22). In the current study, the EGFR proteins were over-expressed in (41.7%) of breast cancer patients, and there was a highly significant difference in the EGFR expression between study groups (P=0.0001); this result was comparable to the result of studies carried out by Nieto, Nawaz (23) in 2007 and Lv, Xie (24) in 2011. When comparing the clinicopathological features and EGFR overexpression in positive and negative tumors. Significant association of EGFR overexpression with age and histologic type of breast cancer were observed. We found EGFR-positive tumors were increased by age and were most common in females more than 56 years old, and EGFR was more prevalent in Ductal carcinoma with 85.2% versus 7.4% in invasive ductal carcinoma; this in disagreement with a study that showed that EGFR over-expressed tumors cases were most common in females under the age of 50 years old and invasive ductal carcinoma (25). While no significant association was noted with tumor grade, her2, and TNBC, this agrees with a study by Hashmi, Naz (25), which showed no significant positive association between EGFR overexpression and triple-negative breast cancer and tumor grade.

Regarding the association between the presence of HMTV *env* and EGFR expression, the results of our study showed no significant association between them. Previous research reveals that MMTV integration in mouse and human cell lines is entirely random. However, some sites were discovered to repeat in distinct samples, indicating that sequence-specific bias in integration exists. According to a study by Sadia, Gomez (26), about sixteen non-random MMTV integration sites were discovered in human breast carcinoma tissue. These integrations sites of the virus have been discovered in or near the following locations: transcription gene promoter, proteins of DNA repair, growth factors, and tumor suppressor genes, providing an opportunity for transformation and overall survival (26). Further research on the viral integration sites may discover a relationship between HMTV sequences and breast carcinoma.

DAXX was first discovered in the cytoplasm as an apoptosis-inducing protein linked to the transmembrane death domain (Fas). It was subsequently clarified to have an anti-apoptotic function (27). Recently, accumulating data has proposed that irregular Expression of DAXX is correlated to oncogenesis. For instance, nearly 43% of pancreatic neuroendocrine cancers have mutant ATRX or DAXX genes. However, the role of the DAXX signaling pathway in malignant diseases is still controversial, for research indicating both repressive and pro-cancerous impacts (28-33). In the current study, we detected the Expression of DAXX by immunohistochemistry in 60 malignant cases and 20 benign breast tumors and found a significant difference between DAXX (+) and DAXX (−) in study groups observed as (P=0.002). DAXX loss expression was found in 55% of BC versus 15% in benign tumors (Table 6); this indicates that DAXX has a defense function, and its loss may lead to cancer progression.

Furthermore, statistical analysis for comparison of DAXX expression levels with clinicopathological parameters of 60 malignant breast cases, as seen in (Table 8) revealed no significant associations between expression levels of DAXX and tumor histo-pathological features (tumor grade, Her2, and TNBC). This conclusion is consistent with their findings from a

study conducted in China [31] but disagrees with the same study about the correlation between the Expression of DAXX and both age and histologic types of Breast cancer Since our study found a significant difference between DAXX and age (*P*=0.031) we observed Patients' DAXX levels had been found to decrease as they get older. A significant association was noted between DAXX expression level and histological types (*P*-value=0.007). Overexpression was associated with benign tumors and ductal carcinoma, and loss expression was observed in invasive ductal carcinoma.

In a comparison of HMTV positive samples and DAXX expression, no association was noted (*P*-value>0.05), indicating that the presence of the virus did not affect the regulation of DAXX expression. This could be connected to the virus integration sites in the cell's genome. These results suggested that DAXX expression level may have a potential role in breast carcinoma's development, progression, and metastasis. The current study found HMTV sequences in the breast carcinoma tissues of Iraqi females. Further evaluation of larger sample size is required to demonstrate the likely proper causal role of HMTV in human breast cancer progression. Moreover, a negative association was found between HMTV and DAXX and EGFR Expression, whereas a significant positive association was noted between EGFR over-expression and cancerous cells. The study result showed a significant association between loss expression of DAXX and malignant tumors; therefore, DAXX depletion may play an essential role in chemotherapy and endocrine therapy resistance. EGFR and DAXX play a role in cancer development mechanisms and may be used as predictive markers for breast carcinoma. However, another study with a larger population is recommended to confirm these results.

Authors' Contribution

Study concept and design: H. A. N.
Acquisition of data: W. N. I.
Analysis and interpretation of data: S. A. A. A.
Drafting of the manuscript: H. A. N.
Critical revision of the manuscript for important intellectual content: S. A. A. A.
Statistical analysis: W. N. I.
Administrative, technical, and material support: H. A. N.

Ethics

The ethical committee of Basrah Medical College and the Basrah Health Directorate gave their approval for this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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