Detection of XIST Gene Deletion by Fluorescence in Situ hybridization in Breast Cancer Patients

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Abstract:

Background: XIST gene is a long noncoding RNA that has been implicated strongly in the process of X chromosome inactivation during early embryogenesis. Its expression is frequently lost in some female's cancer. In breast cancer, XIST gene deletion is a point of much interest, and its role as a prognostic marker is yet unclear.

Aim of the Work: is to detect XIST gene deletion by fluorescence in situ hybridization and to correlate this deletion with the clinicopathological data of breast cancer patients.

Subjects and Methods: This research was a prospective study conducted on sixty recently diagnosed female breast cancer cases who underwent mastectomy at South Egypt Cancer Institute, Assiut University, in the period from December 2019 to December 2021, and 30 apparently healthy breast tissues from mastectomy specimens due to proliferative breast diseases or granulomatous mastitis were included as a control group. XIST gene deletion was detected by fluorescence in situ hybridization.

Results: XIST gene was significantly deleted in patients with breast cancer (P<0.001). This deletion was significantly associated with older age (P = 0.026), positive nodal metastasis (P = 0.005), and advanced tumor stage (P=0.028) but was not related to tumor size (P = 0.501) or CA 15.3 (P = 0.905). Also, XIST gene deletion was more prevalent among patients with triple-negative breast cancer (P = 0.009).

Conclusion: XIST gene was deleted among BC patients. XIST gene deletion was more prevalent among patients with TNBC.XIST gene deletion could be considered as an important predictive biomarker in breast cancer and an indicator of poor clinical outcome.

Keywords: Breast cancer, XIST gene deletion, Fluorescence in situ hybridization

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Introduction:

According to data from the World Health Organization (WHO), breast cancer is the most common cancer that affects women globally. It is the second major cause of cancer-related deaths among American women and a major cause of mortality in developing nations [1]. The incidence in Egypt is predicted to increase dramatically over the coming years as a result of the country's increasing population, demographic changes, and adoption of a more Westernized style of life [2].

Normal development of the breast and mammary stem cells are controlled by several signaling pathways. HER2 and estrogen receptors are two examples of signaling pathways that regulate stem cell proliferation, differentiation, death, and cell motility. It has been hypothesized that breast cancer may originate from mammary cancer stem cells due to the obvious parallels between normal development and the molecular development of breast cancer [3].

Although the quality of healthcare and diagnostic modalities is improving, the prevalence of BC is

increasing. The traditional examination of tumor characteristics includes a biopsy and histological study of tumor tissue, which in recent years has been accompanied by analysis of molecular biomarkers such as next-generation sequencing, microRNA, in situ hybridization, and RT-qPCR to improve the results [4]. Recently, growing evidence suggests that noncoding RNAs and epigenetic controls may be important to the development of breast cancer as well as its metastasis, particularly in triple-negative cases [5].

In female mammals, the human X-inactive specific transcript, or XIST, encodes a 17 kb long noncoding RNA (lncRNA), which lies on one of the two X chromosomes from which it was transcribed to start gene silencing and prevent gene dosage imbalance between males and females [6].

There is strong evidence that XIST is involved in the proliferation, differentiation, and genetic maintenance of human cells, in addition to its well-established role in X chromosome inactivation (XCI) during early embryogenesis. Therefore, it has been suggested that dysfunctional expression of XIST may play a pathologic role in cancer, possibly being related to changes in gene expression caused by changes in the stability of heterochromatin [7].

There is increasing evidence that the development and progression of tumors may be caused by abnormal XIST expression in somatic cells. As an example, in mouse hematopoietic cells and human mammary epithelial cells, genetic deletion of XIST promotes the development of highly aggressive myeloproliferative neoplasms and mammary malignancy, respectively. This suggests that XIST expression from the inactive X chromosome plays a role in saving somatic cells from oncogenesis [8].

XIST loss has been shown in certain studies to affect the differentiation of human mammary stem cells (MaSCs) and to promote the formation of highly tumorigenic carcinomas [3].

Advances in genetic technology have made it possible to rapidly expand our understanding of the genomic expression pattern in cancer and normal cells. A thorough understanding of XIST is necessary for the use of XIST as a therapeutic target and diagnostic biomarker for clinical cancer therapy [9].

The main objective of the current study is to detect the XIST gene deletion by fluorescence in situ hybridization in breast cancer patients and its relation with the clinical and pathological criteria of these patients.

Patients and Methods:

Patients:

This research was a prospective study conducted on sixty recently diagnosed female breast cancer cases who underwent mastectomy at South Egypt Cancer Institute, Assiut University, in the period from December 2019 to December 2021, and 30 apparently healthy breast tissues from mastectomy specimens due to proliferative breast diseases or granulomatous mastitis were included as a control group.

The research program was approved by the ethical committee, faculty of medicine, Assiut University (IRB no. 17100894). All subjects were included after having an informed consent.

The mastectomy specimens from these female patients include invasive carcinomas in different stages. Patients with other malignant diseases, male gender, female with other sex linked diseases, and those who received chemotherapy for BC were excluded from the study.

Methods:

Clinical examination of all patients included the size of the primary tumours, regional lymph node status, hormonal status as ER, PR and HER 2 neu status. In addition, the tumour marker as CA 15.3 was also done.

Cytogenetic analysis:

Samples were 2 Slides of formalin fixed paraffin-embedded tissue (thickness: 3 μm).

I. Procedure steps:

A. Slide preparation:

The first step is slide deparaffinization that was done by baking FFPE overnight at 56 °C. Then, the slides were immersed in xylene and dehydrated in absolute ethanol. The second step is slide pre-treatment by using 50 ml of tissue pre-treatment solution that was heated until it was 98°C–100°C. The third step is enzyme digestion that was performed by covering the tissue with Enzyme Reagent. Then, the slides were washed in distilled water. Slides were dehydrated in a series of concentrations of ethanol.

B. FISH procedure:

The hybridization step was done by applying 5 μL of XIST probe mixture to the slide. Hybridizer settings were denaturation temperature: 80°C, denaturation time: 5 minutes, hybridization temperature: 37°C, and hybridization time: 12 – 16 hours. Washing of the slides was done by the immersion of the slides in 70 ml of 2x SSC (wash #2) then in 70 ml of 0.4x SSC/0.3% NP-40 (wash #1), and finally in wash 2. Then the slides were dehydrated in a series of concentrations of ethanol. The slides were dried in air and darkness in a closed box. Counterstaining and cover slipping were done by applying 5 μl of DAPI counterstain to the target area, and a glass coverslip was applied. Then, the slides' images were captured by Zeiss Axio Imager Z2 fluorescent microscope using Isis FISH Imaging System (MetaSystems GmbH, Altlussheim, Germany). The filters that were used for image capture were DAPI, FITC and Texas Red®.

Analysis and interpretation:

- Signal pattern interpretation:

Normal pattern	Abnormal pattern
2 Red, 2 Green (2R 2G)	Any other pattern

- A total of 200 interphase nuclei were scored per slide. XIST gene deletion is defined as results above the cut-off for XIST probe.
- The normal cut-off value of XIST probe was calculated by analysis of 50 interphase nuclei from each of five normal female breast specimens that were cut at 3 µl and then the inverse beta function was calculated [10].
- Normal cut-off value of XIST probe is 9% (for 1R 2G pattern) [10].

Statistical analysis:

All statistics were performed using SPSS (statistical package for the social sciences; SPSS Inc., Chicago, IL, USA) version 22. Statistically speaking, when the data were not normally distributed, means, standard deviations (SD), medians, and ranges were applied in addition to percentages (number of occurrences) and frequency distributions (percentages) to characterize the data. The student t-test was used to compare quantitative variables for normally distributed data, and the Mann-Whitney test was used for non-normally distributed data. To compare categorical variables, a Chi-square ($\chi 2$) analysis was performed; if the anticipated frequency was less than 5, the Fisher Exact test was utilized instead. The correlation between various variables was determined using the Pearson correlation test. P-values are always two-tailed and have a significance level of 0.05.

Results:

Demographic characteristics:

Table 1 shows both studied groups are females with matched ages and shows no significant difference between them (48.25 ± 11.32 vs. 43.83 ± 9.34, P = 0.068) among both studied groups, respectively.

Table (1): Demographic characteristics of the studied participants (n=90)

Variables	Patients (n=60)	Controls (n=30)	P value
Age (years)			0.068
• Mean ± SD	48.25 ± 11.32	43.83 ± 9.34	
• Range	21 – 70	24 – 57	
Sex			----
• Female	60 (100.0)	30 (100.0)	

Quantitative data are presented as mean ± SD and range; qualitative data are presented as number (percentage). Significance defined by $p < 0.05$

Pathological details:

The pathological data of the studied participants was summarized in table 2.

Hormonal status:

About one-third of the studied cases (33.3%, 20 cases) have positive estrogen receptors, 17 cases (28.3%) have positive progesterone receptors (17 cases were double positive ER & PR), and 13 cases (21.7%) have Her2 neu overexpression. For luminal subtypes, 10 cases (16.7%) were luminal A, 10 cases (16.7%) were luminal B, another 10 cases (16.7%) were Her2 overexpression, and fifty percent of the studied cases (30 cases) were triple negative breast cancer, as shown in Table 3.

Table 2. The pathological data of the studied participants

Variables	Patients (n=60)	
Pathological type	n	%
• IDC, NOS	52	(86.7)
• IDC, NOS with medullary features	1	(1.7)
• IDC, NOS with mucinous differentiation	3	(5.0)
• Metaplastic carcinoma	1	(1.7)
• Mixed IDC, NOS and invasive papillary carcinoma	1	(1.7)
• Extensive DCIS with foci of microinvasive duct carcinoma	1	(1.7)
• Invasive lobular carcinoma	1	(1.7)
Tumor grade		
• Grade II	55	(91.7)
• Grade III	5	(8.3)
Tumor stage		
• IA	4	(6.7)
• IB	2	(3.3)
• IIA	15	(25.0)
• IIB	11	(18.3)
• IIIA	17	(28.3)
• IIIC	11	(18.3)
TNM (Tumor size)		
• T1	10	(16.7)
• T2	40	(66.7)
• T3	6	(10.0)
• T4	4	(6.7)
TNM (lymph node metastasis)		
• N0	17	(28.3)
• N1	16	(26.7)
• N2	12	(20.0)
• N3	11	(18.3)
• Nx	4	(6.7)

IDC: Invasive duct carcinoma, NOS: not otherwise specified, DCIS: ductal carcinoma in situ. Qualitative data are presented as number (percentage).

Table (3): Hormonal status of the studied 60 females with breast cancer

Variables	Patients (n=60)	
Estrogen receptors	n	%
• Negative	40	(66.7)
• Positive	20	(33.3)
Progesterone receptors		
• Negative	43	(71.7)
• Positive	17	(28.3)
HER2		
• Negative	47	(78.3)
• Positive	13	(21.7)
Luminal subtypes		
• Luminal A	10	(16.7)
• Luminal B	10	(16.7)
• HER2 over expression	10	(16.7)
• Triple negative	30	(50.0)

Qualitative data are presented as number (percentage).

CA15.3 and XIST genes:

The mean CA15.3 tumour marker was significantly higher among the studied breast cancer cases compared to matched controls (17.19 U/mL ± 14.08 vs. 11.50 U/mL ± 3.32, P = 0.032). Also, we found that XIST was significantly reduced in tumor samples compared to controls [among the patient group, 24 BC cases (40.0%) have XIST gene deletion and 36 BC patients (60.0%) have no XIST gene deletion compared to all controls samples that didn't show XIST gene deletion (P<0.001)]. The median of XIST gene deletion among BC cases was 14.3% and ranged from 10 to 25% (table 4). No significant correlation was observed between XIST gene deletion and tumour biomarker (CA 15.3) among BC cases (P =0.905) (Table 5).

Table (4): Comparison of CA 15.3 and XIST gene deletion among both studied groups (n=90)

Variables	Patients (n=60)	Controls (n=30)	P value
CA15.3			**0.032**
• Mean ± SD	17.19 ± 14.08	11.50 ± 3.32	
• Median (range)	12.4 (4.7–69.6)	11.3 (5.5 – 19.6)	
XIST gene deletion			**<0.001**
• Negative	36 (60.0)	30 (100.0)	
• Positive	24 (40.0)	0 (0.0)	
• Mean ± SD	14.40 ± 5.29		
• Median (range)	14.3 (5 – 25)		

XIST: X-inactive specific transcript. Quantitative data are presented as mean ± SD and median (range); qualitative data are presented as number (percentage). Significance defined by p < 0.05

Table (5): The correlation between XIST gene deletion and tumor marker (CA 15.3) of the studied females with breast cancer (n=24)

Variables		XIST gene deletion
CA 15.3 (n=24)	**R**	-0.026
	p value	0.905

XIST: X-inactive specific transcript. *Significance defined by p < 0.05, r=correlation coefficient

XIST gene deletion and relation to demographic and clinical data of the studied BC cases

Table 6 shows that XIST gene deletion was more prevalent among older patients (P = 0.026), patients with advanced tumour stage (P=0.028), and those with positive nodal metastasis (P = 0.005), but there was no difference in XIST gene deletion in relation to tumour size (P = 0.501).

Table (6): The relation between XIST gene deletion, demographic and clinical data of the studied 60 females with BC

Variables	Patients with no XIST gene deletion (n=36)	Patients with XIST gene deletion (n=24)	P value
Age			**0.026**
• < 45 years	14 (38.9)	3 (12.5)	
• ≥ 45 years	22 (61.1)	21 (87.5)	
Grade			1
• Grade II	33 (91.7)	22 (91.7)	
• Grade III	3 (8.3)	2 (8.3)	
Tumor stage			**0.028**
• Stage I	6 (16.7)	0 (0.0)	
• Stage II	17 (47.2)	9 (37.5)	
• Stage III	13 (36.1)	15 (62.5)	
TNM (tumor size)			0.501
• Early	31 (86.1)	19 (79.2)	
• Advanced	5 (13.9)	5 (20.8)	
TNM (lymph node metastasis)			**0.005**
• Negative	15 (41.7)	2 (8.3)	
• Positive	21 (58.3)	22 (91.7)	

XIST: X-inactive specific transcript. Qualitative data are presented as number (percentage). Significance defined by p < 0.05

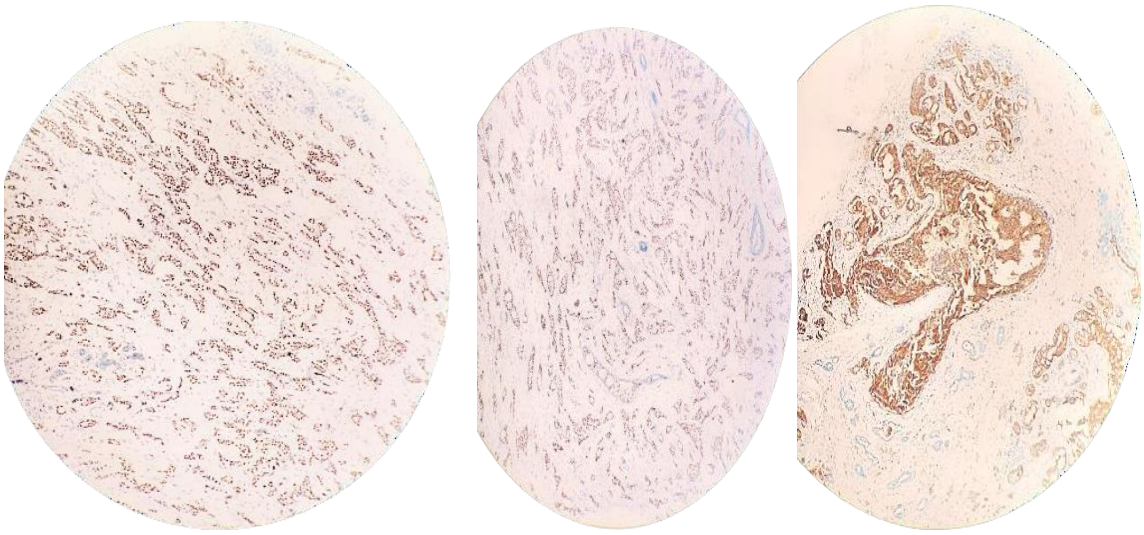
XIST gene deletion and the hormonal status of the studied BC cases

Table 7 shows that XIST gene deletion was higher among patients with negative Estrogen receptors (P=0.025), and those with triple negative BC (P = 0.009), while there was no significant difference in XIST gene deletion in those with negative progesterone receptor (P = 0.102) or those with Her2 neu receptor negative (p = 0.443).

Table (7): The relation between XIST gene and the hormonal status of the studied 60 females with BC

Hormonal status	Patients with no XIST gene deletion (n=36)	Patients with XIST gene deletion (n=24)	P value
Estrogen receptors			**0.025**
• Negative	20 (55.6)	20 (83.3)	
• Positive	16 (44.4)	4 (16.7)	
Progesterone receptors			0.102
• Negative	23 (63.9)	20 (83.3)	
• Positive	13 (36.1)	4 (16.7)	
HER2			0.443
• Negative	27 (75.0)	20 (83.3)	
• Positive	9 (25.0)	4 (16.7)	
Hormonal status			**0.009**
• Luminal A	10 (27.8)	0 (0.0)	
• Luminal B	6 (16.7)	4 (16.7)	
• Her2neu overexpression	7 (19.4)	3 (12.5)	
• Triple negative	13 (36.1)	17 (70.8)	

XIST: X-inactive specific transcript. Qualitative data are presented as number (percentage). Significance defined by p < 0.05



(A) **(B)** **(C)**

Figure (1) (A) ER: strong positive nuclear expression X200, (B) PR: strong nuclear expression X200, (C) Her2: Positive strong membranous staining X200.

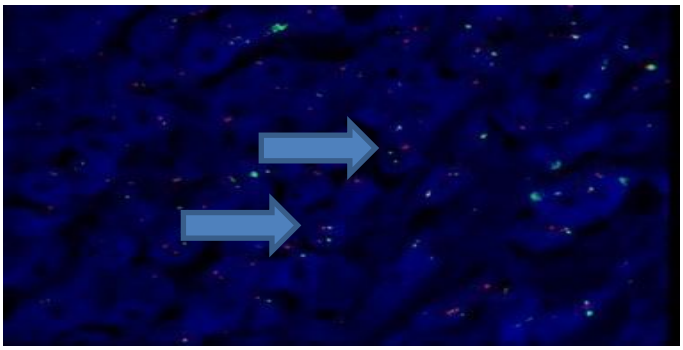


Figure (2) a triple negative breast cancer case with no XIST gene deletion

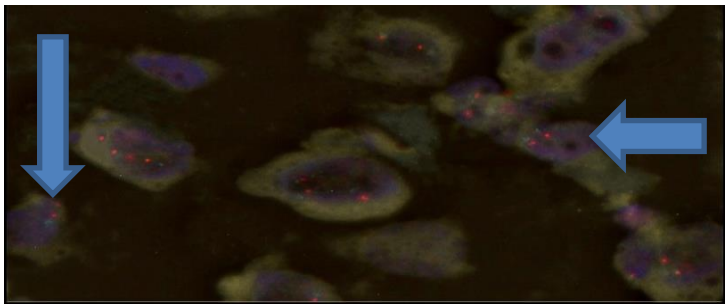


Figure (3) a Luminal type B breast cancer case with no XIST gene deletion

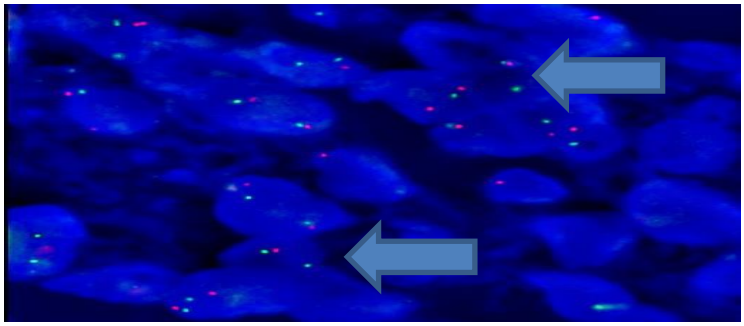


Figure (4) a triple negative breast cancer case with XIST gene deletion

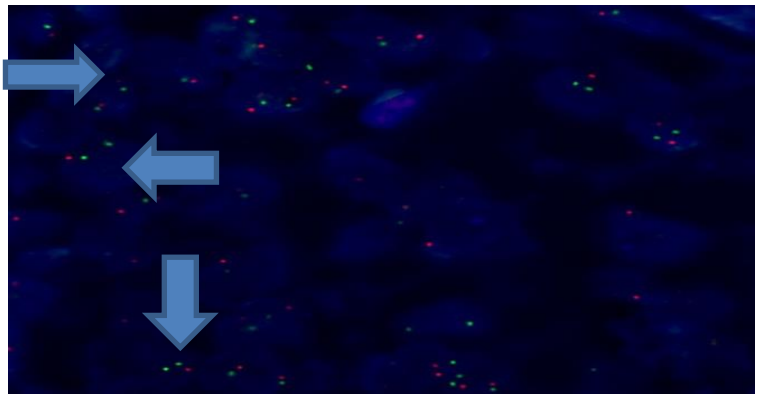


Figure (5) another triple negative breast cancer case with XIST gene deletion

Discussion:

One of the earliest long non-coding RNAs to be recognized is X-inactive–specific transcript (XIST), which plays a significant role in the X inactivation process. Growing evidence supports the role of lncRNA XIST as a regulator of cell growth and development. Numerous studies show that this transcript may play a role in the development of cancer in various tissues. XIST can function as a molecular sponge for miRNAs to change the expression of miRNA targets in addition to interacting with chromatin-modifying molecules [11].

The majority of research has suggested that XIST has an oncogenic role. Human glioblastoma stem cells are among the cells in which it was suggested to be an oncogene, and it was hypothesized that it had a significant impact on the disease's cell division [12]. Various studies have revealed contradictory findings in the cases of osteosarcoma, renal cell carcinoma, ovarian cancer, hepatocellular carcinoma, and breast cancer [11].

In the present prospective study, we aimed to detect XIST gene deletion by fluorescence in situ hybridization in breast cancer patients who were admitted to the South Egypt Cancer Institute and to correlate this deletion with the clinicopathological data of the patients. In our study, the mean age of patients at presentation was 48.25 ± 11.32 years and ranged from 21 to 71 years. This is similar to the median age of 49–52 years of breast cancer patients in Arab countries (including Egypt) that was found by Najjar and Easson (2010) [13] and Rostom et al. (2022) [14], while it was lower than that in the United States of America (USA) (61 years), which was found by Verdial et al. (2017) [15]. The median age at diagnosis varies by race and ethnicity. The age difference between the USA and Egypt may be partially explained by the younger population in the latter or cultural differences [14, 16]. Egyptian older women are less likely to seek medical advice than younger women, compared to their counterparts in the USA [14].

In this study, we observed a significant deletion of XIST in tumor samples compared to healthy-matched controls. This finding supports the hypothesis that XIST gene was abnormally deleted in breast cancer.

Evaluation of XIST gene in breast cancer gives controversial results between studies. In line with the current finding, previous researches supported that XIST expression was significantly decreased in breast cancer, and overexpression of XIST increased cell apoptosis and inhibited the growth, migration, invasion, and proliferation of breast cancer cells. Xing et al. (2018) found that knockout of XIST in mice’s mammary glands accelerated primary tumor growth as well as metastases in the brain [17]. The results of Zheng et al. (2018) showed that in breast cancer tissues

and cell lines, XIST was markedly down-regulated. In another way, overexpressing XIST dramatically reduced the proliferation, migration, and invasion of breast cancer cells through the miR-155/CDX1 axis [18]. Liu et al. (2016) found that tumor metastasis was markedly increased by XIST knockdown, particularly in the brain. Their results strongly suggest that lncRNA XIST plays a critical role in tumor progression and metastasis by inducing epithelial-mesenchymal transition related gene upregulation [19].

Again, more studies support this hypothesis, as a study by Liu et al. (2021) has reported that XIST and UBAP1 (Ubiquitin Associated Protein 1) are downregulated in BC cells. They concluded that forced up-regulation of XIST has attenuated proliferation, migration and invasion of these cells, and accelerated cell apoptosis [20].

On the other hand, Zong et al. (2020) has reported up-regulation of XIST in BC cells, parallel with down-regulation of miR-125b-5p and up-regulation of NLRC5 (Nucleotide Like Receptor C). Breast cancer cells' ability to proliferate, migrate, and invade has been dramatically inhibited by XIST silencing. It has been demonstrated that XIST sponges miR-125b-5p and subsequently affects the expression of NLRC5 [21]. Moreover, a study by Zhang et al., (2020) has found that cells resistant to doxorubicin in breast cancer have higher levels of XIST expression. Furthermore, XIST up-regulation increases ANLN (Anillin, Actin Binding Protein) expression, which prevents doxorubicin-treated breast cancer cells from apoptosis and promotes cell proliferation. XIST acts as a sponge for miR-200c-3p, which regulates ANLN expression [22].

This difference of XIST gene expression among different studies could be attributed to difference in the patient's characteristics as (age, ethnicity, hormonal status, and their tumor stage at the time of diagnosis), as all these factors could influence the genetic characteristics of the patients and in turn XIST gene deletion.

The present study found that the XIST gene deletion was more prevalent among older aged patients. In line with the current study Abdelmohsen et al. (2013) stated that XIST was one of the recognized long noncoding RNAs examined for their involvement in the senescence of cells and showed down-regulation of gene expression during senescence [23].

In the current study, we compared the XIST gene deletion among patients with different clinical stages of BC and results indicated that the XIST gene was significantly deleted in patients with advanced tumor stage (III) than in patients with early tumor stage (I-II) (P value 0.028). XIST gene was also remarkably deleted in patients with positive lymph node metastasis than in those without nodal metastasis (P value 0.005). Thus, XIST may serve as a tumor suppressor in breast cancer. Similar finding was reported by Zheng et al. (2018) whose results indicated that the level of XIST was significantly lower in stage III-IV patients than in stage I-II patients. The expression level of XIST was also remarkably lower in patients with lymph node metastasis than in those without metastasis [18].

It is well known that increased tumor size and advanced nodal status are established independent prognostic factors of poor outcomes [24]. Based on the finding of the current study we can conclude that XIST deletion is associated with poor outcome among BC patients. More researches are needed in this era to establish the prognostic role of XIST deletion among BC patients.

In TNBC, a number of lncRNAs are found to be tumor-driving oncogenic lncRNAs, and a smaller number are found to be tumor-suppressive lncRNAs; they are all specifically implicated in the development of cancer [25]. However, the regulatory functions and molecular mechanisms of XIST in TNBC remain unclear. In the current study, we detected that XIST was significantly down-regulated in TNBC. This finding was supported by Li et al. (2020), who demonstrated down-regulation of XIST in TNBC cells. In these cell lines, up-regulation of XIST caused apoptosis while inhibiting cell growth and the epithelial-mesenchymal transition (EMT). It has been found that in these cells, XIST targets miR-454. Their study was one of the first studies to verify that XIST functioned as a tumor suppressor in TNBC [26].

The impact of XIST on malignancies and especially BC is complex, including many tumorigenic regulatory networks and tumor-associated miRNAs. So, a sufficient understanding of XIST in molecular biology and cytogenetic studies is needed and will be helpful for the utilization of XIST as a diagnostic biomarker and therapeutic target for clinical cancer therapy.

Conclusion:

XIST gene was deleted among BC patients. XIST gene deletion was more prevalent among patients with TNBC. XIST gene deletion could be considered as an important predictive biomarker in breast cancer and an indicator of poor clinical outcome. Thus, this research provided new evidence for XIST-mediated pathway as an effective target for BC management and the development of new treatment methods involving gene therapy. We suggest searching for a suitable target therapy to XIST gene deletion and introducing this type of therapy in the management of BC.

List of abbreviations:

BC	Breast cancer
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
IDC	Invasive duct carcinoma
lncRNA	Long noncoding RNA
NSCLC	Non-small cell lung cancer
PR	Progesterone receptor
SCC	Saline sodium citrate
SD	Standard deviations
SPSS	Statistical package for the social sciences
TNBC	Triple-negative breast cancer
WHO	World Health Organization
XCI	X chromosome inactivation
XIST	X-inactive–specific transcript

Competing interests:

The authors declare that they have no competing interests.

Acknowledgements: (Not applicable)

Ethics approval and consent to participate:

The protocol was approved by the Institutional Review Board of Faculty of Medicine, Assiut University (approval number: 17100894, 13 January 2020 date of approval). Our study conformed to all requirements as governed by the declaration of Helsinki.

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