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Original Research Article

Role of Topoisomerase II Alpha Gene Status on Selection of Anthracycline Therapy in Triple Negative Breast Cancer

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Abstract

Triple negative breast carcinoma [TNC], is peculiar to have hormone receptors negative [ER and PR] and lose HER2 receptor reactivity by immunostaining. Generally, TNC tumors carry a bad prognosis. Little is to be perceived about TOP2A gene amplification in triple negative breast cancer. This information may be of importance for the expectation of the influence of therapy. Thus the objective of this study is to evaluate topoisomerase II alpha gene status in triple negative breast cancer.

A cross sectional study was done, from July 2014 to February 2015 in Oncology hospital of Baghdad Medical Complex. Only 28 patients with triple negative breast cancer were included [ER-, PR-, her2/neu-] other molecular types of breast cancer were excluded. All 28 cases were successfully hybridized using dual-color chromogenic in situ hybridization [DC-CISH][Zyto- Vision] probe kit for determination of topoisomerase II alpha gene status Patients mean age was [47.7 ± 8.8 SD]. Tumor stage was intermediate [stage II] in [57%] while 36% of cases were in stage III. Tumor grade was 46% for grade II and 54% for grade III. Regarding Ki-67, 54% of cases express Ki-67 more than 14% in comparison to 46% of them the expression was less than 14%.Regarding topoisomerase II alpha gene amplification, gene amplification was noticed in single case only as [3.6%] and it was low amplification. So TNBC is always associated with insignificant alterations of topoisomerase 2 alpha gene and thus may predicts poor response to anthracycline therapy in this molecular group of breast cancer.

Key Words:TOP2A, CISH, immunohistochemistry, molecular subtypes of breast cancer

دورحالة الجين Topoisomerase II A في اختيار العلاج أنثراسيكلين في سرطان الثدي الثلاثي السلبي.

الخلاصة

سرطان الثدي الثلاثي السلبي [TNC]، هوهو نوع من سرطان الثدي يكون فيه هرمون مستقبلات سلبي [ER& PR] وتفقد HER2 مستقبلات التفاعل في الصبغات المناعية. عموما، [TNC] يحمل مستقبل سيء في تطور المرض القليل هو أن ينظر إليها عن التضخيم TOP2A الجينات في سرطان الثدي الثلاثي السلبي. قد تكون هذه المعلومات ذات الأهمية بالنسبة لتوقع تأثير العلاج . الهدف من هذه الدراسة هو تقييم topoisomerase IIA جين في سرطان الثدي الثلاثي السلبي.

تم إجراء دراسة مقطعية، من يوليو ٢٠١٤ إلى فبراير ٢٠١٥ في مستشفى الأورام في مدينة الطب في بغداد أدرجت ٢٨ فقط المرضى الذين يعانون من سرطان الثدي ثلاثي السلبية [HER/neu−،PR− ،−ER] تم استبعاد أنواع الجزيئية أخرى من سرطان الثدي . تم تهجين كل ٢٨ حالة بنجاح باستخدام مولدات اللون المزدوج اللون في الموقع التهجين [Zyto] [Zytovisison −DC−CISH] طقم التحقيق لتحديد حالة topoisomerase IIAجين.

كان معدل عمر المرضى [٤٧.٧ ± ٨.٨. تم تشخيص كل الحالات .كانت مرحلة الورم وسيطة [المرحلة الثانية] في [٥٧٪] في حين أن ٣٦٪ من الحالات في المرحلة الثالثة. كان الورم درجة ٤٦٪ للصف الثاني و ٥٤٪ للصف الثالث. وفيما يتعلقبKi−67, ٥٤٪ من الحالات أكثرمن ١٤٪ مقارنة مع ٤٦٪ منهم كان أقل من ١٤٪ .فيما يتعلق topoisomerase IIA التضخيم الجينات، وقد لوحظ التعبير التضخيم الجيني في حالة واحدة فقط كما [٣.٦ ٪] وكان التضخيم المنخفض . لذلك يرتبط TNBC بتغيرات غير مهمة معtopoisomerase IIAجين، وبالتالي قد يتوقع ضعف الاستجابة للأنثراسيكلين العلاج في هذه المجموعة الجزيئية لسرطان الثدي.

الكلمات المفتاحية : الجين TOP2A، CISH التهين الموضعي الصيغي ، تقنية المناعة الكيميائية النسيجية، المجموعات الجزيئية لسرطان الثدي.

Introduction

One million women around the world are detected to have breast cancer each year. Breast cancer is the predominant cancer in females [1]. Breast cancer is a congruous disease with different morphological types, molecular characteristics, and compliance to treatment [2]. Four distinct biologic subtypes involving luminal A, luminal B, HER2-positive and basal-like including triple negative breast cancer (TNBC) were identified. [2]

The last type forming 15% of invasive ductal breast carcinoma. These tumors are frequently estrogen receptor (ER)-negative, estrogen receptor [PR]-negative, human epidermal growth factor receptor 2 (HER2)-negative, cytokeratin 5/6 positive, and/or epidermal growth factor receptor (EGFR)[human epidermal growth factor receptor 1] (HER1)-positive by Immunohistochemistry (IHC) [3].

TNBCs account for 15% - 20% of whole types of breast cancers which are distinguished by of ductal histology, high grade and high proliferative capacity and mitotic count. They are notably more aggressive than their counter parts and unevenly affect child bearing young women [4]. TNBC carry bad prognosis, and it have a risk to re-appear locally and of low opportunity of breast cancer patient to survive [5]

They are celebrated to be ER/PR and HER2/neu negative because of low expression of the luminal and HER2 gene. Nearly 77% of basal- like breast cancers are triple-negative thus, triple negative (TN) and basal breast cancer are not synonymous [6].

Several factors can participate to assess the fate of patient with breast cancer and to be dealt with significantly like tumor stage, grade, lympho-vascular invasion and histological type of breast cancer.3

The TNBC is clinically heterogenous and lacks established certain prognostic markers. Nevertheless, several molecular markers have important predispositions as targets for the systemic therapy of breast cancer.

Ki-67 is a proliferation marker that is identified as a distinct predictive and prognostic factor in primitive stages of breast cancer [7]. High Ki-67 expression is associated with better Response to chemotherapy but with poor prognosis that is similar to the TNBC features [8].

Additional factors like the proliferative index, ploidy, presence of P53, cytokeratins (CK), (EGFR),or topoisomerase II alpha (TOP2A) alterations, may be helpful for prognostic assessment or for the evaluation the fulfillment of treatment [9]. The TOP2A gene, located on chromosome 17q21-22, it encodes topoisomerase II alpha, is a molecular target for anthracyclines so it is an important marker that determine the response to treatment with chemotherapeutic agents [10]. TOP2A anomalies [whether the gene is amplified or lost] are detected in up to 30-90 % of HER2 amplified breast cancer and the detection of the amplification highly predominant. Recent reviews published that TOP2A amplification was detected in 2.78

% HER2 non-amplified breast cancers [10]. TNBC patients treated with Adjuvant anthracycline who demonstrate loss of amplification of TOP2A gene show bad compliance to treatment [8]. Patients with a pathologic full response to anthracycline chemotherapy [neoadjuvant]show generally well prognosis irrespective of molecular subtype of breast cancer and they may demonstrate TOP2A amplification [11]. The existences of wide ranges of technologies that can study and deal with multiple genes status facilitate a better comprehension of breast cancer as variable different biologically and molecularly distinct diseases [11].

In situ hybridization is a technique that make a benefit of complementary DNA, RNA or even modified nucleic acids strand with labels (called probe); these probeis are useful to localized a specific RNA or DNA sequence in a section of tissue (in situ) for determining the organization, regulation, and function of genes. *In situ* hybridization was innovated by Joseph G. Gall and Mary-Lou Pardue [12]. Chromogenic *in situ* hybridization is considered as a cytogenetic technique that form a sort of unity of the revealing the chromogenic signals by in situ hybridization and immunohistochemistry [IHC] techniques for detection of protein expression of that gene [13,14]. It was developed in 2000 as substitutive to fluorescent in situ hybridization [FISH] to highlight HER2/neu oncogene amplification [13]. Both CISH and FISH are identical principally as both methods used hybridization technique for detection and determination the existence, loss, or even amplification of certain locations on DNA [1]. However, CISH technique is considered much more practicable and feasible in diagnostic laboratories due to it make a benefit of bright-field microscopes rather than the costly and sophisticated fluorescence microscopes used in FISH [15]. Dual color-CISH is a modification of CISH technique that required two distinct probes on single slide [16]. It is a well-substantiated technique for the identification of HER-2/neugene amplification although it is occasionally described to be less efficient than FISH[17]. In this technique, one probe will bind the control or what is called the reference which is represented by chromosome 17 centromere (CEN17); while the other probe binds the sequence of interest which is represented by HER-2/neu or other gene. A particularized therapy depended on the molecular biology markers of tumor is the tendency in clinical practice nowadays [5].

This fact to be known is essential to assess the extent of response to treatment, as the amplification of TOP2A is described to be a dose-dependent sensitivity to anthracycline therapy in breast cancer. So the objective of this study is to evaluate topoisomerase II alpha gene status in triple negative breast cancer and its correlation to certain clinico-pthological parameters.

Materials and Methods

A cross sectional study was done, from July 2014 to February 2015. Twenty eight cases were collected through this period from oncology hospital in Baghdad Medical city. Nearly all patients came from Baghdad, middle and south of Iraq. In oncology hospital, after receiving paraffin embedded blokes of tissue, sections were reviewed and tumor classified according to the World Health Organization [WHO] classification [18]. Staging of all collected cases of breast cancer done depending on histopathological picture according to American Joint Committee on Cancer [AJCC] [19]. Grading of ductal carcinoma was carried out following the recommendations of Scarf, Bloom and Richardson [20].

For all cases received in this hospital immunohistochemical assay were done including four markers (estrogen receptors, progesterone receptor, her2/neu and ki67). According to the results of immuno-histochemically analysis, Only Patients with

triple negative breast cancer (ER-, PR-, her2/neu-) are included in this study which was 28 cases.

Chromogenic in situ hybridization was performed in Central Public Health Laboratory/Baghdad. According to inclusion criteria, total collected 28 cases were successfully hybridized using dual-color chromogenic in situ hybridization (DC-CISH) (Zyto-Vision) probe kit for determination of topoisomerase II alpha gene status by using dual-color chromogenic hybridization.

CISH was accomplished for all cases [n = 28] depending the protocol of dual-color CISH technology of Zyto-Vision. As a technique it facilitates highly specific and obvious picture due to the peerless Zyto-Vision_ repeat subtraction technique and is peculiar to be of high sensitivity for detection of topoisomerase II alpha gene amplification. As a technique, it is a novel and may be useful in routine diagnostic works; the so-called ZytoDot- 2C protocol is conducted according to the manufacturer instructions [21].

Preparatory steps:

After Incubation the slides at 70_C for 10 min, dewaxed twice in xylene (10 min). Hydration in graduation of alcohol [100% (2x), 96% (2x), 70% (2x) Aqua dest (2x)] 5 min for each. Peroxidase in 3% H2O2 (5 min). Washing (3x) in Aqua dest (2. Min). Incubation in pretreatment solution (98˚C]) for 15 minutes, and wash(3x) in Aqua dest (2 min each). Adding pepsin solution was for 3.5 min followed by other wash step. and then drying (20 min).

Denaturation and probe hybridization:

The ZytoDot Topo II alpha .CEN 17 Probe (P) was vortexed and 10 microliters was added to each slide prior the slides were cover-slipped and sealed with Fixum. Add Horseradish peroxidase [HRP] polymer to detect HER2 and AP polymer detects CEN 17.

On a hot plate, denaturation was performed (74˚C for 5 min). The slides should be rapidly transferred to chamber [humidity chamber] and be hybridized overnight oven at 37º C.

Post-hybridization and detection process:

The glue was removed and cautiously by emerging the slides in (SSC) buffer for five min. Then the slides incubated for 5 min in (SSC) wash buffer at 78˚C (water-bath), washed (3x) in aqua dest for 2 min. Blocking agent added and incubated at (25˚C) for 10 min. Blocking agent was removed and Anti-Digoxigeninanti-DNP mix added while slides were incubated in chamber (humidity chamber) at 37˚C for 30 min., after that washing 3X in (TBS)wash buffer for 2 min. and then apply HRP Polymer and incubate at 37º C for 30 min. wash 3X in (1x) (TBS) wash buffer for 2 min. AP polymer mix had been applied to the slides and then the slides were incubated at 37˚C for 30 min, wash with TBS for 2 min.

Before incubation of the slides in a dark chamber at room temperature for 15 min;6 drops of AP-Red Solution were added and then the slides carried into a jar (staining jar) and underwent washing in tap water for 2 min. Horse raddish peroxidase -Green Solution was added and again incubation of slides in a dark chamber at 25˚C done and for 2 min., washing them in TBS buffer for 1 min., and then stained with Haematoxylin for 5 seconds, after that the slides were transferred with staining jar and washed in running tap water for 2 minutes, and finally the slides underwent dehydration in 100% ethanol 3X /30 seconds and incubation was done twice time in xylene and for 30 seconds. Slides were rapidly cover-slipped with DPX. Examination of slides was done by light microscope using (x10), (x20) and (x40) objective lenses. For signal counting the (x100) objective lens was used.

Results

Twenty eight (28) cases were successfully hybridized applying topoisomerase II alpha genes probe. All the patients were females.

Ethical committee approval was granted. Patients mean age was (47.7 ± 8.8 SD) and range of 33-64 years. All cases are diagnosed as invasive ductal carcinoma (NOS)except single case of medullary carcinoma. Tumor stage was intermediate (stage II) in fifty seven percent of cases (57%) while 36% of cases were in stage III. Tumor grade was 46% for grade II and 54% for grade III and no case was detected of grade I (Table 1).

A 20 cases were associated with axillary lymph nodes metastasis. The distribution of nodal metastasis was as follows: two patients were considered as (N3) as they have more than nine lymph nodes were involved by tumor metastasis, ten patients had (4-9) positive axillary nodes (N2), and remaining eight patients had a positive axillary node as (N1) i.e. 1-3 positive axillary lymph nodes, while eight patients (28.6%) had negative axillary nodes [N0]. Regarding Ki-67, 54% of cases express Ki-67 more than 14% in comparison to 46% of them the expression was less than 14%.

Regarding topoisomerase II alpha gene amplification, gene amplification was noticed in single case only as (3.6%) and it was low amplification.

Table 1: Clinico-pathological parameters, Ki-67 and Topoisomerase II alpha gene status in 28 patients with Triple negative breast cancer

Characteristics	No.	%
Histological type		
IDC	**27**	**96.4%**
Medullary carcinoma	**1**	**3.6%**
Stage		
Stage I	**2**	**7%**
Stage II	**16**	**57%**
Stage III	**10**	**36%**
Grade		
GI	**/**	**/**
GII	**13**	**46**
GIII	**15**	**54**
Lymph node involvement		
N0	**8**	**28.6**
N1	**8**	**28.6**
N2	**10**	**35.7**
N3	**2**	**7.14**
Ki-67		
>14%	**15**	**53.6%**
< 14%	**13**	**46.4%**
Topoisomerase II alpha gene		
Amplified	**1**	**3.6%**
Not amplified	**27**	**96.4%**

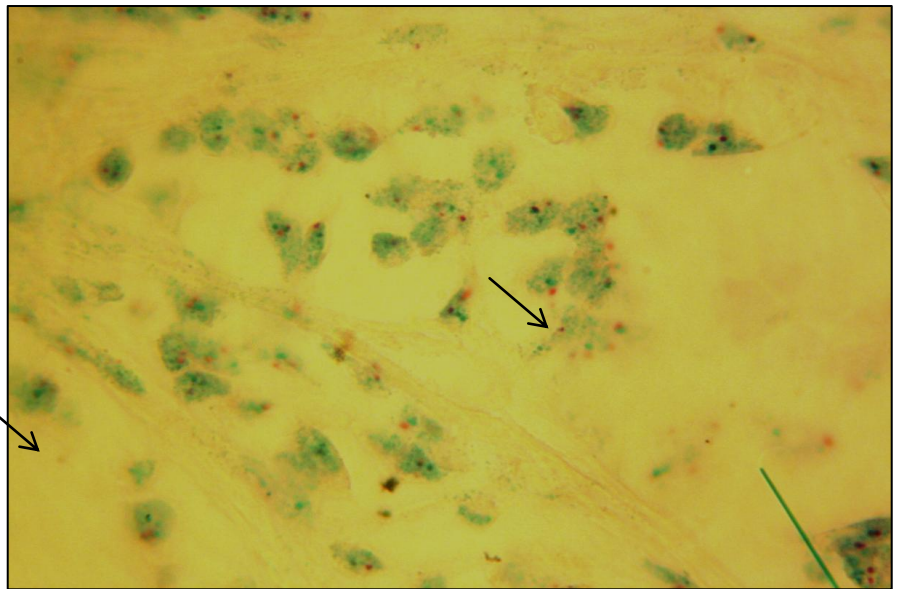

Figure 1: Invasive ductal carcinoma of the breast [diploid topoisomerase II alpha /chromosome 17 centromere by Chromogenic In Situ Hybridization]. The arrows point to normal [not amplified] diploid dots-like intra nuclear signals. Green signal for topoisomerase II alpha gene and red signal for chromosome 17 centromere [oil immersion x1000].

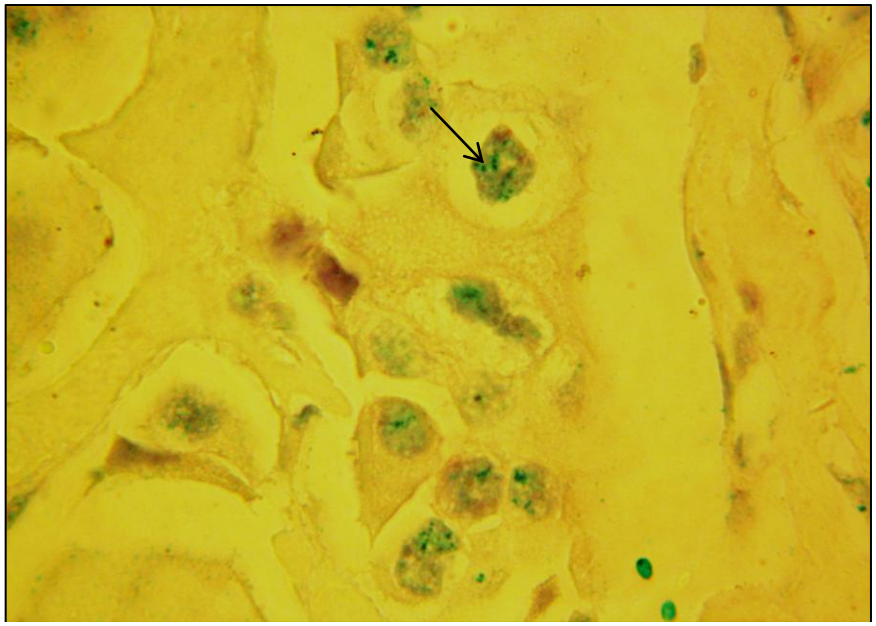

Figure 2: Invasive ductal carcinoma[low-amplified topoisomerase II alpha gene by Chromogenic In Situ Hybridization]: the arrow shows that there are multiple small dots like intra nuclear greenish signals of her2/neu gene and doubled intranuclear red small signals for chromosome 17 centromere. her2/neu / CEN 17 ratio was between 2.3- 4 [oil immersion x1000].

Discussion

Recent advances in the medical and molecular sciences have led to an advanced classification of breast carcinomas, which depend on variations in the patterns of gene expression that derived from cDNA microarrays. So according to this

classification, three main types emerged including; luminal A& B, HER2/neu and basal like type [triple negative]; in which having a clear correlations with prognosis

and response to treatment [22]. However, immunohistochemistry can be used to determine the more complex status of gene expression [23]. Actually cDNA microarray is not considered as a standard and optimal method to classify breast carcinomas needed in routine in clinical practice and determination of suitable therapy. So patients that have ER negative, PR negative and Her2/neu negative by immunohistochemistry is considered to be triple negative group that have high risk for recurrence [24].

According to the results of this study mean age of patients was 47.7 and it is near to that recorded in Francesco *et al* [25] in which 81% of cases were invasive ductal carcinoma [NOS] in comparison to 96.4% of cases in our study.

In this study 57% of patients were in stage II, 54% of patients were in grade III, 35.7% of cases were in N2. According to the study [22] the higher percentage of patients were in grade III, N1and 22 patients showed T2 and thirty three were T3 disease.

Other retrospective studies on patients with TNBC breast cancer, the mean age of patients 48.4, those patients were more likely to present with high histological tumor grade, high stage, most of them presented with more than five lymph nodes involvement. Proliferative index of those patients was more than 14% in more than 60% of cases [26, 27].

The mild differences among these studies and the current study may be attributed to variations of sample size, type of antibodies used whether monoclonal or poly clonal which can affect the specificity of the results, technical factors, geographical variations and hereditary factor is important to be considered. Regarding the grade, the differences may be due to subjectivity in assessment the degree of differentiation of malignant cells in between pathologists.

Regarding topoisomerase II alpha gene status, the gene was low amplified in one [3.6%] case out of 28 cases in this sample. In other study [28] topoisomerase alpha gene amplification was 4.5% of cases. These results can be explained according to many literatures [29-31] which states that topoisomerase 2 alpha gene amplification was uncommonly detected in her2/neu not amplified breast cancer. So TNBC is always associated with insignificant alterations of topoisomerase 2 alpha gene and thus may predicts poor response to anthracycline therapy in this molecular group of breast cancer

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