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Evaluation of Tamoxifen Therapy in Breast Cancer Sudanese Patients A Clinical Pharmacogenomics Study in Radio Isotope Center Khartoum (RICK)

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ABSTRACT

Tamoxifen is a non-steroidal anti-estrogen drug widely used in the treatment of breast cancer and metabolized by the common isozyme CYP2D6. This study was set out to evaluate this current clinical practice using pharmacogenetic techniques. to assess the use and outcomes of tamoxifen treatment. The study was done in Radiation and isotope center Khartoum (RICK) with experimental pharmacogenetic work for the selected patient's blood samples, using PCR-RFLP method. Tamoxifen showed relapses independent to treatment duration with hot flashes as major side effects, but has good quality of life. Pharmacogenomically, patients using tamoxifen (n=56), only (26) samples passed the test, where (80.8%, n=21) were found to be intermediate metabolizers, whilst only (15.4%, n=4) were poor metabolizers and only (3.8%, n=1) were found to be extensive metabolizers. The majority of Sudanese women patients have tamoxifen resistance with high relapses of disease, this due to the intermediate biotransformation by *CYP2D6* among Sudanese ladies so genotyping polymorphism is significant regarding the drug response.

KEY WORDS: Breast Cancer, Tamoxifen, Pharmacogenomic, Pharmacogenic.

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INTRODUCTION

Tamoxifen (TAM), is a selective estrogen receptor modulator; use as adjuvant and metastatic settings of breast cancer, it reduced risk of breast cancer recurrence with (5 years) of adjuvant therapy^{1,2,3}.

Tamoxifen Chemical structure

Tamoxifen is the generic name for the drug sold as Nolvadex, Istubal, Valodex⁴.

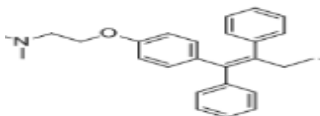


Figure No.1: Tamoxifen chemical structure This adapted from⁵.

Polymorphic enzymes responsible for the biotransformation of TAM

TAM is extensively cleared by hepatic and intestinal metabolism via oxidative and conjugating enzymes *Cytochrome P450*⁶ which is a super gene family of drug-metabolizing enzymes responsible for the metabolism of approximately (90%) of human drugs⁷, TAM metabolize by *CYP2D6* gives major primary metabolite 4-hydroxy-TAM formation⁸ which is also converted to endoxifen via hepatic flavin-containing monooxygenase, on other this enzyme catalyse TAM metabolized into tam-N-oxide⁹ which at last yield endoxifen by *CYP2D6* as illustrated in figure no.2.

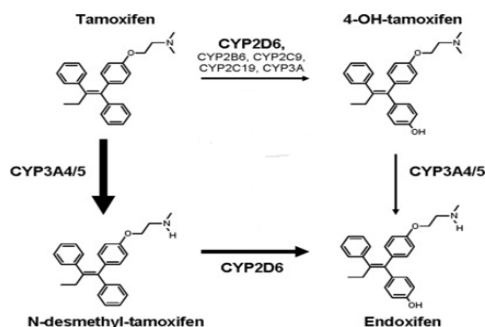


Figure No.2: CYP2D6 Mediation in Biotransformation of Tamoxifen metabolites this adapted from¹⁰

Following continuous treatment in humans, the serum level of Tamoxifen reaches steady state after approximately 4weeks¹¹. Because of its high ant estrogenic potency, endoxifen may play an important role in the clinical activity of tamoxifen¹². The P450-mediated biotransformation of tamoxifen is important in determining both the excretion of the drug and its conversion to the active metabolite¹³.The mutations in the *CYP* genes may cause absence of enzyme, diminished enzyme

expression, enzyme with altered substrate specificity or increased enzyme expression^{14,15,16}. Thus determination of these genetic polymorphism may be of clinical value in predicting adverse or inadequate response to certain therapeutic agents and in predicting increased risk of environmental or occupational exposure linked disease¹⁷. Even if *CYP2D6* polymorphism represents an excellent example of the potential clinical implications of pharmacogenetic research,^{18,19}. In the tumor cells, tamoxifen metabolites bind to estrogen receptors, have also been suggested to contribute to inter-individual variability to tamoxifen benefits²⁰.

Mechanism of tamoxifen action and Pharmacologic tolerance

Tamoxifen metabolizing genes polymorphisms affect the plasma concentration of tamoxifen metabolites²¹. Since the effects of tamoxifen are primarily mediated through the *ER*, the degree of *ER expression* is a strong predictor of responses to tamoxifen, so both pharmacogenomics effects and pharmacological interactions may alter the metabolism and potentially the efficacy of tamoxifen^{2,22,23,24}.

Objective

This study was set out to evaluate this current clinical practice using pharmacogenetic techniques. to assess the use and outcomes of tamoxifen treatment.

MATERIAL AND METHODS

Study population

Breast cancer patients were diagnosed based in clinical examination as well as mammography and pathological examination. This study is hospital based cross sectional retrospective and prospective study in period April 2014-april 2017 in Sudanese female

Selection criteria

Data collection form: Sources of data will include: clinical examination (done by the physician in the respective hospitals), a questionnaire and laboratory investigations, and ethical clearance obtain from ethic committee of Ministry of health Khartoum state while initial permissions were obtained from authorities of the study areas (RICK) Also patients' written informed consent was obtained from each participant prior the enrolment into the study. A simple questionnaire is designed and used for each patient; Medical records were reviewed to confirm complete demographic characteristics present, disease status and treatment. Questionnaires were administered during the face-to-face interviews, which were conducted in English translated orally to Arabic language.

Inclusion and exclusion criteria

Patients must be women with breast cancer adult with any type and stage at age more than (15years) at the time of surgery. Patients must have adequate blood counts and adequate kidney and liver function.

Software and computer facilities

The Thomson and Reuters Citation Management Program Endnote version .3.The Statistical Package for Social Sciences (SPSS) version16.The web-based bioinformatics program Nebcutter (URL: source).The Internet database: NCBI, Primer 3-Plus. Microsoft office package 2007 Enterprise, Gel mega, digital camera and software in a computer.

Pharmacogenomics investigations methods

The CYP2D6 polymorphism genotyping determination methods.

Blood sample collection

From a total of all the study participants (n=56) patients used tamoxifen, Human blood samples from breast cancer patients were collected into an EDTA containing tube, then stored in a refrigerator till extraction at (-20⁰c). Then CYP2D6 DNA containing gene extraction and purification using GF-1 protocol version 3.1

Interpretation of PCR method

According to manufacture CYP2D6 kit (from Sac ace technologies- Casera –Italy) manual, the PCR product length for CYP2D6 should be (352bp), to be visualized after staining with Ethidium bromide.

CYP2D6 polymorphism genotyping determination method

First Suitable enzyme selection: From a total of (56) patients' blood sample, only 27 (50%) sample exhibited promising result. After that the Digestion reactions was performed

Statistical analysis methods

Suitable statistical software such as SPSS V.16 was used for analysis, while appropriate statistical tests were performed to compare results. Results were considered significant, at level of (P \leq 0.05). The data were cleaned and checked for consistency before entering it for analysis.

RESULTS&DISCUSSIONS

Liver CYP2D6 enzyme polymorphism genotyping determination results

DNA extraction and purification results (Gene Template)

From a total of (56) breast cancer patients treated with Tamoxifen, only (27) (48.2%) blood sample were successfully ready-made for the tentative genes of Tamoxifen- metabolizing enzyme CYP2D6. All samples produced DNA with different purities (≥ 1.7) expressed as ratio of *DNA/Protein* using Nano-drop Spectrophotometry, whilst the electrophoresis gel plate was shown in figure no. 3.

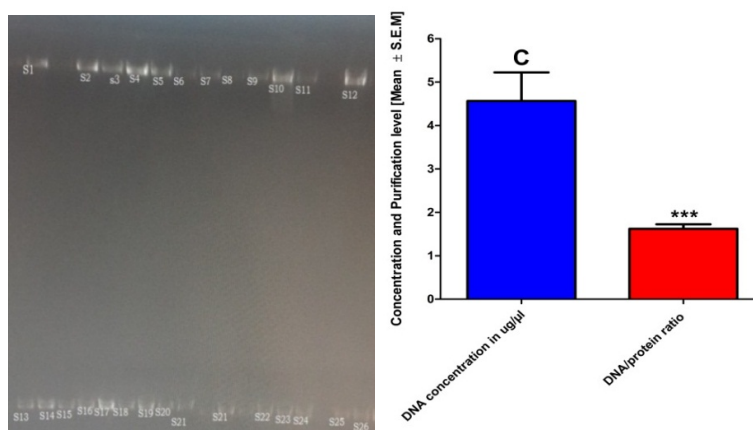


Figure No.3: DNA after extraction it was load in agarose gel (2%) using loading dye, whilst the right graph showed the level of DNA purification Alone (Blue bar) in comparison relative protein expressed (Red bar). The level of significance was donated by stars where p-value ($P \leq 0.001$) and C=control. The detailed data were provided at (Appendix No V page 143)

Although, a huge effort was done in this study in a repeated subsequent manner using different methods, the of results revealed that, only half of the blood samples eligible to be used in polymorphism determination, because the samples used from a population with great varieties and the used primer cannot match all of them with similar degree, Besides, this great variety of the samples arise from two reasons, the ethnic diversity that reflect the exact Sudan condition as an Afro-Arab country with different tribes and the study was conducted at RICK that lies at the capital of Sudan, Khartoum.

In addition to, the DNA different purities, may be due to contamination with salts and a trial was done to solve the problem by ethanol precipitation which may result in an overestimation of the nucleic acid concentration and/or negatively influence downstream analysis as mentioned by Wilming to 2017²⁵.

CYP2D6 DNA containing gene amplification results

Primer selection for CYT2D6 genes results

Using the bioinformatics techniques, the *CYT2D6* primer was designed with a length of (20 base pairs) size molecular weight of 352 as in table no.1.

Table 1: Sequences and properties of designed PCR primers used to amplify the CYT2D6 used in this study

Primers	Primer sequence (5'-3')	Length	TM (°C)
Forward	5-CAA GAA GTC GCT GGA GCA GT-3	20	60.5
Reverse	3-CTG CAG AGA CTC CTC GGT CT-5	20	62.5

Product size (bp) 352

DNA amplification process (RT-PCR) results

At optimum amplification cycle with the selected temperature of (57.5 ° C), and all the other protocol parameters were set as default, DNA product containing *CYT2D6* genes of (352 bp) was obtained with good integrity and high Purity comparable to the ladder, See figure no. 4.

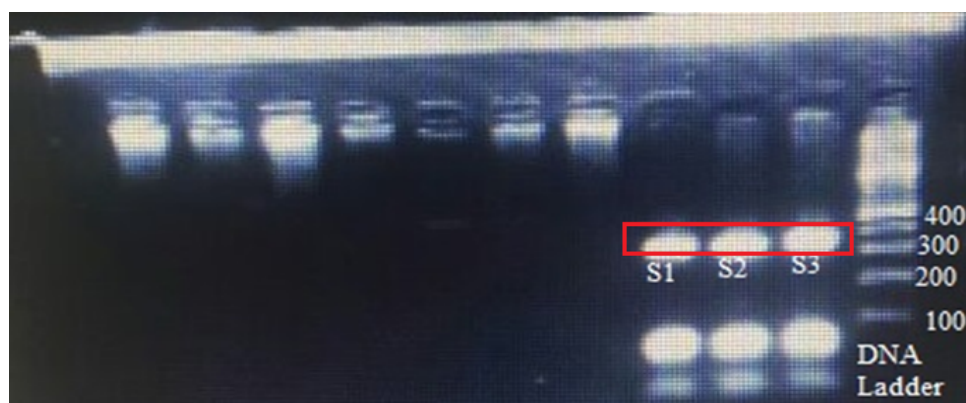


Figure No.4: Electrophoretic gel plate of the produced DNA derived from a patient blood sample (n= 26), after amplification compared to the *DNA ladder* during PCR process, the produced DNA molecular weight was lied between the 300-400 appears as band intensities, besides the ignored rest diameters' light bands were sometimes lids below the required ones.

Using bioinformatics techniques, a primer was designed *CYP2D6* guided by that used by ¹⁰ findings and specifications with a different molecular weight and at different annealing temperature, but it was succeeded to produce a product with (352 bp), and digested with *BstNI* restriction enzyme.

Digestion reactions and polymorphism genotyping determination

Biochemical reaction between the *CYP2D6 DNA-containing gene* extracted from the patients treated with Tamoxifen (n=56) with the selected enzyme *BstN1*, only (n=26) pass the test, where

(80.8%, n=21) were categorized as an intermediate metabolizer, (15.4%, n=4) are poor metabolizers and only (3.8% n=1) are extensive metabolizers, see table no. 2.

Table 2: Three main genotypes distribution of CYP2D6 gene polymorphism in Tamoxifen treated patients blood samples (n=56).

CYP2D6	Tamoxifen cases n=56	P-value
Genotyping	percentage/frequency	
EM	1(1.8%)	0.000
IM	21(37.5%)	
PM	4 (15.4%)	
Missed	30(53.6%)	

* P = < 0.05 P≤0.01, ***, P≤0.001; EM=Extensive metabolizes; IM=intermediate metabolizes; PM=Poor metabolizes.

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Patterns of genetic polymorphism of CYP2D6

The PCR product (352 bp) from patients' blood samples of n=27 was digested with the suitable selected enzymes and gave single fragment (352 bp), two fragments (200 and 152 bp) and three fragments (352, 200 and 152 bp), as shown in figure no.5.

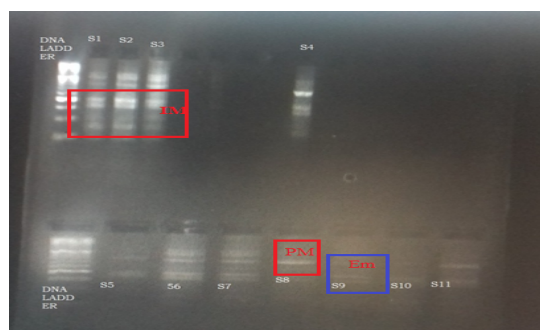


Figure No.5: CYP2D6 PCR products after restriction digestion withBstN1 on 3% agarose gel. In upper row Lane 1 DNA Ladder100 bp, S1, S2, S3=IM and S4 = PM Genotype, S5, S6, S7, S8, S10 and S11=IM, S9 = EM Genotype.

Genotyping studies among Sudanese breast cancer patients using tamoxifen (n=56) to categorize their enzyme activity pattern(s) regarding the tamoxifen, biotransformation to its active metabolite responsible for the desire activity, as effective cancer hormonal therapeutic agent revealed that, the absence of restriction site, gave a (352 bp) fragment, indicating an allele, in which heterozygotes showed IM genotypes with (334, 200 and 152 bp) bands and wild type EM genotypes showed (200 bands) and (152 bp) fragments Frequencies of CYP2D6EM, IM, and PM genotypes: These findings categorized the Sudanese as; more than three quarter are heterozygous, less than quarter have absence of restriction site and the minimum number of them are fully functional enzyme, this indicate the heterozygous contribution in breast cancer disease, this is supported by Surekha 2010who concluded that significant increase in heterozygous frequency in breast cancer indicates its disadvantage¹⁰.

Relation of Tamoxifen metabolizing genes

Polymorphisms vs. patients' factors

A. Percentage of CYP2D6 genotypes vs. family history breast cancer patients

Intermediate metabolizer manifested the highest frequency in all types of family history breast cancer patients but mainly in –ve family history while both other types (IM and PM) appeared only in –ve family history as structured in figure no.6.

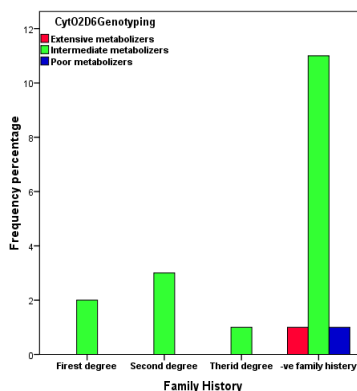


Figure No.6: Frequency distribution of CYP2D6 genotypes vs. family history in breast cancer (n=56)

Intermediate metabolizer manifested the highest frequency in all types of family history breast cancer patients but mainly in –ve family history while both other type (IM and PM) appear in –ve family history only; i.e. heterozygous genotypes frequency increase in non-familial history breast cancer this is antagonized by ¹⁰ who determined that, significant frequency of IM increased with

familial history breast cancer patients so suggested influence of CYP2D6 gene on other cancer susceptibility genes¹⁰.

B. Percentage of CYP2D6 genotypes vs. grades of breast cancer

The intermediate metabolizer genotypes possessed the highest frequency than other genotypes in all grades but the poor metabolizer appeared only in **grade IV** as shown in figure no. 7.

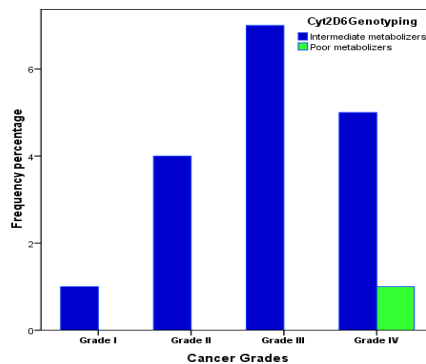


Figure No.7: Frequency distribution of CYP2D6 genotypes vs. grades of breast cancer (n=56).

In different breast cancer grades the IM give the highest percentage than other genotypes but the poor metabolizer appeared only in grade IV, that mean the IM, and PM contribute with the highest grades of breast cancer; which supported by Kalyana 2010 who suggested that PM and IM genotypes were associated with advanced stages of the disease¹⁰ and Hyung 2011 observed that PM group showed more aggressive nodal status²⁶.

CYP2D6 genotypes vs. hormonal receptors status of breast cancer cases

Intermediate metabolizer genotypes distributed among all hormonal receptors mainly *HER +ve* while extensive mainly in *ER+ve* as illustrated in figure no. 8.

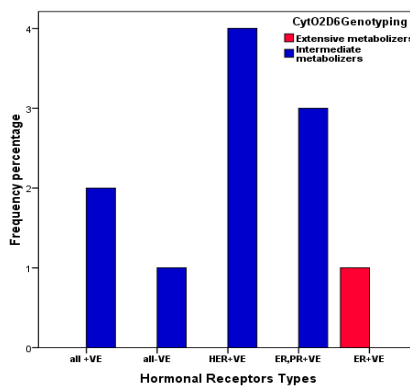


Figure No.8: Frequency distribution of CYP2D6 genotypes vs. hormonal receptor status of breast cancer case (n=56).

The heterozygous genotypes is highly distributed among all hormonal receptors mainly HER+ve while EM only appear in ER+ve so the type of receptor associated with genetic polymorphism, whilst literature review revealed no relevant data were reported

C. *CYP2D6* genotypes vs. breast cancer relapses

Patients having IM genotypes showed highest frequency in breast cancer relapses with minimum number of them possess EM as shown in figure no. 9.

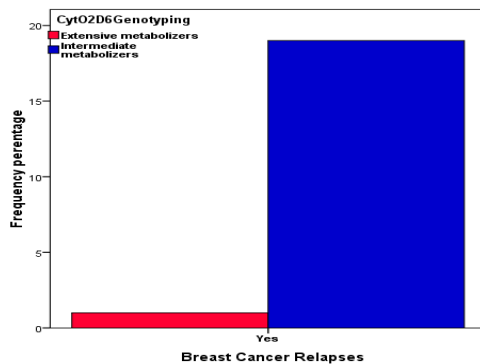


Figure No.9: Frequency distribution of CYP2D6 genotypes vs. breast cancer relapses (n=56)

Tamoxifen is an antiestrogen use to treat breast cancer it active metabolize form endoxifen which catalyze by enzyme called CYP2D6.

CYP2D6 gene is the most important polymorphic genes and its genotype frequency, ¹⁰.

Patients with IM genotypes showed the highest frequency of relapses with minimum number of them possesses EM, i.e. there is an appositve correlation between heterozygous and breast cancer relapses which was antagonized by Timothy 2011who structured that; there were no association between CYP2D6 inhibition and breast cancer recurrence ²⁷.

CONCLUSION

The genotyping polymorphism is significant regarding the drug response and optimum dose regimen determination was necessary for better therapy and less adverse drug reactions.

Tamoxifen mono-therapy for breast cancer must be limited in Sudan, because it gives a moderate action in all cancer grades among Sudanese ladies especially in those have *HER+ve* one, and completely loss its activity in minimum number of them in advance stages. Although it gives full activity in patients possess *ER+ve receptors*, Sudanese people may have different genetic maps and of great interest regarding the pharmacogenomics investigations.

There is a negative correlation between heterozygous and treatment outcomes.

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