Afr. J. Med. Med. Sci. (2022) **5**1(1)**,** 43-49

The presence of some TNFα and receptor polymorphic forms might reduce the risk of breast cancer in Nigeria

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Abstract

Introduction: Breast Cancer remains a global health problem and therefore its early detection and good diagnostic biomarkers are important for successful treatment and prevention. Tumour necrosis factor alpha Single Nucleotide Polymorphisms (TNFαSNPs) have been implicated in the presentation of some human cancers. This preliminary study was carried out to determine for the first time, the role of TNFα SNPs in breast cancer amongst Nigerian women and its possible use as risk assessment tool for breast cancer.

*Methods***:** Participants (200) were recruited from the University College Hospital, Ibadan for this study. Genotyping of TNFα and its receptor SNPs from purified DNA in breast cancer and control samples were carried out using Polymerase Chain Reaction - Touch down technique with allele specific primers.

Results: Five alleles showed a significant association for a reduced risk of breast cancer TNFα 488G (P=0.0140 OR=0.236, 95%CI= 0.07542 to 0.7391), TNFα 380G (P=0.0369, OR= 0.5108, 95%CI= 0.5108 to 0.9251), TNFα 308A P=0.0149, OR = 0.3271, 95%CI=0.1373 to 0.7794), and TNFR1A+IV56+10 -G (P= 0.0024, OR= 0.3535, 95%CI= 0.1851 to 0.6753) TNFα 1032C(P= 0.0158, OR=2.077 CI=(1.180 to 3.653) with very **high heterozygosity** observed among SNPs

Conclusion: Specific genotypic variations in TNFα-SNPs might be a potential risk factor for breast cancer among Nigerian population, raising the possibility of the use of TNFα-SNPs as bio-markers for early risk indicators for breast cancer in Nigeria.

Keywords: *Tumour necrosis factor alpha, single Nucleotide polymorphisms, breast cancer*.

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Résumé

Introduction : Le cancer du sein reste un problème de santé mondial et, par conséquent, sa détection précoce et ses bons biomarqueurs de diagnostic sont importants pour un traitement et une prévention réussis. Les polymorphismes nucléotidiques simples du facteur de nécrose tumorale alpha (TNFαSNP) ont été impliqués dans la présentation de certains cancers humains. Cette étude préliminaire a été réalisée pour déterminer pour la première fois le rôle des SNP du TNFα dans le cancer du sein chez les femmes nigérianes et son utilisation possible comme outil d'évaluation du risque de cancer du sein.

Méthodes : Les participants (200) ont été recrutés à l'University College Hospital d'Ibadan pour cette étude. Le génotypage du TNFα et de ses récepteurs SNP à partir d'ADN purifié dans des échantillons de cancer du sein et de contrôle a été effectué en utilisant la technique de réaction en chaîne par polymérase - Touch down avec des amorces spécifiques d'allèles.

Résultats : Cinq allèles ont montré une association significative pour un risque réduit de cancer du sein TNFα 488G (P=0,0140 OR=0,236, 95 % IC= 0,07542 à 0,7391), TNFα 380G (P=0,0369, OR= 0,5108, 95 % IC= 0,5108 à 0,9251), TNFα 308A P=0,0149, OR = 0,3271, IC à 95 %=0,1373 à 0,7794) et TNFR1A+IV56+10 -G (P= 0,0024, OR= 0,3535, IC à 95 %= 0,1851 à 0,6753) TNFα 1032C (P = 0,0158, OR = 2,077 IC = (1,180 à 3,653) avec une très forte hétérozygotie observée parmi les SNP *Conclusion* : Des variations génotypiques spécifiques des TNFα-SNP pourraient être un facteur de risque potentiel de cancer du sein au sein de la population nigériane, ce qui soulève la possibilité d'utiliser les TNFα-SNP comme biomarqueurs pour les indicateurs de risque précoces de cancer du sein au Nigeria.

Mots clés : *Facteur de nécrose tumorale alpha, polymorphismes nucléotidiques uniques, cancer du sein*.

Introduction

Cancer is a major source of morbidity and mortality globally [1] and it is caused by fundamental defects in the regulation of cell division [2]. Over the years, many theories have been put forth to explain cancer, but it is now known that most, if not all, cancers arise from defects in DNA sequences [2]. Experimental data from laboratory animals have shown that, the same extent of DNA damage or the same mutations in oncogenes and tumour suppressor genes in different hosts can result in wide range of cancers due to effect of numerous polymorphic tumour susceptible genes [3]. Epidemiological data also showed that susceptibility to common, spontaneous cancers in humans can be influenced considerably by multiple polymorphic host genes with relatively weak effects. This points to the fact that in addition to hereditary familial cancer syndromes, the spontaneous cancer is also under strong genetic control [3].

Breast cancer is one of the major cancers causing death in women worldwide [4] and it develops as a result of complex interactions between genetic and environmental factors. Breast cancer survival rates differ greatly worldwide, from 80% or more seen in North America, Sweden and Japan to around 60% in middle income countries and below 40% in low income countries [5]. The low survival rates in developing countries has been attributed to lack of early detection programmes, inadequate diagnosis and treatment facilities, resulting in a high proportion of women presenting with late-stage disease. Research on new and effective early diagnostic methods for breast cancer might however help improve breast cancer treatment and increase breast cancer survival rate in developing countries. Breast cancer has the highest mortality rate in Nigeria (26.5%) [6], hence there is an urgent need for interventions in terms of early diagnosis and treatment facilities.

Earlier studies on genetic factors associated with breast cancer showed that tumour suppressor genes, BRCA 1 and BRCA 2 are the two most commonly mutated genes associated with early onset and familial breast cancer [7]. Further studies confirmed that only a small minority of patients with breast cancer actually develop as a result of inheritance of germline mutations in BRCA 1 and 2 genes [7]. In a study conducted by Fackenthal *et al*. [8], it was found that only 7·1% and 3·9 % among 434 Nigerian breast cancer patients studied had mutations in BRCA 1 and 2 genes respectively, indicating that some other genetic factors may be responsible for the development of breast cancer in the remaining patients

Other genetic factors that have been implicated in the development of breast cancer are Single Nucleotide Polymorphisms (SNPs) of numerous genes. SNPs are the most common genetic variation in human genome, and these variants have been continually studied due to their effects on biological function [4]. SNPs of genes such as Tumour Necrosis Factor Aphla (TNFα) whose protein product is a pro-inflammatory cytokine have been suggested to promote growth of tumour cells. The most widely studied polymorphic forms are the TNFα- 238 and TNFα- 308 at the promoter region of the gene [9]. The common G allele of TNFα-238 and the rare TNFα- 308A polymorphic forms are associated with high TNF production and it has been observed that these particular polymorphic forms have an apparent protective role against a range of tumours among Chinese population [10-11], however a controversial study conducted on Chinese women found an association of TNFα- 308A with metastasis in patients with triple negative of hormonal test breast cancer [11], with no conclusive reasons for the observation. Marsh *et al.* [12] also found TNFα SNPs (TNFα-+488A and TNFα-859T) to be associated with the risk of having bladder cancer among Caucasian population. Zuo *et al.* [13] found an associated of TNFα-308 and TNFα-857 with the risk of cervical cancer in Southwest China. This study was conducted to determine the role of TNFα-SNPs as part of genetic factors responsible for breast cancer development in Nigeria and whether TNFα-SNPs could be included as potential biomarkers in the early detection of breast cancer in Nigeria in order to facilitate early treatment and increase the survival rate of breast cancer in Nigeria.

Methods

Study Site

A baseline case-control study was conducted using histologically confirmed breast cancer patients registered in University of Ibadan College Hospital (UCH). This study was approved by the University of Ibadan and University College Hospital (UI/UCH) Ethics Committee prior to sample collection. All ethical requirements were met during the course of this study.

Sample collection and DNA extraction

A total of 200 individuals between ages 20-70 years were recruited for the study (100 breast cancer patients age matched with 100 controls). Blood

sample (5ml) was collected from each participant for DNA extraction using Qiagen kit for whole blood extraction, and the extraction was done at Genetics and Bioethics Research Unit, Institute for Advanced Medical Research and Training (IAMRAT), University College Hospital (UCH), Nigeria. Blood samples (5ml each) were collected once from the enrolled participants for DNA extraction. The blood samples were collected in ethylene-diamine-tetraacetic acid (EDTA) bottles to prevent coagulation and each bottle was well labelled. To 3ml of each blood sample, 6ml of Red blood cell (RBC) lysis solution was added to lyse all red blood cells in the sample. Then the mixture was spun and supernatant was decanted. This step was repeated twice using 3ml RBC lysis solution till residue was clear enough. To the clear residue, 3ml White blood cell lysis solution was added (to break open all the white blood cells releasing the nuclei contents), the mixture was allowed to homogenise at room temperature overnight. RNAse (25µl) was added at 37ÚC for 30minutes to digest all RNA contaminants. Proteinase K (25µl) was also added at 55ÚC for one hour to digest all protein contaminants. Protein precipitation solution of 1.5ml was added to each sample to precipitate out protein contaminants. DNA of each sample was precipitated in iso-propanol, washed in ethanol and dissolved in Tris-EDTA (TE) buffer.

The extracted DNA was quantified using Nanodrop spectrophotometer and the stability of the DNA was check on 1%agarose gel. The optical density (OD) ratio of the DNA used was 1.8-2.0. Extracted DNA samples in TE buffer were later stored in -20 freezer.

Genotyping of TNFα-SNPs

Genotyping of TNFα-SNPs was done using Polymerase Chain reaction (PCR). ~50ng/µl DNA concentration was used with a master mix of 20 mM Tris-HCL, 22mM KCL, 22 mM NH_4Cl, 1.8 mM $MgCl_2$, 5%Glycerol, 0.05%Tween® 20, 0.06% IGEPAL® CA-630, 0.2 mM dNTPs , 25units/ml One Taq® DNA Polymerase (BioLabs® inc) and 10mM of each primer to make up a total reaction volume of 10µl for each sample.

A Touch down (TD) programme with varied annealing temperatures of 65 ÚC -55ÚC and 56 ÚC -46 ÚC was employed for the PCR at Bio-Science Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The TD programme used was: 94·0 ÚC for 2mins, followed by 35 cycles of 93·0 ÚC for 15 secs, 65·0 ÚC -56 ÚC / 56 ÚC -46 ÚC for 20 secs, 72·0 ÚC for 30 secs, 72·0 ÚC for 5mins and 10·0 ÚC "

Primer sequences used for genotyping TNFα SNPs were obtained from previous studies [12-13] and TNFR1A+IV56+10 SNP primer sequences were designed using Web-based Allele-Specific PCR assay designing tool for detecting SNPs and mutations [14]. Polymerase chain reaction products were then electrophoresed on 1·5% agarose gels and visualised with UV illuminator. Results were read and scored.

Statistical analysis

Data were entered on excel spread sheet and statistical analyses were performed using SPSS statistic software (16·0) and Haploview genetic software (4·2). Genotyping results were sored as 1 for presence of TNFα-SNP in a sample and 0 for its absence and Chi square was used to determine the difference in genotyping results of TNFα-SNPs in breast cancer patients and control at $\alpha=0.05$. Haploview software was used to determine if the genotypes of TNF and it's receptor SNPs follows Hardy-Weinberg rule or not.

Results and Discussion

Breast cancer is a global health problem with more cases being diagnosed with increase in mortality rate especially in developing countries like Nigeria where diagnosis and treatment facilities are inadequate [6]. Gene polymorphisms are part of genetic factors that have been implicated as risk factors in the development of breast cancer. Tumour Necrosis Factor alpha Single Nucleotide Polymorphisms (TNFα-SNPs) are part of such polymorphisms that have been associated with different types of cancers, including breast cancer among various populations.

In this baseline case-control study the role of TNFα and its receptor-SNPs in breast cancer among Nigerian women patients registered in University of Ibadan College Hospital (UCH) was investigated.

The presence of TNFα and its receptor-SNPs was detected through visible bands seen on gel electrophoresis for both controls and breast cancer samples (Fig. 1). The frequencies of TNFα and its receptor-SNPs varied among controls and breast cancer patients. Interestingly, it was found that 380A which had previously been described to be linked to the protective roles and high production of TNFα against tumour development, showed a reduced risk and high frequency among control compared to breast cancer cases in this study; as well as TNFα-488G, 380G, 1032C and TNFR1A+IV56+10 -G ($p<0·05$), while TNFα 1032C indicated an increased risk with odd ratio 2.077 (Table 1).

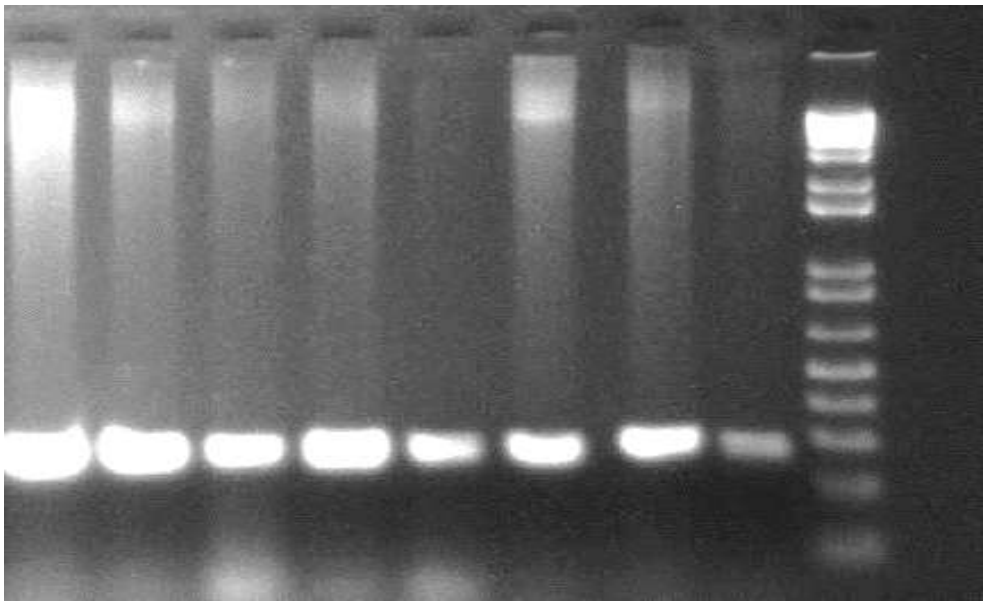

Fig 1. Amplification product of both control (C1-C4)and breast cancer (B1-B4) samples agarose gel for TNFα 859C

Table 1: Showing differences in presence of TNF alpha and receptor SNPs in both breast cancer patients and control

S / N	Allele	cases	control	P value	odd ratio	confidence interval	P value summary
1	488G	85	96	0.0140	0.2361	0.07542 to 0.7391	Significant
2	488A	86	87	1.0000	0.9179	0.4076 to 2.067	Not significant
3	238G	91	96	0.2507	0.4213	0.1253 to 1.416	Not significant
4	238A	81	87	0.3350	0.6370	0.2956 to 1.373	Not significant
5	308A	79	92	0.0149	0.3271	0.1373 to 0.7794	Significant
6	308G	85	90	0.3928	0.6296	0.2682 to 1.478	Not significant
7	859C	86	85	1.0000	1.084	0.4931 to 2.383	Not significant
8	859T	81	81	1.0000	1.0000	0.4933 to 2.027	Not significant
9	380G	58	73	0.0369	0.5108	0.2820 to 0.9251	Significant
10	380A	80	84	0.5813	0.7619	0.3689 to 1.574	Not significant
11	TNFR1+IV56+10 G	35	55	0.0024	0.3535	0.1851 to 0.6753	Significant
12	TNFRA1+1V56+10 A	28	34	0.4173	0.7285	0.3847 to 1.380	Not significant
13	1032C	44	62	0.0158	2.077	1.180 to 3.653	Significant
14	1032T	73	71	0.8750	0.9055	0.4882 to 1.680	Not significant

These results indicate the absence of TNFα-308A, 488G, 380G, 1032C and TNFR1A+IV56+10 - G can be possible risk factors for breast cancer in Nigeria. Kohaar *et al.* [15] established an association between TNFα 308 and breast cancer among Indian population. Guojiang *et al.* [16] also found an association between TNFα-308AA and breast cancer among premenopausal women while others found no association with breast cancer. Yang *et al*. [17] found a negative association between TNFα 308 and breast cancer in Caucasian populations just as found in this study in Nigerian population. As earlier said, studies on other cancer types had also found similar associations, Zuo *et al*. [13] found an association of TNF α308 G/A and TNFα 857C/T with increased risk of cervical cancer and no association with TNFα 238 G/A, TNFα 863 in South west China and Marsh *et al*. [12] found a significant association between TNF polymorphisms TNFα 488A and TNF 859T and risk of bladder cancer.

For the first time in Nigerian population, this study observed a high heterozygosity in SPNs of TNFα genotyped (Fig 1-6). This result is a deviation from Hardy-Weinberg's rule (Table 2). and a similar observation once occurred in Ivoirians population in a study by Santovito *et al*. [18]

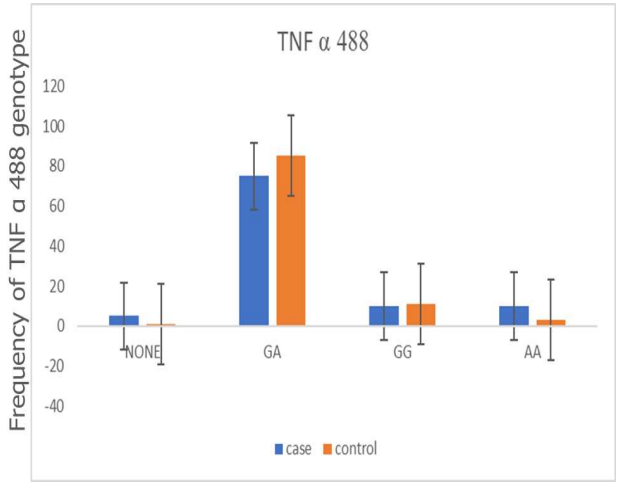

Fig: 2a

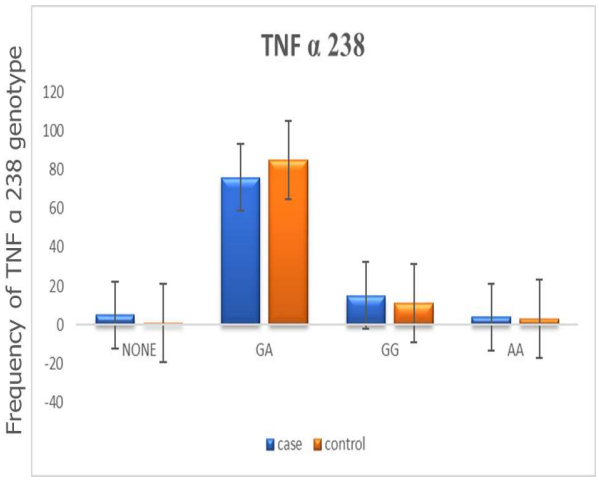

Fig: 2b

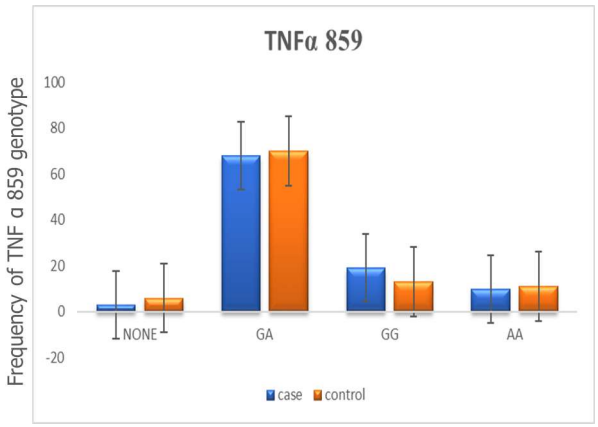

Fig.: 2c

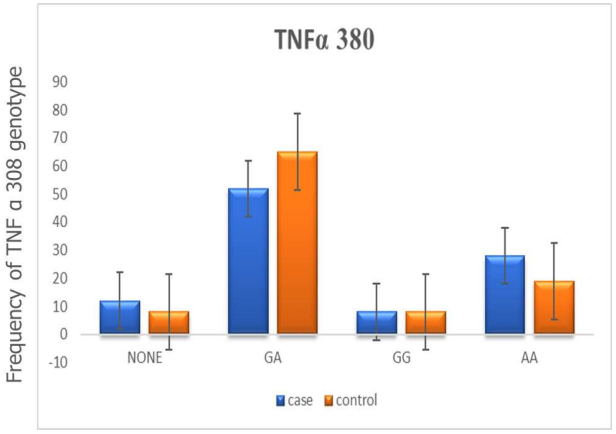

Fig.: 2d

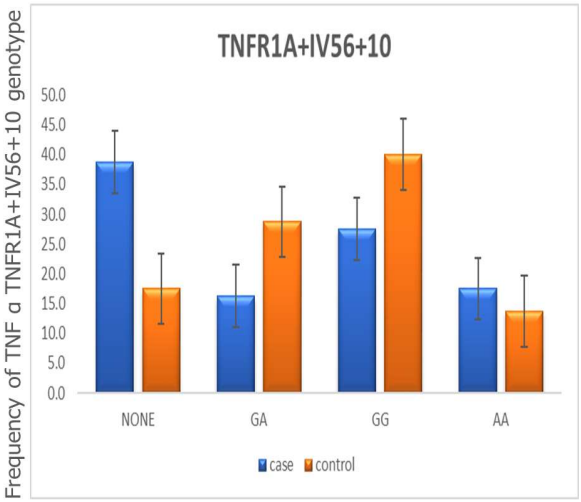

Fig.: 2e

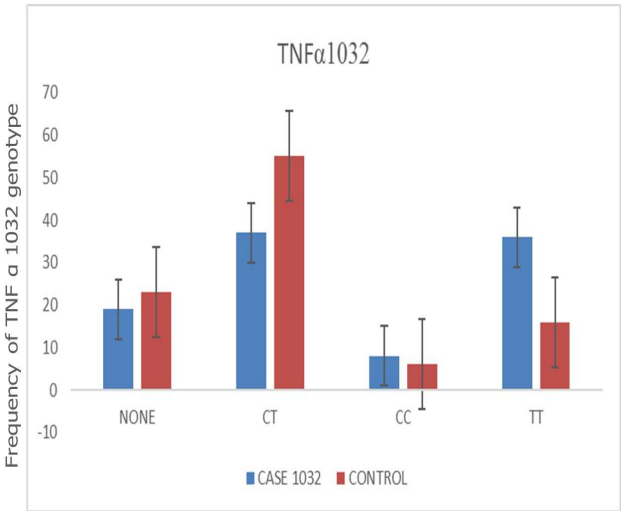

Fig. 2f

This genotypic occurrence may likely be as a result of evolutionary events resulting in a selection for heterozygote genotypes within the population which although not yet linked to breast cancer but may be important in proportionating the expression of TNF gene for Survival.

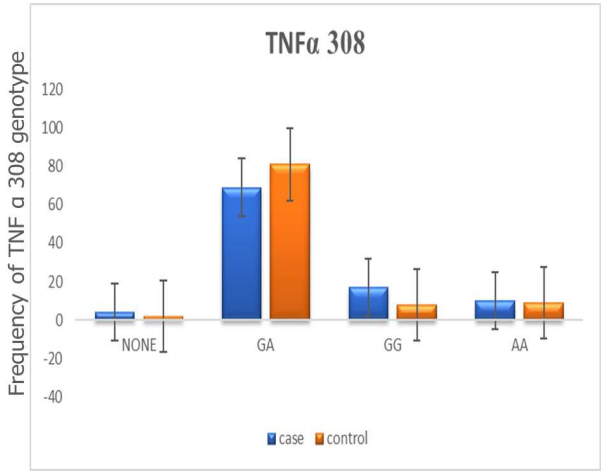

Fig.: 2g

Fig 2.: C1-C4/C5-C8 represent the control samples while B1-B4/B5-B8 represent breast cancer samples.

Table 2: Linkage analysis using Haploview genetic software

	Name Alleles	Position ObsHET	PredHET	HWpval	MAF
1	rs1800610 (TNFá488)	31543827	0.743 0.493	4.0786E-13	0.441 G:A
2	rs9282876 (TNFá859)	31574585	0.693 0.499	5.4113E-8	0.475 C:T
3	rs1800750 (TNFá380)	31575186	0.644 0.493	2.4926E-5	0.441 A:G
4	rs1800629 (TNFá308)	31575254	0.787 0.5	1.8255E-16	0.498 A:G
5	rs361525 (TNFá238)	31575324	0.807 0.494	9.7796E-21	0.443 G:A
6.	Rs1800693 (TNFR1+IV56+10)	6330843	0.313 0.468	7.0E-4	0.374 G:A
7.	Rs1799964 (TNFá1032)	31575254	0.58 0.473	0.0093	0.383 T:C

This study showed that TNFα-308A, 488G, 380G might be associated with reduced risk of having breast cancer in Nigeria. These alleles are known to promote the expression of TNFαin the body. These results agree with earlier studies and further confirm the dual role played by TNFα in tumour cells. It destroys tumour vasculature in high does and in low doses it acts as a vasodilator and chemo-attractant for cells responsible for the inflammatory responses thereby promoting development of tumour stroma [12].

This study therefore, suggests further research on TNFα and its receptor SNPs as possible risk assessment tool for breast cancer among Nigerian women.

Acknowledgments

We appreciate the staffs of Genetics and Bioethics Unit Institute for Advanced Medical Research and Training (IAMRAT) for their contributions to the success of this study. We also appreciate those who consented during sample collection and we appreciate the staffs of Bioscience Unit and International Institute of Tropical Agriculture (IITA).

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Received = 8/01/2020
Accepted = 8/09/2020