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Risk Factors and Molecular Study of Vimentin Gene (VIM), Associated with Female Breast Cancer in Khartoum, Sudan

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Abstract: *Background*: The aim of this study was to investigate the possible risk factors and mutations in *VIM* gene among Sudanese's breast cancer women in Khartoum State. *Methods*: This case-control study involved 45 patients with breast cancer and 45controls. It was conducted across three hospitals and two laboratories in Khartoum State. A structural questionnaire was used to obtain data regarding age, family history, menarche, marriage, menopause, pregnancy, nulliparous and parous women, breast-feeding, use of fertility or contraceptive drugs and grade of the disease. DNA from patient and control tissues was extracted using extraction kits. PCR was conducted to amplify *VIM* gene using specific primers. PCR products were sequenced in order to detect the mutation in *VIM* gene. Data was analyzed using Pearson's Chi-square tests to identify risk factors associated with breast cancer. *Results*: The study showed that the main risk factors associated with breast cancer were family history with first degree relative, menarche, irregularity of menarche, reproductive factors such as pregnancy, breast-feeding and nulliparous. DNA sequencing revealed no mutations in *VIM* gene associated with breast cancer in Sudanese women in Khartoum State. *Conclusion*: The association of other risk factors such as menopausal status, age of menopausal, oral contraceptive birth control and fertility hormones needs more illumination and further work. Other genes associated with breast cancer can be investigated.

Keywords: Breast Cancer, Risk Factors, Sudan, *VIM* Gene

1. Introduction

Breast cancer remains the most common malignancy in women across the world [1]. Although the incidence rate is higher in Europe and the United States the death rates are insignificant [2]. The incidence of breast cancer varies from 89.7 per 100,000 in East Africa to 19.4 per 100,000 individuals in West Europe [3]. Although breast cancer incidence is lower in Sub-Saharan African countries than in developed countries, the cancer picture in Sub-Saharan Africa and especially in Sudan is changing. Lately, breast cancer incidence and mortality rates are increasing. It is the most common cancer in Sudanese women living in Khartoum State [4]. A high standard of mortality rates (ASR) for breast cancer in women living in Khartoum State -using the age-standardized rates per 100 000 person-year between 1966 and 2000, were 60.8 and 66.8 per 100000, respectively. This data is higher than other studies reported in black women in East Africa, Harare, Zimbabwe (46.8 per 100,000, 2006– 2010) and in Kampala, Uganda (31.0 per 100,000, 1991–2006) [5]. In Sudan, Khartoum State, for the period from 2009–2010 the first data from National Population-based Cancer Registry (NCR) was established. The data revealed a total of 6771 incident cases of cancer, 3125 (46.2%) were men and 3646 (53.8%) were women [6].

Several epidemiological risk factors such as age, family history, menarche, pregnancy, nulliparous and porous women, menopause, Hormonal birth control, fertility drugs, breast feeding and marriage, different diagnosis stages of

disease are considered as significant factors for breast cancer development among women in Africa [2, 7, 8].

Genetics plays a limited but important role as a risk factor for breast cancer. About 5%-10% of breast cancer cases demonstrate an autosomal dominant inheritance. Many researchers in breast cancer had identified specific genes that potentially confer a high risk of developing breast cancer such as *BRCA1*, *BRCA2* and *VIM* gene [9].

VIM gene has gained great importance as a canonical marker of epithelial-mesenchymal transition (EMT), also known to maintain cellular integrity and provide resistance against stress [10]. A variety of processes occurring in cells undergoing malignant transformation and metastasis involve *VIM* gene [11]. Evaluating the expression pattern of *VIM* gene in normal and cancer tissues can be of great value in tumor diagnosis and prognosis [12]. In Sudan there was scarce research about the mutations in *VIM* gene [13].

The aim of this study was to identify some risk factors and their association with developing breast cancer among Sudanese women such as age, family history, menarche, marriage, menopause, pregnancy, number of children, breastfeeding and using of fertility or contraceptivedrugsand which stage at diagnosis is common. In addition to investigating the mutations in exon one of *VIM* gene.

2. Material and Methods

2.1. Study Area and Target Groups

This was a Case Control study. An Ethical clearance was obtained from Ministry of Health – Khartoum. The study was conducted in different hospitals (Asia and Bahri Hospitals) and two laboratories (Ribat Hospital Laboratory and National Health laboratory). The study was carried during 2018-2019. The studied groups were 45 female with tumor as cases and 45 females with benign tumor or no malignancy seen in the breast as control. Both groups were matched on age (± 3 years), diagnosis hospital (same hospital), and examination time (within 2 months). After receiving their consents for participation in the study, a structural questionnaire including age, family history, menarche, marriage, menopause, pregnancy, Nulliparous and parous women, breastfeeding, use of fertility or contraceptives (Hormonal birth control)drugs was done.

2.2. Molecular Study

Tissue samples for molecular analysis were obtained after positive (cases) or negative (controls) breast cancer confirmation. DNA was extracted using manufacturer Kit (i-taq-iNtRon biotechnology. Korea). The isolated DNA was quantified using Nano drop spectrophotometer and gel electrophoresis.

2.2.1. Primer Design for VIM Gene
The researcher designed primers (using primer-3 software):
-Forward5'-GGCTCAGATTCAGGAACAGC- 3'.
-Reverse primer 5'- AGCCTCAGAGAGGTCAGCAA - 3'.

2.2.2. Polymerase Chain Reaction (PCR)
DNA was added to Maxime PCR Premix kit (i-taq-iNtRon biotechnology. Korea) with 1 μL forward primer and 1 μL reverse primer. The total volume was adjusted to 20μL using deionized water. A control reaction containing sterile deionized water, but without template DNA was always performed to confirm the absence of contamination. Amplification conditions were set as, A denaturing step at 94°C for 2 min, 40 cycles of: denaturation (94°C for 20 sec), annealing (58°C for 30 sec) and extension (72°C for 1min), and with a final extension step at 72° C for 7 min.

2.3. Data Analysis

2.3.1. Statistical Analysis
Statistical package of social science (SPSS) version 21 was used for analysis of the data to study the relationship between variables and breast cancers among the two groups.

2.3.2. In Silico Analysis
The PCR products for each sample were gel purified prior to sequencing analysis. Sequencing of PCR was performed using automated sequencer (Macrogen Co. Lt., Seoul, Korea). Quality control of the nucleotides chromatogram was done using Finch TV software version 1.4.0 to ensure that all ambiguous sites are correctly called and determine the overall quality [14]. Then nucleotides sequences were searched for sequence similarity using nucleotide BLAST. Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software [15].

3. Results

Ninety women were enrolled in this study, 45with breast cancer considered as cases and 45 women who were negatively diagnosed were selected as control.

3.1. Demographic Data

Data regarding different factors such as age, family history, menopausal status, age at menopause, use of fertility or contraceptives (Hormonal birth control)drugs and stage of breast cancer were investigated. Most cases (73.3%) were above 40 years old (Figure 1) and (66.7%) had a family history of breast cancer. About 46.6% had a first degree relative diagnosed with breast cancer (Figure 2).
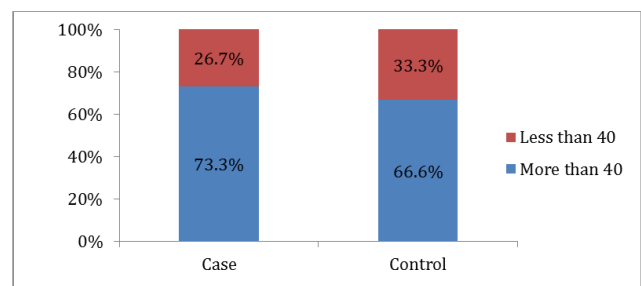

Figure 1. *Classification according to Age in Control and Case Groups.*
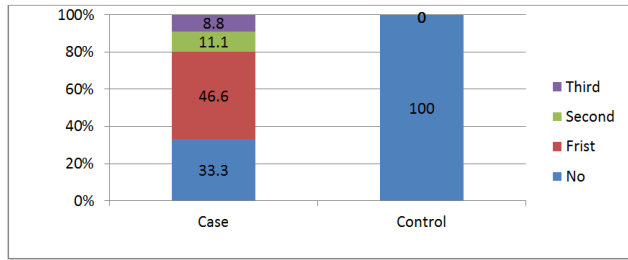
Figure 2. The Degree of Kinship within Family.

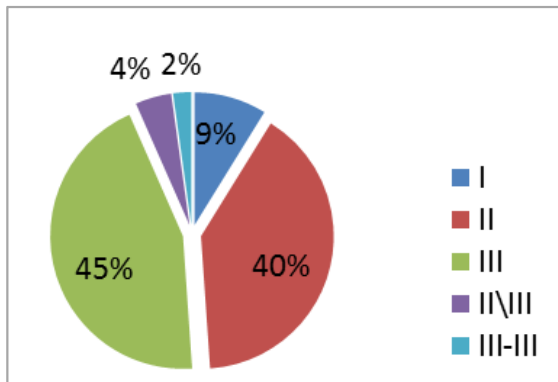The patients were at different stages of cancer as shown in Figure 3

Figure 3. Classification of patients according to different stages of cancer.

The data obtained regarding the various questions asked in questionnaire are shown in Table 1.

Table 1. Data regarding different risk associated factors.

Parameter	Case	Control
A) Age of First Menarche		
1- Less than 14 years	31.1%	73.3%
2- More than 14 years	68.8%	26.6%
B) Regularity of Menarche		
1- Yes	26.6%	62.2%
2-No	73.3%	37.7%
C) Marital Status		
1- Yes	42.2%	82.2%
2 –No	57.7%	17.7%
D) Age of marriage		
1- Less than 18 years	24.4%	73.3%
2- More than 18 years	17.7%	8.8%
3- unmarried	57.7%	17.7%
E) Menopause Status		
1- Yes	42.2%	37.7%
2-No	57.7%	62.2%
F) Age of Menopause (years)		
1- Non	51.1%	62.2%
2-35-44	11.1%	17.7%
3- 45- 54	28.1%	20%
4- more than 55	8.8%	0
G) Pregnancy		
1- Married but No pregnancy occurred	22.2%	8.8%
2- Yes	20%	71%
3- Unmarried	57.7%	22.2%
H) Age of pregnancy		
1-Unmarried	57.7%	22.2%
2- No pregnancy with marriage	22.2%	4.4%
3-Less than 18	6.6%	64.4%
4-More than 18	13.3%	8.8

Parameter	Case	Control
I) Number of Children		
1-Unmarried	55.5%	22.2%
2- 1-5	17.7%	55.5%
3- 6-10	4.4%	20%
4- Married with no children	22.2%	2.2%
J) Breast feeding		
1-Non applicable	11.1%	11.1%
2- Yes	8.8%	64.4%
3-No	80%	24.4%
K) Drugs used		
1- None	66.6%	46.6%
2- Fertility drugs	4.4%	22.2%
3- Contraceptives (hormonal birth control)	28.8%	31.1%

3.2. Association Between Different Risk Factors and Breast Cancer

A significant association ($P < 0.00$) was found between family history and breast cancer. The results among the breast cancer patients revealed that 46% had a first degree relative with the disease.

In addition, women with older age at menarche (>14 years old) were found to be at higher risk ($P < 0.00$) for breast cancer (68%) than women with younger age of menarche (<14 years old). The regularity of the menarche 33 (73%) was also considered as a significant risk factor ($P < 0.0.01$). Unmarried women demonstrated a higher risk of breast cancer than others ($P < 0.00$)**.**

Another important risk factor was the age of marriage and age of pregnancy. Women married and got pregnant at younger age (<18 years old) were found to be at lower risk ($P < 0.00$) than women married at older age (>18 years old). There was a significant association between breast cancer and breastfeeding ($P < 0.00$) were 80% of the cases were not breast feeding. Furthermore, women with more children (parous women) were found to be at lower risk to develop breast cancer compared with women without children (Null-parous women) ($P < 0.00$). The results showed most of the cases in this population were diagnosis at late stage 45% (III) 40% (II).

3.3. Analysis of Molecular Results

Following extraction and conducting PCR using the specific primers, the results of vimentin gene gave a band size of ~172bp (Figure 4).
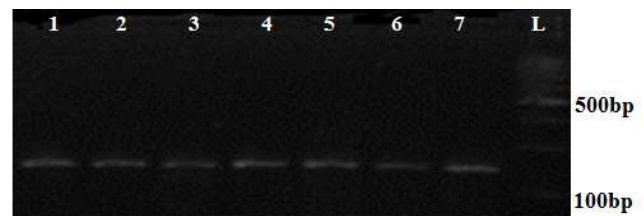

Figure 4. Agarose gel electrophoresis of amplified part of VIM gene.

Lane L: 100bp molecular ladder, Lane 1-6: Positive *VIM* gene ~172bp

No mutations in Vimentin (*VIM*) gene among Sudanese breast cancer women was identified after sequencing. The data showed 100% identity to *VIM* gene sequence retrieved
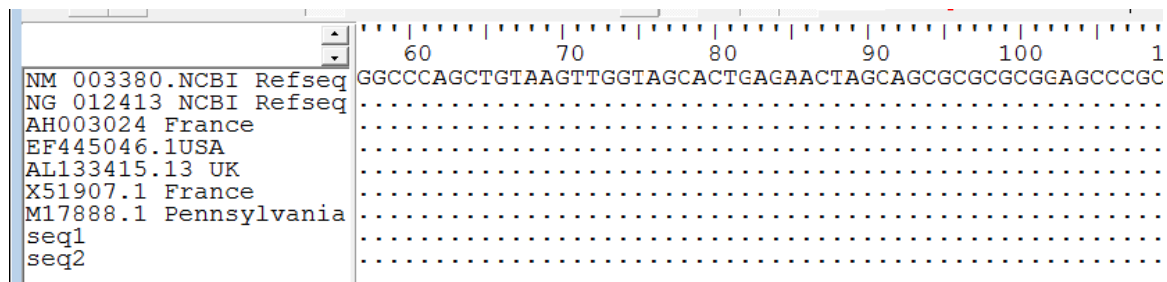
from GenBank. (Figure 5)

Figure 5. *DNA Sequences Alignment of VIM Gene for Samples with reference sequences from NCBI Database, with 100% Identity using BioEdit software.*

4. Discussion

This study was a case-control study involving 90 Sudanese women living in Khartoum state conducted during the period 2018- 2019. In total, 50% of the cases were diagnosed with breast cancer and 50% were chosen as control with no malignancy seen.

In this study (42.2%) of the patients were found to be in a postmenopausal age (>40 years old) This is in agreement with Mohamed *et al*., who stated that the breast cancer is higher among postmenopausal women [16]. This observation disagreed with the higher incidence of breast cancer in younger women observed in 52 of 196 black American women (26%) vs. 48 of 300 American cases [17]. Similarly, in a British study conducted to examine data collected from 445 patients newly diagnosed with breast cancer showed that black British patients were significantly younger (p=0.001) with median age of 46 compared with 67 for the white patients with breast cancer [18].

In this study, family history of breast cancer was found to be a significant factor for developing the disease. This observation is similar to the study conducted in north east China showing that familial risks have a largely genetic basis on breast cancer [19].

This study also compared between first, second and third degree of relation of family relative and found strong significant association between females with cancer with a first-degree family member. This is similar to a study in Sweden which suggested that familial cancers had early onset mainly in individuals whose parents were affected [20].

Despite the early menarche (age below 14 years) which was not a significant risk factor for breast cancer among the studied population, epidemiological studies in Germany have a contradictory results which showed a decrease of breast cancer risk by later age at menarche. Generally, the risk decreased by 10–24% with each year of delay in menarche [21]

This study also revealed that most of the cases were unmarried or married after 18 years of age. This group had a higher risk for breast cancer when compared with married women especially before 18 years old. These results disagree with study done in the eastern region of Saudi Arabia, where it was observed that most breast cancer cases who were married under 18years of age compared to the control group [22]. Since there is no previous study in this context in

Sudan, the late marriage will be perceptible risk factor of getting breast cancer in the future in Sudan.

Reproductive factors, like pregnancy and breastfeeding have been reported to have a protective role against breast cancer [23]. In Moroccan women there was clear significant relation between with full-term pregnancy and breast cancer compared with non-pregnant women [24].

Nulliparous women had a higher risk for breast cancer compared to porous women in this population. A meta-analysis of large Scandinavian epidemiological studies found that nulliparous women have a 30% increased risk compared with women who have had at least 1 full-term pregnancy [24].

According to results of the present study, breast feeding is of a protective factor against breast cancer in Sudanese women. These findings are consistent with a large collaborative studies in India which perceived that women who were breastfeeding for longer time have a lower risk by 4.3% for every 12 months of breastfeeding [25]. The clear explanation of this finding is the delay of the return of regular ovulatory cycles and decrease endogenous sex hormone levels [25].

Another possible explanation relates to structural changes that occur in the breast following lactation and weaning, meaning induced mammary differentiation which confers protective effects against breast cancer, also differentiated cells are comparatively more resistant to be transformed into cancerous cells [25].

The majority (88.8%) of women presented in this population were diagnosed at stage III that was similar to the proportion of women with late-stage of breast cancer (III) from 50% to 27% in white women, and from 60% to 32% in black women at in the USA between 1973 and 2011 [26]. Four South African studies examined racial differences, which consistently showed a higher percentage of late-stage of breast cancer in black Africans (range 74-91%) than white Africans (30–44%) [26]. These findings may be attributable to the fact that patients ignore symptoms and do not access medical care at an early stage. In addition, this might due to a lack of education, a skeptical attitude towards western medicine, cultural customs that exclude and isolate diseased individuals [26].

Most of cases were not at menopausal status so the effect had no clear significant in this population. A study in USA carried during 2004 revealed that menopause will increase the risk of breast cancer [8].

The use of contraceptives (hormonal birth control) and oral fertility drugs had no clear significance association with breast cancer in this study. These result have the same view with Dutch study that showed women currently taking combined oral contraceptives have slight effect to devolved disease compared to never users, but there is no evidence of an increase in the risk (10 years after stopping the use of drugs) [27]. It disagrees with epidemiological studies from the Women's Health Initiative (WHI) which had a randomized trial and provided convincing evidence that the effect of oral contraceptives or hormone replacement therapy (HRT) increases breast cancer risk, but depending on strongly of hormonal constituents, duration of HRT use and time of cessation [27].

This study was conducted to investigate mutations in exon one of VIM gene and breast cancer in women. No mutations were found in exon one of VIM gene. The study of Noh et al in Maryland explained how VIM had been linked to the malignant transformation and metastatic spread of cancer cells [28].

Other observations of the structural of similarities between VIM and the proto-oncogenes c-fos, c-jun, Raf and the v-mos oncogene indicated vimentin-dependent control of gene expression can promote transformation of primary cells into malignant cancer cells [29].

Two reports showed that over expression of VIM protein is correlated with breast cancer and increased invasive behavior and promotion of migration of mammary epithelial cells [29-30].

Vimentin-associated migration in pre-malignant breast cancer cells has been shown to be induced by H-Ras-V12G and Slug. Interestingly, the presence of VIM during Epithelial-Mesenchymal Transition (EMT) leads to up regulation of the receptor tyrosine kinase Axl that enhances the migratory behavior of breast epithelial cells [31].

Korschinget al., (2005) used immunohistochemistry technique for VIM protein study and 15 other differentiation markers in invasive breast cancer tissue samples, and concluded that neither EMT nor myoepithelial histogenesis could fully explain the origin of the vimentin-expressing cells in the tissues [29].

5. Conclusion

This study has revealed that family history with first degree relative, menarche, irregular of menarche, reproductive factors such as pregnancy, breast-feeding and nulliparous are the strongest risk factors associated with breast cancer for Sudanese women. No mutations in exon one of *VIM* gene among Sudanese breast cancer women were found (viewing 100% identity to *VIM* gene retrieved from GenBank).

6. Recommendations

Other risk factors playing an important role in breast cancer such as menopausal status, age of menopausal, contraceptive (hormonal birth control) and oral fertility drugs needs illumination and further work to show their association with breast cancer. It is recommended to increase the number of samples, RT-PCR for *VIM* gene expression in breast cancer women in Sudan and whole exons sequencing (exome) of *VIM* gene.

Also it is advisable to increase family awareness and knowledge about early marriage, pregnancy, natural breast-feeding.

Acknowledgements

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