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The Study of BRCA 1 and BRCA 2 Gene Mutations in Benign and Malignant Lesions of the Breast.

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Abstract
Understanding the role of BRCA 1 and BRCA 2 gene mutations is critical in the study of breast carcinogenesis. The aim was to study was to investigate BRCA 1 and BRCA 2 gene mutations in benign and malignant lesions of the breast. A case-controlled retrospective study done with 10-formalin fixed and paraffin wax embedded tissue blocks retrieved from pathology archives (5 benign fibroadenoma and 5 malignant invasive ductal carcinoma) confirmed cases of the breast. BRCA 1 gene SNPs of fibroadenoma, indel was the most prevalent with 100% and no gene mutation in transversion and transition. BRCA 1 gene SNPs of invasive ductal carcinoma. The most prevalent gene mutation was transversion (45%). In functional mutation of BRCA 1 gene of fibroadenoma, no gene mutation in fibroadenoma. BRCA 1 gene of invasive ductal carcinoma the most prevalent was missense (71%). BRCA 2 gene SNPs of invasive ductal carcinoma recorded mutation in transition (100%). In the comparison of BRCA gene SNPs of fibroadenoma, the most prevalent was indel (100%), invasive ductal carcinoma the most prevalent was transition (50%). The comparison of BRCA gene functional mutation shows that missense (70%) was the most prevalent. BRCA 1 and BRCA 2 gene mutation can be implicated in breast carcinogenesis.

Keywords: BRCA 1 and 2, Fibroadenoma, Invasive Ductal Carcinoma.

Introduction

Breast cancer is the most common form of cancer in women globally, occurring in both developed and developing nations. It accounts for 25% of all new occurrences of cancer in women and is the second greatest cause of mortality in industrialized nations, behind lung cancer (Dubey *et al.,* 2021). Nigeria accounts for 100,000 of the 681,000 new cases of cancer diagnosed in Africa each year (Jemal *et al.,* 2011). Any changes or mutations in this gene can raise the chance of getting breast, ovarian, and prostate cancer (Ekundina *et al.*, 2023). Breast cancer, which appears in the mammary glands, is a collection of several malignancies. Breast cancer is the most prevalent cancer in women, but it can also affect men. In the later decades of life, breast cancer incidence is rising. Women over 50 experience 75% of the cases, while women over 65 experience 50% of the cases (Momenimovahed *et al*., 2019). Breast cancer is an unknown origin, although there is a risk factor for its occurrence. It is understood that certain risk groups of people have a higher risk of having breast cancer when it comes to malignancy in general (Ferlay *et al.,*2015). The BRCA1 and BRCA2 genes are the two most common genes in breast cancer and ovarian cancer that are autosomal dominant and have a high penetrance (Ayub *et al*., 2014).

The genes BRCA1 and BRCA2 function as tumor suppressors and create tumor suppressor gene (TSG) proteins, earning them the moniker TSGs. Breast, ovarian, and prostate cancer risk can be enhanced by alterations or mutations in the BRCA1 gene, which is found on chr17q. One of the acrocentric chromosomes in men, chr13q, houses the BRCA2 gene. Any changes or mutations in this gene can raise the chance of getting breast, ovarian, and prostate cancer. A BRCA1 gene mutation increases the risk of acquiring breast cancer in women by 60% to 80% and

increases the likelihood that men will also develop prostate cancer. BRCA2 germ-line mutations are seen in approximately 35% of families with early-onset breast cancer in women and also causes an increased risk of ovarian cancer in women and breast cancer in men (Ayub *et al*., 2014).

Materials and Methods

Tissue Blocks

In this retrospective study, a total of 10 tissue blocks were selected from the pathology archives of the Federal Teaching Hospital Ido-Ekiti (FETHI). All 10 tissue blocks retrieved were fixed in formalin and embedded tissue blocks consisting of 5 benign fibroadenoma and 5 malignant invasive ductal carcinomas of the breast.

DNA Extraction:

The following protocols were adopted for extracting good quality DNA from paraffin embedded tissue. Each tissue was ground in sterile mortal and pistil with 500 µl of extraction buffer before being poured into a sterilized Eppendorf tube and 33 µl of 20% Sodium Dodecyl Sulphate (SDS) was added, vortexed briefly, and incubated in a water bath at 65ºC for 10 minutes. 10µl of 5M potassium acetate was then added at room temperature, vortexed, and centrifuged at 10000g for 10 minutes. The supernatant was collected in another Eppendorf tube, and 300l of cold iso-propanol was added. The mixture was gently mixed, and it was kept at -20ºC for 60 minutes. The DNA was sedimented by centrifugation at 13000g for 10 minutes, after which the supernatant was gently decanted, and the pellet was not disturbed. After washing the DNA pellet with 500 µl of 70% ethanol, it was centrifuged at 10000g for 10 minutes. Ethanol was decanted, and the DNA was air-dried at room temperature until there was no trace of ethanol left in the tube. To preserve and suspend the DNA, the pellet was resuspended in 5µl of Tris EDTA buffer (Odeyemi *et al.,* 2020).

Polymerase Chain Reaction

The cocktail of preparation for PCR sequencing comprised of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM MgCl2, 1µ l of 10 mM dNTPs mix, 1 µl of 10 pmol each primer (table 3.1), and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 35µ l with sterile distilled water 15 µl DNA template. PCR was performed in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile comprising of an initial denaturation for 5 minutes at 94°C, followed by 30 cycles made up of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 60 seconds; and a final termination for 10 minutes at 72°C (Odeyemi *et al*.,2020)

Integrity Test

To validate the amplification, the integrity of the amplified DNA of about 1.5 Mb gene fragment was tested on an Agarose gel of about 1%. The buffer (1XTAE buffer) was prepared and then used to make the 1.5 percent agarose gel. The suspension was microwaved for 5 minutes. The molten agarose was allowed to cool to 60ºC before being stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray, and molten agarose was poured into the tray. The wells were formed by allowing the gel to solidify for 20 minutes. The 1XTAE buffer was poured into the gel tank, barely submerging the gel. After loading the 100bp DNA ladder into well 1, two microliters (2 µl) of 10X blue gel loading dye (which gives the samples color and density to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells. The gel was electrophoresed at 120V for 45 minutes, photographed, and then visualized using ultraviolet trans-illumination. The sizes of the PCR products were estimated by comparing them to the mobility of a 100bp molecular weight ladder that was run in the gel alongside the experimental samples. (Odeyemi *et al*.,2020)

Purification of Amplified Product

The amplified fragments were ethanol purified after gel integrity to remove the PCR reagents. In a new sterile 1.5 µl tube Eppendorf, 7.6 µl of Na acetate 3M and 240 µl of 95 percent ethanol were added to each about 40 µl PCR amplified product, mix thoroughly by vortexing, and store at -20°C for at least 30 minutes. Centrifugation for 10 minutes at 13000 g and 4°C, followed by supernatant removal (invert tube on trash once), after which the pellet was washed with 150µ l of 70% ethanol and mixed, then centrifuged for 15 minutes at 7500 g and 4°C. Remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 minutes before resuspending in 20 µl of sterile distilled water

and storing in -20ºC prior to sequencing. The purified fragment was tested on a 1.5 percent Agarose gel at a voltage of 110V for about 1 hour to confirm the presence of the purified product and quantified using a thermo scientific nanodrop model 2000. (Odeyemi *et al.*,2020)

Sequencing

The amplified fragments were sequenced using an Applied Biosystems Genetic Analyzer 3130xl sequencer and the BigDye terminator v3.1 cycle sequencing kit, according to the manufacturer's instructions. For all genetic analyses, the Bio-Edit software and MEGA 6 were used

Photomicrography

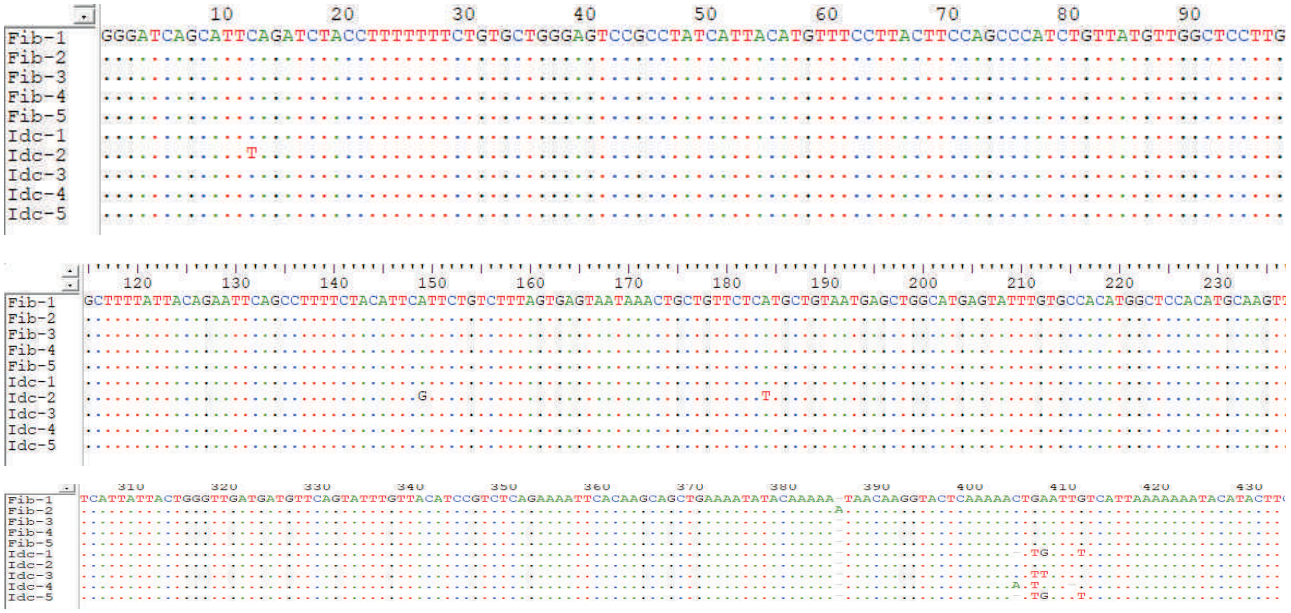
The stained sections were examined under a LEICA research microscope (LEICA DM750, SWITZERLAND) interfaced with a digital camera (LEICA ICC50). Digital photomicrographs of stained sections for the histomorphology on the tissue blocks studied were taken at various magnifications and reported for morphological changes.

Data Analysis:

The results obtained from this study were analyzed using graphs, tables and charts.

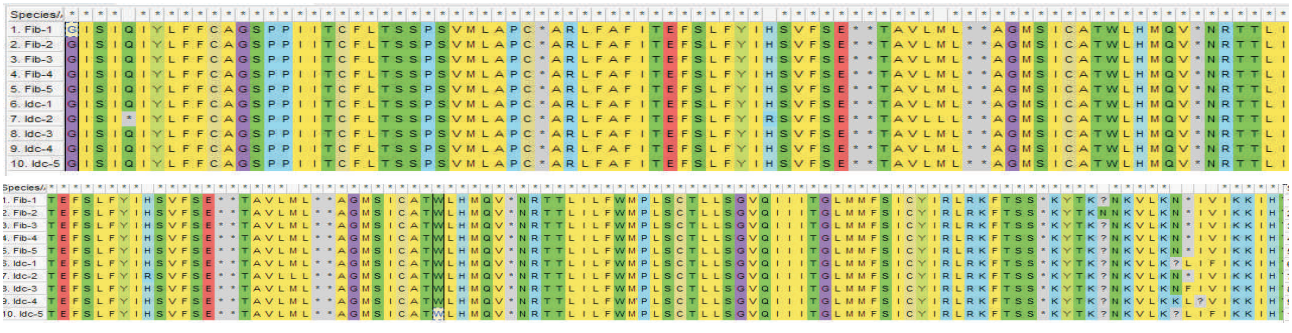
Results

Figure 1a: Sequence alignments showing regions of nucleotide difference within the BRCA1 gene regions.



INTERPRETATION: Virtual representation showing alignments within BRCA 1 nucleotide sequence.

Figure 1b: Amino Acid Alignments Showing Regions of Functional Mutation as a Result of Single Nucleotide Mutation Along the BRCA 1 Gene Regions



Ligands: *IDC: invasive ductal carcinoma,* FIB: fibroadenoma

Interpretation: This shows an amino acid alignments of the functional mutation

Table 1: Pairwise Genetic matrix showing the genetic difference within the *BRCA 1 gene* regions.

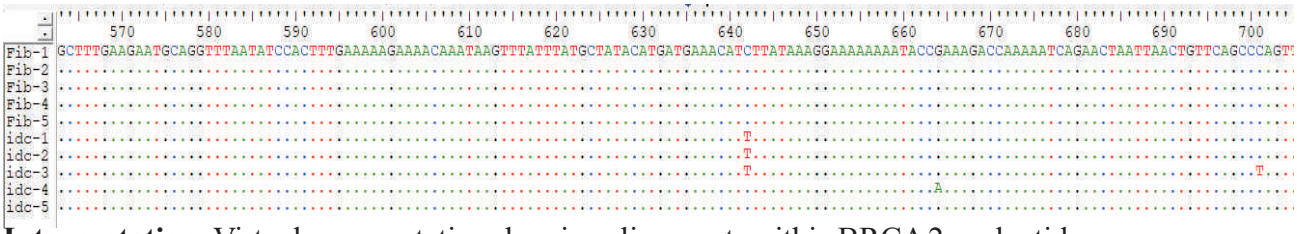
	Fib-1	Fib-2	Fib-3	Fib-4	Fib-5	Idc-1	Idc-2	Idc-3	Idc-4	Idc-5
Fib-1										
Fib-2	0.0000									
Fib-3	0.0000	0.0000								
Fib-4	0.0000	0.0000	0.0000							
Fib-5	0.0000	0.0000	0.0000	0.0000						
Idc-1	0.0068	0.0068	0.0068	0.0068	0.0068					
Idc-2	0.0068	0.0068	0.0068	0.0068	0.0068	0.0136				
Idc-3	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0113			
Idc-4	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0113	0.0045		
Idc-5	0.0068	0.0068	0.0068	0.0068	0.0068	0.0000	0.0136	0.0045	0.0045	

Genetic difference within and between groups as along the BRCA 1 gene

	within group	between group
Fib	0	0.00586
Idc	0.01	0.00586

Interpretation: The table shows that there is no genetic difference within the group in fibroadenoma but in invasive ductal carcinoma, there is genetic difference which is 0.01.There is genetic difference between the group in both fibroadenoma and invasive ductal carcinoma which is 0.00586.

Figure 2a: Sequence Alignments Showing Regions of Nucleotide Difference Within The *BRCA 2 Gene* Regions.



Interpretation: Virtual representation showing alignments within BRCA 2 nucleotide sequence.

Figure 2b: Amino Acid Alignments Showing Regions of Functional Mutation as a Result of Single Nucleotide Polymorphism (Snp) Mutation Along the BRCA 2 Gene Regiom.

Interpretation: This shows the virtual representation of amino acid alignment with regions of functional mutation because of SNP mutation along the BRCA 2 gene regions.

Table 2: Pairwise Genetic matrix showing the genetic difference within the *BRCA1 gene* regions

	Fib-1	**Fib-2**	**Fib-3**	**Fib-4**	**Fib-5**	**idc-6**	**idc-7**	**idc-8**	**idc-9**	**idc-10**
Fib-1										
Fib-2	0.000									
Fib-3	0.000	0.000								
Fib-4	0.000	0.000	0.000							
Fib-5	0.000	0.000	0.000	0.000						
idc-6	0.001	0.001	0.001	0.001	0.001					
idc-7	0.001	0.001	0.001	0.001	0.001	0.000				
idc-8	0.003	0.003	0.003	0.003	0.003	0.001	0.001			
idc-9	0.001	0.001	0.001	0.001	0.001	0.003	0.003	0.004		
idc-10	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.003	0.001	

Genetic difference within and between groups as along the BRCA2 gene

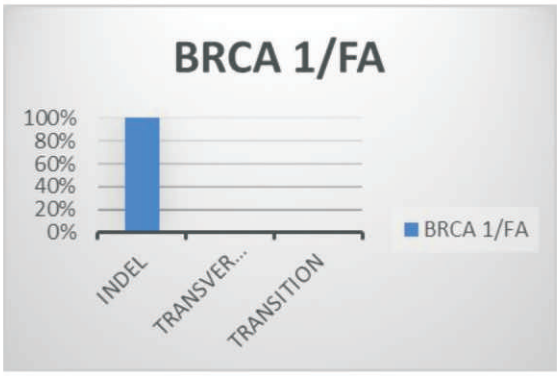
	within group	between group
Fib	0	0.00142
Idc	0	0.00142

Interpretation: This table shows that there is no genetic difference within the group in fibroadenoma and invasive ductal carcinoma but there is genetic difference between the group in both fibroadenoma and invasive ductal carcinoma which is 0.00142.

Table 3: Summary table showing the effect of Mutation along the BRCA Gene regions

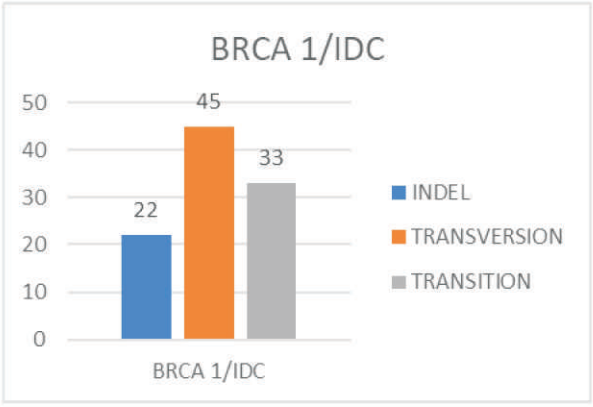
Description	*Location*	*Gene type*	*% occurrence*	*Specimen*	*Mutation type*	*Mutation type Description*
A	386(9:1)	BRCA 1	1(4.8)	Fib	Indel	Inserted Asparagine
C	405(4:2)	BRCA 1	2(9.5)	Idc	Indel	deleted Asparagine
T	411(9:1)	BRCA 1	1(4.8)	Idc	Indel	Deletion of Isoleucine
C:A	405(7:1)	BRCA 1	1(4.8)	Idc	Transversion	Missense mutation Changing Asparagine to Lysine
A:T	183(9:1)	BRCA 1	1(4.8)	Idc	Transversion	Silent mutation retaining serine
G:T	407(3:2)	BRCA 1	4(19.0)	Idc	Transversion	Missense mutation Changing stop code to leucine
G:T	412(4:1)	BRCA 1	2(9.5)	Idc	Transversion	Missense mutation Changing Valine to Phenylalanine
A:G	150(9:1)	BRCA 1	1(4.8)	Idc	Transistion	Silent mutation retaining Serine
C :T	12(9:1)	BRCA 1	1(4.8)	Idc	Transistion	Missense mutation Changing Glutamate to stop code
A:G:T	408(8:1:1)	BRCA 1	2(9.5)	Idc	Transistion	Missense mutation Changing leucine to phenylalanine.
C:T	642(7:3)	BRCA 2	3(14.3)	Idc	Transistion	Silent mutation retaining Isoleucine
C:T	771(9:1)	BRCA 2	1(4.8)	Idc	Transistion	Missense mutation Changing Glutamate to Lysine
G:A	664(9:1)	BRCA 2	1(4.8)	Idc	Transistion	Missense mutation Changing Proline to Leucine

Figure 3: A graph showing SNPs frequency in Fibroadenoma of BRCA 1 gene.



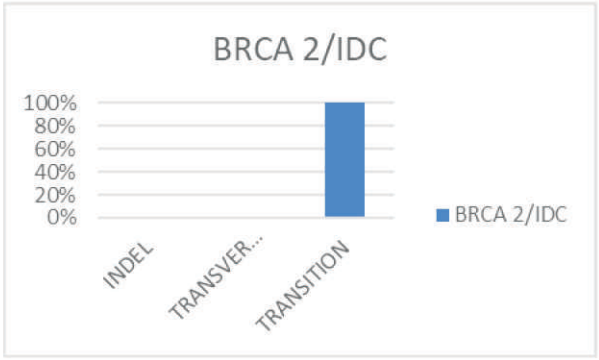
This graph shows the SNPs in fibroadenoma of BRCA 1 gene, it is observed that the most prevalent mutation is indel (100%) while transversion and transition is 0%.

Figure 4: A graph showing SNPs frequency in invasive Ductal carcinoma of BRCA 1 gene



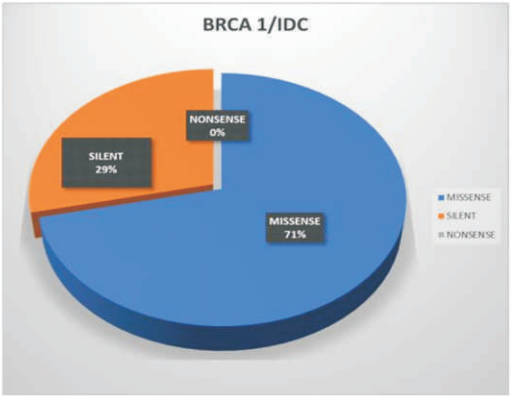
This graph shows the SNPs in Invasive Ductal Carcinoma of BRCA 1 gene, the most prevalent type of mutation observed was transversion which was (45%) while indel (22%) and transition was (33%).

Figure 5: A graph showing SNPs frequency in Invasive ductal carcinoma of BRCA 2 gene.



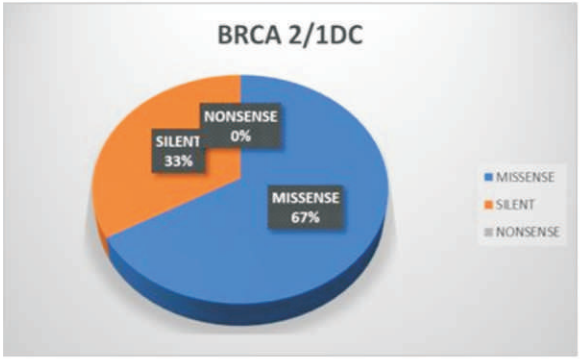
This graph shows the SNPs in invasive ductal carcinoma pf BRCA 2, the most prevalent observed was transition (100%) while transversion and indel was 0%.

Figure 6: A pie chart showing the frequency of the functional mutation type of BRCA 1 Gene in Invasive Ductal Carcinoma.



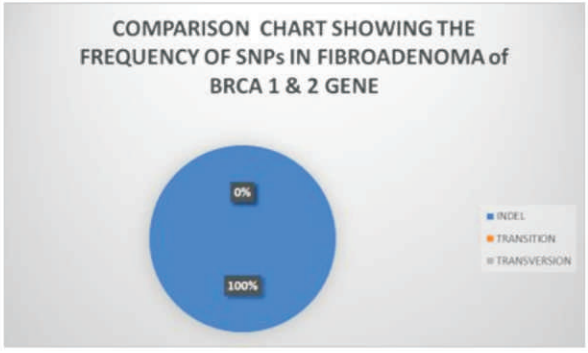
This chart indicates that Missense (71%) was observed to have the highest frequency in BRCA 1 followed by silent (29%) and nonsense (0%)

Figure 7: A pie chart showing the frequency of functional mutation type of BRCA 2 in Invasive Ductal Carcinoma.



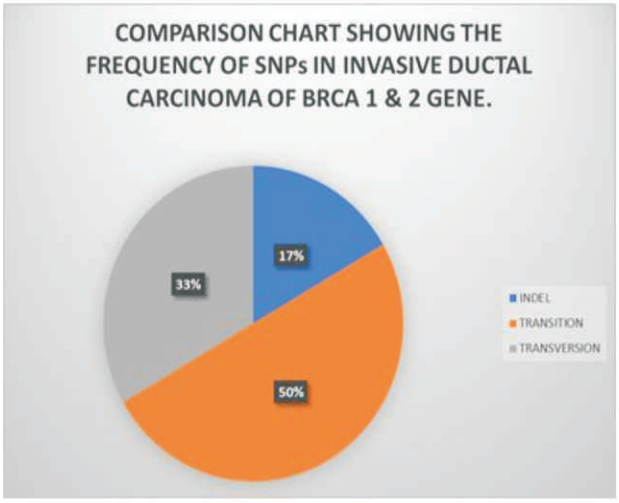
This chart indicates that Missense (67%) was observed to be the most prevalent type of mutation in BRCA 2 followed by silent (33%) and nonsense (0%).

Figure 8: A comparison pie chart showing the frequency of SNPs in fibroadenoma of BRCA gene



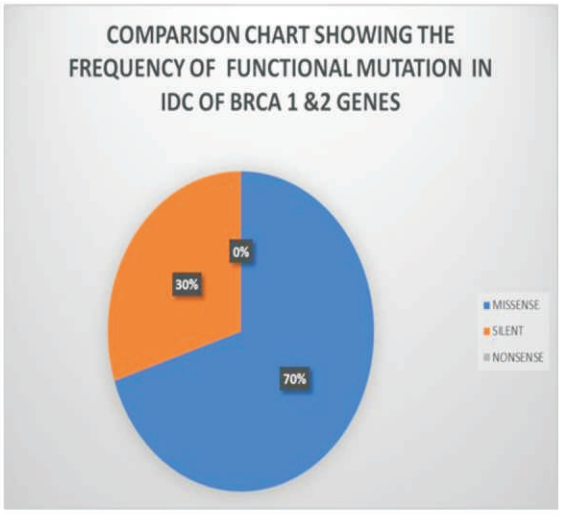
This pie chart shows the comparison frequency of single nucleotide polymorphism in fibroadenoma of BRCA gene.it was observed that most prevalent was indel (100%) and transition and transversion (0%)

Figure 9: A comparison pie chart showing the frequency of SNPs in invasive ductal carcinoma of BRCA genes



This pie chart shows the comparison frequency of single nucleotide polymorphism in invasive ductal carcinoma of BRCA gene.it was observed that most prevalent was transition (50%), transversion (33%) and indel (17%).

Figure 10: A comparison pie chart showing the frequency of functional mutation in invasive ductal carcinoma of BRCA gene



This pie chart shows the comparison frequency of functional mutation in invasive ductal carcinoma of BRCA gene.it was observed that most prevalent was missense (70%) followed by silent (30%) and nonsense (0%).

Discussion

This study shows that the Missense mutation in BRCA1 gene which changes stop code to leucine appears to be the most frequent mutation type at 4(19.0) followed by silent mutation in BRCA 2 gene at 3(14.3) retaining Isoleucine and Missense mutation changing leucine to Phenylalanine at 2(9.5). Missense mutation in BRCA 1 gene changing stop codon to leucine, it was observed that the missense mutation instead of having a stop codon was substituted to leucine. This process led to the production of shorten aberrant protein that is not likely to be functional. And when this stop codon was translated or an amino acid coded by the stop codon, there was an addition of extra amino acid (Leucine) to the list of already existing amino acids which caused an alteration (Change) in the normal proteins' configuration. This process then led to the development of BRCA proto-oncogene that advanced to BRCA Oncogene then to BRCA Oncoprotein that is implicated in breast cancer. Because when a gene has not undergone any modification, it cannot become an Oncogene. When gene has become an abnormal gene, abnormal protein is produced and is formed which cannot perform its specific function. An outcome which is in alignment with the reports of (Gupta *et al*., 2019) as well as Niederhuber *et al*. (2019) who studied the genomic analysis of 100 breast cancers for mutations in the coding exons of 21,416 genes and revealed up to 6,964 single-base substitutions (68.0% missense, 6.1% nonsense, 2.3% splice site, <1.0% stop codon, 23.5% silent mutations). This could be due to the genetic versatility of BRCA 1 and 2 Oncogenes in breast cancer. The breast is a unique target organ for carcinogenesis because of the proximity of fat tissue and embedded epithelial cells.

While the silent mutation in BRCA 2 gene of invasive ductal carcinoma retained Isoleucine and Missense mutation changing leucine to Phenylalanine as such still maintaining its normal genomic sequence with an intact functional response.

On single nucleotide polymorphism (SNPs) in fibroadenoma of BRCA 1 gene, it was observed that the most prevalent mutation was indel (100%) while transversion and transition were at 0% each. This shows that the indel mutation would have an increased prevalence in fibroadenoma than other mutation types. This simply means that the Missense mutation cannot take place with the above pattern. This result showed that the Indel mutation is more prevalent in Fibroadenoma than the transition and transversion gene mutation which explains why Fibroadenoma is benign when compared with malignant.

This was in alignment with the reports of (Lee and Soltanian, 2015; Klinger *et al*., 2019). In their study it was estimated that about 10% of the world's female population suffer from fibroadenoma once in a lifetime. It is also suspected to be the reason why the peak age for fibroadenoma in Caucasian women is in their 20s whereas in African, American, Hispanic and other women of colour, it is found earlier in late teens. Unlike the SNPs in Invasive ductal Carcinoma, where the most prevalent type of mutation observed was transversion at (45%) followed by transition (33%) and then indel at (22%). Feng *et al*. (2018) also reported invasive ductal carcinoma as one of the most common forms of invasive breast cancer that accounts for 55% of breast cancer incidence upon diagnosis, according to statistics from the United States in 2004. Research has shown that Transversion mutation tends to undergo substitution more than other mutation types, which was observed in the result.

Invasive ductal Carcinoma, the frequency of the functional mutation type of BRCA 1 showed that Missense (71%) was observed to have the highest frequency followed by silent (29%) and nonsense (0%) while in BRCA 2 gene, Missense (67%) was the most prevalent type of mutation followed by silent (33%) and then nonsense at (0%). This was observed in the reports of (Zheng *et al*., 2018; Pitt *et al*., 2018) which stated that mutation in BRCA1 gene is considered as the main cause of hereditary breast cancer unlike the BRCA2, and it is responsible for 40–45% of total hereditary breast cancer development, they further stated that over 858 BRCA1 mutations have been confirmed to have a significant clinical impact on cancer susceptibility (Mehrgou and Akouchekian, 2016; Mannu *et al.,*2020). And this could be attributed to the fact that the inheritance of germ-line mutations in autosomal dominant susceptibility genes appears to be responsible for most cases of breast cancer. Also, due to the fact that more of the base pair substitutions in the DNA sequence occurred in BRCA 1 gene, this is also in line with the

report of (Dale and Park, 2010). As a result of this, most breast tumors arising in patients with germ-line BRCA2 gene mutation have been found to exhibit loss of the wild-type BRCA2 allele, this gene is believed to function as a tumor suppressor (Zhang and Li, 2018). SNPs studied in invasive ductal carcinoma of BRCA1 gene showed that (45%) were transversion, (33%) transition while (22%) were indel. This pattern is perfectly in alignment with the study carried out by Brown, (2012) on Single nucleotide polymorphisms(SNPs) which states that transversion mutation are more likely ($C \leftrightarrow A$ or $C \leftrightarrow G$, $T \leftrightarrow A$ or $T \leftrightarrow G$) or ($A \leftrightarrow C$ or $A \leftrightarrow T$, $G \leftrightarrow C$ or $G \leftrightarrow T$) than transition mutation (purine ($G \leftrightarrow A$) or another primidine instead of one primidine ($C \leftrightarrow T$) owning to the fact that substituting a single ring structure is more likely than substituting a double ring for a single ring caused by mismatches of the complementary base in the newly synthesized strand during DNA replication (Brown, 2012; Mannu *et al*., 2020). In single nucleotide polymorphisms of BRCA 2 gene, there was 100% transition and 0% transversion and indel. This is in agreement with the study done by (Simchoni *et al*.,2016; Bellacosa *et al*.,2020; Wang *et al*.,2021) in their study,408 samples were analyzed and it was observed that SNPs showed 100 percent of transition mutation pattern in BRCA 2 gene specifically in invasive ductal carcinoma but there was no detectable evidence of other mutation unlike the BRCA1 gene.

Comparing the frequencies of functional mutation in invasive ductal carcinoma between BRCA 1 and 2 genes, result showed that the most prevalent was Missense at (70%) followed by silent (30%) and then nonsense mutation at (0%). This is in agreement with the study done by (Kuchenbaecker *et al*.,2017) which in their study stated that BRCA1 single nucleotide polymorphisms (SNPs) could only exert their potential effects through missense polymorphism and silent polymorphism and could slightly modify BRCA1 and 2 protein functions or stabilize them. Similarly, Single nucleotide polymorphism (SNPs) could also alter BRCA1 or 2 expressions by acting on transcription, splicing or translation. It is not known yet whether the few reported frequent BRCA1 missense polymorphisms alter BRCA1 or 2 functions or stabilizes them. While the frequency of single nucleotide polymorphism in fibroadenoma of BRCA genes indicates that the most prevalent was indel (100%) and transition and transversion (0%), a familiar pattern in alignment with the reports of (Lee and Soltanian, 2015; Kuchenbaecker *et al*., 2017).

However, this study also shows that in comparison with the frequencies of single nucleotide polymorphism in invasive ductal carcinoma of BRCA genes, it was observed that most prevalent was transition (50%), transversion (33%) and indel (17%) but this is not in agreement with the report of (Brown, 2012, Godet and Gilkes,2017) on SNPs which states that transversion mutation are more likely to undergo substitution than others, hence the specific cause of this pattern is unknown. In this nucleotide sequence, Some of the SNPs have turned to functional mutation (Missense) and this accumulated to the formation of abnormal gene that has produced an abnormal protein that could not take up its assigned function of tumor suppression and genomic stability.

Histological examination further supported the evidence observed in mutational pattern of BRCA 1and 2 breast cancer showing that there was an enlargement of the stroma around the tubular ducts in peri canalicular pattern. The ducts were lined by luminal epithelial cells and outer myoepithelial cells. Also, the luminal epithelial cells show hyperplasia in the mammary cell architecture, this is diagnostic of fibroadenoma. While invasion through the basement membrane of breast duct, it is an indicative of invasive ductal carcinoma tissue.

Conflict of Interest

The authors declare no conflict of interest.

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