Study of ATM (ataxia-telangiectasia mutated) gene profile in Breast cancer patients among Mizo population.

Abstract

Dissertation submitted in fulfillment of the

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Master of Philosophy

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By

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Registration No. MZU/M.Phil/359 of 26.5.2017

Under the Supervision of

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Declaration of the Candidate

I, **Indira Sonar**, hereby declare that the subject matter of this dissertation entitled "***Study of ATM (ataxia- telangiectasia mutated) gene profile in Breast cancer patients among Mizo population***" is a record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the dissertation had not been submitted by me for any research degree in any other University/Institute.

This is being submitted to Mizoram University for the award of the degree of Master of Philosophy in Biotechnology.

Dr. Joyotrimony Bhattacharya
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(Department of Biotechnology)

Indira Sonar
(Candidate)

(Prof. N. Senthil Kumar)
(Supervisor)

CERTIFICATE

This is to certify that the dissertation entitled **"*Study of ATM (ataxia-telangiectasia mutated) gene profile in Breast cancer patients among Mizo population*"** submitted to Mizoram University for the award of Master of Philosophy in Biotechnology by **Indira Sonar** Registration No. *MZU/M.Phil/359 of 26.05.2017*, Research scholar in the Department of Biotechnology, is a record of research, based on the results of the experiments and investigations carried out independently by him during the period from 2016 to 2017 of study, under my guidance and supervision and has not been previously submitted for the award of any Indian or foreign University.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for the purpose of submission of M.Phil dissertation.

Place: **Prof. N Senthil Kumar**

Date: **(Signature of the supervisor)**

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(Indira Sonar)

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Abbreviation

ATM- Ataxia-Telangiectasia Mutated.

A-T- Ataxia-telangiectasia.

NCRP- National Cancer Registry Program.

ICMR- Indian Council of Medical Research.

ICAR- Indian Council of Agricultural Research.

WHO- World Health Organization.

OR- Odd Ratio.

NCBI - National Centre for Biotechnology Information.

SPSS - Statistical Package for the Social Sciences.

HOPE- Have (y)Our Protein Explained.

MDR- Multifactor dimensionality reduction.

Ref.Seq- Reference Sequence.

AGVGD- Align GVGD.

HR- Hormone Receptor.

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1. INTRODUCTION

Breast cancer is a malignant tumour originated from the breast cells. Generally, breast cancer starts developing in the cells of the lobules or in the ducts. It can even develop in the stromal tissues, which comprises of the fatty and fibrous connective tissues of the breast. According to Murthy et al. (2009), breast cancer is the third leading cause for cancer death, and leads as the second most common cancer in India. According to NCRP (2013), Mizoram, the North-Eastern state of India has a highest age standardised breast cancer rate of 14.1 per 1,00,000 population. Studies have revealed that intake of alcohol, tobacco and peculiar food habits, especially high fat intake are important risk factors for breast cancer (Ghatak et al., 2013; Sieri et al., 2008).

Strong genetic background is the backbone for breast cancer. Hereditary breast cancer reveals to display familial aggregation and is linked up with the early age onset and an elevated incidence of bilateral occurrence. Although the breast cancer susceptibility genes BRCA1 and BRCA2 were discovered, but in 1994 a total of 18 breast cancer-associated susceptibility genes have been classified. These genes include low penetrance (FGFR2, LSP1, MAP3K1, TGFB1, and TOX3), moderate penetrance (ATM, BRIP1, CHEK2, PALB2, and RAD50) and high penetrance (CDH1, NBS1, NF1, PTEN, TP53, and STK11).

Worldwide Scenerio of Breast cancer

In India, the incidence of breast cancer is on the hike and among females it is ranging in first position, pushing cervical cancer to the second position. According to the data obtained from ICMR, it is reported that one in twenty-two women in India during her lifetime is likely to suffer from breast cancer, but in America the figure is definitely more, where one in eight women is arrested by this deadly type of cancer (Ferlay et al., 2008).

About 9 million new cancer cases are estimated and diagnosed every year, and that each year over 4.5 million people die from cancer in the world. According to the reports of ICAR (2003), incidence of breast cancer comprises 10.4% in the worldwide among women, which categorises it to be the second most common type of non-skin cancer after lung cancer and the fifth most common cause of cancer death. WHO (2006) reported that in 2004 about 5,19,000 deaths occurred in the worldwide. However, Muss et al. (2009) revealed that breast cancer is more frequent in women than in men, though males have a poor outcome due to late diagnosis. In India per year, number of new cancers is about 7 lakhs and each year over 3.5 lakhs people die of cancer. In these 7 lakhs cancer, about 2.3 lakhs of cancer are due to the effect of tobacco.

Reports of National Cancer Registry Program (NCRP) revealed that in the urban parts of India, breast cancer has concurred cervical cancer as it is the most common cancer that has been diagnosed among women. Globally, 11.1 per 100000 age standardized mortality rate for breast cancer in India have been observed. In India, the mortality rates for breast cancer are in hike as compared to the incidence rates. Due to the lack of or limited access to the early detection services and treatment, there is a poor survival rate in India for breast cancer.

ATM gene structure and function

ATM gene is located at chromosome number 11q22.3. It is made up of 66 exons, out of which 62 encodes a protein of 3056 amino acids (Savitsky et al., 1995). It is an active serine/ threonine kinases. ATM is encoded by a protein family known as PI3K-related protein kinases (PIKK) (Abraham, 2004). These proteins consist of a domain that is similar to phosphatidylinositol 3-kinase. Studies of Bosotti et al. (2000) concluded that ATM consists of C-terminal FAT domain (FRAP, ATM, TRAPP) with a FATC domain which is a highly conserved 35 residue tails. However, this domain is important for ATM kinase activity regulation and also for binding the regulatory proteins as suggested by Jiang et al. (2006). ATM also consists of N-terminal HEAT domain which functions in two different ways: (1) influences the communication with the other proteins (Perry and Kleckner, 2003) and (2) a zone important for binding of substrate (Fernandes et al., 2005). Other putative motifs reported by Lavin et al. (2004) include incomplete leucine zipper and a proline-rich region that binds c-Abl.

ATM plays a lead role in the DNA repair of double strand breaks. The DNA damage response includes damaged DNA recognition, protein repair recruitment, cell cycle checkpoints signalling, regulation of transcriptomes and apoptosis activation. In normal functioning cells, ATM remain as inert dimers or multimers. ATM, in response to DNA double strand breaks, dissociates into highly active monomers (Bakkenist and Kastan, 2003). During this procedure, autophosphorylation of ATM occurs on Ser1981 and initiates a signalling cascade in the site of DNA damage by phosphorylation of multiple response of DNA damage and cell-cycle proteins, which include proteins for breast cancer susceptibility genes: TP53, BRCA1 and CHEK2.

Swift et al. (1986) reported that Ataxia-telangiectasia (A-T) is an autosomal recessive condition with a frequency of 1 in 40 000 to 1 in 3,00,000 in Caucasian populations. Inactivation of ATM gene leads to the condition A-T. A-T mutated patients are estimated to have hundred-fold increased chance of breast cancer as compared to the normal population. According to the reports of Chun and Gatti (2004) and Taylor and Byrd (2005), A-T is characterized by cerebellar ataxia, oculomotor apraxia, choreoathetosis, frequent infections, oculomotor apraxia, immunodeficiency, increased risk of malignancy and ionizing radiation sensitivity.

Ataxia-telangiectasia gene (ATM) was mapped by using genetic linkage analysis in chromosome 11q and in 1995 positional cloning was used for its identification (Gatti et al., 1988; Savitsky et al., 1995). A-T condition was considered to be genetically heterogeneous and delineated into four complementation groups but Savitsky et al. (1995) revealed that all groups were mutated due to mutation in the same gene by using ATM mutation analysis.

According to Martin et al. (2000); Goss et al. (1998) and Nathanson et al. (2001) several risk factors have been defined for breast cancer, which compromises of family history, age, radiation exposure and hormonal factors. Studies of Nathanson et al. (2001) revealed that mutations in lower penetrance genes explain much of the breast cancer hereditary predisposition, apart from BRCA1 and BRCA2. Oncoproteins comprising the

tumor suppressors p53 and BRCA1 are regulated by ATM (Kastan et al., 2000; Gatei et al., 2000).

Epidemiological studies have showed high malignancy frequency, basically breast cancer among A-T blood relative patients (Boder et al., 1963; Swift et al., 1976; Swift et al., 1987; Pippard et al., 1988; Swift et al., 1991; Athma et al., 1996; Inskip et al., 1999; Janin et al., 1999; Olsen et al., 2001). Athma et al. (1996) suggested that A-T heterozygotes have high risk of breast carcinoma than the non-carriers leading to the conclusion that ATM gene mutation carriers compiles for 6.6% of all breast cancer cases.

In United States (US), one in eight women develop breast cancer during her lifetime. Lacey et al. (2002) reported that breast cancer incidence rate is enormously increasing but the rate of mortality has decreased as early detection reduces the mortality rate. Genetic testing may help high-risk individuals to get benefited from more intense monitoring and, in the future, from attenuating or preventive therapies. According to Morrell et al. (1986) AT patients during their short lifetime develop malignancy (38% of A-T homozygotes), mostly leukemia and lymphoma.

Role of ATM gene in Cancer

Ataxia telangiectasia (AT), rare human disease, which has distinctive features like extreme cellular sensitivity to radiation, cerebellar degeneration and a predisposition to cancer. All AT patients contain mutations in the gene ATM. Most other AT-like disorders are defective in MRN protein complex genes. The ATM protein helps in rapid increase in kinase activity which immediately helps in the formation of double-strand breaks (Canman et al., 1998; Banin et al., 1998). The phenotypic manifestation of AT is due to the protein ATM kinase, which involves in repair of DNA, cell death, G_2/M checkpoints and G_1/S, intra-S checkpoint, regulation of gene, initiation of translation, and maintenance of telomere (Kurz et al., 2004). Thus, any defect in ATM has major manifestations in repairing certain types of DNA damage, and hence from improper repair, cancer may result. AT patients have higher breast cancer risk that has been ascribed to interactions of ATM and BRCA1 phosphorylation and the proteins associated to it, following damage to DNA (Chen, 2000).

Pathways of ATM gene (Morgan *et al.,* 2007)

ATM responses two steps to DNA double strand breaks: Rapid Response and Delayed Response.

- **Rapid Response**:

  In the rapid response, activated ATM phosphorylates CHK2, effector kinase, which in turn phophphorylates CDC25A, resulting in degradation and ubiquitination. Thus, accumulation of phosphorylated CDK2-Cyclin takes place and cell cycle progression is blocked.

- **Delayed Response:**

  In the delayed response, the inhibitor of p53 and MDM2 is phosphorylated by ATM.p53 is also phosphorylated by Chk2. Thus, activation and stabilization of p53 elevates the expression of p21, Cdk inhibitor, which inturn keeps Cdk activity low and helps in maintaining long-term cell cycle arrest.

2. Review of Literature

Swift et al. (1987) reported that ataxia-telangiectasia relatives suffer increased rate of breast cancer. This study was done based on the importance to A-T families, but it also had wider significance to 1% population that might be the carriers of A-T predisposing mutation. Therefore, relative modest increase risk in breast cancer carriers equalise to attributable population risk. However, Swift's report confirmed increased breast cancer risk in relatives of ataxia telangiectasia cases, according to epidemiological surveys done on cancer incidence. Easton (1994) reported the relative risk of breast cancer to be 3.9.

Epidemiological studies done by Thompson et al. (2005b) in 1160 relatives of 169 ataxia-telangiectasia patients estimated that overall breast cancer relative risk to be 2.23 (95% CI=1.16–4.28) in comparison to the general population and was estimated to be higher i.e., 4.9 (95% CI=1.90–12.9) in woman under 50 years of age.

Studies suggested that cases of A-T patient relatives have higher chance for breast cancer risk as the frequency of ATM mutation is increased in breast cancer as compared with the controls. But this study did not support the reports of FitzGerald et al. (1997) where ATM mutations were analysed in 401 breast cancer cases and 201 controls. Numerous reasons were put forward to solve this contradiction of ATM in breast cancer between epidemiological factors and this case–control comparison. Gatti et al. (1999) explained that there is a difference between the ATM mutation that results in susceptibility to breast cancer and the ataxia- telangiectasia. However, they reported that there is a difference between missense ATM variants and truncating ATM mutations which had a effect on activity of ATM and hence its susceptibility to cancer. They suggested that carriers of ATM truncations have wild type ATM activity (50%) and a normal phenotype. However, people who have two truncations showed no function of ATM protein and this might result in A-T. Relevantly, they reported that certain missense variants of ATM encode abnormal proteins functionally that results in a dominant negative fashion, which might have more adverse effect on functions of ATM than a single truncating mutation. Thus, in contrast this result in phenotypic

consequences in ATM missense variants carrier i.e., cancer susceptibility. However, Gatti et al. (1988) confirmed that two missense variants would have no abolition of ATM activity to enhance A-T. Therefore, this study explains that there is no enhancement of ATM truncating mutations in breast cancer, but does not explain the elevated breast cancer risk in cases of A-T female relatives

Most of the studies done on ATM are inconclusive, mainly due to two drastic factors. First, small numbers of cases are included as ATM consists of 66 exons and are difficult to screen. Second, very few studies have been conducted to screen the whole ATM gene (cases as well as controls), as a result of which comparision between the frequency and type of identified sequence variants becomes very difficult.

To clarify the role of ATM in breast cancer susceptibility, Renwick et al. (2006) conducted an analysis, where they reported 2 ATM mutations in control and 12 in familial breast cancer cases (P=0.0043) that cause ataxia telangiectasia. These mutations consist: abnormalities of splice-site, truncations and two missense mutation that altered protein function and resulted in ataxia telangiectasia (A-T). The result of this epidemiology studies predicted that mutations in ATM gene causes ataxia telangiectasia (A-T) and in breast cancer it should occur in increased frequency. According to the studies conducted by Thompson et al. (2005b) and Renwick et al. (2006) molecular and epidemiological analysis done on ATM revealed that it is a low penetrance breast cancer susceptibility gene which confers B2-fold risks of breast cancer. In UK population, analysis demonstrated that the prevelance of ATM mutation is similar to CHEK2 and similar risk and incidence prevails for breast cancer in both the genes; ATM and CHEK2 (Renwick et al., 2006; Nevanlinna and Bartek, 2006).

Epidemiological studies revealed that in woman under 50 years of age breast cancer risk in ATM carriers is higher and also, in cases of first-degree relatives of ataxia-telangiectasia as compared to ATM heterozygotes (Olson et al., 2005; Thompson et al., 2005b). Stankovic et al. (1998) reported one particular polymorphism, T7271G, for higher breast cancer risk. This mutation was reported in two families which showed slow ataxia progress, less telangiectasia

and fertility, and low activity of kinase protein (Stankovic et al., 1998; Stewart et al., 2001). Subsequently, in Australian family five woman with breast cancer were reported to have heterozygous T7271G carriers. According to the reports of Chenevix-Trench et al. (2002) mutation in ATM cell lines affected its expression and activity in a dominant negative fashion.

From the studies of Speit et al. (2000) and Neu bauer et al. (2002), it was suggested that ATM heterozygote cells showed intermediate response to ionizing radiation sensitivity that were performed based on cell survival assays, so their studies concluded that radiosensitivity is a distinctive feature for ataxia-telangectasia cells. Although, in vivo condition ATM heterozygote cells exhibit clinically relevant radiosensitivity remains unclear. It has been speculated that in the proportion of breast cancer cases ATM heterozygotes may be over-expressed that resulted in enlarged acute or late reaction of normal tissues that underwent radiotherapy (Angele et al., 2003; Guti errez-Enriquez et al., 2004; Meyer et al., 2004). The response to radiation therapy is of potential clinical importance for increased breast cancer risk in ATM heterozygotes.

Demonstration conducted revealed that predisposition of ATM mutation in breast cancer can be used as a risk stratification in woman without cancer to reduce their breast cancer risk by making medical and lifestyle choices. Therefore, BRCA1 and BRAC2 mutations have a 15-fold breast cancer risk and more than 70% woman with this mutation develop the disease. Subsequently, mutations in ATM gene have a 2 fold breast cancer risk and only 15% woman with this mutation develop the disease. According to Gudmundsdottir and Ashworth (2006), the status of ATM mutation can be used for the breast cancer treatment only if it responses to the effect of radiation and chemotherapy.

Studies done on epidemiological and molecular factors have signified the ATM role in breast cancer and conclude that mutation in ATM leads to A-T in homozygote carriers (biallelic) confer breast cancer susceptibility in heterozygote carriers (monoallelic). ATM is considered to be the fifth gene for DNA repair, along with BRCA1, BRCA2, CHEK2

andTP53, and is shown to be involved in predisposition of breast cancer. BRCA1, BRCA2 and TP53 are associated with higher breast cancer risk, ATM and CHEK2 are associated with modest risk for breast cancer (Antoniou and Easton, 2006).

ATM, low-penetrance susceptibility gene, is implicated as a hereditary breast cancer. The protein, ATM kinase, plays an important role in intrigity of genomes and acts as an activator to cellular responses of DNA double-strand breaks. Studies were done on Chile and Mexican population to interpret the relationship between common variants of ATM and familial breast cancer (Calderon et al., 2014). Polymorphisms IVS24-9delT and IVS38-8T>C and 5557G>A were reported in both the population and concluded that these variants were responsible for increased breast cancer risk. In Chile population, polymorphism 5557G>A was also reported for higher risk of breast cancer by Gonzalez-Hormazabal (2008).

Southey et al. (2016) reported c.7271T>G (p. Val2424Gly) in European population which showed increased breast cancer risk (p=0.0012). In their statistical report, OR was 11.0 (95% CI -1.42 to 85.7). They also concluded that, this variant had no association with ovarian and prostate cancer risk.

Martinez et al. (2016) concluded that mortality rate is high in woman who are single than in married woman patients. Population based study revealed that comparison done between married and unmarried woman, the unmarried woman was more likely to develop the breast cancer risk and were diagnosed with later stage of the disease and hence resulted in death as they had insufficient time for the treatment (Osborne et al. 2005). Parikh et al. (2015) conducted a study in a safety net hospital where they reported that there was over 2-fold increase in mortality due to breast cancer in single compared to married breast cancer patients.

3. Methodology

3.1. Sample collection and processing:

A total of 20 samples [non-familial Breast cancer patients (10 nos.) and healthy controls (10 nos.)] were collected. Breast cancer patients were registered under Mizoram State Cancer Institute, Aizawl, Mizoram. Selection criteria were based only on local ethnic patients without a family history of Breast cancer (first or second-degree relatives on either side of the family). All selected subjects were informed about the objectives of the study, prior to the collection of blood samples. Detailed information on demographic factors including lifestyle and dietary habits, tobacco and alcohol consumption, reproductive and medical history and family history in relation to cancer were collected with the patient consent. The ethical approval has been obtained from ethical committees of Civil Hospital, Aizawl (B.12018/1/13-CH(A)/IEC/33 dtd.15/10/2014) and Institutional Human Ethical Committee, Mizoram University.

3.2. Extraction of Genomic DNA from Blood Sample

Genomic DNA was extracted from peripheral whole blood samples using a kit base standard protocol (QIAamp DNA mini kit (250), catalogue no.51306) and sample were stored in - 20°C.

3.3. PCR amplification

The ATM region (430 bp) was amplified by PCR using primer E29 (5'GTGTATTTATTGTAGCCGACTATCT3') with accession number ENSG00000149311 as described by Ensemble for ATM region. In 25 µl tubes, Polymerase chain reaction (PCR) was carried out in which total reaction volumes, each containing 17.1µl of nuclease free water, 1X of PCR buffer, 0.2pM of each primer, 0.2mMof dNTPs, and 1U of Taq DNA polymerase (Fermentas, Germany). The reaction was performed in thermal cycler which involves initial denaturation of 95ºC for 5mins, followed by 35 cycles each consisting of denaturation at 94ºC for 40secs, annealing at 56ºC for 40secs, extension at 72ºC for 40secs and a final extension at 72ºC for 5mins. The ATM region (527 bp) was amplified using primer: E31(5'CCCATCTTGTAGTAGTACCTTACATAGTTA3'). In 25 µl PCR tubes, Polymerase

chain reaction (PCR) was carried out in which total reaction volumes, each containing 17.1µl of nuclease free water, 1X of PCR buffer, 0.2pM of each primer, 0.2mM of dNTPs, and 1U of Taq DNA polymerase (Fermentas, Germany). The reaction is performed in thermal cycler which involves initial denaturation of 95ºC for 5mins, followed by 35 cycles each consisting of denaturation at 94ºC for 40secs, annealing at 54.1ºC for 40secs, extension at 72ºC for 40secs and a final extension at 72ºC for 5mins.

The ATM region (512 bp) was amplified using primer E50 (5'-GATGCTTAGGAAGGTGTGTGA-3'). In 25 µl PCR tubes, Polymerase chain reaction (PCR) was carried out in which total reaction volumes, each containing 17.1µl of nuclease free water, 1X of PCR buffer, 0.2pM of each primer, 0.2mM of dNTPs, and 1U of Taq DNA polymerase (Fermentas, Germany). The reaction is performed in thermal cycler which involves initial denaturation of 95ºC for 5mins, followed by 35 cycles each consisting of denaturation at 94ºC for 40 secs, annealing at 53.6ºC for 40 secs, extension at 72ºC for 40 secs and a final extension at 72ºC for 5 mins. The amplification products of PCR (10 µl) were subjected to electrophoresis in a 1 % agarose gel in 1×TAE buffer at 80 V for 30 min, stained with Ethidium Bromide (0.5µg/ml), and images were examined in gel documentation systems. PCR products were further stored at -20ºC for sequencing.

3.4. Sequencing and Sequence analysis

All PCR products were sequenced to ensure reading accuracy from the opposite direction, which was performed in DBT- Advanced State Level Biotech Hub, Mizoram University. Sequence analysis was performed using FinchTV 1.4 software (Geospiza, Inc., USA) and DNA Baser Sequence Assembler v 3.2 (2012) software. Ensemble Transcript of the ATM was used to compare the sequences using the sequence analysis tool BLAST (NCBI, Bethesda, USA). Using the NCBI database, genomic sequence variations was identified. Sequence differences obtained between cancer and healthy blood samples was recorded as ATM polymorphisms or mutation. The polymorphism recorded was then verified against Ensemble database version HG 37. Sequence variants which were not found in that database were reported as new polymorphism/mutation. ATM gene card database was used to check all ATM exons.

3.5. Statistical Analysis

SPSS version 20.0 was used for doing descriptive and logistic regression analysis. P value of <0.05 at 95 % confidence interval (CI) was taken to be significant. Logistic regression analysis was done for crude odd ratio (OR) and 95 % confidence interval (CI) determination for breast cancer risk factor. Logistic regression analysis was done to control for confounding variables like marital status, reproductive history, activities, food habits, consumption of sahdah, tuibur, khaini, gutkha, cigarette and alcohol. $\chi 2$ test was done using Hardy-Weinberg equilibrium for any deviation from allele frequencies that are expected. Statistical packages, SPSS version 20.0 (SPSS Inc. Chicago, USA), was used for the analysis. Analyses were performed using R-console statistical package ver3.3.0 (The R Foundation for Statistical Computing). MDR analysis was used to further validate our logistic regression based study on epidemiological factors interaction using a model-free approach and interaction entropy graph was also plotted.

4. Results

4.1 DNA Isolation, PCR analysis and Sequence analysis:

The results of the DNA isolation and PCR product has been shown below (Fig.'s 1,2 and 3). Genomic sequence variations were identified using the NCBI database. Two mutations A>C and C>T were identified at locus 67189 and 67137, respectively which has been shown below (Fig.'s 4 and 5).

Mutation result showed that there was a change in amino acids for A>C and C>T, where the amino acid Histidine changes to Proline (A>C) and amino acid Lycine changes to Phenylalanine (C>T). Pathogenicity effect for both the mutations were checked using Poly-Phen2 score which showed possibly damaging with a score of 0.668(A>C) and 1.000(C>T) (Fig.'s 6 and 7). The results of above description have been shown in Table 1.

Results from HOPE software showed the changes in the ontology of the amino acid (Fig.'s 8 and 9). Fig.8 shows that the mutant residue (Proline) is smaller than the wild-type residue (Histidine). The wild-type residue is more hydrophobic than the mutant residue. The mutation introduces a more hydrophobic residue at this position and so this results in loss of hydrogen bonds and disturb correct folding. And in Fig. 9 the mutant residue (Phenylalanine) is bigger than the wild-type residue (Lycine). The hydrophobicity of mutant residue is higher than the wild type residue.

4.2 Demographic factors associated with breast cancer

Correlation study showed that factors like age at menopause, age at first delivery, breast feeding, night duty and consumption of sahdah are contributing to breast cancer risk which has been shown in Table 2.

Logistic regression analysis was done for confounding factors where we found tuibur (P=0.050) to have a significant role for increased risk of breast cancer which has been shown in Table 3. MDR analysis graph result has been shown in Fig. 10 and it was done to find the demographic interaction with breast cancer risk and thus early age marriage showed increase breast cancer risk (15-20 age).

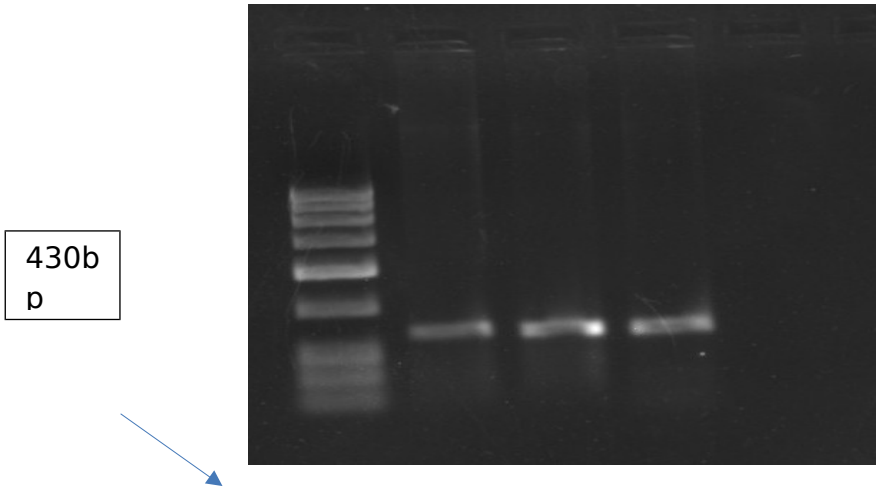


Fig. 1. ATM (430 bp) of A-T patients amplified by PCR.

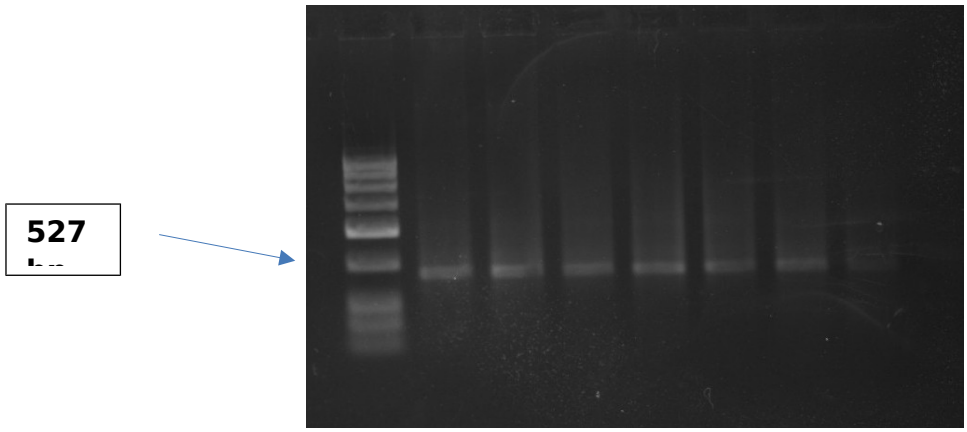


Fig. 2. ATM (527 bp) of A-T patients amplified by PCR.

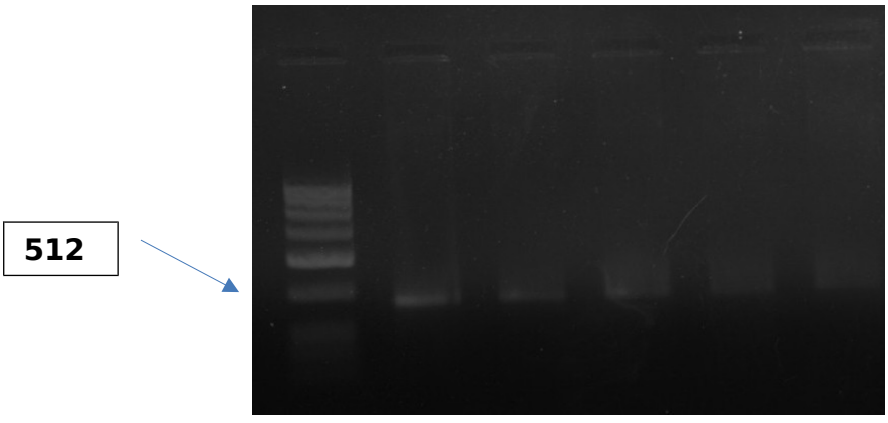
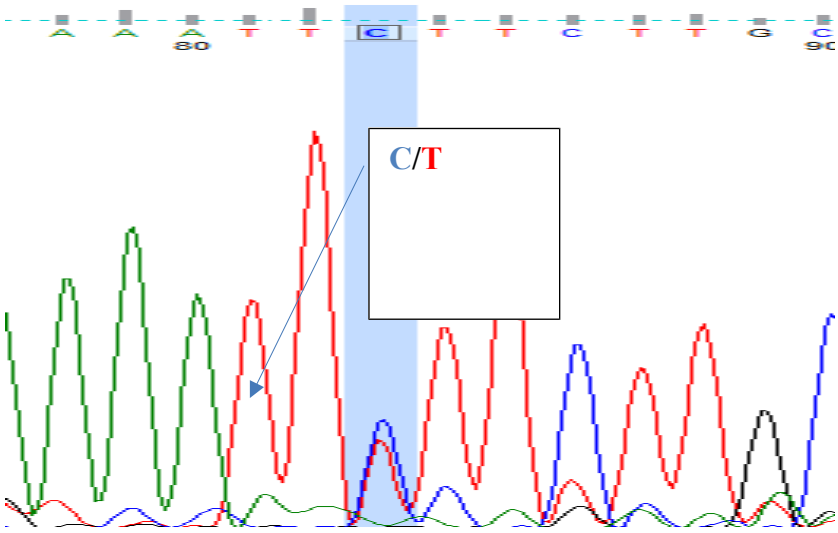
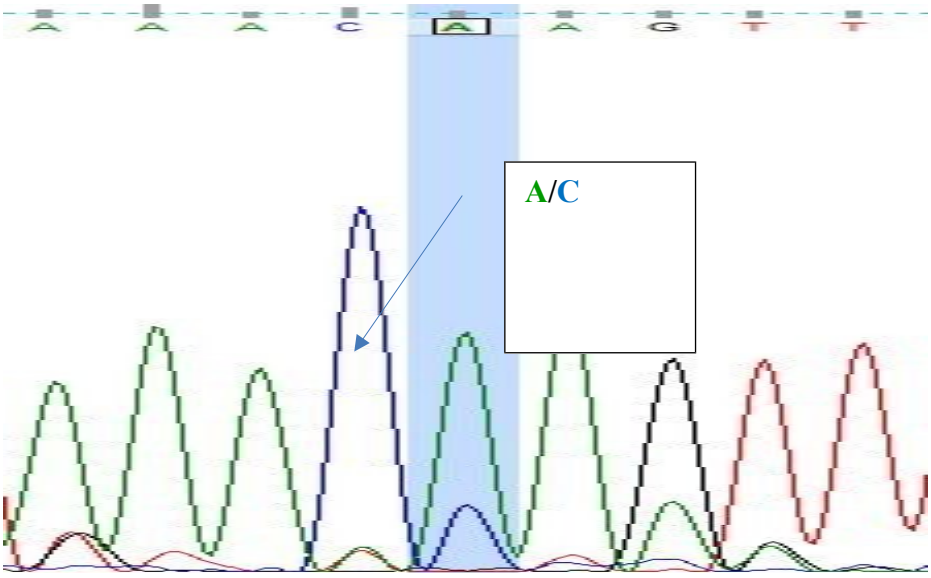


Fig. 3. ATM (512 bp) of A-T patients amplified by PCR.



Blue Colour= wild type Red Colour= Variation

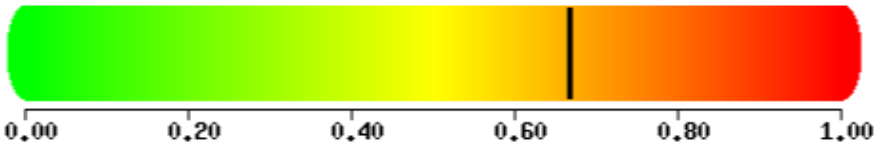
Fig. 4. ATM mutation in C67137T



Green Colour=wild type Blue Colour=Variation

Fig. 5. ATM mutation in A67189C.

Table 1. Variation and Mutation in ATM region of breast cancer samples.



Locus/gene	Base change	Ref. Seq	Codon no.	Codon position	Amino acid change	Polyphen score 2	SNP & GO	Mutation tasting	AGVGD
67189	A>C	A	1436	2	Histidine-Proline	0.668	Neutral	Disease causing	C15; pathogenic
67137	C>T	C	1419	1	Lycine-Phenylalanine	1	Neutral	Disease causing	C0; pathogenic

Fig. 6. Polyphen2score of A>C.

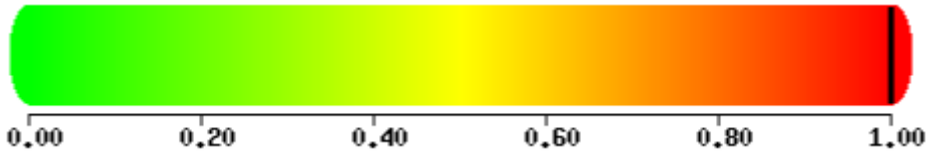
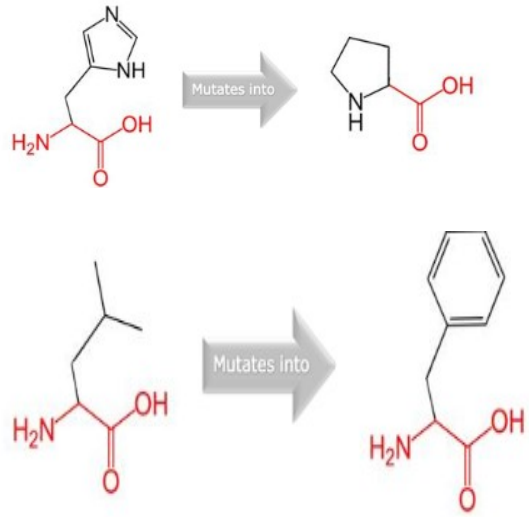


Fig. 7. Polyphen2score of C>T.



Histidine Proline Lycine Phenylalanine

Wild type residue Mutant residue Wild type residue Mutant residue

A>C C>T

Fig. 8(A>C) and **Fig**. 9(C>T). HOPE showing ontology of the amino acids change.

Table 2. Risk Estimation of Odd Ratio and CI (95%).

Factors	Criteria	C.I. (95%)	Odd Ratio
Age at Menarche	Middle	0.03/13.19	0.83
	too early	0.01/3.67	0.25
Age at Menopause	already	0.32/21.55	2.33
Age at First Delivery	late	0.11/12.36	1.16
	not yet	0.17/58.01	2.33
Breast Feeding	no feeding	0.67/133.96	6
Birth Control Pills	normal	0.11/6.25	0.85
Abortion	once	0.11/11.72	1.14
Exercise	less	0.21/78.76	3
	Never	0.28/15.66	2
Night Duty	Yes	0.18/54.04	2.25
Sahdah	Less	0.07/7.77	0.83
	more	0.31/25.92	2.5

Factors like **age at menopause, age at first delivery, breast feeding, night duty** and **consumption of sahdah** are contributing to breast cancer risk.

Table 3. Logistic regression analysis for confounding factors.

Sl. No.	Criteria	Control	Breast Cancer	Odd Ratio	C.I.(95%)	Significance
Marital Status	married	9(47.4)	1(100)	0.000	0	1
	single	10(52.6)	0(0)			
Age at Menarche	late	1(33.3)	2(66.7)	0.436	1.66-0.11	0.22
	middle	3(37.5)	5(62.5)			
	Too early	6(66.7)	3(33.3)			
Age at Menopause	Not yet	7(53.8)	6(46.2)	1.000	4.49-0.22	1
	already	2(33.3)	4(66.7)			
	2	1(100)	0(0)			
Age at Delivery	early	7(53.8)	6(46.2)	1.314	3.09-0.55	0.53
	late	2(50.0)	2(50.0)			
	Not yet	1(33.3)	2(66.7)			
Abortion	No	8(53.3)	7(46.7)	1.977	10.91-0.35	0.43
	Once	2(50.0)	2(50.0)			
	twice	0(0)	1(100.0)			
Birth Control Pills	No pills	6(46.2)	7(53.8)	0.544	2.64-0.11	0.45
	Less	3(50.0)	3(50.0)			
	more	1(100.0)	0(0.0)			
Breast Feeding	No feeding	9(56.2)	7(43.8)	3.857	45.57-0.32	0.28
	feeding	1(25.0)	3(75.0)			
Breast Feeding Duration	0	0(0.0)	1(100.0)	1.253	4.74-0.33	0.73
	1	1(100.0)	0(0.0)			
	0year	8(57.1)	6(42.9)			
	More no. Of feeding	1(25.0)	3(75.0)			
Exercise	Everyday	6(60.0)	4(40.0)	1.443	3.84-0.54	0.46

	Less	1(33.3)	2(66.7)			
	never	3(42.9)	4(57.1)			
Night Duty	No	9(52.9)	8(47.1)	2.250	29.76-0.17	0.53
	yes	1(33.3)	2(66.7)			
No. of children	0	0(0)	1(100.0)	1.000	7.47-0.13	1
	Children	9(56.2)	7(43.8)			
	Less	0(0.0)	1(100.0)			
Sahdah	Never	5(55.6)	4(44.4)	1.261	2.47-0.64	0.5
	Normal	3(60.0)	2(40.0)			
	heavy	2(33.3)	4(66.7)			
Tuibur	Never	4(33.3)	8(66.7)	0.237	0.99-0.05	0.05
	Less	1(33.3)	2(66.6)			
	Normal	3(100.0)	0(0.0)			
	heavy	2(100.0)	0(0.0)			

Tuibur (P=0.05) have a significant role for increased risk of breast cancer. Significant values which is equal to 0.05 shows the more risk for any cancer type.

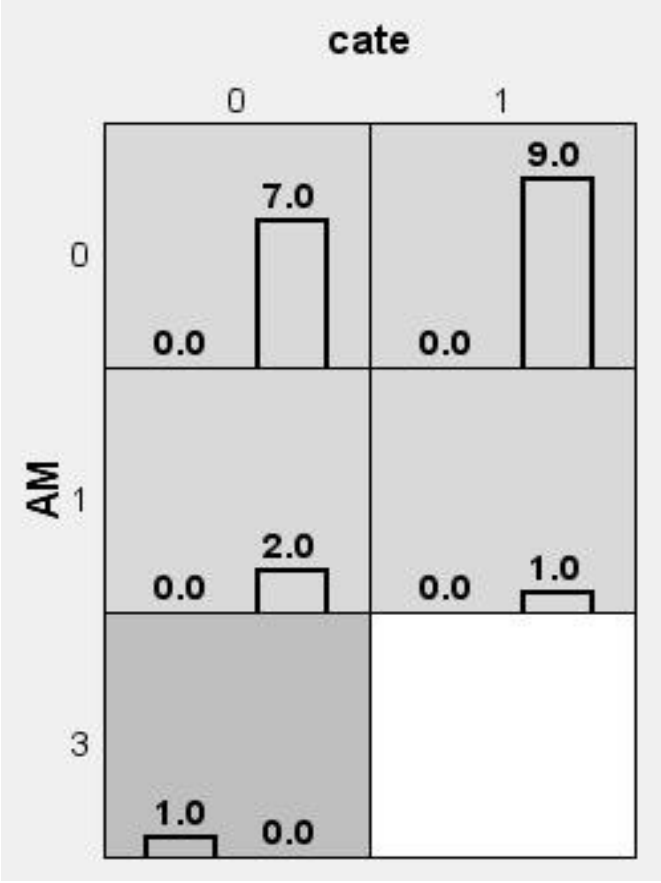


Fig. 10. MDR analysis graph showed that **early age at marriage** can increase the risk of breast cancer cases.

AM – Age at Marriage.
Cate – Category.

In category 0 represents **control** (healthy) data while 1 represents **breast cancer data**. For marital status, 0 represents **too early age**, 1 represents **middle age** while 3 represents too **late** or **single**. In the above fig., it says that patients who got married at early age had high risk of developing the breast cancer.

5. Discussion

In this report, we selected 50% non-familial breast cancer and 50% healthy cases to investigate the role of ATM in breast cancer. Remarkably, ATM mutation is present in our entire non-familial breast cancer cases.

Fletcher (2010) reported S49C, S707P, F858L, P1054R and L1420F polymorphisms in Caucasian population. Polymorphism S2855R was reported by Tavtigian (2009) in Australian population. Paglia (2009) reported Asp2597Tyr polymorphism in French population. Again, in 2002 Tavtigian published S217L and Al541Thr polymorphism in Australian population. Dork (2001) found out S707P polymorphism in Lower Saxony, a region in the north of Germany, population. However, in our recent study we did not found out all these polymorphism in Mizo population, rather we found out two mutations H1436P and L1419F in this population, which are not yet reported in any other population.

In mutation, H1436P, there was an amino acid change Histidine to Proline at codon position 2 and codon number 1436.The mutation had Polyphen2 score 0.668(A>C) with a

possibly damaging effect. It has a pathogenic effect and causes the disease i.e. it has higher risk for causing breast cancer.

In mutation, L1419F, there was an amino acid change Leucine to Phenylalanine at codon position1and codon number 1419.The mutation had Polyphen2 score 1.000(C>T) with a possibly damaging effect. It has a pathogenic effect and causes the disease i.e. it has higher risk for causing breast cancer.

Histidine is an essential amino acid in our body. It is a precursor of histamine and has vasodilatory properties and triggers allergic reactions that is responsible for inflammatory processes In our present finding the amino acid Histidine gets mutated, so our body cannot produce histamine from the decarboxylation of histidine, which is a metabolic pathway involving enzyme catalyst histidine decarboxylase and the pathway gets disturbed. As histamine enhances the formation of RBCs and WBCs, thus it increases the defensive system in our body. Histamine is an essential amino acid which has a good antioxidant and thus it is capable of neutralizing the harmful action of free radicals. Thus, it is capable to counteract the toxins produced by the bodys own metabolism, and also from other external agents such as heavy metals, contaminated air, radiation damage etc. Histidine is vital to human life. Firstly, the nitrogen present in the histidine side chain helps to capture protons from other molecules which shuttles them away and regenerate inactive enzymes. .However, this allows the enzyme to become active again thus by opening up a receptor site, or a site that allows another molecule to bind and be used for another purpose. It can easily capture protons since it is positively charged and shuttle them away for use. But if any changes occurs in this amino acid then the above function may get disturbed and thus may lead to mutation which in turn affects the whole body function of a human being.

Lysine is an essential amino acid. It is very important and is necessary in various aspects like maintaining a good state of our connective tissues, formation of collagen, production of hormones, enzymes and antibodies, synthesize carnitine with the help of methionine.It ensures the formation of our defenses, increases immunity, keeping our body antibodies and prevents the occurrence of infection. Lysine is necessary for proper growth as it stimulates growth hormone. It plays an important role as an antioxidant in contributing to cell repair caused by free radicals. As lysine is vital to our life, so it provides glucose to the body though

metabolism. In our present finding the amino acid Lysine gets mutated so it cannot be metabolized into acetyl-CoA to form adenosine triphosphate, the energy currency of the body and thus thepathway gets disturbed. This metabolism takes place in citric acid cycle in animals. But if any changes occurs in this amino acid then the above function may get disturbed and thus may lead to mutation which in turn affects the whole body function of a human being.

In our epidemiological studies, we concluded that those women who were not married had higher risk for breast cancer compared with those who were married. And also, our findings concluded that factors like age at menopause, age at first delivery, breast feeding, night shift and consumption of sahdah had adverse effect on the increase risk for breast cancer. Martinez et al. (2017) also suggested that death rate was higher in breast cancer patients who were unmarried as compared to the married woman, but also concluded that benefits of survival varies by tumour subtype and across ethnic/racial groups. According to J de Graaff et al. (1977), it was reported that woman who got married and delivered child at early age had increased risk for cervical cancer.

According to Trentham-Dietz et al. (2014), breast feeding significantly showed reduced association with breast cancer risk among premenopausal status. However, 47 epidemiologic studies analyses suggested that breast cancer risk was reduced in association with breast feeding regardless of menopausal status (CGHFBC, 2002). Breast feeding for longer duration may be effective for reduced breast cancer incidence among all the age group, however they may not be the major approaches for prevention of breast cancer, but, may provide better opportunities to understand the behind these associations with breast cancer to decrease the cancer burden in the future (CGHFBC, 2002). According to Bernstein et al. (2006) and CGHFBC (2002), it was reported that breast feeding for a prolonged period is associated with decreased risk of HR positive and HR negative breast cancer.

Studies conducted by CGHFBC, (2012) reported that more the age of the woman at menopause risk of breast cancer increases by 3%. According to the reports of Key et al.

(2011), woman whose menopause occur after 55 ages have 30% higher chance of breast cancer risk than woman of 45 age. The reason behind this is the amount of estrogen exposed to a woman in her lifetime; as the amount of estrogen exposed to a woman is directly proportional to the increased risk of breast cancer. According to the researchers from University of Minnesota School of Public Health in Minneapolis reported that women seem to be at increased risk of developing bladder cancer that go through menopause at early age.

Studies showed that woman who deliver their first child at the age of 35 or more tend to have more risk of breast cancer as compared to woman who delivers at younger age (Willett et al., 2014). Also, woman who deliver at the age of 35 or more have less risk of breast cancer as compared to woman who had never delivered a child (Colditz et al., 2000). Ai-Jing Luo et al. (2016) reported that women with older age at first birth relates to an increased risk of pancreatic cancer.

Studies of Steven et al.(1992) showed that night shift work increases the risk of breast cancer in woman as light suppress melatonin secretion at night time. In 2007, World Health Organisations International Agency for Research in Cancer (IARC) revealed that shift work involves disruption of circadian as a probable human (breast) carcinogen. Studies done by Johnni et al. (2011) on Danish nurses showed increased odd ratios for breast cancer among night shift workers as compared to the day workers. Akerstedt et al. (2015) showed that there was increase in breast cancer risk in night shift workers but only after relatively long-term exposure. But in 2016, studies conducted by Ruth et al. suggested that night shift work may have little or no effect on increase breast cancer risk. According to the literature survey done by Brudnowska et al. (2011), it was concluded that night shift is not associated with breast cancer only but with other cancers also like colorectal cancer, endometrial cancer, prostate cancer and non-Hodgkin's lymphoma.

To our knowledge, the present study is a novel finding in terms of the possible role of ATM mutations in breast carcinogenesis. In conclusion, these two novel mutations can be used for early diagnosis marker for breast cancer risk in Mizo population, Northeast Indian population. However, our epidemiological studies cannot fully support the cause of higher

breast cancer risk, so further studies involving large number of samples to validate more epidemiological factors for breast cancer risk is required.

6. Summary and conclusion

The Present study was designed with the aim to identify the candidate ATM gene for early breast cancer diagnosis in Mizo population.

Genomic DNA was extracted from blood samples of ten unrelated breast cancer and ten healthy women. The ATM coding regions were amplified by stepdown PCR and were subjected to direct sequencing by Sanger method. And then statistical analyses were carried out to study the demographic factors associated with breast cancer.

Two novel mutations (67189 A>C, 67137 C>T) were identified at coding position 1436 and 1419 where the amino acids Histidine changes to Proline and Lycine changes to Phenylalanine, respectively.

In codon position, 1419 the protein features were affected in phosphorserine domain. The wild type residue was more hydrophobic than the mutant residue for codon position 1436.

Pathogenicity effect of two novel mutations were checked by using Poly-Phen2, in which the mutation was predicted to be possibly damaging with a score of 0.668(A>C) and 1.000(C>T).

Our results showed that these two novel mutations can be used for early diagnosis marker for breast cancer in Mizo population. Our epidemiological studies revealed that factors like age at menopause, age at first delivery, breast feeding, night shift and consumption of sahdah had adverse effect on the increase risk for breast cancer in Mizo population. Our findings also concluded that consumption of tuibur (P=0.05) had a significant role in increase breast cancer risk. According to our MDR analysis, we also concluded that early age at marriage can increase the risk of breast cancer cases in Mizo population.

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