Original Article

Evaluation of Ataxia–Telangiectasia Mutated IVS10 Mutation in Breast Cancer Along with Clinicopathological Parameters

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ABSTRACT

Background: Breast cancer is the most common cancer in women worldwide, with an estimated 2.26 million new cases diagnosed in 2020. The important genes associated include BRCA1, BRCA2, CHEK2, PTEN, TP53, and ataxia–telangiectasia mutated (ATM). ATM is responsible for repairing double-strand breaks in DNA making it a significant candidate in breast cancer predisposition. ATM variant, c.1066-6T>G, has been associated with an increased risk of breast cancer in some but not all studies. The Indian studies on the allele IVS10-6T>G are very limited. The present study was undertaken to evaluate the associations between c.1066-6T>G ATM gene variant and breast cancer incidence in Indian women and its correlation with histological grade, stage, and surrogate molecular classification. **Materials and Methods:** Routine histopathological processing was done after adequate fixation of the specimen followed by staining with hematoxylin and eosin and immunohistochemistry for ER, PR, Her2neu, and Ki67. Single-nucleotide polymorphism for ATM allele IVS10-6T>G was studied after DNA extraction, polymerase chain reaction amplification, and restriction enzyme digestion. **Results:** All cases were found to be negative for ATM allele IVS10-6T>G mutation. Maximum number of patients (19 cases; 52.78%) had pT2 stage tumor followed by 11 patients (30.56%) with pT3. Majority of cases were luminal B (11; 30.56%) followed by triple negative (10; 27.78%). **Conclusion:** Although the results obtained by mutational analysis in the present study are not in agreement with the previous study on Indian women it agrees with the numerous previous studies and meta-analyses done on women with breast carcinoma in the Western world.

KEYWORDS: *Ataxia–telangiectasia mutated allele IVS10-6T>G, breast cancer, single-nucleotide polymorphism*

INTRODUCTION

Breast cancer is the most common cancer in women worldwide, with an estimated 2.26 million new cases diagnosed in 2020. This represents about 11.7% of all new cancer cases and 25% of all cancers in women. The mortality rate of breast cancer was 6.9% with 0.68 million cases worldwide in 2020. For the 1st time, female breast cancer has become the most commonly diagnosed cancer surpassing lung cancer, in particular due to high prevalence in low- and middle-income countries.[1]

As per the GLOBOCAN data 2020, in India, breast cancer accounted for 13.5% (178,361) of all cancer cases and 10.6% (90,408) of all deaths with a cumulative risk of 2.81.[2]

There are a number of recognized risk factors for breast cancer development including hormonal, reproductive, menstrual history, age, lack of exercise, alcohol, radiation, obesity, and genetic mutations.[3] The important genes associated with breast cancer include

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BRCA1, BRCA2, CHEK2, PTEN, TP53, and ataxia–telangiectasia mutated (ATM).

ATM, which is expressed in a wide variety of tissues, is located on chromosome 11q22-23. The gene is approximately 150 kb in length possessing 66 exons with a 12-kb transcript. The open reading frame of 9168 nucleotides encodes a nuclear phosphoprotein of 3056 amino acids.[4]

Mutations in the ATM gene are responsible for the rare autosomal recessive chromosomal instability disorder ataxia–telangiectasia (A-T). The function of ATM protein in repairing double-strand breaks in DNA makes it a significant candidate in breast cancer predisposition.[5] The role that the ATM protein itself plays in breast cancer susceptibility is of keen interest as most studies of A-T mutation-affected families have demonstrated an excess risk of breast cancer associated with heterozygosity for the ATM mutations ranging from three- to seven-fold overall.[6-14]

In contrast, mutation screening of the ATM gene conducted within a series of breast cancer cases and controls has produced mixed results.[6,15-20] It has been hypothesized that these differences may reflect allelic heterogeneity of ATM and that only a specific class of variants with the potential to dominantly interfere with the product of the wild-type allele contributes to breast cancer risk in heterozygotes.[21] Among the different variants evaluated, the V2424G (C.7271 T>G) and ATM D1853V missense variants were associated with the maximum and least risks of breast cancer, respectively.[22-28]

Another ATM variant, c.1066-6T>G, has also been associated with an increased risk of breast cancer in some but not all studies.[29,30]

The Indian studies on the allele IVS10-6T>G are very limited. Only one study conducted by Syeed *et al.* could be found after extensive search of the literature.[31]

Due to this paucity of literature on Asian population, the present study was undertaken to evaluate the associations between c.1066-6T>G ATM gene variant and breast cancer incidence in Indian women and to correlate the presence of this variant with histological grade, stage, and surrogate molecular classification of breast cancer in them.

MATERIALS AND METHODS

It was a hospital-based cross-sectional study which included all newly diagnosed cases of breast carcinoma operated over an 18-month period. All patients who received any neoadjuvant therapy or were diagnosed with other breast lesions apart from primary carcinomas of the breast were excluded from the study. Routine histopathological processing was done after adequate fixation of the specimen. Sections were stained with hematoxylin and eosin followed by immunohistochemistry for ER, PR, Her2neu, and Ki67 according to the standard procedure.[32] The clinicopathological details such as age, size of lump, nodal status, family history, contralateral breast involvement, histopathological type, and modified Bloom-Richardson grade were recorded.

DNA extraction

DNA was extracted by column method using Invitrogen PureLink Genomic DNA Purification Kit (Thermo Scientific, USA) with around 25 mg of breast tissue. DNA was quantified using NanoDrop 2000 Spectrophotometer with 260/280 ratio taken as the criteria for purity.

Amplification by polymerase chain reaction and restriction digestion

Polymerase chain reaction (PCR) was carried out to amplify ATM gene in a final volume of 20 μl containing ×10 reaction buffer, 2 mM dNTPs, $MgCl_2$, ×10 bovine albumin serum, Taq DNA polymerase, and 50 ng/μl genomic DNA along with forward and reverse primer in an Applied Biosystems Veriti 96-Well Thermocycler. To detect the ATM IVS10-6T>G variant, a 193-bp PCR product was amplified with forward (5'-ACAGCGAAACTCTGGCTCAAA-3') and reverse primer (5'-TGATCTTTTATTACTTCCCAGCCTAGT-3') obtained from Integrated DNA Technologies, Belgium.

Cycling conditions were 95°C, followed by 35 cycles of 30 s of denaturation at 95°C and 30 s of annealing at 54°C, with a final extension for 7 min at 72°C. The size and integrity of PCR products were checked by electrophoresis of 10 μl of the reaction product on a 1.2% agarose gel.

Digestion was performed in a total volume of 20 μl with 10 μl of 193-bp product, 1 μl 4U of FastDigest RsaI restriction enzyme (Thermo Scientific, USA) in a reaction tube with ×10 fast digest green buffer at 37°C for 15–30 min. The digested products were separated on a 0.7% agarose gel stained with ethidium bromide, and the genotype was determined by the banding pattern observed.

The DNA bands were visualized at 302 nm by a UV transilluminator. The variant allele was identified by the presence of 135- and 58-bp fragments while the wild-type allele which lacks the RsaI restriction site was identified by a single 193-bp product.

RESULTS

Demographic parameters

Out of 36 cases, majority were in the 4th (36.11%) and 5th (25%) decades. The mean age of patients was 44.14 ± 12.6 years. Twelve cases were postmenopausal (33.3%) while the remaining were premenopausal. A positive family history of breast cancer was observed in 7 cases (19.44%). Bilateral disease was seen in 6 cases (16.67%).

Clinicopathological parameters

Tumor size in the study ranged from 1.7 to 12 cm with majority tumors (20 cases – 55.56%) having tumor size between 2 and 5 cm. Based on the modified Bloom-Richardson grading, the tumors were classified into Grades I (5 cases – 13.89%), II (18 cases – 50%), and III (13 cases – 36.11%) [Figure 1].

Maximum number of patients (19 cases – 52.78%) had pT2 stage tumor followed by 11 patients (30.56%) with pT3, 4 patients (11.11%) with pT1, and only 2 patients (5.56%) with pT4 stage.

Eighteen cases (50%) demonstrated nodal metastasis with 13 cases with N1 status (36.11%) and 2 (5.56%) and 3 (8.33%) cases of N2 and N3, respectively. Twenty-six cases (72.22%) had no distant metastasis, while for 10 cases (27.78%), metastatic disease status was unknown.

The cases were classified into Stages I–IV based on the AJCC 8th edition with maximum number of cases in Stage II (14; 38.88%). Table 1 depicts the distribution of cases based on the AJCC 8th edition.

The surrogate molecular classification was evaluated in all cases based on immunohistochemistry for ER, PR, Her2neu, and Ki67. Figures 2-4 depict the luminal A, luminal B, and Her2-enriched subtypes, respectively.

Molecular analysis

All cases (100%) were found to be negative for ATM allele IVS10-6T>G mutation. Figure 5 depicts the gel documentation of the mutational analysis of ATM IVS10-6T>G.

DISCUSSION

In the present study, maximum patients (36.11%) belonged to the age group of 31–40 years, with the mean age being 44.14 ± 12.60 years. Robinson *et al.* showed that the mean age of their subjects was 55.7 years, which is a decade later than the mean age found in Indian women.[33] Similarly, in a study on women suffering from breast cancer in a cohort from South India, conducted by Antony *et al.*, the mean age of incidence was found to be 50.85 years.[34] Thus, the age distribution in our study differs significantly from the previous Western data, although it is similar to the studies done on Indian breast cancer previously. Thus, it is quite evident that breast cancer occurs at a younger age in Indian women as compared to the women in the West.

We found that majority of the cases (24/36 patients, 66.67%) were premenopausal. This finding differs from the findings of previous Indian studies, such as by Chopra *et al.* and Malvia *et al.* who found major burden of the disease to be among the postmenopausal women.[35,36] In a study by Reinier *et al.* on 61,844 women, they recorded that 61% were postmenopausal.[37] This difference from the Western literature might be

Table 1: Combined pathological tumor-node-metastasis staging (American Joint Committee on Cancer, 8th ed..)

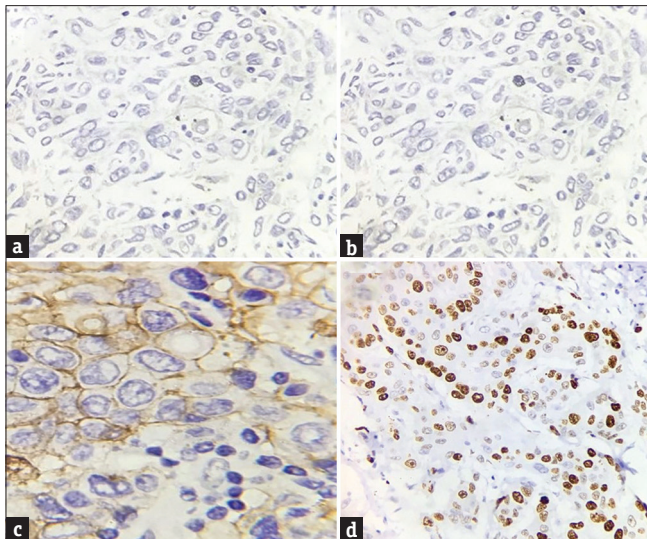| AJCC stage	Frequency (%)
I A	4 (11.11)
I B	0
II A	14 (38.88)
II B	4 (11.11)
III A	11 (30.55)
III B	0
III C	3 (8.33)
IV	0
Total	36 (100)

AJCC: American Joint Committee on Cancer
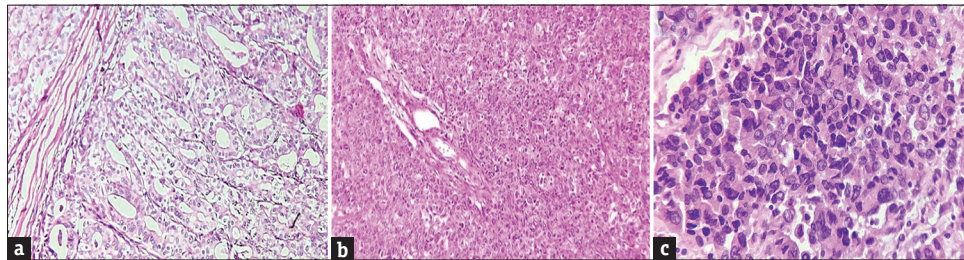
Figure 1: Modified Bloom-Richardson grading: (a) Grade I infiltrating ductal carcinoma showing proliferation of atypical cells in the form of tubules (H and E, ×10), (b) Grade II infiltrating ductal carcinoma with tumor cells arranged in sheets as well as tubules (H and E, ×10), (c) Grade III infiltrating ductal carcinoma. The tumor cells are mainly in the form of sheets with marked nuclear pleomorphism. The nuclei of the tumor cells are large and hyperchromatic with the presence of atypical mitosis (H and E, ×40)
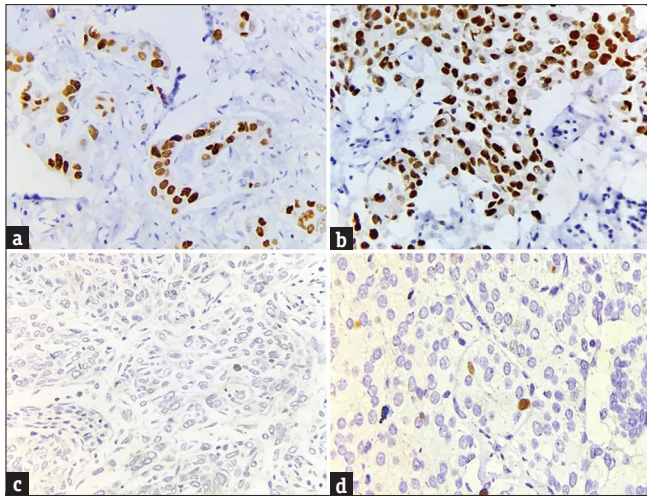
Figure 2: Surrogate molecular classification- Luminal A Subtype. (a) Positive nuclear immunopositivity for ER. (10X), (b) Positive nuclear immunopositivity for PR. (10X), (c) Negative expression for Her2. (10X), (d) Ki67 proliferation index is <14%. (10X)

Figure 3: Surrogate molecular classification- Luminal B Subtype. (a) Complete membranous positivity for Her2. (40X), (b) Ki67 proliferation index is <14%. (10X), (c) Positive nuclear immunopositivity for ER. (10X), (d) Positive nuclear immunopositivity for PR. (10X)
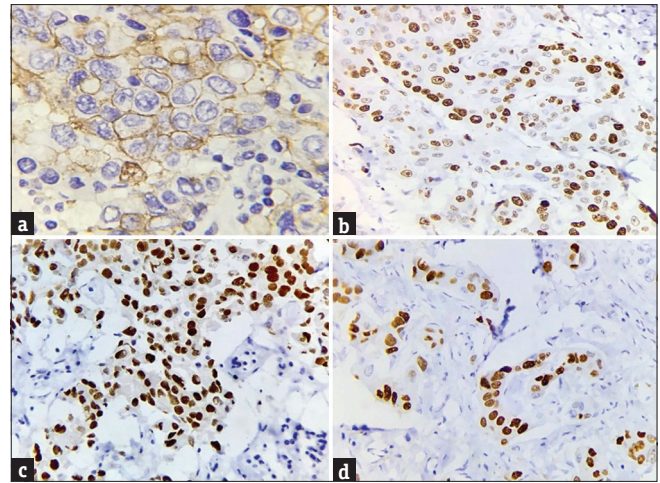

Figure 4: Surrogate molecular classification- Her2Neu enriched Subtype. (a) Positive nuclear immunopositivity for ER. (10X), (b) Positive nuclear immunopositivity for PR. (10X), (c) Complete membranous positivity for Her2. (40X), (d) Ki67 proliferation index is <14%. (10X)

Figure 5: Gel documentation image of mutational analysis. Lanes 1–10 show bands at 193 bp, signifying the absence of mutation. NC: negative control. M: 100-bp ladder sequence

reflective of the trend of breast cancer occurring at a younger age in India.

The overall global prevalence of family history in breast cancer is found to be 33%, as estimated in the study by Larsen *et al*.[38] In Indian scenario, Saxena *et al*. found a positive family history in 20.6% of cases. In our study, we found 19.44% having a significant family history, which is comparable with the observations by Saxena *et al*.[39] Thus, the prevalence of family history in Indian breast cancer patients is significantly lower than their Western counterparts.

In our study, six cases (16.67%) had contralateral breast involvement. Our findings differ from observations

by Abraham *et al*., who showed the prevalence of bilaterality in breast cancer to be 0.04% only, in their cohort of Indian breast cancer patients.[40] A study conducted by Lehman *et al*. showed that 30 of 969 breast cancer cases (3.1%) had contralateral breast involvement.[41] The higher proportion of contralateral breast cancer in our study compared to the Western studies may be due to the fact that breast cancer patients in India present to the hospitals late in the progression of the disease.

According to the study by Leong *et al*., majority of carcinoma breast cases in the West presented in Stages I and II of disease, whereas in India, 45.7% presented in advanced stages (Stages III and IV).[42,43] However, in our study, maximum number of patients were in Stage II (14 cases, 38.88%) followed by Stage III A (11 cases, 30.55%). Thus, the findings of our study are in agreement with previous Indian data, and highlight that breast cancer in our country tends to present at a more advanced stage.

The present study showed that the majority of cases (50%, 18) were of Grade II differentiation. This is

in agreement with studies conducted on breast cancer in Indian as well as Western women in the past.[43-45]

Observations made by Kumar *et al.* in their study showed that luminal A subtype was most prevalent (34%), followed by basal-like/triple-negative subtype (25%). Luminal B and Her2/neu subtypes had the same prevalence, i.e. 18% each.[45] Studies conducted on the women suffering from breast cancer in developed regions of the world such as the study by Strand *et al.* have shown that among 478 cases, 45% of cases were luminal A subtype, followed by 42% luminal B.[46] Only 8% belonged to triple-negative subtype, with Her2Neu-enriched subtype being the least common (5%). In our study, however, we found that majority of the cases were of luminal B (11 cases, 30.56%), followed by 10 cases (27.78%) which were triple negative. Eight cases (22.22%) were in Her2-enriched subtype, and 7 cases (19.44%) belonged to luminal A subtype. Our findings thus show a higher prevalence of luminal B and Her2Neu-enriched subtypes. Overexpression of the protein and/or amplification of the Her2 gene have been reported in approximately 20%–30% of breast cancers, as observed by Kumar *et al.*, which is similar to the prevalence obtained in our study, i.e. 22.22%.[45] Our findings are thus in agreement with previously published literature, thereby pointing toward the fact that overall breast cancer in India is of the more aggressive type.

Assessment of the size of the tumor was made in the present study, and it was found that the maximum number of patients (55.56%) had tumor size in the range of 2–5 cm. This can be correlated with the study by Kumar *et al.*[45] who also found that 55.4% of tumors were in the range of 2–5 cm. The average size of the tumor in our study was 4.47 cm, while in the study by Kumar *et al.*, it was found to be 3.4 cm. However, Strand *et al.* found that the average tumor size in 2012 cases examined by them was 1.69 cm, which is significantly lower than that found in Indian literature.[45] This may be because of better screening protocols in the developed nations and higher awareness about breast cancer among the population which leads to early presentation and diagnosis of patients.

ATM allele IVS10-6T>G has been extensively studied worldwide, but to the best of our knowledge, there is an extreme paucity of Indian work on this mutation. Its role in breast carcinoma among Indian women has not been studied extensively. Only a single study was found on ATM IVS10-6T>G after extensive literature search.

In the present study, we attempted to elucidate this mutation and found that out of 36 breast cancer patients included in our study, none (0/36) showed the presence of this mutation.

A case–control study conducted by Syeed *et al.* on high-risk population of Kashmiri women analyzed the presence of ATM IVS10-6T>G in 130 breast cancer patients and 220 female healthy controls. They found that majority (68.4%) of the breast cancer patients were homozygous for T/T variant, 21.5% of patients were heterozygous for T/G variant, and 10% of patients were homozygous for GG variant. They concluded that ATM IVS10-6T>G is associated with sufficiently high risk of breast cancer as it was present in 21.5% of their cases.[31]

Several studies on this mutation have been conducted in the Western world. A hospital-based study conducted by Dörk *et al.* on German women showed that 7/1000 (0.7%) cases showed the presence of ATM IVS10-6T>G.[20] Several other large as well as small-scale studies showed similar findings. In a study conducted by Chenevix-Trench *et al.*, it was found that none out of the 262 Australian breast cancer patients included in their population-based study showed the presence of ATM allele IVS10-6T>G.[47] Marouf *et al.* evaluated the expression of ATM variants in Moroccan women and found no expression of ATM IVS10-6T>G in patients or controls.[48] Thus, their findings are in agreement with the findings of the present study. Lei *et al.* carried out a hospital-based study on non-BRCA-1/2 cases and unselected controls, and subsequently found that 2 out of 768 (0.3%) cases showed the presence of ATM allele IVS10-6T>G.[49]

Broeks *et al.* included 1000 cases and 500 controls in their hospital-based study in Germany and found 7/1000 (0.7%) cases harboring this mutation.[50]

Lindeman *et al.* found that 7 of 495 patients (1.4%) were heterozygous for the IVS10-6T>G variant while the carrier rate in unselected Australian women with no family history of breast cancer was reported to be 0.83% (*P* = 0.4).[51] Similarly, in a study conducted by Szabo, on breast cancer in Australian women, it was found that 9 out of 1172 (0.8%) cases showed the presence of this mutation.[52] Thompson *et al.* conducted a population-based study in Australia, and found that 3 out of 302 (1.0%) cases and 7 out of 707 (1.0%) controls exhibited this mutation.[53]

Bernstein *et al.* found that the IVS10-6T>G mutation of ATM was present only 1 out of 511 (0.2%) bilateral and in 8 out of 638 (1.3%) unilateral breast cancer cases.[54] Soukupova *et al.* also conducted a study among breast carcinoma patients in the Czech Republic. They found that only one out of 161 (0.6%) of which 114 were

selected based on non-BRCA1/2 status, and two out of 183 (1.1%) controls were positive for the presence of this mutation.[55]

Ding *et al.* conducted a meta-analysis of 11 studies including 8831 cases and 4957 controls. The carrier frequency of the ATM IVS10-6T>G mutation was found to be 0.5% (45/8,831) in patients with breast cancer and 0.7% (38/4957) in healthy controls. When all the 11 studies were pooled into the meta-analysis, there was no evidence for a significant association between IVS10-6T>G mutation and breast cancer risk (odds ratio = 0.87, 95% confidence interval = 0.55–1.37). In the subgroup analyses by source of controls and family history with BRCA1/2 status, no significant association was found in any subgroup of population. When sensitivity analyses were performed, all the results were not materially altered. Their meta-analysis strongly suggested that IVS10-6T>G mutation is not associated with increased breast cancer risk.[56] The meta-analysis does not include any study conducted on Asian population on the allele IVS10-6T>G.

CONCLUSION

The mutational analysis results obtained in our study although not in agreement with the previous study on Indian women but they are concordant with the numerous previous studies and meta-analysis done on women suffering from breast carcinoma in the Western world.[31] The reason for the difference from a previous Indian study could be the different geographic and ethnic distribution of our patients, and a relatively smaller sample size. The conflicting results obtained in the two studies carried out on the Indian population point toward the need to carry out future larger studies with a greater sample size to ascertain the exact significance and prevalence of this mutation in breast cancer.

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Conflicts of interest
There are no conflicts of interest.

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