RESEARCH COMMUNICATION

Codon 72 and G13964C Intron 6 Polymorphisms of TP53 in Relation to Development and Progression of Breast Cancer in India

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Abstract

The p53 protein is at the center of cell regulatory pathways influencing transcription and activity of several replication and transcription factors. In exon 4 of the gene TP53, a codon 72 polymorphism causing an Arg/Pro substitution has been reported in breast and other cancers. This substitution is in the putative SH3 binding domain of p53 protein, influencing binding capacity and thereby functional properties. In the present investigation of a relatively large series of cases in India, the frequency of the homozygous arginine genotype (33.2%) was significantly higher in the breast cancer group as compared to controls (19.6%), \( \chi^2 = 11.791 \) (P=0.003). Patients with premenopausal breast cancer had a more elevated frequency (41.1%) than postmenopausal cases (25.4%) although the genotype frequency distribution did not show significant variation with respect to hormonal receptor status. Elevation was greatest in patients in advanced stages of cancer. The heterozygote frequency (Arg/Pro) was also found to be increased in overweight and obese women with breast cancer. TP53 codon 72 polymorphism might predispose individual for the development of breast cancer as well as to bad prognosis. Intronic variants may affect gene regulation through aberrant splicing or through disruption of critical DNA-protein interaction. While no significant association was observed with relation to CC genotype as well as C allele of G13964C intron polymorphism with breast cancer, the C allele frequency showed association with respect to other risk confounding factors which might play role in progression of breast cancer.

Keywords: TP53 codon 72 - TP53 intron 6 (G13964C) - polymorphisms - breast cancer - receptor status

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Introduction

The human TP53 gene is located on the 17p13.1 and spans 16-20 kb DNA. The gene has 11 exons coding for an mRNA of 2.2-2.5 kb and a protein of approximately 53 kDa of 393 amino acids. Both exon-intron organization of the gene and aminoacid sequence of the protein is conserved among species. TP53 is a DNA-binding protein with transcription regulatory activities, and it is divided into three domains, the amino-terminal domain containing the activation domain, the central core containing sequence-specific DNA binding domain and multifunctional carboxy-terminal domain.

A polymorphism has been demonstrated at codon 72, where the Proline (Pro) is frequently replaced with Arginine (Arg) at exon 4 of TP53 gene. Both forms are morphologically wild type and do not differ in their ability to bind to DNA in a sequence-specific manner. This substitution is in the putative SH3 binding domain of P53, influencing binding capacity and thereby functional properties of TP53. Codon 72 lies within a proline-rich domain of TP53 required for growth suppression and apoptosis. It comprises five PxxP SH3 (SRC-homology-3) binding motifs, one of which is lost when proline is replaced with arginine. This substitution could account for the difference that these two TP53 variants exhibit in E6-mediated degradation, transcription activation and induction of apoptosis, and could explain the implication of codon 72 polymorphism in carcinogenesis (Thomas et al., 1999). The Arg allele increases the ability of TP53 to locate to mitochondria and induce cellular death, whereas the Pro allele exhibits a lower apoptotic potential and an increased cellular arrest in G1 of the cell cycle (Bergamaschi et al., 2006). Thus, this can be considered as an important gain of function polymorphism at cellular level. Increased levels of TP53 activity protects against cancer at the cost of premature aging (van Heemst et al., 2005), indicating role of TP53 gene expression not only in local cancer development but also degenerative processes in the whole organism. The TP53 Arg72Pro polymorphism might influence longevity, prognosis after cancer diagnosis and risk of cancer. This polymorphism has been shown

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to have varying ethnic and geographical distribution. The association of 72 codon polymorphism with breast cancer risk had been studied and the results were inconsistent (Greenblast et al., 1994). It has been proposed that p53Arg protein is more susceptible to inactivation through the E6-ubiquitin pathway than the p53Pro isofrom, and that TP53 Arg/Arg homozygosity is associated with increased risk of developing HPV-associated cervical cancer (Storey et al., 1998). One study showed associations of TP53Arg with advanced lung cancer and support the observation, that the Arg allele is preferentially retained in Arg/Pro germline heterozygotes (Papadakis et al., 2002).

Variations/mutations in intronic sequences have been found to exert impact on the gene expression/transcription/stability of mRNA of these genes (Goessl et al., 1997). Some introns contain regulatory sequences such as silencers, enhancers i.e., binding sites for elements that regulate the level of gene expression and thus also affect protein synthesis (Laure et al., 1994). Intronic variants may influence gene regulation through aberrant splicing or through disruption of critical DNA protein interaction. The TP53 G13964C intron 6 variant does not involve splice site or enhancer hence it might not influence expression pattern. Buller et al., (1995) confirmed that the variant was not associated with over expression. However, intron 6 seems to be the mutational hotspot in patients with familial tumors of Li-Frameni spectrum, who were not carriers of any other missense mutation in coding region of TP53. However, the mechanism by which these variants lead to altered genetic variation still remains obscure.

One of the probable explanations could be that the site lies adjacent to CpG rich Alu repeat sequence thus making it vulnerable to higher rate of mutation. Functional analysis using an in vitro cell survival assay demonstrated that lymphoblast cell lines derived from patients with the G13964C variant exhibited a reduced level of apoptosis after chemotherapy and prolonged cell survival following DNA damage (Lehman et al., 2000). The association of TP53 G13964C intron variant with breast cancer risk had been studied and the results were inconsistent (Anna Marsh et al., 2001). Several other germline mutations in intron 6 have been described. The 13964 G>A sequence variant was identified in six affected members of a Li-Fraumeni family, and in only one out of 184 healthy controls (Avigad et al., 1997). Whereas strong TP53 protein immunoreactivity was detected in both normal and tumour tissues from these patients, no aberrant mRNA expression was detected. Further, various other mutations in the region 13487-13494 were reported (McDaniel et al., 1991; Peller et al., 1995). Taken together these studies imply that intron 6 of the TP53 gene could be a mutation hot spot. These nucleotide changes may act via novel mechanisms of gene regulation that appear to be important for tumor formation.

Hence the present study aimed to observe the association of codon 72 and intron 6 polymorphisms with respect to breast cancer development.

Materials and Methods

A group of 250 breast cancer patients were selected for study. 250 healthy and age matched women without family history of breast cancer or any other cancers were selected to serve as control group. Cases were chosen from Nizam’s Institute of Medical Sciences after confirmed diagnosis. The diagnosis of breast cancer was established by pathological examination, mammmography, Fine needle aspiration (FNAC) and biopsy. Epidiological history such as age at onset of breast cancer, diet, socioeconomic status, occupation, reproductive history, family history and consanguinity were taken through personal interview with breast cancer patients using specific proforma. The patients were screened for receptor status of estrogen, progesterone and HER-2/neu by immunohistochemical assay. Clinical history such as size of the tumor, presence of auxiliary nodes, extent of metastasis, stage and type of the breast cancer, chemotherapeutic drugs used and prognosis of the disease was collected with the help of oncologist. Informed consent was taken from all patients and controls included in the study.

Five milliliters of blood was collected in an EDTA vaccutainer from patients as well as controls. DNA was isolated (Nuremberg et al., 1991) and used for amplification of codon 72 polymorphism and G13964C intron 6 polymorphisms of TP53 gene.

TP53 codon 72 polymorphism

PCR-RFLP was done for identification of codon 72 polymorphism using specific primers (Nur Buyru et al., 2003). The amplified product was digested with 5 units of Bsh 1236I enzyme (Fermentars) at 37°C for overnight and electrophoresed on 2% agarose gel. The recognition site (CGCG) of the restriction enzyme is present only in the arginine encoding allele. The proline allele is identified by the presence of single fragment of 131 bp and the arginine allele by 81 and 50 bp.

TP53 G13964C intron 6 polymorphism

PCR-RFLP was done for identification of G13964C intron polymorphism using specific primers (Anna Marsh et al., 2001). The amplified product was digested with 4 units of HhaI enzyme (New England Biolabs) at 37°C for overnight and electrophoresed on 2% agarose gel. The GG genotype is indicated by the presence of 98 and 33 bp fragments and CC genotype which lacks the restriction site by 131 bp fragment.

Statistical analysis

The results were analyzed using appropriate statistical tests by SPSS Version 14. Odds ratio was estimated to calculate the relative risk for each genotype to develop disease. Differences in genotype frequency distribution between disease and control groups was done using 2*2 χ² and χ² test for heterogeneity. Multivariate analysis was done for both polymorphisms with respect to the clinical variables.

Results

The mean age at onset of the breast cancer in the present study was 47.6 years. The genotype distributions were analysed with respect to risk confounding factors...
such as menopausal status, body mass index, and hormone receptor status (estrogen receptor, progesterone receptor), stage of the tumor and familial history.

**TP53 codon 72 polymorphism**

Both the disease and control groups were in Hardy-Weinberg equilibrium with respect to the TP53 codon 72 polymorphism. The frequency of homozygous Arginine genotype (33.2%)/Arginine allele (57.6%) was increased significantly in breast cancer patients when compared to controls (19.6%/47.2%); \( \chi^2 = 11.791 (P=0.003^*) \) (see Table 1). The OR for genotype Arg/Arg vs Pro/Pro = 2.3714 (CI 1.409 – 3.9912) was found to be significant. Patients with premenopausal breast cancer had elevated frequency of homozygous Arginine genotype (41.1%) as compared to postmenopausal breast cancer patients (25.4%) with corresponding increase in Arginine allele frequency (61.7%). No significant association was observed with familial incidences of breast cancer. The heterozygote (Arg/Pro) as well as Arg allele frequency was found to be elevated in overweight (46.2%) and obese (60%) women with breast cancer. The frequency of homozygous Arginine genotype did not show significant association with breast cancer cases when compared to the controls. Premenopausal patients had elevated frequency of C allele (7/12:58.3%) as compared to postmenopausal patients (5/12:41.7%). The C allele frequency was elevated in non-familial cases (2.6%). The breast cancer patients with high BMI has elevated frequency of the C allele (8/11:72.7%) when compared to lower BMI (3/112:27.3%). The C allele frequency was elevated in estrogen (7/9:77.8%) and progesterone (5/9:55.6%) receptor negative breast cancer patients. When stage of the disease was considered, C allele frequency was increased in advanced stages of breast cancer (6/10:60%) as compared to early stages of the disease (4/10:40%).

**Multivariate analysis of TP53 codon 72 and intron 6 polymorphisms with different clinical factors was done.** No significant association was observed between Arg/GC and Arg/Pro/GC with breast cancer. The Arg/GC genotype frequency was found to be elevated in breast cancer patients who were postmenopausal (66.7%), non-familial (66.7%), high BMI (66.7%), estrogen (80%) and progesterone receptor positive status (80%). We observed only one breast cancer patient who is having Arg/Pro/CC genotype of intron 6 and that patient is premenopausal , non-familial, high BMI, estrogen receptor negative and progesterone receptor positive with advanced stage of the breast cancer. Other combinations like Arg/GG, Pro/ GG and Arg/Pro/GG showed increased frequency with respect to risk confounding factors and it might be due to lower frequency of CC genotype which is the rare allele.

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**TP53 G13964C intron 6 polymorphism**

No significant association was observed with CC genotype and C allele of G13964C intron polymorphism and breast cancer. This might be due to very low frequency of CC genotype as only 1 case was observed to have CC genotype in our study. The breast cancer patient with CC genotype is premenopausal breast cancer, estrogen receptor negative, progesterone positive receptor status, overweight and with advanced stage of breast cancer. As CC genotype frequency was very less we calculated the allele frequencies. The frequency of C allele did not showed any significant association with breast cancer cases when compared to the controls. Premenopausal patients had elevated frequency of C allele (7/12:58.3%) as compared to postmenopausal patients (5/12:41.7%). The C allele frequency was elevated in non-familial cases (2.6%). The breast cancer patients with high BMI has elevated frequency of the C allele (8/11:72.7%) when compared to lower BMI (3/112:27.3%). The C allele frequency was elevated in estrogen (7/9:77.8%) and progesterone (5/9:55.6%) receptor negative breast cancer patients. When stage of the disease was considered, C allele frequency was increased in advanced stages of breast cancer (6/10:60%) as compared to early stages of the disease (4/10:40%).

**Table 1. Allele Frequency of TP53 codon 72 Polymorphism with Respect to Breast Cancer and Epidemiological/ Clinical Parameters**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Arg n</th>
<th>Arg %</th>
<th>Pro n</th>
<th>Pro %</th>
<th>Chi square (p value)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>288</td>
<td>57.6</td>
<td>212</td>
<td>42.4</td>
<td>10.428 (0.001*)</td>
<td>1.520 (1.184-1.950)</td>
</tr>
<tr>
<td>Controls</td>
<td>236</td>
<td>47.2</td>
<td>264</td>
<td>52.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menopausal Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>153</td>
<td>61.7</td>
<td>95</td>
<td>38.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td>135</td>
<td>53.6</td>
<td>117</td>
<td>46.4</td>
<td></td>
<td></td>
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<tr>
<td>Familial History</td>
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<td></td>
<td></td>
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<tr>
<td>Familial</td>
<td>60</td>
<td>40.5</td>
<td>88</td>
<td>59.5</td>
<td></td>
<td></td>
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<tr>
<td>Non-Familial</td>
<td>152</td>
<td>43.2</td>
<td>200</td>
<td>56.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
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<td>&lt;20</td>
<td>18</td>
<td>64.3</td>
<td>10</td>
<td>35.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-26.4</td>
<td>37</td>
<td>68.5</td>
<td>17</td>
<td>31.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.4-30</td>
<td>110</td>
<td>52.9</td>
<td>98</td>
<td>47.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;30</td>
<td>53</td>
<td>58.9</td>
<td>37</td>
<td>41.1</td>
<td>5.090 (0.165)</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>104</td>
<td>57.8</td>
<td>76</td>
<td>42.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>110</td>
<td>62.5</td>
<td>66</td>
<td>37.5</td>
<td>0.642 (0.423)</td>
<td>0.821 (0.537-1.255)</td>
</tr>
<tr>
<td>Progesterone receptor</td>
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<td></td>
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<tr>
<td>Positive</td>
<td>103</td>
<td>59.2</td>
<td>71</td>
<td>40.8</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>111</td>
<td>55.0</td>
<td>91</td>
<td>45.0</td>
<td>0.525 (0.469)</td>
<td>1.189 (0.790-1.791)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>I</td>
<td>11</td>
<td>50</td>
<td>11</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>105</td>
<td>54.7</td>
<td>87</td>
<td>45.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>84</td>
<td>57.5</td>
<td>62</td>
<td>42.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>63</td>
<td>65.6</td>
<td>33</td>
<td>34.4</td>
<td>3.720 (0.293)</td>
<td></td>
</tr>
</tbody>
</table>
The frequency of Arginine genotype (33.2%) was increased significantly in breast cancer as compared to controls (19.6%). It has been suggested that the TP53 codon 72 polymorphism might influence expression of the TP53 gene since the substitution occurs within the p53 transactvation domain (Gottlieb et al., 1996). The codon 72 is located in the hydrophobic region of the protein which determines its conformation, DNA-binding and transcriptional activity essential for growth suppression. A recent study demonstrated the influence of TP53Arg72Pro in DNA repair capacity, showing that TP53 72Pro variant activates several TP53 dependent target gene 72Arg s involved in DNA repair and DNA damage repair much more efficiently than the variant expressing cells (Siddique et al., 2006). These contradictory results could be explained by the differential effects of this alteration in p53 function. Several in vitro evidences have demonstrated that both TP53Arg72Pro variants may selectively regulate specific cellular functions. The present study reports significant association of homozygous Arginine genotype with the breast cancer and our results were in accordance with other studies from North India, where Arg/Arg homozygotes in the Ladakhi sample was statistically significant compared to other populations (P = 0.0014 with Punjabi samples, P = 0.0025 with Rajasthani Gujar, P = 0.0009 with Rajasthani Rajput samples, and P = 0.012 with Kashmiri samples (Kunzang et al., 2002).

Arginine allele frequency was found to be increased in Turkish as well as in Jewish breast cancer patients when compared to controls (Nur Buyru et al., 2003; Ohayon et al., 2005), whereas among Japanese breast cancer cases, Pro72 was significantly elevated in breast cancer than controls (Huang et al., 2003). No significant association of codon 72 polymorphism and breast cancer risk in Tunisian and Russian individuals was reported (Mabrouk et al., 2003; Suspitsin et al., 2003). The meta-analysis and regression study identified from 50 articles showed no evidence of association or heterogeneity for preinvasive lesions. For invasive cervical cancer with undefined histology, the Arg/Arg genotype was not found to affect risk (OR, 1.195% confidence interval (CI), 0.9 –1.3). However, a slightly increased risk was observed for squamous cell carcinoma (OR, 1.5; 95% CI, 1.2–1.9) and adenocarcinoma (OR, 1.7; 95% CI, 1.0 –2.7) (Anita et al., 2004). Arginine is known to induce apoptosis with faster kinetics than Proline but not a strong inducer of transcription. Premenopausal patients had elevated frequency of Arginine genotype (41.1%) as compared to postmenopausal patients (25.4%) with corresponding increase in Arg allele frequency, which might suggest that individuals with Arginine allele are at risk for early onset of breast cancer. The genotype frequency distribution of codon 72 polymorphism did not show significant variation with respect to hormonal receptor status (estrogen receptor and progesterone receptor) except for slight increase of Arg/Pro heterozygotes in patients positive for progesterone receptor status (54.0%) whereas Arg allele frequency was increased in estrogen receptor negative (62.5%) and progesterone receptor positive (59.2%) status breast cancer cases.

When stage of the disease was considered, Arginine genotype and allele frequency was elevated in patients with advanced stages (47.1% & 65.6%) of the breast cancer as compared to early stages. However, earlier studies reported association of Proline allele with breast cancer (Weston et al., 1997). It had been proposed that TP53 codon 72 polymorphism might affect the function of TP53 mutations and confer a growth advantage to tumors in which another mutation resides on the Arginine allele (Langerod et al., 2002). The heterozygote frequency Arg/Pro was found to be elevated in overweight (46.2%) and obese (60%) women with breast cancer. No significant association was observed with familial incidence of breast cancer.

The polymorphism lies within Proline rich region that is known to be related to growth suppression and apoptotic function of the protein. Functional data have shown that Arginine allele induces apoptosis 5-fold better than the Proline allele due to its efficient localization to the mitochondria (Dumont et al., 2003). Hence it had been suggested that Arginine homozygotes might respond more favorably to radiation or chemotherapy when compared to Proline allele. These favorable effects of Arginine allele may, however, be reversed by higher frequency of somatic TP53 mutation on this allele (Bonafe et al., 2003). The retention of the Arginine allele with loss of Proline allele in the tumor tissue was associated with reduced survival in heterozygous breast cancer patients. It was observed that, the breast tumors of homozygotes for Proline allele had a lower frequency of somatic TP53 mutations than tumors of Arginine homozygotes and heterozygotes (Noma et al., 2004).

In the present study, the CC genotype was observed in one patient out of 249 and also in two members of control group. The patient with CC genotype had premenopausal breast cancer and the tumor was found to be of estrogen receptor negative and progesterone receptor positive status. The patient had very high BMI and tumor progressed to advanced stage. As CC genotype frequency was very less we calculated the allele frequencies. The frequency of C allele did not showed any significant association with breast cancer cases when compared to the controls. The present study was in accordance with that of another study (Fiszer-Maliszewska et al., 2003), who confirmed that intronic mutation G13964C does not confer risk to develop breast cancer. On contrary, a study on Polish population indicated that TP53 G13964C was not observed as a germline mutation associated with high-risk cancer (March et al., 2001). No significant association with G13964C mutation was observed in Australian breast cancer patients (Varley et al., 2001). The 13964 G>C variant was detected at an elevated frequency in familial breast cancer patients from North America. Using immortalised lymphoblastoid cell lines derived from 13964 G>C variant carriers, the investigators showed that the mutation is functionally active and results in an inhibition of apoptosis and prolongation of cell survival in vitro upon DNA damage in response to cisplatin, suggesting that this mutation affects breast cancer risk. However, strict segregation of this variant with the disease...
has not been shown (Lehman et al., 2000).

Premenopausal patients had elevated frequency of C allele (7/12:58.3%), which suggest that that carriers of C allele are at risk of developing breast cancer at younger ages. The C allele frequency was elevated in non-familial cases (2.6%) which is in accordance with German breast cancer study (Liu et al., 2004). The breast cancer patients with high BMI has elevated frequency of the C allele (8/11:72.7%) which suggest that the individuals with higher BMI and C allele carriers might have risk of developing breast cancer. The C allele frequency was increased in estrogen (7/9:77.8%) and progesterone (5/9:55.6%) receptor negative breast cancer patients as well as in patients with advanced stages of breast cancer (6/10:60%) which might suggest that carriers of C allele breast cancer patients will have bad prognosis of the disease as the G13964C variant exhibited a reduced level of apoptosis after chemotherapy and prolonged cell survival following DNA damage (Lehman et al., 2000).

In agreement with the earlier report the present study did not showed any association with CC genotype or C allele of G13964C mutation with breast cancer. The C allele frequency showed association with the other risk confounding factors of breast cancer which might play role in the prognosis of breast cancer.

Multivariate analysis of TP53 codon 72 and intron 6 polymorphisms with different clinical factors has revealed interesting results. In our study we did not observed any breast cancer patients with both Arginine and CC genotype. We considered only Arg/DC and Arg/Pro/DC combinations for the discussion as Arg and C alleles were risk alleles for the development of breast cancer. No significant association was observed between Arg/DC and Arg/Pro/DC with breast cancer. When Arginine and GC combinations were combined there was an elevated frequency of Arg/Pro/GC with breast cancer. When Arginine and GC were risk alleles for the development of breast cancer. No significant association was observed between Arg/GC and Arg/Pro with breast cancer patients. Multivariate analysis of TP53 codon 72 and intron 6 polymorphisms in breast cancer patients. Gynecol Oncol., 58, 368-374.


March A, Spurdle AB, Turner BC, et al (2001).The intronic G13964C variant in p53 is not a high-risk mutation in breast cancer study (Liu et al., 2004). The breast cancer patients with high BMI have elevated frequency of the C allele (8/11:72.7%) which suggest that the individuals with higher BMI and C allele carriers might have risk of developing breast cancer. The C allele frequency was increased in estrogen (7/9:77.8%) and progesterone (5/9:55.6%) receptor negative breast cancer patients as well as in patients with advanced stages of breast cancer (6/10:60%) which might suggest that carriers of C allele breast cancer patients will have bad prognosis of the disease as the G13964C variant exhibited a reduced level of apoptosis after chemotherapy and prolonged cell survival following DNA damage (Lehman et al., 2000).

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In conclusion our findings showed that the association of Arginine genotype with breast cancer in the present study might indicate that TP53 codon 72 polymorphism might predispose individual for the development of breast cancer as well as to bad prognosis. No significant association with CC genotype/C allele of G13964C mutation with breast cancer was observed but it might be association with bad prognosis of the breast cancer.

Acknowledgments

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