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Allelic polymorphism in codon 72 of p53 gene: prognosis value, survival rates, and their association with breast cancer

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Summary

Purpose of investigation: There are controversial findings to establish relationship between genotype polymorphism of codon 72 of *P53* gene, its prognosis value, and survival rate of the patients in breast cancer. For the first time this study has shown such relationship in Sabzevar, Iran. *Materials and Methods:* A descriptive analytical case–control study was conducted on 160 people (80 patients and 80 controls). DNA was extracted and codon 72 of the p53 gene was amplified. The genotype of the p53 gene was determined by electrophoresis, samples were sequenced, and all patients were followed up for 30 months. *Results:* The frequency of heterozygote arginine/proline was 49 (30.6%) and 51(31.9%) in the patients and controls, respectively. Homozygote arginine/arginine had frequency of 29 (18.1%) in the patients while it was 15 (9.4%) in controls. Homozygote of proline/proline was two (1.3%) in the patients and 14 (8.8%) in controls. The sequencing results were consistent to PCR and electrophoresis results. *Conclusions:* This is the first study in the region which shows relationship between genotype polymorphism, survival rate, and its prognosis value in breast cancer. The authors showed that homozygote proline/proline in controls was significantly higher compared with that in the patients. They may therefore, conclude that detection of allelic polymorphisms of codon 72 of the p53 gene including arginine/arginine could be a risk factor predisposition for breast cancer and valuable tool for determining prognosis, progress, and treatment purposes.

Key words: Breast cancer; Allelic polymorphism; Codon 72.

Introduction

Breast cancer is one of the most leading causes of deaths in the world and the second one in western countries [1]. The prevalence of the disease is also increasing in Asian populations [2]. In 90% metastasized patients, the resistance to routine chemotherapies has been reported [3]. Different epigenetic or genetic factors [4] have been assumed to be involved in the process of the disease including inhibition in apoptosis and impairment in the repair of DNAs [5, 6]. The p53 gene, located on the short arm of chromosome 17 with 11 exons has been identified for its role to inhibit impairments in cycle and growth of cells [7] in apoptosis, transcription, and senescence [8 ,9]. Several studies including the authors' previous study [10] showed that polymorphism and some mutations in the p53 gene play roles in changes which lead to malignancy in colorectal [11] breast, lung, and bladder cancers [12, 13]. It has been suggested that polymorphism in the codon 72 of the exon 4 of the p53 gene has link to breast cancer. It has two allelic forms which lead to produce arginine (CGC) and proline (CCC) in p53 protein respectively [14]. It has been also reported that the risk of breast cancer has link with homozygocity of CGC genotype, arginine/arginine phenotype [15]. In contrast, some studies concluded that there is no such association between breast cancer and the polymorphism in the codon 72 of the p53 gene [16-18]. However, an association between homozygocity of proline (CCC) and the prognosis of breast cancer has been reported in Finland by Tommiska *et al.* [19].

Controversial results from different parts of the world may be due to the possible roles for geographical, regional or race links between the p53 gene polymorphism and the risk of breast cancer. It is worthy to indicate that recent studies showed a decrease in the average age to suffer from breast cancer in Iran [20]. Although life expectancy has been increased in parallel to decreasing of mortality, the overall prevalence has been mounted [21]. The present study therefore, aimed to determine such possible effect of having a specific polymorphism in codon 72 of p53 gene including arginine/arginine, proline/proline, or arginine/proline genotypes on the prevalence status of cancer and as risk factors in breast cancer.

The authors followed up all patients for 30 months to examine that if there was any relationship between those polymorphic genotypes and survival rates because, to the best of their knowledge, there is no report in the region of such research. The prognosis value of determination of such polymorphisms and their utilization in early diagnosis and treatment, particularly when younger women are more prone to this cancer, were also examined.

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Materials and Methods

Patients and controls

The study was a descriptive, analytical case control which was conducted on 160 samples including 80 patients with breast carcinoma and 80 matched healthy controls. The ethical committee of the university approved the study. After describing the study's nature, aims and possible benefits for improvement to prevent or cure the disease a written consent form was given from each involved sample.

For pathological diagnosis of the breast carcinoma, five sections with five-µm thickness were prepared from each patient. A peripheral blood sample at 1.5 ml was taken from each healthy matched control, homogenized, and stored in the tubes containing 0.5 molar ethylene diamine tetra acetic acid (EDTA) at -20ºC.

DNA extraction and PCR

DNA was separately extracted from each patient and control using standard kit. Codon 72 of the p53 gene was amplified using two specific primers by PCR technique. Specific primers for proline and arginine were prepared and lyophilized. They were diluted using deionized sterilized water to the required weight/volume based on manufacturer's instructions. The five mmolar dNTP solution (stock ten mmolar) was used. The sequences for proline and arginine primers were as follows respetively:

Forward: 5'GCCAGAGGCTGCTCCCCC3' 3'
Reverse: 5'CGTGCAAGTCACAGACTT
Forward: 5' TCCCCCTTGCCGTCCCAA3' 3'
Reverse: 5' CTGGTGCAGGGGCCACGC

During the optimized PCR process 59ºC was used for 50 seconds to amplify proline and arginine. The concentrations of materials used were as follows: DNA at five mM, primers and chloride magnesium at 0.5 mM, arginine at two mM and proline at five mM. The exon 4 of the p53 gene containing codon 72 was amplified in 35 cycles at the same condition for all collected samples.

Histopathological experiments

All diagnosed cancerous tissues after surgery were fixed with 10% formalin and renewed after four hours. Tissue passage was done by a processing tissue device after 24 hours. Sections with five-µm thickness were prepared for all samples using rotational microtome. For background staining hematoxylin, eosin, a specific monoclonal rabbit antihuman PTEN antibody and avidin-biotinolated immunoperoxidase were used. To localize the antigenic determinants a citrate buffer at 0.9% and hydrogen peroxidase at 3% were added to all samples and kept at 37º C for 30 minutes to inhibit endogenous peroxidase. After five times washing with phosphate buffer saline (PBS), streptoavidin conjugated with horse radish phosphate (HRP) was added to all sections to oxidize diaminobenzidine (DAB) which stained cells to brown.

Grades and stages

For determination of grades and stages, all samples were examined by two separate pathologists. Microscopy was carried out using a motic microscope equipped with advanced motic plus software with both 100 and 400 magnifications [22]. Histological grading was separately performed by microscopy based on the three following parameters: mitotic activity, nuclear pleomorphism, and the extent of tubule formation. Grades were then proposed in three groups: 1, 2, and 3 [23]. Tumor stages in the breast cancer were designated in numbers from 0 to 4 when 0 is for in situ carcinoma and Stages I, II, III, and IV are for the four consequent stages [24].

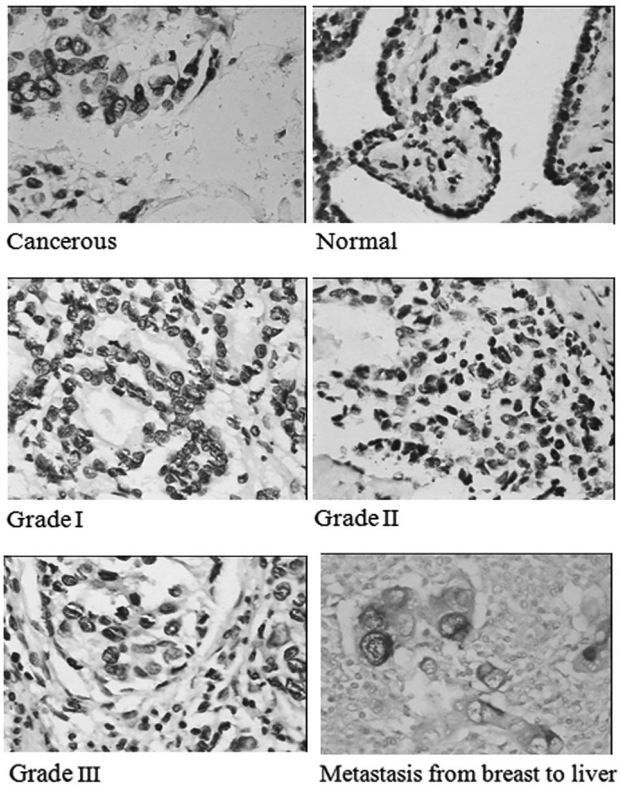

Figure 1. — Cross sections (four µm) from healthy controls and patients. Above: left cancerous, right normal. The following figures show different grades and metastatic section in the liver. All samples, normal and cancerous, were stained by H/E for the background and specific staining using streptoavidine conjugated with HRP which is able to oxidize (DAB). This staining method colored cells brown so that they can be distinguished with each other and other components. The magnification was 100 except for figures showed grades and metastatic pictures for which 400 magnification scales were utilized.

Electrophoresis

Electrophoresis was briefly set as follows: PCR product at five µl was mixed with one µl of loading dye and inserted into 1.5% agarose gel. For DNA staining, ethidium bromide was used and the results were photographed using gel documentation device. The genotype for each sample was then identified based on the base pairs (bp) of each bind compared with the standard marker.

Sequencing: Before sequencing each DNA sample was prepared by cutting the selected binds and was then amplified by PCR and the resultants were then sent to South Korea for sequencing.

Follow up: To evaluate relationship between the survival rate of patients with breast cancer and the genotypic mutation characteristics of codon 72, all patients were followed up for 30 months. For this purpose the authors contacted close relatives of all patients at proper intervals to ask and check the status of each one.

Statistics

Data was analyzed using SPSS software version 15. The Pearson chi-square and Fisher exact tests were used when required to explore associations between genotype and histological parameters. Using Kaplan Meier method, survival rate of patients with

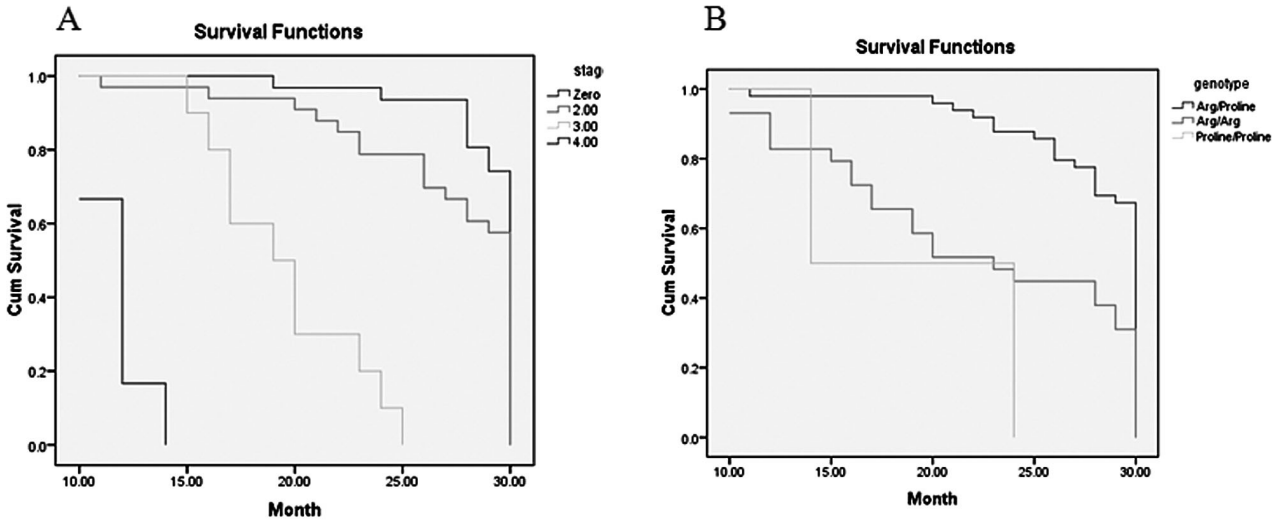

Figure 2. — The association between survival rates and the stages of cancer. For calculation Stages 0 and I were accumulated and designated as Stage I and the other as II, III, and IV. During follow up, the stages of the disease were measured and as expected patients with higher stages had lesser survival rate. A: Association between survival rates and the stages of the breast cancer. B: Association between survival rates and genotypes of the breast cancer. Genotype 1 was designated as arginine/proline, genotype 2 was designated as arginine/arginine, and genotype 3 was designated as proline/proline.

different genotypes and groups in higher and lower cancer stages was explored. Differences were tested by Mantel-Cox log-rank test. The results were considered to have significant difference when the p value was < 0.05 through all experiments.

Results

In the present study all 80 cancerous patients were diagnosed with a breast carcinoma. The minimum and maximum ages in patients were 20 and 86 years. In the healthy controls their ages were between 23 and 80 years. The average age for patients and controls were 47.22 ± 12.95 and 48.02 ± 12.48 years, respectively.

Stages

Of 80 patients, 31 (38.8%) cases were in the Stages of 0 (in situ carcinoma) and I who had cancerous cells limited to one or some lobules and ducts and there was no sign of metastasis in fatty tissues, lymph nodes or cells surrounding the location of the carcinoma. There were 33 (41.3%) samples in Stage II. They showed metastasis in the nearby tissues such as close lymph nodes. The number in Stage III was ten (12.5%) with metastatic cells in the regional lymph nodes. The other six patients had carcinoma in Stage IV and metastasis was observed in three patients in their lungs, one in her second breast and the remaining two ones in their liver (Figure 1).

Grades

There were 13 (16.3%) samples with grade 1, 45 (56.3%) with grade 2, and 22 (27.5%) with grade 3 (Figure 2). Regarding the grade and the menopausal age, the results showed significant differences between women who were at or less than 45 years old and older than 45 years. For example, a significant difference ($p < 0.007$) was seen between ten (12.5%) non-menopausal women and in three (3.8%) menopausal ones with grade 1. There were 22 (27.5%) non-menopausal and 23 (28.8%) menopausal cases with grade 2. However, for all women with grade 3, 17 (21.4%) menopausal ones had significantly higher rate ($p <$

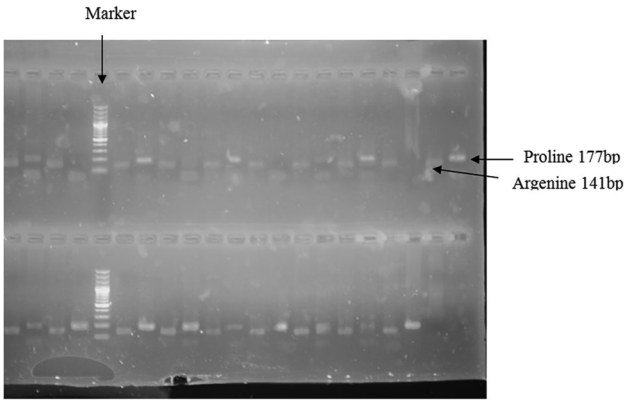

Figure 3. — The PCR products of codon 72 of p53 gene in exon 4 for proline/proline, arginine/arginine and proline/arginine in samples with breast cancer. The marker ladder was a standard ladder of 100 bp. The allele for proline/proline was 177 bp.The allele for arginine/arginine was 141.

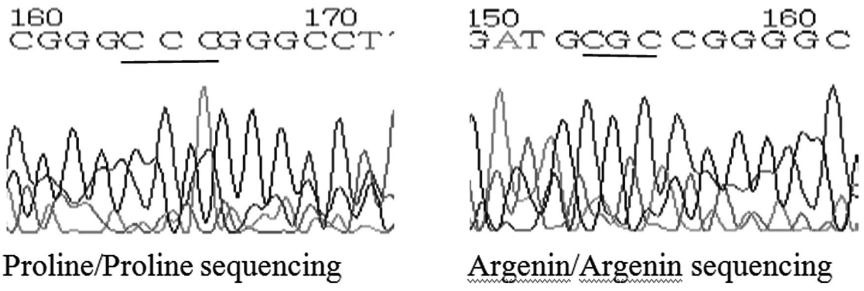

Figure 4. — The results for identifying sequencing of codon 72 polymorphism of p53 gene. Left above: The figure shows the genotype CGC which encodes proline. Right above B: The figure shows the genotype CCC which encodes arginine.

Table 1. — *The frequency of the genotype codon 72 of p53 gene expression in the different tumor stages in the cells of breast cancer and normal tissue. The data were statistically tested to find any significant differences.*

Variables	Arg/pro No. (%)	Arg/Arg No. (%)	Pro/Pro No. (%)	Statistical relationship
Cancer	49 (30.6%)	29 (18.1%)	2 (1.3%)	$p < 0.001$
Normal	51 (31.9%)	15 (9.4%)	14 (8.8%)	
Stage of cancer in situ carcinoma				$p < 0.001$
and I	22 (27.5%)	9 (11.5%)	0 (0%)	
II	26 (32.5%)	7 (8.8%)	0 (0%)	
III	1 (1.3%)	8 (10%)	1 (1.3%)	
IV	0 (0%)	5 (6.3%)	1 (1.3%)	
Tumor grade				$p < 0.01$
I	7 (8.8%)	6 (7.5%)	0 (0%)	
II	34 (42.5%)	11 (13.8%)	0 (0%)	
III	8 (10%)	12 (15%)	2 (2.5 %)	
Age < 45	55 (34%)	20 (12.5%)	12 (7.5%)	$p = 0.124$
Age > 45	45 (28%)	24 (15%)	4 (2.5%)	

0.007) compared with five (6.3%) non-menopausal women. There were no significant differences ($p < 0.44$) between the stage of the carcinoma and either non-menopausal or menopausal situation.

The association between the survival rates in the patients with arginine/proline genotype was higher than that in those who had arginine/arginine genotype. Patients with proline/proline genotype had the lowest survival rate compared with other checked genotypes (Figure 2).

Genotypic polymorphism

The authors analyzed the frequency and distribution of different arginine and proline genotypes. Their alleles had 141 and 177 base pairs respectively. The frequency of heterogeneous arginine/proline genotype was 49 (30.6%) and 51 (31.9%) in the patients and healthy controls, respectively, with no significant differences. The frequency of arginine/ arginine was 29 (18.1%) in the cancerous samples whereas it was 15 (9.4%) in the healthy controls, which showed a higher rate in the patients compared with the controls but, there was no significant difference. However, the frequency of homogeneous proline/proline genotype in the healthy controls with 14 (8.8%) cases was significantly higher than that in the patients with two (1.3%) cases ($p < 0.001$)(Table 1). Below we have brought the genotypes followed by their consequent stages in accordance with their frequency: proline/proline at Stage IV arginine/arginine at Stage III, arginine/proline at Stage II<, and Stage I, respectively. The results for bands of arginine and proline alleles are shown in Figure 3.

Sequencing results

The results of sequencing for homozygous arginine/arginine, proline/proline, and heterogenous arginine/arginine were consistent with the results given by PCR and electrophoresis in the patients and healthy controls (Figure 1). Moreover, the results of grading and staging were also consistent in terms of having significant or non-significant differences between genotypes in the patients and controls (Figure 4).

Follow up

Association between the survival rate and the genotypic mutation characteristics of codon 72 of p53 gene were examined for all patients. All patients were followed up for 30 consequent months by interval contacts with their closed relatives. It was expected that patients who were in higher stages had lesser survival rate compared to those who were in lower stages. During follow up, 38 patients out of 80 ones died. Of 38 dead 20 had hemozygote arginine/arginine, 16 had heterozygote arginine/proline, and two patients had proline/proline genotypes. The highest survival rate was observed in patients with arginine/proline genotype compared with others. The second higher survival rates were for cases with arginine/arginine whereas two patients with proline/proline genotype had the lowest survival rate among the examined cases though they were only two cases.

Discussion

Studies have shown that the risk of breast cancer mortality may be affected by genetic and epigenetic factors [25] including polymorphisms in the p53 gene. In this gene codon 72 on exon 4 have two distinct alleles which encode arginine (CGC) and proline (CCC), respectively, in the p53

protein structure [26]. The present results showed that in the healthy people, the homozygote proline/proline was significantly higher compared to the patients. In contrast, the homozygote arginine/arginine genotype in the patients with breast carcinoma was higher compared with the healthy controls. The authors also found that there was slightly higher heterozygote proline /arginine in the healthy people compared with the patients.

The survival rate in the patients with arginine/proline genotype was higher than that in those who had arginine/arginine. So, it seems that patients with arginine/arginine had more chance to suffer from cancer. Cases with proline/proline genotype had the significantly lowest survival rate compared with other checked genotypes. However, it may be assumed that, the reason could be the number of the cases as they were only two. More importantly they showed the highest stages, one at the stage III and another one at the stage IV. These findings suggest that we need to perform more investigations with a bigger sample size to cover other possibilities**.**

The significant association between the grades and the stages with the genotype was also observed. However, there was no such association in women regarding menopausal or non-menopausal status and the distinct genotype. Some studies in Brazil and Greece were in agreement with the present results as they reported a significant link between the presence of arginine/arginine and the risk of the breast cancer [15]. In contrast, Vijayraman *et al.* from Maduria have reported that there was no such link between the arginine/arginine, proline/proline, and arginine/proline genotypes of p53 gene and the breast cancer [27]. However, it is worthy to note that in that study, sample size was smaller than the present. They had only 100 samples (50 patients and 50 controls) whereas the present authors examined 160 people, including 80 with cancer and 80 that were healthy.

An association between proline/proline genotype for exon 4 of codon 72 has been shown by a group in Austria [28] which demonstrated similar findings with the present results. They also showed that the frequency of heterozygous proline/arginine was close to each other in the cancerous and healthy people. However, unlike the present study, they suggested that proline/proline had a role in breast cancer. In Japan, it has been also reported that people with proline/proline for codon 72 of p53 gene had lesser life expectancy than those with either proline/arginine or arginine/arginine genotypes [29]. Again in agreement with the present results, a study in Saudi Arabia claimed that there was a positive link between the initiation of the breast cancer and the arginine/arginine genotype. In addition, they proposed that there is a possible protective role for arginine/proline genotype [30].

According to above studies, it may be suggested that the stage status of the disease had more important role in the survival rate compared to the genotype status (Figure 4). These controversial results may reflect the possibility of different functionality for genotypic polymorphism of codon 72 which may be influenced by other factors, such as geographical and regional epigenetic specifications or even other unknown reasons. Further investigations, therefore, may be required to clarify and justify such controversies. In addition, these results are the reason why many studies have focused on the roles of codon 72 of p53 gene worldwide, particularly in the case of breast cancer. Similar to the present results in another study, Gochhait *et al.* showed that arginine/arginine genotype was more prevalent in the women with breast cancer compared with healthy ones [31].

Moving to another insight, it has been also shown that responses to the breast cancer treatment with anthracycline can be influenced by polymorphism of codon 72 of p53 gene in addition to pathological characteristics [25]. In this regard Wegman *et al.* in Sweden have reported that patients with breast cancer who had estrogen receptors and proline allele had shown better response to tamoxifen against the breast cancer than those who carried other alleles of codon 72 [32]. They also showed that patients who had arginine/arginine genotype had the same response to the treatment with either tamoxifen or non-tamoxifen chemotherapy. Therefore, these results suggested that lack of proline allele in the patients resulted in the better effective response to non-tamoxifen chemotherapy [32]. Schneider *et al.* who worked on neck and head cancers in Germany believed that the role of polymorphism of codon 72 of p53 gene is due to its role in apoptosis in malignant cells. Their study showed that tumors with arginine/arginine were resistant to apoptosis while tumors with proline/arginine were susceptible to the same apoptosis process [33]. Like the present results, their study confirmed the importance of some aspects of oncogenesis for arginine/arginine allele.

Conclusion

Controversial findings in breast cancer studies in different regions [34] suggested that there are probably regional patterns for the appearing and presence of a particular genotype of codon of 72, which plays role in oncogenic processes. The present authors may assume that, due to the effect of epigenetic factors in genotypic changes and consequently in different functions, more experimental wider works are required to identify the genotypic situation of codon 72 in any area where breast cancer is endemic and prevalent. The importance of breast cancer, the realty that there are no sufficient studies to clarify possible roles of polymorphism in codon 72 in different regions and populations, and many other controversial results, would encourage researchers to design comprehensive studies focusing on the factors affecting the survival rate, life expectancy, and related regional factors. These findings may lead to earlier diagnosis and subsequently the findings would be the best effective prevention and treatment protocols and hence would lead to less mortality due to breast cancer.

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