A preliminary study of microsatellite instability analysis in different genotypes of p53 codon 72 in breast invasive ductal carcinomas

Mehdi Nikbahkt Dastjerdi[1], Ph.D. ,Masoumeh Faghani[2], Ph.D., Mansour Salehi[3], Ph.D., Mohammad Rabbani[4], Ph.D.

Dept. of Anatomical Sciences, Medical school, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: The polymorphic variants at codon 72 of the p53 gene, encoding either proline or arginine at residue 72, produce marked change in the structure of p53. From the evidence that the DNA mismatch repair system and p53 interact to maintain genomic integrity, we hypothesized that the codon 72 variation may influence the prevalence of microsatellite instability; a feature of malignancies associated with mismatch repair deficiency in breast invasive ductal carcinoma.

Methods: TP53 codon 72 genotypes were detected by PCR using specific primer pairs for amplifying the Proline or the Arginine Alleles. Then, the frequencies of microsatellite instability (MSI) were analyzed in three genotypes of P53 codon 72 using genomic DNAs from 120 specimens of breast ductal carcinomas by testing the BAT-26 marker .

Results: From 120 specimens, 73(60.8%) was Arg/Arg, 31(25.8%) Arg/Pro and 16(13.3%) Pro/Pro. MSI analysis revealed that 24.2% of the tumors (29 patients) was microsatellite instability-positive and 75.8% (91 patients) was microsatellite instability -negative. The frequency of microsatellite instability in the Arginine/Arginine, Arginine/ Proline and Proline/Proline genotypes were 14 (19.2%), 12 (38.7%) and 3 (18.8%) respectively. A significant difference in distribution of MSI was found for the Arginine/ Proline genotype compared with (grouped) Arginine/Arginine and Proline/Proline genotypes (P=0.028).

Conclusion: Our findings suggested that breast invasive ductal carcinomas arising in individuals with p53 codon 72 heterozygosity (Arginine/Proline) may be preferentially prone to microsatellite instability more than other genotypes.

Keywords
Microsatellite instability, polymorphism, P53, breast invasive ductal carcinoma.

Introduction

Breast cancer is the most prevalent malignancy in women [1]. Recent studies indicate that a substantial fraction of breast tumors have frequent microsatellite instability (MSI) [2]. MSI represents a mutational process of insertions and deletions and is characterized by variations in the length of microsatellite repeats in the tumor DNA relative to the germline length. It is one form of genomic instability and a hallmark of the DNA replication error (RER) phenotype due to the inactivation of mismatch repair (MMR) genes[3]. The MSI phenotype is well established in some colon, gastric, pancreatic and endometrial cancers, whereas its oc-

1. **Corresponding author,** Associate Professor, Dept. of Anatomical Sciences, Medical school, Isfahan University of Medical Sciences, Isfahan, Iran. Email: nikbakht@med.mui.ac.ir, Tell: +98311 7922403
2. Assistant Professor, Dept. of Anatomical Sciences, Medical school, Guilan University of Medical Sciences, Rasht, Iran
3. Associate Professor, Dept. of Genetics, Medical school, Isfahan University of Medical Sciences
4. Professor, Dept. of Biotechnology, Pharmacy School, Isfahan University of Medical Sciences.

currence in breast cancer still remains to be clarified; has been reported in some breast cancer patients with variable frequency (0-33%)[2].

TP53 gene, located on chromosome 17p13, is one of the most mutated genes affecting many types of human cancers[4]. In addition to mutations, several polymorphisms in the wild-type p53 gene locus have been detected which could alter its function[5].

The most commonly polymorphism in the general population which associated with cancer development is the codon 72 Arginine (Arg) to Proline (Pro) substitution [6] .

There is evidence that the MMR system and p53 interact to maintain genomic integrity7. Furthermore, studies provided evidence for co-operation between MMR system and p53 in promotion of cell cycle arrest, cell death and tumorigenesis [8]. Moreover, MMR-deficient cells exhibit defects in the activation of p53 family after exposure to alkylating agents or cisplatin [9]. Because of functional differences between the two polymorphic variants of p53 which could alter its function; we hypothesized that the arg72/pro72 variation in p53 may influence the presence of MSI in patients who had developed breast cancer. Thus we undertook the present study in order to explore a possible association between presence of MSI with different genotypes at codon 72 of TP53 tumor suppressor gene.

Methods

Population and samples

We performed a study on 120 specimens of breast ductal carcinomas and the corresponding adjacent cancer-free breast tissues attending Alzahra, Sina, Seydolshohada and Sepahan Hospitals (Isfahan) over the period 2006-2007.

DNA isolation from breast carcinomas and cancer-free breast tissues

Genomic DNA from the tumors and corresponding adjacent cancer-free breast tissues was prepared using High pure PCR Template preparation DNA isolation kit (Roche, Germany), according to manufacturer's instructions.

PCR amplification of TP53 codon 72 polymorphism

The TP53 codon 72 Pro allele were detected by PCR using the primer pair p53Pro+/ p53Pro- (p53Pro+:5'-GCCAGAGGCTGCTCCCCC; and P53Pro-: 5'-CGTGCAAGTCACAGACTT) and the p53 codon 72 Arg allele by the primer pair p53Arg+/p53Arg- (p53Arg+: 5'-TCCCC-CTTGCCGTCCCAA and p53Arg-: 5'-CTG-GTGCAGGGGCCACGC)[7]. Between 100 to 300 nanograms DNA was used as template in a 25 μl PCR reaction mixture containing 1.5 μmol MgCl2, 1 U Taq polymerase (Sinagen) and 2 μmol either of the primer pairs.

PCR cycling conditions were carried out with an initial denaturation step for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30s at 60 °C (for Arg) or 54 °C (for Pro) and 30 s at 72 °C. A final extension step was performed at 72 °C for 5 min. The PCR reaction was done separately for each of the two polymorphic variants. The amplified products were subjected to electrophoresis on 1% agarose gel in 1× TBE buffer and visualized on a transilluminator using ethidium bromide.

PCR-SSCP of the BAT-26 poly(A) Tract for detection of MSI

It previously established that analysis of the BAT-26 poly(A) tract within the hMSH2 gene was sufficient to establish the MSI status of tumors [10]. Thus we did the nonisotopic detection of deletions in the BAT-26 poly(A) mononucleotide repeat using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis for detection of MSI in the tumor samples. The DNA obtained from the corresponding adjacent cancer-free breast specimens was used as control.

Primers used for amplification of the BAT-26

Genotype	N(%)	MSI		P value
		Positive N (%)	Negative N (%)	
A/p	31 (25.8)	12 (38.7)	19 (61.3)	
A/A	73 (60.8)	14 (16.9)	59 (83.1)	0.028*
P/P	16 (12.5)	3 (13.3)	13 (81.3)	

A/A: Arg/Arg genotype; A/P: Arg/Pro genotype; P/P: Pro/Pro genotype; N : number
MSI: Microsatellite Instability
* χ2 test: Arg/Pro genotype compared with (grouped) Arg/Arg and Pro/Pro   genotype

Table 1. Distribution of Microsatellite instability in different genotypes of TP53 codon72 among breast cancer cases

mononucleotide repeat were as follows [10]:

Forward 5'.TGACTACTTTTGACTTCAGCC 3'
Reverse 5'. AACCATTCAACATTTTTAACCC 3'

PCR conditions comprised 5 min denaturation at 94°C, 2 min annealing at 45°C, 2min extension at 70°C, followed by 32 cycles of 1 min annealing at 45°C, 1min extension at 70°C and 30s denaturation at 94°C. The program was terminated by 5 min extension at 70°C. The PCR product was denatured into single-strand DNA by heating in formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylenecyanol) for 5 min at 94°C and then separated on a mini-gel electrophoresis apparatus (Farayand Danesh, Iran) using nondenaturing 15% acrylamide (60:1, acrylamide:bisacrylamide) gels containing 5% glycerol. Gels were run at ambient temperature and without cooling. Gels were run for 2.5 h at 90 V. Silver staining were carried out as previously described. The presence of additional, faster migrating bands compared with normal tissue DNA were indicative of the presence of a shortened BAT-26 poly(A) tract and therefore of the MSI+ phenotype.

Statistical analyses

Associations between qualitative variables were evaluated using the χ2-test. Statistical significance level was set to P ≤ 0.05.

Results

This analysis included 120 invasive breast ductal carcinomas and adjacent normal breast tissue. The age of 120 patients ranged from 23-79 years. Mean age was 50.4±10.6 years in MSI positive patients and 48.6±14.1 years in MSI negative ones. No correlation was observed between MSI and age of the patients (P=0.532).

Detection of TP53 codon 72 polymorphism by allele specific PCR was successfully conducted in all invasive breast ductal carcinomas. Resulting PCR products were either 177bp (Fig.1)   for proline allele or 141bp (Fig.2) for arginine allele. Of 120 specimens, 73 (60.8%) were Arg/Arg, 31(25.8%) Arg/Pro, and 16 (13.3%) Pro/Pro (Table1). The MSI study was performed in 120 patients by analysis of size variations within the BAT-26 poly(A) tract. Silver stain PCR-SSCP analysis showed an specific PCR product of 121 bp. Those cases with an unequivocally distinct additional band or shifts in the tumor tissue DNA compared with normal tissue DNA were recorded and classified as MSI- positive(fig 3). MSI analysis revealed that 29 patients (24.2%) were MSI-positive and 91 patients (75.8%) were MSI-negative. MSI was more frequently observed in tumors arising in Arg/pro genotype(Table 1).A significant difference in distribution of MSI was found for the Arg/Pro genotype compared with (grouped) Arg/Arg and Pro/Pro genotypes (P=0.028).

Discussion

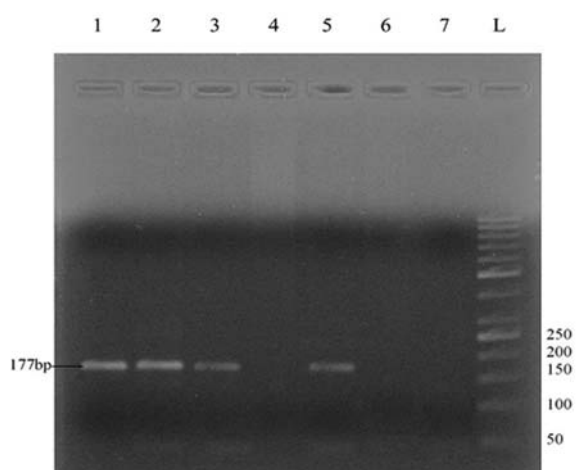MSI which caused by mutations such as insertions or deletions in microsatellite repeats is

Fig. 1. Detection of the p53 codon 72 polymorphism by PCR amplification. The PCR products were electrophoresed on 1% agarose gel.

Lane 1, 2, 3, 5: Positive for Pro Allele (177bp band);

Lane 4, 6: Negative for Arg Allele,

Lane 7: Negative Control,

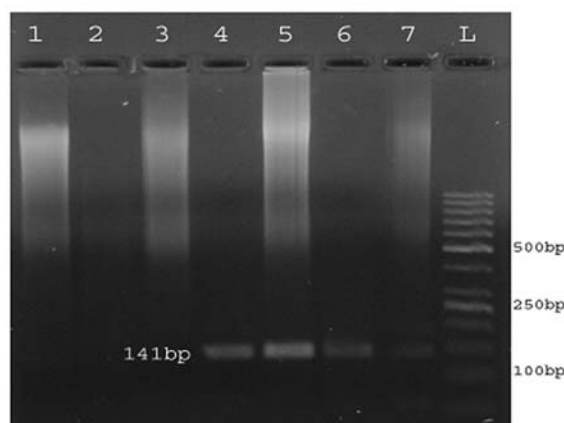L: Gene ruler TM DNA Ladder. The sizes of the bands are indicated beside the gel photo.

Fig. 2. Detection of the p53 codon 72 polymorphism by PCR amplification. The PCR products were electrophoresed on 1% agarose gel.

Lane 4, 5, 6, 7: Positive for Arg Allele (141bp band),

Lane 2, 3: Negative for Arg Allele

Lane 1: Negative control

L: Gene ruler TM DNA Ladder. The sizes of the bands are indicated beside the gel photo.

resulted from a failure of the MMR system to edit errors made during DNA replication [2]. The MMR genes play a major role in the correction of DNA damage. Mutations in cell cycle control and MMR genes disable cells to repair damaged caused by genetic alterations. Loss of MMR proteins apparently is an important event in the process of carcinogenesis or in a primary step of tumor progression in certain-type of cancer [22]. A defective MMR system increases the rate of replicative errors throughout the genome of cancer cell, and there are evidences that some tumors with apparent MMR defects may also have MSI 23. Also, loss of MMR may result in loss of cell cycle control and/or resistance to apoptosis, both of which promote neoplastic transformation [24]. Therefore, detection of MSI, a functional marker of MMR defects, might be useful for determination of these defects. The MMR system is inactivated either by hypermethylation of the promoter, or because of germ-like mutations in MMR genes [2]. Determining whether particular cancers have MSI may have significant biological and
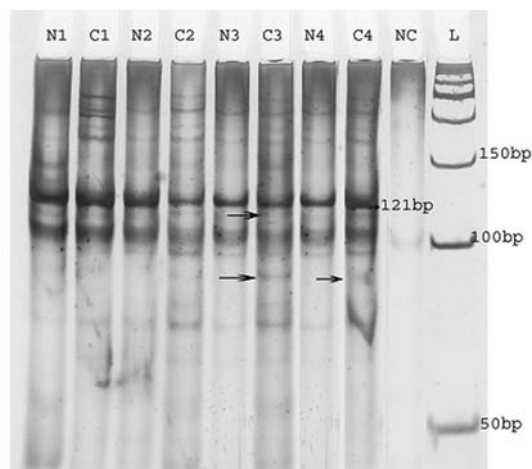

Fig. 3. Detection of the microsatellite instability by PCR amplification of BAT26. The PCR products were electrophoresed on 15% acrylamide gel and were stained with silver nitrate staining:

N: Normal Tissue

C: Cancer Tissue

NC: Negative Control

L: Gene ruler TM DNA Ladder. The sizes of the bands are indicated beside the gel photo.

In lane C3 two shifted band and in lane C4 one shifted band were shown microsatellite instability (arrow head).

clinical importance. First, recognition of MSI could help understanding of the pathogenesis and potentially in planning for prevention of cancer. Second, MSI may be of prognostic importance and may also be predictive of tumor responsiveness to certain chemotherapeutic drugs, such as alkylating agents. And finally, the ability to detect MSI-positive cells in clinical samples may be a useful diagnostic tool for recognizing rare cancer cells in these samples [25]. Many repeated sequences have the potential to demonstrate instability. Thus an unequivocal indicator which demonstrates instability regions has not been described yet. There are several studies in the literature investigating MSI in breast carcinoma at different loci by using different microsatellite markers. However, all studies comprise low numbers of patients and the frequency of alterations are quite low for most satellites. Thus, using a large number of microsatellites does not necessarily contribute to the diagnostic accuracy. In most MSI positive tumors, the 26 deoxyadenosines in the BAT-26 poly(A) tract in the intron 5 of MSH2 gene are typically shortened by 4-16 bp in length [26]. It has been showed that silver-stained SSCP gels with only 8 cm in length can readily detect deletions of as little as 3 bp in BAT-26. Together with the observation that screening for BAT-26 deletion is more than 99% accurate for the identification of MSI status [10], the reported frequency of BAT-26 polymorphisms in pure Caucasian populations (0.08%) is sufficiently low [27]. False positives though, should not to be a concern in the routine analysis of MSI. The BAT-26 appears to undergo significant deletions in the large majority of tumors with MSI, as demonstrated by comparison with alterations to several different microsatellite loci. The hypersusceptibility of BAT-26 to deletion in tumors with the MSI phenotype obviates the need to examine a panel of five different microsatellite loci as was proposed [10].

The exon four of Tp53 is one of the largest exons of this gene, lies close to the central region, and is important for DNA-specific binding [29]. A common p53 polymorphism, encoding either Pro or Arg at residue 72, produces characteristic change in the structure of p53. This polymorphism also lies in a part of p53 that is involved in the induction of apoptosis [30].

In this study we first investigated the genotype frequencies of TP53 codon 72 in 120 invasive breast ductal carcinomas. Then we analysed the MSI in three genotypes of this codon. MSI analysis revealed that 29 patients (24.2%) were MSI-positive and 91 patients (75.8%) were MSI- negative. There is a considerable variation in the frequencies of MSI reported in breast carcinomas .Such variability may depend on the series of tumors analyzed, microsatellites examined, and the number of altered markers used to define MSI. In contrast, no MSI was found by other authors using an extended group of markers and analyzing breast cancer tumors and cell lines [25]. Such variability suggest that MSI appear to play a role only in the pathogenesis of some breast cancer types.

In this study we showed also a significant association between the p53 codon 72 variation and presence of MSI in the invasive breast ductal carcinomas. MSI was more frequently observed in tumors arising in Arg/ pro genotype. These findings supported the notion of a cooperation between p53 and the MMR system and indicated that heterozygosity for p53 codon 72 may increases genomic instability at the nucleotide level. Three possible explanations may account for the p53 dependent increase in MSI. First, heterozygosity of p53 codon 72 may reduce genomic instability at the nucleotide level by mediating repair, either directly or indirectly. This is supported by the observation that p53 is capable of attach to insertion/deletion loops, the DNA lesion associated with MSI [31]. A second possibility is that the Arg72 allele is preferentially mutated and retained in various human tumors arising in Arg / Pro heterozygotes, and that the p53 mutant plays as a more

potent inhibitor of p73 as a member of p53 family which has apoptotic function when p53 has Arg72 rather than Pro72 [32] . These findings suggest that this polymorphism acts as an intragenic modifier of mutant p53 behavior and has an effect on the biological role of p53. Third possibility is that p53 heterozygotes may leads to the upregulation of MMR defects. In this study we did not consider MMR defects in relation to detection of p53 genotypes or MSI and it is thus an important issue for future studies.

At present, the significance of the p53 codon 72 polymorphism remains obscure, both in terms of cancer epidemiology and pathobiology. Additional comprehensive studies using a spectrum of excised human carcinoma tissue samples from greater numbers of tumors will be needed to elucidate the association between polymorphic residue within p53 and microsatellite behavior of MMR in human carcinogenesis.

Conclusion

Our findings suggested that breast invasive ductal carcinomas arising in individuals with p53 codon 72 heterozygosity (Arginine/ Proline) may be preferentially prone to microsatellite instability more than other genotypes.

Acknowledgement

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