RESEARCH ARTICLE

Detection of p53 Common Intron Polymorphisms in Patients with Gastritis Lesions from Iran

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Abstract

Background: p53 alterations have been implicated in the development of many cancers, such as gastric cancer, but there is no evidence of p53 intron alterations in gastritis lesions. The aim of this study was to investigate the p53 intron alterations in gastritis along with p53 and mismatch repair protein expression and microsatellite status. Materials and Methods: PCR-sequencing was conducted for introns 2-7 on DNA extracted from 97 paired samples of gastritis lesions and normal adjacent tissue. Abnormal accumulation of p53 and mismatch repair proteins was investigated using immunohistochemistry. In addition, microsatellite status was evaluated with reference to five mononucleotide markers. Results: Gastritis cases included 41 males and 56 females in the age range of 15-83 years, 87.6% being *H.pylori* positive. IVS2+38, IVS3ins16 and IVS7+72 were the most polymorphic sites. Their minor allele frequency values were as follows: 0.38, 0.21 and 0.06, respectively. Samples with GG genotype at IVS2+38 and CT at IVS7+72 had no insertion. Moreover, most of the stable samples (91.9 %) had a G allele at IVS2+38. All of the samples were IHC negative for p53 protein, microsatellite stable and expressed mismatch repair proteins. p53 alterations were prominent in the H. Pylori+ group, but without statistical significance. Conclusions: According to our results, some p53 polymorphisms such as IVS2+38, IVS3ins16 and IVS7+72, because of their correlations together or with microsatellite status may contribute to gastritis development. However, so far effects on p53 expression and function remain unclear. Therefore, a comprehensive survey is needed to delineate their biological significance.

Keywords: *Helicobacter pylori* - gastritis - p53 gene - intron - polymorphism - gastric cancer

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Introduction

p53 gene alterations appear to be key factors in the development of gastric cancer (Fenoglio et al., 2003; Whibley et al., 2009). The human p53 gene is located on the chromosome 17, coding for a protein of about 53 kDa composed of 393 amino acids (Bai et al., 2006). p53 is a DNA-binding protein with transcription regulatory activities and as a tumor suppressor gene is essential for preventing aberrant cell proliferation and maintaining genome integrity following genotoxic stress (Brusa et al., 2003; Brueckl et al., 2004). Following various intra and extracellular stimuli, such as DNA damage or hypoxia, wild type p53 is activated and emerges as a pivotal regulatory protein which triggers G_1/S arrest through induction of $p21^{cip1/kip1}$ protein which per se binds to and inhibits CDK2 (cyclin dependent kinase 2) from an association with cycling E. By the way p53 induces programmed cell death (apoptosis) in some cell types (Carstens et al., 2004).

Introns are integral elements of eukaryotic genomes that perform various important functions such as alternative splicing and also actively participate in gene regulation and evolution (Furihata et al., 2002; Xinarianos et al., 2002).

Accurate RNA splicing requires the absence of mutations in the cis-acting consensus elements known to be involved in RNA splicing i.e., the conserved sequence at the intron-exon junctions and the branch point (Sogame et al., 2003; Thongsuksai et al., 2010). Intron point mutations can lead to aberrant mRNA splicing which result in the production of a truncated (if no) protein, representing an alternative mechanism for inactivation.

The intron sequences in the p53 have been implicated in the regulation of gene expression and in DNA protein interactions through putative sequences for binding. For example, a sequence in the intron 4 is recognized by p53 intron 4-binding protein and some other consensus sequences which are recognized by transcription factors such as Sp1 (Smith et al., 1996) and through which introns

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can modulate p53 expression.

In Iran, gastric cancer still is a fatal disease and prevalent with about 7,300 new cases every year (Mehrabian et al., 2010) in spite of dramatic decrease of its incidence in most of the western and Japanese populations (Malekzadeh et al., 2009). Gastric cancer is a multi-factorial disease and develops as a result of continuous cell damage caused by exposure to different carcinogens (Malekzadeh et al., 2009). According to Correa's cascade (Correa, 1988) gastritis is a precancer lesion toward gastric cancer. In this model of carcinogenicity, accumulation of genetic and epigenetic abnormalities, enable precancerous lesions such as gastritis to grow into neoplasm and ultimately gastric cancer. Therefore, evaluation of molecular events during gastritis development will be useful in the early detection of patients prone to gastric cancer and subsequently prevention from it become more severe.

Several genetic events such as mutations or amplification of proto-oncogene as well as allelic deletions of tumor suppressor genes have been described in this sequence of premalignant changes (Boussioutas et al., 2003), but their exact level /order in which they work is still unclear.

p53 has an important role in genomic stability. Therefore, it seems necessary to examine a marker showing genomic stability such as microsatellite status. Microsatellite instability (MSI) is a genome-wide alteration characterized by a global instability of repetitive microsatellite sequences (Wang-Gohrke et al., 2002). Hence, we decided to evaluate p53 and the Microsatellite status together to find additional insights into their potential effect on gastritis development and probable interplay among these processes during the gastritis genesis.

Regarding the importance of p53 as an essential brake in cell cycle progression and also the critical role of intron regions for normal translation of proteins, clearly disruptions of p53 function through intron alterations may have a salient effect on the integrity of cells and confer a selective advantage for the tumor cells.

To our knowledge, there is only one report about the intron alterations of p53 in familial gastric cancer on a small Japanese population (Yamada et al., 2007) and no information about gastritis, so the purpose of this study was to characterize p53 intron variations in order to elucidate its correlation with clinicopathological aspects of gastritis lesion.

Materials and Methods

Patients

This study was approved by the ethics and scientific committee of our institution. The patients were informed about the aims of this study and considered competent to make the decision as voluntary. Two sets of samples (for histological examination according to the update Sydney classification and for DNA extraction) were taken from a gastritis lesion and normal endoscopic appearance from each patient who had undergone endoscopic evaluation of upper gastrointestinal tract in the Taleghani hospital (Tehran-Iran). Patients with present or previous neoplastic disease, previous gastric surgery, and gastric or duodenal ulcers were excluded.

DNA extraction

The DNA from gastric biopsies was extracted using DNeasy kit and QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions.

PCR amplification and mutation analysis using direct sequencing

PCR-sequencing carried out for evaluation of p53 introns 2-7 using primers spanning intron splicing site as follow: 5' TCTCAGACACTGGCATGGTG 3' and 5' GGCAAGGGGGACTGTAGATG 3'for introns 2 and 3, 5' CTAGCAGAGACCTGTGGGAAG 3' and 5' CACTGACAGGAAGCCAAAGG 3' for introns 3 and 4, 5' TTGTTTCTTTGCTGCCGTC 3' and 5' CCCCCTACTGCTCACCTGG 3' for introns 4-6, 5' GCGACAGAGCGAGATTCC 3' and 5' CTGAGTGGGAGCAGTAAGGAG 3' for introns 6 and 7.

PCR was performed in the reaction containing 1x PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM of each dNTP and 0.5 U Taq polymerase. The PCR program was as follows for intron 2 and 3: An initial cycle of 5 min at 94°C and then 30 cycles of 94°C for 30s, 62.1°C for 30 s, 72°C for 45 s that was concluded by 10 min at 72°C for the final extension. For other introns PCR program was the same with that of intron 2 and 3 with the exception of annealing temperature: 59.5°C, 63 °C, 62.1°C for intron 4, 5 and 6 and 7 respectively. Sequencing was done using ABI 3130xl Genetic Analyzer.

Microsatellite instability analysis

DNA extracted from gastritis tissue and blood was analyzed for MSI using five microsatellite markers: NR-27, NR-21, NR-24, BAT-25 and BAT-26. Briefly PCR products of foregoing markers were analyzed (fragment analysis) using ABI 3130xl Genetic Analyzer. Fragment analysis of the PCR products allowed determination of either expansions or reductions of the microsatellite repeats. The samples were classified as MSI-high, if ≥ two markers demonstrating instability, or MSI-low, when only one marker demonstrated instability (Buhard et al., 2004).

Immunohistochemical analysis of mismatch repair enzymes and p53 protein

Immunohistochemical (IHC) staining for products of mismatch repair (MMR) genes, MLH1, MSH2 and MSH6 was done according to previously described method (Molaei et al., 2010). Intramucosal lymphocytes were used as positive controls. Indeed, IHC for p53 protein was done as previously described (Najjar et al., 2011) using monoclonal antibody against p53 (clone DO-7, DAKO A/S, Denmark) which detected both the wild and mutant types.

Statistical analysis

SPSS13 software (chi-square test, fisher exact test

and ANOVA) was used to evaluate the association of p53 nucleotide alterations and clinicopathological findings, MSI and IHC. For All tests the significance level was set at 5%.

Results

Findings about patients

Histological examination confirmed 97 paired samples (gastritis and normal) were included in the study, 41 male (42.3%, mean age: 44.5±17.) and 56 female (57.7%, mean age: 42.6±15.) in the age range of 15-83 years. Gastritis tissues were classified by our pathologists as follow: 33 patients with moderate active chronic gastritis, 39 patients with moderate chronic gastritis, 21 patients with severe active chronic gastritis and four patients with severe chronic gastritis.

DNA sequencing

According to our study nucleotide changes was seen in intron regions as follow: IVS2+38C>G (rs1642785), IVS3+40-41ins16 (ACCTGGAGGGCTGGGG, rs17878362), IVS3-29C>A (rs1788332), IVS6+31A>G (rs34949160), IVS7+72C>T (rs1294778), IVS7+92T>G (rs129510) (Table 1).

IVS2+38 (Figure 1), IVS3+40-41ins16 (Figure 2) and IVS7+72 C>T (Figure 3) were more polymorphic than other, their minor allele frequency was as follow 0.38, 0.21 and 0.06 respectively (Table 1).

According to our study 66% of patients had no insertion (Figure 2C) and 7.2% had homozygous insertion (Figure 2A) while the others (26.8%) were heterozygous (Figure 2B).

31 gastritis samples together with normal adjacent tissue have ancestral alleles, 10 male and 21 female (p 0.389).

There was a significant association between IVS3+40-41ins16 and IVS2+38 (p<0.001) and IVS2+38 (0.032). Samples with GG genotype at IVS2+38 and CT at IVS7+72 had no insertion. Also, samples with CC at IVS7+72 had TT alleles at IVS7+92 (p<0.001) and AA at IVS6+31 (p 0.009). Polymorphism at IVS2+38 was in association with IVS3-29 and IVS6+31 (p 0.003 and 0.007 respectively)

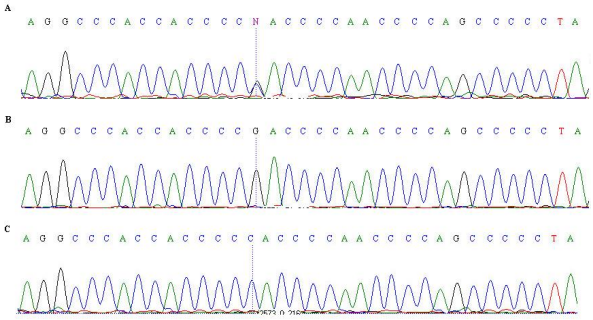

Figure 1. DNA Sequence of Intron 2 of p53 Gene showing IVS2+38 C>G Alteration. A) Sequence with heterozygout polymorphism B) homozygous GG and C) Homozygous CC. N shows the site of alteration

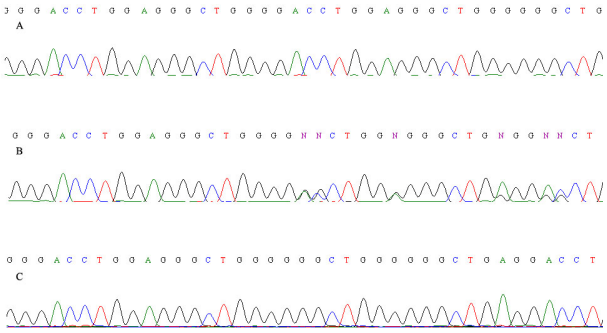

Figure 2. Sequencing Data Showing the 16 bp Insertion, the Starting Nucleotide was Shown NN. A) Sequence with no insertion B) Heterozygous insertion C) Homozygous insertion

Table 1. Types and the Frequencies of Nucleotide Changes in Intron 2-7 of p53 in Gastritis Lesion

Intron position	IVS2+38	IVS3,40-41ins16 Homo /hetro	IVS3,40-41ins16	IVS3-29	IVS6+31	IVS7+72	IVS7+92
RS	1642785	17878362		17883323	34949160	12947788	12951053
Type of alteration	C>G	Insertion		C>A	A>G	C>T	T>G
Frequency	GG:33(34.0)	Without insertion	64(66.0)	CC:90(92.7)	AA:96(99)	CC:81(87.1)	TT:81(94.2)
	CC: 9(9.3)	Heterozygous insertion	26(26.8)	CA:7(7.3)	AG:1(1.0)	CT:12(12.9)	GG:1(1.2)
	CG:55(56.7)	Homozygous insertion	7(7.2)				GT:4(4.7)
Allele frequency	G: 0.62		0.79	C:0.96	A:0.99	C:0.94	G:0.97
	C: 0.38		0.21	A:0.04	G:0.01	T:0.06	T:0.03

*Frequencies of the alterations were reported as number (%)

Table 2. Statistical Correlation between the Frequency of p53 Alterations in *H.Pylori* Positive and Negative Groups (chi ^{2} test)

		IVS2.38			Insertion			IVS 3.29		IVS 6.31		IVS 7.72		IVS 7.92		
		CC	CG	GG	Ye	No	Hetero	CC	CA	AA	AG	CC	CT	TT	GG	GT
HP positive	N	9	50	26	6	55	24	78	7	84	1	71	10	77	1	4
	%	10.6	58.8	30.6	7.1	64.7	28.2	91.8	8.2	98.8	1.2	87.7	12.3	93.9	1.2	4.9
HP negative	N	0	5	7	1	9	2	12	0	12	0	11	1	11	0	0
	%	0	41.7	58.3	8.3	75	16.7	100	0	100	0	91.7	8.3	100	0	0
P value			0.123			0.699			0.384		0.876		0.569		0.702	

*The number of patients was reported in the rows 2 and 4 and the percentage in rows 3 and 5 respectively for HP positive and HP negative. The third row contains p-value for difference between HP positive and negative groups

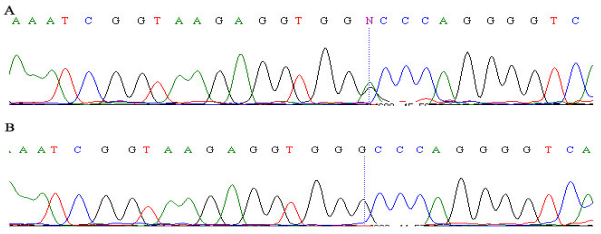

Figure 3. DNA Sequence of Intron 7 of p53 Gene (IVS7+72 C>T), the Sequence was Read in Reverse Direction. A) Heterozygous CT B) homozygous CC. N shows the site of alteration

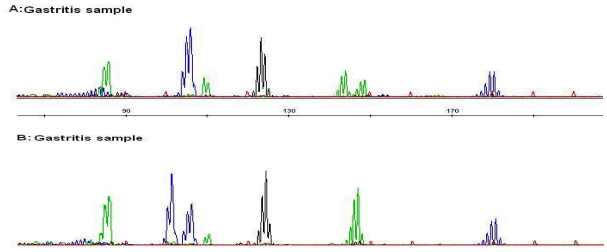

Figure 4. Electropherogram Profiles of BAT-26, BAT-25, NR-24, NR-21, and NR-27 in DNA from Gastritis lesion. Polymorphism of NR-21 marker (A) BAT-25 marker (B). Similar picks were seen in the normal tissues (not shown)

MSI and IHC analysis of p53 and mismatch repair genes expression

Nuclear staining was not seen in gastritis and normal adjacent tissues. Gastritis samples have stable microsatellite, but nine patients had polymorphism in normal DNA with the variant allele for NR-21 (Figure 4A) and one for Bat-25 (Figure 4B). The samples with polymorphic allele for NR-21 were mutated, but the sample with Bat-25 variant had no alteration. IHC showed expression of MMR proteins in the all of the samples.

Most of the stable samples (91.9 %) had allele G at IVS2+38 (p 0.032). Also, most of the stable samples had not insertion and this association was statistically significant (p 0.008).

p53 alterations and clinicopathological findings

According to specific staining for *H.Pylori* (HP), 85 samples (87.6%) were positive and the other 12 (12.4%) samples were negative. p53 alterations were more prominent in the HP^+ group than the HP^- group, but this difference was not statistically significant (Table 2). Also, statistically there was no association between p53 intron variations and degree of inflammation (activity), age and gender.

Discussion

Gastric cancer is the fourth most common cancer in Iran with the 5-year survival rate of 23.6%, and the median life expectancy of 19.9 months (Zeraati et al., 2005). Therefore it seems necessary to investigate the degree to which p53 gene polymorphisms contribute to the pathogenesis of gastritis. Early detection of p53 alterations (and other genes involved in tumorigenesis) in precancerous lesions such as gastritis may be useful for the early detection of patients prone for gastric cancer and prevention of gastric cancer. Also, such studies will promote our understanding about the role of the p53 gene in the natural history of gastric carcinogens.

The entire profile of the alterations of p53 gene in gastritis and gastric cancer has not been fully detailed. Most of the previous studies examined only p53 exonic regions and there is only one report on intron alteration of p53 in gastric cancer (Yamada et al., 2007). In the foregoing study IVS3Ins16, IVS2+38C>G, IVS3-29C>A, IVS7+72C>T, IVS7+92T>G were reported from Japan in search for novel germ line p53 mutation in familial gastric cancer (Yamada et al., 2007).

Intron variants may affect mRNA splicing (Davis et al., 2009), gene regulation (Shamsher et al., 2000) and DNA protein interactions especially binding of transcription factors (Smith et al., 1996). Therefore, genetic changes within the non coding regions may serve as an alternative mechanism for p53 inactivation or weakening its performances which may result in gastritis lesions.

The 16 bp insertion in intron 3 of p53 (p53Ins3) found to be associated with increased risk of several cancers such as colorectal (Gemignani et al., 2003), lung (Wu, et al., 2002), breast (Wang-Gohrke et al., 2002; Koshiol et al., 2009), cervical (Koshiol et al., 2009) and ovary cancer (Angelopoulou et al., 1998; Wang-Gohrke et al., 1999). According to our study most of patients had no insertion while the other was prominently heterozygous. However, this alteration was not associated with clinicopathological findings of the current study. Exon and intron 3 of p53 are only 112 base pair and maybe an increase of 16 base pair in the length alters mRNA splicing and expression, thus affecting p53 functions. Previous study using a couple of algorithms and *in silico* analyses did not predict any splicing site in this region and therefore differential splicing of the pre-mRNA, but the basal level of p53 mRNA decreased in the cell lines having insertion (both hetero and homozygous) in compare with normal allele (Gemignani et al., 2003).

Therefore, future studies would be required to delineate the consequence of this variation on p53 function and its correlation with molecular and clinical processes during gastritis development toward gastric cancer.

There is no information about the importance of IVS2+38 polymorphism during the development of gastritis lesions. According to our finding IVS2+38 polymorphism was significantly associated with polymorphisms at IVS3-29 and IVS6+31. Currently, we have no explanation about the probable cause and consequence of this correlation. However, previous work on the cervical cancer revealed that this polymorphism is associated with increased risk of cervical intraepithelial neoplasia/HPV persistence (Koshiol et al., 2009). Also, significant differences were found among the distributions of the genotypes in blood samples compared to the corresponding ovarian cancer tissue (Maunakea et al. 2010).

To our knowledge this is the first report about the frequency of IVS+72 variations in gastritis lesion. This polymorphism may be a risk factor for oral neoplasms (Li et al., 2005). It deserves to do a comprehensive study about its role during gastric cancer development (from gastritis

toward gastric cancer) and its effect on p53 stability, function and mRNA expression.

Samples with GG allele at IVS2+38 and CT at IVS7+72 have no insertion at IVS3Ins16. Also, samples with CC at IVS7+72 had TT allele at IVS7+92 and AA at IVS6+31. These findings show that these alleles exist simultaneously and belong with the same allelotype. Maybe some condition(s) mediates the occurrence of these alleles together or even co–occurrence of these alleles confers an important characteristic to the gastritis.

Despite some sequence variations in p53 intron regions, no nuclear staining for p53 protein was seen in gastritis and normal adjacent tissues. Generally (not always) the presence of immunoreactive p53 indicates mutant p53 protein that is more stable than wild type and therefore it accumulates in the nucleus. Apparently, the polymorphic changes that we've seen in the intron regions have little (if no) effect on protein expression. Maybe these alterations (which reside in the sits not important for splicing) result in the protein with the same stability as wild type and therefore in negative IHC as seen in the previous work (Shiao et al., 1994; Najjar et al., 2011). According to other work, precancerous lesions such as gastritis, intestinal metaplasia (IM) and dysplasia, p53 protein was expressed at low levels (if no) while 33-43.5% of cancer tissues had overexpressed p53 (Romiti et al., 1998; Li et al., 2005). Apparently detection of p53 protein accumulation by IHC is first seen in IM or dysplasia (Romiti et al., 1998; Li et al., 2005) and to some extent, depend on the type of genetic alterations.

In line with former findings about infrequency of MSI in gastritis lesion (Kashiwagi et al., 2000; Kim et al., 2002; Li et al., 2005), the status of microsatellite in gastritis lesion was stable and few samples had variant alleles. The probable explain, is that MSI arises from loss of mismatch repair system whiles our samples express MMR proteins. Maybe MMR defects and subsequent MSI happen later during malignant transformation of gastric mucosa. For example, MSI was reported up to 9.3% for intestinal metaplasia and 26.7% for gastric carcinoma (Hamamoto et al., 1997; Leung et al., 2000; Kim et al., 2002).

Considering the significant association between allele G at IVS+38 and microsatellite stability, it could be concluded that p53 gene with these alleles confer stabilizing effect on the genome more than other alleles.

In line with previous work on p53 exon alterations (Murakami et al., 1999; Najjar et al., 2011), p53 alterations were prominent in HP^+ group than HP^- group. Apparently the HP-related inflammatory processes lead to high levels of nitric oxide and other inflammatory compounds such as reactive oxygen species which interact selectively with genomic DNA and result in p53 and other genes' alterations (Higashimoto et al., 2000).

In this study most of our samples were stable microsatellite and altered p53. This finding is in line with other studies which state p53 gene alterations appeared to be rarely accompanied with MSI (Yamamoto et al., 1999).

We could not find any correlation between p53 polymorphisms and gastritis clinicopathological aspects, perhaps the most important polymorphisms are those that alter protein function through changing its structure, not alterations in the site beyond splicing-important sites as we detect. However some p53 polymorphisms such as IVS2+38, IVS3ins16 and IVS7+72, because of their correlation with together or with microsatellite status, may contribute in the gastritis development. More studies on these polymorphisms in the next steps toward gastric cancer opens new windows for better insight about the molecular basis of gastric cancer.

To our knowledge this is the first comprehensive study on gastritis lesion regarding the size of population and molecular evaluations such as sequencing of p53 introns 2-7, microsatellite status, p53 and MMR proteins expression. In overall we found some variations in the introns, so far their functional effects have never been reported and their significance during RNA splicing and DNA protein interactions remain to be elucidated. Also, it can be concluded that the effective p53 alterations are more frequent in the next step and further genetic and epidemiological studies of this p53-positive gastritis with intron variations is needed to shed light on this precursor lesion of gastric cancer.

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