P53- The Molecular Guardian Crashes in Gastric Adenocarcinomas - A Study in an Ethnic Kashmiri Population

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Abstract

Genetic instability underlies the etiology of multistep gastric carcinogenesis. The p53 mutations observed in tumors represent the expression of such instability by allowing the accumulation of genetic alterations caused by multiple mechanisms. The present study was conducted to investigate the nature and frequency of TP53 mutations in patients with gastric adenocarcinomas of Kashmir valley. Tumor samples from 30 patients with primary gastric adenocarcinomas undergoing radical gastrectomy were evaluated. The mutational status of the p53 (exons 5 to 8) was screened by PCR-SSCP analysis followed by direct sequencing. Of all 30 gastric adenocarcinomas including ten intestinal types and twenty diffuse types, 20% patients (6/30) harbored mutations in the p53 gene. Overall, twenty-one mutations were found in TP53 in 30 patients included in this study. Mutations were found at codon 142 (3 cases) of exon 5, codon 144 (1 case) in exon 5, codon 147 (1 case) in exon 5, codon 157 (1 case) in exon 5, codon 169 (2 cases) in exon 5, codon 170 (3 cases) in exon 5, codon 172 (1 case) in exon 5, codon 173 (3 cases) in exon 5, codon 179 (3 cases) in exon 5, codon 180 (1 case) in exon 5, codon 213 (1 case) in exon 6, the insertional mutation was between codon 216 & 217 (1 case) in exon 6 and codon 267 in exon 8 (1 case). The mutation pattern comprised of 12 insertions, 6 substitutions (all transversions) and 3 deletions. All the twelve insertions represented frame-shift mutations. The six single-base substitutions leading to aminoacid substitution included four missense mutations and a single silent mutation. The mutation effect was found to be significant (p<0.05). This study exhibited significant amount of mutation in exon 5 (OR=90.25 and p<0.05 within the CI of 12.47-652.89) of TP53 in the gastric adenocarcinoma patients from Kashmir valley. Comparison of mutation profile with other ethnic populations and regions reflected different differences and similarities indicating co-exposure to a unique set of risk factors. The differences could be due to exposure to explicit environmental carcinogens, different lifestyle, dietary or cultural practices of Kashmiris being an ethnic population that need further investigations. The direct sequencing results, therefore, shall help in understanding the molecular events associated with progression and metastasis in gastric carcinoma.


Keywords: Gastric cancer; p53; Kashmir; India

Abbreviations: PCR: Polymerase Chain Reaction; SSCP: Single Strand Conformational Polymorphism

Introduction

Gastric cancer is the 2nd most common tumor in the world which represents bulk of global cancer burden. It has a very poor prognosis and is the second most common cause of death from cancer worldwide. This is attributable to its bleak 5 year survival rate, and is the second most common cause of death from cancer representing bulk of global cancer burden. It has a very poor prognosis has

show a significant decrease in gastric cancer occurrence in their off-springs, suggesting that the cause is related to environmental factor starting early in life [13-15].

Demographically, Kashmir is one of the three provinces of the Jammu and Kashmir State in north India, situated at an altitude of 1800-2400 m above sea level. It comprises of a non-migrant population who have special social, personal and dietary habits that endowed this population with a common ethnic origin. The exact prevalence in Jammu & Kashmir is not known since whatever little work has been done on gastro-esophageal cancers in this part of the world was hospital-based and no population-based epidemiologic studies have been undertaken. In Kashmir, the clinical experiences have revealed a very high prevalence of gastric cancer, although

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there is scarcity of data available at present in this regard. The age
standardized incidence rates for gastric cancer were 36.7/100,000
per year among men & 9.9/100,000 per annum in the women [16].
These figures were three to six times higher than those recorded
by cancer registries in Bangalore, Madras and Bombay. Thus, among
north Indian states Kashmir has a high incidence of gastric cancers.

Multiple genetic alterations underlie the multi-step progression
to cancer. Genomic instability induced by these genetic alterations
that determine replication errors could contribute to the inactivation
of tumor suppressor genes and increase the mutation rate. The
identification of a growing number of genes, primarily tumor
suppressor genes contribute to the development and progression of
human solid tumors [17-20]. Structural alterations of the p53 product
have been frequently detected in a wide variety of human tumors
[21,22]. The recessive or dominant negative mutations instigate the
loss of p53 function, which can affect the DNA-binding domain of
the protein (exons 5 to 9), as well as the interaction with other cellular
or viral oncoproteins [23,24]. The wild-type p53 protein exerts
pleiotropic effects through the transcriptional activation of different
target genes that control important checkpoints in the modulation
of cell cycle progression [25-30]. The accumulation of wild-type p53
protein results in two pathways: Cell cycle arrest and programmed
cell death, which are mutually involved in tumor suppressor functions
[31]. TP53 induces a transient suppression of the cellular growth at
the G1/S checkpoint [32] and causes an irreversible induction of
the pathways leading to p53-dependent programmed cell death [33,34]
and DNA repair. Therefore, p53 mutations lead to disruption of these
pathways conferring a selective growth advantage for tumor cells
resulting in increased proliferation activity and tumor development
[35,36]. Besides, mutated p53-bearing cells have altered controls
through the cell cycle progression and prevent apoptosis. Thus, may
play a role in the mechanisms of resistance to chemotherapeutic
agents [37-40].

According to the classification by Lauren [41], stomach
cancer is classified into two main histological types: diffuse and
intestinal. Several studies in the last decade [42-44] point to precise
combinations of genetic and epigenetic alterations that differ in both
subtypes, although a few of them appear to be common as well. The
most evident genetic changes found in both types of gastric cancers
include loss of heterozygosity, hypermethylation of several genes
in addition to mutational abnormalities of p53 tumor suppressor
gene [44-47]. These alterations are also frequently observed in
pre-cancerous lesions such as intestinal metaplasia and dysplasia,
which are precursors of the intestinal type of gastric cancer [45,48].
Most mutations of p53 gene or genetic and/or epigenetic changes
of upstream and/or downstream located genes in the p53 network
result in a loss of function of the wild-type gene product. However,
most but not all mutant p53 proteins have a prolonged half-life and
accumulate in cells [49,50]. Both p53 accumulation and its absence
in the nucleus of malignant cells could thus be used as a valuable
prognostic marker and predictor of clinical outcome of gastric
tumors [45,50-52].

The present study is aimed at evaluation of involvement of
TP53 gene mutation in incidence and development of gastric cancer
in patients from Kashmir valley. This study also addresses the
documentation of the data, the first on gastric cancers from this part
of the world. Further, to analyze the differences and similarities in
the mutation profiles from various regions, a comparison was made
between the mutation patterns of TP53 obtained in present study
and the data compiled at International Agency for Research on Cancer
(IARC) TP53 data base (http://www.iarc.fr/p53/homepage.htm) [57].

Material and Methods

Patients and samples

Patients attending Gastroenterology and Surgery Departments at
Sheri-I-Kashmir Institute of Medical Sciences, Srinagar, Jammu &
Kashmir, India, between July 2008 and July 2009 for gastric cancer
management were recruited for the study, with prior informed
consent. Patient underwent endoscopy and histopathological
examination to establish the clinical profile. Tumor samples from
patients with primary gastric adenocarcinomas undergoing radical
gastroctomy were evaluated. A total of 30 gastric cancer cases under
study included surgically resected gastric tissue (which incorporated
tumor tissue, normal tissue and lymph nodes wherever involved)
was collected from these sporadic gastric cancer cases. All samples
were snap frozen at -70°C until analysis. A questionnaire was used
to collect information on clinico-epidemiological characteristics of
the patients including age, family history of the disease, body mass,
provisional diagnosis, lymph nodes involved, clinical tumor stage and
histopathological grade of tumor.

DNA isolation, PCR-SSCP analysis and sequencing

High-molecular-weight DNA was isolated from single cell
suspension of blood and tissue samples of gastric cancer patients by
Proteinase-K digestion and phenol-chloroform extraction [53].

PCR amplification using 4 set of primer pairs (Table 1) were used
to amplify exons 5 through 8 (DNA binding domain) of TP53. PCR
was carried out in MJ Research Minicycler at respective annealing
temperature (Table 1) using standard protocol. SSCP analysis of PCR
product was carried out on 6% non-denaturing Polyacrylamide gel
(PAG) utilizing either non-radioactive silver staining or radioactive
procedures [54-56]. In non-radioactive SSCP analysis [55], PCR
products mixed in denaturing buffer (95% formamide, 10mM NaOH,
0.05% xylene-cyanol FF and 0.05% Bromphenol blue) in 1:1 ratio were
heat denatured at 95°C for 5 minutes, immediately cooled on ice for
20min, 6 µl of which were loaded on 6% PAG and electrophoresed
in 0.5X Tris-borate EDTA buffer at ±17°C at 4W constant power for

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon</th>
<th>Nucleotide positions in genomic DNA</th>
<th>Primer sequence*</th>
<th>Annealing Temperature (°C)</th>
<th>Product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Exon5</td>
<td>13005-13024 13295-13314</td>
<td>1)TGTTCACTTTG 2)AGAGGAGGATGAGGAACAG</td>
<td>55</td>
<td>310</td>
</tr>
<tr>
<td>p53</td>
<td>Exon6</td>
<td>13271-13280 13475-13494</td>
<td>1)TGTTGGCCAGGAGGTCCTCACAG 2)TGAGGAGGACCTGACCAACCA</td>
<td>62</td>
<td>224</td>
</tr>
<tr>
<td>p53</td>
<td>Exon7</td>
<td>13941-13960 14158-14177</td>
<td>1)CTGGTCACAGG 2)AGGGGTGCGGCGGGAACCCAG</td>
<td>62</td>
<td>237</td>
</tr>
<tr>
<td>p53</td>
<td>Exon8</td>
<td>14442-14461 14579-14598</td>
<td>1)TGCTCAGTAAGTGTAACTCA 2)GCTGTGTCCTACCTGCCTAGT</td>
<td>58</td>
<td>156</td>
</tr>
</tbody>
</table>

*1) Sense primer  2) Antisense primer

Table 1: Primers used for screening different exons of TP53.
18-22h. Gels were then silver stained. In radioactive SSCP analysis, radiolabelled PCR products (using α-32-pCTP) mixed in denaturing loading buffer (95% formamide, 20mM EDTA, 0.05% xylene-cyanol FF and 0.05% Bromophenol blue) in 1:10 ratio were heat denatured at 95°C for 5 minutes, 3µl of which were loaded on 5% PAGE and electrophoresed at 200V in 0.5X Tris-borate EDTA buffer at ±17°C for 18-22h. Gels were then transferred onto 3mm Whatman paper, covered with saran wrap and dried in vacuum drier at 90°C for 1h. The saran wrap was then replaced by X-ray film and kept at -70°C for 48h. The mobility shift in DNA bands were visualized by developing the x-ray film in a developer.

Statistical analysis

All statistical analyses were performed using S-PLUS software. Chi-square test for homogeneity of proportions was used to determine significance of mutation pattern and mutation effect data. Odds ratio was utilized to determine associations of presence of mutations with various Clinico-epidemological characteristic such as age, provisional diagnosis, lymph nodes involved, clinical tumor stage and histopathological grade of tumor. Statistical significance was considered when p≤0.05. The prevalence and pattern of TP53 mutations obtained in patients from Kashmir was compared with compiled data reported for gastric cancer in IARC TP53 mutation database, release 9, 2004 (http://www.iarc.fr/p53/homepage.htm).

Results

Mutational screening of exons-5 to 8 of TP53 gene in 30 sporadic tumors included in this study exhibit TP53 mutations in 6/30 (20%) patients with gastric adenocarcinoma. Five out of six patients exhibited somatic mutations in nature. However, one patient did exhibit germline mutation. Overall, the six patients revealed a total of 21 mutations; these were exhibited at codon 142 (3 cases) of exon 5, codon 144 (1 case) in exon 5, codon 147 (1 case) in exon 5, codon 157 (1 case) in exon 5, codon 169 (2 cases) in exon 5, codon 170 (3 cases) in exon 5, codon 172 (1 case) in exon 5, codon 173 (3 cases) in exon 5, codon 179 (3 cases) in exon 5, codon 180 (1 case) in exon 5, codon 213 (1 case) in exon-6, the insertional mutation was between codon 216 & 217 (1 case) in exon-6. The mutational pattern included 12 insertions, 6 substitutions (all six were transversions) and 3 deletions (Table 3). The mutational pattern data of TP53 revealed a high % age of age, provisional diagnosis, lymph nodes involved, clinical tumor stage and histopathological grade of tumor. Statistical significance was considered when p≤0.05. The prevalence and pattern of TP53 mutations obtained in patients from Kashmir was compared with compiled data reported for gastric cancer in IARC TP53 mutation database, release 9, 2004 (http://www.iarc.fr/p53/homepage.htm).

Statistical analyses were performed using Pearson χ2 test and Fisher’s exact test. The data were computerised and statistical tests were performed with the program, Statistical Package for Social Sciences (SPSS version 10.05). The tests were considered significant when their overall p values were below 0.05.

Table 3: Clinico-epidemiological details and nature of TP53 mutation spectrum in sporadic gastric adenocarcinoma patients from Kashmir valley.
The study exhibited significant amount of mutation in exon-5 (90.48%) and only 9.5% in exon-6 of TP53 in the gastric adenocarcinoma patients from Kashmir valley.

The mutational effect data revealed significantly high % of age of frameshift mutations (71.4%) (15/21) compared to missense (23.8%) and only 4.8% occurred in two different patients. Significant amount of mutations were found in exon-5 (p=0.025 and p>0.05 within the CI of 12.47-652.89) of TP53 while exons-7 and 8 did not show any mutation at all. Further, in exon-5 an insertion (426lnsA) at nucleotide# 13105 in codon 142 resulting in frame-shift mutation was identified in three different patients and another insertion (510lnsT) at nucleotide# 13189 also resulting in frame-shift mutation in codon170 occurred in three different patients. A deletion (518delT) at nucleotide# 13197 in codon 173 resulting in frame-shift mutation was identified in two different patients. In exon 5, four different mutations (1 missense and 3 frameshift) at codon 142, 144, 179 and 180 were found in same patient (Table 3). None of the TP53 mutation bearing patients harboured mutations at the hot-sprouts reported in gastric cancers as per UMD TP53 database (http://p3.free.fi/Database/p53_dbase.html). However, all the mutations identified at various codons in our study tally to either the moderate (between 11-100) or maximum (>100) number of mutations as indicated by the distribution of p53 mutations in various cancers.

The presence of TP53 mutations when compared with various clinico-epidemiological attributes of gastric adenocarcinoma patients showed some association, though statistically not significant, of TP53 mutation with age, positive lymph node status of patient as well as clinical tumor stage (II & III) and moderately and rural/urban status of patient (Table 5).

**Discussion**

The occurrence of TP53 mutations in gastric adenocarcinoma patients in high and low incidence and racially diverse populations has been well established. However, the information regarding the involvement of this gene in the incidence of and predisposition to gastric cancer in ethnic Kashmiri families is lacking. Of particular mention is the fact that Kashmir is a province of the north Indian state of Jammu and Kashmir situated at an altitude of 1800-2400 m above sea level with extremely cold climatic conditions and comprises of a non-migrant population who have special social, personal and dietary habits that endowed this population with a unique ethnic origin. Thus, this study focused on the evaluation of involvement of TP53 gene mutation in incidence and development of gastric cancer in patients from Kashmir valley is significant and warrants documentation, the first on gastric cancers from this part of the world.

Various investigators have examined the mutational profile of gastric cancers by examining exons-2 through 11, although most studies restrict their examination to exons-5 through 8. The reported incidence of p53 mutations in invasive carcinomas ranges from a low of 0% to a high of 76.9% [58,59]. Nonetheless, our study revealed the overall frequency of mutations in TP53 to be 20% in gastric adenocarcinoma patients of Kashmir which is comparable with the existing reports in the literature [60-71]. In the R12 release of IARC [57] mutation prevalence data base for TP53, the overall mutation rate in gastric cancers was documented to be 45%. It is likely that these differences in the frequency of TP53 mutations in gastric cancers are due to such factors, as ethno-geographic diversity of populations studied, small sample size, differences in exposure to endogenous or exogenous carcinogens, differences in life style & food habits, social and cultural differences, which are yet obscure but their role in the molecular events associated with progression and pathogenesis of human cancers is worth addressing in the future studies.

The mutational spectrum of p53 in gastric cancers is wide. The mutations in exons 5 through 8 of TP53 in our study on the gastric adenocarcinoma patients of Kashmir were found to be unequally distributed. On comparison, a high percentage of mutation was found in exon-5 (90.48% (19/21) Vs 36.28% (78/215) reported in IARC and exon-6 (9.52% (2/21) Vs 15.35% (33/215) reported in IARC). Surprisingly, no mutation was found in exons-7 and 8 [0% Vs 22.32% (48/215) & 23.72% (51/215) reported in IARC]. Five out of six patients exhibited mutations somatic in nature. However, one patient exhibited a single germline mutation (Table 2). This patient exhibited a germline mutation at codon169 in exon-5 nucleotide# 13185. There are several sites where mutations are more common than others. More than one mutation was present in a single tumor- insertions 426lnsA at nucleotide# 13105 in codon142 and 510lnsT at nucleotide# 13189 in codon170 resulting in frame-shift mutation, each occurred in three different patients, deletion (518delT) at nucleotide# 13197 in codon173 also resulting in frame-shift mutation was identified in two different patients. In exon-5, four different mutations (1 missense and 3 frameshift) at codons 142, 144, 179 and 180 were found in the same patient. Similar results displaying multiple mutations in a single tumor were also documented by Flejou et al [72] as within a given tumor there can be heterogeneity of the p53 mutational status [73].

A peculiarity of TP53 mutation pattern data of Kashmiri patients was significantly high prevalence of insertions (57.1%) compared to 2.07% documented in the IARC mutation pattern data on all gastric cancers, R12 release [57]. The frequency of the deletion mutation (14.3%) found in Kashmiri patients was also higher than the reported...
frequency from rest of compiled world data of 6.63%. Mutations resulting in loss or gain of nucleotide base pairs may represent the second highest endogenous mutagenic event for p53 gene in human cancers [74]. Insertions and deletions in the p53 gene can be explained by slipped-mispairing mechanism as proposed for germinal mutations of a small number of eukaryotic genes [75]. Almost all deletions and insertions could be due to DNA sequence features of monotonic base runs, adjacent or nonadjacent repeats of short tandem sequences, palindromes and runs of purines or pyrimidines (homocopolymer runs) as increased length of monotonic runs correlates positively with increased frequency of events. Thus, deletions and insertions in the p53 tumor suppressor gene may reflect both spontaneous and carcinogen-induced mutagenesis. The mutation effect data revealed significantly high %age of frameshift mutations (71.4%) (15/21) compared to 5.46% reported in the IARC mutation pattern data [57] but low frequency of missense mutations (5/21) (23.8%) and silent mutations (1/21) (4.8%) as against 71.6% and 8.53% documented in IARC mutation effect data [57]. Complex frameshift mutations can be explained by the formation of quasi-palindromes, with mismatch excision and replication using one strand of the palindrome as a template.

The overall frequency of substitutions mutation was 28.6% (6/21) was observed in our study, all these substitutions were base transversions. A large fraction of the p53 mutations in gastric adenocarcinomas of the ethnic Kashmiri population are base transversions, a type of mutation that is infrequent in other tumors aside from lung [76], breast [77] and hepatocellular [78] carcinomas. To our knowledge this is the first report implicating the role of base transversions in gastric cancers. Thus, the different mutation spectrum with high transversions may imply that the exogenous mutagens outweigh the endogenous processes in this cancer.

C>G>C mutations found in our study were equivalent at CpG or non-CpG sites (16.7%), an observation unique to our population. In contrast, the IARC mutation spectrum data on all gastric cancers document a higher frequency of 35.1% at CpG sites Vs 23.2% at non-CpG sites [IARC R12 release, 2007] [57]. Presence of alkyl nitrosamine in food stuffs, leading to O'-alkyl guanine adducts and base mispairing during replication, resulting in G>A (or C>T on the other strand of the DNA) transition [79] has been implicated in the etiopathogenesis of oesophageal and gastric carcinomas [80,81,82]. However, to establish a correlation between the enhanced transversions in our patients and the presence of carcinogens already reported or unreported to be present in the local food stuffs needs further investigations.

In summary, mutation pattern of TP53 revealed certain peculiarities in having maximum mutations in exon-5, high frequency of deletions and insertions besides no mutation at hotspot codons. The study, therefore, suggests TP53 as a potential molecular marker and prognostic tool. Nevertheless, these observations need further investigations in a bigger cross section of the gastric cancer patients. In future, it will be interesting to explore if exposure to particular environmental carcinogens, different life style, dietary and cultural practices adopted by Kashmiris could generate the mutation pattern observed in present study. More complete analysis of all p53-coding exons would give a more thorough picture of mutational patterns of the DNA-binding domain and focus on the factors that eliminate the p53 DNA binding function and thus would alter p53 function and cell cycle kinetics every time they occur.

References
interacting protein Cipl is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75: 805-816.


