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 190 (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 287 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 aminocacid substitution included four missense mutations and a single silent mutation. The mutation effect data 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 both 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.

Conclusions: p53 gene mutation incites the pathogenesis of human gastric adenocarcinomas.

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, of about 25% and has not changed appreciably in 60 years. In 2001 alone, stomach cancer affected 850,000 people, of which 522,000 men and 328,000 women died of stomach cancer [1]. Globally, incidence of gastric cancer shows a wide geographic variation; being particularly high in Japan, Chile, Costa Rica, Colombia, China, Portugal, Iceland, Finland and Scotland [2], considerably lower in US, UK, Canada, Greece, New Zealand, Sweden and Honduras [3] Two-thirds of these cases occur in the developing countries [4]. In Asia, areas with a low incidence include India, Pakistan and Thailand [5] Incidence rates in men are twice those in women, in both low-risk and high-risk areas. However, in most countries there has been a considerable decline in both the incidence and mortality of gastric cancer during the past six decades [6,7]. Thus, since 1930, the annual mortality rate in US has dropped from about 38%-7% per 100,000 populations for men and from 28%-4% per 100,000 for women due to the identification of several predisposing factors in recent years [8,9]. Overall, it still remains among the leading killer cancers representing 3% of all cancer deaths [10-12] Migrants from high to low incidence countries show a significant decrease in gastric cancer occurrence in their offsprings, 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...
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 genotoxic 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 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 Sher-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 gastrectomy 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 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 Minicyclet 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% Bromophenol 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

| Gene | Amplicon | Nucleotide positions in genomic DNA | Primer sequence* | Annealing Temperature (°C) | Product size(bp) |
|------|----------|------------------------------------|------------------|--------------------------|-----------------|
| p53  | Exon5    | 13005-13024                        | 1)TGGTCCACCTTG TGCCCTGACT 2)AGCAATCAGTGAGGAATCAG | 55             | 310             |
| p53  | Exon6    | 13271-13280                        | 1)TGGTGCCCAGGGTTCCCAG 2)TGAGGGGACCTGACAAACCA | 62             | 224             |
| p53  | Exon7    | 13941-13960                        | 1)CTGTGCCACAG GTCCTCCCAA 2)AGGGGCTACGCGGCAACCCAGA | 62             | 237             |
| p53  | Exon8    | 14442-14461                        | 1)TCCGAGAAGTGGGATAATCTA 2)GCTTGCTACCTCGCCTAGT | 58             | 156             |

*1) Sense primer  2) Antisense primer
was considered when p-stage and histopathological grade of tumor. Statistical significance as age, provisional diagnosis, lymph nodes involved, clinical tumor mutations with various Clinico-epidemological characteristic such as odds ratio was utilized to determine associations of presence of mutation pattern and mutation effect data. Chi-square test for homogeneity of proportions was used to determine significance of mutation pattern and mutation effect data. Written on paper,
(p = 0.011). This 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 mutation effect data revealed significantly % age of frameshift mutations (71.4%) (15/21) compared to missense (23.8%) plus silent mutations (4.8%) (p=0.0002) [Table 4]. All missense mutations occurred in heterozygote state. Significant amount of mutations were found in exon-5 (p=0.0025) 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 (426InsA) at nucleotide # 13105 in codon 142 resulting in frame-shift mutation was identified in three different patients and another insertion (510InsT) 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-spots reported in gastric cancers as per UMD TP53 database (http://p53.free.fr/Database/p53_database.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).

| MUTATION PATTERN DATA (21) (%) | MUTATION EFFECT DATA (21) (%) |
|-------------------------------|-------------------------------|
| Insertions                    | Mis sense                     |
| Substitution                  | Frame-shift                   |
| G>C>T A                      | 1 (16.7%)                    |
| A>T>A                        | 1 (16.7%)                    |
| C>G>G>C                      | 1 (16.7%)                    |
| C>G>G>C at CpG               | 1 (16.7%)                    |
| T>A>G>C                      | 1 (16.7%)                    |
| C>G>A>T                      | 1 (16.7%)                    |
| Deletion                      | Silent                        |
|                              | 1 (4.8%)                     |
|                              |                              |
| χ² = 9.00                    | χ² = 22.28                   |
| df = 2                       | df = 2                       |
| p-value=.001                 | p-value=.000                 |

Table 4: Mutation pattern and mutation effect data of TP53 in gastric cancer patients from Kashmir valley.

| Feature                  | Mutant TP53 (%) | 95% CI    | OddsRatio |
|--------------------------|-----------------|-----------|-----------|
| Age                      |                 |           |           |
| ≤45yrs                   | 0/10            | 0.027-2.526 | 0.259     |
| >45yrs                   | 6/20 (66.6%)    |           |           |
| Rural                    | 5/24 (20.83%)   | 0.124-13.967 |           |
| Urban                    | 1/6 (16.67%)    |           |           |
| Lymph node/s involved    |                 |           |           |
| Yes                      | 5/6 (83.3%)     | 0.091-11.028 | 1.00      |
| No                       | 1/6 (16.7%)     |           |           |
| Clinical Tumor stage     |                 |           |           |
| II                       | 1/10 (10%)      | 0.033-3.327 | 3.327     |
| III                      | 5/20 (25%)      |           |           |

Table 5: Association of TP53 gene mutation with Clinico-epidemiological features of gastric adenocarcinoma patients of Kashmir (n=30).

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 ethnographic and geographic diversity of populations studied, small sample size, differences in exposure to endogenous or exogenous carcinogens, differences in lifestyle and food habits, social and cultural differences, which are yet ubiquitous 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 426InsA at nucleotide# 13105 in codon142 and 510InsT at nucleotide# 13189 in codon170 resulting in frame-shift mutations, 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 \(4^\text{th}\) age of frameshift mutations (71.4%) (15/21) compared to 5.4% reported in the IARC mutation pattern data [57] but lower 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\text{G} \rightarrow C\text{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 O6-alkyl guanine adducts and base mispairing during replication, resulting in G\rightarrow A (or C \rightarrow G) 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.

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