

RESEARCH COMMUNICATION

Lack of p16 Gene Mutations in Gastric Cancers in Kashmir

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Abstract

Background and Aim: The focus of the study was to investigate the frequencies of homozygous deletions and mutations of p16 gene in gastric carcinomas in the Kashmiri population. **Methods:** A total of 84 gastric carcinoma patients were screened by the single strand conformation polymorphism (SSCP) technique and later by DNA sequencing to detect mutations of the p16 gene. Also PCR was applied further to further detect any homozygous deletions. **Results:** SSCP and DNA sequencing performed encompassing all the three exons of p16 gene could not detect any mutations in any of 84 cases. Though we could observe mobility shifts in SSCP of two samples, subsequent DNA sequencing did not show any mutation. Further PCR could not detect any homozygous deletion in P16 in any case. **Conclusion:** Though Kashmir is a high incidence area of gastric carcinomas, p16 gene mutations /or deletions do not appear to be involved.

Keywords: p16 gene - mutation - homozygous - SSCP - gastric carcinomas - N-Nitroso compounds - Kashmir

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Introduction

Gastric carcinogenesis is a multistep process composed of genetic and epigenetic alterations (Tahara, 1993). Gastric cancer can develop in any part of the stomach and may spread throughout the stomach and to other organs; particularly the oesophagus and the small intestine. As per Cancer, World Health Organization (Feb 2006), Stomach cancer causes nearly one million deaths worldwide per year. In the global incidence stomach cancer is second most common tumour (Chan and Rashid, 2006). India, overall, is deemed to be a low incidence (<6.7 /100,000) country but there are regions, like Kashmir, with a particularly high incidence (Sipponen et al., 1983; Diwani et al., 1988; Khuroo et al., 1992). Clinical experience has revealed very high prevalence of gastric cancer in Kashmir. It is suspected that several risk factors are involved including diet, gastritis, smoking, intestinal metaplasia and Helicobacter pylori infection. It is more common in men.

Kashmir valley is one of the high incidence areas (Khuroo et al., 1992) where different environmental and dietary habits play an overwhelming role in the development of gastric cancer. These include intake of sun-dried vegetables of Brassica family (Hakh), pickled vegetables (Anchar) and hot salted tea, which contains potentially high content of carcinogenic compounds like nitrosamines (Kumar et al., 1992). Personal habits

like smoking of hukka/cigarette increases the risk of developing gastric cancer (Diwani et al., 1988; Khuroo et al., 1992). In Kashmir, the most important specific food habit is consumption of large quantities of hot salted tea. The use of sodium bicarbonate at the time of boiling the tea leaves and the further addition of common salt to the prepared tea cause one to suspect that the tea does more than cause thermal injury to oesophageal epithelium. Common salt (NaCl) is a well known irritant of gastric epithelium and has been considered a risk factor for gastric cancer (Khuroo et al., 1992). The presence of N-nitroso compounds (found in most of the customary dietary items of a native Kashmiri) in the stomach has been incriminated as a possible etiological factor in the genesis of gastric cancer (Mirvish et al 1972; Siddiqi et al 1988). As per Census of India 1981, Series 8, Jammu and Kashmir; one of the factors include the peculiar geography of the valley (situated at an altitude of 1800-2400 m above the sea level), with severe cold winter may have a bearing on the etiology.

Several genetic and epigenetic alterations have been suggested to play important roles in the carcinogenesis pathway, affecting oncogenes, tumour suppressor genes, apoptosis-regulating or mismatch repair genes. (Igaki et al., 1994). The various genetic changes associated with the development of gastric cancer include inactivation of several tumour suppressor genes (mutation of the p53 gene, dysregulation of cell-cycle control in G1 by several

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mechanisms like inactivation of p16^{MTS1}, alterations of RB, amplification of Cyclin D1 and activation of oncogenes (e.g., EGFR, c-MYC). (Mandard et al., 2000).

Chromosome 9p21 shows a high rate of heterozygous deletion and homozygous deletion in many tumour types, including gastric cancers, indicating that this region harbours tumour suppressor genes (Serrano et al., 1993; Kim et al., 1994). P16 gene, a tumour suppressor protein located at chromosome 9p21, inhibits the function of cyclin D1/CDK4 and CDK6 complex, and cause p53-independent G1 arrest through the phosphorylation of pRb (Serrano et al., 1993; Lukas et al., 1995). Inactivation of p16 gene by different mechanisms is commonly found in human cancers (Cairns et al., 1995; Igaki et al., 1995; Tien et al., 1997) which includes mutations, homozygous deletion and promoter hypermethylation.

In the present investigation we have searched for deletion and/or mutation of the CDKN2A/p16 genes in gastric cancer in an attempt to correlate gene alterations with the origin and progression of the tumour.

Materials and Methods

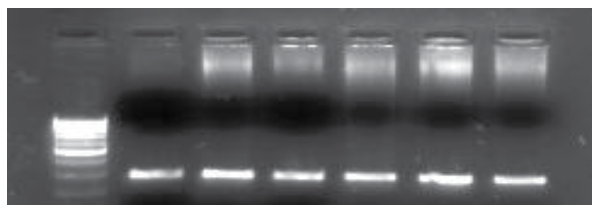
Under sterile conditions, fresh tumour specimens and their adjacent non-tumour normal appearing tissues were obtained in the course of surgery from 84 patients with primary GC, admitted in the department of general surgery at the Sher e Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India. A written informed consent was obtained from each patient for inclusion in this study.

DNA extraction

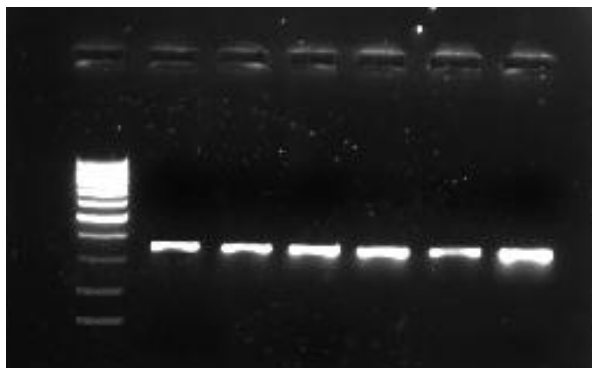
Paired tissue samples, tumor and normal, resected from the patients in the general surgery department were snap-frozen and immediately stored at -70. DNA was extracted using the standard phenol chloroform method.

Polymerase chain reaction

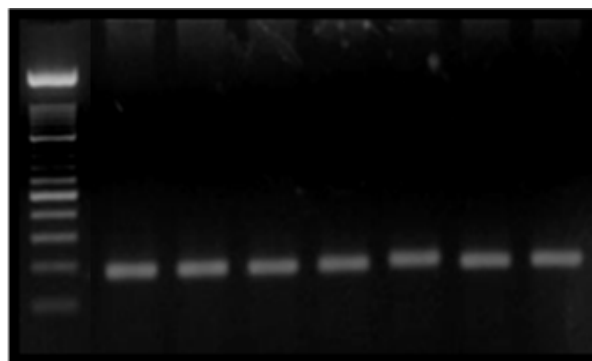
The three exons of p16 gene were amplified by employing 3 sets of primers which were, exon1 5'GCTG CCGAGAGGGGAGAGCAG3'exon1R5'GCGCTACC TGATTCCAATTC3', exon2 5'CATCTGTCTCTCTG GCAG3', exon2R5'CTCAGATCATCAGTCCTCAC3', exon3 5'CCGGTAGGGACGGCAAGAGA3', exon3R5'CTG TAGGACCTCGGTGACTGATGA3'. PCR amplification was performed in 50 μ L reaction volume containing 1X PCR buffer containing 1.5mM MgCl₂, 200 μ M of each dNTPs, 10 pmoles of each forward and reverse primer, 50-100ng of DNA, 1U taq DNA polymerase. PCR reaction was carried out in a thermal cycle machine (Biorad-USA) as follows: one cycle at 94°C for 5', 35 cycles at 94°C for 30s, annealing at 58°C for 30 s for exon 1, 53°C for 30s for exon 2, 60°C for 30s for exon 3, extension at 72°C for 30 s. This was followed by a final extension step of 7 min at 72°C. 6 μ L of PCR product was loaded into the gel, and a 100 bp DNA ladder was used as a marker. The PCR products were electrophoresed at voltage of 100 V on 2 % agarose gel for 30 min and visualized under UV illumination using an ethidium bromide stain (Figure 1). SSCP was performed on all samples. The PCR products exhibiting abnormally migrating bands were submitted to



PCR products of p16 gene Exon 1(280bp). M:marker 100 bp



PCR products of p16 gene Exon 2(347bp). M:marker 100 bp



PCR products of p16 gene Exon 3(169 bp).M:marker 100 bp

Figure 1. Showing PCR Products

manual DNA sequencing.

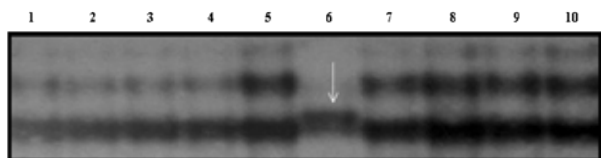
Results

In this study a total of 84 samples of confirmed GC patients were enrolled from 2008-09. The mean age of the patients was 57 years who were between 40 & 75 years. None of the patients had received either chemotherapy or radiotherapy prior to surgery. All of the primary tumours were pathologically confirmed to be GC cases; among which there were 64 males and 20 females. The male/female ratios in this investigation were 3:1. Patients underwent endoscopic, radiologic and histopathologic examination to establish the clinical profile. Histopathological grades and clinical staging were evaluated according to standard criteria by two expert pathologists. Among 84 GC cases, 72(85.71%) were smokers and rest non smokers 12/84(14.29%). 51 of 84 patients had dysphagia while as rest 33 patients had no such condition. 35/84(41.67%) patients were confirmed histopathologically as stage I-II while as 49/84(58.33%) were of stage III-IV. 25(29.76%), 40(47.62%), and 19(22.62%) patients of total 84 cases were histologically proven to be well differentiated, moderately and poorly differentiated GCs, respectively (Table 1).

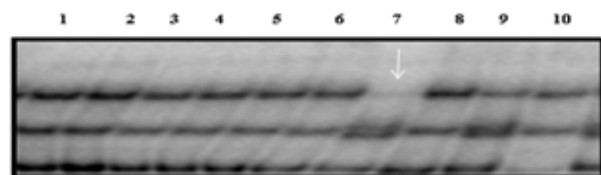
Table 1. Different Characteristics of Gastric Cancer Cases

Characteristics	Subgroup	Patients n=84(%)
Dwelling	Rural	65(77.38)
	Urban	19(22.62)
Age	<60 yrs	50(59.52)
	≥ 60 yrs	34(40.48)
Gender	Male	64(76.19)
	Female	20(23.81)
Smoking status	Smoker	72(85.71)
	Non smoker	12(14.29)
Use of snuff	Yes	11(13.10)
	No	73(86.90)
Dysphagia	Grade I,II,III	51(60.71)
	No dysphagia	33(39.29)
TNM Staging	Stage I & II	35(41.67)
	Stage III & IV	49(58.33)
Histological grade	WD	25(29.76)
	MD	40(47.62)
	PD	19(22.62)
Pathological type	Intestinal	67(79.76)
	Diffuse	12(14.29)
	Mixed	05(5.95)
Consumption of hot salted tea/day	1-3 cups	11(13.10)
	3-6 cups	25(29.76)
	>6 cups	48(57.14)

WD: Well Differentiated; MD: Moderately well Differentiated; PD: Poorly Differentiated



PCR-SSCP of exon 2 in gastric biopsies, showing altered band mobility at position 6 i.e sample GT 16



PCR-SSCP of exon 2 in gastric biopsies, showing altered band mobility at position 7 i.e sample GT 52

Figure 2. Showing Altered Band Mobility

In the present study, PCR technique was used to detect deletions/mutations in the three exons of the gene CDKN2A/p16. No deletion/mutation was detected in any exon of the CDKN2A/p16 genes. Single-strand conformation polymorphism analysis allowed the screening of the samples to be sequenced. Only two (GT16, GT52) showed a different migration (Figure 2)

for exon 2 of gene CDKN2A/p16, but no alteration was detected in the base sequence of the exon by nucleotide sequencing. Thus, no mutation or deletion was detected in any of the analyzed exons.

Discussion

There have been relatively few reports on p16INK4a status in stomach cancers and most have come to the conclusion that p16INK4a is rarely deleted or mutated in the primary tumors. Even in cell lines, the incidence of homozygous deletion is relatively low, but this may be because the studies have examined the same limited number of cell lines (Igaki et al., 1994; Akama et al., 1996; Fushida et al., 1996). In this study contrary to our expected results, we could not detect any deletion or any somatic mutation in 84 GC cases in all the three exons of p16 gene.

Our results corroborate with the study of Igaki et al (1995) and suggest that deletion/mutation is not an important mechanism of CDKN2A/p16 gene inactivation in primary gastric tumours. Therefore, other mechanisms of inactivation, such as methylation of promoter region and improper functioning of proteins, may be considered in order to estimate the real contribution of this gene to gastric cancer development and progression. Although the p16 gene has been known to be homozygously deleted in a variety of cancer cell lines and primary tumors, the status of the p16 gene has not been well studied in gastric carcinoma. To date, there have been 3 studies of the p16 gene in gastric carcinoma cell lines and primary tumors. (Igaki et al., 1994; 1995; Sakata et al., 1995). The deletional study by Southern blot analysis in gastric carcinoma cell lines showed deletion in 2 (22%) of a total of 9 cell lines, (Igaki et al 1994) and the mutational status of the p16 gene by PCR-SSCP in primary tumors showing no mutation in a total of 64 gastric adenocarcinoma (Igaki et al., 1995). Gastric cancer is among the leading causes of mortality and least genetic studies have been done in this region and tempted our group to study this gene (Young et al., 1997). There have been no reports of the mutation frequency of the p16/ CDKN2 gene in primary gastric adenocarcinomas. The present results clearly show that the mutation of the p16/CDKN2 gene is quite low, and even absent, in the surgical specimens of gastric carcinomas. The reason for a high frequency of p16/CDKN2 gene alteration in cell lines but not in primary tumors remains unknown.

Owing to the high incidences of gastric cancer in Kashmir than the rest of India we tried to look for the genetic alteration in p16 gene but contrary to our expectations we could not detect any mutation/or deletion in any case. This implies that other genetic factors are responsible for development of gastric cancer which needs to be further evaluated.

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