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RAPID COMMUNICATION

A study on *p53* gene alterations in esophageal squamous cell carcinoma and their correlation to common dietary risk factors among population of the Kashmir valley

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Abstract

AIM: To systematically examine the extent of correlation of risk factors, such as age, consumed dietary habit and familial predisposition with somatic *Tp53* molecular lesion causal to elevate carcinogenesis severity of esophageal squamous cell carcinoma (ESCC) among the Kashmiri population of Northern India.

METHODS: All cases (n = 51) and controls (n = 150) were permanent residents of the Kashmir valley. Genetic alterations were determined in exons 5-8 of *Tp53* tumor suppressor gene among 45 ESCC cases histologically confirmed by PCR-SSCP analysis. Data for individual cancer cases (n = 45) and inpatient controls (n = 150) with non-cancer disease included information on family history of cancer, thirty prevailing common dietary risk factors along with patient's age group. Correlation of genetic lesion in *p53* exons to animistic data from these parameters was generated by Chi-square test to all 45 histologically confirmed ESCC cases along with healthy controls.

RESULTS: Thirty-five of 45 (77.8%) histologically characterized tumor samples had analogous somatic mutation as opposed to 1 of 45 normal sample obtained from adjacent region from the same patient showed germline mutation. The SSCP analysis demonstrated that most common *p53* gene alterations were found in exon 6 (77.7%), that did not correlate with the age of the individual and clinicopathological parameters but showed significant concordance (P < 0.05) with familial history of cancer (CD = 58), suggesting germline predisposition at an unknown locus, and dietary habit of consuming locally grown *Brassica* vegetable "Hakh" (CD = 19.5), red chillies (CD = 20.2), hot salty soda tea (CD = 2.37) and local baked bread (CD = 1.1).

CONCLUSION: Our study suggests that somatic chromosomal mutations, especially in exon 6 of *Tp53* gene, among esophageal cancer patients of an ethnically homogenous population of Kashmir valley are closely related to continued exposure to various common dietary risk factors, especially hot salty tea, meat, baked bread and "Hakh", that are rich in nitrosoamines and familial cancer history.

Key words: Case-controls; Esophageal squamous cell carcinoma; Dietary carcinogens; *p53* alterations

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INTRODUCTION

Among human cancer, esophageal carcinogenesis appears to be a complex multi-step process with a multi-factorial etiology, where environmental, geographical, and genetic factors appear to play major roles[1]. Apparently 400 000 new cases of esophageal cancer occur each year, and esophageal cancer as such is the sixth leading cause of

cancer death worldwide[2]. Extended epidemiological studies in high incidence areas, such as Northern Iran, Northern China and South Africa, provide evidence that exposure to specific diet-related nitroso compounds in parallel with nutritional deficiencies and consuming food contaminated by mycotoxins are the most important determinants of the disease[3]. Also in some parts of India, esophageal cancer is alarmingly rising and is as such the third leading cancer in men and fourth leading cancer in women in these regions[4]. Reports from Southern India suggest that esophageal squamous cell carcinoma (ESCC) occurs in more than 80% of cases in chronic tobacco smokers that is further potentiated by heavy use of alcohol and additional prevailing risk factors, including nutritional factors and vitamin deficiencies[5]. In the Kashmir valley, belonging to the Northern part of India, esophageal cancer has been reported to exceed 40% of all cancers, however, very few reports have associated this malignancy with specific risk factors prevalent in the area[6].

With the advent of molecular biology, new strategies are being carried out for the prevention and treatment of cancer. Cumulating evidence indicates that changes in both dominant oncogene and tumor suppressor genes are likely for malignant transformation of normal cell[7]. Among these genetic abnormalities, *p53* tumor suppressor gene, a critical regulator of cell growth, differentiation and apoptosis, is frequently affected in most human cancer[8]. The frequencies of *Tp53* alterations in esophageal carcinoma vary from 26% to 87% that occur in multiple sites throughout the open reading frame, which are mostly limited to DNA binding domain that spans exon 5 through exon 8[9]. The current study was initiated to find out the risk factors of esophageal cancer in Kashmiri inhabitants and to correlate *p53* alterations in ESCC with prevalent risk factors.

MATERIALS AND METHODS

Collection of tumor samples and data from case-control population

Hospital and general population-based case-control study of permanent inhabitants of Kashmir was performed between March 2003 and December 2005. Cases were selected from the Government Medical College, Srinagar, Kashmir and controls from three districts of Kashmir valley, India viz. Srinagar, Anantnag and Pulwama. From each of the three districts, 150 healthy individual families (controls) were randomly selected and evaluated along with selected patients on a detailed questionnaire for demographic information as well as exposure to etiological factors, including dietary nitrosoamines, intake of pickled vegetables, meat, hot salty tea, source of water, *etc* as well as the incidence of any family history of cancer in them. Family history of cancer was defined as the presence of esophageal cancer in one or more relatives for both cases and controls. One hundred milligram of tumor specimen and other 100 mg of macroscopically normal adjacent esophageal tissues were obtained from each selected ESCC patient (cases) that were admitted in different Wards of Government Medical College, Srinagar, Kashmir and were diagnosed for the first time for such type of cancer. The selected cases diagnosed for the first time for ESCC having no chemotherapy or radiotherapy prior to biopsies were interviewed 3 d prior to the surgery. From 51 histopathologically confirmed ESCC samples, information related to histopathological grade *etc* was collected from the Division of Pathology, Government Medical College, Srinagar. For evaluating the association between the common dietary risk factors and genesis of ESCC in Kashmiri inhabitants, the dietary habits of all the 45 cases were compared with two groups of controls (Group II and Group III), each representing 45 normal subjects taken from total of 150 healthy individual families. Group II represented the controls with family history of cancer and group III represented the controls without any family history of cancer.

DNA extraction and PCR amplification

High molecular genomic DNA from the fresh ESCC and from adjacent normal tissue samples was extracted using DNAzol reagent (Imperial Bio-Medics, India). The extracted DNA was used for PCR amplification reactions. PCR primers used were same as used previously for the amplification of 5-8 exons of *TP53* gene[19]. Out of total 51 ESCC cases, 6 with apparent cancer of other origin were excluded from PCR amplification and only 45 were used for the further study. All the reactions were carried out in a total volume of 50 μL in Techgene (0.2 mL) PCR System (Techne, UK). Typical PCR conditions were as follows: denaturation at 94℃ for 10 min, followed by 35 amplification cycles at 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min. An elongation step at 72℃ for 10 min was added to the final cycle for all the exons. PCR products were separated on 40 g/L agarose gel continuously and visualized by 600 g/L ethidium bromide staining.

SSCP analysis

Non-radioactive SSCP was performed as described previously[10] with slight modification. A 60 g/L non-denaturing gel conditions were used with 60 mL/L glycerol (dissolved in 0.5 × TBE buffer). Aliquots of amplified products were mixed with equal amounts of denaturating buffer (500 mL/L deionized formamide and 500 mL/L glycerol with 0.5 g/L xylene cynol and 0.5 g/L bromophenol blue). Samples were denatured at 95℃ for 5 min, kept on ice until loading on the 60 g/L non-denaturating gel and run in vertical electrophoretic plates (Banglore Genei, India) with 0.5 × TBE as running buffer at 150 *V* for 3-4 h at room temperature. SSCP fragments were visualized by silver staining.

Statistical analysis

Analysis of the data was performed on the original data by using chi-square (χ^2) test to determine correlation between *p53* alterations in ESCC and dietary risk factors as well as with clinicopathological status of ESCC tissue samples. $P < 0.05$ was considered statistical significant.

RESULTS

Samples, clioncopathological parameters and p53 mutations

Forty-five cases without any apparent cancer of other

Table 1 Correlation between clinicopathologic findings and p53 genetic alterations (n = 45)

Factors	patients *n* (%)	*p53* alterations		[1]*P* value
		Positive (%)	Negative (%)	
Age (yr)				0.848
< 60	10 (22.2)	8 (80)	2 (20)	
> 60	35 (77.7)	27 (77.14)	8 (22.8)	
T-status				0.128
T1	5 (11.1)	3 (60)	2 (40)	
T2	7 (15.5)	3 (42.8)	4 (57.1)	
T3	22 (48.8)	19 (86.3)	3 (13.6)	
T4	11 (24.4)	8 (72.7)	3 (27.2)	
N-status				0.114
N0	15 (33.3)	10 (66.6)	5 (33.3)	
N1	30 (66.6)	26 (86.6.3)	4 (13.3)	
M-status				0.2
M0	25 (55.5)	18 (70)	7 (30)	
M1	20 (44)	15 (75)	5 (25)	
Cell-differentiation				0.21
Well		25 (75.7)	8 (24.2)	
Moderate		3 (42.8)	4 (57.1)	
Poor		3 (60)	2 (40)	

[1]Chi-square (χ^2) test with Fisher's exact test was used to test the frequency distribution of *p53* alteration.

origin, and 150 inpatient controls were enrolled in the current study, among which most of the cases (77.8%, 35/45) were older than 60 years (Table 1). Thirty-three of 45 (73.3%) tissue samples were histologically confirmed to be well-differentiated, 15.5% (7/45) moderately differentiated and 11.1% (5/45) poorly differentiated cancer. Regarding depth of tumor invasion, 5 (11.1%) cases belonged to T1, 7 (15.5%) cases belonged to T2, 22 (48.8%) cases belonged to T3 and 11 (24.4%) cases belonged to T4. Our results demonstrated no difference in the incidence of *p53* alterations among the patients below and above 55 years. With respect to *p53* alterations and depth of tumor invasion, T3 and T4 demonstrated higher frequency of mutations (> 72%) as compared to T1 and T2. However, statically no positive correlation was found between tumorous *p53* alterations and age (P = 0.848), depth of tumor (T) invasion (P = 0.128), lymph node metastasis (M) (P = 0.114) and cell differentiation (P = 0.210) (Table 1).

Risk factors of esophageal cancer

In the general population (controls)-based survey representing 50 individual families, each from three districts of Kashmir valley, matched for different life styles and dietary habits revealed some common food habits that were also common in the selected ESCC cancer patients (Table 2). Total 51 ESCC cases, 45 cases without cancer of any other origin and represented by Group I showed 35 (77.7%) cases with family history of esophageal cancer. Two groups of controls were used representing 51 families, with one group designated as Group II showing the family history of cancer and Group III without any family history of cancer. Interestingly, more than 80% subjects in Group I and Group II showed the same pattern of dietary habits i.e. heavy consumption of hot salty tea, local baked bread, meat, red chillies as well as consumed "Hakh", a locally grown green leafy vegetable belonging to the *Brasicca* family, very heavily (0.57 kg/person per wk) as compared to subjects in group III. Statistically, we also could not find any remarkable difference quantity-wise in consumption of Hakh, hot salty tea, baked bread, meat and red chillies between the group I and Group II. However, both of these two groups differed significantly from the Group III with respect to consumption of these aforementioned food items at very lower amounts (Table 2).

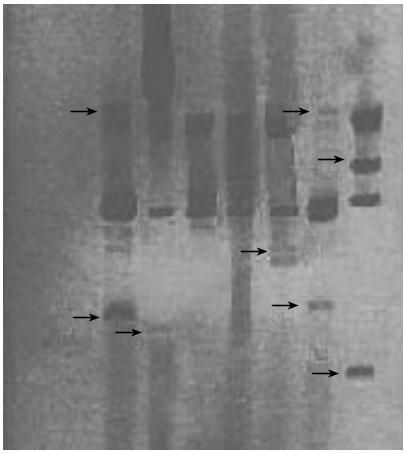

Figure 1 Single-strand conformational polymorphism (SSCP) of p53 exon 6 in ESSC specimens showing 8 anomalous bands with altered mobility and indicates the presence of *p53* mutation (arrows).

Correlation of p53 alterations in ESCC with the prevalent risk factors

Interestingly, 32 of 35 (91.42%) tumor samples from selected 45 cases (Group I) with family history of cancer showed positive for *p53* alterations as compared to only 3 of 10 (30%) samples from cases without any family history of cancer. On SSCP analysis, a total of 82 anomalous bands from these tumor DNA of 35 ESCC patients in exons 5-8 with single exon and multiple exon alterations were observed (Table 3). One case also showed an anomalous band in DNA from both normal constitutional DNA as well as tumor DNA. Statistically, the genetic alterations in 5-8 exons of *p53* gene in total of 45 cases differer significantly with respect to each other (χ^2 = 11.25, P = 0.0105) and most of the mutations (77.7%) were confined to exon 6 (Figure 1), followed by exon 7 (38.9%), exon 8 (33.3%) and exon 5 (27.7%) in decreasing order. Statistically, *p53* alterations, especially in exon 6, were closely related to familial history of cancer (CD = 58), suggesting germline predisposition at an unknown locus, and also to prevalent dietary habit of consuming locally grown *Brassica* vegetable "Hakh" (CD = 19.5), red chillies (CD = 20.2), hot salty soda tea (CD = 2.37) and local baked bread (CD = 1.1).

DISCUSSION

Reports indicate that the Kashmir valley of Jammu and Kashmir State of India ranks among the highest incidence area for esophageal cancer in the world[6]. Despite the gravity of the problem, very little work has initiated in the area. In the current study, 77.7% patients were older than 60, and thus indicating ESCC to be the malignancy of adults with 80% of them found to seek treatment in the

Table 2 Some specific dietary habits of three groups consisting each of 51 individuals

Dietary group	Group I	Group II	Group III	At P values < 0.05, CD
Baked bread (Kandroo Roti)	41/51 (5 roti/d per person)	50/51 (4 roti/d per person)	45/51 (2 roti/d per person)	1.1
Hot salty tea with soda	43/51 (5 cups/d per person)	51/51 (4 cups/d per person)	48/51 (2 cups/d per person)	2.37
Meat	48/51 (0.82 kg/wk per person)	51/51 (0.75 kg/wk per person)	48/51 (0.500 kg/wk/person)	0.25
Red chillies	51/51 (78 g/wk per person)	50/51 (90 g/wk per person)	30/51 (38 g/wk per person)	20.2
Brassicia olerecia (Hakh)	51/51 (0.35 kg/wk per person)	51/51 (0.57 kg/wk per person)	51/51 (0.55 kg/wk per person)	19.5

advanced stage of their cancer and diagnosed for the first time[5]. These results indicate the lack of general awareness of this disease among the general population and remain a major health concern that needs immediate medical attention in the area.

The important cause for high incidence of disease in the Kashmir valley may be defined on the basis that unlike Ladakh and Jammu constuinties of Jammu and Kashmir state that are the low incidence area for ESCC, the Kashmiri population on account of climatic conditions practice an exclusively different dietary life-styles, including use of hot salty tea, red chillies, baked bread, meat, high-nitrate diet especially locally grown *Brassica* leaves, the Hakh (Table 2). To our knowledge, scant literature that have been documented till date suggests diet-related *N*-nitroso compounds in parallel with nutritional deficiency to be most important determinants of disease in this area[11,12]. Our investigation provides evidence that the controls with family history of cancer (group II) followed more or less the same dietary habits as that of patients (Group I), indicating consumption of such types of dietary risk factors rich in nitroso compounds associated with carcinogenesis of ESCC. It is noteworthy that Group II subjects developed much more chance of getting ESCC than subjects from Group III that consumed very low quantity of such foods (Table 2). Thus, in accordance with earlier findings, our results also suggest that there is a chance of higher rate of new esophageal cancer in the population that is exposed to common dietary risk factors than those without such exposures[13]. Among such dietary risk factors, heavy consumption of hot salty soda tea provides chronic irritation of esophageal epithelium causing predisposition of carcinogenic substances to initiate tissue-specific malignant transformation and at the same is also a rich source of carcinogens like *N*-nitrosopropline and nitrosopipecolic acid[11]. On individual basis, the daily dietary nitrate intake (237 mg) in Kashmir valley is much higher than the values reported for most of the Western countries (i.e., Germany 75 mg/d and Britain 95 mg/d) and that through consumption of "Hakh" alone that seems to have also a great importance as potential source of nitroso compounds and their precursors[14,15]. Thus, the results clearly indicate that on daily basis, the Kashmiri population gets exposed to high amounts of nitrate, nitrite and precursor of nitroso compounds that are in high enough quantity to be one of the responsible dietary risk factor for this malignancy in the area[11,14]. Additionally, high consumption of red chilli pepper in the region also indicates higher risk for them towards gastric cancer than non-consumers[6]. Thus exposure of Kashmiri inhabitants to such high or even to low levels of these dietary compounds throughout the life could be involved in the carcinogenesis of esophagus in this high-risk area.

Table 3 Frequencies of *p53* genetic alternations detected by PCR-SSCP in esophageal squamous cell carcinoma (n = 45)

	p53 exons 5-8							
	Exon 5		Exon 6		Exon 7		Exon 8	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
No. of cases (%)	13	32	35	10	18	27	15	30
	28.8	71.1	77.7	22.2	40	60	33.3	66

Most of the researchers now suggest various food-borne carcinogens associated with the genesis of ESCC through the involvement of *p53* tumor suppressor gene. Such reports thus suggest a molecular epidemiology approach to investigation of cancers for which the causes have remained elusive[6,17]. In accordance to earlier reports, interestingly, our study could not find any positive association between clinicopathological variables and *p53* alterations in the collected samples[18] (Table 1). On the other hand, current study, in contradiction to earlier report, demonstrates most of the alterations (14/18) confined to exon 6 of *p53* gene[1]. These results are in consonance with most of the other earlier reports that showed more than 50% of mutations confined to exon 6 of *p53* gene among selected ESCC cancer patients[19].

Finally, this preliminary investigation of tumor gene alterations in patients from Kashmir valley thus supports a large body of epidemiological observations, pointing to dietary mutagenic carcinogenesis and family history of cancer peculiar to populations at high risk of esophageal cancer. The results clearly indicate that such somatic alterations of *p53* gene, especially in its exon 6, in ethnically homogenous population of Kashmir valley are closely related to specific dietary habit and family history of cancer[6]. However, family history of cancer depends on many factors and cannot as such conclude that the positive

familial history of cancer is due to genetic susceptibility. It is finally suggested that the gradual change in dietary and cultural features in the population should produce a significant decrease in esophageal carcinogenesis in the area, and at the same time, in order to draw some logical conclusion, further studies with large set of samples and data are strictly warranted from this part of India.

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