RESEARCH COMMUNICATION

The ECRG1 290Arg/Gln Polymorphism is Related to Risk of Esophageal Squamous Cell Carcinoma in Kashmir

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Abstract

***ECRG1* (esophageal cancer related gene 1) is a novel candidate member of the tumor suppressor gene family previously found to be down regulated in human esophageal cancer (ESCC). So far no evidence regarding the role of the *ECRG1* gene in this cancer has been reported from the Kashmir valley, located on the border of the high risk 'esophageal cancer belt'. A case control study was therefore carried out with genomic DNA from 165 newly diagnosed ESCC patients (cases) and 200 control subjects. DNA was analyzed for *ECRG1* polymorphisms by RFLP PCR, gel electrophoresis and direct sequencing. A statistically significantly increased risk of ESCC was found to be associated with the ECRG1 *Arg/Gln* and *Gln/Gln* genotype occurrence compared to the *Arg/Arg* genotype (odds ratio (OR) 1.698, 95% confidence interval (CI) 1.112–2.593); P= 0.0138) was observed. Statistically significant results were also obtained between the *ECRG1* polymorphism and histophathological grade, smoking, dysphagia, low fruit/vegetable intake and salt tea consumption.**

Keywords: ECRG1 - genetic risk - esophageal SCC - RFLP - PCR

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Introduction

Primary esophageal carcinomas are the most common malignant tumors of the esophagus. Histopathologically the two distinct subtypes are squamous cell carcinoma and adenocarcinoma. These along with carcinoma of the gastroesophageal junction account for more than 90% of primary carcinomas (Parkin et al., 2005). Worldwide squamous cell carcinoma remains the most common type of cancer affecting esophagus (Gregory et al., 2006). Esophageal cancer represents third most common gastrointestinal malignancy worldwide (World Cancer Research Fund, 1997). It is the 8th most common malignancy, and 6th most common cause of cancer death worldwide (Parkin et al., 2005; Wu et al., 2006). Apparently four lakh new cases of this cancer occur each year.

The exact cause of ESCC is unknown; however lot of work has been carried on role of various gene mutations, and polymorphisms on esophageal mucosal cancers. Previous studies suggest that esophageal cancer related gene 1 (*ECRG1*), which is a novel candidate of tumor suppressor gene family is expressed in normal esophagus, liver, colon and lung, but the expression is seen to be downregulated in tumors, especially in ESCC, and their adjacent tissues (Liu et al., 1990).

Kashmir valley, the north most part of India, has been reported as a high incidence area of esophageal cancer. Cancer of esophagus accounts for 40% of all types of cancer in the valley (Murtaza et al., 2006). Age-adjusted incidence rate per 105 individuals per year is 43.6 and 27.9 in males and females, respectively (Khuroo et al., 1992). It is present on the border of high risk 'esophageal cancer belt'. Various etiological factors like dietary habits, nutritional deficiency, environmental and carcinogenic exposure, lifestyle, viral infection, excessive use of alcohol and tobacco and ethnicity which can be considered to be manifested as genetic predisposition, contribute to esophageal carcinogenesis (Katiyar et al., 2005). Analysing the population of Kashmir, it is found to be predominantly non-migrant with unique social, personal and dietary habits. The male:female ratio of EC in Kashmir has been reported as 1.5:1 (Khuroo et al., 1992), with males at a higher risk similar to other areas in India, as observed in the NCRP report 2005.

Diet has been observed to play an important role in the development of esophageal cancer (Khuroo et al., 1992; Phukan et al., 2001). The population in Kashmir has predominant non vegetarian eating habits and consume mostly mutton and beef. Apart from the aforementioned foods, consumption of locally grown vegetables, fruits, red chillies and lotus stem (Nadru) is high especially in the Northern region. People consume some special food items like dried and pickled vegetables, sundried (Hokh

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Gaard) and smoked fish (phari), mixed spice cake (wur) and vegetable food colourant (saffron, mawal) (Katiyar et al., 2005). Literature suggests that diet related N-nitroso compounds in parallel with nutritional deficiencies to be the most important determinants of esophageal carcinoma in this area (Siddiqi et al., 1988; Chang-Claude et al., 1997) which is in good agreement with the observations of Singer et al., 1986 in China. It is to be noted here that the drying of the food stuff in open sun is prevalent and such foods contain significant amount of n-nitroso compounds (Kumar et al., 1992).

ECRG1 has been cloned by the effective mRNA differential display technique through comparing the differential gene expression between normal esophageal epithelium and esophageal cancer cells from high incidence families of ESCC (Su et al., 1998). It has 1375 base pairs, contain 10 exons, spanning 54,014bp on chromosome 4q 13.2, and has a 1254 bp open reading frame encoding a 418 amino acid polypeptide, it begins with an ATG start codon and ends with a stop codon present in the ECRG1 sequence (Su et al., 1998). The protein product has been suggested to be of 45kD. Bioinformatics analysis indicates that the product of ECRG1 is a member of the membrane-anchored serine protease family (Netzel-Arnett et al., 2003) which contains three conserved tandem serine protease domains (His, Asp and Ser), that play a key role in proteolytic activity, which participates in proteolytic reactions that are essential to a diverse range of physiological and pathological processes (Rawlings and Barrett, 1994). As has been hypothesized previously the 290Arg→Gln polymorphism might be associated with increased risk of ESCC because this SNP might affect the function of ECRG1 protein (Boassa and Yool, 2003; Shiokawa et al., 2005). Therefore, we carried out a case-control study in our population to examine this hypothesis, we also investigated whether there is a link between various risk factors and *ECRG1* genotype.

Materials and Methods

Study Population

This study included 165 endoscopically and histologically confirmed ESCC patients (cases) from Department of Medicine, Government Medical College Srinagar and Cardiovascular and Thoracic Surgery Department of Sheri Kashmir Institute of Medical Sciences Srinagar. Another 200 patients, having no previous history of esophageal cancer or cancer elsewhere in the body were included in the study, these served as controls and they were matched to cases in all respects [age (±8 years)]. Patients both cases and controls, had been recruited between July 2008 and January 2010 in the hospitals.

At recruitment, informed consent was obtained from each subject, and personal data from each participant regarding demographic characteristics, such as sex, age, and other related risk factors including smoking were collected via questionnaire. The collection of blood samples for this study was approved by the appropriate Institutional Ethics Committees. DNA was extracted from the blood samples using modified salting out method (Nasiri et al., 2005).

Genotype analysis in cases and controls

Previously reported PCR primers corresponding to the sequences of *ECRG1* gene were used in the amplification, forward primer 5´-CAGGGCTTAGCGCTCTGTTA-3´and reverse primer 5´-GCTCATATACTTTGGGCAGCTT-3´ produced a 354 bp fragment(Li et al., 2006). The amplification reaction was carried out in 50µl reaction volume in a 0.2ml PCR tubes(axygen) consisting of 70 ng template DNA, 0.4 μM each primer(Sigma), 0.2 mM each deoxynucleotidetriphosphate (dNTP)(cinnagen), 2.0 mM $MgCl_2$ and 1.0 U Taq DNA polymerase with 1x reaction buffer (Biotools). For PCR amplification, the standard program used was as follows: one initial denaturation step of 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; and a final elongation step of 7 min at 72°C. As the gain of a MspI restriction site occurs in the polymorphic allele, so the PCR product was digested using MspI (Fermentas) restriction enzyme to distinguish the Arg290Gln polymorphism. The 290Gln/Gln genotype gave a single band representing the entire 354 bp fragment, the variant 290Arg/Arg genotype resulted in the formation of two fragments of 232 and 122 bp, and the heterozygous 290Arg/Gln genotype gave all of three fragments of 354, 232 and 122 bp on 2% agarose gel stained with ethidium bromide (Figure 1A). Three genotypes revealed by MspI digestion were confirmed by DNA sequencing (Figure 1B represents represent the complementary sequences of the

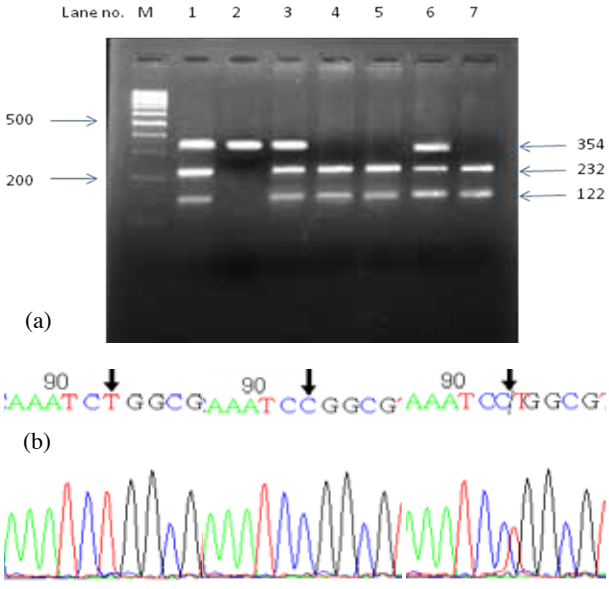

Figure 1. Analysis of the *ECRG1 Arg290Gln* Polymorphism. (a) Representative gel picture showing PCR–RFLP analysis of the ECRG1 genotypes in genomic DNAs of study subjects with the restriction enzyme MspI. M, DNA size markers; Subjects 1, 3 and 7, Arg/Arg genotype; Subjects 2 and 6, Arg/Gln genotype; Subjects 4 and 5, Gln/Gln genotype. (B) Partial DNA sequence of three different allelic PCR products analyzed directly with an DNA sequencer ABI prism 310 automatic sequencer [These sequences represent the complementary sequences of the actual sequences documented for ECRG1 by various research groups, as such the actual G to A transition at the nucleotide 869 (codon 290) in exon 8 (869 G→A) is seen to be represented by complementary C/T substitution]

Table 1. General Characteristics of the Population

Characteristics		Cases (165)	Controls (200)	P value
Age	≤60	93 (56.4)	103 (51.5)	0.804
	>60	72 (43.6)	97 (48.5)	
Gender	Female	70 (42.4)	83 (41.5)	0.862
	Male	95 (57.6)	117 (58.5)	
Dwelling	Urban	22 (13.3)	35 (17.5)	0.275
	Rural	143 (86.7)	165 (82.5)	
Smoking/Snuff	Ever	141 (85.5)	158 (79.0)	0.11
	Never	24 (14.5)	42 (21.0)	
Salt Tea Consumption	≤3 cups/day	55 (33.3)	86 (43.0)	0.059
	>3 cups/day	110 (66.7)	114 (57.0)	
Economic Status	≤5000/month	135 (81.8)	155 (77.5)	0.31
	>5000/month	30 (18.2)	45 (22.5)	
Fruit & Vegetable	Very Low	110 (66.7)	146 (73.5)	0.188
	Moderate/High	55 (33.3)	54 (37)	

Table 2. Genotypic and Allelic Frequencies of *ECRG1* among Case and Controls and Their Association with Risk of ESCC

Gene	Variants	Cases (n=165)	Controls (n=200)	O.R (95% CI)
ECRG1	Arg/Arg	88 (53.65)	132 (66.0)	1.00
	Arg/Gln	66 (40.0)	60 (30.0)	1.65 (1.061-2.566)
	Gln/Gln	11 (6.0)	8 (4.0)	2.62 (0.79-5.33)
	Arg/Gln + Gln/Gln	77 (46.6)	68 (34.0)	1.698 (1.1124-2.593)

actual sequence).

Statistical analysis

Statistical analysis on the data was performed using GraphPad Prism version 5.0 software. The χ^2-test (Pearson's χ^2) was used to examine differences in demographic variables, distribution of genotypes. The association between genotype and risk of ESCC was estimated by calculating odds ratios (ORs) and their 95% confidence intervals (95% CIs). A P value of <0.05 was used as a criterion for statistical significance.

Results

In the present study 165 blood samples from ESCC patients and 200 blood samples from healthy controls were used. The patients comprised 95 males and 70 females (M/F ratio = 1.36) and the control subjects consisted of 118 males and 82 females (M/F ratio = 1.44). General characteristics of the ESCC patients and controls are given in Table 1. No significant age or gender related differences were observed between the groups (P > 0.05) suggesting that the frequency matching was adequate. Allelic and genotypic frequencies of three variants (*Arg/Arg*, *Arg/Gln*, *Gln/Gln*) resulting from the SNP in exon 8 of *ECRG1* gene are given in the Table 2.

Allelic frequencies and genotype distributions of the *ECRG1 Arg290Gln* polymorphism in cases are shown in Table 3. The allelic frequencies for occurrence of *ECRG1 290Arg* and *290Gln* were 73.7% and 26% among controls, and 81% and 19% among ESCC cases, respectively, with the *290Gln* allele being significantly more prevalent in cases than in the controls, suggesting that subjects carrying at least one *290Gln* allele had an increased risk for the development of ESCC compared with subjects carrying the 290 *Arg/Arg* genotype (O.R = 1.698, 95% CI= 1.1124 - 2.593, P< 0.013), because the *290Gln/Gln* genotype was relatively infrequent, it was combined with the *290Arg/Gln* genotype as a group for analysis.

On examining the effect of *ECRG1* genotype by stratifying it against potential variables such as age, sex, smoking status etc. Statistically significant results were obtained between the polymorphism and histopathological grade, smoking, dysphagia, low fruit/vegetable intake and salt tea consumption. On contrary no statistically significant results were observed for *ECRG1* polymorphism with respect to age, gender, dwelling and economic status, other important factors determining predisposition to the cancer (see Table 3).

Table 3. Clinicopathological Characteristics of Esophageal Cancer Patients with Reference to the DNA Polymorphism in the Esophageal Cancer Related Gene 1

Variables		Total n=165	Arg/Arg n=88	Arg/Gln n=66	Gln/Gln n=11	χ^2, P value
Age	≤60	93 (56.36)	49 (55.68)	36 (54.54)	8 (72.72)	1.303, 0.52
	>60	72 (43.63)	39 (44.31)	30 (45.45)	3 (27.27)	
Gender	Female	70 (42.42)	36 (40.90)	28 (42.42)	6 (54.54)	0.744, 0.689
	Male	95 (57.57)	52 (59.09)	38 (57.57)	5 (45.45)	
Dwelling	Urban	22 (13.33)	14 (15.90)	5 (07.57)	3 (27.27)	4.248, 0.119
	Rural	143 (86.66)	74 (84.09)	61 (92.42)	8 (72.72)	
Smoking/ Snuff	Ever	137 (83.30)	79 (89.72)	48 (72.72)	10 (90.9)	8.29, 0.015
	Never	28 (16.96)	9 (10.22)	18 (27.27)	1 (9.09)	
Dysphagia	Yes	126 (76.36)	69 (78.4)	53 (80.30)	4 (36.36)	10.52, 0.0051
	No	39 (23.63)	19 (17.04)	13 (19.69)	7 (63.63)	
Economic Status	≤5000/month	135 (81.81)	76 (86.36)	51 (77.27)	8 (72.72)	2.75, 0.25
	>5000/month	30 (18.18)	12 (13.63)	15 (22.72)	3 (27.27)	
Fruit & Vegetable Intake	Very Low	110 (66.66)	51 (57.95)	50 (75.75)	9 (81.81)	6.597, 0.0369
	Moderate/High	55 (33.33)	37 (42.04)	16 (24.24)	2 (18.18)	
Family History	Familial	8 (4.84)	1 (1.13)	6 (9.09)	1 (9.09)	5.63, 0.05
	Sporadic	157 (95.15)	87 (98.86)	60 (90.9)	10 (90.90)	
Hitopathology Grade	PD	66 (40.0)	48 (54.54)	17 (25.75)	1 (9.09)	17.72, 0.0001
	MD/WD	99 (60.0)	40 (45.45)	49 (74.24)	10 (90.9)	
Salt Tea consumption	≤3cups/day	55 (33.33)	38 (43.18)	14 (21.21)	3 (27.27)	8.386, 0.015
	>3cups/day	110 (66.66)	50 (56.81)	52 (78.78)	8 (72.72)	

Discussion

Although some work has been carried out to elucidate the role of *ECRG1* in esophageal cancer (Wang et al., 1998; Zhao et al., 2004; Li et al., 2006; Bachmann et al., 2009), no report regarding the role of this gene in ESCC is available from this region. Taking into account the impact of reduced function of a tumor suppressor protein resulting from the amino acid substitution in the conserved catalytic domain of the protein (Boassa and Yool, 2003; Shiokawa et al., 2005), we assessed the most important SNP of *ECRG1* (*Arg290Gln*) in an ethnic Kashmiri population for the first time and found that ESCC patients with at least one Gln allele were at an increased risk of developing the cancer as compared to the subjects carrying 290 *Arg/Arg* genotype.

Since smoking is a risk factor for ESCC and effects the expression and function of many tumor suppressor genes, it was found that *ECRG1* polymorphism has a significant association with smoking status in Kashmiri population. This observation is consistent with the previous observations by Li et al., 2006, who found a multiplicative joint effect between smoking and *ECRG1* polymorphism. Higher risk of ESCC among smokers with the variant *ECRG1* genotype may be attributed to the normal esophageal cells or initiated esophageal cancer cells resulting from exposure to tobacco carcinogen such as the nitrosonornicotine (NNK) (Wynder and Hoffmann, 1994). In the present study 90.9% of the patients with Gln/Gln genotype were smokers. .

Low fruit and vegetable intake has been shown as a strong risk factor for development of ESCC in a number of studies (Cheng and Day, 1996; De Stefani et al., 2000). Anti-carcinogenic factors found in those food include ascorbic acid, vitamin E, carotenoids, flavonoids, phytosterols, indoles, fiber, among others (World Cancer Research Fun- IARC, 1997). Consumption of hot salted tea (noon chai) in bulk has been considered as a potential risk factor for the development of ESCC in Kashmir (Siddiqi et al.,1989; Kumar et al., 1992). This has been attributed to probable exposure to nitroso compounds, amines and nitrates present in the dried tea leaves (Siddiqi et al., 1992; Murtaza et al., 2006). Interestingly, when we stratified our data on *ECRG1* polymorphism with respect to consumption of fruits and vegetables and degree of consumption of hot salted tea, we found higher percentage of ESCC patients with Gln/Gln genotype (81.81 and 72.72% respectively), consumed lesser amounts of fruits and vegetables and more than 4 cups of hot salted tea. Since all the above given factors have been well established to have a strong role to play in the development of ESCC in most of the cases, it is suggested that these factors may in turn increase the possibility to develop malignancy under the condition of lower tumor suppressor *ECRG1* function . In addition, effect of the *ECRG1 290Arg/Gln* and *290Gln/Gln* genotypes on risk of ESCC seemed to be more pronounced in cases with higher tumour grade than those having lower tumor.

In conclusion, our study demonstrated a significant association between the *ECRG1* genetic polymorphism (occurence of at least one Gln allele) and ESCC in Kashmiri population, suggesting a possible role for *ECRG1 Arg290Gln* polymorphism in the etiopathogenesis of ESCC in this region. However the evaluation of the impact of the polymorphism of *ECRG1* on the development and prognosis of the disease has to be explored in the future.

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