Role of *CYP2E1* genotypes in susceptibility to colorectal cancer in the Kashmiri population

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Abstract

Cytochrome P450 2E1 (CYP2E1) is a key enzyme involved in the metabolic activation of procarcinogens such as N-nitrosoamines and low-molecular-weight organic compounds. The main aim of this study was to determine whether *CYP450 2E1* polymorphisms are associated with the risk of colorectal cancer (CRC). We investigated the genotype distribution of the *CYP2E1* gene *RsaI* and a 96-base pair (bp) insertion in 86 CRC cases in comparison with 160 healthy subjects. We found the frequency of the *CYP2E1 RsaI* genotype to be 53.5 per cent (46/86) for c1/c1, 17.4 per cent (15/86) for c1/c2 and 29.1 per cent (25/86) for c2/c2, and the *CYP2E1* 98-bp insertion frequencies to be 63.9 per cent (55/86) for non-insertion (i/i), 22.1 per cent (19/86) for heterozygous insertion (i/I) and 36.0 per cent (12/86) for homozygous insertion (I/I) among CRC cases. We also found the *CYP2E1 RsaI* c2/c2 and *CYP2E1* 98-bp heterozygous i/I genotypes to be significantly associated with an increased risk of CRC ($p = 0.01$). We suggest that *CYP2E1* polymorphisms are involved in the susceptibility to developing CRC in the ethnic Kashmiri population.

Keywords: *colorectal cancer, CYP2E, polymorphism, Kashmir*

Introduction

Colorectal cancer (CRC) is one of the major causes of mortality and morbidity, and is the fourth most common cancer in men and the third most common cancer in women worldwide.[1] Kashmir has been reported as being a high-incidence area for gastrointestinal (GIT) cancers.[2,3] In the Kashmir valley, CRC represents the third most common GIT cancer after oesophageal and gastric cancers.[4–6]

Epidemiological studies on various populations have shown that an increased risk of developing GIT cancers is associated with diet.[2,7,8] One important hypothesis that has received a large amount of attention is that N-nitroso compounds from dietary sources are involved in the carcinogenesis of GIT cancer.[9,10] It is known that most exogenous (xenobiotics) and endogenous chemical carcinogens require biotransformation to activated forms to be carcinogenic.[11,12] Most of the enzymes involved in drug metabolism are genetically polymorphic, and these polymorphisms may affect enzyme activity or inducibility.[13–15]

The cytochrome P450 2E1 gene (*CYP2E1*) is located on chromosome 10q26.3. It is 18,754 base pairs (bp) long, consists of nine exons and eight introns and encodes a 493-amino acid protein. CYP2E1 belongs to the cytochrome P450 superfamily.[16] It is a natural ethanol-inducible enzyme and is

of great interest because of its role in the metabolism and bioactivation of many low molecular weight compounds, including ethanol and acetone, drugs such as acetaminophen, isoniazid, chlorzoxazone and fluorinated anaesthetics and many procarcinogens such as benzene, N-nitrosoamines, vinyl chloride and styrene.[17–20]

CYP2E1 contains six restriction fragment length polymorphisms, of which the *RsaI* polymorphism (*CYP2E1*5B*; C-1054T substitution) and the 96-bp insertion in its 5′-flanking region have drawn much interest.[16,20,21] The *RsaI* polymorphism has been shown to affect the transcriptional level of the gene. The variant type of this polymorphic site can enhance the transcription and increase the level of CYP2E1 enzymatic activity *in vitro*.[22] The variant allele of the 96-bp insertion polymorphism has been shown to express greater transcriptional activity.[23]

We carried out a case–control study in our population to determine if these *CYP2E1* polymorphisms are associated with an altered risk of developing CRC. We also investigated whether there was a link between the clinicopathological variables and the *CYP2E1* genotype, and hence their role in CRC predisposition.

Materials and methods

Study population

This study included 86 CRC cases. All patients were recruited from the Department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences, Kashmir. Blood samples were collected from 160 age- and sex-matched individuals with no signs of any cardiac disease, to serve as external controls. The mean age of both patient and control groups was 52 years (see Table 1 for details).

Data on all CRC patients were obtained from personal interviews with patients and/or their guardians, and their medical records. All patients and/or guardians were informed about the study and their willingness to participate was recorded on a predesigned questionnaire (available on request). The collection and use of blood samples (from patients and controls) for this study had been previously approved by the appropriate institutional ethics committee.

Table 1. Frequency distribution analysis of selected demographic and risk factors in colorectal cancer cases and controls

Variable	Cases (n = 86)	Controls (n = 160)	p Value
Age group			
≤50	30 (34.9%)	56 (35.0%)	1
>50	56 (65.1%)	104 (65.0%)	
Gender			
Female	37 (43.0%)	72 (45.0%)	0.764177
Male	49 (67.0%)	88 (55.0%)	
Dwelling			
Rural	59 (68.6%)	104 (65.0%)	0.565659
Urban	27 (31.4%)	56 (35.0%)	
Smoking status			
Never	31 (36.0%)	75 (46.8%)	0.102256
Ever	55 (64.0%)	85 (53.2%)	
Pesticide exposure			
Never	33 (38.4%)	75 (46.8%)	0.200325
Ever	53 (61.6%)	85 (53.2%)	

DNA extraction and polymerase chain reaction

DNA extraction was performed using the ammonium precipitation method. Genotyping for the *CYP2E1 RsaI* and 96-bp insertion polymorphisms was determined by the method described by Morita *et al*.[21] The oligonucleotide primers used for the amplification of the target regions are listed in Table 2. The polymerase chain reaction (PCR) was carried out in a final volume of 25 μl, containing 50 ng genomic DNA template, 1× PCR buffer (Fermentas, Glen Burnie, MD), with 2 mM $MgCl_2$, 0.4 μM of each primer (GenScript, Piscataway, NJ), 50 μM deoxynucleotide triphosphates (dNTPs) (Fermentas) and 0.5 U DNA polymerase (Fermentas). For PCR amplification, the standard programme was used as follows: one initial denaturation step at 94°C for 7 minutes, followed by 30 denaturation cycles of 30 seconds at 94°C, 45 seconds of annealing at X°C (See Table 2) and 45 seconds of extension at 72°C for 35 cycles, followed by a final elongation cycle at 72°C for 7 minutes.

The PCR product of *CYP2E1 RsaI* was 413 bp in length and was then digested with 2 U *RsaI* in a reaction mixture of 20 μl for 3 hours at 37°C.

Table 2. Primers for *CYP2E1* gene polymorphism

Target codon	Sequence	Amplicon (bp)	T_m (°C)
CYP2E1* 5B RsaI	F5′-CCAGTCGAGTCTACATTGTCA-3′ R5'-TTCATTCTGTCTTCTAACTGG-3′	413 bp	55
***CYP2E1* 96-bp insertion**	F5′-GTGATGGAAGCCTGAAGAACA-3′ R5′-CTTTGGTGGGGTGAGAACAG-3′	729 bp for insertion 633 bp for non-insertion	66

T_m, melting temperature.

The digestion resulted in fragments of 352 bp and 61 bp for the c1 allele. The PCR product of the *CYP2E1* 96-bp insertion allele was 729 bp in length and that of the non-insertion allele was 633 bp in length.

DNA amplicons, as well as the digestion products, were electrophoresed through a 2–3 per cent agarose gel (Genie, Bangalore, India) for resolution. The genotypes of >20 per cent of the samples were reassessed in a double-blind manner by two independent researchers, to confirm the results. A positive control for each polymorphism was used for 50 per cent of the samples.

Statistical analysis

The observed frequencies of the above genotypes in patients with CRC were compared with controls using chi-square or Fisher exact tests when the expected frequencies were small. The chi-square test was used to verify whether the genotype distributions were in Hardy–Weinberg equilibrium. Statistical significance was set at $p \leq 0.05$. Statistical analyses were performed using PASW version 18 software.

Results

A total of 86 CRC cases and 160 control subjects were included in this study. The patients comprised 49 males and 37 females (M/F ratio = 1.32) and the control subjects consisted of 88 males and 72 females (M/F ratio = 1.2). The mean age in patient and control groups was 52 years. No significant gender- or age-related differences were observed between the groups ($p > 0.05$). Furthermore, of 86 confirmed cases of CRC, 81 were sporadic, four were familial adenomatous polyposis and one case was hereditary non-polyposis (Lynch Syndrome) CRC. All but one case had an adenocarcinoma, one had squamous cell carcinoma of the basal cell type. Thirty-six patients had carcinoma in the colon and 50 carcinoma in the rectum. Fifty-nine were rural- and 27 urban-living, and 55 were smokers and 31 non-smokers (see Table 1 for further details).

Among the CRC cases, we found the frequency of the *CYP2E1 RsaI* genotype to be 53.5 per cent (46/86) for c1/c1, 17.4 per cent (15/86) for c1/c2 and 29.1 per cent (25/86) for c2/c2, while the frequency in the general control population was 70.0 per cent (112/160) for c1/c1, 12.5 per cent (20/160) for c1/c2 and 17.5 per cent (28/160) for c2/c2. The association between the *CYP2E1 RsaI* polymorphism and the CRC cases was found to be significant ($p < 0.05$) (Table 3). Furthermore, for the 96-bp insertion polymorphism of *CYP2E1*, the genotype frequencies were 63.9 per cent (55/86) for non-insertion (i/i), 22.1 per cent (19/86) for the heterozygous insertion (i/I) and 13.9 per cent (12/86) for the homozygous insertion (I/I), while in the general control population the frequency of i/i was found to be 81.3 per cent (130/160), 7.5 per cent (12/160) for i/I and 11.3 per cent (18/160) I/I. The association of the *CYP2E1* 98-bp insertion polymorphism with the CRC cases was also found to be significant ($p < 0.05$) (Table 3). In individual patients it was found that the *CYP2E1 RsaI* c2/c2 and *CYP2E1* 96-bp heterozygous i/I genotypes were both associated with an increased risk of CRC ($p = 0.01$ and 0.0009, respectively).

Analysis of the *CYP2E1 RsaI* and 98-bp insertion polymorphisms with that of the clinicopathological parameters also revealed significant associations with many parameters (Tables 4 and 5). The *CYP2E1 RsaI* c2/c2 genotype was associated

Table 3. Genotype frequencies of *CYP2E1* polymorphism in cases and controls

CYP2E1 genotype	Cases ($n = 86$)	Controls ($n = 160$)	OR (95% CI); χ^{2a}; p value[b]	χ^2; p value (overall)
RsaI				
c1/c1 (wild-type)	46 (53.5%)	112 (70.0%)	1.0 (ref)	**6.81; 0.03**
c1/c2	15 (17.4%)	20 (12.5%)	1.8 (0.86–3.87); 0.11; 0.15	
c2/c2 (variant)	25 (29.1%)	28 (17.5%)	**2.17 (1.14–4.11); 0.01; 0.01**	
c1/c2 or c2/c2	40 (46.5%)	48 (30.0%)	**2.02 (1.17–3.48); 0.009; 0.01**	
96-bp insertion				
i/i (non-insertion)	55 (63.9%)	130 (81.3%)	1.0 (ref)	**12.1; 0.002**
i/I	19 (22.1%)	12 (7.5%)	**3.74 (1.7–8.23); 0.0006; 0.0009**	
I/I (insertion)	12 (13.9%)	18 (11.3%)	1.57 (0.79–3.49); 0.25; 0.29	
i/I or I/I	31 (36.0%)	30 (18.8%)	**2.44 (1.31–4.41); 0.003; 0.003**	

[a] Pearson value; [b]Fisher exact value. Significant p values are shown in bold.

significantly ($p < 0.05$) with age, nodal status (Table 4) and tumour grade, and the *CYP2E1* 96-bp i/I genotype was associated significantly ($p < 0.05$) with tumour location (Table 5).

Discussion

As reported previously in various studies on the Kashmiri population,[2,4,5] it has been proven beyond doubt that this population is exposed to a special set of environmental and dietary risks, including exposure to nitroso compounds, amines and nitrates, reported to be present in local foodstuffs, most of which have been shown to contain important irritants and carcinogens.[3,6]

In the present study, we assessed the two most common single nucleotide polymorphisms of *CYP2E1*, *RsaI* and the 96-bp insertion, in an ethnic Kashmiri population for the first time, as the role of these polymorphisms in relation to the risk of CRC has not been reported from this part of the world.

Although a number of studies have been carried out around the world on the association between the *CYP2E1* polymorphism and cancer risk, the findings have been inconsistent.[24] Some studies demonstrated that the common genotype or alleles (i.e. *RsaI* and the 96-bp insertion) confer a greater risk of oral and pharyngeal,[25] oesophageal,[26] liver[27] and lung[28] cancers. In other studies, however, an increased risk of oral,[29] nasopharyngeal,[30] liver[31] and colorectal[32,33] cancers was observed with the rare genotype or allele carriers (i.e. the variant form of these two genotypes).

In the present study, we found the frequency of the *CYP2E1 RsaI* genotype to be 53.5 per cent (46/86) for c1/c1, 17.4 per cent (15/86) for c1/c2 and 29.1 per cent (25/86) for c2/c2 among CRC cases, and the *CYP2E1 RsaI* polymorphism to be significantly associated with the risk of CRC ($p < 0.05$). The overall risk of CRC was found to be 2.17 times higher in case of c2/c2 homozygous state. These results were consistent with those of Kiss *et al.*[32] and Yu *et al.*[33] Kiss *et al.* found the *CYP2E1* c2 allele to be significantly associated with CRC (odds ratio 1.91, 95 per cent confidence interval 1.05–3.52) in a Hungarian population.[32] Yu *et al.* found the *CYP2E1* c2 allele to be a susceptibility factor for CRC.[33] This may be because of the increased transcriptional activation of the c2 variant of the *CYP2E1* gene, with elevated expression levels of *CYP2E1* mRNA and protein.[34] We also found a significant association between the *CYP2E1 RsaI* genotype and age (>50), nodal status (involved) and tumour grade (C + D). Furthermore, a recent meta-analysis by Zhou *et al.* also revealed the *CYP2EI RsaI* c2/c2 genotype to be associated with an increased risk of CRC.[20]

In the case of the 98-bp insertion polymorphism, we found that the genotype frequencies were 63.9 per cent (55/86) for non-insertion (i/i), 22.1

Table 4. Association between *CYP2E1* (*RsaI*) polymorphism and clinicopathological characteristics

Variables	Cases ($n = 86$)				
	$n = 86$	c1/c1 46 (53.5%)	c1/c2 15 (17.4%)	c2/c2 25 (29.1%)	χ^2; p value
Age group					
≤50	30 (34.9%)	21	5	4	***6.29; 0.04***
>50	56 (65.1%)	25	10	21	
Gender					
Female	37 (43.0%)	20	6	11	0.07; 0.96
Male	49 (67.0%)	26	9	14	
Dwelling					
Rural	59 (68.6%)	27	11	21	5.0; 0.08
Urban	27 (31.4%)	19	4	4	
Smoking status					
Ever	55 (64.0%)	29	8	18	1.45; 0.48
Never	31 (36.0%)	17	7	7	
Tumour location					
Colon	36 (41.9%)	19	5	12	0.84; 0.65
Rectum	50 (58.1%)	27	10	13	
Nodal status					
Involved	48 (55.8%)	24	4	20	***11.34; 0.003***
Not Involved	38 (44.2%)	22	11	5	
Tumour grade					
A + B	38 (44.2%)	22	11	5	***11.34; 0.003***
C + D	48 (55.8%)	24	4	20	
Pesticide exposure					
Ever	53 (61.6%)	26	8	19	3.13; 0.20
Never	33 (38.4%)	20	7	6	
Bleeding PR/constipation					
Yes	60 (69.8%)	32	10	18	0.13; 0.93
No	26 (30.2%)	14	5	7	
Tumour type[a]					
Mucinous	33 (38.5%)	20	6	7	1.84; 0.39
Non-mucinous	52 (60.5%)	25	9	18	

[a]One was squamous cell carcinoma. Significant p values are shown in bold.

per cent (19/86) for heterozygous (i/I) and 36.0 per cent (12/86) in case of homozygous insertion (I/I) among CRC (86) cases and heterozygous i/I genotype to be associated with increased risk of CRC ($p = 0.0009$). The overall risk of CRC was found to be 3.74 times higher in the case of the i/I heterozygous state. These results are different from those found in a previous study on CRC;[21] however, the present study is only the third such study on this polymorphism to have been carried out. The other study in this field examined the relationship between the 96-bp insertion polymorphism and cancer risk, and was carried out in Japan by Itoga *et al.*,[35] who found an increased risk of oesophageal cancer, but not of lung cancer, in individuals who had two variant 96-bp insertion alleles.

We also found a significant association between the *CYP2E1* i/I heterozygous genotype and the

Table 5. Association between *CYP2E1 (96 bp)* polymorphism and clinicopathological characteristics

Variables	Cases ($n = 86$)				
	$n = 86$	i/i 55 (63.9%)	i/I 19 (22.1%)	I/I 12 (13.9%)	χ^2; p value
Age group					
≤50	30 (34.9%)	19	7	4	0.05; 0.97
>50	56 (65.1%)	36	12	8	
Gender					
Female	37 (43.0%)	26	8	3	2.0; 0.36
Male	49 (67.0%)	29	11	9	
Dwelling					
Rural	59 (68.6%)	38	13	8	0.03; 0.98
Urban	27 (31.4%)	17	6	4	
Smoking status					
Ever	55 (64.0%)	35	10	10	3.01; 0.22
Never	31 (36.0%)	20	9	2	
Tumour location					
Colon	36 (41.9%)	22	5	9	***7.38; 0.02***
Rectum	50 (58.1%)	33	14	3	
Nodal status					
Involved	48 (55.8%)	28	12	8	1.53; 0.46
Not Involved	38 (44.2%)	27	7	4	
Tumour grade					
A + B	38 (44.2%)	27	7	4	1.53; 0.46
C + D	48 (55.8%)	28	12	8	
Pesticide exposure					
Ever	53 (61.6%)	38	9	6	3.62; 0.16
Never	33 (38.4%)	17	10	6	
Bleeding PR/constipation					
Yes	60 (69.8%)	42	11	7	3.15; 0.20
No	26 (30.2%)	13	8	5	
Tumour type*					
Mucinous	33 (38.5%)	22	7	4	0.27; 0.87
Non-mucinous	52 (60.5%)	32	12	8	

*One was squamous cell carcinoma. Significant p values are shown in bold.

tumour location (rectum). This finding was in line with that of a case–control study carried out in Hawaii, where the authors showed an increased risk of CRC, especially of rectal cancer, in those having at least one 96-bp insertion.[36] This was hypothesised to be because of a high intake of red meat, which increases the endogenous production of N-nitroso compounds in the intestine. The Kashmiri population is also known to consume high quantities of red meat.

Furthermore, it has been found that both the *RsaI* c2 and 96-bp I alleles are fairly common in Asian populations compared with Caucasians. In the present study, we found the frequencies of *RsaI* c2 and 96-bp I alleles to be of 30.0 per cent and 18.8 per cent, respectively (see Table 3). These frequencies are similar to those found for these alleles in other Asian populations. The frequencies of the *RsaI* c2 allele were 22 per cent in Japanese,[21] 4 per cent in Caucasians[36] and 15 per cent in Hawaiians.[36]

The frequencies of the 96-bp insertion allele were 23 per cent in Japanese,[21] 15 per cent in Taiwanese,[37] 10 per cent in African-American,[37] and 2 per cent in Caucasian[37] subjects.

Conclusion

We conclude that there is a significant relationship between the *CYP2E1 RsaI* and 96-bp insertion polymorphisms and the risk of CRC in the ethnic Kashmiri population. We also observed a significant correlation between the *RsaI* c2/c2 variant and 96-bp i/I heterozygous genotype with various clinicopathological variables in this population. These correlations now need to be authenticated in a large sample study, in order to discern racial differences and determine the aggressiveness of CRC.

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