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ACE Polymorphism in Colorectal Cancer Patients of Kashmiri Population – A Short Report

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Abstract: *Aims*: The angiotensin-converting enzyme (ACE) gene in humans has an insertion-deletion (I/D) polymorphic state in intron 16 on chromosome 17q23. This polymorphism has been widely investigated in different cancers and has been implicated as the risk factor for the development of various cancers. To investigate the ACE I/D genotype frequency in CRC cases in Kashmiri population and to correlate it with the known clinicopathological characters of CRC cases.

Methods: We designed a case control study, where 86 CRC cases were studied for ACE I/D polymorphism against 150 controls taken from general population. The polymorphisms of ACE gene were investigated using polymerase chain reaction for detection of an I/D polymorphism.

Results: Among CRC (86) cases we found the frequency of ACE DD genotype to be 41.86 % (36/86), II 13.95% (12/86) and DI 44.2% (38/86). There was no significant association between the ACE I/D genotype with any of the known clinicopathological characters.

Conclusion: The ACE I/D polymorphism is not a significant risk factor in the CRC carcinogenesis in our population.

Keywords: Colorectal Cancer, *ACE*, Polymorphism, Kashmir.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading malignancies and is the major cause of mortality and morbidity worldwide. CRC is the fourth most common cancer in men and the third most common cancer in women worldwide [1]. So far now Kashmir has been reported as a high-incidence area of GIT cancers [2,3]. In Kashmir valley CRC represents the third most common GIT cancer after esophageal and gastric [4,5].

Angiotensin-converting enzyme (ACE), a key zinc metallo-enzyme of the rennin-angiotensin system is widely distributed in body [6]. The ACE catalyzes the conversion of angiotensin I into the biologically active peptide, angiotensin II, which is involved in the control of fluid-electrolyte balance and systemic blood pressure [7]. Angiotensin II is a potent vasoconstrictor and also mediates cell growth and proliferation by stimulating various cytokines and growth factors [8].

The ACE gene is located on long arm of chromosome 17 (17q23.3), the gene is 21 kilo bases (kb) long and comprises 26 exons and 25 introns. More than 160 *ACE* gene polymorphisms have been reported so far and most of which are single nucleotide polymorphisms (SNPs). Only 34 of these polymorphisms are located in coding region of this gene [9]. Rigat *et al*., [10] is the first to report the insertion/deletion (I/D) polymorphism of ACE. This polymorphism is characterized by the presence (insertion) or absence (deletion) of a 287 bp *Alu*Ya5 element inside intron 16 producing three genotypes (*II* homozygote, *ID* heterozygote and *DD* homozygote). Although the I/D polymorphism is located in a non-coding region (i.e. intron) of the ACE gene, several investigators have found that the *D* allele is related to increased activity of ACE in serum. The highest serum ACE activity was seen in the *DD* genotype while the lowest was seen in the *II* genotype [11].

Several diseases including coronary heart diseases [12], stroke [13], hypertension [14] and diabetes mellitus [15] have been shown to be associated with the ACE I/D polymorphism. A number of studies have been carried out lately on association of ACE I/D polymorphism with various cancers proving that angiotensin II is a key enzyme involved in neovascularization, cell proliferation, inflammation, and cell adhesion [16-21]. Ace gene polymorphism has been reported to be implicated in pancreatic [22], prostate [23,24], breast [25], lung [26], gastric [27], esophageal [28] as well as in CRC [18,29,30].

Therefore, we carried out a case-control study in our population to determine if this ACE I/D polymorphism is associated with an altered risk of developing CRC. We also investigated whether there was a link between the clinicopathological variables with the ACE genotype and hence their role in CRC predisposition.

MATERIALS & METHODS

Study Population

This study included 86 CRC cases. All patients were recruited from Department of Surgery of our Institute. Blood

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Table 1. Primers for ACE Gene Polymorphism

Target Codon	Sequence	Amplicon (bp)	T_m (°C)
ACE	F 5'-CTGGAGACCACTCCCATCCTTTCT -3' R 5'- GATGTGGCCATCACATTCGTCAGAT -3'	490bp for II 190bp for DD	58
ACE I	F5-TGGGACCACAGCGCCCGCCACTAC -3' R5- TCGCCAGCCCTCCCATGCCCATAA-3'	335bp	67

samples of 160 age and sex matched cases with no signs of any cardiac disease or diabetes were collected to serve as external controls. The controls were previously interviewed and only those who fulfilled the predetermined age/sex group were selected after obtaining the consent to participate in the study. The controls belonged to the same racial Kashmiri population of which the cases belonged. 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 or guardians and medical records. All patients or guardians were informed about the study and their will to participate in this study was taken on predesigned questionnaire (Available on request). The collection and use of blood samples (from patients and controls) for this study were previously approved by the appropriate Institutional Ethics Committee.

DNA Extraction & Polymerase Chain Reaction

DNA extraction was performed using Ammonium Acetate Method. Polymorphism in intron 16 of the *ACE* gene was assessed by polymerase chain reaction (PCR) under conditions that have been previously described

The specific segment of ACE gene was amplified by using the previously reported primers (Salem AH *et al*., 2009) which amplified 190bp amplicon in case of homozygous DD genotype, 490bp in case of homozygous II genotype and both in case of heterozygous DI genotype (Fig. **1**). All samples that were identified initially as a *DD* genotype were reanalyzed using an insertion-specific primer pair, as reported by Lindpaintner *et al*. [31], except that the annealing temperature was 67°C. A 335 bp band was obtained only in the presence of the *I* allele and no bands were detected for samples with *DD* genotype. PCR was carried out in a final volume of 25 µL containing 50 ng genomic DNA template,

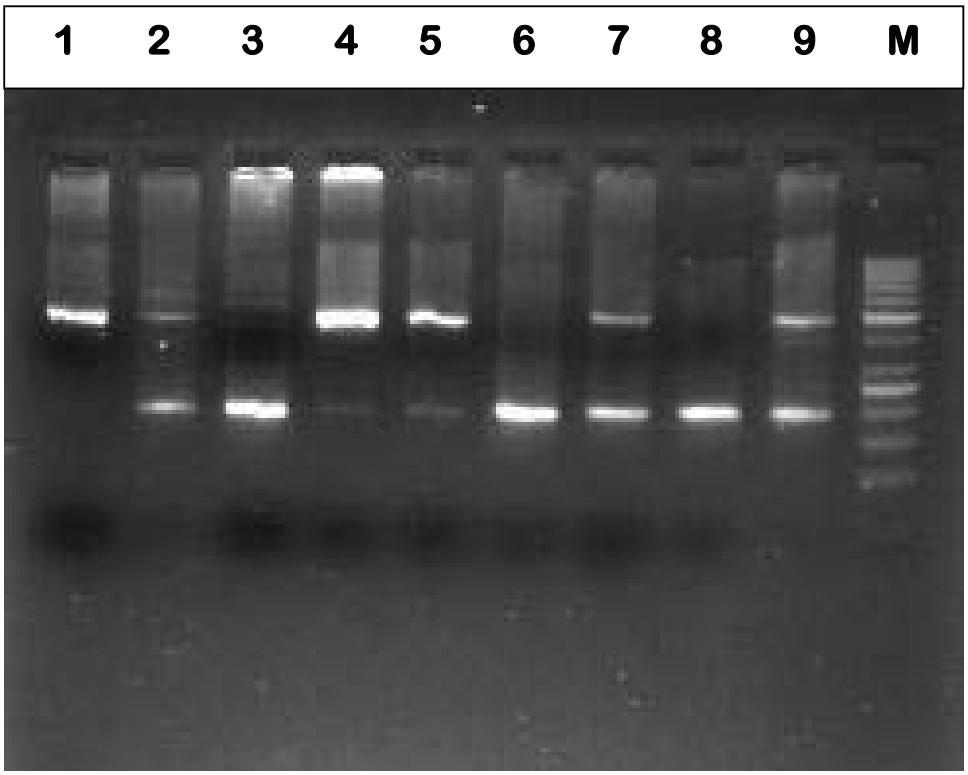

Fig. (1). Representative gel picture of *ACE* DI polymorphism by differential amplification of intron 16 of the *ACE* gene.
Lane M: 50bp ladder.
Lane 1 shows II form,
Lanes 2, 4, 5, 7 & 8 show DI form and.
Lanes 3, 6 & 8 show DD form.

1X PCR buffer (Fermentas) with 2 mM $MgCl_2$, 0.4 µM of each primer (Genescript), 50 µM dNTPs (Fermentas) and 0.5 U DNA polymerase (Fermentas). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 7 min, followed by 30 denaturation cycles of 30 s at 94°C, 45 s of annealing at 58°C, and 45s of extension at 72°C, followed by a final elongation cycle at 72°C for 7 min.

DNA amplicons were electrophoresed through a 2-3% agarose gel for resolution. The genotypes of >20% of the samples were double blindly reassessed to confirm the results by two independent researchers. A positive control for each polymorphism was used for 50% of samples.

Statistical Analysis

Observed frequencies of genotypes in hypertensive patients were compared to controls using chi-square or Fisher exact tests when expected frequencies were small. The chi-square test was used to verify whether genotype distributions were in Hardy-Weinberg equilibrium. Statistical significance was set at $P < 0.05$. Statistical analyses were performed with 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). Mean age in patient and control groups was 52 years. No significant gender- or age-related differences were observed between the groups ($P > 0.05$). Further more, out of 86 confirmed cases of CRC, 81 cases were sporadic, 4 were FAP and one case was HNPCC. All but one case was adenocarcinoma and only one was squamous cell carcinoma (SCC) of basal cell type, 59 rural and 27 urban, 36 cases had carcinoma in colon and 50 in rectum and 55 were smokers and 31 non smokers. See Table **2** for further details.

In this study, among CRC (86) cases we found the frequency of ACE DD genotype to be 41.86 % (36/86), II 13.95% (12/86) and DI 44.2% (38/86), while as in general control (160) population the DD frequency is 53.75 % (86/160), II 13.13% (21/160) and DI 33.13% (53/160). The association of ACE I/D polymorphism with the CRC cases was not found to be significant ($p> 0.05$) (Table **3**).

We also found the hazard ratio of ACE DI genotype in CRC cases to be 1.7 times and of ACE II genotype in CRC to be 1.36 times that of general control population; indicating thereby that ACE I allele is associated with the increased risk of CRC development (Table **3**).

Furthermore, analysis of the ACE I/D polymorphism with that of the clinicopathological parameters did not reveal any sort of significant association. However, we observed that the DI genotype was more common in higher tumor grade cases (24/38; 63.2%) and rectal cancers (25/38; 65.8%) though not of significant levels statistically.

DISCUSSION

Kashmir also called *Pir-e-Waer* is one of the picturesque places on earth located in the northern part of India in between the Himalayas is home of one of the oldest ethnic population that has been proven beyond doubt to be exposed to a special set of environmental and dietary risks which include consumption of sun-dried and smoked fish and meat, dried and pickled vegetables, red chilli, Hakh (a leafy vegetable of Brassica family), hot noon chai (salted tea) and Hukka (water pipe) smoke [2,4,5]. As previously reported, the etiology and incidence of various GIT cancers in this population have been attributed to a probable exposure to nitroso compounds, amines and nitrates are reported to be

Table 2. 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%)	1.00
>50	56 (65.1%)	104 (65.0%)	
Gender			
Female	37 (43.0%)	72 (45.0%)	0.76
Male	49 (67.0%)	88 (55.0%)	
Dwelling			
Rural	59 (68.6%)	104 (65.0%)	0.56
Urban	27 (31.4%)	56 (35.0%)	
Smoking status			
Never	31 (36.0%)	75 (46.8%)	0.10
Ever	55 (64.0%)	85 (53.2%)	
Pesticide Exposure			
Never	33 (38.4%)	75 (46.8%)	0.20
Ever	53 (61.6%)	85 (53.2%)	

Table 3. Genotype Frequencies of ACE Intron 16 Gene Polymorphism in Cases & Controls

ACEs Genotype	Cases (n= 86)	Controls (n=160)	OR (95% CI)	χ^2; P Value
DD	36 (41.86 %)	86 (53.75%)	1 (Ref)	3.47; 0.17
DI	38 (44.2%)	53 (33.13%)	1.7 (0.96-3.02)	
II	12 (13.95%)	21 (13.13%)	1.36 (0.61-3.06)	
DI+II	50	74	1.61 (0.95-2.74)	

present in these local food stuffs most of which have been shown to contain important irritant and carcinogens [3,32].

CRC being the world's third most common malignancy is also paving its way in Kashmiri population where it has increased in the last two decades to become the third most common malignancy of GIT after esophageal and gastric [5].

In one of the pioneer studies by Rocken *et al.* [29]. On ACE I/D polymorphism it was observed that ACE expression levels and hence the DI genotype was directly correlated with the tumor size, thus implicating the ACE as one of the risk factors to CRC development. Also in another retrospective study by the same lab on gastric cancer it was found that ID genotype of the ACE gene influences the tumor development and advancement to metastasis [27]. Also it has also been shown that ID or II genotype were at a greater risk of developing CRC than that of DD carriers [18]. Hence taking these observations into account we designed this prospective cohort study to observe the association of ACE I/D genotype and CRC risk in our population.

We observed the frequency of ACE DD genotype to be 41.86 % (36/86), II 13.95% (12/86) and DI 44.2% (38/86) in CRC cases from our Kashmiri population which are in tune with the frequencies reported by other authors [18,27]. However statistically there was no significant association between the ACE I/D polymorphism with the CRC cases. These results were similar to one of the study where no relation was found between the ACE I/D polymorphism and the risk of CRC development [33,34].

However, we found cases with DI genotype to be 1.7 times and cases with II genotype 1.36 times more predisposed to CRC than that of DD genotype, overall risk of development of CRC for I allele was 1.61 times. These results were also similar to what has been already reported [18,33].

We also found the DI genotype to be more common in higher tumor grade cases (24/38) and rectal cancers (25/38), highlighting the role played by ACE in development of higher advanced tumors as has been reported previously by Rocken *et al.* [29]. As we know ACE helps in the synthesis of Angiotensin II which in turn has been found to be a potent angiogenic factor that may participate in tumorigenesis by promoting angiogenesis in cancer cells by neovascularization, cell proliferation and cell adhesion *via* altered expression of angiotensin II type 1 (AT1R) and type 2 receptor (AT2R) [17,25]. This may somehow explain the relationship between the II and DI genotype with that of the higher tumor grade.

CONCLUSION

Hence in our study we conclude that although there is a correlation between the ACE I/D genotype with the higher tumor grade but the polymorphism is not a significant risk factor in the CRC carcinogenesis in our population. Thus we conclude that the ACE I/D polymorphism is negatively related to the development and progression of CRC in our Kashmiri population, these results conform to the previous studies [33,34].

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