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ASSOCIATION OF ALU-ACE AND CYP1A1 WITH COLORECTAL CANCER

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ABSTRACT

Colorectal cancer (CRC) defined as the cancerous growths in the colorectal, rectum and appendix is also referred to as colorectal cancer or large bowel cancer. In the last two decades, studies have demonstrated that CRC cells undergo major epigenetic alterations. Among which, genetic variants in oncogenes have been extensively investigated as the essential role in cancer aetiology. The ACE I/D and CYP1A1 polymorphisms were detected by PCR-RFLP. Analysis of the data was carried out using Epi Info 5 software. Pooled odds ratios and relative risk were calculated by the random-effects method. Multifactor Dimensionality Reduction (MDR) analysis was performed to study case-control data and gene-gene interactions. Considering Alu-ACE, the inter group heterogeneity was found to be ($\chi^2 = 11.1576$; d.f. =2; $(0.01>p>0.001)$, significant value when observed between colorectal cancer patients and controls. Risk estimates show a significant association of ID phenotype (RR = 1.85), an increased risk of 85% and more, indicating that individuals with ID phenotype are 1.8 times more likely to get the disease when compared with the other phenotypes of the Alu-ACE polymorphism. Risk estimates show a significant association of m1m2 and m2m2 phenotypes with colorectal cancer individuals (RR = 1.23 and 1.36) respectively. In MDR analysis, the two-factor interaction model was the best model, which shows that there was an interaction between the two SNP's ($p \leq 0.01$). This study concludes that I/D polymorphism in the ACE gene may confer the risk of colorectal cancer.

KEYWORDS: Colorectal, Alu- ACE, CYP1A1, Polymorphism.

INTRODUCTION

Colorectal cancer (CRC) defined as the cancerous growths in the colorectal, rectum and appendix is also referred to as colorectal cancer or large bowel cancer. Colorectal carcinoma (CRC) accounts for 9.4% of total cancer cases.[1] It is a commonly diagnosed cancer in both men and women and represents the third most common form of cancer and the second leading cause of cancer-related death in the world.[2] Each year, a global incidence exceeding 1.2 million new cases emerge and 600,000 deaths occur.[3] It is more common in developed than developing countries.[4] The initiation and progression of colorectal cancer development is modulated by the gradual accumulation of both environmental and genetic factors.[5] The mechanism of this form of carcinogenesis is still not fully understood. Despite environmental agents such as cigarette smoking, dietary, alcohol consumption and obesity, found to be major risk factors for colorectal cancer, only a fraction of individuals exposed to these factors develop colorectal cancer during their lifetime,[6-8] suggesting that genetic factors play an important role in the development of colorectal cancer. In the last two decades, studies have demonstrated that CRC cells undergo major epigenetic alterations.[9] Among which, genetic variants in oncogenes have been extensively investigated as the essential role in cancer aetiology.[10]

Angiotensin-converting enzyme (ACE), a major participant in the rennin-angiotensin system converts angiotensin I to the vasoconstrictor angiotensin II.[11] ACE has been implicated in the pathology of several carcinomas such as breast, prostate, endometrial and oral cancer, since its inhibition suppresses tumor growth and angiogenesis.[12-17] The ACE gene is located on human's chromosome 17q23 that consists of 26 exons and 25 introns.[18] The insertion deletion (I/D) polymorphism in this gene refers to an Alu repetitive sequence 287 bp long, in intron 16, resulting in three genotypes, DD and II homozygotes and ID heterozygotes. The I/D polymorphism is reported to determine circulating and tissue ACE levels, such that individuals homozygous for the D allele have higher tissue and plasma ACE concentrations than heterozygotes and II homozygotes.[18]

The Human Genome Project identified 57 human Cytochrome P450 enzymes (CYPs), ordered into 18 families and 43 subfamilies by sequence similarities.[20] These genes code for enzymes that play a role in: metabolism of drugs, foreign chemicals, arachidonic acid and eicosanoids; steroid synthesis and metabolism; cholesterol metabolism and bile-acid biosynthesis; vitamin D synthesis and metabolism; retinoic acid hydroxylation; and those of

still unknown function.[21] Moreover, CYP expression alters various downstream signal-transduction pathways and such changes can be precursors to malignancy.[21] Three polymorphisms of the *CYP1A1* gene have been examined with regard to cancer susceptibility: a T→C transition in the 3′-untranslated region which creates a *Msp*I restriction site (*CYP1A1*2A*, rs 4646903) and an A→G transition in exon 7 leading to a substitution of valine for isoleucine at codon 462 (*CYP1A1*2C*, rs 1048943).[22-25] The CYP1A1 m4 allele located in exon 7, exchanges threonine 461 with asparagine (rs 1799814).[26] These polymorphisms have been associated with increased activity of the enzyme, and increased carcinogen activation would be expected to increase the risk of cancer. Several studies reported an increased risk of colorectal cancer associated with *CYP1A1*2A*[23] and *CYP1A1*2C*[27] while others showed no association of either *CYP1A1*2A* or *CYP1A1*2C* with colorectal cancer [28-31] or adeno adenomas.[32] Considering the extensive role of ACE and CYP1A1 (MspI) in the pathogenesis of cancers, this study was performed to estimate the association between these polymorphisms and colorectal cancer risk in patients with colorectal adenocarcinoma and healthy controls.

MATERIALS AND METHODS

Cases were the patients with histologically confirmed colorectal adenocarcinoma who were admitted in Government King George General Hospital, Visakhapatnam, Andhra Pradesh for surgical treatment. The study was ethically approved for collecting blood samples from the human subjects by the local [Andhra University] ethics committee. Eligible cases were 30 men and 30 women aged 20 to 74 years at time of diagnosis; had no prior history of partial or total removal of the colorectal, inflammatory bowel disease; and were mentally competent to give informed consent. Sixty controls were randomly selected from the study area by frequency-matching with respect to gender. Eligibility criteria for controls were the same as described for the cases except that they had no prior diagnosis of colorectal cancer. Blood samples were collected in sterile vials containing 15% EDTA as an anticoagulant from both controls and patients for DNA isolation. DNA was isolated by salting out method.[33]

The ACE I/D polymorphism was detected by PCR as described by Bather.[34] The primers used were: 5'- CTGGAGACCACTCCCATCCTTTCT-3' as forward primer and 5'-GATGTGGCCATCACATTCGTCAGAT -3' as reverse primer. Amplification resulted in a combination of a 490 bp product and / or a 190 bp product depending on the presence or absence of the ACE I- allele fragment, respectively. The CYP1A1 polymorphism found in

the 3-flanking region was detected by PCR-RFLP as described by Kawajiri.[35] The PCR product was subjected to restriction digestion using MspI enzyme. The primers used were: 5'-CAGTGAAGAGGTGTAGCCGCT-3' and 5'-TAGGAGTCTTGTCTCATGCCT-3'. The CYP1A1 polymorphisms were classified as homozygous for m1/m1 (CYP1A1 A genotype which produced a 340-bp band), heterozygous for m1/m2 (CYP1A1 B genotype which produced 340, 200 and 140-bp bands), or homozygous for m2/m2 (CYP1A1 C genotype which produced 200 and 140-bp bands) alleles. The amplified products were separated on 2% agarose gels stained with ethidium bromide and visualized under a UV Trans illuminator.

Analysis of the data was carried out using Epi Info 5 software. In addition, the gene frequencies were estimated by using maximum likelihood methods[36] and goodness of fit between the observed and expected phenotype frequencies were tested.[37] Genotype frequencies were checked for deviation from Hardy–Weinberg equilibrium and were not significantly different from those predicted. Odds ratios and 95% confidence interval (95% CI) were calculated to assess the strength of the relationship between different phenotypes of the two single nucleotide polymorphisms ACE and CYP1A1 with colorectal cancer. Pooled odds ratios and relative risk were calculated by the random-effects method.[38] For odds ratio, confidence interval was calculated. Increased risk was calculated using the formula: Increased Risk = (Relative Risk – 1.00) x 100. The significance level was 5%.

Multifactor Dimensionality Reduction (MDR) analysis was performed using MDR software (v. 3.0.2) to study case-control data, gene-gene interactions and gene-environment interactions.[39,40] Best models with possible combinations of the polymorphisms were considered based on 10-fold cross validation and maximum testing accuracy. Once MDR identifies the best combination of factors, the final step is to determine which multifactor levels (genotypes) are high risk and which are at low risk using the entire data set. This final evaluation is conducted with a threshold ratio that is determined by the ratio of the number of affected individuals divided by the number of unaffected individuals in the data.

RESULTS

60 cases presenting colorectal cancer and 60 cases of age and sex matched healthy controls were included in the present study. Distribution of phenotype frequencies and allele frequencies for Alu - ACE and CYP1A1 markers in colorectal cancer patients and controls were shown in Tables 1 and 2.

Table -1: Distribution of Alu ACE and CYP1A1 polymorphism phenotypes in colorectal cancer patients and controls.

System	Phenotype	CRC Patients		Controls	
		Observed	Expected	Observed	Expected
Alu – ACE	**II**	18.00	22.69	18.00	22.69
	ID	38.00	28.41	38.00	28.41
	DD	4.00	8.90	4.00	8.90
	Total	**60.00**	**60.00**	**60.00**	**60.00**
		χ^2 = 6.9043* (0.01>p>0.001)		**χ^2 = 6.9043*** (0.01>p>0.001)	
CYP1A1	**m1m1**	18.00	17.18	30.00	28.15
	m1m2	28.00	29.85	22.00	25.90
	m2m2	14.00	12.97	8.00	5.95
	Total	**60.00**	**60.00**	**60.00**	**60.00**
		χ^2 = 0.2355 (0.70>p>0.50)		**χ^2 = 0.9151** (0.50>p>0.30)	
Inter Group χ^2		**Alu – ACE**		11.1576*	
		CYP1A1		5.3562	

Table -2: Distribution of Alu-ACE and CYP1A1 allele frequencies in colorectal cancer patients and controls.

System	Allele	CRC Cancer	Controls
Alu - ACE	I (wild)	0.6150 ± 0.0487	0.6650 ± 0.0472
	D (mutant)	0.3850 ± 0.0487	0.3350 ± 0.0472
CYP1A1	m1 (normal)	0.5350 ± 0.0025	0.6850 ± 0.0464
	m2 (mutant)	0.4650 ± 0.0025	0.3150 ± 0.0464

In Alu-ACE gene, the frequency of the I and D alleles in controls were 66% and 33% with observed genotype frequencies of 50%, 33% and 17% for II, ID and DD respectively. The frequency of I and D alleles in patients were 61% and 38% with observed genotype frequencies of 30%, 63% and 7% respectively. The study group showed the predominant occurrence of ID phenotype in colorectal cancer patients. The chi- square test for homogeneity was found to be significant in patients (χ^2 = 6.9043; d.f = 1; 0.01 > p > 0.001) and non-significant in controls (χ^2 = 3.7284; d.f = 1; 0.10 > p > 0.05). The inter group heterogeneity was found to be (χ^2 = 11.1576; d.f. =2; (0.01>p>0.001), significant value when observed between colorectal cancer patients and controls, indicating that patients group deviate from Hardy Weinberg equilibrium.

Genotype frequency obtained from CYP1A1 gene analysis in patients with colorectal cancer revealed that the majority of them were m1m2 heterozygotes (47%) followed by m1m1 homozygotes (30%) and m2m2 homozygotes (23%). The control group had genotypic

frequency of 50% homozygotes (m1m1) followed by the frequencies of 37% of m1m2 and 13% of m2m2 phenotypes. The allelic frequency in patient group was 53% of m1 and 46% of m2 and the control group showed 68% of m1 allele and 31% of m2 allele. The study group showed the predominant occurrence of m1m2 phenotype in colorectal cancer patients. The chi- square test for homogeneity was found to be non-significant in patients ($\chi^2 = 0.2355$; d.f = 1; $0.70 > p > 0.50$) and controls ($\chi^2 = 0.9151$; d.f = 1; $0.50 > p > 0.30$). The inter group heterogeneity was found to be ($\chi^2 = 5.3562$; d.f. =2; $(0.10>p>0.05)$, non-significant value when observed between colorectal cancer patients and controls, indicating that patients group was not deviated from Hardy Weinberg equilibrium.

Table 3: Test of Association, Relative Risk, Odds Ratio Estimates of Alu- ACE Phenotypes in colorectal cancer patients and controls

ACE Phenotype	Control (n)	Colorectal Cancer				
		(n)	RR	OR	95% CI	χ^2 values
II vs (ID+DD)	30	18	0.64	0.43	0.19 – 0.97	5.0000*
ID vs (II+DD)	20	38	1.85	3.45	1.53 – 7.88	10.8100*
DD vs (II+ID)	10	4	0.54	0.36	0.09– 1.35	2.9100

Test of association of Alu-ACE phenotypes with the disease condition compared to the control group, the odds ratio and relative risks for each genotype versus the other two are shown in Table 3. ID heterozygotes were at an increased risk of colorectal cancer, with an overall odds ratio of 3.45 (95 percent Confidence Interval: 1.53, 7.88; p = 0.001) by the method of Dersimonian and Laird. Homozygotes (II & DD) were at a decreased risk of colorectal cancer, with an overall odds ratio of 0.43 and 0.36 (p = 0.02; 0.08). In the present study, Risk estimates show a significant association of ID phenotype with colorectal cancer individuals (RR = 1.85). The result shows an increased risk of 85% and more, indicating that individuals with ID phenotype are 1.8 times more likely to get the disease when compared with the other phenotypes of the Alu-ACE polymorphism.

Table 4: Test of Association, Relative Risk, Odds Ratio Estimates of CYP1A1 Phenotypes in Colorectal Cancer and Control Groups

CYP1A1 Phenotype	Control (n)	Colorectal Cancer				
		(n)	RR	OR	95% CI	χ^2 values
m1m1 vs (m1m2+m2m2)	30	18	0.64	0.43	0.19-0.97	5.0000*
m1m2 vs (m2m2+m1m1)	22	28	1.23	1.51	0.68-3.35	1.2300
m2m2 vs (m1m1+m1m2)	8	14	1.36	1.98	0.70-5.73	2.0000

Test of association of CYP1A1 phenotypes with the disease condition compared to the control group, the odds ratio and relative risks for each genotype versus the other two are shown in Table 4. Homozygotes m2m2 and heterozygotes m1m2 were at an increased risk of colorectal cancer, with an overall odds ratio of 1.98 and 1.51 by the method of Dersimonian and Laird. Homozygotes (m1m1) were at a decreased risk of colorectal cancer, with an overall odds ratio of 0.43 (p = 0.02). In the present study, Risk estimates show a significant association of m1m2 and m2m2 phenotypes with colorectal cancer individuals (RR = 1.23 and 1.36) respectively. The result shows an increased risk of 20% and 36% more, indicating that individuals with m1m2 and m2m2 phenotype are more likely to get the disease when compared with the other phenotypes of the CYP1A1 polymorphism.

Table 5: Results of MDR analysis on genetic factors.

No. of loci	Polymorphism Model	Testing Accuracy	CVC	Prediction error (%)
1	Alu-ACE	0.65	10/10	35.0*
2	Alu-ACE, CYP1AI	0.65	10/10	35.0*

*P≤0.01 based on 1000 permutations.

Table 6: Distribution of high-risk and low-risk genotypes in the best two locus model.

Pattern	Multilocus–genotype combinations		Number of		Case/control ratio	Association with colorectal cancer
	Alu-ACE	CYP1A1	cases	Controls		
1	II	m1m1	18	30	0.6	Low-risk
2	ID	m1m2	28	20	1.4	High-risk
3	ID	m2m2	10	0	∞	High-risk
4	DD	m1m2	0	2	0.0	Low-risk
5	DD	m2m2	4	8	0.5	Low-risk

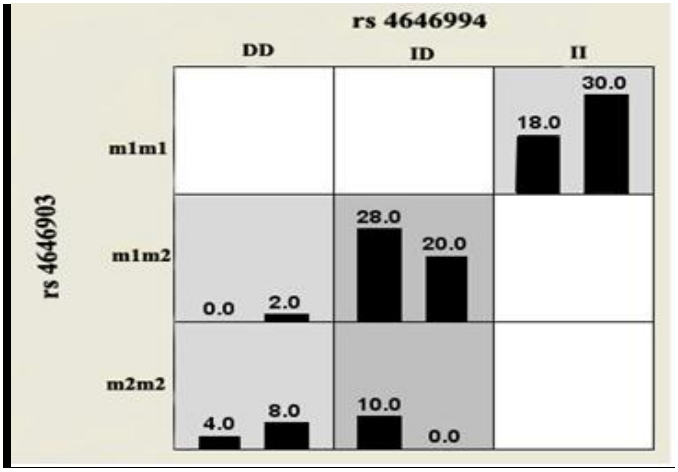

Figure 1: An MDR Analysis of the Two-factors (Alu-ACE and CYP1A1) - Interaction Model of colorectal cancer.

In the cell in the figure, the left bands represent the disease case, and the right bands represent the control case. High-risk combinations are depicted as darkly shaded cells, low-risk combinations as lightly shaded cells.

MDR software was used to analyze the interaction of the 2 factors that may affect the colorectal cancer and the results were detailed in Tables 6 and 7. We found that the cross-validation (CV) consistency of the two-factor model (Alu- ACE and CYP1A1) was maximal (10/10) and the accuracy of the test samples was the highest (0.65). Permutation testing was used to perform a hypothesis test and evaluate its statistical significance. Thus, the two-factor interaction model was the best model, which shows that there was an interaction between the two SNP's ($p \leq 0.01$). Thus the 1-way and 2-way models are both found to be significant. Table 6 summarizes the two locus genotype combinations associated with high risk and with low risk, along with the corresponding distribution of cases and of controls, for each multilocus genotype combination. The cell is labelled as either high risk if the case–control ratio reaches or exceeds a predetermined threshold (for example, ≥1) and low risk if it does not reach this threshold (Fig 1). `The interaction information analysis revealed a strong synergism between the two SNPs markers Alu- ACE and CYP1A1contributing to colorectal cancer.

DISCUSSION

Numerous studies have shown the association of these genetic polymorphisms with CRC.[41-52] However, the results remained inconsistent.

Human ACE is the key enzyme in the renin-angiotensin system, which works in the regulation of blood pressure, the number of red blood cell, cardiovascular homeostasis and serum electrolytes. In recent years there were more evidences indicating that ACE was associated with the pathogenesis of cancer, even it was the trigger events at least in some group of patients with cancer. It may influence tumor cell adhesion, proliferation, migration, angiogenesis and metastatic behaviours. ACE has been associated with several types of cancer including breast, prostate, endometrial and oral carcinomas by influencing tumor cell proliferation, tumor cell migration and angiogenesis.[14-17,53] The I/D polymorphism in intron 16 of the ACE gene is the most extensively studied polymorphism. This polymorphism is based on insertion or deletion of a 287-bp Alu sequence, leading to a change in the plasma ACE level. Growing number of studies have been published to investigate the associations between this polymorphism with cancer risk; however, the results were inconsistent and

conflict. Some studies investigating the ACE I/D polymorphism in various carcinomas have revealed an association of the high expression D allele with risk for carcinogenesis.[14-17] Our data was in agreement with this study. Our results showed an increased risk of 85% and more, indicating that individuals with ID phenotype are 1.8 times more likely to get the disease when compared with the other phenotypes of the Alu-ACE polymorphism. These studies investigated sex hormone-related neoplasias and suggested that the increased ACE expression affected oncogenesis mainly by facilitating angiogenesis, due to the higher production of angiotensin II. Nevertheless, there are several reports that have not found a correlation between the ACE I/D polymorphism and disease risk.[48] In contrast, in a study investigating the role of ACE in oral cancer it was suggested that oral oncogenesis was driven through a bradykinin-related pathway and not through angiotensin II.[53] In some studies, the D allele and the DD homozygosity have been associated with an increased risk in different diseases, such as end-stage renal disease (ESRD) in patients with DN, myocardial infarction, coronary disease and atherosclerotic plaque calcification, left ventricular dysfunction after myocardial infarction, lung cancer and colorectal cancer and in proliferative retinopathy in type 1 diabetes.[54-57] However, the exact mechanism of how and why the DD genotype represents a favourable genetic factor for the onset of the disease still remains unknown. Butler (2000)[55] provides plausible explanations on the DD-ACE genotype in cardiovascular disease. The author suggests that this genotype may cause alterations in endothelial function, influencing the underlying metabolic control mechanisms, such as insulin resistance, and also the vascular responsiveness to Ang II.

CYP1A1 is a member of the CYP1 family and participates in the metabolism of a vast number of xenobiotic, as well as endogenous substrates.[58] CYP1A1 plays a key role in phase I metabolism of polycyclic aromatic hydrocarbons and in oestrogen metabolism. The dysfunction of CYP1A1 can cause damage to DNA, lipids and proteins, which further results in carcinogenesis.[59] Polymorphisms of the CYP1A1 enzymes may contribute to the variable susceptibility to carcinogenesis by altering the level of gene expression or messenger RNA stability, resulting in highly inducible activity of the enzyme.

Several studies have suggested the CYP1A1 polymorphisms were associated with elevated risks of prostate cancer, oesophageal cancer and head and neck cancer, endometrial, lung, cervical, oral, head and neck, ovarian, gastric, breast and colorectal.[52,60-74] It has been shown that alcohol intake and cigarette smoking are two important risk factors for colorectal

cancer.[64] Different cancers with different carcinogenic mechanisms and environmental exposures had disparate responses to CYP1A1 genotypes (MspI/ Ile462Val). It has been shown that CYP1A1 Ile462Val polymorphism can increase the activity of enzymes and activation of carcinogens may increases the risk of colorectal cancer[75,76] increase breast cancer risk in Caucasians,[77] is a risk factor for oesophageal cancer in Asians but not in Caucasians.[78] However, Ile/Val polymorphism is not related with the increased risk of oesophageal carcinoma and prostate cancer.[79-81]

The MspI polymorphism C/C genotype is associated with an increased risk of cervical cancer. Interestingly, Gutman and co-workers[82] reported that the CYP1A1 MspI C/C polymorphism is unlikely to be a major risk factor for cervical cancer. In the subgroup analysis by ethnicity, the MspI C/C polymorphism was found to confer an increased cancer risk among Asians and mixed population but not Caucasians or Africans. The effects of the two genotypes of each CYP1A1 polymorphism are diverse according to subgroup analysis stratified by ethnicity, cancer type and source of control. The meta-analysis by Zheng suggests that CYP1A1 *Ile 462 Val* polymorphism was a low penetrance susceptibility gene in colorectal cancer development. On the contrary, CYP1A1 *MspI* polymorphism does not seem capable of modifying colorectal cancer risk. [71] Our data was in agreement with this study. In a study by Sivaraman et al.,[23] an eightfold increased risk of colorectal cancer was observed with the *Msp*I *CYP1A1* rare allele among Japanese living in Hawaii. The increased risk estimates reported in our study for *CYP1A1* are 20% and 36% more, indicating that individuals with m1m2 and m2m2 phenotype are more likely to get the disease when compared with the other phenotypes of the CYP1A1 polymorphism. Moreover, the interaction information analysis of gene-gene interactions revealed a strong synergism between the two SNPs markers Alu- ACE and CYP1A1contributing to colorectal cancer.

In summary, this study suggests that I/D polymorphism in the ACE gene may confer the risk of colorectal cancer. More studies would be of great value to explore the interaction between the ACE I/D polymorphism and cancer risk. Further studies of patients in larger numbers and of different ethnic backgrounds may be necessary to elucidate the association between the ACE I/D gene polymorphism and increased risk of colorectal cancer. Moreover, the studies of gene-gene and gene-environment interactions between these polymorphisms and CRC risk should also be performed and considered.

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