Genetic Polymorphisms of FOXO3a Gene in Colorectal Cancer among North Indian Population

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Abstract

Background Colorectal cancer (CRC) heritability is determined by the composite relations between inherited variants and environmental factors. In developing countries like India CRC incidence rates have been increasing specially. In the present study, we focused on the distribution of FOXO3a gene polymorphisms in North Indian colorectal cancer patients.

Methods A case–control study was conducted on 900 samples including 450 colorectal cancer patients and 450 age matched controls. We genotyped the SNPs rs2253310 and rs4946936 via Polymerase Chain Reaction-Restriction fragment length polymorphism (RFLP) analysis and Polymerase Chain Reaction-single stranded conformation polymorphism (SSCP) procedure followed by sequence detection.

Results A significantly increased risk of CRC was observed with rs4946936 genotype (P= 0.0393; OR= 1.405 CI=1.051-1.879). GT haplotype although not reaching statistical significance appeared to be at higher “risk” haplotype (OR- 1.164, 95%CI= 0.967~1.401), while as other haplotypes CC (OR- 0.893, 95% CI=0.665~1.200]), CT (OR- 0.806, 95%CI= 0.616~1.055) and GC (OR- 0.994, 95%CI= 0.809~1.221) were found to be “protective” for developing colorectal cancer.

Conclusion This study lends support for an increased risk of CRC associated with the rs4946936 polymorphism. Nevertheless, statistically significant association between rs2253310 genotypes and CRC risk was not observed.

Introduction

Cancer is the second leading cause of death before 70 years of age according to World Health Organization (WHO). Worldwide the rates of cancer incidence and mortality are rapidly growing. Due to the transmission of life style, the cancers associated with the poverty and infections are being displaced by with those that are more prevalent in developed countries [1]. It was estimated that there will be an increase of 30% new cases of colorectal cancer in next coming years (*GLOBOCON*-2018). In terms of incidence rate, CRC ranks third whereas second in case of mortality (*GLOBOCON*-2018). A diet high in red or processed meat consumption has been reported associated with an increased risk of colon cancer, but not rectal cancer [2]. In India, CRC is the fifth most common cancer following breast, cervix/uteri, lip/oral cavity, and lung cancer [3].

The mammalian Forkhead box O (FOXO) transcription factor family is the evolutionary conserved 110 amino acid protein, identified by winged-helix structure of their DNA binding domain [4]. FOXO family includes FOXO1, FOXO3 (FOXO3a), FOXO4 and FOXO6 that are identified in humans. The FOXO subfamily is concerned with repair of DNA damage, cell survival and cell proliferation [5–7]. FOXO3a has been extensively studied as a crucial protein that regulates several essential cellular functions like cell cycle arrest, oxidative stress and apoptosis. It has been an important target to inhibit the progression of cancer cells. In many systems FOXO3 has been emerged as a tumor suppressor gene [8]. and therefore is an important target to inhibit cancer cell progression. Breast cancer, prostate cancer, gliomas and CRC

have also been associated with the deregulated expression of FOXO3a. In metaplastic colorectum, liver metastasis and primary CRC, loss or reduced abundance of FOXO3a was seen [9].

Studies of genome-wide association using single nucleotide polymorphism (SNP) analysis have successfully identified loci for different quantitative characteristics. Single nucleotide polymorphisms are known to modulate levels of DNA damage, DNA repair capacity and cancer risk [10, 11]. Recent studies have shown that FOXO3a polymorphisms lead to cell cycle control loss leading to the formation of cancers like prostate cancer and acute lymphoblastic leukemia [12]. Only a few studies investigated that the variant alleles are at considerable higher risk of bladder or breast cancer [13, 14]. Therefore, the purpose of this study was to find the association of FOXO3a SNPs (rs2253310 and rs4946936) in CRC.

Materials And Methods

This study was approved by the Jamia Millia Islamia and Sher-e-kashmir Institute of Medical Sciences Bioethics Committee. Informed consent was obtained from all participants of the study. The total sample consisted of 450 colorectal cases (CRC group) and 450 apparently healthy (Healthy group) subjects. The information of age, smoking status, gender, tissue grade, tumor node involvement etc. is summarized in Table 3.

Sample collection, DNA isolation and Genotyping of SNP:

Blood samples were collected in EDTA coated vials. Human Genomic DNA was extracted as per laboratory protocol from control and case subjects using Phenol-Chloroform method. DNA was quantified using NanoDrop ND-1000 spectrophotometer. Extracted DNA was amplified by PCR for rs2253310 and rs4946936 using the oligonucleotide primers given in the Table1. 100ng of isolated DNA, 0.5µl of forward and reverse primers, 200µl of 200 µM of each deoxynucleotide triphosphates (dNTPs) (deoxyadenosine triphosphate [dATP], deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) along with 1 unit of DNA polymerase was used. PCR cycling procedures were as follows: 95°C for 5minutes, 34 cycles of 94°C for 30 seconds, 58°C (rs2253310) and 59°C (rs4946936) for 30 seconds and elongation at 72°C for 30 seconds. The final amplicon elongation was performed at 72°C for 5 minutes. PCR products were visualized by electrophoresis on 1.5% agarose gel containing 2 µl of ethidium bromide.

Analysis of PCR products using RFLP and SSCP

rs2253310C>G was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) FIG. 1(a) and (b). The PCR product (321bp) was digested by DpnI restriction enzyme (THERMO SCIENTIFIC) at 37°C overnight. The C- allele carrying PCR product is cleaved once by the enzyme giving rise to two fragments (127bp and 194bp), where as the G-allele gave only one band

(321bp). The digested products were separated by electrophoresis on 2.5% ethidium bromide-stained agarose gels and visualized under UV transilluminator.

rs4946936C>T was analyzed using PCR-RFLP. The PCR products of (224bp) were then digested with BfmI (THERMO SCIENTIFIC) overnight at 37°C for 1-16 hours as per the manufacturers protocol. The sizes of the restriction fragments of the PCR products were 152bp and 72bp for the wild type homozygote (CC), 224bp for the TT genotype and 224bp, 152bp and 72bp for the heterozygote (CT). Since sequence variation often goes undetected, because each enzyme used scans only a small number of potentially variable nucleotide positions, therefore, single-strand conformation polymorphism (SSCP), further provided useful alternative for the direct analysis of sequence variation.

SSCP assay was performed in 16µl reaction mixture containing 4µl of PCR product and 12µl of denaturing loading dye (95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05% bromophenol blue, and 0.05% xylene cyanol) and heat- denatured at 95°C for 10 min and afterward immediately snap-chilled on ice prior to polyacrylamide gel electrophoresis (PAGE). Single-stranded amplicons were loaded onto 8 % polyacrylamide gel(29:1 acrylamide to bisacrylamide) containing 50 mM of Tris– borate (pH 7.5) and 2.5 mM of EDTA prepared with 1× Tris–borate–EDTA (TBE) buffer and electrophoresed for 12-14 h at constant temperature. The gel was subjected to silver nitrate staining to visualize the SSCP bands (Fig. 1(c) and 2(c). Samples demonstrating differences in band-shifts with respect to the wild-type bands were categorized as mutants.

To analyze the genotype of rs2253310 and rs4946936 in a more accurate and fast manner, we used direct sequencing method to confirm the results of RFLP and PCR coupled SSCP. The genotypes of all the 10% samples, determined by direct sequencing Fig. 1(d) and 2(d) showed the exact pattern as shown by RFLP and SSCP. Discrepancies were not observed.

Statistical analysis

Statistical package for social sciences (SPSS) software version 17.0 was used for data analysis. 3x2chi-square test was performed to compare the overall distribution of genotype and allelic frequencies of cases and controls. The odd ratios (OR) were calculated from genotypic frequency and allelic frequency with 95% confidence interval (95%CI) with adjustment for age. All P-values were corrected for multiple comparisons according to Bonferroni method. LD pattern and population haplotype frequencies for the SNPs were estimated using SHEsis (freely available software). Fisher’s exact test was performed for determining the association of haplotypes with diseased condition.

Results

Electrophoresis interpretation by RFLP and SSCP of rs2253310 Fig.1 (a),(b),(c) and rs4946936 Fig.2(a), (b),(c) indicated that DNA samples that presented two-low weight bands contained the polymorphism, whereas samples with only one low-weight band suggested the absence of polymorphism. Sequencing of

the samples validated the results representing the homozygus wildtype, heterozygus and homozygus variant genotypes.

Chi square test were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy–Weinberg equilibrium). Association between the genotypes distribution and risk for CRC was evaluated by calculating the odd ratios (ORs) and their 95% confidence interval (95% CIs) using logistic regression analysis. Online SHEsis (http://analysis.bio-x.cn/myAnalysis.php) was applied for haplotype prediction and analysis. Association between each individual FOXO3a polymorphism and risk for colorectal cancer are summarized in Table 3. We hypothesized that FOXO3a may play role in colorectal cancer progression, remarkable association between rs4946936 SNP and colorectal cancer was seen (p=0.0393) whereas, rs2253310 did not show any statistical significance associated with CRC (p=0.1213).

In FOXO3a rs2253310 polymorphism, the allelic and genotypic frequency on comparison between CRC and normal patients (Table 2) were very similar. In both the groups, about 56.7-61.1% of individuals were homozygous GG, 33.3-31.8% were GC heterozygote and the remaining 10-7.1% carried the CC genotype. No association was found between FOXO3a rs2253310 genotypes and any of the clinical parameters except the strong association revealed with nodal status (p=0.03), tumor grade (p=0.046) and gender (p=0.022).

In FOXO3a rs4946936, we found the frequency of the T allele (60%) to be higher in patients than in controls (35.6%) and therefore a risk factor for susceptibility to CRC. No significant association was seen between FOXO3a TC genotype for age (p= 0.335). TC genotype seems to be associated with good prognosis.

Haplotype analysis and linkage disequilibrium

Estimated FOXO3a SNP haplotype frequencies for rs2253310 and rs4946936 are shown in Table 4 using SHEsis program which determines the most probable haplotypes based on allelic frequencies. Our data suggested that GT haplotype although not reaching statistical significance appeared to be at higher “risk” haplotype (OR- 1.164, 95%CI= 0.967~1.401), while as other haplotypes CC (OR- 0.893, 95% CI=0.665~1.200]), CT (OR- 0.806, 95%CI= 0.616~1.055) and GC (OR- 0.994, 95%CI= 0.809~1.221) were found to be “protective” for developing colorectal cancer. Although the two haplotype frequencies were not found in strong LD but the genotypes seem to be associated with good prognosis. No evidence for the statistically significant linkage disequilibrium was found between rs2253310 and rs4946936 FOXO3a SNPs in our cohort of patients.

Discussion

FOXO3a is known to play the role of central transcription factor mediating multiple physiological and pathological processes involving cell cycle progression [15], apoptosis [16] and survival [17] and therefore

indicating its role in various diseases, particularly cancer. In addition, FOXO3a is closely known to be associated with human longevity [18]. FOXO3a has been associated with the development of muscle atrophy, Parkinson's disease, premature ovarian follicle, and heart diseases. In CRC, the gene FoxO3a has an ambiguous function. A study found that inactivation of FoxO3a promoted progression of the tumor [19].

The current study, to the best of our knowledge, reports for the first time that the analysis of changes in FOXO3a in North Indian population in the form of polymorphism may increases the susceptibility for CRC. We determined the frequency of the rs2253310 and rs4946936 polymorphism in carcinoma of the colorectal cancer by comparing tumor DNA data with that of a group of control subjects. Both of these polymorphisms have not been examined in relationship with any cancer disease. Our study revealed that rs4946936 is strongly associated with colorectal cancer. This SNP which is located in 3' UTR is characterized by C to T conversion. We found that C to T change significantly contributed to increased risk of CRC. Our results demonstrated that the patients with FOXO3a rs4946936 TC (OR: 1.244, 95% CI: 0.934–1.657) genotype has correlation with risk of CRC when compared with CC genotype after adjustment for age. The current population showed an increased colorectal cancer risk for the summarized parameters except for the age. Significant difference was found between clinicopathological parameters and rs4946936 SNP and no significant association was found for rs2253310.

Being case-control study using limited number of subjects, the polymorphism meaningful for the risk of the target disease may show null results due to low minor allele frequencies. In addition, FOXO3a contains other SNPs that might be associated with colorectal cancer were not investigated in the current study.

Conclusion

In conclusion, we found that rs4946936 SNP may be novel polymorphism of the FOXO3a gene associated with CRC. Larger, well designed studies and genome wide association studies are needed to confirm these findings.

Declarations

Acknowledgement:

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Conflict of Interest:

None

Author contribution:

LU and ZIB contribute equally, designed the experimental work and wrote the manuscript. BZ and KI helped in performing the experiments. RAW provided the hospital support and samples. MMAR designed the study and Manuscript.

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Tables

Table 1
Primers used for genotyping FOXO3a SNPs.

SNPs	Primer Sequences	PCR product length	Genotype (size of digested fragments	
			in base pairs)	
rs2253310	F5'GAGCTTGCTTTGGAGATGCA3' R5'CCCAGTCACTCACATAGTCCT3'	321bp	DpnI	GG 321 GC127,194,321 CC 127,194
rs4946936	F5'GGGTCCTGAGAACTTCTGAGT3' R5'GACATTCTGTAAGACATTCTGCCT3'	224bp	SfcI	TT 224 TC 224, 152, 72 CC 152, 72

Table 2
Genotype and allele frequencies of FOXO3a Polymorphisms in Cases and Controls.

rs2253310	Cases(N = 450)	Controls(N = 450)	[a]p	[b]p	aOR(95% CI)
CC	255(56.7%)	275(61.1%)	0.1213		
CG	150(33.3%)	143(31.8%)		0.257	1.0341(0.807–2.228)
GG	45(10%)	32(7.1%)		0.09	1.517(0.934–2.461)
CC Vs CG + GG				0.121	1.451(0.904–2.330)
C%	27%	23%		0.217	1.210(0.894–1.638)
G%	73%	77%			
rs4946936					
TT	171(38%)	160(35.6%)	0.0393		
TC	201(44.7%)	234(52%)		0.135	1.244(0.934–1.657)
CC	78(17.3%)	56(12.4%)		0.022	1.405(1.051–1.879)
TT Vs TC + CC				0.447	1.111(0.847–1.457)
T%	60%	35.60%		0.682	0.946(0.723–1.236)
C%	40%	64.44%			

*p < 0.025 values significant after bonferroni coefficient.

OR odds ratio, CI confidence interval

[a]p value for 3x2 χ2 test of comparison of overall genotype frequencies between cases and controls.

[b]p value and corresponding age-adjusted OR (aOR) with 95% CIs[aOR(95% CI)] for comparison of genotype frequencies between cases and controls by logistic regression analysis (age is not adjusted in allele frequency comparisons).

Table 3
Association of FOXO3a rs2253310, rs4946936 SNPs with clinicopathological, life style and environmental characteristics of colorectal cancer patients from North India.

Characteristic	rs2253310			rs4946936		
	Genotype(n)	P Value	aOR(95% CI)	Genotype(n)	P Value	aOR(95% CI)
Smoking	CC 28/177		1.000.	TT 95/76		1.000.
Yes/No	CG 73/77	0.359	0.983(0.947–1.020)	TC 116/85	0.06	0.956(0.934–0.979)
	GG 141/114	0.591	0.990(0.956–1.026)	CC 31/47	0.001	0.950(0.921–0.980)
	CG + GG (214/191)	0.47	0.987(0.954–1.022)	TC + CC (147/132)	0.08	0.957(0.937–0.978)
Alcohol	CC 24/21		1.000.	TT 71/00		1.000.
Yes/No	CG 59/91	0.538	0.990(0.960–1.022)	TC 87/114	0.001	0.971(0.953–0.988)
	GG 100/155	0.372	0.987(0.959–1.016)	CC 25/53	0.051	0.979(0.956–1.003)
	CG + GG (159/246)	0.39	0.987(0.959–1.016)	TC + CC (112/167)	0.002	0.973(0.957–0.990)
Dwelling	CC 30/15		1.000.	TT 92/79		1.000.
Rural/Urban	CG 71/79	0.06	0.962(0.923–1.002)	TC 102/99	0.04	0.950(0.929–0.972)
	GG 142/113	0.207	0.976(0.939–1.014)	CC 49/29	0.179	1.022(0.990–1.055)
	CG + GG (213/192)	0.129	0.972(0.936–1.008)	TC + CC (151/128)	0.001	0.963(0.943–0.984)
Tumor Size	CC 25/20		1.000.	TT 77/94		1.000.
> 5cm	CG 67/83	0.649	0.992(0.959–1.026)	TC 88/113	0.05	0.964(0.946–0.983)
≤ 5cm	GG 107/148	0.365	0.987(0.958–1.016)	CC 34/44	0.432	0.989(0.963–1.016)
	CG + GG (174/231)	0.428	0.988(0.959–1.018)	TC + CC (122/157)	0.001	0.971(0.954–0.989)
Tumor Location	CC 27/18		1.000.	TT 65/106		1.000.
Colon/Rectum	CG 45/105	0.052	0.967(0.935-1.000)	TC 71/130	0.03	0.968(0.951–0.985)

Characteristic	rs2253310			rs4946936		
	CC 95/160	0.089	0.973(0.944–1.004)	CC 31/47	0.809	0.997(0.973–1.021)
	CG + GG (140/265)	0.064	0.971(0.942–1.002)	TC + CC (102/177)	0.003	0.976(0.960–0.992)
Nodal Status	CC 24/21		1.000.	TT 108/63		1.000.
Involved/	CG 95/55	0.03	1.038(1.004–1.073)	TC 121/80	0.06	0.952(0.932–0.973)
Not involved	GG 156/99	0.04	1.04091.002–1.080)	CC 46/32	0.937	0.999(0.973–1.026)
	CG + GG (251/154)	0.021	1.043(1.006–1.080)	TC + CC (167/112)	0.20	0.955(0.935–0.976)
Tumor Grade	CC 17/28		1.000.	TT 72/99		1.000.
I + II	CG 71/79	0.046	1.031(1.001–1.062)	TC 88/113	0.001	0.9700(0.953–0.988)
III + IV	GG 107/148	0.449	1.010(0.985–1.036)	CC 35/43	0.221	0.983(0.956–1.011)
	CG + GG (178/227)	0.208	1.016(0.991–1.042)	TC + CC (123/156)	0.009	0.977(0.960–0.994)
Age	CC 30/15		1.000.	TT 90/81		1.000.
> 50	CG 81/69	0.202	0.972(0.930–1.016)	TC 133/68	0.335	0.987(0.960–1.014)
≤(50)	GG 156/99	0.587	0.988(0.948–1.031)	CC 44/34	0.788	1.005(0.969–1.043)
	CG + GG (237/168)	0.392	0.982(0.943–1.023)	TC + CC (177/102)	0.512	0.991(0.966–1.017)
Hypertension	CC 26/19		1.000.	TT 57/114		1.000.
Yes/No	CG 57/93	0.207	0.979(0.947–1.012)	TC 72/129	0.003	0.976(0.959–0.992)
	GG 89/166	0.107	0.975(0.946–1.005)	CC 43/35	0.01	1.040(1.009–1.071)
	CG + GG (146/259)	0.119	0.976(0.947–1.006)	TC + CC (115/164)	0.11	0.987(0.972–1.003)
Gender	CC 21/24		1.000.	TT 90/81		1.000.
Male/Female	CG 81/69	0.022	1.045(1.006–1.084)	TC 102/99	0.065	0.952(0.930–0.974)

Characteristic	rs2253310			rs4946936		
	GG 140/115	0.105	1.025(0.995–1.057)	CC 50/28	0.052	1.033(1.000-1.068)
	CG + GG (221/184)	0.041	1.033(1.001–1.065)	TC + CC (152/127)	0.002	0.966(0.945–0.987)
P < 0.00125,P values significant after bonferroni coefficient.						
aOR age adjusted odds ratio, CI confidence interval.						
P value and corresponding age-adjusted OR (aOR) with 95% CIs[aOR(95% CI)] by logistic regression analysis.						

Table 4
Haplotypes of two SNPs of FOXO3 (rs2253310 and rs4946936) in colorectal cancer cases and controls.

Haplotypes	Cases	Control	P Values	Odd ratio (95%CI)
C C*	94.06(0.105)	104.01(0.116)	0.453616	0.893 [0.665 ~ 1.200]
C T*	112.94(0.125)	135.99(0.151)	0.115606	0.806 [0.616 ~ 1.055]
G C*	251.94(0.280)	252.99(0.281)	0.956122	0.994 [0.809 ~ 1.221]
G T*	441.06(0.490)	407.01(0.452)	0.107957	1.164 [0.967 ~ 1.401]
OR: Odd Ratio; CI: Confidence interval				
P Value and corresponding OR with 95% CI for Fisher's exact test.				
Frequency < 0.03 in both control and cases has been dropped.				

Figures

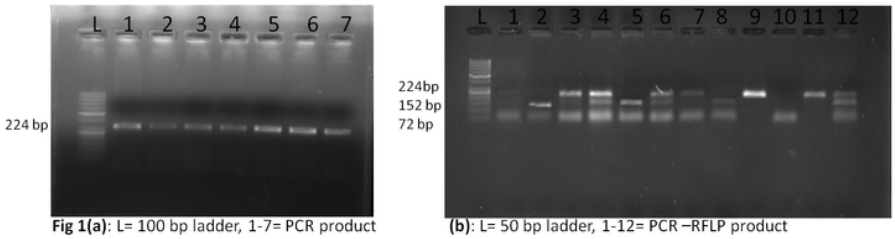
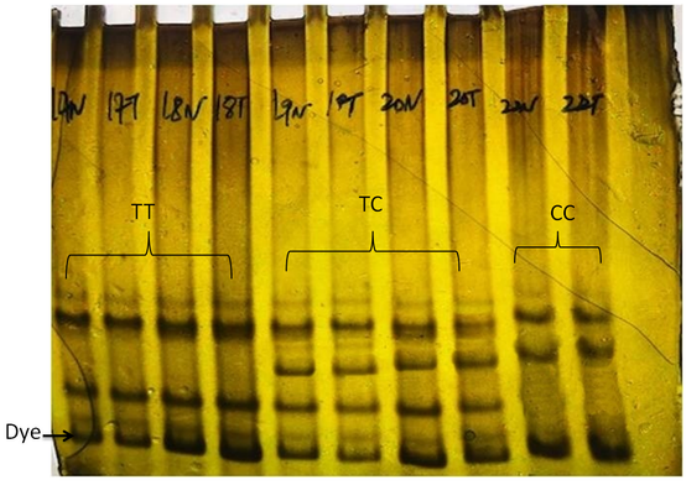
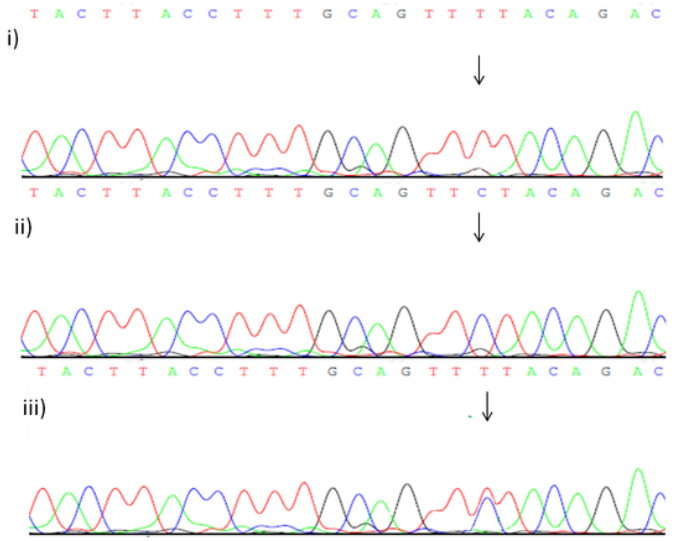


Fig 1(a): L= 100 bp ladder, 1-7= PCR product

(b): L= 50 bp ladder, 1-12= PCR –RFLP product



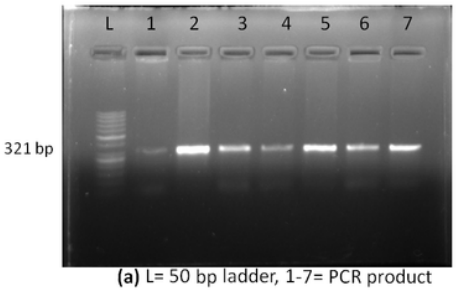
(c): Silver stainning, rs4946936



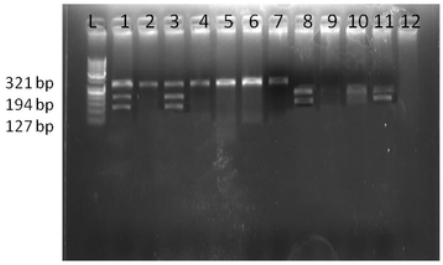
(d) : Sequencing results of rs4946936. Arrows indicate polymorphic sites of genotypes. a) Homozygous wild b) Homozygus variant C) Heterozygus

Figure 1

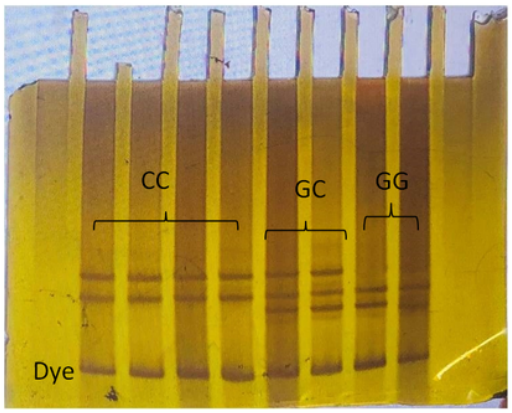
a,b, c show Electrophoresis interpretation by RFLP and SSCP of rs2253310 d- the exact pattern as shown by RFLP and SSCP



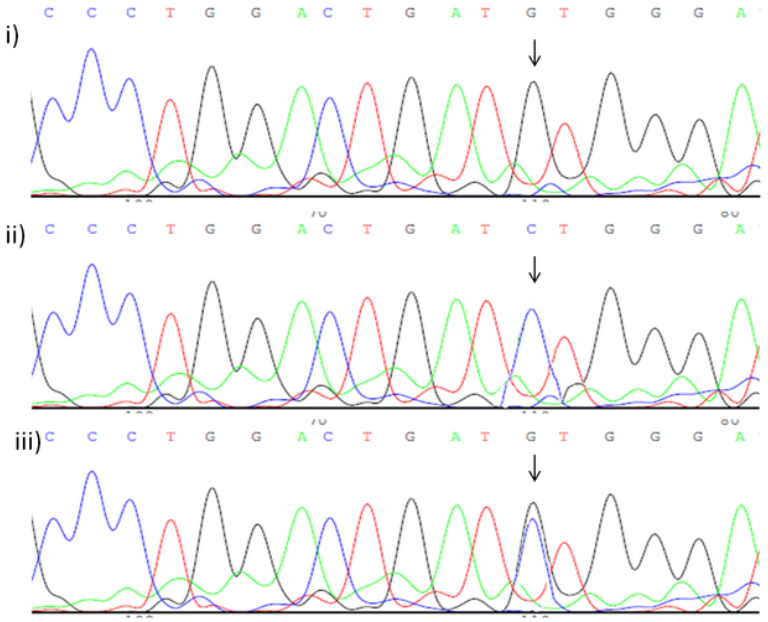
(a) L= 50 bp ladder, 1-7= PCR product



(b): L= 50 bp ladder, 1-12= PCR –RFLP product



(C): Silver stanning, rs2253310



(d) : Sequencing results of rs2253310. Arrows indicate polymorphic sites of genotypes. a) Homozygous wild b) Homozygus variant C) Heterozygus

Figure 2

a,b,c show Electrophoresis interpretation by RFLP and SSCP of rs4946936 d- the exact pattern as shown by RFLP and SSCP