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Original Research Article

Association between PARK2 Intronic SNP rs10945859 and Colorectal Cancer in North Indian Population

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ABSTRACT

Background: PARK2 intron (PACRG regulatory region) polymorphism rs10945859 has been associated with diseases such as Leprosy, Parkinson's etc. However association of this polymorphism with cancer remains unexplored. Therefore in this study we evaluated the association (if any) of this polymorphism and colorectal cancer (CRC) in North Indian subjects.
Methods: A total of two hundred subjects (100 colorectal cancer and 100 healthy individuals) of North Indian origin were scored for this polymorphism using (amplification created restriction site) PCR-RFLP method. Freely available online tools SFmap and SpliceAid were used to predict splicing factor binding sites.
Results: Statistically non-significant protection was observed for TC (OR: 0.794, 95% CI: 0.281-2.246, P= 0.664) and CC genotypes (OR: 0.891, 95% CI: 0.323-2.458, P= 0.823), while overall no difference of allele or genotype frequencies was found between healthy and CRC subjects for rs10945859. Out of all characteristics studied alcohol drinking (P=0.021) and smoking (P=0.035) characters showed statistically significant difference among CRC subjects. Gain of NOVA1 and hnRNP K and loss of SRp20 splicing factor binding sites were observed using *in-silico* tools.
Conclusion: Our results demonstrate that there was statistically non-significant protection due rs10945859 polymorphism and no overall difference between genotype and allele frequencies. However since the sample size used in this study was small, future studies with larger sample size are required.
Key-Words: PARK2, PACRG, rs10945859, RFLP, Colorectal cancer

INTRODUCTION

PARK2 (Parkin) is a E3 ubiquitin ligase involved in targeting different proteins to proteasome mediated degradation via polyubiquitination. [1] Deletion or mutation in PARK2 gene results in early-onset Parkinsonism (EOPD) & autosomal recessive juvenile Parkinsonism (ARJP). [2,3] PACRG (parkin co-regulated gene protein) shares common overlapping promoter polymorphisms with PARK2 and both genes have been shown to be associated with Leprosy. [4,5] PACRG is also suggested to be involved in Parkinson's pathogenesis. [6] PARK2 and PACRG-are shown to possess tumor suppressive nature. [7,8] Alterations in PARK2 have been implicated in multiple cancers such as non-small cell lung cancer, cervical cancer and other cancers. [9-12] Promoter methylation of this overlapping region is also shown to be involved in acute lymphoblastic leukemia and chronic myeloid leukemia. [13] Polymorphisms in the PARK2 and PACRG genes have been positively associated with Leprosy, Typhoid and Parkinson's. [5,14,15]

PARK2 intronic (PACRG regulatory region) rs10945859 polymorphism in the overlapping region has been associated with chronic HCV infection and Leprosy. [5,16] Among different cancers colorectal cancer (CRC) is the third most frequently occurring cancer in the world. Till date no study has evaluated the association (if any) of rs10945859 polymorphism with any cancer type or specifically for CRC. Therefore to find evidence whether this polymorphism is associated with CRC or not, we conducted a case control-study using 200 unrelated subjects (100 CRC patients and 100 healthy controls) from North India.

MATERIALS AND METHODS

Ethics statement

The approval of the study was obtained from the Institution Ethics Committee of G B Pant Hospital New Delhi and the Institutional Human Ethical Committee of Jamia Millia Islamia, New Delhi. All the participants provided their written informed consent for inclusion in this study.

Primer Designing

The sequence of PACRG/PARK2 overlapping region and polymorphism details of rs10945859 was retrieved from NCBI (http://www.ncbi.nlm.nih.gov). Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) was used for designing of primers. Amplification-created restriction site method was used for genotyping this polymorphism. The sequence of primers and amplified region were as following.

- FP5'- TTATCTGGACTTGCTGGCACT -3'
- RP5'- GATCCAGCTGTTTCCCAGTGGAGC CATTCCAGTTTTCCTGCAGGACAA TT -3'

TTATCTGGACTTGCTGGCACTAGTGT
AACTATTTTCTACAACGTTCTATTTCA
TAACTTTCTAGTACTGCCACAGACAT
CTTTCTAAAATACAACCTGTTCATAA
TGCTCTTTTGTAGATAATGGTTCAGT
GACTCCTCCCTATCGCCAACTTTAAA
CCTACCTCCTTGACAAAGCTTATCTG
GTGTGCCTGCAGCCTCACCAGATACC
TTTCATTCTATTCTCTTCTCTCCCCTC
CATGCCTGTCTAGAAGGCCGCCTTCA
CCTAGTTAACTCCCACTTGTCCTGCA
GGAAAACTGGAATGGCTCCACTGGG
AAACAGCTGGATC

The polymorphic site contains either T/C as major and minor allele. The bold letter c in the above sequence represents the minor allele at the polymorphic site. The underlined original "c" residue near to polymorphic site was replaced with "a" by introducing a mismatch "T" (underlined) in the reverse primer. This change upon amplification resulted in formation of MfeI restriction endonuclease recognition site CAATTG whenever the minor allele was present. Whenever major allele "T" was present the site will read as TAATTG, which was not recognized with this restriction enzyme.

DNA extraction and Genotyping

Under aseptic conditions blood samples were collected and genomic DNA was isolated from peripheral blood leucocytes using the standard phenol-chloroform method. Extracted DNA were checked and quantified by using 0.8% agarose and NanoDrop ND-1000 spectrophotometer respectively. Appropriate dilutions of DNA were made for further experiments. Genotyping was performed using PCR-RFLP method were 326 bp region was amplified. For PCR 50 µl reaction was setup in a 200 µl PCR tubes. The reaction mixture consisted as follows, 5 µl of 10X reaction buffer (with 1.5mM $MgCl_2$) , 4 µl of dNTP (2.5mM), 4 µl of primer pair each of 12 µM, 2 µl (50 ng/µl) template, 3 U/ul of Taq polymerase (Himedia) & 30 µl milliQ water. PCR cycle was as follows - 95 ºC for 5 min and 32 cycles of 95 ºC for 30 sec., 59 ºC for 40 sec, and 72 ºC for 40 sec, final extension for 72 ºC for 5 minutes. PCR product was digested overnight at 37 ºC in a water bath with 1 U of the MfeI enzyme in total reaction volume

of 50 µl as per manufacturer's protocol (Fermentas, Thermo Fisher, USA). The digested products were resolved on 3.5% agarose gels at 70 v. for 120 min. and visualized via EtBr staining. Randomly selected samples were crosschecked with direct sequencing also.

Computational prediction of potential splicing factor binding site

To assess whether the intronic SNP was a splicing regulatory sequences or not, we used freely available online tools SFmap and SpliceAid for the prediction of splicing factor binding sites. The SFmap, a web server utilizes a modified Hamming distance formula to define a match between a splicing factor sequence query and a target sequence. The distance scores are then standardized and a Z-score is obtained for calculating the significance of each query relative to a background model which is then compared to a threshold value to give a probable prediction. We predicted the potential splicing factor binding to the SNPs (rs10945859) using both SFmap and SpliceAid.

RESULTS

Through amplification-created restriction site method we were able to introduce the MfeI restriction enzyme site in the PCR product. Upon digestion with MfeI the homozygous T allele gave a single product of 326 bp while the homozygous C allele gave a major product of 276 bp and a digested 50 bp product while the heterozygous condition gave both 326 bp and 276 bp products (Figure 1).

In healthy subjects the genotype frequencies were 44% for TT, 48% for TC and 8% for CC genotypes while major (T) and minor (C) allele frequencies were 68% and 32% respectively. In CRC subjects the genotype frequencies were 49%, 41% and 10% for CRC subjects while allele frequencies were 69.5% and 30.5% respectively. Even after age adjustment no significant association was found between rs10945859 genotypes and allele frequencies with incidences of CRC (Table 1). The genotype and allele frequencies followed Hardy-Weinberg equilibrium (Table 1). Statistically non-significant protection was observed from odds ratio (OR) for TC (OR: 0.794, 95% CI: 0.281-2.246, P= 0.664) and CC genotypes (OR: 0.891, 95% CI: 0.323-2.458, P= 0.823) (Table 1). In clinicopathological, life style and environmental characteristics analysis of CRC subjects only alcohol drinking (P=0.021) and smoking (P=0.035) were found to be significantly different among CRC subjects (Table 2).

Computational analysis of intronic SNP for splicing factor binding site

Gain or loss off splicing factor binding site was predicted first with SFmap online tool for SNP rs10945859 where we found loss of one SRp20 splicing factor binding site and gain of NOVA1 splicing factor binding site due to minor allele "C". Whereas with second online tool SpliceAid we found gain of splicing factor heterogeneous nuclear ribonucleo protein K (hnRNP K) binding site due to the presence of minor allele "C" (Figure 2).

Table 1. Genotype and allele frequencies of rs10945859 in Colorectal Cancer Cases and Controls

rs10945859	Cases	Control	[a]p	[b]p	[c]p	aOR(95% CI)
TT	49 (49%)	44(44%)	0.303,0.742	0.594	0.372	1.304(0.728-2.335)
TC	41(41%)	48(48%)			0.664	0.794(0.281-2.246)
CC	10(10%)	8(8%)			0.823	0.891(0.323-2.458)
TT VS TC+CC					0.478	1.223(0.701-2.133)
T%	69.5	68				
C%	30.5	32			0.478	1.047(0.577-1.902)

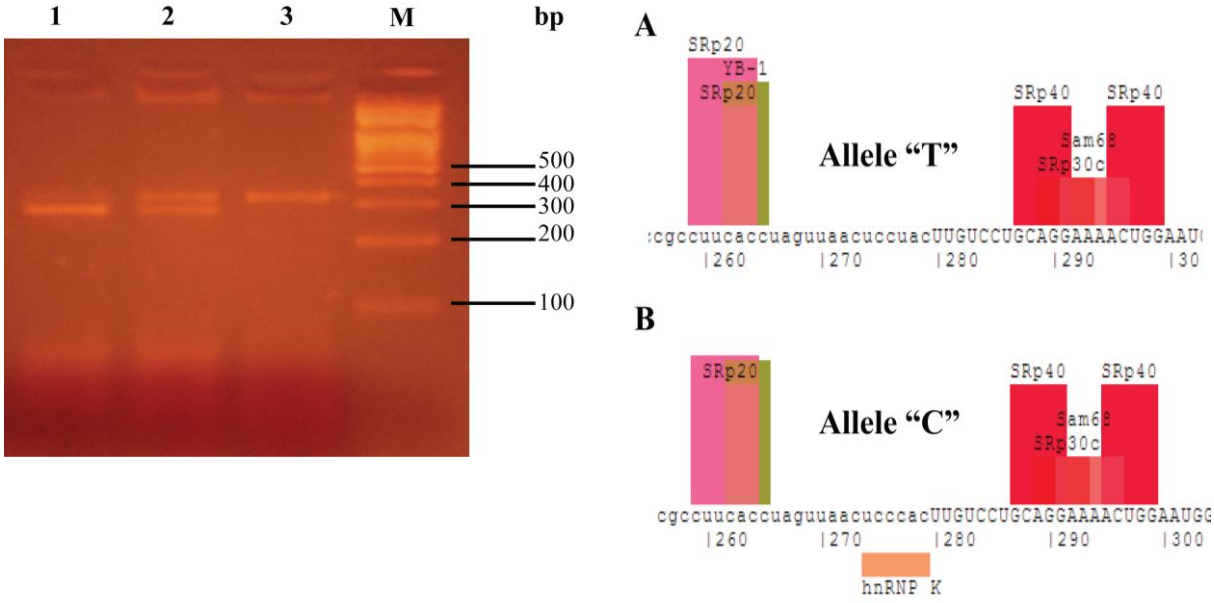
[a]p value for Hardy–Weinberg equilibrium testing

[b]p value for 2 x3 $\chi 2$ test of comparison of overall genotype frequencies of cases and controls

[c]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 2.Association of PARK2/PARCG rs10945859 SNP with clinicopathological, life style and environmental characteristics of colorectal cancer patients from North India.

Characteristics	Genotype	aOR(95% CI)	P value
Alcohol	TT (21/28)	1.000.	
Yes/No	CT (18/23)	0.967(0.916-1.020)	0.217
	CC (6/4)	0.885(0.798-0.982)	0.021
	CT+CC(24/27)	0.961(0.922-1.001)	0.058
smoking	TT(15/34)	1.000.	
Yes/No	CT(16/25)	0.961(0.919-1.006)	0.087
	CC (5/5)	0.892(0.802-0.992)	0.035
	CT+CC(21/30)	0.952(0.914-0.992)	0.019
Dwelling	TT(29/20)	1.000.	
Rural/Urban	CT(20/21)	0.952(0.908-1.000)	0.048
	CC(6/4)	0.946(0.869-1.031)	0.208
	CT+CC(26/25)	0.960(0.923-0.999)	0.043
Pesticide	TT(19/30)	1.000.	
Yes/No	CT(17/24)	0.967(0.920-1.017)	0.189
	CC(3/7)	0.943(0.858-1.037)	0.224
	CT+CC(20/31)	0.967(0.927-1.009)	0.119
Tumor Location	TT(23/26)	1.000.	
Colon/Rectum	CT(26/15)	0.960(0.921-1.000)	0.051
	CC(7/3)	0.953(0.897-1.012)	0.118
	CT+CC(33/18)	0.958(0.924-0.993)	0.02
Nodal Status	TT(29/20)	1.000.	
Involved/Not involved	CT(21/20)	0.997(0.959-1.037)	0.888
	CC(7/3)	0.925(0.862-0.993)	0.031
	CT+CC(28/23	0.984(0.953-1.017)	0.34
Tumor Grade	TT(27/22)	1.000.	
I+II/III+IV	CT(21/20)	0.987(0.949-1.026)	0.498
	CC(4/6)	0.946(0.869-1.031)	0.208
	CT+CC(25/26)	0.985(0.952-1.020)	0.398
Age	TT(21/28)	1.000.	
≥(50) ≤(50)	CT(18/23)	0.967(0.916-1.020)	0.217
	CC(4/6)	0.889(0.788-1.003)	0.056
	CT+CC(22/29)	0.968(0.928-1.009)	0.123
Hypertension	TT(26/23)	1.000.	
Yes/No	CT(20/21)	0.972(0.931-1.015)	0.203
	CC(5/5)	0.943(0.880-1.011)	0.101
	CT+CC(25/26)	0.971(0.936-1.007)	0.111
Gender	TT(26/23)	1.000.	
Male/Female	CT(24/17)	0.976(0.938-1.015)	0.222
	CC(6/4)	0.943(0.880-1.011)	0.101
	CT+CC(30/21)	0.973(0.941-1.007)	0.117
aOR age adjusted odds ratio, CI confidence interval			
P value and corresponding age-adjusted OR (aOR) with 95% Cls[aOR(95% CI)] by logistic regression analysis			



DISCUSSION

SNP based genome screens have identified different locations at different chromosomes, that may be involved in predisposition to prostate, breast cancers as well as CRCs. [17-19] The detection of the susceptibility variants has established the principle of the "common variant–common disease" model, but it is possible that several or many more parallel variants remain to be found. Once a large enough number of such genes has been identified, it will become possible to apply information about them in the clinical setting. Parkin locus has been implicated in different cancers such as ovarian cancer, small cell lung carcinoma and cervical cancer. [9,11,20] Till date no association between any PARK2 polymorphism and incidence of CRC have been reported. SNP rs10945859 in the PARK2 gene is one such SNP which could prove useful in predicting the clinical outcome in cancer. To evaluate the potential of rs10945859 SNP in this study we genotyped 100 CRC and 100 healthy unrelated subjects from North India.

Genotyping of rs10945859 SNP was done with RFLP by altering one base in the reverse primer, a common strategy utilized for genotyping of many SNP's. The studied population was found to be under Hardy-Weinberg equilibrium (0.742). The minor allele frequency for this SNP was found to be 0.32 in healthy individuals of this studied group of our population which was higher than the reported frequency for other population (0.23) and global MAF (0.22). [16] The variation found in allele frequency could be due to smaller sample size studied in this case. Logistic regression analysis even after age adjustment showed no overall significant association between rs10945859 and CRC in the studied group. Although the odds ratio obtained for TC (OR: 0.794, 95% CI: 0.281-2.246, P= 0.664) and CC genotypes (OR: 0.891, 95% CI: 0.323-2.458, P= 0.823) suggests protection due to this allele but since the sample number was small no statistical significance was observed. Some well-established lifestyle features which are known to aid in cancer development namely alcohol drinking and smoking also showed significant difference within CRC subjects.

In-silico splicing factor binding analysis revealed gain and loss of different splicing factors binding sites. Gain of splicing factor NOVA1 and hnRNP K binding site due to minor allele suggest there potential in regulating alternative splicing of PARK2. Interestingly both NOVA1 and hnRNP K are known to regulate alternative splicing in a neuron specific manner. [21,22] hnRNP K is a component of intronic splicing enhancer complex and is known to complete with early spliceosomes component U2AF65 and regulate alternative splicing during neuronal differentiation. [21] On the other hand NOVA1 is known to be essential for alternate splicing and neuronal viability. [22] Thus unknown alternatively spliced isoform of PARK2 may be present depending on cell type and involvement of this polymorphism might be more prevalent in brain tumors and cancers of neuronal origin.

In conclusion based on the results obtained we encourage extensive analysis of this polymorphism in as larger sample size along with wet lab experiments for further validations.

Conflict of Interests: The authors declare no competing interests.

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