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Full Length Research Paper

Loss of heterozygosity (LOH) of deleted in colorectal cancer (*DCC)* gene and predisposition to colorectal cancer: Significant association in colorectal cancer patients of Kashmir

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Loss of heterozygosity (LOH) of deleted in colorectal cancer (*DCC)* gene, a putative tumour suppressor gene has been implicated in development of various cancers. Here we aim to study the frequency of LOH of *DCC* gene in colorectal cancer (CRC) patients of Kashmir and further to assess any association with etio-pathological factors. Genomic DNA from 80 confirmed CRC tissue samples as well as adjacent normal tissues were used in this study. LOH of *DCC* gene was detected using two intronic microsatellite markers, D18S8-M2 and variable number tandem repeat (VNTR) by restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) respectively. LOH in *DCC* was observed in 26 of 80 (32.5%) at VNTR and 19 of 80 (23.75%) at D18S8-M2 region in CRC cases. The combined frequency of LOH of *DCC* gene of two markers aggregated to 56.25 % (45 of 80) of CRC cases. In this study it is found that LOH has a frequency of 56% in patients with CRC and is highly frequent in patients with higher stage/grade in CRC.

Key words: Colorectal cancer, restriction fragment length polymorphism, loss of heterozygosity, microsatellite markers.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women. An estimated 16,650 cases and 7,050 deaths from CRC are expected to occur among African Americans in 2011 (American Cancer Society). In Kashmir valley frequency of CRC is showing an increasing trend. As per a recent hospital based survey conducted by Arshad et al. (2011) (Data under revision) colorectal cancer is ranked fourth most common cancer in Kashmir with a frequency around 11% of all cancers. As per this study the ratio of male: female is 1:1 with an ASR (Age standardized rate) incidence 4×10^5/Year. *DCC* a putative tumour suppressor gene has been mapped on the long arm of chromosome 18 (18q). *DCC* has high homology with NCAM, one of the adhesion molecules abundantly expressed in nervous tissues, it has been reported to play an important role in cell-cell or cell-extracellular matrix interaction during development and differentiation (Fearon et al., 1994). Some reports strongly indicate that *DCC* is important for cell

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Abbreviations: ASR, Age standardized rate, ***DCC*,** deleted in colorectal cancers; **VNTR,** variable number tandem repeat; **MSI,** microsatellite instability; **RFLP,** restriction fragment length polymorphism.

Table 1. Primer sets used in PCR-LOH assays.

Primer set	Microsatellite markers	Primer sequence	Annealing temperature (°C)	Amplicon size (base pairs)	Reference
01	VNTR	5'-GATGACATTTTCCCTCTAG-3' 5'GTGGTTATTGCCTTGAAAAG-3'	56	150-210	Dian-Chun et al. (1998)
02	MspI RFLP (D18S8-M2)	5'TGCACCATGCTGAAGATTGT-3' 5'AGTACAACACAAGGTATGTG-3'	56	396	

differentiation (Hedrick et al., 1994), particularly in the differentiation of neuronal cells (Laelor et al., 1992). In normal conditions *DCC* induced apoptosis limits cellular lifespan in the intestinal crypt and thereby inhibits the initiation of malignant transformation. Transfection of *DCC* cDNA into a human cell line lacking *DCC* expression suppresses tumour growth and results in apoptosis and cell cycle arrest (Klingelhutz et al., 1995). Tanaka et al. (1991) reported that tumorigenicity in human colon carcinoma cells can be suppressed by introduction of normal chromosome 18 revealing tumour suppressor functions of *DCC*. Alterations in *DCC* gene like mutations (Kathleen et al., 1994) reduced expression (Christelle et al., 2001; Goi et al., 1998). LOH (Adrienne et al., 2011) has been implicated in the development of various cancers including CRC. Loss of function of *DCC* gene can be assessed by detecting instability in microsatellite markers. Microsatellites are short repetitive sequences of DNA that are scattered throughout the genome and are stably inherited, unique to each individual and have low inherent mutation rate (Weber et al., 1989; Hearne et al., 1992). Several studies have shown that alterations due to mutations in the simple repeat sequences or microsatellites are feature unique to a number of cancers (Sturzeneker et al., 2000; Arzimangogou et al., 1998). Many reports on colon cancer have found varying length of microsatellite DNA in tumour tissues as compared to matching normal tissue. This variation in length of microsatellite represents a mutational process of insertion or deletion within tumour DNA (Brentnall et al., 1995). Loss of heterozygosity (LOH) at a constitutional heterozygous locus indicates the probability of loss of a tumour suppressor gene, which might promote neoplastic progression (Lasko et al., 1991; Fearon et al., 1990).

In this study conducted for the first time in Kashmir (Northern part of India), we analyzed frequency of LOH of *DCC* gene in colorectal cancer patients in relation to sequence variation of two microsatellite markers, D18S8-M2 located in the polymorphic locus D18S8, localized to 18q21.3 (Parry et al ., 1991) and VNTR region lying within intronic region of *DCC* gene.

MATERIAL AND METHODS

Sample collection

Paired tumour and adjacent normal tissue were collected from eighty patients of CRC who had undergone surgery at the Sher-I-Kashmir Institute of Medical Sciences (SKIMS) between 2009 and 2011. Almost all the patients with left colon carcinoma had attended the hospital with a clinical presentation of bleeding per rectum and those with right colon carcinoma were positive for occult blood. Grading of cancers was performed according to the WHO classification and staging by the 1997 TNM classification guidelines (UICC, 1997). Diagnostic slides prepared from fresh tumour samples were reviewed by a panel of two expert pathologists to confirm diagnosis and ensure uniformity of classification criteria (Table 2). This study was approved by the ethical committee of SKIMS.

DNA extraction

Tissue samples (both tumour and adjacent normal) collected were snap-frozen immediately and stored at -70°C. DNA was extracted by standard *Proteinase K*, Phenol/chloroform method as described by Blin et al. (1976).

PCR-loss of heterozygosity

PCR amplification was carried out in a 25 µl volume with a concentration of 50 ng of genomic DNA,1 × PCR buffer containing 1.5 mM $MgCl_2$, 100 µM each of dATP, dGTP, dTTP, dCTP,1.5 unit of *Taq DNA polymerase* (Biotool, Madrid, Spain), 1 µM of forward and reverse primers (Table 1). The priming region for D18S8-M2 was located within specific tumour suppressor gene possessing a polymorphic *Msp*I site within the region designated as D18S8-M2, and was detected by RFLP. After an initial denaturation at 95°C for 5minutes, 35 cycles of 94 °C for 40 seconds, a specific annealing temperature 56°C for 40 s, and 72°C for 40 s and with the final extension at 72°C for 7 min were performed. The amplified PCR products for D18S8-M2 (396bp) was digested by *Msp*I restriction enzyme and analyzed on 8% polyacrylamide gel (Figure 1) whereas amplified product of VNTR region was directly run on 8% PAGE (Figure 2) and photographed under ultraviolet light. In this study only informative cases were

Table 2. Relation of clinico pathological variables with LOH.

Variable	Cases (n=80)	LOH –ve [%]	LOH+ve [%]	P-value
Grade(differentiation)				
Well differentiated	51(63.75%)	31(60.78%)	20 (39.21%)	0.00019*
Mod/Poorly differentiated	29(36.25%)	4(13.79%)	25 (86.20%)	
Clinical staging				
Stages I-II	47(58.75%)	25(53.19%)	22(46.80%)	0.044*
Stages III-IV	33(41.25%)	10(30.30%)	23(69.69%)	
Location				
Colon	42(52.5%)	15 (37.71%)	27 (64.28%)	0.12
Rectum	38(47.5%)	20 (52.63%)	18 (47.36%)	
Dwelling				
Rural	46(57.5%)	22 (47.82%)	24 (52.17%)	0.393
urban	34(42.5%)	13 (38.23%)	21 (61.76%)	
Age				
<50	33(41.25%)	14 (42.42%)	19 (57.57%)	0.8412
≥50	47(58.75%)	21 (44.68%)	26 (55.32%)	
Sex				
Male	43(53.75%)	21(48.83%)	22(51.16%)	0.323
Female	37(46.25%)	14(37.83%)	23(62.16%)	

χ^2 was used to calculate the p-value of the variables. *p-Value<0.05 was considered statistically significant.

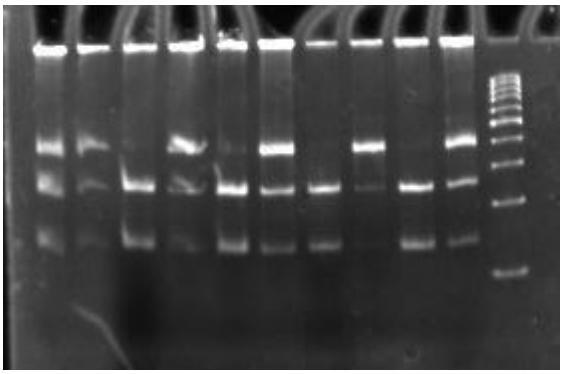

Figure 1. Showing LOH of *DCC* gene at M2 region detected with PCR-RFLP. Lane M: 100bp DNA ladder, N=Normal; Lane T= Tumor. LOH^{+ve} in Informative cases.LOH^{-ve} samples showed no loss of heterozygosity.

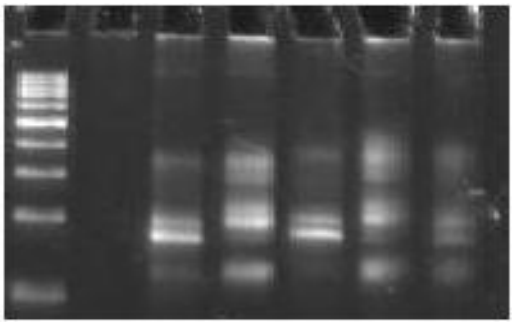

Figure 2. Showing LOH of *DCC* gene at VNTR region. Lane M=100bp DNA ladder, N= Normal; T=Tumor. Strong allelic imbalance is seen in tumor showing range of bands (150-200bp) not in adjacent normal tissues, with dominance of the larger 200-base pair allele.

included (cases in which normal samples were heterozygous at M2-D18S8 marker) (Figure 1), whereas uninformative cases (cases in which normal sample showed no heterozygosity) were excluded from the study. Digested product of D18S8-M2 region yielded products of size 396,257 and 139 bp. LOH was considered positive for samples with absence of 396 bp band and presence of 257 and139 bp (Figure1). PCR product of VNTR when run directly on 8% PAGE generated a spectrum of alleles ranging in size from150 to 210bp (Figure 2) depending on insertion or deletion.

Statistical analysis

Chi-square was used to compare the LOH positive and LOH negative samples of *DCC* gene with the etio-pathological parameters. P-value<0.05 was considered statistically significant.

RESULTS

Loss of heterozygosity of *DCC* gene was determined by

Table 3. Percentage of cases with and without loss of heterozygosity at two different markers.

Markers (n=80)	LOH-ve	LOH+ve	P-value
D18S8M2	61 (76.25%)	19 (23.75%)	0.21
VNR	54 (67.56%)	26 (32.50%)	

χ^2 was used to calculate the p-value of the variables.

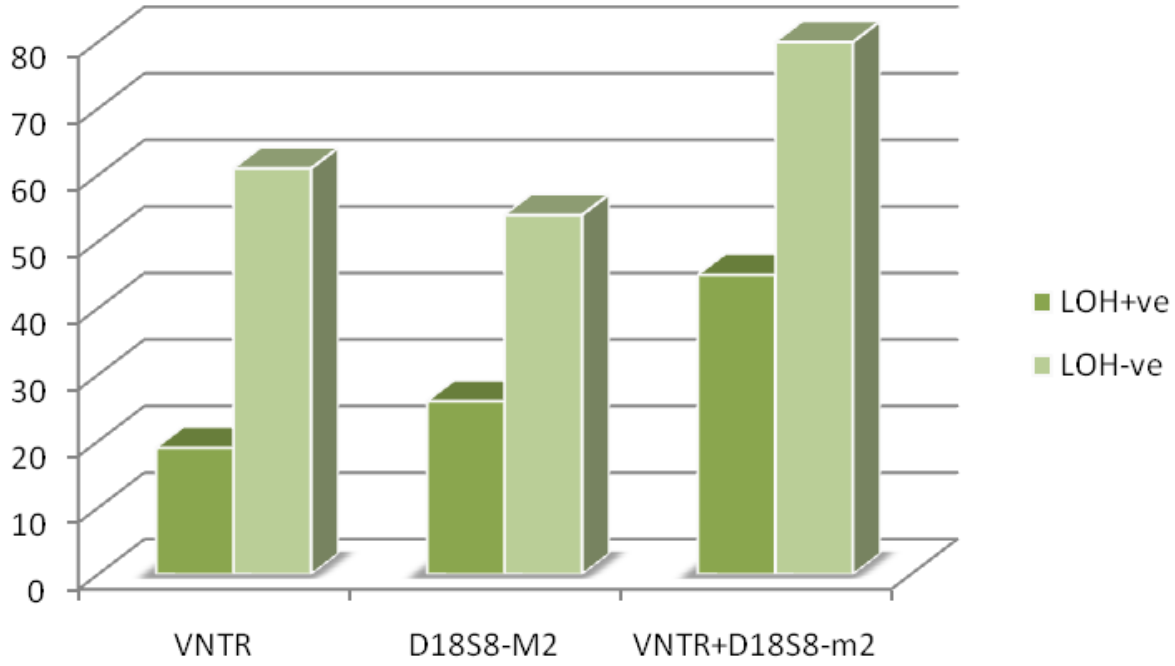

Chart 1. Represents frequency distribution of LOH at two markers D18S8-M2 and VNTR of *DCC* gene.

PCR-LOH assay in 80 samples of colorectal carcinoma and corresponding adjacent normal tissue. Mean age at the time of diagnosis was 52 years (range 30 to 80) with male: female ratio of 1:1.All the tumour samples included in this study were histopathologically confirmed cases of CRC. Histopathological findings of the CRC cases revealed 51 of 80 (63.75%) as well differentiated grade and 29 of 80 (36.25%) as moderately/poorly differentiated grade. LOH at both D18S8-M2 and VNTR markers was observed as 39.21% (20/51) in samples with well differentiated grade and 86.20% (25/51) in moderately/poorly differentiated samples. 47 of 80 (58.75%) cases of CRC were of stage I-II and 33 of 80 (41.25%) of stage III-IV. LOH was found in 46.80% (22/47) and 69.69% (23/33) at both the markers in stage I-II and III-IV respectively (Table 2). The overall combined frequency of LOH at two markers (D18S8-M2 and VNTR) in CRC cases was observed to be 56.25% (45/80) (Table 3 and Chart 1). LOH of *DCC* was found to be highly frequent in patients with higher stage/grade of CRC and this association was found to be significant ($p<0.05$). However no association of LOH was observed with any of the etiological parameters as depicted in Table 2.

DISCUSSION

In this study frequency of LOH of *DCC* gene was studied in CRC patients using two markers one within the polymorphic locus D18S8 localized to 18q21.3 and the other in VNTR. In our report the frequency of LOH in *DCC* gene was observed in 26 of 80 (32.5%) at VNTR and 19 of 80 (23.75%) at D18S8-M2 region in CRC cases. The combined frequency of LOH of *DCC* gene of two markers aggregated to 56.25% (45 of 80) of CRC cases and this observation was either in tune with few related studies or in discordance with other reports. Allelic deletion of *DCC* has been reported as 65% (Bruce et al., 2007); 12% (Mustafa et al., 2007) and 78% (Shih et al., 2005) in colorectal carcinoma whereas Latil et al. (1994) and Gima et al. (1994) reported 33 and 26% allelic losses of 18q in prostate cancer and endometrial carcinomas respectively. Tomonao Gima et

Table 4. Frequency of loss of heterozygosity of *DCC* in different cancers**.**

Cancer type	Frequency of LOH	References
Esophageal cancers	17/72 (24%)	Ying et al. (1992)
Esophageal cancers	20/26 (77%)	Robert et al. (1992)
Colorectal cancer	11/17(65%)	Bruce et al. (2007)
Colorectal carcinoma	5/43(12%)	Mustafa et al. (2007)
Colorectal carcinoma	162/207(78.7%)	Shih-Ching et al. (2005)
Bladder cancer	16/45(36%)	Hiroshi et al. (1996)
Gastric cancer	18/51 (35.3%)	Dian-Chun et al. (1998)
Colorectal carcinoma	8/15 (53%)	Schmitt et al. (1998)
Colorectal carcinoma	21/38(55.3%)	Dian-Chun et al. (1995)
Testis Cancer	10 /36(28%)	Hong-Qi et al. (1995)
Endometrial carcinomas	1/7 (14%)	
Cervical carcinomas	I/11 (9%)	Enomoto et al. (1995)
Ovarian tumours.	2/6 (33%)	
Colorectal carcinoma	5/17 (29%)	Cawkwell et al. (1994)
Colorectal carcinoma	43/57(75%)	Kathleen et al. (1994)
Colorectal carcinoma	12/39 (31%)	Lacopetta et al. (1994)
Endometrial carcinomas	16/61(26%)	Tomonao G et al. (1994)
Prostate cancer	6/20(33%)	Latil A et al. (1994)
Prostatic carcinomas	5/11 (45%)	Xiang et al. (1993).
Colorectal carcinoma	7 / 11 (63%)	Rei kikudii Yet al. (1992)
Esophageal cancers	16/67(24%)	Huang et al. (1992)
Gastric cancers	14/23(62%)	Uchino et al. (1992)
Our study	45/80 (56%)	

al. (1994) reported 26% frequency of allelic losses at one or more chromosome 18q loci in endometrial carcinomas (Table 4). Their study suggests that the target for allelic loss on chromosome 18q seen in different cancers is the *DCC* gene, and that inactivation of this gene may be critical for the development of most carcinomas.

Tumour suppressor genes like *DCC* play an important role in etiology of different human cancers (Koiti et al., 2002; Duman-Scheel, 2009). The *DCC* gene encodes several different protein products as a result of alternative splicing (Patrick and Eric, 2004). The expression of *DCC* is limited to the basal layer, which is an active, and yet, regulated zone of cell proliferation (Liu et al., 2011). Recent studies have proposed that *DCC* functions as a tumor suppressor by promoting apoptosis (Duman-Scheel, 2009). Christelle et al. (2001) proved that *DCC* drives cell death independently of both the mitochondria-dependent pathway and the death receptor/caspase-8 pathway. LOH at 18q is associated with decreased *DCC* expression and has been linked to many other types of cancer, including neuroblastomas, hematologic malignancies, gastric, endometrial, prostate, ovarian, esophageal, testicular, breast, and glial cancers (Adrienne et al., 2011).

Even though mutations of tumour suppressor genes have been hypothesized to act recessively at cellular level so that both maternal and paternal copies of gene must be inactivated in order for growth suppressive function to be eliminated (Knudson et al., 1985), but *DCC* does not confirm to this recessive gene model and loss of single allele without mutation in remaining allele leads to reduced expression of this protein resulting in altered adhesion properties of cell (Vogelstein et al., 1988). Sato et al. (2001) reported frequent loss of expression without sequence mutations of the *DCC* gene in primary gastric cancer. In colorectal carcinoma, the accumulation of multiple genetic alterations such as mutation of oncogenes (K-ras), 1p deletion, 8p deletion, and LOH of 17p and 18p are believed to contribute to carcinogenesis starting from colorectal adenoma to carcinoma (So¨reide et al., 2006). Results of our study are consistent with some of the earlier studies whereas few studies (Table 4) report more frequency of LOH in CRC. The reason of this bias in our results may be due to the consideration of only two markers in our study to detect LOH of *DCC* gene.

The data demonstrates that LOH of *DCC* gene occurs more frequently in late stage carcinoma than in early stages ($p<0.05$). Many studies including Liu et al. (2011) and Mustufa et al. (2007) suggested that the loss of expression of *DCC* occurred more frequently in the cases of later clinical stages and higher pathological grades in ovarian cancers and colorectal cancers.

However our study could not find any correlation of LOH of *DCC* with any other etiological factors.

Conclusion

In this study it is found that LOH has a frequency of 56% in patients with CRC and is highly frequent in patients with higher stage/grade in CRC. Our study concludes that LOH of *DCC* gene may be one of the genetic events involved in the development of colorectal cancer in Kashmiri population.

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