Loss of Heterozygosity Status Analysis in Colonic Cancer Cells in a Group of Bangladeshi Patients

R Paul[1], M Kamal[2], F Begum[3], T Saha[4], S Afroz[5], MN Islam[6]

Abstract

Background*:** Colorectal cancer is a multifactorial disease process. A cross-sectional descriptive study was conducted among 35 cases of colorectal cancer patients to see the loss of heterozygosity (LOH) status analysis in those colonic cancer cells. Environmental and genetic factors both contribute to colorectal cancer. Etiology contributing from environmental factors includes dietary factors, obesity, alcohol intake, smoking and life style changes. The molecular events that leads to colorectal carcinoma is heterogeneous and includes genetic and epigenetic abnormalities. ***Methods: It is now believed that two pathogenitically distinct pathways namely the APC/b- caterin pathway and the microsatellite instability pathway are responsible for the development of colorectal cancer. Loss of heterozygosity in a cell represents the loss of normal function of one allele of a gene in which the other allele was already been inactivated. This term is mostly used in the context of oncogenesis. An inactivating mutation in one allele of a tumor suppressor gene that occurs in the parent's germ line cell is passed on to the zygote resulting in an offspring that is heterozygous for that allele. LOH occurs when the remaining functional allele in a somatic cell of the offspring becomes inactivated by mutation. This could cause a normal tumor suppressor gene to loss it's normal activity and results tumorogenesis. LOH is a common phenomenon in a variety of human cancers like colorectal carcinoma, urinary bladder cancer, osteosarcoma, prostatic carcinoma, cervical carcinoma, small cell lung cancer, non-small cell lung carcinoma, pancreatic carcinoma, breast carcinoma, malignant pituitary tumor and retinoblastoma. ***Results*:** LOH was most frequently found in chromosome 19p region (11.4% cases), followed by chromosome 12p (8.5% cases) and 4p (8.5% cases) and 5q regions (8.5% cases). ***Conclusion*:** Loss of heterozygosity in colonic cancer patients in our country was most frequently found in chromosome 19p region followed by chromosome 12p, 4p and 5q regions.

Key words: Colonic cancer, Loss of Heterozygosity.

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Introduction

Colorectal carcinoma is the most common type of gastrointestinal cancer. Genetic susceptibility may be the soil on which subsequent environmental factors act[1]. The dietary factors predisposing to a higher incidence of colorectal cancers are (1) excess dietary calorie intake relative to requirements, (2) a low content of unabsorbable vegetable fiber, (3) intake of red meat, (4) a corresponding high content of refined carbohydrates, (5) diet high in total fat or saturated fat, (6) decreased intake of protective micronutrients. (7) deficiencies of vitamins A, C, E which act as free radicle scavengers, may compound damage caused by oxidants. In addition to dietary modification aspirin or other NSAIDs have also protective effect against colorectal carcinoma[2].

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The genetic and epigenetic pathways involved in colorectal carcinogenesis are:

APC/b-catenin pathway : This is characterized by chromosomal instability that results in stepwise accumulation of mutations in a series of oncogene and tumour suppressor gene. The molecular evolution of colon cancer along this pathway occurs through a series of morphologically identifiable stage. Initially there is a localized colon epithelial proliferation. This is followed by small adenoma formation that progressively enlarge, become more dysplastic and ultimately develop into cancer. APC

gene acts as a gate keeper protein, as it regulates the level of b- catenin, an important mediator of the Wnt /b- catenin signaling pathway. This signaling pathway plays an important role in normal intestinal epithelial development. It is also involved in the development of colorectal carcinoma[2].

Loss of p^{53}: Losses at chromosome 17p have been found in 70% to 80% of colon cancer. These chromosome deletion affect the p^{53} gene, suggesting that mutation in p^{53} occur late in colon carcinogenesis.

K-RAS mutation: The K- RAS gene is the most frequently observed activated oncogene in adenoma and colon cancer. K- RAS plays a role in intracellular signel transduction and is mutated in approximately 50% of carcinoma.

Loss of SMADS: A common allelic loss in colon cancer is on 18q21.SMAD2 and SMAD4 involved in TGF-b signaling are located on 18q21. Lack of SMAD4 increases gastrointestinal tumorigenesis.

Telomerase activity: Telomeres plays a role in stabilizing the chromosome. They shorten with each cell division until cell senescence develop. Telomerase activity is required to maintain telomere stability and hence cell immortality, a prerequisite for all cancer cells. In colorectal carcinoma there is increased telomerase activity.

Microsatellite instability pathway: It is characterized by genetic lesions in DNA mismatch repair genes. Defective DNA repair is caused by inactivation of DNA mismatched repair genes. Inherited mutations in any of five genes that are involved in DNA repair are responsible for the famial syndrome of HNPCC. These human mismatch repair genes , hMSH2, hMLH1,MSH6, hPMS1, hPMS2 are involved in genetic proof reading during DNA replication and have earned the moniker of caretaker genes. The majority of mutations involved (90%) MSH2 and MLH1. Mutations in the mismatched repair genes cause alteration of microsatellites, leading to microsatellite instability. Tumours associated with microsattllite instability tend to be mucinous or poorly differentiated , with a prominent host response, a circumscribed growth pattern and right sided location[3].

DNA methylation abnormalities (Hypomethylation and Hypermethylation) : In addition to genetic mutation, this epigenetic change has been included as an alternative mechanism to cancer development.

Loss of heterozygosity in colorectal cancer: Loss of heterozygosity in colorectal cancer was first reported by Vogelstein et al[4]. It was then explored by many investigators. Attempts to establish the relationship between colorectal cancers and allele losses at different chromosome loci have been made. In one study over colorectal cancer, the frequency of allelic losses were determined at 17p (28%), 18p (26%), 18q and 5q (25%), 9q and 15q (24%), 8p (23%) and 14q (20%) throughout the genome and reported additional six different LOH areas as 4q (42%), 1p (36%), 11q (31%), 17p(28%), 18p (26%) and 1q (25%) in sporadic colorectal carcinoadenomas. PTPRJ (protein tyrosine phosphatise receptor type J) on chromosome band 11p11 is a deleted found gene among several cancer types including colorectal cancer. The observation of high frequency LOH on chromosome 1 in patients with sporadic colorectal cancer is described by Jen-Kou Lin et al[5]. may suggest the presence of putative tumour suppressor genes associated with sporadic colorectal cancer on 1p36.31-36.33. By high resolution deletion mapping, two high frequency regions of LOH (4q12-21.1 and 4q25-31.1) were detected, which may contribute to locate TSGs on chromosome 4q involved in carcinogenesis and progression of sporadic colorectal carcinoma Yaw KT et al [6]. High frequency LOH is associated with high metastatic potential of colorectal cancers. LOH has a high frequency of 56%in patients with CRC and is highly frequent in patients with higher stage/grade in CRC.

Objectives

To detect the loss of heterozygosity status in the colonic cancer cells of these cases.

Methods

A cross sectional study was carried out in 35 specimen of colorectal carcinoma patients in department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka during the period of May 2010 to January 2011. Cases were collected from Bangabandhu Sheikh Mujib Medical University (BSMMU) and private hospitals and clinics of Dhaka city. The loss of heterozygosity status analysis was carried out in collaboration with the Department of Health Studies, Biological Sciences Division (BSD) of The University of Chicago Medical Center, Chicago , IL , USA and BSMMU, Dhaka.

Inclusion criteria

a. Histologically confirmed cases of adenocarcinoma of the colon.

b. Cases with complete clinical information and investigation report who underwent surgical resection.

c. Clinically suspected inflammatory diseases which were subsequently diagnosed as colorectal carcinoma after histological examination.

d. Availability of fresh unfixed colorectal cancer specimen.

e. Availability of normal uninvolved colonic tissue away from the tumour.

Exclusion criteria

a. Clinically suspected colorectal carcinoma subsequently proved to be non-malignant lesions after histological examination.

b. Non Hodgkin lymphoma and other non epithelial tumors of the colon.

c. Cases without clinical data and investigation report.

d. Patients who had cancers both in the right and left colon, either synchronously or metachronously.

e. Formalin fixation or delay in receiving specimen.

Sample Tissue collection for LOH study

Paired unfixed tissue samples one each from tumour and healthy mucosa were taken in DNAse free 1.5 ml eppendorf tube (Ambion Catalogue # AM12450, Ambion company, USA). Dimention of the tissue blocks were 4 to 5 mm. These eppendorf were kept at -80°c and sent to the department of Health Studies, Biological Sciences Division of The University of Chicago Medical Center, Chicago , IL , USA in dry ice.

DNA extraction and LOH study was done at the Department of health studies (BSD), The University of Chicago, Chicago, IL, USA under agreement between the department of pathology, BSMMU and Department of health studies (BSD), The University of Chicago, Chicago, IL, USA.

DNA extraction from colonic tissue:

DNA extraction and subsequently high-density SNP (Single nucleotide polymorphism) genotyping microarray was done in the molecular genomics lab of University of Chicago, IL, USA. DNA extraction from the colonic tissue was done using Gentra Purgene Tissue Kit (Catalogue# 158667, QIAGEN, USA, www.qiagen.com)

Genome-wide SNP Genotyping assay:

Oligonucleotide-based SNP (Single nucleotide polymorphism) genotyping array was used to generate genotype, copy number and LOH data. Illumina's Human Cyto12 v2.1 Bead Chip was used that had a total of 299,140 markers of which 292,482 were SNPs and the rest 6,658 were CNV probes. Assay was done according to manufacturer's protocol.

Statistical analysis:

Histopathological portion: All the necessary and relevant data were recorded methodically and meticulously as far as possible in the clinical proforma. Relevant data were analyzed by standard statistical method.

Loss of heterozygosity status analysis part: For SNP based genotyping, Bead Chip was used that had a total of 299,140 markers of which 292,482 were SNPs and the rest 6,658 were CNV probes. For CN (copy number) analysis background correction was done with adjustment for fragment length and probe sequence, but no normalization was done. The log2 ratio of the signal intensity was used for calculation of the CN. For detection of CN change regions, the Hidden Markov Model (12) was used with maximum probability of 0.995, genomic decay of 10,000,000, and b = 2. Maximum probability specifies the probability of retaining the same state between neighboring observations. The genomic decay describes how quickly (expressed in base pairs) the Hidden Markov Model retention of state will decay toward the initial probability. Specifies the Gaussian bandwidth of the distribution from which observations are drawn. Higher values of would expect more noise but may not detect the smaller region. Smaller values will result in more regions. The reported regions contain at least 10 probe sets. The CN change regions were mapped to cytoband regions and the length was calculated from the start to the end regions.

The paired LOH regions were calculated assuming the maximum probability of 0.99, genomic decay of 10,000,000, and genotype erro b = 0.01. The length of the region was calculated from the start to the end regions.

Results

Histopathological diagnosis

Most of the tumours 25(85.7%) were adenocarcinoma among the 30 patients. On the other hand 5 (14.3%) were mucinous adenocarcinoma.

Loss of heterogygosity status analysis:

Table-I: showed the paired LOH analysis data.

Chromosome	Cytoband	Samples	Samples	Length (bp)	% Samples	Heterozygous rate	Markers
19	19p13.11 - 19p12	C55_T C56_T C78_T C89_T	4	4240691	11.4286	0.073997	598
19	19p13.3	C55_T C56_T C78_T C89_T	4	2863400	11.4286	0.057095	451
19	19p13.2 - 19p13.13	C55_T C56_T C78_T C89_T	4	1647356	11.4286	0.047269	238
19	19p13.11	C55_T C56_T C78_T C89_T	4	1444601	11.4286	0.076191	210
19	19p13.2	C55_T C56_T C78_T C89_T	4	1389933	11.4286	0.065476	210
19	19p13.3	C55_T C56_T C78_T C89_T	4	1058394	11.4286	0.070552	163
19	19p13.2	C55_T C56_T C78_T C89_T	4	692471	11.4286	0.072072	111
12	12q24.11 - 12q24.13	C55_T C56_T C89_T	3	2540641	8.57143	0.071053	380
4	4p16.3 - 4p16.2	C55_T C56_T C89_T	3	1562906	8.57143	0.070748	245
5	5q32	C55_T C56_T C76_T	3	1512221	8.57143	0.070370	90
12	12p13.33	C55_T C89_T C90_T	3	1007112	8.57143	0.049275	115
19	19p13.3 - 19p13.2	C55_T C78_T C89_T	3	955984	8.57143	0.000000	158
19	19p12	C55_T C56_T C89_T	3	876928	8.57143	0.044331	391
12	12p11.21 - 12p11.1	C55_T C89_T C90_T	3	799453	8.57143	0.061111	120
19	19p13.12	C55_T C78_T C89_T	3	770591	8.57143	0.000000	117
19	19p13.2	C55_T C56_T C89_T	3	746272	8.57143	0.093567	114
19	19p13.12	C55_T C78_T C89_T	3	731223	8.57143	0.003268	102
12	12p11.22 - 12p11.21	C55_T C89_T C90_T	3	721291	8.57143	0.079772	117
12	12p12.1	C55_T C89_T C90_T	3	698349	8.57143	0.077626	73
12	12p12.3	C56_T C89_T C90_T	3	687344	8.57143	0.045268	81
5	5q33.1	C55_T C56_T C76_T	3	666240	8.57143	0.060109	61
12	12p12.3	C56_T C89_T C90_T	3	658135	8.57143	0.012048	83
19	19p13.3	C55_T C78_T C89_T	3	622512	8.57143	0.000000	99
5	5q32 - 5q33.1	C55_T C56_T C76_T	3	596269	8.57143	0.039216	51
12	12p12.3	C55_T C89_T C90_T	3	528183	8.57143	0.007576	88
19	19p13.11	C55_T C78_T C89_T	3	516429	8.57143	0.004219	79

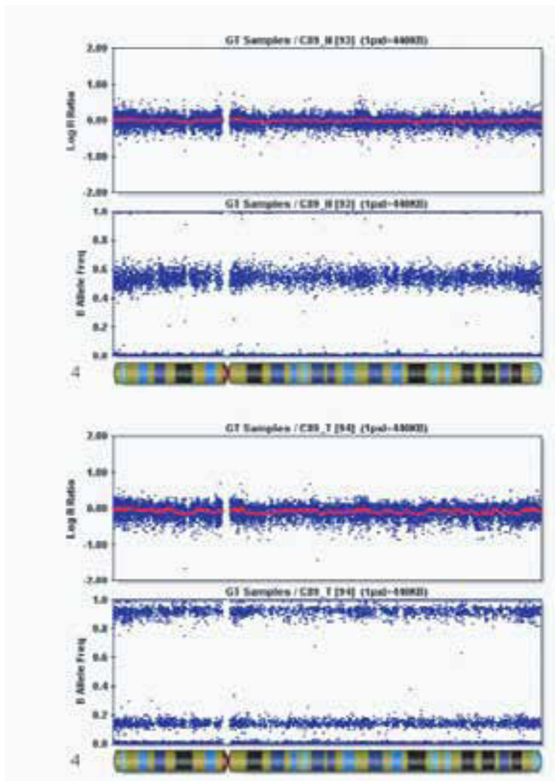

Figure-1: shows (C89) Chr4 copy neutral LOH

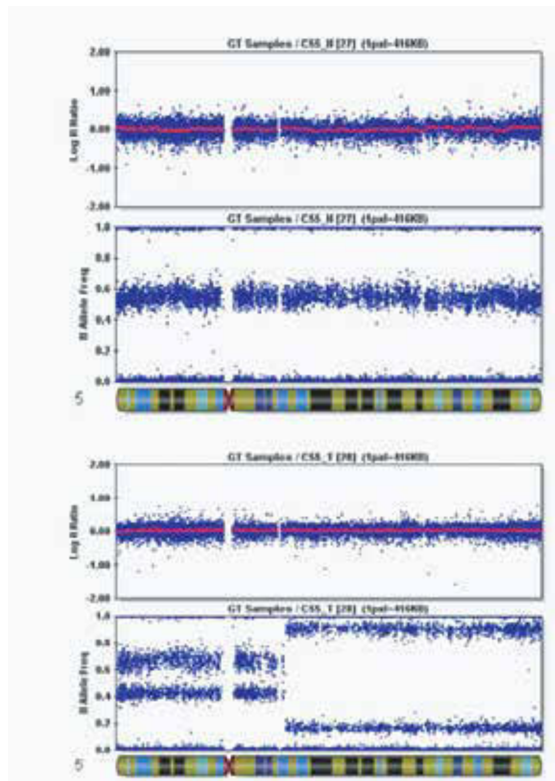

Figure-2: shows (C55) Chr5q copy neutral LOH

Discussion

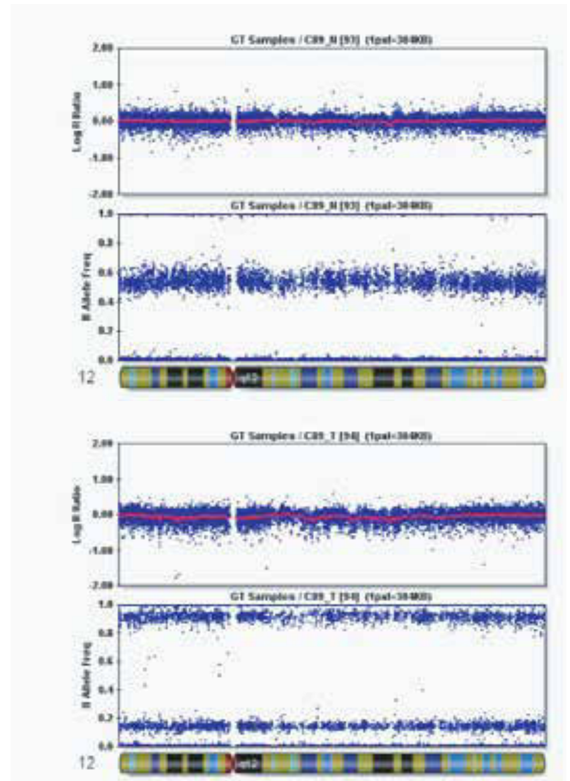

Figure-3 : shows (C89) Chr12 copy neutral LOH

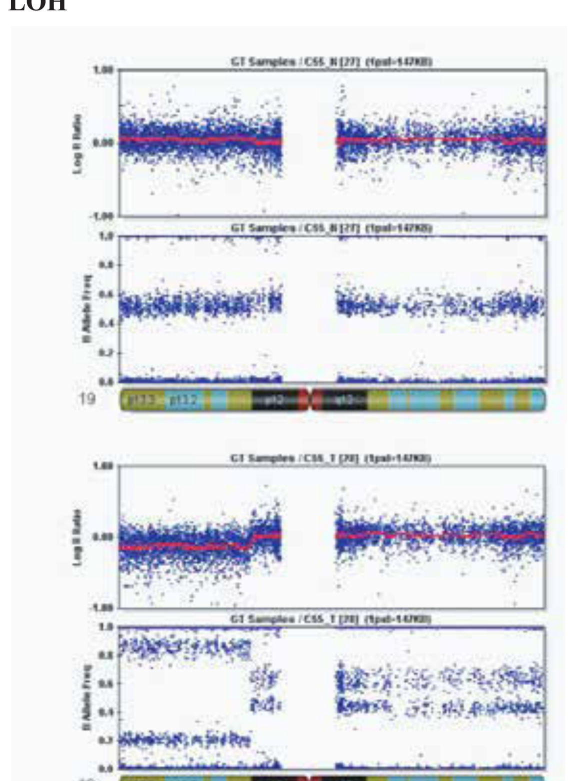

Figure-4: shows (C55) Chr 19p Del with LOH

Discussion

On histological examination of 35 colorectal cancer cases, 30 (85.7%) cases were adenocarcinoma (NOS) and five (12%) cases were mucinous adenocarcinoma.

Most of the colon cancer is adenocarcinoma, (30=85.7%) observed in my study. This finding is similar to the findings described by Yaw et al[6], 25 (84.16%), (88%), 5 (74%), Keating et al[7] 16 (94.7%) and Pahlavan et al[8] 26 (90%).

Loss of heterozygosity is the most common molecular genetic alteration observed in human cancers. LOH analysis is a sensitive genetic method that is used to detect micro deletions on chromosomes. A frequent deletion in a chromosomal region may suggest the existence of a candidate tumour suppressor gene.

In the model of colorectal tumorigenesis, mutational inactivation of tumor suppressor genes predominates. Higher density SNP array can be used effectively to detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions. In addition, more markers increase confidence in a detected event. If multiple adjacent SNPs show a consistent change, the confidence in the call is much higher than when it is based on a single SNP[9].

Loss of heterozygosity (LOH) of tumor suppressor genes was observed at various loci on different chromosomes like 1p, 1q, 4q, 5q, 8p, 9q, 11q, 12p, 14q, 15q, 17p, 17q, 18p, 18q and 22q in colorectal cancer[10].

In one study over colorectal cancer, the frequency of allelic losses were determined at 17p (28%), 18p (26%), 18q and 5q (25%), 9q and 15q (24%), 8p (23%) and 14q (20%) throughout the genome Jen-Kou Lin et al[5]. Mao et al. (2006) also reportedmore six different LOH areas as 4q (42%), 1p (36%), 11q (31%), 17p(28%), 18p (26%) and 1q (25%) in sporadic colorectal carcinoadenomas.

In the present study including 35 patients of colorectal cancer, paired loss of heterozygosity status analysis (colorectal carcinoma tissue compared to corresponding normal colonic mucosa) revealed a number of genomic regions with LOH in cancer genome. Mapping these regions to genomic location shows that LOH was most frequently found in chromosome 19p region (11.4% cases), followed by chromosome 12p (8.5% cases) and 4p (8.5% cases) and 5q regions (8.5% cases). Regarding LOH loci in chromosome this present study also correlates with the other studies that observed by Ozaslan et al[10] and Jen-Kou Lin et al[5]. The candidate genes lost in these regions presumably of recessive character , remaining to be discover in future.

Conclusion

In this stydy Loss of heterozygosity in colonic cancer patients in our country was most frequently found in chromosome 19p region followed by chromosome 12p, 4p and 5q regions.

Larger number of cases and available facilities for genomic data analysis interpretation in our country would provide light to find out actual pathogenic factor of having CRC.

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