



RAPID COMMUNICATION

Tumor suppress genes screening analysis on 4q in sporadic colorectal carcinoma

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locus. In tumors larger than 5 cm in diameter, LOH frequency was significantly higher than tumors that were less than 5 cm (56% *vs* 13.79%, $P = 0.01$). On D4S1534 locus, LOH was significantly associated with liver metastasis (80% *vs* 17.25%, $P = 0.012$). No relationship was detected on other locus compared with clinicopathological features.

CONCLUSION: 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.

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Key words: Loss of heterozygosity; Colorectal carcinoma; Chromosome; 4q; Tumor suppressor gene

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Abstract

AIM: To search candidate tumor suppressor genes (TSGs) on chromosome 4q through detecting high loss of heterozygosity (LOH) regions in sporadic colorectal carcinoma in Chinese patients.

METHODS: Thirteen fluorescent labeled polymorphic microsatellite markers were analyzed in 83 cases of colorectal carcinoma and matched normal tissue DNA by polymerase chain reaction (PCR). PCR products were electrophoresed on an ABI 377 DNA sequencer. Genescan 3.7 and Genotype 3.7 software were used for LOH scanning and analysis. Comparison between LOH frequency and clinicopathological factors were performed by χ^2 test.

RESULTS: Data were collected on all informative loci. The average LOH frequency on 4q was 28.56%. The D4S2915 locus showed highest LOH frequency (36.17%). Two obvious deletion regions were detected: one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). None case showed complete deletion of 4q, most cases displayed interstitial deletion pattern solely. Furthermore, compared with clinicopathological features, a significant relationship was observed between LOH frequencies on D4S3018

INTRODUCTION

Colorectal Cancer (CRC) is one of the most common malignant tumors threatening people's health^[1,2]. After lung cancer in men and breast cancer in women, it is the most common cause of cancer related death. Improvement in its prognosis will not be achieved without a better understanding of its etiology and tumor molecular biology. In recent years, the genetic basis of human tumors has been increasingly elucidated. As a model for both multistep and multipathway carcinogenesis, colorectal neoplastic progression provides paradigms of both oncogenes and tumor suppressor gene (TSG) in epithelial tumors^[3,4]. The latter changes predominate. In addition to the allelic loss noted on chromosome 5q, 17p and 18q, many other chromosome losses can be observed in colorectal

carcinoma. Regions from chromosome 1q, 4p, 6p, 6q, 8p, 9p and 22q were lost in 25%-50% of the cases studied^[3].

Chromosome losses in colorectal tumor were first detected by cytogenety, later, by probes of restriction fragment length polymorphisms (RFLPs), and now by loss of heterozygosity (LOH) in analyzing allelic loss so far. The loss of TSGs is believed to be one of the key steps to carcinogenesis of CRC^[5]. The loss of one allelic at a specific locus is caused by deletion mutation or loss of a chromosome from a chromosome pair^[6]. When this occurs at a TSG locus where one of the allelic is already abnormal, it can result in neoplastic transformation. The LOH analysis, based on polymorphic microsatellite DNA, has currently become an effective and powerful tool currently to find informative loci and then to find candidate TSGs^[7,8].

Many studies reported frequent loss on 4q in couples of carcinoma. But much less studies has been done on 4q in colorectal carcinoma. In this study, we performed LOH analysis using high resolution microsatellite markers on chromosome 4q in 83 sporadic colorectal carcinoma cases, in an effort to identify additional TSGs involved in colorectal tumorigenesis.

MATERIALS AND METHODS

Patient sample and DNA extraction

This study was based on eighty-three consecutively collected tumors, including 40 males and 43 females, from unrelated patients with CRC, treated at the Surgical Department in Shanghai First People's Hospital, China. The patients' ages ranged from 31 to 84 years with a median of 66. The cancerous tissue and adjacent normal tissue were fresh frozen. These tissues were cut into cubes of approximately 2 mm³ and immediately frozen in liquid nitrogen. DNA was extracted using standard methods with proteinase K digestion and phenol/chloroform purification^[9]. All patients were confirmed by pathology, and were staged by Duke's criterion. Each patient gave his or her informed consent for the use of his or her tissue in this study.

Microsatellite markers and polymerase chain reaction (PCR)

Initially, 83 cases of CRC were analyzed by PCR using thirteen microsatellite markers which map to 4q. DNA samples were analyzed as tumor and matched normal pairs using primers of the following microsatellite loci (hereditary location): centromere--D4S3000 (4q12)--D4S3018 (4q13.2)--D4S2915 (4q21.1)--D4S1534 (4q21.23)--D4S2986 (4q23)--D4S407 (4q28.1)--D4S430 (4q28.1)--D4S2939 (4q31.1)--D4S3046 (4q32.2)--D4S2979 (4q32.3)--D4S415 (4q34.3)--D4S1535 (4q35.1)--qter (Figure 1). The average hereditary distance from genothon map is 10.4cM (centi-Morgan)^[10].

LOH analysis

A portion of each PCR product (0.5 µL) was combined with 0.1 µL of Genescan 500 size standard (PE

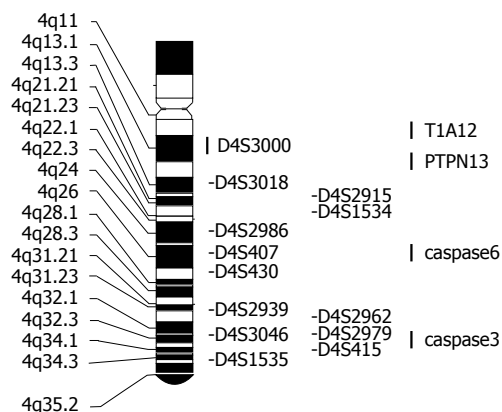


Figure 1 Microsatellite markers on chromosome 4q and putative TSGs.

Applied Biosystems Foster city CA, USA) and 0.9 µL of formamide loading buffer. After denaturation at 96°C for 5 min, products were electrophoresed on 5% polyacrylamide gels on an ABI 377 DNA sequencer (PE Applied Biosystems Foster city CA, USA) for 3 h. Genotype 3.7 software displays individual gel lanes as electropherograms with a given size, height, and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a LOH (Figure 2). Most amplification of normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygosity) and those PCR reactions in which fragments were not clearly amplified were scored as not informative. The LOH frequency of a locus is equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency of chromosome 4q is the average value of each locus LOH frequency.

Statistical analysis

Date was statistically analyzed using the SPSS software package, version 11.5 (SPSS Inc). Comparisons between LOH and clinicopathological data were performed by χ^2 test. $P < 0.05$ was considered as statistically significant.

RESULTS

LOH analysis of CRC on 4q

Eighty-three CRCs were analyzed for LOH at thirteen marker loci spanning chromosome 4q. All loci got informative messenger (Table 1). Tumors exhibiting wide microsatellite instability were excluded from deletion mapping. The average LOH frequency on 4q was 28.56%. Seventy-two samples (86.75%) showed LOH event on at least one locus. The D4S2915 locus showed highest LOH frequency (36.17%). Two distinct region of frequent allelic loss were observed on chromosome 4q, one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). None case showed complete deletion of 4q,

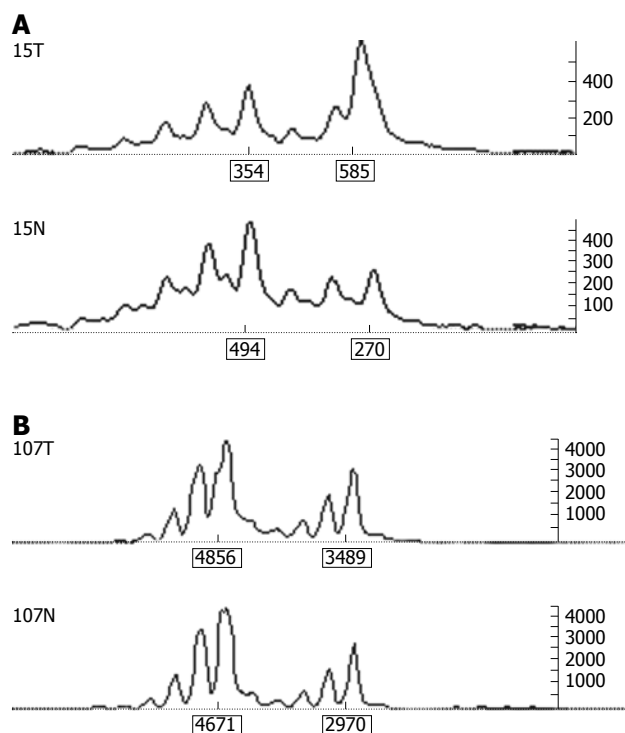


Figure 2 LOH demonstrations. **A:** The typical peak of LOH: Allele ratio = $(T1/T2)/(N1/N2) = (354/585)/(494/270) = 0.33 < 0.67$; **B:** The normal peak: Allele ratio = $(T1/T2)/(N1/N2) = (4856/3489)/(4671/2970) = 0.67 < 0.89 < 1.5$. T: Tumor; N: Normal.

most cases displayed interstitial deletion pattern solely. It can be speculated that putative TSGs may locate on 4q12-21.1 and 4q25-31.1.

Relationship between clinicopathological features and LOH on 4q

Compared with clinicopathological features, by statistics, a significant relationship was observed between LOH frequency on D4S3018 and tumor diameter. LOH frequency in tumors larger than 5 cm in diameter (56%, 14/25) was significantly higher than tumors that were less than 5 cm in diameter (13.79%, 4/29; $P = 0.01$). On 4S1534 locus, LOH frequency was 80% (4/5) in cases with liver metastasis, which was significantly higher than that without liver metastasis (17.25%, 5/29) ($P = 0.012$). No relationship was detected on other locus compared with these clinicopathological features.

DISCUSSION

Inactivation of TSGs appears to be one of the genetic mechanisms involved in the development of CRC^[3,11,12]. Deletion of TSGs occurs frequently in human malignances. Such events can be detected using markers from the region of genomes that include a TSG. Allelic loss detected as LOH have been proved useful for mapping regions of DNA that contain TSGs. LOH at specific chromosomal regions strongly suggests the existence of TSGs at the relevant segment.

Studies conducted thus far have focused on known TSG, particularly, APC, p53, DCC, MCC, Smad4 and so

Table 1 LOH frequency statistics result on 4q

Marker	Location	LOH case	Normal case	Informative case	Distance (cm)	LOH rate
D4S3000	4q12	22	42	78.05	-	34.38
D4S3018	4q13.2	18	36	65.06	9.7	33.33
D4S2915	4q21.1	17	30	56.63	10.6	36.17
D4S1534	4q21.23	9	25	40.96	7.5	26.47
D4S2986	4q23	7	39	55.42	10.6	15.22
D4S407	4q25	17	35	62.56	10.7	32.69
D4S430	4q28.1	15	35	60.24	11.1	30
D4S2939	4q31.1	21	38	71.08	14.4	35.59
D4S2962	4q31.23	16	43	71.08	12.1	27.12
D4S3046	4q32.2	16	39	66.27	13.3	29.09
D4S2979	4q32.3	7	25	36.78	12.6	21.87
D4S415	4q34.3	14	36	60.24	6.6	28
D4S1535	4q35.1	14	45	71.08	14	23.73

on. The genetic events that led to the development and progression of CRC on chromosome 4q have not been elucidated. There were a lot of evidence that multiple genetic abnormalities on 4q contribute to development of a series of tumors, and that supported the presence of TSGs in the long arm of chromosome 4.

Loss of chromosome 4q has been found in the majority of breast carcinoma^[13], cervical cancer (4q21-q25)^[14]. In oral squamous cell carcinoma^[15] and relapsed childhood acute lymphoblastic leukemia^[16], 75% and 20% cases showed LOH on at least one of the loci, with frequent loss region centered on 4q25 in the former. In esophageal adenocarcinoma, Hammoud *et al*^[17] found LOH frequency was 54.5%-65% on 4q21-35 with a lower resolution allelotyping studying. Rumpel *et al*^[18] found LOH was 80% on at least one marker on 4q, and discerned three non-overlapping areas (4q21.1-22, 4q32-33, 4q35), whose total spanning length is quite coincident with Hammoud^[17]. Hurst *et al*^[19] found that in bladder cancer, 4q loss was the most frequent loss (83%) in all arms of chromosome by microarray-based comparative genome hybridization, with the minimal region of deletion at 4q13.1 and 4q31.3. This result was compatible with previous report of LOH analysis in bladder tumors^[20,21]. In malignant mesothelioma and small cell lung cancer, two non-overlapping loss region on 4q were at 4q33-34 (> 80%) and 4q25-26 (> 60%)^[22]. In primary small cell lung cancer, Cho *et al*^[23] defined five commonly deleted region all more than 50% on 4q, namely, 4q24, 4q27-28.3, 4q33, 4q34.1 and 4q34.3-35.2. In the research of hepatocellular carcinoma, much work has been done. Piao *et al*^[24] made a detailed deletion mapping and identified seven independent, frequently lost regions. In fact, a high frequency of LOH on chromosome 4q in hepatocellular carcinoma has been reported by several study groups^[25-27]. Recent years, LOH frequency was found 33.3% (at 4q26-27)^[28], 50%^[29], and 38%^[30] for at least two adjacent markers in hepatocellular carcinoma. These study results strongly suggested that 4q may locate more than one putative TSG.

The research of LOH on 4q in colorectal carcinoma was much less in this decade. LOH at telomere markers have been reported less than 10% on 4q^[31]. Analysis of

mid-position microsatellite markers on 4q arm showed moderate of allelic imbalances (< 25%) in colorectal tumors^[32]. Comparative genomic hybridization analysis of a serial of colorectal adenomas and carcinomas identified chromosome 4 losses as of the recurrent alterations observed in malignant stages^[33]. Arribas *et al.*^[34] found frequent loss on 4q21-28 in CRC by using AP-PCR method. However, detailed mapping of the long arm of chromosome 4 has not been reported in colorectal carcinoma thus far.

With high resolution markers, we performed detailed LOH analysis and observed two obvious deletion regions: one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). The first region included 4q12-21.1, 4q13.1^[19], furthermore, the region was adjacent with 4q21-25^[14], 4q21-35^[17], 4q21.1-22^[18], 4q33-34^[22] detected before. The second region was adjacent with 4q21-25^[14], 4q25^[15], 4q31.3^[19], covered by 4q21-35^[17], and completely spanned 4q32-33^[18], 4q25-26^[22], 4q27-28.3, 4q33^[23], 4q26-27^[28], partially overlapped with 4q21-28^[34], 4q24-26^[35] as well. Because of different LOH judge criteria and different density markers used, the minimal deletion region in different tumors displayed discrepancy.

LOH on chromosome 4q seems to be correlated with aggression features and a late genetic event involved with the progression rather than the initiation of cancer. In hepatocellular carcinoma, a study by Okabe *et al.*^[36] showed that the high rate of LOH on 4q are associated with a poor differentiation of tumors, vascular invasion and intrahepatic metastasis in hepatocellular carcinoma. A similar finding was made by Konishi *et al.*^[37], LOH was found in 71% of poorly differentiated hepatocellular carcinoma. Rashid *et al.*^[29] found 4q was preferentially lost in hepatocellular carcinoma containing p53 mutation, that is, LOH of 4q was present in 75% of hepatocellular carcinoma with, but only 25% of hepatocellular carcinoma without a p53 gene mutation ($P = 0.01$), indicating a possible interaction between p53 gene mutation and 4q loss in the pathogenesis of hepatocellular carcinoma. Loss of 4q34-35 regions showed a significant association with alcohol intake ($P = 0.05$) and with high grade of differentiation ($P = 0.02$) in hepatocellular carcinoma^[30]. Investigators^[22] thought TSG on 4q play an important role in pathogenesis of malignant mesothelioma and small cell lung cancer, because non-small cell lung cancer had lower frequency at this region. Similarly, the result of primary bladder cancer correlated allelic loss with advanced tumor stage and grade of the lesions^[14]. Piao *et al.*^[24] found five regions among seven common deleted regions was associated with tumor differentiation, suggesting that allelic loss on chromosome 4q is a late genetic event involved in the progression rather than in the initiation of hepatocarcinogenesis. Pershouse *et al.*^[35] reported that the allelic deletion involving 4q may represent an early event in head and neck squamous cell carcinoma oncogenesis.

A significant relationship was detected between LOH frequency on D4S3018 and tumor diameter. LOH

frequency in tumors larger than 5 cm was significantly higher than tumors less than 5 cm in diameter ($P = 0.01$). On D4S1534 locus, LOH rate was significantly associated with liver metastasis ($P = 0.012$). It can be speculated that these events were associated with progression after tumorigenesis, indicating a late event in colorectal carcinoma.

No statistical significant correlation was found between the presence of LOH and clinicopathological data in oral squamous cell cancer^[15], esophageal adenocarcinoma^[17] and in Taiwanese hepatocellular carcinoma^[28].

By scanning GeneMap' 99 database, and searching www.gdb.org, four genes should be studied further. PTPN13 (4q21.3) is a Fas-associated protein, tyrosine phosphate that binds to a negative regulatory domain in the FAS protein and inhibits Fas-induced apoptosis^[38]. Caspase3 (4q34) and caspase6 (4q24-35), mammalian homologues of Ced-3 gene, are responsible for cleavage and inactivation of key homeostatic protein during apoptosis^[39]. T1A12/mac25 (4q12-13) expression is abrogated during breast cancer progression concomitant with LOH on chromosome 4q, indicating its tumor suppressor function^[40]. All of these three genes are putative TSGs.

In summary, this is the first detailed LOH analysis on chromosome 4q in CRC by using high resolution microsatellite markers. We detected two non-overlapping deletion region (4q12-21.1 and 4q25-31.1), which may contain TSGs. Additional attempts will aide in the discovery of the genes inactivated by these deletion events. The elucidation of the biochemical property of these gene products will be an important step in understanding the biology of this highly lethal and common cancer.

COMMENTS

Background

Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations as the tumor progresses to advanced stages. The classic mechanism of tumor suppressor gene (TSG) inactivation is described by two-hit modes in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in loss of heterozygosity (LOH) at multiple loci. LOH is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of TSGs predominates.

Research frontiers

Most genome-wide scans for LOH have been conducted at low resolution with a relatively small number of polymorphic markers. For example, an average of 120 microsatellites have been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype was approximately 280 polymorphic markers before the year 2000. SNPs are the most common form of sequence variation in human genome, occurring approximately every 1200 bp. High density mapping of genetic losses reveals potential tumor suppressor loci and might be useful for clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance. Higher density SNP array can effectively detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions.

Innovations and breakthroughs

A great deal of evidence supported the presence of TSGs in the short arm of chromosome 4. Fewer studies have been reported in colorectal cancer. Previous allelotyping analysis of cancer by many groups was used with a relatively low density of markers. By deletion dense markers mapping, authors detected two obvious high frequency LOH regions spanning D4S3013 and D4S405 locus in colorectal cancer (CRC). Candidate TSG might be located between D4S3017 and D4S2933 (about 1.7 cm).

Applications

This method could be used to detect some major allelic loss regions in genome-wide scans of LOH in patients with CRC.

Terminology

LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome non-disjunction and mitotic non-disjunction followed by republication of the remaining chromosomes, mitotic recombination and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display a complete loss. However, more than half of the losses are associated with only a partial loss of a chromosome rather than a whole chromosome. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor TSGs and to characterize different tumor types, pathological stages and progression.

Peer review

This is a report that describes the LOH events on 4q in sporadic CRC in Chinese patients, further studies will benefit from this paper. The data presented is clear and concise in the text.

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