Are there tumor suppressor genes on chromosome 4p in sporadic colorectal carcinoma?

Hai-Tao Zheng, Li-Xin Jiang, Zhong-Chuan Lv, Da-Peng Li, Chong-Zhi Zhou, Jian-Jun Gao, Lin He, Zhi-Hai Peng

Abstract

AIM: To study the candidate tumor suppressor genes (TSG) on chromosome 4p by detecting the high frequency of loss of heterozygosity (LOH) in sporadic colorectal carcinoma in Chinese patients.

METHODS: Seven fluorescent labeled polymorphic microsatellite markers were analyzed in 83 cases of colorectal carcinoma and matched normal tissue DNA by PCR. PCR products were eletrophoresed on an ABI 377 DNA sequencer. Genescan 3.7 and Genotype 3.7 software were used for LOH scanning and analysis. The same procedure was performed by the other six microsatellite markers spanning D4S3013 locus to make further detailed deletion mapping. Comparison between LOH frequency and clinicopathological factors was performed by χ² test.

RESULTS: Data were collected from all informative loci. The average LOH frequency on 4p was 24.25%, and 42.3% and 35.62% on D4S405 and D4S3013 locus, respectively. Adjacent markers of D4S3013 displayed a low LOH frequency (< 30%) by detailed deletion mapping. Significant opposite difference was observed between LOH frequency and tumor diameter on D4S412 and D4S1546 locus (0% vs 16.67%, \( P = 0.041 \); 54.55% vs 11.11%, \( P = 0.034 \), respectively). On D4S403 locus, LOH was significantly associated with tumor gross pattern (11.11%, 0, 33.33%, \( P = 0.030 \)). No relationship was detected on other loci compared with clinicopathological factors.

CONCLUSION: By deletion mapping, two obvious high frequency LOH regions spanning D4S3013 (4p15.2) and D4S405 (4p14) locus are detected. Candidate TSG, which is involved in carcinogenesis and progression of sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm).

INTRODUCTION

Colorectal cancer (CRC) constitutes the second most common neoplasm in Western countries and is the third leading cause of cancer-related death, the overall 5-year survival rate is approximately 45%[1]. Improvement in its prognosis can 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 provide paradigms of both oncogenes and tumor suppressor gene in epithelial tumors[2,3]. The latter changes predominante. In addition to the allelic loss on chromosome 5q, 17p and 18q, many other chromosome losses can be observed in colorectal carcinoma. Regions on chromosome 1q, 4p, 6p, 6q, 8p, 9p and 22q were lost in 25%-50% of the colorectal tumor cases studied previously[2].

Chromosome losses in colorectal tumor were first detected by cytogenesis, later, by probes of restriction fragment length polymorphisms (RFLP) and now by loss of heterozygosity (LOH) in analyzing allelic loss. The key steps to carcinogenesis of colorectal cancer[4]. The loss of one allelic at specific locus is caused by deletion mutation or loss of a chromosome from a chromosome pair[5]. When this occurs at a tumor suppressor gene locus where one of the allelic is already abnormal, it can result
in neoplastic transformation. The LOH analysis based on polymorphic microsatellite DNA has become an effective and powerful tool currently to find informative loci and candidate tumor suppressor genes\(^6\)\(^7\). Most investigations concentrated on defining the minimal regions of loss of specific chromosomes in various cancers in an effort to identify the putative tumor suppressor genes targeted by the loss\(^8\).

In this study, we first analyzed the LOH events on chromosome 4p using seven microsatellite markers and made further refined deletion mapping analysis spanning D4S3013 locus in 83 sporadic colorectal carcinoma cases in an attempt to identify additional candidate tumor suppressor genes involved in colorectal tumorigenesis.

**MATERIALS AND METHODS**

**Patient sample and DNA extraction**

This study was based on consecutively collected tumors in 83 patients with colorectal cancer, including 40 males and 43 females, 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 control tissue (> 10 cm) were freshly frozen. The tissues were cut into cubes of approximately 2 mm\(^3\) and immediately frozen in liquid nitrogen. Each patient gave his or her informed consent for the use of his or her tissue in this study. 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 criteria.

**Microsatellite markers and PCR**

Initially, 83 cases of colorectal cancer were analyzed by PCR using seven microsatellite markers (Shanghai Biology Technology Company, China) which map to chromosome 4p. DNA samples were analyzed as matched normal and tumor pairs using primers of the following microsatellite loci (hereditary location/heterozygote): pter-D4S412 (4p16.3/76)-D4S2935 (4p16.1/62)-D4S1599 (4p16.1/81)-D4S303 (4p15.33/76)-D4S3013 (4p15.2/84)-D4S391 (4p15.2/85)-D4S405 (4p14/85). The average hereditary distance was 8.65 cm\(^{10}\) (Figure 1A). As the D4S3013 locus showed high LOH frequency (35.62%), six additional microsatellite markers map to chromosome 4p15 were employed to further investigate LOH. The same DNA samples were then analyzed as matched pairs for the following microsatellite markers (location/heterozygote): pter-D4S2926 (4p15.32/80)-D4S3017 (4p15.31/77)-D4S3013 (4p15.31/77)-D4S2933 (4p15.32/80)-D4S3017 (4p15.31/77)-D4S3013 (4p15.31/77). The average hereditary distance was restricted within 1.03 cm\(^{10}\) (Figure 1B).

**LOH result analysis**

A portion of each PCR product (0.5 μL) was combined with 0.1 μL Genescan 500 size standard (PE Applied Biosystems Foster City, CA, USA) and 0.9 μL formamide loading buffer. After denaturation at 96°C for 5 min, products were electrophoresed on 5% polyacrylamide gels using an ABI 377 DNA sequencer (PE Applied Biosystems Foster City, CA, USA) for 2.5 h. Genotype 3.7 software display 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 loss of heterozygosity (Figure 2). Most amplified 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 4p is the average value of each locus.

**Statistical analysis**

Comparisons between LOH and clinicopathological data were performed by \(\chi^2\) test. \(P < 0.05\) was considered as statistically significant.

**RESULTS**

**LOH analysis of colorectal cancer on 4p**

Eighty-three colorectal cancers were analyzed for LOH at the seven marker loci spanning chromosome 4p. All loci got informative messengers. The average LOH frequency on 4p was 24.25%. Sixty-three samples (75.90%) showed
In colorectal tumors, previous allelic typing\textsuperscript{[28]} cytogenetic\textsuperscript{[25,27]} and comparative genomic hybridization\textsuperscript{[28]} studies have reported moderate losses (0%-30%) of chromosome 4. These data have not raised special interest in this chromosome as a candidate to harbor a tumor suppressor gene, therefore, colorectal cancer investigations at least one LOH event. Two distinct regions of frequent allelic loss at 4p15.2 and 4p14.5 (4p14) locus on chromosome were detected (Table 1). The LOH frequency was 35.62% and 42.3%, respectively. This suggested that putative tumor suppressor genes may be located near 4p15.3 and 4p14.5 loci.

**LOH deletion mapping results on 4p15 encompassing 4p15.31**

The chromosome region spanning 4p15 loci on 4p15 was investigated using a saturation mapping strategy with another 6 microsatellite markers that are closely located within this region (Figure 2). To detect putative tumor suppressor genes easily, we limited average hereditary distance to 1.03 cm. Forty samples (48.19%) putative tumor suppressor genes easily, we limited average hereditary distance to 1.03 cm. Forty samples (48.19%) showed at least one LOH event. The average LOH frequency spanning 4p15.31 was 24.2% (Table 2). We showed at least one LOH event. Two distinct regions of frequent allelic loss at 4p15.2 and 4p14.5 (4p14) locus on chromosome were detected (Table 1). The LOH frequency was 35.62% and 42.3%, respectively. This suggested that putative tumor suppressor genes may be located near 4p15.3 and 4p14.5 loci.

**Relationship between clinicopathological features and LOH on 4p**

On 4p14 loci, no LOH was detected in patients with tumor larger than 5 cm in diameter (0/27), while in patients with tumor less than 5 cm in diameter, LOH frequency was 14.29% (5/35; \( P = 0.041 \)). On the contrary, on 4p15 locus, LOH frequency was 35.29% (6/17) in the former; and only 10% (3/30) in the latter locus \(( P = 0.030)\). Notably, on 4p13 locus, LOH was significantly associated with tumor gross pattern. In tumor of the massive, ulcerative and encroaching pattern, the LOH frequency was 10%, 0%, 33.33%, respectively \(( P = 0.030)\). No significant relationship was found between clinicopathological features and LOH on other loci (data not shown).

**DISCUSSION**

Inactivation of tumor suppressor genes appears to be one of the genetic mechanisms involved in the development of colorectal cancer\textsuperscript{[11,12]}. Deletion of tumor suppressor genes occur frequently in human malignancies. Such events can be detected using markers from the region of genome that include a tumor suppressor gene. Allelic deletions detected as LOH have been proved useful for mapping regions of DNA that contains tumor suppressor genes, i.e., LOH at specific chromosomal regions strongly suggests the existence of tumor suppressor genes at the relevant segment.

A great deal of evidence supported the presence of tumor suppressor genes in the short arm of chromosome 4. These include the reversion of the immortal phenotype by chromosome 4 transfer\textsuperscript{13} and the frequent occurrence of losses in or near the 4p14-4p16 region in bladder cancer\textsuperscript{[6]}. LOH has been observed at distal 4p in sporadic neuroblastoma with an incidence ranging from 20% to 29%\textsuperscript{[10,12]}. Using array comparative genomic hybridization, Hurst et al\textsuperscript{[9]} reported the loss frequency of 4p to be 52% in bladder cancer. More importantly, Shirapurkar et al\textsuperscript{[8]} observed the loss frequency of > 50% at 4p15.1-4p15.3 in malignant mesothelioma and lung carcinoma. LOH on 4p was 21% and > 30% in differentiated adenocarcinoma of stomach as well\textsuperscript{[19,20]}. Head and neck squamous cell carcinoma, invasive cervical cancer and acinic cell carcinoma also showed a high allelic loss frequency\textsuperscript{[21,22]}.
have not included a detailed analysis of loss in this chromosome. Choi et al.[29] reported a LOH frequency of 24%-30% at just several loci on chromosome 4 in colorectal cancer. Later, Arribas et al.[10,11] used AP-PCR method and suggested chromosome 4p14-4p16 may contain tumor suppressor gene, because LOH frequency on D4S2397 was as high as 35%. These reports indicate that 4p14-4p16 region displayed frequent loss in a couple of cancers, so 4p14-4p16 region is of important value for TSG screening.

D4S3013 locus region, 4p15.2, was concordant with several reports in other tumors before.[14,16,20,21] In this study, we investigated the LOH on 4p in 83 sporadic cases of colorectal cancer. The results showed putative tumor suppressor gene may harbor adjacent to D4S405 and D4S3013 locus. We made further detailed deletion mapping spanning D4S3013 locus, and found that the surrounding markers of D4S3013 displayed a low LOH frequency (< 30%). Therefore, we speculate that the candidate TSG may be located between D4S3017 and D4S2933, about 1.7 cm in hereditary distance.

We found several loci were significantly associated with clinicopathological features. On D4S412 locus, no LOH was detected in patients with tumor larger than 5 cm in diameter, while in patients with tumor less than 5 cm in diameter, the LOH frequency was 14.29% (P = 0.041). On the contrary, on D4S1546 locus, the LOH frequency showed opposite phenomenon. On D4S403 locus, LOH was significantly associated with tumor gross pattern. Similarly, Arribas et al.[11] found solely at the D4S2937 locus was indicative of a shorter disease-free survival (P = 0.027). Choi et al.[29] found 4p loss was significantly associated with early onset of colorectal cancer. The effect of 4p loss on the early-onset disease is unlikely to be the result of tumor aggressiveness, because 4p loss was not found to be correlated with cancer-related death. Nishizuka et al.[28] found 4p LOH had an essentially similar frequency in early and advanced differentiated adenocarcinoma. The differential behavior of LOH at different markers suggested that distinct mechanisms and/or selection presses participate in the mutational event that affect this chromosomal region during the tumorigenic process.

Regarding allelic loss at 4p, cholecystokinin type A receptor (CCK-AR) gene maps near D4S2397,[12,31] (Figure 1). Recent reports have suggested that cholecystokinin receptor may function as a tumor suppressor gene.[14,36]

In summary, we investigated LOH on 4p in sporadic colorectal carcinoma in Chinese patients and detected two high deletion regions encompassing D4S3013 (4p15.2) and D4S405 (4p14). Candidate TSG, involved in sporadic colorectal carcinoma on chromosome 4p, may be located between D4S3017 and D4S2933 (about 1.7 cm). Further related gene screening and functional studies may contribute to the identification of the tumor suppressor gene in these regions.

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