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Original Article

Identification of Tumor Suppressor Loci on the Long Arm of Chromosome 4 in Primary Small Cell Lung Cancers

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Recent cytogenetic studies have indicated that loss of the long arm of chromosome 4 is a frequent event in small cell lung cancer (SCLC), which suggests the presence of tumor suppressor genes there. To precisely map tumor-suppressor loci on chromosome 4q for further positional cloning efforts, we tested 15 primary SCLCs. Forty two polymorphic microsatellite markers located on chromosome 4q were used in the microsatellite analysis. We found that 12 (80%) of the 15 tumors exhibited loss of heterozygosity (LOH) in at least one of the tested microsatellite markers, and that 3 (25%) of these 12 tumors exhibited a larger area of deletion on chromosome 4q. Frequent LOH, defined as occurring in more than 50% of the tumors, was observed in five commonly deleted regions on 4q, namely 4q24, 4q27-28.3, 4q33, 4q34.1, and 4q34.3-35.2. Of these 5, LOH at 4q33 was the most frequent (61.5%). Six (40%) of the 15 tumors exhibited shifted bands in at least one of the tested microsatellite markers. Shifted bands occurred in 3.7% (23 of 630) of the loci tested. Our data demonstrated that at least five tumor-suppressor loci exist on the long arm of chromosome 4 and that they may play an important role in the development and progression of primary small cell lung cancer tumorigenesis.

Key Words: Small cell lung cancer, chromosome 4, LOH, tumor suppressor

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INTRODUCTION

Bronchogenic carcinoma, the leading cause of cancer-related death in the world, is classified into two distinct histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter which includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. SCLC accounts for 18 - 29% of all bronchogenic carcinomas and carries a very poor prognosis because of wide dissemination in the early stage.[1]

Carcinogenesis results from the accumulation of multiple genetic alterations such as the activation of oncogenes and the inactivation of tumor suppressor genes. The latter involves mostly small mutations of one of the allele and loss of a large part of the other allele,[2,3] a process which can be identified by loss of heterozygosity (LOH). Thus, LOH is a marker of chromosomal regions that may harbor tumor suppressor genes.[4]

Many studies based on somatic LOH have already led to the discovery of several tumor suppressor genes, such as the retinoblastoma (Rb) gene, p53 gene, adenomatous polyposis coli (APC) gene, and fragile histidine triad (FHIT) gene. However, the molecular mechanisms of SCLC are not fully understood yet.

Cytogenetic studies have reported 4q deletions in SCLC cell lines[5] and the frequent occurrence of LOH on chromosomes 3p, 4q, 5q, 10q, 13q, 17p, and 22q in SCLC.[4,6,7] This means that these chromosomes contain tumor suppressor genes which play an important role in the development

or progression of SCLC. Recently, Kim et al. have identified five tumor suppressor loci, three on chromosome 9p[8] and two on chromosome 10q, in primary SCLCs.[9]

Previous studies have shown that LOH on chromosome 4q is frequently seen in various cancers.[10-12] The regions commonly deleted in these tumors have been identified at 4q21 - q26 regions. Additional regions such as 4q11-12, 4q12-23, and 4q35 have been reported in hepatocellular carcinoma.[13] However, detailed mapping of the long arm of chromosome 4 has not been reported in primary SCLC so far.

In an effort to define the tumor suppressor loci on chromosome 4q in SCLC, we performed detailed deletion mapping of chromosome 4q in 15 primary SCLCs using microsatellite analysis with 42 highly polymorphic markers.

MATERIALS AND METHODS

Tissue specimens

Fifteen primary SCLCs and adjacent normal tissues were obtained from surgical resections performed at Severance hospital, Yonsei University College of Medicine, Seoul, Korea. Paraffin-embedded tissue blocks were sectioned using a microtome. A 4μm section from each block was stained with hematoxylin and eosin (H&E) and reviewed by a pathologist (Dr. D. H. Shin) to confirm the diagnosis and locate the tumor areas. The tumor cells were able to be microdissected at normal cell contamination levels of less than 30%. The normal control tissues were dissected using the same approach.

DNA extraction and microsatellite analysis

Dissected tissues were digested in 200μl of 50 mM Tris-HCl (pH 8.0) containing 1% SDS-proteinase K and incubated at 42℃ for 12 - 24 h. Digested samples were purified, and DNA was precipitated as described previously.[14] For microsatellite analysis, 42 highly polymorphic markers on the long arm of chromosome 4 were obtained from Research Genetics (Research Genetics, Huntsville, AL, USA) (Table 1). One of the prmers for each marker was end-labeled with [γ -^{32}P] ATP (3,000 Ci/mmol; Buckinghamshire, UK) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA, USA). PCR reactions were carried out in a 12.5μl volume containing 20 ng of genomic DNA, 1% DMSO, 200μM dNTP, 1.5 mM $MgCl_2$, 0.4μM of PCR primers which included 0.1μM γ-^{32}P-labeled primer, and 0.5 units of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD, USA). DNA was amplified for 35 cycles, each consisting of 95℃ for 30s, 52 - 60℃ for 1 min, and 70℃ for 1 min, in a temperature cycler (Hybaid; Omnigene, Woodbridge, NJ, USA) in 500μl plastic tubes, followed by a 5 min extension at 70℃. The PCR product was separated on a 6% polyacrylamide-urea-formamide gel, which was then autoradiographed. LOH was defined as having occurred when the intensity as measured by visual inspection in either of the two alleles was reduced by more than 50% as compared with that in the normal control panels. The presence of shifted bands was assessed by the appearance of clear novel alleles that were not observed in the normal tissue control panels.

RESULTS

To define the presence of putative tumor suppressor genes on 4q in SCLC, we selected 42 highly polymorphic microsatellite markers on the long arm of chromosome 4 and found that 12 (80%) of the 15 tumors exhibited LOH in at least one of the tested microsatellite markers. In addition, 3 (25%) of these 12 tumors were found to contain larger area deletions on chromosome 4q. Frequent LOH was observed in the area between *D4S1647* and *D4S421* (50%), between *D4S1615* and *D4S3039* (50%), between *D4S1595* and *D4S2431* (61.5%), between *D4S2417* and *D4S1584* (53.8%), and between *D4S171* and *D4S1652* (53.8%)(Fig. 1 and 2). Six (40%) of the 15 tumors were shown to have shifted bands in at least one of the tested microsatellite markers. Of the 630 tested loci 23 (3.7%) demonstrated shifted bands and the percentage of altered loci in the six tumors ranged from 2.5 - 40% (average 10.5 ± 16.53).

Table 1. Frequency of LOH on the Long Arm of Chromosome 4 in 15 Cases of Primary SCLC

Marker	Location	No. of informative	No. of LOH	LOH (%)
D4S1627	4p13-14	9	4	44.4
D4S398	4q12-13.2	11	4	36.4
D4S399	4q12-13.2	11	2	18.2
D4S2367	4q13.3	10	3	30
D4S392	4q12-13.3	10	3	30
D4S400	4q21	3	0	0
D4S395	4q21.23	13	4	30.8
D4S423	4q22.3	10	3	30
D4S414	4q22.1-22.3	9	1	11.1
D4S2634	4q23-24	9	3	33.3
D4S1647	4q24	10	6	60
D4S421	4q24	4	2	50
D4S411	4q24	10	2	20
D4S406	4q25	10	3	30
D4S1611	4q26	7	2	28.6
D4S402	4q26-27	11	2	18.2
D4S430	4q27	10	4	40
D4S1615	4q27	9	4	44.4
D4S3039	4q28.3	10	4	40
D4S175	4q31.1	10	3	30
D4S1644	4q31.21-31.22	9	1	11.1
D4S424	4q31.22	10	4	40
D4S1625	4q31.23	9	2	22.2
D4S413	4q32.1	5	1	20
D4S3033	4q32.1	13	4	30.8
D4S3046	4q32.1	12	3	40
D4S1566	4q32.3	4	1	25
D4S2368	4q32.3	8	2	25
D4S2979	4q32.3	10	3	30
D4S1595	4q33	13	4	30.8
D4S2431	4q33	9	5	55.6
D4S415	4q34.1	11	3	27.3
D4S2417	4q34.1	10	5	50
D4S1607	4q34.1	13	4	30.8
D4S1584	4q34.1	7	4	57.1
D4S2951	4q34.1	13	4	30.8
D4S1554	4q34.1	12	5	41.7
D4S408	4q34.2	8	3	37.5
D4S3047	4q34.2	13	5	38.5
D4S171	4q34.3	9	3	33.3
D4S426	4q35.2	10	3	30
D4S1652	4q35.2	6	4	66.7

DISCUSSION

A striking feature of lung cancer is the large number genetic changes found in clinically evident lung cancer. However, cytogenetics and the technique of comparative genome hybridization (CGH) have identified many areas of chromosomal deletion and nonreciprocal translocation indicating the presence of undiscovered tumor suppressor genes and areas of amplification indi-

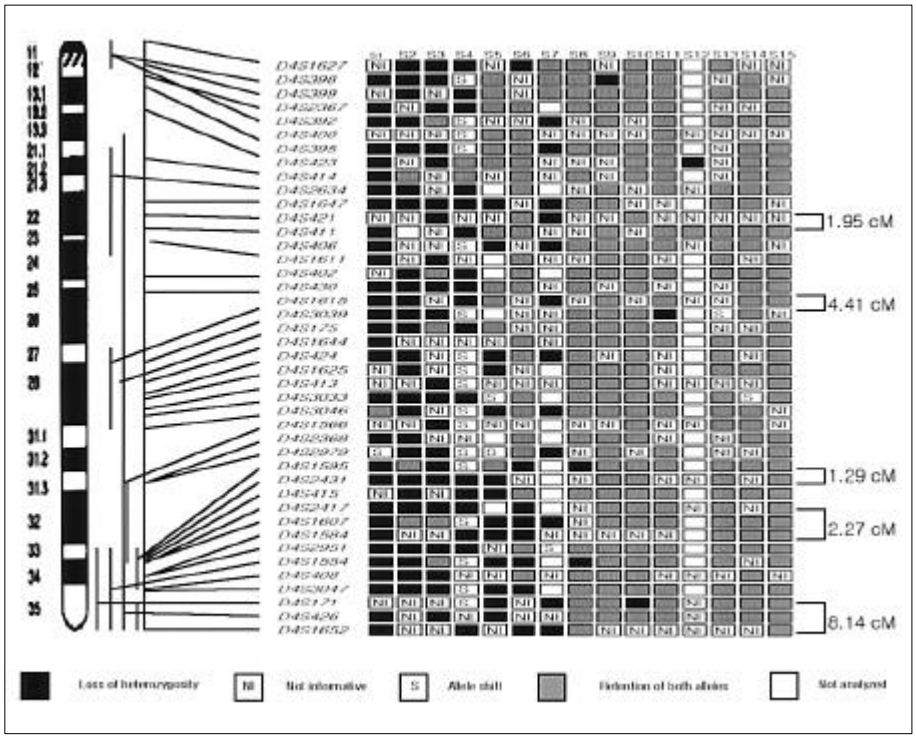

Fig. 1. Deletion mapping at chromosome 4q in primary small cell lung cancers. Names of microsatellite markers are at left side of the figure and tumors are given on the top of the figure. A total 12 (80%) tumors exhibited LOH at more than one region on chromosome 4q. The deleted regions are indicated on the right side of the figure. They are between 4q24, 4q27-28.3, 4q33, 4q34.1 and 4q34.3-35.2.

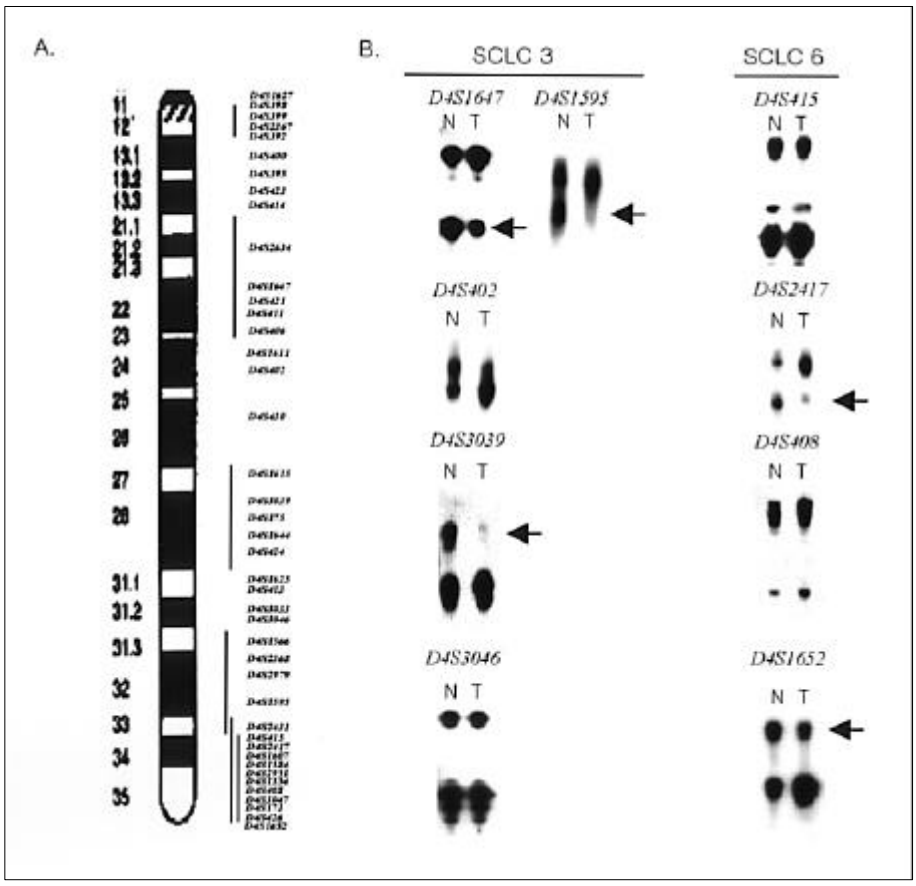

Fig. 2. Examples of deletions observed in primary small cell lung cancers. A. The order of microsatellite markers used in this study. B. LOH at markers D4S1647, D4S3039 and D4S1595 in tumor SCLC3 and D4S2417 and D4S1652 in tumor SCLC6, while retention at markers D4S402 and D4S3046 in tumor SCLC3 and D4S415 and D4S408 in tumor SCLC6. N indicates normal tissues and T indicates tumor tissues.

cating positions of undiscovered oncogenes. In addition, comparison of tumor DNA with the DNA from normal tissue from the same patients, when tested with probes detecting polymorphic markers, has also demonstrated LOH at many of these same loci.

SCLC has revealed frequent deletions on chromosomes 3p, 4q, 5q, 10q, 13q, 17p, and 22q.[4,7] Petersen et al.,[7] using CGH analysis, found that deletions were observed on chromosome 10q in 94% of tumors and on chromosomes 4q, 5q, 13q and 17p in 86% of tumors. These high incidences of chromosomal loss indicated that the inactivation of multiple growth-inhibitory pathways contributes to the aggressive phenotype of SCLC. Kim et al.[9] reported that at least two tumor suppressor loci by LOH analysis for chromosome 10q were represented in primary SCLC, which plays an important role in SCLC development and progression.

Functional evidence for putative tumor suppressor genes on chromosome 4 has been provided by the observation of a change of cell morphology in culture and a suppression of PA-1 tumorigenicity in nude mice with introduction of human chromosome 4 into the PA-1 human teratocarcinoma cell line.[15] Evidence for a cell senescence-related gene(s) on human chromosome 4 was also presented by the normal chromosomal transfer into three immortal human cell lines (HeLa, J82, T98G).[16] Recently, the transfer of a human chromosome 4, retaining the region surrounding the epidermal growth factor (EGF) locus at 4q24-26, into U251 human glioblastoma multiforme cells was demonstrated to cause reversion of the tumorigenic phenotype of the parental U251 cells.[17] Thus, the recent studies have focused on defining deletions associated with chromosome 4q in a variety of human solid tumors, including hepatocellular,[13,18] uterine cervical,[10] head and neck squamous cell carcinoma,[11,19] as well as Hodgkin's lymphoma.[20]

LOH on chromosome 4q seems to be correlated with aggressive features and a late genetic event involved with the progression rather than the initiation of cancers. A study by Okabe et al.[21] showed that the high rates of LOH in chromosome 4q are associated with a poor differentiation of tumors, vascular invasion, and intrahepatic metastasis in hepatocellular carcinoma. A similar finding was made by Konish et al. in their determination that LOH on chromosome 4q was found in 71% of poorly differentiated carcinomas and 20 - 25% of well differentiated carcinomas.[22] These findings demonstrated that the development and progression of hepatocellular carcinoma resulted from the accumulated inactivation of tumor suppressor genes and that these genetic changes occurred in the late stages of hepatocarcinogenesis.[13,21] Furthermore, a similar result had been obtained in the study of primary bladder carcinoma undertaken to correlate allelic loss of a commonly deleted 14 cM critical region on 4q34.2 - 35.2 with advanced tumor stage and grade of the lesions.[12]

Among our samples, only one with an extensive stage showed a much higher (93.5%) rate of LOH in tested markers with the informative result than the other samples of limited stage. Therefore it is apparent from our results that LOH on 4q affects the late stage in SCLC.

Our study demonstrated that five independent commonly deleted regions were defined on chromosome 4q in primary SCLC; 1.95 cM long from *D4S1647* to *D4S421* (50%) on 4q24, 4.41 cM long from *D4S1615* to *D4S3039* (50%) on 4q27-28.3, 1.29 cM long from *D4S1595* to *D4S2431* (61.5%) on 4q33, 2.27 cM long from *D4S2417* to *D4S1584* (53.8%) on 4q34.1, and 8.14 cM long from *D4S171* to *D4S1652* (53.8%) on 4q34.3-35.2.

As well as the highest frequency of deletion at 4q33, another two non-overlapping regions, 4q34.1 and 4q34.3-35.2, were defined near 4q33. 4q32-35 is the region that has been suggested as harboring tumor suppressor gene(s) in various tumors such as esophageal carcinoma,[23,24] SCLC,[7] malignant mesothelioma,[25] hepatocellular carcinoma,[26,27] and urinary bladder cancer.[12] Deletion of 4q32-34 is correlated with the immortalization of human keratinocytes from head and neck squamous cell carcinoma in tissue culture.[28] Since cellular immortalization is one of the characteristics of malignant transformation, it is possible that a gene involved in the regulation of cell senescence is a target of inactivation in cancers with genetic deletion at this locus.[24]

In our study, the other two of the five commonly deleted regions, 4q24 and 4q27-28.3, are

distinctly distinguished from the 4q25-26 residing *EGF* gene. 4q24 is one of three non-continuous regions of LOH found by Nagai et al. in hepatocellular carcinoma.[26] High frequency LOH was also found in uterine cervical carcinoma in this area which contains the ADH5 locus.[10] Because chromosome 4q contains genes encoding growth factors or genes expressed predominantly in the liver such as albumin, alcohol dehydrogenase (ADH3), fibrinogen and UDP-glucuronyltransferase, the deletion of this region might profoundly alter cell growth conditions and hepatocyte functions.[18,26] Also deletion of the 4q21-26 region including the ADH5 locus at 4q21-25 as well as the ADH3 locus at 4q21-23 plays an important role in the progression of urinary bladder carcinoma.[12]

4q27-28.3 is a region reported by several investigators to be frequently deleted. Piao et al.[27] reported that 4q27 was found to be the highest loss region (71.4%) in hepatocellular carcinoma. Microsatellite analysis by Hammond et al.[29] revealed that the fibronectin locus, FGA, on the 4q28 region displayed the highest LOH in hepatocellular carcinoma. These data suggest that the 4q28 region might carry a gene important in liver proliferation. The results of our study are concordant with these reports. Fibronectin, a glycoprotein, can be found in soluble form in the plasma, on the cell surface and as a component of the extracellular matrix. Invading and disseminating tumor cells come into contact with a variety of extracellular molecules that constitute the extracellular matrix. The deletion of the fibronectin locus may be related with the aggressive features displayed by these cancers such as tumor invasion and dissemination.

To conclude, our results have confirmed the presence of at least five tumor suppressor loci on chromosome 4q, loci which may play an important role in the development and progression of primary SCLC.

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