

**Identification of  
five distinct tumor suppressor  
loci on the long arm of  
chromosome 15  
in primary small cell lung cancer**

**Thesis by**

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**Identification of  
five distinct tumor suppressor loci  
on the long arm of chromosome 15  
in primary small cell lung cancer**

**Directed by Professor Se Kyu Kim**

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**Abstract**

**Identification of tumor suppressor loci on the long arm of  
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(Directed by Professor Se Kyu Kim)

Small cell lung cancer (SCLC) frequently shows a loss of heterozygosity (LOH) on chromosome 15q. In order to define the commonly affected



region on chromosome 15q, we tested 23 primary SCLCs by microsatellite analysis.

By analyzing 43 polymorphic microsatellite markers located on chromosome 15q, we found that 14 (60.8%) of 23 tumors exhibited a LOH in at least one of the tested microsatellite markers. Two (14.3%) of the 14 tumors were found to have more than a 50% LOH among the tested informative markers on chromosome 15q. LOH was observed in five commonly deleted regions on 15q. Of those regions, LOH from *D15S1012* to *D15S1016* was the most frequent (47.8%). LOH was also observed in more than 20-30% of tumors at four other regions, from *D15S1031* to *D15S1007*, from *D15S643* to *D15S980*, from *D15S979* to *D15S202*, and from *D15S652* to *D15S642*. Four of the 23 tumors exhibited shifted bands in at least one of the tested microsatellite markers. Shifted bands occurred in 3.2% (29 of 914) of the loci tested.

Our data suggests the presence of at least five tumor suppressor loci

on chromosome 15q in SCLC, and further that these may play an important role in SCLC tumorigenesis.

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Key Words: small cell lung cancer, LOH, microsatellite marker, chromosome 15

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**I. INTRODUCTION**

Bronchogenic carcinoma is the most common cancer in terms of

incidence, possibly due to the strong association between lung cancer and cigarette smoking and it remains a lethal disease of world wide concern.<sup>1</sup>

The poor prognosis of lung cancer has barely changed over the last decades.

Lung cancers can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for approximately 25% of all new cases of lung cancer, and has a very aggressive clinical course with frequent widespread metastases during the early stage of the disease. Untreated patients with limited- and extensive-stage SCLC have a median survival of 3 and 1.5 months, respectively.

Lung cancers are characterized by a multiple genetic alterations, such as the amplification of oncogenes and deletion or loss-of-function mutation of both alleles of tumor suppressor genes during disease development.<sup>2,3</sup>

The inactivation of tumor suppressor genes can be identified by LOH, which is a marker of chromosomal regions containing tumor suppressor

gene(s).<sup>4</sup>

Recent cancer research has focused on delineating the chromosomal location(s) of the putative tumor suppressor gene(s) involved in the malignant behavior of many tumors, including SCLC. As a result, Kim et al. identified three tumor suppressor loci on 9p,<sup>5</sup> two on 10q,<sup>6</sup> five on 5q,<sup>7</sup> and three on 4q<sup>8</sup> in SCLC by microsatellite analysis. However, although deletions on several chromosomes have previously been reported in SCLC, they are insufficient to define SCLC tumorigenesis.

SCLC was shown to have a complex pattern of chromosomal alterations, including 55-70% deletions on 15q, in a cytogenetic study by Petersen et al.,<sup>9</sup> and in an allelic deletion study by Stanton et al.<sup>10</sup> These results suggest that the allelic deletions of chromosome 15q are candidate regions for the harboring of novel tumor suppressor genes whose inactivation is important for SCLC pathogenesis.

To determine the potential presence of tumor suppressor gene(s) on

15q in SCLC, we tested 23 primary SCLCs by intensive microsatellite analysis.

## **II. MATERIALS AND METHODS**

### **1. Tissue specimens**

Twenty-three primary SCLCs and adjacent control tissues were obtained from bronchoscopic biopsy specimens, and surgical resections were done at the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Paraffin-embedded tissue blocks were sectioned to 4- $\mu$ m using a microtome. Sections were stained with hematoxylin and eosin (H&E) and reviewed by a pathologist to confirm the diagnosis and to locate tumorous and tumor-free areas. The tumor cells were microdissected with normal cell contamination levels of less than 30%. The normal control tissues were dissected using the same approach.

## **2. DNA Extraction**

Dissected tissues were digested in 200  $\mu\ell$  of TE9 buffer [1M Tris-HCL(pH 8.9), 0.5M EDTA, 4M NaCl] containing 20  $\mu\ell$  10X SDS-proteinase K and incubated at 42 °C for 12-24h. Digested products were boiled for 10 mins at 100 °C to inactivate enzymes, and purified by treating with phenol-chloroform. DNAs were ethanol precipitated in the presence of 2  $\mu\ell$  glycogen, dried at room temperature and then resuspended in ultra-pure water. The concentration of DNAs was measured by UV spectrophotometer at 260nm. DNAs were stored at 4 °C.

## **3. Microsatellite analysis**

For microsatellite analysis, following 43 microsatellite markers on the



long arm of chromosome 15 were obtained from Research Genetics (Research Genetics, Huntsville, AL, USA): *D15S122*, *D15S986*, *D15S822*, *D15S1002*, *D15S1048*, *D15S165*, *D15S1031*, *D15S1010*, *D15S1007*, *D15S971*, *D15S118*, *D15S1012*, *D15S994*, *D15S659*, *D15S1028*, *CYP19*, *D15S1016*, *D15S195*, *D15S117*, *D15S643*, *D15S1036*, *D15S974*, *D15S1020*, *D15S153*, *D15S983*, *D15S980*, *D15S114*, *D15S984*, *D15S1023*, *D15S211*, *D15S1041*, *D15S205*, *D15S655*, *D15S979*, *D15S202*, *D15S127*, *D15S652*, *D15S130*, *D15S207*, *D15S533*, *D15S966*, *D15S87*, *D15S642*.

One of the primers of each marker was end-labeled with [ $\gamma$ -<sup>32</sup>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 final volume of 12.5  $\mu$ l containing 20 ng of genomic DNA, 1% DMSO, 200  $\mu$ M dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.4  $\mu$ M of PCR primers, which included 0.1  $\mu$ M  $\gamma$ -<sup>32</sup>P-labeled primer, and 0.5 units of Taq DNA

polymerase (GIBCO-BRL, Gaithersburg, MD, USA).

DNA was amplified for 35 cycles, consisting of 95 °C for 30 sec, 52-60 °C for 1 min, and 70 °C for 1 min in a thermal cycler (Hybaid; Omnigene, Woodbridge, NJ, USA) in 500  $\mu$ l plastic tubes, followed by a final 5 min extension at 70 °C.

PCR products were mixed with 2X sample buffer, boiled for 3 min at 95 °C and then immediately stored in ice. These products were separated on a 6% polyacrylamide-urea-formamide gel, and then autoradiographed.

LOH was defined as more than a 50% reduction in the intensity of either of the two alleles by visual inspection versus normal control panels.

Shifted bands were determined by the appearance of alleles not present in normal tissue control panels.

### III. RESULTS

We used 43 microsatellite markers which are highly polymorphic on the long arm of chromosome 15, and found that 14 (61%) of 23 cases exhibited LOH in at least one of the tested microsatellite markers (Fig. 1) and 2 (SCLC1, SCLC2) of these 14 tumors showed more than 50% LOH among the tested informative markers, suggesting large areas of deletion (Fig. 1, and 2). Frequent LOH was observed in the areas from *D15S1031* to *D15S1007* (25%), from *D15S1012* to *D15S1016* (47.8%), from *D15S643* to *D15S980* (21.7%), from *D15S979* to *D15S202* (33.3%), and from *D15S652* to *D15S642* (23.8%) (Table1, and Fig.1, and 2). Four (17.4%) of 23 cases exhibited shifted bands for at least one of the tested microsatellite markers, especially for SCLC15. Shifted bands occurred in 3.2% (29 of 914) of the loci tested (Fig. 1, and 2).

**Table 1 . The summary of frequency of allelic loss in small cell lung cancer.**

<b>Primer</b>	<b>Location</b>	<b>No. of amplified</b>	<b>No. of Informative</b>	<b>No. of LOH</b>	<b>% LOH</b>
D15S122	15q11.1-11.2	18	8	2	25
D15S986	15q11.1	20	11	0	0
D15S822	15q11.2	20	16	2	13
D15S1002	15q11.2	20	10	1	10
D15S1048	15q13.2	21	12	1	8
D15S165	15q12-13.1	20	6	1	16
D15S1031	15q13.3	21	14	3	14
D15S1010	15q	16	8	2	25
D15S1007	15q15.1	21	14	2	14
D15S971	15q15.3	18	18	0	0
D15S118	15q15.3	21	9	0	0
D15S1012	15q15.3	22	11	3	27
D15S994	15q15.2	19	10	3	30
D15S659	15q21.1	18	13	3	23
D15S1028	15q21.1	21	13	2	15
CYP19	15q21-22	21	17	5	6
D15S1016	15q21.2	21	11	3	21
D15S195	15q	21	11	1	9
D15S117	15q21.3	21	10	1	10
D15S643	15q21.3	20	16	3	19
D15S1036	15q21.3	21	13	2	15

<b>Primer</b>	<b>Location</b>	<b>No. of amplified</b>	<b>No. of Informative</b>	<b>No. of LOH</b>	<b>% LOH</b>
D15S974	15q	23	23	2	9
D15S153	15q22.3	20	9	2	22
D15S983	15q23	19	14	3	21
D15S980	15q23	17	6	2	33
D15S114	15q23	22	17	0	0
D15S984	15q	20	9	2	22
D15S1023	15q	20	11	2	18
D15S211	15q	9	6	1	17
D15S1041	15q	22	10	1	10
D15S205	15q25	21	15	3	20
D15S655	15q26.1	11	7	1	14
D15S979	15q26.1	21	15	4	27
D15S202	15q26.1	20	10	4	40
D115S127	15q26.1	19	13	1	8
D15S652	15q26.2	19	11	2	18
D15S130	15q26.2	21	14	2	14
D15S207	15q	19	9	2	22
D15S533	15q	21	18	2	11
D15S966	15q	21	14	1	7
D15S87	15q26-pter	21	9	3	33
D15S642	15q26.3	20	16	2	13

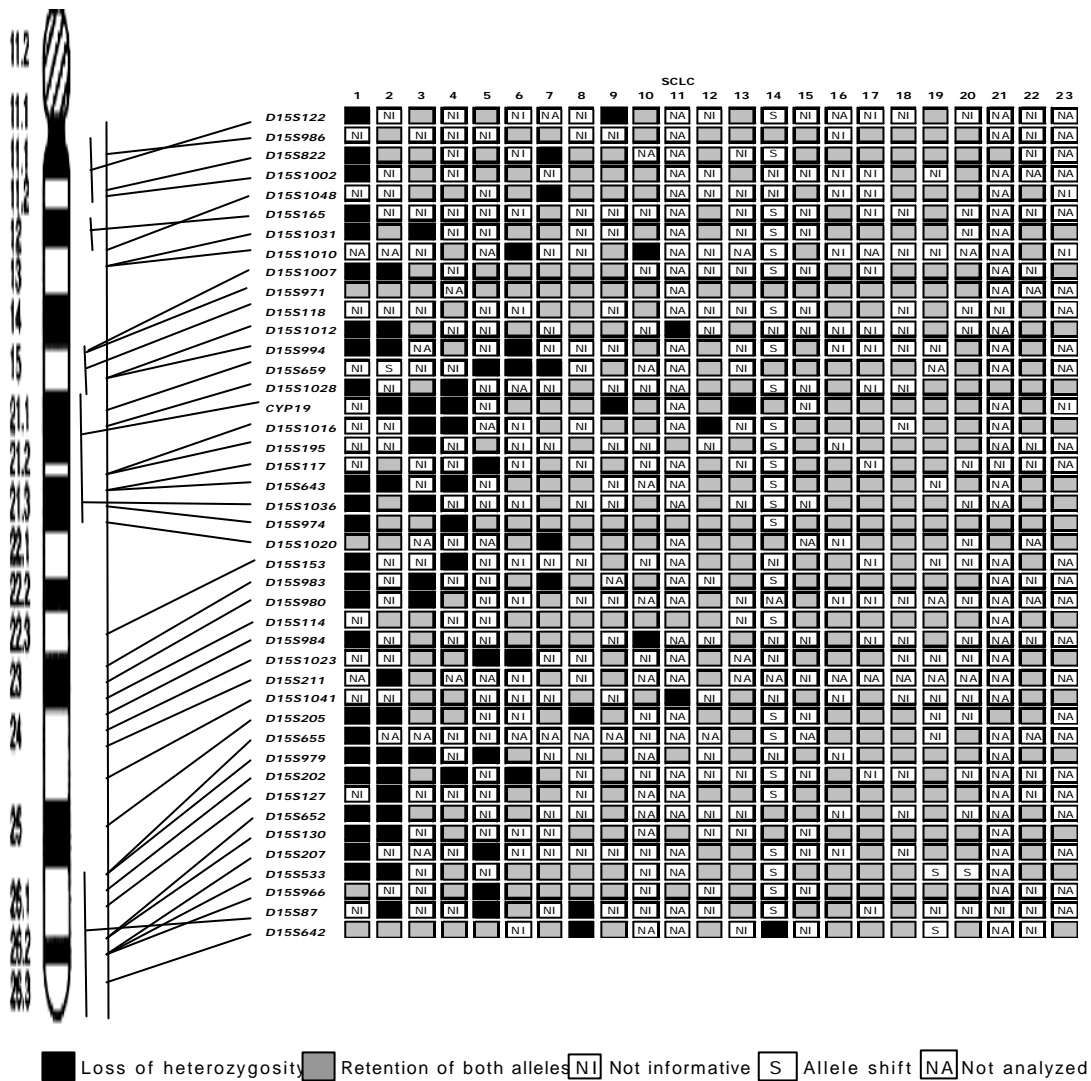


Fig. 1. Deletion mapping on chromosome 15q in primary small cell lung cancers. Names of microsatellite markers are at left side of the figure and tumors are given on the top of the figure. A total 14 (61%) tumors exhibited LOH at more than one region on chromosome 15q. The deleted regions are indicated on the right side of the figure.

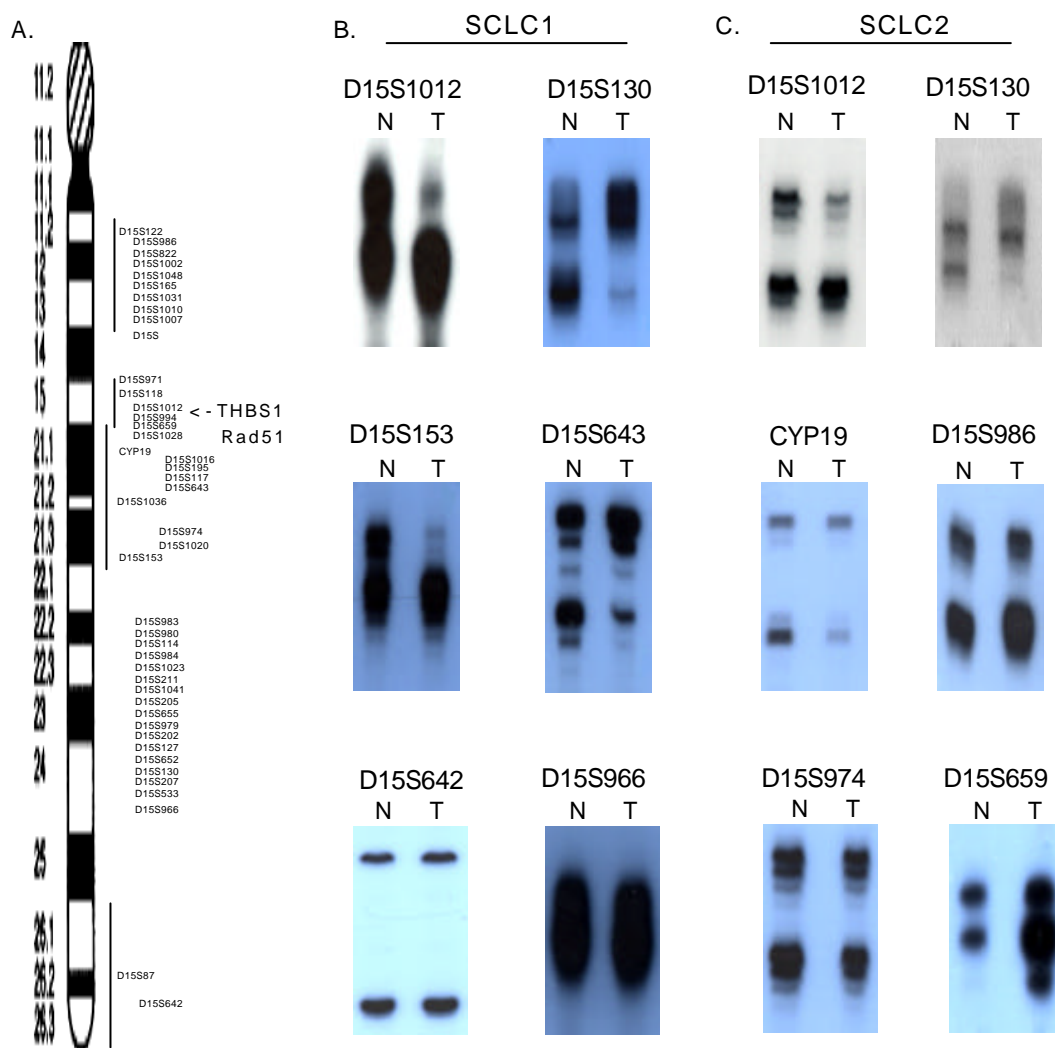


Fig. 2. Examples of deletions observed in primary small cell lung cancers. A. The order of microsatellite markers used in this study. B. LOH at markers D15S1012, D15S130, D15S153 and D15S643 in tumor SCLC1 and D15S1012, D15S130 and CYP19 in tumor SCLC2, while retention at markers D15S642 and D15S966 in tumor SCLC1 and D15S986 and D15S974 in tumor SCLC2. Shifted band at markers D15S659 in tumor SCLC2. N indicates normal tissues and T indicates tumor tissues.

## IV. DISCUSSION

The dismal prognosis of SCLC is due to its biologic behavior, which results in dissemination to regional lymph nodes and/or distant metastasis sites in more than 90% of patients at the time of initial presentation, and because the disease is virtually incurable with current treatment modalities.<sup>11</sup> Surgery is inappropriate as a primary treatment in SCLC; systemic chemotherapy and radical radiotherapy are preferable. As a result, it is difficult to get enough tissues for research purposes, which significantly limits active researches on SCLC.

The karyotypes of SCLC exhibit extensive abnormalities. Whang-Peng et al. showed the first consistent cytogenetic abnormality in lung cancer, deletions on chromosome 3p in virtually all SCLC cell lines examined.<sup>12</sup> As suggested by the extensive cytogenetic abnormalities, LOH is common in lung cancers, especially in SCLC. Kim et al. reported frequent deletions



on chromosomes 4q,<sup>8</sup> 5q,<sup>7</sup> 9p,<sup>5</sup> and 10q<sup>6</sup> in primary SCLCs. More recently, genome-wide allelotypes of lung cancer, based on high throughput PCR-based methods for detecting DNA polymorphisms, have been reported, which found that the chromosomal arms with the most frequent LOH were 1p, 3p, 4p, 4q, 5q, 8p, 9p (*p16*), 9q, 10p, 10q, 13q (*Rb*), 15q, 17p (*p53*), 18q, 19p, Xp, and Xq.<sup>13</sup>

Previously, a cytogenetic study by Petersen et al. indicated a variety of patterns of chromosomal alterations, including 55% deletions of 15q in SCLC.<sup>9</sup> An allelic deletion study by Stanton et al.<sup>10</sup> also revealed allelic losses of >70% of 15q in SCLC. These suggest that allelic deletions of 15q could be candidate regions for the harboring of novel tumor suppressor genes whose inactivation is important for SCLC pathogenesis.

Allelic deletions of 15q are reported to be correlated with some hereditary diseases, such as Prader-Willi or Angelman syndrome,<sup>14</sup> hereditary spastic paraplegia,<sup>15</sup> and reading disability (dyslexia),<sup>16</sup> and a

rare connective tissue disorder, Marfan's syndrome.<sup>17</sup> In addition, a recent report suggested that the region between markers *GABRB3* (15q11.2) and *15S165* (15q12-q13.1) on chromosome 15q is associated with a major susceptibility locus that influences triglyceride concentrations.<sup>18</sup>

However, in terms of the development and progression of cancer, recent studies have focused on defining deletions associated with chromosome 15q in a variety of human solid tumors, including neuroblastoma,<sup>19</sup> gastric neuroendocrine tumor,<sup>20</sup> esophageal adenocarcinoma arising in Barrett's epithelium,<sup>21</sup> pancreatic acinar cell carcinoma,<sup>22</sup> and papillary serous peritoneal carcinomas.<sup>23</sup>

Chromosomal alterations of 15q seem to be correlated with both early and late genetic events in cancers.

15q chromosomal gains were found in adrenocortical tumors of childhood,<sup>24</sup> prostate carcinomas,<sup>25</sup> and in oral squamous cell carcinomas.<sup>26</sup> Aubele et al. reported that the early stages of breast cancer

(ductal carcinoma *in situ*; DCIS) and atypical ductal hyperplasia, but not lymph node metastasis were mainly characterized by DNA gains of 15q,<sup>27,28</sup> which may represent early events in the carcinogenic process. They suggested that the proto-oncogene products c-Src1 (15q23-25) may play a role in this process, and that the detection of DNA gain and loss in early lesions indicates that activation of proto-oncogenes and the inactivation of tumor suppressor genes has already occurred in proliferative lesions adjacent to invasive carcinoma of the breast.

However, the LOH of chromosome 15q also seems to be correlated with aggressive features, and a late genetic event involved in the progression rather than the initiation of cancers.

A study by Richard et al. showed that deletions at several chromosomes including 15q were present in poorly differentiated carcinomas and estrogen receptor-negative tumors, and it was suggested that they contribute to a more aggressive tumor phenotype in breast cancer.<sup>29</sup> Wick

et al. also reported that the frequency of 15q loss was significantly higher in metastatic cases of breast cancer.<sup>30</sup> Furthermore, similar results had been obtained, namely, that deletions of 15q were more frequent in stages pT2-4 (cancer cells reaching the muscular bladder wall) than in stage pT1 carcinomas in urinary bladder cancer (invasion limited to the lamina propria),<sup>31</sup> and 15q losses had been proposed to be late events in most high-grade ovarian carcinomas, but to occur in a subset of low-grade ovarian carcinomas.<sup>32</sup>

Among our samples, only one with an extensive stage (SCLC5) did not show any difference of LOH frequency in tested markers with the informative result compared to the other samples with limited stage. It is not apparent from our results that a LOH on 15q affects the late stage in SCLC. However, we think that our sample size was not large enough to determine the impact of LOH on 15q in terms of its influence during the stages of SCLC carcinogenesis.

Our study demonstrated that five independent commonly deleted regions were defined on chromosome 15q in primary SCLCs; a 4.28 cM length from *D15S1031* to *D15S1007* (25%) on 15q13.3-15.1, a 11.99 cM length from *D15S1012* to *D15S1016* (47.8%) on 15q15.3-21.2, a 19.49 cM length from *D15S643* to *D15S980* (21.7%) on 15q21.3-23, a 2.24 cM length from *D15S979* to *D15S202* (33.3%) on 15q26.1, and a 32.12 cM length from *D15S652* to *D15S642* (23.8%) on 15q26.2-26.3.

As well as the highest frequency deletion at 15q15.3-21.2, another two non-overlapping regions, 15q21.3-23 and 15q13.3-15.1 were defined by the present study. 15q11-21 is a common region reported by several investigators to be frequently deleted.

15q11.1-15 is a region that has been suggested to harbor tumor suppressor gene(s) in a variety of human solid tumors, including human malignant mesothelioma,<sup>33,34</sup> prostate cancers,<sup>35</sup> ovarian cancers,<sup>36</sup> parathyroid adenomas,<sup>37</sup> and metastatic tumors of the breast, lung, and

colon.<sup>30</sup> These data suggest that the loss and/or inactivation of a putative tumor suppressor gene in 15q11.1-15 contributes to the development or progression of malignant mesothelioma as well as other types of tumors.

15q15 is a region that contains the thrombospondin-1 (*THBS1*) gene, which is believed to have tumor suppressor properties via an ability to inhibit angiogenesis,<sup>38</sup> and *RAD51*.<sup>39</sup>

THBS1 is a multifunctional glycoprotein with p53- and Rb-regulated angiogenic inhibition properties and is one of the major secreted proteins of human platelets and is an extracellular matrix component of a variety of cells including vascular endothelial cells and tumor cells. THBS1 has been reported to modulate platelet aggregation, wound healing, protease activity and cellular functions, such as adherence, motility, and growth.<sup>39</sup>

THBS may also contribute to tumor progression<sup>40</sup> and metastasis formation,<sup>30</sup> as a potent antiangiogenic factor, and might play a role during the later stages of carcinogenesis, because its production correlates

inversely with tumorigenesis and metastatic potential in melanoma, lung and breast carcinoma cell lines.<sup>41</sup>

*RAD51*, which is located at 15q15.1, is a potentially relevant gene in this region. *RAD51* is the human homologue of the bacterial *RecA* gene whose product participates in the repair of double-strand breaks and in chromosomal disjunction. Moreover, mutations in the mouse *Rad51* gene have been associated with severe chromosomal loss in actively dividing cells,<sup>42</sup> and deletions and numerical losses appear to be the predominant forms of genomic imbalance in malignant mesothelioma.<sup>39</sup> Recurrent losses from 13q12-14 occur with mutations of *Rad51*, because the *BRCA2* gene product is an essential co-factor in the RAD51-dependent DNA repair of double-strand breaks.<sup>43</sup> The presumed role of the *Rad51* gene in tumor development stems from the demonstration that the gene interacts with the breast tumor suppressor genes, *BRCA1* and *BRCA2*.<sup>43-</sup>

Kersemaekers et al. suggested that a novel tumor suppressor gene may be present on 15q21 in carcinoma of the uterine cervix.<sup>47</sup> In their study, the highest frequency (27%) of LOH was at CYP19, one three markers they used. Although the distribution of deletions did not enable them to identify a very smaller region of deletion, they suggested the presence of a tumor suppressor gene on 15q in HPV-related cervical cancer tumorigenesis. 15q21 is known to harbor the fibrillin 1 gene (*FBN1*), and a recent report suggested an association between *FBN1* and a rare connective tissue disorder, Marfan's syndrome,<sup>17</sup> and a malignant mesothelioma in two affected brothers not exposed to asbestos.<sup>48</sup> *FBN1*, which is responsible for Marfan's syndrome, maps approximately 5-cM distal from the shortest region of overlap on 15q15. *FBN1* encodes a glycoprotein that is a major constituent of 10-12 nm microfibrils in the extracellular matrix.<sup>49</sup> The mutated *FBN1* gene also seems to be associated with multisystemic connective tissue disease, such as



scleroderma, or systemic sclerosis.<sup>50</sup> Compared with controls, fibrillin 1 protein synthesized by systemic sclerosis fibroblasts is unstable. Systemic sclerosis fibroblasts showed increased glycosaminoglycan and collagen synthesis in response to TGF stimulation compared with normal fibroblasts.<sup>51,52</sup> Recently, Bisconti et al. reported a case of malignant mesothelioma in two affected brothers without asbestos exposure, which seems to be associated with chromosomal deletions of 15q.<sup>48</sup> They suggested that *FBN1* might play some role in the malignant transformation associated with multisystem connective tissue disorder.

15q21-22 is an area, which contains *SMAD3* and *SMAD6*. To date, over half a dozen human Mad homologues (*SMADs*) have been identified. *SMAD4*(*DPC4*) is part of the TGF signaling pathway, and TGF signals from the membrane to the nucleus via *SMAD* proteins. TGF receptor activation results in *SMAD2* and *SMAD3* phosphorylation, which then form heteromeric complexes with *SMAD4*. Inhibitory *SMADs*, *SMAD6*

and SMAD7, can prevent TGF signaling by interacting either with the receptor or with SMAD2 and SMAD3. The encoding sequences for these proteins are organized in two gene clusters, one at 18q21 (*SMAD2*, *SMAD4*, and *SMAD7*) and the other at 15q21-22 (*SMAD3* and *SMAD6*).<sup>53</sup> Losses of 15q stress the importance of SMADs inactivation in SCLC carcinogenesis.

15q26-qter is a region frequently deleted in 35% of parathyroid adenomas.<sup>37</sup> In our study, two distinct separate regions of 15q26.1 and 15q26.2-26.3 were found.

15q25-q26 is associated with the regulation of body weight. Fat-free mass consists mostly of skeletal muscle and bone tissues, and the linkages on chromosomes 7p, 15q (15q25-q26), and 18q are probably related more to the skeletal muscle component of fat-free mass than to bone mineral density.<sup>54</sup>

To conclude, our results suggest the presence of at least five tumor

suppressor loci on chromosome 15q, which may play important roles in the development and progression of primary SCLC.

## V. CONCLUSION

To identify the tumor suppressor loci on chromosome 15q, we used 43 highly polymorphic microsatellite markers on the long arm of chromosome

15. Our conclusions are as follows:

1. Fourteen (60.8%) of 23 cases exhibited LOH in at least one of the microsatellite markers tested.
2. Two (SCLC1, SCLC2) of these 14 tumors showed more than 50% LOH among the tested informative markers, suggesting large areas of deletion.
3. Frequent LOH was observed in the area from *D15S1031* to *D15S1007* (25%), from *D15S1012* to *D15S1016* (47.8%), from *D15S643* to *D15S980* (21.7%), from *D15S979* to *D15S202* (33.3%), and from *D15S652* to *D15S642* (23.8%).

4. Four (17.4%) of 23 cases exhibited shifted bands in at least one of the tested microsatellite markers, especially in SCLC15.
5. Shifted bands occurred in 3.2% (29 of 914) of the loci tested.

Our data suggest the presence of at least five tumor suppressor loci on the long arm of chromosome 15, and that they may play important roles in SCLC tumorigenesis.

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15

15

가 , .

45

23 15 43

PCR - LOH

23 LOH가 1 14

60.8% . LOH가 14

50% 2 (SCLC1, SCLC2) 14.3%

5 LOH

, D15S1031 D15S1007 4.28 cM 25%,

D15S1012 D15S1016 11.99 cM 47.8%,

D15S643 D15S980 19.49 cM 21.7%, D15S979

D15S202 2.24 cM 33.3%, D15S652

D15S642 32.12 cM 23.8%

Shifted bands 23 4 SCLC15 60%

altered loci가 . Shifted bands 914 loci

3.2% 29 loci . 15

5 가 ,

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