

Polymorphic Sites within the *MCC* and *APC* Loci Reveal Very Frequent Loss of Heterozygosity in Human Small Cell Lung Cancer

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Abstract

Using single-strand conformation polymorphism we have found two polymorphic sites, AAC to AAT at codon 511 (exon 12) and GCT to GCG at codon 708 (exon 15), in the *MCC* gene. These sites and an *RsaI* polymorphic site in *APC* allowed us to study 23 human small cell lung cancer (SCLC) and 7 non-small cell lung cancer samples for allele loss. Of the 23 SCLC samples, 21 (91%) were informative for one or more of these markers, and we found allele loss in more than 80% (17 of 21). In non-small cell lung cancer samples, 5 of 7 (71%) were informative, and reduction or loss of one allele was found in 2 of 5 (40%). Seven cases were informative for both genes, loss of heterozygosity occurred for both genes in five, one retained heterozygosity for both, and one SCLC had loss of heterozygosity for *APC* but not for *MCC*. We conclude that loss of heterozygosity occurs frequently for *MCC* and *APC* in lung cancer of all histological types and is very frequent in SCLC. This suggests the presence of tumor suppressor gene(s) in the *MCC/APC* region of 5q21 involved in human lung cancer.

Introduction

Several studies have demonstrated loss of heterozygosity of the long arm of chromosome 5 (at 5q21) in sporadic colon cancer and mutations of genes in this region in the germ line of some patients with familial adenomatous polyposis and Gardner's syndrome (1, 2), indicating that abnormalities of 5q may be the earliest genetic alteration in some forms of colon cancer. Further evidence for the importance of genes located on chromosome 5 in growth control comes from the transfection experiments of Tanaka *et al.* (3), where suppression of tumorigenicity of colon cancer cell lines was found after transfecting an intact chromosome 5. Recently, two genes, *MCC*⁴ and *APC/DP2.5*, were described within 5q21 (1, 4, 5). Interestingly, the *MCC* gene was found to carry somatic abnormalities exclusively in sporadic colon cancer samples (5), while the *APC* gene was mutated in the germ line of FAP/GS patients as well as being somatically mutant in several colon cancer samples (6, 7). These data demonstrated the involvement of the *MCC* and *APC* genes in the pathogenesis of colon cancer; however, the function of these genes remains unknown.

In lung cancer, chromosomal deletions and/or LOH on chro-

mosomes 3p, 9p, 11p, 13q, and 17p have been reported (8-12) as well as a high frequency of inactivating abnormalities of the *Rb* (chromosome 13q) and *p53* (chromosome 17p) tumor suppressor genes (13, 14). Chromosome 5 abnormalities have not been stressed. In fact, in the initial allelotyping screen of lung cancer by Yokota *et al.* (9) only 1 of 13 informative cases show chromosome 5 marker loss (9). The particular probes used (*D5S2* and *FMS*) have now been mapped to the 5q33-qter region. A recent study by Ashton-Rickardt *et al.* (15) looking for loss of heterozygosity in resected lung cancer specimens using probes all along chromosome 5q detected about 20% allele loss, but this study evaluated only two cases of SCLC (15). At the time this report appeared, we had evaluated whether the 5q21 locus is a target for genetic abnormalities in small lung cancer using polymorphisms within the *MCC* and *APC* genes themselves. We examined 23 SCLC and 7 NSCLC lung cancer tumor and cell line samples matched with normal autologous tissues for LOH. In both genes, these analyses were carried out on polymorphic DNA sites using SSCP analysis and diagnostic digestion of PCR products by specific restriction enzymes. In agreement with Ashton-Rickardt *et al.* (15), we find that 5q21 shows occasional loss of one allele in NSCLC. However, in SCLC we find that the incidence of allelic deletion in the *MCC/APC* region was extremely high, >80%, focusing attention on this portion of chromosome 5 in the search for tumor suppressor gene(s) which play a role in the development of lung cancer.

Materials and Methods

Lung Cancer and B-Lymphoblastoid Cell Lines, SCLC Tumors, and Autologous Normal Tissues. The tumor cell lines used in this study were established in the National Cancer Institute-Navy Branch laboratory from tumor samples obtained from SCLC and NSCLC patients on approved clinical protocols as described (16, 17). B-lymphoblastoid cell lines established from blood samples of the same SCLC and NSCLC patients were utilized as the source of constitutional DNA for loss of heterozygosity analysis. NSCLC lines H1437, H1648, H2009, H2087, and H2122 were derived from adenocarcinomas, and H2052 was from a mesothelioma. The histological subtype of H1993 is not available. Samples from SCLC tumors and normal autologous tissue were obtained at autopsy from National Cancer Institute-Navy Branch patients, frozen on dry ice, and stored at -70°C until use. Genomic DNA and total RNA from cell lines were prepared by the guanidine isothiocyanate/CsCl method (18). DNA from finely minced tumor and normal tissue samples was purified by proteinase K digestion, extracted with phenol/chloroform, and precipitated by ethanol (18).

PCR/SSCP Analysis and Diagnostic Enzymatic Digestion of the *MCC* and *APC* Loci. The PCR/SSCP method (19, 20) was modified to screen for LOH at chromosome 5q21 to analyze highly polymorphic sites contained in exons 12 and 15 of the *MCC* gene. Primers used for the PCR amplification were those published by Kinzler *et al.* (5), which were synthesized with an ABI 380B DNA synthesizer (Applied Biosystems). The primers for exon 12 were ATGTTGATTAATCCGTTGGC

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⁴ The abbreviations used are: *MCC*, mutated in colon cancer; *APC*, adenomatous polyposis coli; LOH, loss of heterozygosity; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

(5') and ACCCCAGAGCAGAAGGCT (3'), and those for exon 15 were GGCCTAACTGGAATGTGT (5') and GCCCAGATAAACAC-CAGC (3'). Each PCR amplification was carried out using 1 µg of genomic DNA in a final volume of 10 µl, which was labeled during the reaction with 0.5 µl (5 µCi) of (α-³²P)dCTP (>3000 Ci/mmol; Amersham), and 50 ng of each primer were combined with buffer and nucleotides as recommended by Perkin Elmer/Cetus. PCR conditions consisted of 1 cycle at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 56°C for 30 s. The reactions were processed as previously described (21) and analyzed on an 8% nondenaturing acrylamide gel.

An *RsaI* polymorphic site in APC gene exon 11 has been previously reported (6). The APC gene exon 11 PCR products were obtained using 1 µg of genomic DNA, with the same conditions as described above for the MCC gene. The primers used for this amplification are as follows: exon 11, sense, GGACTACAGGCCATTGCAGAA; exon 11, anti-sense, GGCTACATCTCCAAAAGTCAA (22). The entire APC PCR reaction was then digested with 2 units of *RsaI* at 37°C for 12 h. The digestion products were electrophoresed on a 10% nondenaturing acrylamide gel at 120 V for 2 h.

Direct Sequencing of Genomic DNA/PCR Products. The direct sequencing of double-strand genomic DNA fragments obtained by PCR has been carried out for MCC exons 12 and 15 as previously described (23). The primers used for sequencing were within the amplified fragment and were designed on the basis of the MCC DNA sequence received from Drs. B. Vogelstein and K. Kinzler.

Results

SSCP and Direct Sequencing Analyses Reveal Two Highly Polymorphic Sites in Exons 12 and 15 of the MCC Gene. SSCP analysis of MCC exons 12 and 15 performed in a search for mutations in lung cancer samples revealed band shifts in some samples but not in others (Figs. 1 and 2). Direct sequencing of the genomic DNA/PCR products of MCC exons 12 and 15 obtained from tumor and normal autologous samples showed the presence of two polymorphic sites not previously described (sequencing data not shown). One was located at codon 511 (exon 12) as a single nucleotide substitution AAC to AAT. The second, a GCT to GCC substitution, occurred in exon 15 at

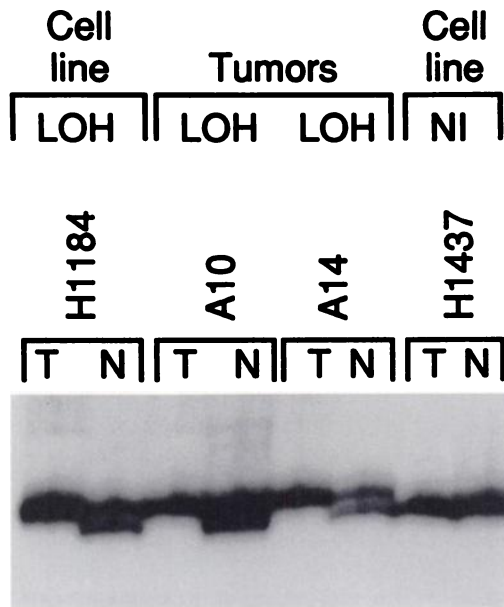


Fig. 1. Examples of detection of LOH of chromosome region 5q21 by SSCP analysis of MCC exon 12 PCR products in SCLC and NSCLC cell lines and autopsy-derived SCLC tumors matched with normal autologous tissue controls. The two bands represent the alternative alleles. NI, not informative; T, tumor DNA; N matched normal DNA.

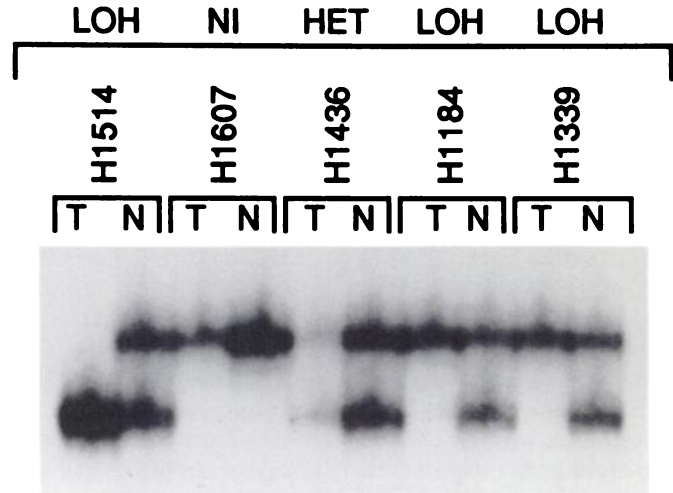


Fig. 2. Detection of LOH of chromosome 5q21 by SSCP analysis of MCC exon 15 PCR products in a representative panel of SCLC cell lines matched with normal autologous tissue controls. The two bands represent the alternative alleles. NI, not informative; HET, heterozygous (informative but not lost); T, tumor DNA; N, matched normal DNA.

codon 708. Neither resulted in an amino acid substitution. These sites were independently discovered and communicated to us by Drs. Kenneth Kinzler and Bert Vogelstein. These highly polymorphic sites (informative in 52% and 42%, respectively, of our samples) were used in connection with a previously described APC exon 11 polymorphic site (6, 22) to screen SCLC and NSCLC samples for allele loss on chromosome 5q21.

Detection of LOH by SSCP Analysis of MCC Exons 12 and 15. SSCP analysis for exon 12 (Fig. 1) and exon 15 (Fig. 2) was carried out on a total of 30 lung cancer DNA samples (Table 1). In the 23 SCLC samples, the MCC exon 12 polymorphic site was heterozygous (and thus informative) in 15 (65%), while the exon 15 site was informative in 11 (47%). Six cases were informative at both sites. In seven NSCLC cell lines, the MCC exon 12 site was informative in four (57%), and exon 15 was informative in three (43%), while one case was informative for both markers. Combining the data from both sites, we found that LOH for the MCC gene was present in 14 of 17 (82%) informative SCLC samples and in 2 of 5 (40%) informative NSCLC samples.

RsaI Digestion of APC Exon 11 Demonstrates LOH for Chromosome 5q21. To analyze LOH for the APC gene, we used a previously described polymorphic site located in exon 11 which created a new restriction site for *RsaI* (6, 22). Digestion of genomic DNA PCR products (Fig. 3) allows the assessment of the frequency of LOH for the APC gene (Table 1). We found that 10 SCLCs were informative for the APC exon 11 site and 7 (70%) had LOH. In NSCLC cell lines, one case was informative and it showed LOH. Combining the data from both the MCC and APC genes, LOH at these 5q21 loci occurs in 17 of 21 (80%) informative SCLCs and in 2 of 5 (40%) informative NSCLCs.

Discussion

Allele loss at one or more of MCC/APC 5q21 loci was detected in 80% of 21 informative SCLCs and in 40% of 5 informative NSCLCs, suggesting the presence of a tumor suppressor gene(s) in this region that is involved in the pathogenesis of lung cancer. This frequency of allele loss in SCLC is the highest reported for this region in any cancer, including colon.

In fact, the frequency of LOH for chromosome 5q in colon cancer is about 40% (24). Boynton *et al.* (25) have recently shown that human esophageal cancer specimens show a similar high frequency of allele loss, 77% (25). Ashton-Rickardt *et al.* (15) studied 43 surgically resected lung cancers (only 2 of which were SCLC) in which they found 21% LOH in 33 informative cases using several chromosome 5q markers (15). Only one of the probes used in their analysis was within the *MCC* or *APC* genes. Likewise, we found LOH at the *MCC/APC* loci in 3 of 10 informative resected primary NSCLC samples (data not shown). It appears from our data that SCLC tumors, taken at autopsy, and SCLC cell lines (most of which were derived from metastatic sites) have a much higher frequency of loss than NSCLC tumors or cell lines. We found approximately equal rates of loss from SCLC cell lines and uncultured tumor specimens, implying that our results are not an artifact of *in vitro* culture. Primary resected SCLC samples are clinically very difficult to obtain, and our SCLC tumors collected at autopsy represent more advanced-stage tumors than those that undergo resection. Thus it may be that loss on 5q is a later event involved in SCLC tumor progression.

Several genes besides *MCC* and *APC* have been mapped to this region of chromosome 5q: the *FER* gene, which had been identified as a tyrosine kinase gene homologous to *src*; the *TBI* gene, sharing similarities with the *ADP*, ATP carrier/translocator protein family; the *TB2* gene, with no similarities with other known genes; and the *SRP 19* gene (1, 4, 26). In one case

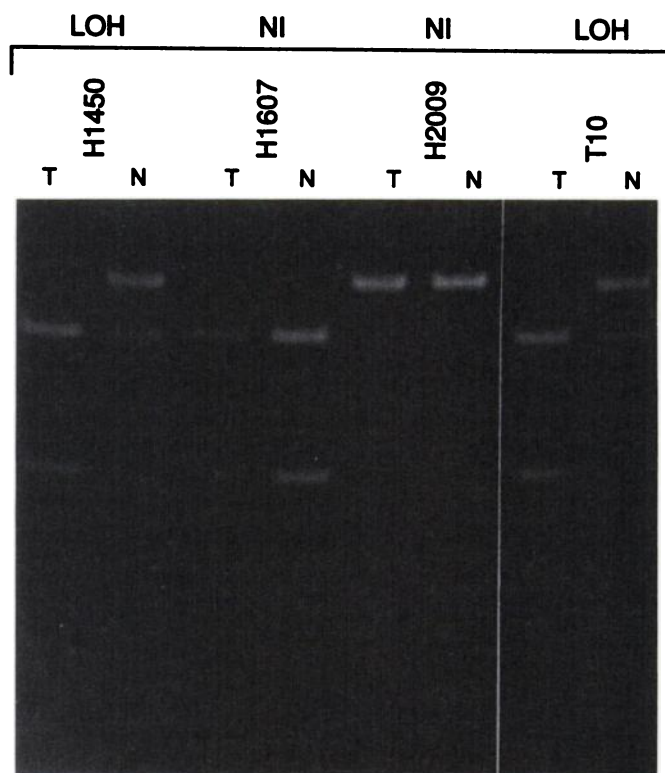


Fig. 3. Examples of detection of LOH of chromosome region 5q21 by *RsaI* digests of *APC* exon 11 PCR products from cell lines and an SCLC tumor. *NI*, not informative; *T10*, autopsy-derived tumor DNA from sample A10; *T*, tumor DNA; *N*, matched normal DNA.

Table 1 *MCC* and *APC* allele loss in lung cancer samples

Summary of LOH in lung cancer samples by SSCP analysis of *MCC* gene exons 12 and 15, and digestion by *RsaI* enzyme of *APC* gene exon 11.

	Samples ^a	5q21 polymorphic sites		
		<i>MCC</i> gene		<i>APC</i> gene
		Exon 12	Exon 15	Exon 11
SCLC cell lines	NCI-H1173	NI	HET	LOH
	H1184	LOH	LOH	NI
	H1339	LOH	LOH	NI
	H1417	LOH	LOH	NI
	H1436	HET	HET	NI
	H1450	LOH	LOH	LOH
	H1514	LOH	LOH	NI
	H1607	LOH	NI	NI
	H2141	NI	NI	LOH
	SCLC tumor samples from autopsy	A1	NI	NI
A2		LOH	NI	NI
A3		LOH	NI	LOH
A6		NI	NI	NI
A7		LOH	LOH	LOH
A8		NI	LOH	NI
A10		LOH	NI	LOH
A11		HET	HET	HET
A12		NI	NI	HET
A13		LOH	NI	NI
A14		LOH	LOH	NI
A15		LOH	NI	NI
A16	NI	NI	NI	
A20	NI	NI	LOH	
NSCLC cell lines	NCI-H1437	NI	HET	NI
	H1648	LOH	NI	NI
	H1993	HET	HET	NI
	H2009	NI	NI	NI
	H2052	HET	HET	NI
	H2087	LOH	NI	LOH
	H2122	NI	NI	NI

^a Lung cancer samples as described: the number of the cell lines (NCI-H) identify lung cancer cell lines established at the National Cancer Institute-Navy Oncology Branch, while SCLC autopsy (A) tumor numbers are arbitrary. *NI*, normal tissue DNA not heterozygous and thus tumor sample was not informative; *HET*, tumor retained heterozygosity; *LOH*, tumor showed loss of heterozygosity at site.

(SCLC cell line H1173) LOH was present for the *APC* gene but not for *MCC*. This could indicate the involvement of one of these other genes or of *APC* and not *MCC*, or result from partial deletions (resulting in truncated genes) not involving the entire *MCC* or *APC* genes and thus deserves further study. However, the *MCC* and *APC* genes, which have both been previously shown to be mutated in sporadic colorectal cancer and (for *APC*) in the germ line of FAP/GS patients (6, 7), may be considered the best candidates for tumor suppressor genes on 5q21 involved in lung cancer. Our initial studies evaluating lung cancer cell lines for somatic mutations in some of those exons of the *MCC* and *APC* genes found to be mutated in colorectal cancer and FAP patients, and for the highly conserved tyrosine kinase domain of *FER* gene, have not identified any abnormalities in the remaining allele.⁵ Since most polyps in patients carrying a germ line mutation for *APC* have not shown LOH for this locus, it has been suggested that quantitative effects related to the loss (by mutation) of one allele may be sufficient for adenoma pathogenesis (7, 24). Thus, it is possible that a simple gene dosage effect engendered by loss of one copy of some 5q21 gene is contributory to the development of lung cancer. Still, the possibility exists that somatic mutations in lung tumors occur in the remaining copy of these genes, and more extensive studies are under way.

Frequent involvement of 5q has been previously described in gastric cancer, colon cancer, esophageal cancer, and in the germ line of FAP/GS patients (1, 2, 24). The results presented here indicate that LOH of the *MCC* and *APC* loci on chromosome 5q21 is a common event for lung cancer as well. Further studies should evaluate the frequency of LOH and structural analysis of genes in the *MCC/APC* complex in human lung cancer and

⁵ D. D'Amico and D. Carbone, unpublished observations.

assess the physiological role of these genes in lung cancer cell growth.

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