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Molecular Analysis of a Myc Antagonist, ROX/Mnt, at 17p13.3 in Human Lung Cancers

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The chromosome region 17p13 is known to be frequently deleted in lung cancers. We recently showed the presence of an independent, commonly deleted region at 17p13.3, suggesting that in addition to p53 at 17p13.1 an as-yet-unidentified tumor suppressor gene may reside in this telomeric region. Interestingly, the chromosomal location of a recently isolated novel *myc* antagonist gene, termed ROX/Mnt, coincides exactly with the centromeric border of the commonly deleted region at 17p13.3 in lung cancers. In conjunction with the generally acknowledged roles of *myc* genes in the pathogenesis of lung cancers, these findings led us to investigate whether ROX/Mnt is altered in lung cancers. Despite an extensive search for alterations in 52 lung cancer specimens, somatic mutations of ROX/Mnt could not be identified. We conclude that ROX/Mnt itself is not a frequent target for 17p13.3 deletions in lung cancers and that further explorations are required to identify the putative tumor suppressor gene at 17p13.3.

Key words: ROX — Mnt — 17p13.3 — Lung cancer

Accumulating evidence suggests that genetic changes in tumor suppressor genes probably play a major role in the pathogenesis of lung cancer. Frequent allelic loss is a hallmark of the presence of a tumor suppressor gene in the affected chromosome regions. Cytogenetic and molecular biological analyses have revealed frequent chromosomal deletions in lung cancers on 3p, 5q, 8p, 9p, 11p, 13q, 17p, 18q and 22q.[1)] We and others have reported genetic alterations of candidate tumor suppressor genes in lung cancers, including p16 on 9p, Rb on 13q, p53 on 17p, and *Sma*d2 and *Sma*d4/DPC4 on 18q.[2–7)]

17p13 deletion has been reported to occur frequently in various types of human cancer including lung cancer.[8)] It is thought that the *p53* gene at 17p13.1 is a molecular target for the frequent 17p deletions in lung cancer.[5)] In a previous study, we showed the presence of an independent, commonly deleted region(s) between D17S695 (the telomeric border) and D17S379 (the centromeric border) at 17p13.3 in addition to the p53 locus at 17p13.1, suggesting the presence of an additional tumor suppressor gene at 17p13.3 (submitted for publication).

The *ROX/Mnt* gene was recently isolated as a novel bHLHZip Max-binding protein.[9,10)] ROX/Mnt forms a heterodimer with Max and can function as a transcriptional repressor, efficiently suppressing Myc-dependent activation. Other bHLHZip Max-binding proteins include Myc, Mad, Mxi1, Mad3 and Mad4.[11–14)] Only Myc:Max activates transcription and promotes proliferation, whereas Mad:Max, Mxi1:Max, Mad3:Max and Mad4:Max in addition to ROX/Mnt:Max repress transcription. With the aim of antagonizing Myc, an adenovirus harboring Mad has been constructed for gene therapy and shown to possess inhibitory activity on the proliferation and tumorigenicity of human astrocytomas.[15)] It is interesting that Mxi1 was reported to have mutated in a fraction of prostate cancers with 10q24-25 deletions.[16)] As for *ROX/Mnt*, wild-type *ROX/Mnt* has been shown to suppress Myc+Ras cotransformation of primary cells, whereas *ROX/Mnt* containing a deletion of the Sin3 interaction domain (SID) cooperates with Ras in the absence of Myc to transform cells, suggesting a potential role as a tumor suppressor gene.[10)] Of particular note is that the *ROX/Mnt* gene was mapped at 17p13.3, its 3′-untranslated region coinciding exactly with the D17S379 locus.[9,17)] Thus, the *ROX/Mnt* gene was found to coincide with the commonly deleted region(s) identified by us at 17p13.3. These findings prompted us to investigate whether the *ROX/Mnt* gene may be involved in the pathogenesis of lung cancers.

Tumor samples, together with uninvolved lung tissues, were collected from 52 patients diagnosed histologically as having primary lung cancers (five cases of small cell lung cancer (SCLC), 27 of adenocarcinoma, 17 of squa-

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mous cell carcinoma, one of adenosquamous carcinoma, and two of large cell carcinoma). Three SCLC tumor samples were obtained during necropsy, while two SCLC and 47 non-SCLC tumor samples were collected during surgery. Among these 52 lung cancer specimens, allelic loss at D17S379 was observed in 20 (60.6%) of 33 informative cases, while amplification (higher than 5 fold) of members of the *myc* gene family was observed in 3 cases (c-*myc* in 2, N-*myc* in 1, L-*myc* in 0) (data not shown).

To search for and identify gross alterations in the *ROX/Mnt* gene, Southern blot analysis was carried out using *Hin*dIII and *Eco*RI-digested DNAs as described previously.[18] A cDNA probe, which covered the entire open reading frame (ORF) of the *ROX/Mnt* gene, was prepared by means of polymerase chain reaction (PCR) using the following oligonucleotide primers: SS1 (sense; 5′-CGGCGCGATGAGCATAGAGA) and SAS1 (antisense; 5′-AGCGAATTCATTGGGTGGAATGGTGGCCTT); S3 and AS4; and S5a and AS7a (Table I). No gross alterations of the *ROX/Mnt* gene were found by Southern blot analysis (data not shown). We then performed northern blot analysis using 10 μg of total RNA to search for and identify altered expression of the *ROX/Mnt*. The expression of *ROX/Mnt* transcripts 4.8 kb in size were found to be almost ubiquitous in lung cancer specimens (data not shown).

The entire ORF of *ROX/Mnt* was examined by reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis to search for subtle mutations. The ORF of 1746 bp was divided into eight fragments. Primer sequences used for the fragments are shown in Table I. The second and fifth fragments from the 5′ end of the ORF were examined by nested PCR for higher specificity in amplification (Fig. 1C and Table I). Similarly, the sixth and seventh fragments were amplified by hemi-nested PCR. Annealing temperatures for PCR amplification are also shown in Table I. RT-PCR products were electrophoretically separated on 6% non-denaturing polyacrylamide gels both at 4°C without glycerol and at room temperature in the presence of 5% glycerol. RT-PCR-SSCP analysis yielded nine of 52 primary lung cancer specimens with distinct electrophoretic mobilities in fragment 2 (Fig. 1A). To examine whether these cases indeed carried somatic mutations, repeated RT-PCR-SSCP analysis of both lung cancer specimens and the corresponding normal lungs of these nine cases was conducted, which showed that the mobility shifts were equally present both in lung cancer specimens and in the corresponding normal lungs (Fig. 1B). Three patterns of these mobility shifts were observed. The most frequent pattern was designated as allele 1 (A1 in Fig. 1B). The mobility shift pattern designated as allele 2 (A2) in Fig. 1B was detected in eight of the 52 cases and a rare pattern designated as allele 3 (A3), also seen in Fig 1B, was present in a single case (case 37). Distinctly shifted bands were excised from the RT-PCR-SSCP gels, amplified by PCR, and subsequently cloned into pBluescript SKII(–) (Stratagene) for sequence analysis. Alleles 1 and 2 were found to represent two types of sequence variations at codon 69 (CCA for allele 1 and CCG for allele 2) and at codon 109 (GCG for allele 1 and ACG for allele 2). The former nucleotide substitution did not alter the encoded amino acid (proline), but in the latter case, threonine was substituted for alanine, resulting in a significant change in polarity (Table II). By using *Nae*I and *Sac*II, which recognize these sequence variations, we were able to confirm that they were closely linked in a Japanese population (data not shown). Since there appeared to be no preference in terms of allelic loss in the lung cancers and none of the eight patients had a significant family history, they probably reflect sequence variations among normal subjects. The mobility shift observed in case 37 was

Table I. Oligonucleotide Primers Used for PCR Amplification of *ROX/Mnt*

Sense primer		Antisense primer		Annealing temparature (°C)
S1:	AGCGGATCCAGCCTGACCTGTGCCCG	AS1:	CGGGGGAGCCGGTGGAGACA	60
S2a:	ATACCCTTCCTGTGGAGGAA	AS2a:	CTGCCATTGGGTGGAATGGT	60
S2b:	CATGGAGGCGCCACCCCTGCCT	AS2b:	ATTGGGTGGAATGGTGGCCTTCG	66
S3:	AGCGGATCCTGCTCCCCTACTGCCGGACT	AS3:	GATCCCCCCGGGCCTCTTCTTC	63
S4:	AGCGGATCCTGAAGTTGGCACCAGCTGAAG	AS4:	AGCGAATTCCAGTACGTCCATCCACTGGCT	63
S5a:	AGCGGATCCTGCGGTACATCCAGTCCCTGAA	AS6:	GTGGTAGCTGTGTGGGCGATGA	60
S5b:	GGCTGGCAGAGCTCAAGCAC	AS5b:	GCAGGGTGGACTTCAGCAGC	63
S5a:	AGCGGATCCTGCGGTACATCCAGTCCCTGAA	AS6:	GTGGTAGCTGTGTGGGCGATGA	60
S6b:	TAAGCTGAGCCATCGTCCCCAG	AS6:	GTGGTAGCTGTGTGGGCGATGA	65
S7:	GACGGCTGGGGGTGGCTCCA	AS7a:	AGCGAATTCTGGCTGGAATGTGTGGAGCT	63
S7:	GACGGCTGGGGGTGGCTCCA	AS7b:	CAGGCCGGCCGTGCCGTTGA	63
S8:	GCCCACACCCTCTCGCACCA	AS8:	GGGCCTCTGAGTGGCCTCGT	63

Table II. Sequence Variations Identified within the Coding Region of *ROX/Mnt*

Allele	Sequence variations					
	Codon 69		Codon 91		Codon 109	
	Nucleotide	AA[a)]	Nucleotide	AA[a)]	Nucleotide	AA[a)]
A1	CCA	Proline	ATC	Isoleucine	GCG	Alanine
A2	CCG	Proline	ATC	Isoleucine	ACG	Threonine
A3	CCA	Proline	ATG	Methionine	GCG	Alanine

a) AA, amino acid.

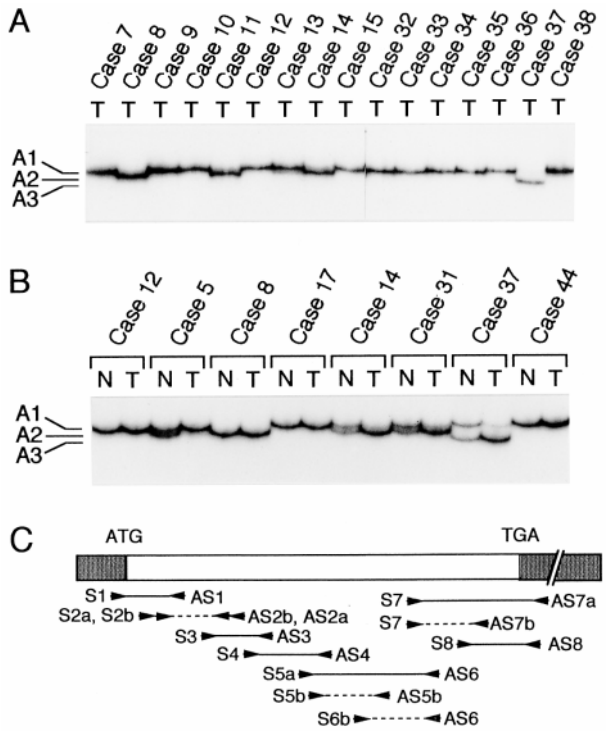

Fig. 1. RT-PCR-SSCP analysis of *ROX/Mnt*. A, Representative results of RT-PCR-SSCP analysis using S2b and AS2b oligonucleotide primers. T, lung cancer specimens. B, Distinct mobility shifts present both in lung cancer specimens (T) and in the corresponding normal lungs (N). A1, A2 and A3 represent three alleles exhibiting distinct mobility shifts. C, Schematic diagram of the strategy for RT-PCR-SSCP analysis of *ROX/Mnt* cDNA. Open box, coding region; shaded boxes, 5′ and 3′ untranslated regions; dotted lines, nested PCR or hemi-nested PCR.

shown to result from a nucleotide substitution at codon 91 (ATC to ATG). This substitution altered the encoded amino acid from isoleucine to methionine (Table II), while this rare allele was shown to be retained in the lung cancer specimens of case 37. It remains to be determined whether this rare allele reflects a germline mutation which can result in the impairment of *ROX/Mnt* function. We thus conclude that the *ROX/Mnt* gene is not a frequent target for genetic alterations in lung cancer. Petersen *et al*. recently reported that no mutation in the *Mxi1* gene was detected in the analysis of 22 SCLC cases.[19] Therefore, *myc* antagonists such as *ROX/Mnt* and *Mxi1* do not appear to be frequently involved in the pathogenesis of lung cancer, in contrast to the well recognized direct involvement of the *myc* genes themselves.

We have suggested previously that there might be three independent tumor suppressor genes on the short arm of chromosome 3, i.e., 3p14.2, 3p21.3 and 3p25.[20] Chung *et al*. also reported that allelic loss on chromosome 3p becomes more widespread during progression from preneoplastic to invasive lesions, suggesting sequential involvement of multiple tumor suppressor genes on the same chromosome arm.[21] Because it has been suggested that chromosome 17p carries multiple tumor suppressor genes, it will be interesting to study whether 17p also exhibits progressive allelic loss during the development of lung cancer. In any case, since our present findings do not support the idea that *ROX/Mnt* is a target gene, it is obvious that isolation of a candidate tumor suppressor gene at 17p13.3 will be a crucial step towards a better understanding of the molecular pathogenesis of lung cancers. Identification of the putative tumor suppressor gene at 17p13.3 should further clarify whether it may also be involved in the pathogenesis of other human malignancies such as breast cancer,[22, 23] ovarian cancer,[24] primitive neuroectodermal tumor,[25] medulloblastoma,[26] astrocytoma,[27] hepatocellular carcinoma,[28] and uterine cervical cancer.[29]

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