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TECHNICAL COMMENTS

Rates of *p16* (*MTS1*) Mutations in Primary Tumors with 9p Loss

A critical area of chromosomal loss at region 9p21-22 has been implicated in the genesis of different types of primary tumors. Initial observations defined deletions of this region in leukemia, gliomas, and cell lines derived from a wide spectrum of human tumors (*1*). In addition, linkage studies pointed to a gene in this area responsible for familial and sporadic melanoma (*2*). Subsequent analysis in human tumors demonstrated loss of heterozygosity (LOH) or homozygous deletion of this region (*3, 4*). Moreover, loss of chromosome 9 was found to occur early in the progression of several of these cancers (*5*).

Recently $p16^{INK4}$, identified as an inhibitor of activated cyclin D–cdk4 complexes (*6*), emerged as a candidate tumor suppressor gene when it was localized to 9p21 and found to be within the critical deleted region (*7, 8*). The demonstration by Skolnick and co-workers (*7*) of point mutations in melanoma cell lines prompted us to examine the role of Multiple Tumor Suppressor gene (*MTS1*) (which encodes *p16*, an inhibitor of cyclin-dependent kinase 4) in a variety of primary tumors. After excluding tumors (*4*) that demonstrated homozygous deletions of the region, we selected 75 primary tumors, all previously mapped by microsatellite markers, that displayed allelic loss of the region on chromosome 9p (Table 1). We then amplified exons 1 and 2 independently from the *p16* gene, cloned the amplified products into a plasmid vector, and sequenced p16 from pooled clones (*9*).

According to Knudson's hypothesis (*10*), if *p16* was the target of the deletion, tumors with a 9p loss should have intragenic mutations of *p16*. This argument was used in the initial observation that *p53* was the critical tumor suppressor gene on chromosome 17. Subsequently, *p53* mutations were found in the majority of carcinomas with 17p loss (*11*). In contrast, we identified only two polymorphisms and two mutations of *p16* among all 75 primary tumors. One prevalent polymorphism, a G→A transition at 436 nucleotides (codon 140), was found in four tumors and resulted in an amino acid change from alanine to threonine. The second polymorphism occurred in one tumor at 172 nucleotides (codon 52) and resulted in the same amino acid change. Both of these changes were present in germline DNA, and the codon 140 polymorphism had been described previously in one melanoma cell line (*7*). Two tumors (one bladder, one head and neck) contained mutations, a CGA→TGA (Arg→stop) at codon 50 (Fig. 1) and a GAG→TAG (GLU→stop) at codon 25, respectively. Both represented new somatic mutations, and both occurred in neoplasms of advanced stage. We tested all 75 primary tumors by comparative multiplex polymerase chain reaction (PCR) (*4*) with exons 1 and 2 of the *p16* gene and found no *p16* deletions. To further exclude small deletions extending into the *p16* gene, we then tested all bladder and kidney tumor and normal DNAs by Southern blot analysis (*12*) and found no homozygous deletions or rearrangements of *p16*.

We sequenced 97% (excluding four codons in exon 3) of the coding region of *p16* and found only two somatic mutations in 75 tumors. We excluded tumors with homozygous deletion of the region containing *p16*, and therefore it is unlikely that we amplified and sequenced normal tissue DNA. Although it is possible that unusual mutations in the promoter or noncoding regions of this gene could be involved in inactivation of *p16*, this would be unlikely as the predominant mechanism of mutation.

So, what is the role of *p16* in primary tumors? The demonstration of mutations

Table 1. Chromosome 9 loss in primary tumors. Primary neoplasms were microdissected to remove nonneoplastic tissue and were scored for allelic loss by microsatellite analysis (*4*). Tumors with partial loss (including the region 9p21-22) or monosomy of chromosome 9 are listed. Lung, nonsmall cell; bladder, transitional cell; kidney, clear cell; head and neck, squamous cell carcinomas; brain, gliomas. *p16/MTS1* was sequenced in all 75 tumors.

Tumor	9p loss	Monosomy	Total
Lung	9	6	15
Bladder	15*	10	25
Kidney	2	9	11
Head and neck	15*	0	15
Brain	4	5	9
Total	45	30	75

*Only one tumor in each of these groups contained a *p16* mutation.

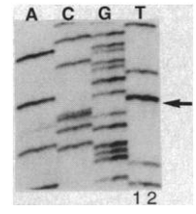

Fig. 1. Autoradiograph of a sequencing gel, demonstrating a mutation in codon 50 of *p16* (CGA→TGA) in a primary bladder tumor (arrow). The C→A transition (lane 1), was confirmed by reamplification, recloning, and resequencing (lane 2). Sequencing primers for exon 2 and other methods are described in (*9*). A, adenine; C, cytosine; G, guanine; T, thymine.

in melanoma cell lines and possibly in dysplastic nevus syndrome argues for some role of *p16* in the initiation or progression of tumors derived from melanocytes. Some data suggest that the region involved in melanoma may be separate from that in other tumor types (*13*). Between 10% and 20% of primary tumors (including bladder, brain, head and neck, and lung) contain homozygous deletions that include *p16*. Excluding these tumors, we found mutations in only a small fraction of primary tumors (with loss of heterozygosity confined to 9p) that would be expected to contain alterations of this gene.

Loss of *p16* may provide an additional growth advantage to some of these neoplasms. The incidence of homozygous deletions is smaller in primary bladder tumors (*4*) than it is in cell lines (*7*). Perhaps tumor cells with *p16* homozygous losses may have a selective growth advantage in tissue culture, as has been reported for neuroblastoma cells with N-myc amplification and other tumor cells with regard to *p53* (*14*). Our own observations suggest, however, that *p16* is not the primary target of 9p21-22 allelic loss in a large number of nonmelanoma primary tumors. Isolation of other candidate genes from this region should lead to the identification of another tumor suppressor gene (or genes) involved in the progression of these tumor types.

Paul Cairns
Li Mao
Adrian Merlo
Daniel J. Lee
Donna Schwab
Yolanda Eby
Kaori Tokino
Peter van der Riet
James E. Blaugrund
David Sidransky
Department of Otolaryngology–Head and Neck Surgery, Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine, 818 Ross Research Building, 720 Rutland Avenue, Baltimore, MD 21205–2196, USA

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9. *p16* was amplified in two segments as described (*17*), and the same amplification primers were used for each exon. UDP ends were added to each primer for rapid cloning into a CloneAmp vector (Gibco-BRL, Gaithersburg, MD). Cloned products were used to transform competent DH5-α bacteria, and pooled clones were sequenced as described [P. van der Riet *et al.*, *Cancer Res.* **54**, 25 (1994)] with the amplification primers used for sequencing.
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12. Ten micrograms of normal and tumor DNA were digested with Eco R1, separated on 1% agarose gels, and transferred by the method of Southern (DNA) blotting. The 500–base pair exon-2 and 350–base pair exon-1 PCR products from *p16* were isolated from agarose gels and then labeled by random priming [S. E. Goelz *et al.*, *Biochem. Biophys. Res. Commun.* **130**, 118 (1985)]. After hybridization with both probes, blots were washed and exposed to film as described (*10*).
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Response: The issue of *MTS1* involvement in human cancer is quantitative: Is it involved in a minority of cases, as the data of Cairns *et al.* suggest, or in a large fraction, as our analysis of tumor cell lines suggests? Resolution of this issue requires comparison of primary tumors with tumor cell lines and reliable assessment of point mutation frequency, as well as homozygous deletion frequency. Analysis of primary tumors can be challenging because of possible contamination from normal cells.

We observed homozygous deletions of the gene *MTS1* in 5 out of 15 bladder cell lines studied (*1*); the figure of 33% is thus based on a relatively small set of tumor lines and it is possible that the real frequency may be somewhat different. Also, deletion frequencies of *MTS1* might differ in primary tumors as compared with tumor cell lines. However, other explanations for the suggested disparity between frequencies in primary tumors and cell lines may be more likely. Cairns *et al.* based their figure for the frequency of homozygous deletions (11%) on analysis of primary bladder tumors that were examined with polymorphic microsatellite repeat markers flanking the *MTS1* gene called *IFNA* and *D9S171* (*2*). From physical mapping experiments in this region, we suspect that *IFNA* and *D9S171* lie at least one megabase from one another (*3*). Cairns *et al.* state that their set of tumor samples has been selected for LOH in this region, with no further homozygous deletions detected by Southern (DNA) blots or multiplex, quantitative PCR with the use of *MTS1* sequences (*2*).

Comparison of a large number of such deletions from different tumors should generate a nested set with deletions that range in size from large (removing considerable flanking DNA) to small (removing only sequences within the gene). When we analyzed homozygous deletions of 9p21 in melanoma cell lines with markers that lie some distance from *MTS1* but within *IFNA* and *D9S171*, we found a frequency of 16/86 detectable deletions (*3*). When we used *MTS1* gene sequences, the frequency was 53/86, an increase of more than threefold. This general result applied to all cancer cell lines we examined (*1*). Thus, we would expect Cairns *et al.* to see an increase in homozygous deletion frequency using *MTS1* itself as a probe compared with the frequency found using *IFNA* and *D9S171*. That they do not see an increase may indicate that homozygous deletions occur in primary tumors in an area of 9p21 that does not include *MTS1*. However, we found no evidence for other nonoverlapping deletions in our analysis of tumor cell lines. Deletion analysis of individual tumor types revealed that homozygous deletions clustered in about the same place in all of the 290 cell lines examined (Fig. 1). Fur-

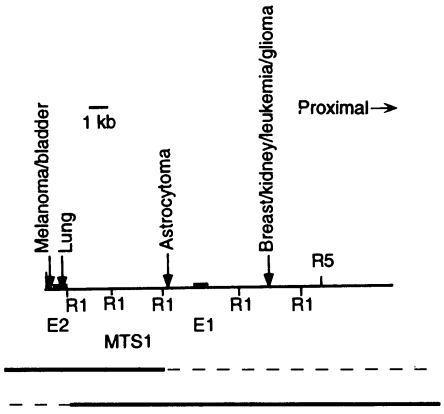

Fig. 1. Restriction map of part of the cosmid c5 showing the location of coding exons 1 and 2 (E1 and E2) from *MTS1* (*1*). The positions of the centers of homozygous deletion (defined as the most frequently deleted marker averaged over nine markers centered around *MTS1*) for different tumor cell types are shown. Only tumor cell line types that had several examples where both deletion breakpoints had been roughly determined were used in the analysis. The two lines below show the approximate extent of the most proximal deletion breakpoint (upper line) observed in the analysis of 290 tumor cell lines and the most distal breakpoint (lower line). Dashes represent lost genomic DNA.

thermore, all homozygous deletions detected among these cell lines removed or impinged on *MTS1*. An alternative explanation for the disparity in deletion frequency is that the technical difficulties of primary tumor analysis may have confounded detection of homozygous deletions.

Cairns *et al.* report a low frequency of *MTS1* somatic point mutations in primary tumors selected for LOH in 9p21 (for example, 1/15 bladder tumors). In unselected primary bladder carcinomas, we observed about this same frequency of point mutations (4), which is also approximately the same as that observed for *MTS1* in bladder cell lines. This finding suggests that *MTS1* point mutations do not occur to a significant extent in cultured bladder tumor cells but, rather, occur during tumor development in vivo. We have found *MTS1* point mutations in many other tumor cell line types (bladder, sarcoma, prostate, pancreas, and lung) and in primary tumor types (melanoma, bladder, and ovary) (4, 5). These data support the hypothesis that *MTS1* has a role in many of the tumor cell line types that suffer homozygous deletions of the region. However, in most tumor cell line types, the rate of homozygous deletion is considerably higher than the rate of point mutation. We conclude that point mutation may not be the predominant mechanism of mutation at this locus; homozygous deletion may be favored.

The results we have obtained with deletion mapping are consistent with *MTS1* being involved frequently in a wide range of tumors. However, there may be a discrepancy between the rate of *MTS1* involvement detected in bladder cancer and the rate of 9p LOH in bladder cancer. A rate of up to 80% LOH of 9p has been reported for superficial bladder carcinomas (6). Using our data, we predict a rate of about 56% (homozygous deletions plus coding sequence mutations) for *MTS1* disruption. This difference (56% as opposed to 80%), if significant, has three possible explanations: (i) a percentage of *MTS1* mutations is not being detected at present (for example, a significant number may occur outside the coding sequence); (ii) the *MTS1* gene is a codominant tumor suppressor gene, that is, loss of one copy has some effect on cell growth; and (iii) other 9p genes may contribute to tumor suppression.

Alexander Kamb
Qingyun Liu
Keith Harshman
Sean Tavtigian
Myriad Genetics, Inc.,
421 Wakara Way,
Salt Lake City, Utah 84108, USA
Carlos Cordon-Cardo
Department of Pathology,
Memorial Sloan-Kettering Cancer Center,
New York, NY 10021, USA
Mark H. Skolnick
Department of Medical Informatics,
University of Utah,
Salt Lake City, UT 84108, USA

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7. *MTS1* (*p16*^{INK4}) has been designated *CDKN2* by the HUGO Nomenclature Committee.

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