Advances in Brief

DPC4, a Candidate Tumor Suppressor Gene, Is Altered Infrequently in Head and Neck Squamous Cell Carcinoma[1]

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Abstract

DPC4, a candidate tumor suppressor gene, was identified recently at chromosome 18q21.1. Frequent homozygous deletion or mutations of the gene were observed in pancreatic carcinomas. To investigate the role of this gene in head and neck squamous cell carcinomas (HNSCCs), we examined 16 HNSCC cell lines from 11 patients and 20 primary HNSCCs for alterations of the gene by sequencing all 11 exons of the gene. Fourteen cell lines from 10 patients showed monomers at marker *D18s46* (18q21.1). Full-length cDNA was detectable in all cell lines by reverse transcription-PCR. A nonsense mutation was identified at codon 526 (GAA to TAA, glutamine to termination) in two cell lines (UMSCC22A and UMSCC22B) derived from the primary tumor and lymph node metastasis of the same patient. Loss of heterozygosity was found in 7 of 15 (47%) informative primary tumors at *D18s46*, whereas only one polymorphism was observed in both tumor and normal tissues from the same patient (at codon 525; ATT to GTT, isoleucine to valine). Our data indicate that although *DPC4* is altered infrequently in HNSCC, it may play some role in the tumorigenesis of a small subset of HNSCCs.*

Introduction

HNSCC[3] is one of the common human cancers, with an incidence of 500,000 cases per year worldwide (1). Recent studies have demonstrated that multiple genetic alterations are involved in the tumorigenic process in HNSCC (2–6). Among these alterations, frequent LOH at many chromosome loci are the most striking, indicating that multiple tumor suppressor genes play important roles in HNSCC tumorigenesis (3, 4).

Recently, Hahn *et al.* (7) reported the identification of a candidate tumor suppressor gene, *DPC4* (for deleted in pancreatic carcinoma), at chromosome 18q21.1 by positional cloning. Frequent homozygous deletions and inactivating mutations accompanying LOH have been found in this region in pancreatic carcinomas, suggesting that the gene may have a tumor suppressor function (7, 8). Previously, loss of 18q was observed frequently in HNSCC by cytogenetic analysis (9, 10), and LOH at 18q was found in about 50% of HNSCCs by microsatellite analysis, including the site of *DCC* (a candidate suppressor gene for colorectal cancer; Refs. 11 and 12). However, no direct evidence proves that *DCC* is the target gene at 18q. To determine whether *DPC4* is the target gene and plays a role in the tumorigenesis of HNSCC, we examined 16 HNSCC cell lines and 20 primary HNSCCs for LOH status at 18q21 and coding sequences of *DPC4*.

Materials and Methods

Cell Lines and Primary Tumors. HNSCC cell lines used in this study included UMSCC10A, UMSCC10B, UMSCC11A, UMSCC11B, UMSCC14A, UMSCC14B, UMSCC17A, UMSCC17B, UMSCC22A, UMSCC22B, and UMSCC38 (gifts from Dr. Thomas E. Carey); 183A, MAD886Ln, and 1483 (gifts from Dr. Peter G. Sacks); TR146 (a gift from Dr. Thomas H. Rupniak); and SqCC/Y1 (a gift from Dr. Michael Reiss). These lines were derived from 11 patients. Cell line pairs designated A and B with the same UMSCC number were derived from the same patient (13). In addition, we used 20 fresh primary tumors from 20 patients who underwent surgery for histologically confirmed HNSCC at The University of Texas M. D. Anderson Cancer Center, along with corresponding blood lymphocytes. Those specimens were immediately stored at −80°C until analyzed.

Preparation of Samples and DNA Extraction. Frozen tumor specimens were cut by using a cryostat. A 4-μm section was made from each tumor and stained with H&E to determine the percentage of tumor cells on the slide (only the tumors with more than 50% of tumor cells were used), and then 20 to 40 12-μm sections were cut. Another 4-μm section was made after the final 12-μm section and was stained to count the percentage of tumor cells. This information was recorded and incorporated into interpretation of the molecular analysis. Tissues were digested in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 1% SDS-proteinase K and incubated at 42°C for 12 to 24 h. DNA extraction from tissues, cell lines, and blood lymphocytes followed the method described previously (14).

LOH Analysis. *D18s46* (Research Genetics, Huntsville, AL), a microsatellite marker at 18q21.1, has been used for LOH analysis. For PCR amplification, one of the primers was end-labeled with [γ-^{32}p]ATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Berverly, MA). PCR reactions were carried out in a 12.5-μl volume, as described previously (14). The PCR products were separated on a 6% polyacrylamide-urea-formamide gel, which was then autoradiographed. LOH was defined as a >50% reduction of the intensity by visual inspection in either of the two alleles as compared to those in normal control panels.

RNA Extraction and RT-PCR. Total RNA was extracted from cell lines according to a method reported previously (15). For RT-PCR, 2 μg total RNA were subjected to reverse transcription with random hexamer, deoxynucleotide triphosphates, and 200 units Superscript II RNase H⁻ reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in a 20-μl reaction volume. PCR amplification was performed by using primers P1 (5′-AAGGATCAAAATT-GCTTCAG-3′) and P2 (5′-ATTGTATTTTGTAGTCCACC-3′) for the *DPC4* cDNA fragment. The product was run on a 1% agarose gel and visualized by ethidium bromide staining.

Sequence Analysis. Individual exons were PCR amplified separately, except exons 2 and 3 and exons 5 and 6, which were coamplified. The primers used for amplifying those exons are as follows: exon 1, P1 and DPC4AS1 (5′-AGAGTATGTGAAGAGATGGAG-3′); exons 2 and 3, DPC4S2 (5′-TG-TATGACATGGCCAAGTTAG-3′) and DPC4AS3 (5′-GCCCCTAACCT-CAAAATCTAC-3′); exon 4, DPC4S4 (5′-TTTTGCTGGTAAAGTAG-TATGC-3′) and DPC4AS4 (5′-CTATGA AAGATAGTACAGTTAC-3′); exons 5 and 6, DPC4S5 (5′-CATCTTTATAGTTGTGCATTATC-3′) and DPC4AS6 (5′-TAATGAAACAAAATCACAGGATG-3′); exon 7, DPC4S7 (5′-TGAAAG TTTTAGCATTAGACAAC-3′) and DPC4AS7 (5′-TGTACT-CATCTGAGAAGTGAC-3′); exon 8, DPC4S8 (5′-TGTTTTGGGTGCAT-TACATTTC-3′) and DPC4AS8 (5′-CAATTTTTTAAA GTAACTATCTGA-
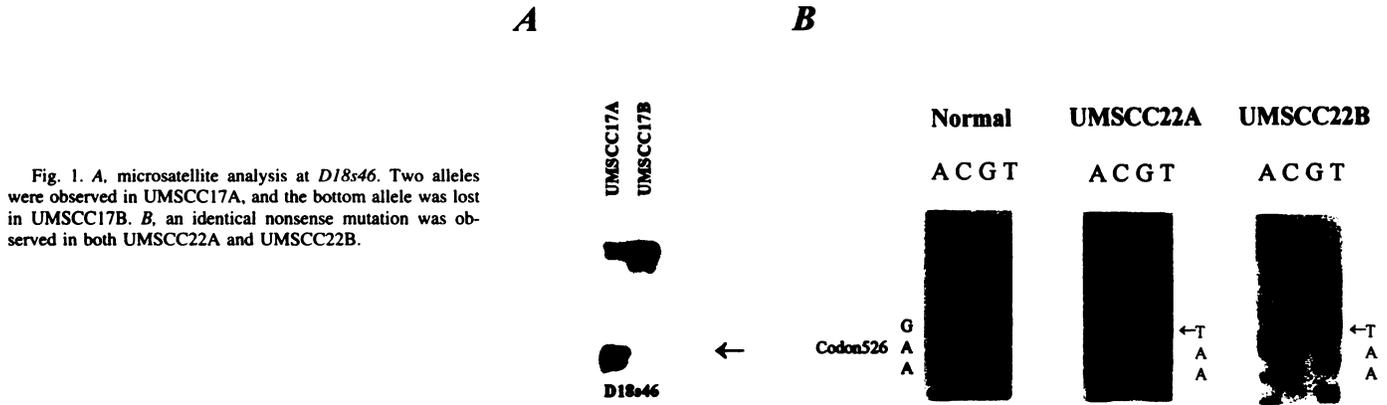
A   **B**

Fig. 1. *A*, microsatellite analysis at *D18s46*. Two alleles were observed in UMSCC17A, and the bottom allele was lost in UMSCC17B. *B*, an identical nonsense mutation was observed in both UMSCC22A and UMSCC22B.

3'); exon 9, DPC4S9 (5'-TATTAAGCATGCTATACAATCTG-3') and DPC4AS9 (5'-CTTCCACCCAGATTTCAATTC-3'); exon 10, DPC4S10 (5'-AGGCATTGG TTTTTAATGTATG-3') and DPC4AS10 (5'-CTGCTCAAA-GAAACTAATCAAC-3'); and exon 11, DPC4S11 (5'-CCAAAAGTGTG-CAGCTTGTTG-3') and P2. Sequencing primers were the same as the PCR primers except that used for exon 6, for which the sequencing primer was 5'-TGGATTTACTGGTCAGCC-3'. Amplified DNA and sequencing primers labeled with [γ-^{32}P]ATP or [γ-^{33}P]ATP as described above were subjected to PCR amplification for 30 cycles using the AmpliCycle sequencing kit (Perkin-Elmer, Branchburg, NJ), according to the manufacturer's protocol. Each amplified product (3 μl) was run on a 6% Long-Range gel (FMC BioProducts, Rockland, ME) and exposed to film. Each mutation identified has been confirmed by a repeat sequence analysis.

Results and Discussion

Fourteen of 16 HNSCC cell lines from 10 patients showed monomers at the marker *D18s46*, which locates as centromeric to *DCC* by radiation hybrid mapping (16). All cell lines expressed full-length DPC4 mRNA by RT-PCR examination, indicating that there is no homozygous deletion at the *DPC4* locus in the cell lines tested. It is interesting that cell line UMSCC17A, from a persistent cancer of the larynx, showed a heterozygous pattern at marker *D18s46*, whereas UMSCC17B, derived from an invasive tumor of the neck of the same patient after radiation therapy to the primary tumor (17), exhibited the LOH pattern (Fig. 1A). Because the two lines were derived from the

same patient, this may indicate the presence of a putative tumor suppressor gene at the region that involves the late tumor progression process; alternatively, the loss at 18q in UMSCC17B is the result of *in vitro* culture.

Because the polymorphism frequency of the marker *D18s46* is 80% (18), we estimated that our cell lines from eight individuals should have had LOH at this region, which encouraged us to examine the *DPC4* gene for a possible mutation on the remaining allele. We sequenced all 11 exons of the gene and identified an identical nonsense mutation at codon 526 (GAA to TAA) in UMSCC22A (derived from a primary tumor of the hypopharynx) and UMSCC22B (derived from a lymph node metastasis), which derived from the same patient (Fig. 1B); these mutations caused a glutamine-to-termination change that made a truncated protein. It has been noted that there is no remaining signal at the normal sequence site (Fig. 1B). These results suggest that the mutation at the *DPC4* gene in these cell lines should have occurred *in vivo* and that the *DPC4* gene has been completely inactivated by a nonsense mutation on one allele and a deletion at another allele in these cell lines.

To further investigate the potential role of *DPC4* *in vivo*, we examined 20 primary HNSCCs in this study. LOH was observed in 7 of 15 (47%) informative tumors at the marker *D18s46* (Fig. 2A). Sequencing all 11 exons of the gene identified a missense mutation at codon 525 (ATT to GTT) in a primary tumor, which caused an
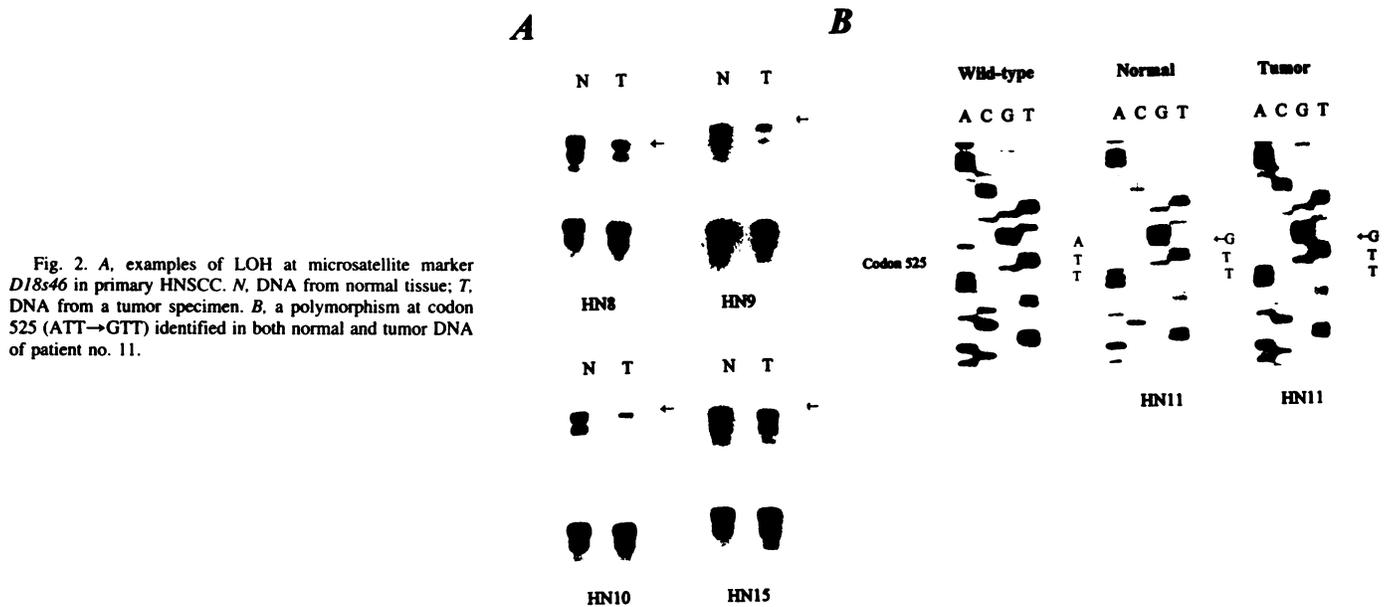
A   **B**

Fig. 2. *A*, examples of LOH at microsatellite marker *D18s46* in primary HNSCC. *N*, DNA from normal tissue; *T*, DNA from a tumor specimen. *B*, a polymorphism at codon 525 (ATT→GTT) identified in both normal and tumor DNA of patient no. 11.

isoleucine-to-valine substitution (Fig. 2*B*). However, the identical nucleotide substitution was also observed in the normal tissue of the same patient, indicating that this alteration may represent a polymorphism (Fig. 2*B*). Since LOH at *D18s46* was also observed in this case, whether this nucleotide substitution has a biological effect in tumorigenesis is unclear.

DPC4 has a sequence similarity to the *Drosophila melanogaster* Mothers against dpp (Mad) protein and sma-2, sma-3, and sma-4. Its function may be similar to that of members of the transforming growth factor β superfamily (7). The greatest similarity to these homologues was seen in exons 1, 2, and 11 of *DPC4* (7). Both nucleotide alterations we identified are located in exon 11 of the gene, inside a highly conserved area, indicating that this region may be important in its tumor suppressor function. In fact, all six mutations at the gene reported a previously altered COOH-terminal part of the protein (7), further supporting the idea that the 3' end of the gene may be more important in its tumor suppressor function. Because the inactivating mutation in the *DPC4* gene was observed in only one HNSCC patient and no homozygous deletion was identified in this study, the high frequency of LOH at the region may not be explained by *DPC4*. Our data suggest that *DPC4* is altered infrequently in HNSCC and may play some role in the tumorigenesis of a small subset of HNSCCs. The frequent LOH observed at this region may be associated with other tumor suppressor gene(s).

Acknowledgments

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