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Chromosome 13q Deletion Mapping in Head and Neck Squamous Cell Carcinomas: Identification of Two Distinct Regions of Preferential Loss[1]

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ABSTRACT

Head and neck squamous cell carcinomas show frequent cytogenetic alterations involving the long arm of chromosome 13. To define the extent of 13q deletions and to identify the minimal areas of chromosome loss, 48 primary squamous cell carcinomas of the head and neck were analyzed for loss of heterozygosity using 11 different polymorphic loci. About 67% of the tumors displayed loss of genetic material at 13q. Most of the cases showed loss of the entire long arm of the chromosome. However, the presence of partial deletions in 10 cases provided evidence of the existence of two preferential sites of chromosome loss at 13q32–ter and 13q14.2–q14.3. The colocalization of the 13q14 minimal region of deletion with the retinoblastoma (*RB*) gene, which has been proposed as an oncosuppressor in diverse tumor types, prompted us to verify the involvement of this gene in the development of head and neck cancer. No significant variation in RB protein or *RB* mRNA expression was detected, thus excluding a role for such a gene in the genesis of this type of tumor. Taken together, our data suggest the existence of two new tumor suppressor genes (one close to and one distal to *RB*), which play a role in the development and/or progression of head and neck squamous cell carcinomas.

INTRODUCTION

HNSCC[3] is a relatively common tumor, which accounts for about 15% of all the neoplasms in the Western world, with a peak of frequency in the North of Italy and France. HNSCCs are usually associated with tobacco and alcohol abuse, but considerable evidence suggests that environmental factors may contribute to the increased frequency of this type of tumor in the developed countries (1–3). The growing epidemiological relevance of HNSCC, together with the limited progress in the surgery as well as in the radiotherapy or chemotherapy of this neoplasm, emphasizes the need for a better understanding of HNSCC biology, which may result in more appropriate clinical approaches. In fact, despite the great emphasis on early diagnosis and the efforts to improve the therapeutic management, HNSCC remains one of the most disfiguring tumors, which heavily affects both duration and quality of life of the patients (4).

Recent cytogenetic and allelotype studies indicate that deletions as well as rearrangements of the long arm of chromosome 13 (13q) are a frequent finding in HNSCC (5–11). Since deletion is one of the major mechanisms by which tumor suppressor genes are knocked out, the frequency of chromosome 13 alterations in HNSCC suggests that one or more oncosuppressors involved in the growth control of the aerodigestive epithelium are located at 13q.

To evaluate the role of chromosome 13 deletions in HNSCC development and to define the precise localization of the putative tumor suppressor genes involved in tumor development, we investigated 48 cases of HNSCC using 11 different polymorphic loci. The analysis showed a remarkably high frequency of chromosome 13q allelic losses (LOH, 67%) and allowed the identification of two putative minimal regions of deletion, tentatively mapped telomeric to *D13S128* (13q32–ter) and at 13q14.2–q14.3 band. The colocalization of the minimal area of loss in 13q14 with the *RB* gene prompted us to verify the involvement of this gene in HNSCC development. None of the cases studied showed any considerable reduction in RB protein expression or any significant transcriptional alteration, irrespective of the presence of deletions at 13q14, thus excluding a role for this gene in the genesis of HNSCC. Taken together, our data suggest the existence of two new tumor suppressor genes (one close to and one distal to *RB*), which may play a role in the genesis and/or progression of HNSCC.

MATERIALS AND METHODS

Samples, DNA, and RNA Extraction. Matched tumors and corresponding normal mucosa were obtained from 48 patients with primary HNSCC. No patient had been treated with chemotherapy or radiotherapy before surgery. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C until extraction of DNA and RNA.

Frozen tissues were powdered with a cell dismembrator. Genomic DNA extraction was performed with a DNA extractor according to the manufacturer's instructions (Applied Biosystem, Foster City, CA). RNA extraction was according to Chomczynsky and Sacchi (12).

RFLP and Microsatellite Polymorphism Analysis. LOH analysis at 13q was performed using a PCR-based approach. All the oligonucleotides used for PCR amplifications were synthesized using an Applied Biosystem synthesizer (Foster City, CA). Polymorphic loci, chromosome location, and amplification primers were as follows: *D13S141* (13q11–12.1): 5′-GTCCTCCCGGCCTAGTCTTA-3′, 5′-ACCACGGAGCAAAGAACAGA-3′ (13); *FLT-1* (13q12): 5′-TTTGGCCGACAGTGGTGTAA-3′, 5′-AGGACCAAACCATGTCTGTC-3′ (14); *D13S139* (13q12.3–14.3): 5′-GTCCTCCCGGCCTAGTCTTA-3′, 5′-ACCACGGAGCAAAGAACAGA-3′ (13); *RB-Bam*HI-RFLP (13q14.2): 5′-CAGGACAGCGGCCCGGAG-3′, 5′-CTGCAGACGCTCCGCCGT-3′ (15); *RB1.20* VNTR (13q14.2): 5′-GTATGAACTCATGAGACAGGCAT-3′, 5′-AATTAACAAGGTGTGGTGGTACACG-3′ (16); *D13S165* (13q14.3): 5′-GTTTCGCCAAGCCTGTT-3′, 5′-GTTGACAATAAAATACGCCACA-3′ (17); *D13S272* (13q14.3): 5′-ATACAGACTTCCCAGTGGCT-3′, 5′-AGCTATTAAAGTTCCCTGGATAAAT-3′ (17); *D13S268* (13q14.3): 5′-TCTCTCAAGCACAAACGC-3′, 5′-CATGAAAGGCACTCACAGA-3′ (17); *D13S131* (13q14.3–22):5′-TACCAACTCTCAGCATTCCCA-3′, 5′-GGATAGTTCTCCCAGTGCTTTTT-3′ (14); *D13S128* (13q32–34): 5′-GGACACAACTGACTTCATAATGAAT-3′, 5′-TTCACCGAAGTGGAAGAGAGAT-3′ (13); and *D13S129* (13q32–34): 5′-ACCAAAACTGCACAAATGCCA-3′, 5′-TGTGTTGCTATAGTTTCCTTATTATAGAAT-3′ (13).

Microsatellite polymorphic loci were tested for LOH as described previously (18). In detail, PCRs were carried out in 10 μl of reaction volume with 10 pmol of each primer, 50 ng of genomic DNA, standard PCR buffer, 200 μM each of dATP, dGTP, and dTTP, 50 μM dCTP, 0.3 μCi [^{32}P]dCTP (3000 Ci/mmol; Amersham, Aylesbury, United Kingdom), and 0.25 units *Taq* DNA polymerase (Promega, Madison, WI). PCR conditions were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C (*D13S* and *FLT-1* polymorphisms) or 58°C (*RB1.20* VNTR) for 30 s, and elongation at 72°C for

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; RB, retinoblastoma; pRB, retinoblastoma protein; LOH, loss of heterozygosity; PDH-1, β subunit of the pyruvate dehydrogenase gene.

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30 s. After amplification, 2 μl of the reaction were mixed 1:1 with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat denatured, and then electrophoresed on a 6% polyacrylamide/7 M urea gel. After electrophoresis, gels were vacuum dried and autoradiographed. Samples were considered informative for alleles separated by more than 4 bp. Ascertainment of LOH was performed by densitometric analysis comparing the ratio between the signal intensity of the two alleles in the neoplastic DNA with the same ratio in the corresponding normal DNA. LOH was scored for reduction in the allelic ratio >50%.

For RFLP of the *RB* locus (*RB-Bam*HI-RFLP), PCRs were performed in 50 μl of reaction volume as described previously (18). After PCR, 25 μl of reaction were digested with 10 units of the appropriate restriction enzyme and then electrophoresed in 4% agarose gel.

RT-PCR. Single-strand DNA was synthesized by oligodeoxythymidylate priming from 1 μg of total RNA using 25 units of AMV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 μl, according to the manufacturer's instructions. After heat inactivation of the reverse transcriptase enzyme, the product was diluted 1:5. A 2-μl aliquot of cDNA was used directly for each PCR. Specific amplifications of two portions of the *RB* and β subunit of the *PDH-1* gene were performed using the primers listed below. The PCR reaction mixture (50-μl final volume) consisted of 2 μl of template cDNA, 200 μM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of each primer, 1 unit of *Taq* DNA polymerase (Promega), and the corresponding reaction buffer.

The mRNA of *RB* was amplified in two overlapping fragments covering the entire coding portion of the gene with the following primers: *RB* set A, bases 220–1830, codon 1–530: 5′-GCACGAGTTGATCCTAGATGAG3′, 5′-AATGGCTTCTGGGTCTGGAAG-3′; and *RB* set B, bases 1775–2712, codon 512–816: 5′-TACTGCAAATGCAGAGACACA-3′, 5′-TCCACCAAGGTCCTGAGATCC-3′.

PCR consisted of 25 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. In semiquantitative RT-PCR, the amplification of the β subunit of the *PDH-1* gene, the transcription of which is almost constant throughout the cell cycle (19), served as an indicator of the relative amounts of cDNA template used for each reaction. The primers were 5′-GGTATGGATGAGGAGCCTGG-3′ and 5′-CTTCCACAGCCCTCGACTAA-3′ (19); PCR consisted of 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The PCR products were separated on 1% (*RB*) or 4% (*PDH-1*) agarose gels.

Immunohistochemistry. pRB was evaluated on formalin-fixed, paraffin-embedded sections using the PMG-245 (Pharmingen, San Diego, CA) and the U392 (UBI, South Lake, UT) monoclonal antibodies, as described previously (20). Briefly, 4-μm sections were treated with the microwave antigen retrieval system (21) and incubated for 1 h at room temperature with the primary antibody (1:100 and 1:300, respectively). Immunostaining was completed using an automated immunostainer (Ventana 320/ES; Tucson, AZ) by the peroxidase-labeled streptavidin method. A nonspecific IgG1 mouse monoclonal antibody was used in all of the experiments as a negative antibody control. Normal tissue known to express the pRB and a pRB-negative retinoblastoma cell line (Y79) were used as controls.

RESULTS

LOH Analysis. Forty-eight cases of primary HNSCC were analyzed for LOH using 11 different polymorphic loci extending from the 13q11 to 13q34 bands. Results are summarized in Fig. 1. All the patients analyzed showed constitutive heterozygosity for at least three polymorphisms. Thirty-two of the 48 cases analyzed (67%) showed LOH for at least one of the polymorphisms tested. Twenty-two cases (46%) carried a wide deletion involving almost the entire long arm of chromosome 13.

In 10 cases, the presence of a partial or interstitial deletion provided information on the minimal areas of preferential chromosome loss. The existence of two discrete regions of deletion was suggested by the comparative analysis of these 10 cases (Figs. 1 and 2). In detail, cases HN6 and HN7 carried a deletion that involved the loci centromeric to *D13S128* (13q32–34), whereas case HN12 showed a deletion involving the loci centromeric to *D13S131* (13q14.3–22). The extension of this area of deletion was progressively restricted to the chromosomal region centromeric to *D13S165* (13q14.3) by the analysis of cases HN10 to HN11 of Fig. 1. The analysis of cases HN177 and TC-35 allowed the identification of this area as telomeric to the *D13S139* locus (13q12.3–14.3) and specifically involving the genetic material flanked by the *D13S139* and *D13S165* loci, as evident by the comparative analysis of cases HN11 to TC-35. A second area of deletion, presumably telomeric to *D13S128* (13q32–34), was found by the analysis of cases HN14, HN34, HN169, and possibly HN6, HN10, and HN12, although a homozygous deletion at locus *D13S128* cannot be excluded in the last three cases.

Immunohistochemical Analysis of pRB. To evaluate the involvement of the *RB* gene as a specific target of the 13q deletions, 18 cases showing LOH involving the 13q14 band and 3 cases with no evident deletion at this region were analyzed for RB protein expression (Fig. 1). A normal staining pattern was observed in all the cases analyzed. pRB nuclear immunoreactivity was observed in the majority (>70%) of neoplastic cells, as well as in the adjacent normal and dysplastic epithelium, when present, and in the stromal cells (Fig. 3). Similar results were obtained with both the antibodies. No staining was present in negative controls and in the pRB-negative retinoblastoma Y79 cell line.

RT-PCR Analysis of RB. Semiquantitative RT-PCR analysis was performed to verify the presence of *RB* transcriptional abnormalities. We sought to amplify the coding portion of the gene into two overlapping fragments of about 1 kb each to magnify the sensitivity of the analysis. In fact, because of the large dimension of the *RB* transcript, Northern blot analysis may miss some relatively small defects, such as small deletions or insertions. According to immunohistochemical data, no significant qualitative or quantitative variations in *RB* mRNA were observed for the 20 cases analyzed (HN5, 7, 10, 16, 18, 20, 22, 28, 30, 32, 24, 38, 57, 139, 169, 170, 177, 178, 182, and 191; data not shown).

DISCUSSION

Cytogenetic studies have pointed out that a considerable fraction of HNSCCs and derived cell lines show aberration at chromosome 13, including loss of the whole chromosome (6–8), small deletions (6–8, 11), as well as formation of 13q isochromosome (22). All these data suggest that the 13q genetic region harbors one or more genes that play a key role in HNSCC development. To corroborate in primary tumors the high occurrence of these chromosomal alterations reported *in vitro* and to define the minimal areas of deletion, we analyzed 48 primary HNSCCs for LOH by microsatellite and RFLP analysis.

The study showed that the loss of the long arm is a frequent finding in HNSCC (67% of the cases analyzed). In agreement with cytogenetic data, most of the tumors lost the whole long arm of chromosome 13, but the presence of partial deletions in 10 cases allowed the identification of two discrete areas of preferential chromosome loss. A first area of deletion was identified to map at the 13q14.2–14.3 band, telomeric to the *RB* locus. In particular, the comparative analysis of cases HN34 and HN11 with cases HN177 and TC-35 precisely localized this preferential area of chromosomal loss as flanked by the *D13S139* and *D13S165* (13q14.3) loci. This region co-maps with the minimal common area of deletion described in two cases of oral cancer by karyotypic analysis (7). Moreover, 5 of 10 cell lines analyzed by Cowan *et al.* (6) showed loss of the genomic portion extending from the centromere to 13q14.3. Taken together, these data strongly support the existence in 13q14.2–14.3 of a tumor suppressor gene specifically implicated in HNSCC.

To evaluate the involvement of the *RB* gene as the specific target of

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CASE	S141	FLT-1	S139	RB	S165	S272	S268	S131	S128	S129
	q11-12.1	q12	q12.3-14.3	q14.2	q14.3	q14.3	q14.3	q14.3-22	q32-34	q32-34
TC-31*	≡	----	■	■	----	----	----	■	■	----
TC-34*	■	----	■	■	----	----	----	■	■	----
TC-43*	≡	----	≡	■	----	----	----	■	■	----
TC-47	■	----	≡	■	----	----	----	■	≡	≡
HN2	■	----	■	■	----	----	----	≡	≡	■
HN3*	■	----	≡	■	----	----	----	■	■	----
HN4*	■	----	≡	■	----	----	----	■	■	----
HN15*	■	----	■	■	----	----	----	■	■	----
HN19	■	----	≡	■	----	----	----	■	■	----
HN20*	■	----	■	■	----	----	----	■	≡	■
HN22	■	----	■	■	----	----	----	■	■	----
HN26	■	----	■	■	----	----	----	■	≡	■
HN45*	■	----	■	■	----	----	----	≡	■	----
HN30	■	----	■	■	----	≡	----	■	≡	≡
HN5	■	----	■	≡	■	----	----	■	≡	■
HN68	≡	≡	■	≡	■	----	----	■	■	■
HN16	≡	----	≡	■	■	----	≡	■	■	----
HN17*	■	----	≡	■	■	----	≡	■	■	■
HN139	≡	≡	■	■	■	■	----	■	■	≡
HN170	----	----	≡	■	≡	■	≡	■	----	≡
HN178	----	----	■	■	■	≡	≡	■	----	≡
HN191	----	----	≡	■	■	≡	■	≡	----	■
HN7*	≡	≡	■	≡	≡	■	≡	■		≡
HN6*	■	■	≡	■	■	■	■	≡		■
HN12*	■	≡	■	■	≡	≡	≡		≡	■
HN10*	≡	----	■	■	■			≡	■	≡
HN14*	≡	■	■	■	≡				≡	■
HN34*	■	■	≡	■				≡		■
HN11*	≡	≡	≡	■			≡	≡	≡	≡
HN177	----	----		■	≡	≡	≡	≡	----	≡
TC-35*				■	≡					≡
HN169	----	----			≡	≡	■	≡	----	■
HN158	≡	≡	≡			≡				
HN18	≡	≡	≡			≡				≡
HN56		≡	≡	≡	≡					
HN57	≡	≡			≡				≡	
HN182	----	----	≡	≡					--------	
TC-38*	≡	----			≡		----			≡
HN47		----			≡	≡	----			
TC-36		----			≡	≡	----			
HN27		----			≡	≡	----			≡
HN32		----			≡		----			
HN38		----					----			≡
HN28		----			≡		----			
TC-37*		----			≡		----			≡
TC-40		----	≡			≡	----		≡	≡
HN40*	≡	----					----		≡	≡
HN102	≡	≡	≡		≡	≡	----			≡

Fig. 1. 13q deletion mapping in HNSCC. *Black box*, LOH; ----, not informative; *white box*, no deletion; *lined box*, not done; *, cases analyzed by immunohistochemistry for pRB expression.

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such a deletion, we performed immunohistochemical analysis of the pRB expression in a series of cases showing 13q14 deletion. Since most of the mutations described for the *RB* gene consist in nonsense mutations or interstitial deletions that lead to the abolishment of pRB synthesis, immunohistochemistry provides a sensitive indication of *RB* involvement (18, 23–25). All the cases tested showed a normal pRB immunostaining pattern, which correlated with a normal transcriptional activity of the gene.

The retention of pRB synthesis and the absence of remarkable qualitative or quantitative alterations of *RB* transcript strongly suggest that *RB* is not the target of 13q14 deletion in HNSCC. The retention of an intact *RB* gene in HNSCC is in line with the recent observation of a mutual exclusivity between *RB* functional inactivation and cyclin D1 overexpression (26). Overexpression of cyclin D1 has been described in a large fraction of HNSCC (27) and is considered a prominent event in the development of this tumor type. Since cyclin D1 expression is strictly dependent on the presence of a functional RB protein, being regulated by it (28), this further supports the notion that the target of chromosome 13q14 deletion in HNSCC is a gene other than *RB*.

Our results are in agreement with the data recently published by Yoo *et al.* (10), who report the frequent deletion of a region broadly mapped to 13q14.1–22 in a series of advanced and recurrent HNSCCs. Because of the retention of normal RB staining pattern, Yoo *et al.* (10) hypothesized a role for the *Brush1* gene. This has been recently indicated as a putative oncosuppressor involved in the 13q deletions in breast cancer. In particular, the *Brush1* mRNA expression has been described to be dramatically reduced or abolished in most of the breast cancer cell lines carrying 13q loss (29). Nevertheless, *Brush1* has been mapped centromeric (13q12) to the hot spot of deletion identified in our study, and consistently, our preliminary data[4] suggest no significant variation in *Brush1* expression levels in tumors compared with normal mucosa, indicating that another gene, still unidentified, is actually the target of the 13q14.2–14.3 deletions in HNSCC.

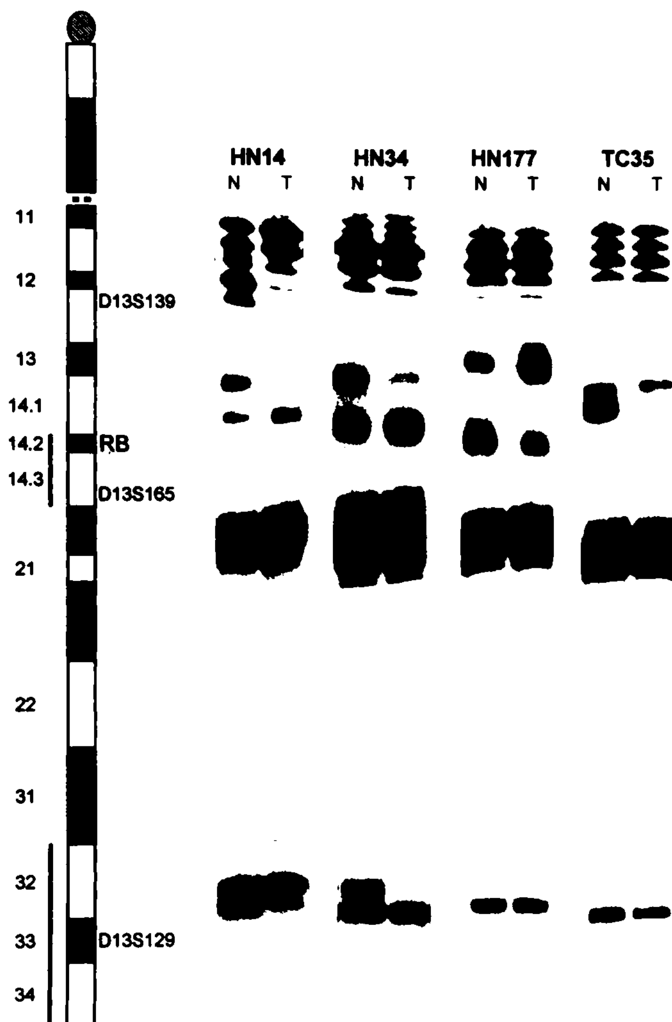

Fig. 2. LOH analysis of four representative HNSCCs. *N* and *T*, DNA samples isolated from normal mucosa and tumoral tissue, respectively. *Left*, some of the polymorphic loci tested and their approximate chromosomal location. *Top*, cases considered. The *bars* on the *left* of the chromosome indicate the two preferential areas of allelic loss (13q14.2–14.3 and 13q32–ter) identified in HNSCC.

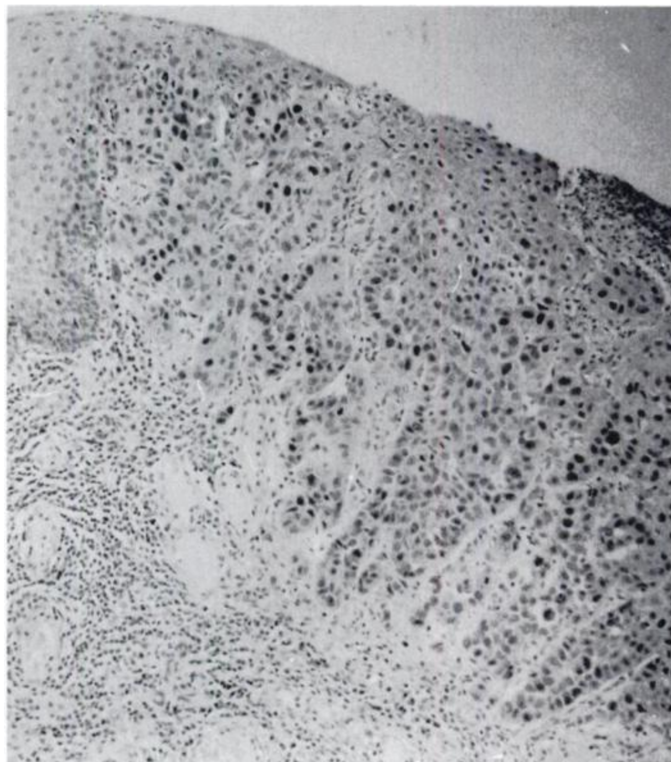

Fig. 3. pRB immunohistochemistry in HNSCC. Strong nuclear immunostaining with the monoclonal antibody PMG-245 in the majority of neoplastic cells of case HN11, despite the presence of a deletion involving the *Rb* locus. ×125, immunoperoxidase with light hematoxylin counterstain.

The existence of an oncosuppressor gene close to *RB* has been suggested in different types of tumors, such as ovary carcinoma, breast carcinoma, and leukemias (30–33). In particular, a putative oncosuppressor (*DBM*) involved in B-cell chronic lymphocytic leukemia has been described to map near the locus *D13S25*, which lies 1.6 cM telomeric to the *RB* gene (30, 32). Similarly, a gene responsible for a subset of hereditary female and male breast cancers (*BRCA2*) has been mapped to a 6-cM interval on 13q12–13 (34).

A second hot spot of deletion, tentatively mapped to 13q32–ter, is a new datum that emerges from our study. This finding, which was not reported by Yoo *et al.* (10) presumably because of the low informativity of the polymorphisms used to screen this region, is in agreement with some cytogenetic studies describing occasional 13qter deletions (7, 8). Interestingly, no case showed selective loss of the telomeric portion of the chromosome. In fact, all of the cases carrying the 13q32–ter deletion also lost the genetic material at the 13q14 band. Thus, the high frequency of the whole chromosome 13q loss and the simultaneous deletion at the 13q14 and 13q32–ter portions suggest that the two putative oncosuppressors may act in cooperation, as described for the *WT1* and *WT2* loci located at 11q13 and 11q15, which are co-deleted in Wilm's tumor (35).

In summary, the deletion of the long arm of chromosome 13 is a common feature in different types of tumor. The present study supports the notion of a role for 13q deletions in HNSCC development and suggests the existence of two putative oncosuppressors, tenta-

[4] R. Maestro, unpublished results.

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tively mapped at 13q14.2–14.3 and 13q32–ter, specifically involved in head and neck tumor development.

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