ORIGINAL ARTICLE

Definition of a Tumor Suppressor Gene Locus on the Short Arm of Chromosome 3 in Squamous Cell Carcinoma of the Head and Neck by Means of Microsatellite Markers

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Background: Tumor suppressor genes are important in the development of head and neck cancer. Using microsatellite markers that map close to the region 3p24-pter, we determined the frequency of allele loss close to this site with a view to narrowing the search for a putative tumor suppressor gene involved in the development of squamous cell carcinoma of the head and neck, which may facilitate future positional cloning techniques.

Design: Laboratory-based project with tumor and normal specimens subjected to molecular genetic analysis. Tumor–normal tissue DNA pairs were analyzed for allelic imbalance and microsatellite instability on chromosome 3p in the region 3p24-pter by the polymerase chain reaction and microsatellite markers D3S1304, D3S656, D3S1252, D3S1293, THRB, and D3S1266.

Setting: Molecular genetics and oncology research laboratory.

Patients: Paired tumor-normal DNA samples were obtained from 46 patients with tumors of the head and neck.

Main Outcome Measures: Detection of loss of heterozygosity and microsatellite instability on chromosome 3 in the region 3p24-p25.1.

Results: We found loss of heterozygosity with at least one marker in 48% of informative cases and loss of heterozygosity or microsatellite instability in 57% of informative cases. The minimal region of loss was found in the region bounded by D3S656 and D3S1293.

Conclusion: A putative tumor suppressor gene in head and neck cancer lies between D3S656 and D3S1293 in the 3p25.1 region.

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TUMORS OF the head and neck are a heterogeneous group and manifest a range of diverse clinical behaviors.[1] Squamous cell carcinoma is the sixth most common malignant neoplasm in the world and accounts for 5% of malignant neoplasms in the Western world.[2] Survival rates for these patients have not improved in the past 20 years, and this fact has stimulated interest in identifying the genetic changes that lead to carcinogenesis in the head and neck.

Human carcinogenesis is known to be a multistep process, and these steps may include inactivation of tumor suppressor genes (TSGs), thus allowing tumor cell proliferation and unrestrained tumor growth.[3,4] This has been well illustrated by the model for colorectal malignant neoplasm, as described by Fearon and Vogelstein.[5] Cytogenetic analyses and loss of heterozygosity (LOH) studies in a range of malignant neoplasms have been used to identify key regions in the human genome that encode TSGs, such as the p53 gene or the "deleted in colonic carcinoma" gene.[6-8] Studies of LOH have also been used extensively to identify regions on chromosomes that may contain new TSGs.[6-8] Normally every gene has two alleles, and these can be represented as two bands on a gel. Allelic imbalance detected as loss or marked reduction (>50%) of one of these allelic bands is termed *loss of heterozygosity*.[7]

Analysis of LOH is analogous to cytogenetic analysis, with the advantage that the resolution is greater if closely spaced markers are used.[7] A number of developments in the field of molecular biology have lent themselves to major improvements in the precision of LOH analysis. For example, the application of the polymerase chain reaction (PCR) allows amplification of small amounts of DNA at least 10 000-fold.[9] In addition, we now know that there are short

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MATERIALS AND METHODS

SPECIMENS

Tumor specimens were collected from 46 patients with tumors of the head and neck who attended the Royal Liverpool (England) University Hospital. These consisted of 16 hypopharyngeal tumors, 12 oral cavity tumors, eight laryngeal tumors, six oropharyngeal squamous cell carcinomas, one skin squamous cell carcinoma, and three salivary tumors. Normal tissue samples were also obtained from the same patients for comparison. The tissue samples were frozen in liquid nitrogen and stored at −70°C. Hematoxylin-eosin staining of representative parts of the specimens demonstrated the ratio of tumor vs normal tissue. The majority of specimens used in this study contained more than 70% tumor tissue, and all contained more than 50% tumor cells.

DNA EXTRACTION

Genomic DNA was extracted from tumor and normal specimens by means of a DNA extraction kit (Nucleon 11, Scotlab, Coatbridge, Scotland) according to the manufacturer's instructions. The isolated genomic DNA samples were stored at 4°C.

PCR AND LOH ANALYSIS

Details of the microsatellite primers in the 3p24-pter region were obtained from the Genome Database, The Johns Hopkins University, Baltimore, Md; the location of these markers is shown in **Table 1**. The microsatellite markers D3S656, THRB, and D3S1252 were obtained from Isogen (Amsterdam, the Netherlands), while markers D3S1304, D3S1293 and D3S1266 were Weissenbach markers obtained from Research Genetics. The PCR reactions were performed in a 25-μL reaction volume and contained 100 ng of genomic DNA, 500-μmol/L deoxynucleoside-triphosphate, 5 pmol each of forward and reverse primers, 0.2 U of *Taq* polymerase (Advanced Biotechnologies, Baltimore, Md), and 5 μL of 10× buffer (670-mmol/L TRIS hydrochloride, pH 8.5; 166-mmol/L ammonium sulfate; 67-mmol/L magnesium chloride; 1.7-mg/mL bovine serum albumin; 100-μmol/L β-mercaptoethanol; 1% (wt/vol) Triton X-100). The reactions were denatured for 5 minutes at 95°C, and the DNA was subsequently amplified for 30 cycles of 95°C for 30 seconds, the appropriate annealing temperature (usually 55°C) for 30 seconds, and 70°C (elongation step) for 1 minute. A sample (10 μL) of the PCR product was then subjected to electrophoresis on a 10% acrylamide gel at a constant voltage of 250 V per hour for 2500 volt-hours. The acrylamide gels were then silver stained, and the DNA bands of each tumor and normal sample pair were observed and compared over a white-light box. Examples of heterozygosity and LOH are shown in **Figure 1** and **Figure 2**.

CLINICOPATHOLOGIC DATA

The LOH data for each microsatellite marker were analyzed and possible associations with tumor site, TNM stage, nodal status, tumor differentiation, history of smoking, history of alcohol intake, clinical outcome, and survival were explored.

STATISTICAL ANALYSIS

Quantitative analysis of our data was carried out with the χ^2 test or Fisher's exact test where appropriate, as well as weighted logistic regression analysis.[16] Survival curves were calculated with the Kaplan-Meier product limit estimate method.[17] Differences in survival times were analyzed by the log-rank method.[18] All analyses were carried out with SAS software.[19]

Table 1. Results of LOH in SCCHN in 3p24-pter Region*

Microsatellite Primers	Map Position on Chromosome	Allele Loss/ Informative Cases, No. (% LOH)
D3S1304	3p25-pter	2/23 (7)
D3S656	3p25.1	12/38 (32)
D3S1293	3p24-p25	9/29 (31)
D3S1252	3p24.2-pter	4/28 (14)
THRB	3p24	2/11 (18)
D3S1266	3p24	0/36 (0)

**LOH indicates loss of heterozygosity; SCCHN, squamous cell carcinoma of the head and neck.*

repetitive DNA sequences scattered throughout the human genome, and these repetitive sequences are called *microsatellites*. Primer pairs that allow PCR amplification of these microsatellites are now available as panels of chromosome markers that effectively allow us to analyze specific areas of interest on any individual chromosome for allelic imbalance. Loss of heterozygosity has been noted in almost all known solid tumors.[7] Certain chromosomes have been implicated in the malignant transformation process of more than one tumor type. For example, LOH on chromosome 3 has been noted frequently in a number of tumor types, including lung cancer[8] and renal cell carcinoma.[10] Allele loss on chromosome 3 has also been implicated in squamous cell carcinoma of the head and neck (SCCHN) in a number of studies.[11-15] In particular, frequent LOH has been noted on the short arm of chromosome 3 (3p), indicating that tumor suppressor genes with a role in the malignant transformation process of SCCHN may be located on the 3p arm.[14,15]

Latif et al[12] suggested that the region 3p13-p25 may be of particular importance in carcinogenesis, and a great deal of interest has been focused on this chromosome as a result. Previous work from our group analyzed SCCHN by means of a panel of microsatellite markers distributed throughout the long and short arms of chromosome 3.[14] The highest incidence of LOH was located to 3p24-p25, suggesting that this region might be the site of a TSG involved in this disease.[14] The aim of this study was therefore to define more precisely the area of LOH in the region 3p24-p25 with the use of new markers that map close to and within this region. The objective of this study was to refine further the area of minimal loss to the 3p24-p25 region, which may contain a putative TSG in head and neck cancer.

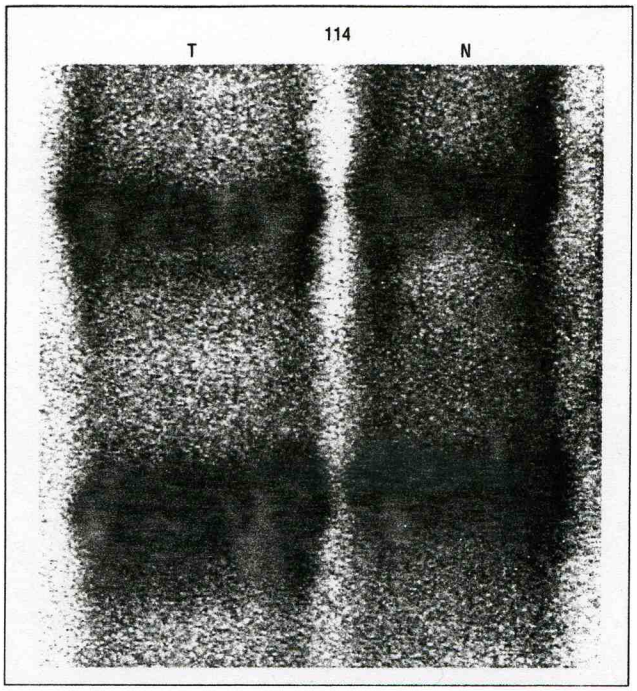

Figure 1. *Example of heterozygosity. T indicates tumor; N, normal.*

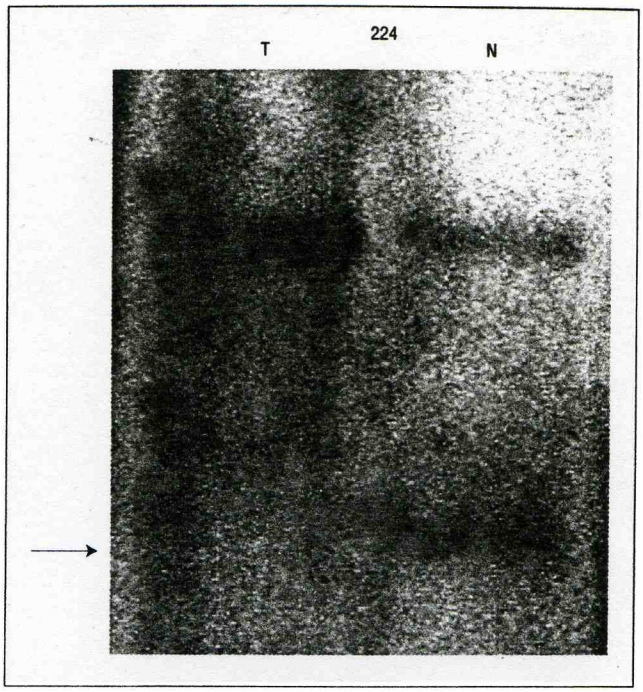

Figure 2. *Example of loss of heterozygosity. T indicates tumor; N, normal, arrow, absence of an allelic band.*

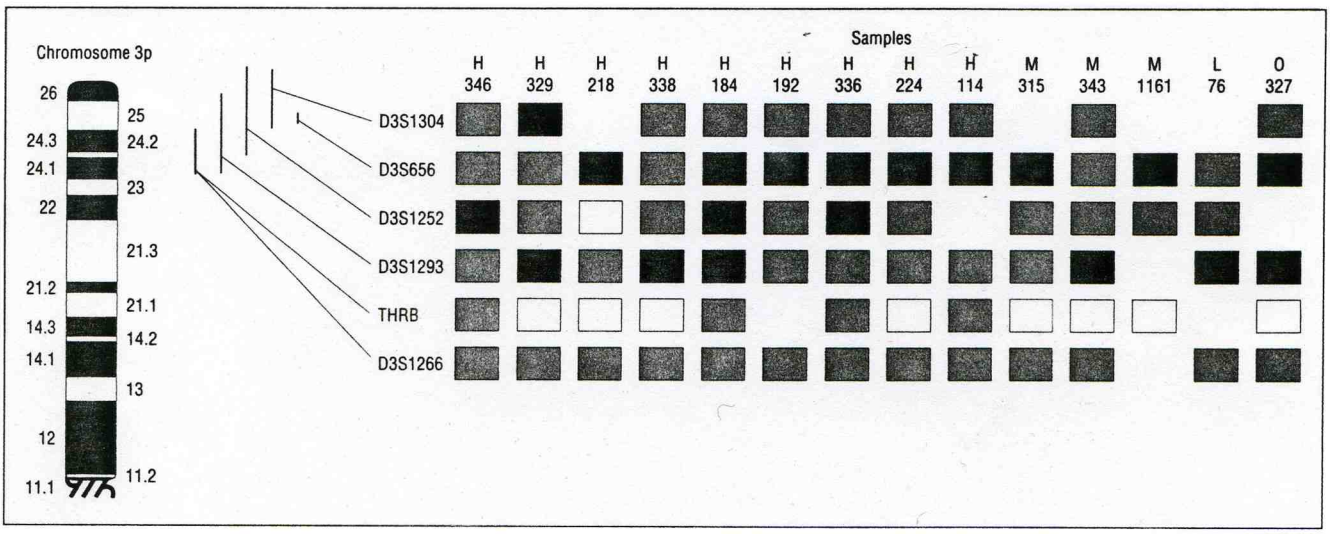

Figure 3. *Karyogram of chromosome 3p showing location of markers in the 3p24-pter region. Pattern of loss of heterozygosity in tumor samples indicates the region of minimal loss. Black squares indicate loss of heterozygosity; gray squares, heterozygous; white squares, homozygous; H, hypopharynx; M, mouth; L, larynx; and O, oropharynx.*

RESULTS

We analyzed 46 head and neck tumor specimens, including 43 SCCHN samples and three salivary tumor specimens, for LOH on chromosome 3 in the region 3p24-pter by means of six microsatellite markers that map close to this locus. The results are tabulated in Table 1. Twenty-one (48%) of 44 informative cases exhibited LOH with at least one marker. In addition, microsatellite instability (designated S) occurred in seven tumors with the use of these markers. Overall, 24 (57%) of 44 informative cases showed either LOH or microsatellite instability with one of these markers.

For further analysis of SCCHN specimens, we excluded the salivary tumors as we believed the behavior of these warranted separate analysis. Overall analysis of LOH in SCCHN specimens excluding salivary tumors showed LOH in 20 (48%) of 42 informative cases and LOH or microsatellite instability in 23 (54%) of 42 informative cases with at least one of these markers. The highest incidence of LOH in SCCHN was obtained with D3S656, with LOH in 12 (32%) of 38 informative cases. The minimal region of loss is shown in **Figure 3**. This shows a karyogram of the short arm of chromosome 3 and the location of the markers analyzed in the 3p24-pter region together with some of the more informative tumors. Results on several tumors shown in Figure 3 indicate that the minimal region of loss is the region bounded by D3S656 and D3S1293. This localized the minimal region of loss to the 3p24-

Table 2. Clinicopathologic Data and LOH* Results in 3p24-pter Region for Each Tumor Site

Site	TNM Stage		Positive Nodes, No.	Histologic Findings		LOH With 3p24-pter Markers, No. (%)
	Stage	No.		Classification	No.	
Hypopharynx (n=16)	2	1	13	Well differentiated	1	10/16 (63)
	3	5		Moderately differentiated	12	
	4	10		Poorly differentiated	3	
Mouth (n=12)	1	2	4	Well differentiated	2	5/12 (42)
	2	1		Moderately differentiated	8	
	4	9		Poorly differentiated	1	
				No data	1	
Larynx (n=8)	1	1	2	Moderately differentiated	0	3/8 (37)
	3	5		Poorly differentiated	7	
	4	2			1	
Oropharynx (n=6)	2	1	3	Moderately differentiated	2	1/6 (16)
	3	1		Poorly differentiated	2	
	4	3		No data	2	
	No data	1				
Salivary (n=3)	...	...	2	Well differentiated	1	1/3 (33)
				Moderately differentiated	1	
				No data	1	
Skin (n=1)	...	...	1	Moderately differentiated	1	1/1

**Loss of heterozygosity.*

Table 3. Association Between Loss of Heterozygosity Using D3S656 and Histologic Findings, T Status, and Smoking History

	No. of Specimens	
	Heterozygous	Loss of Heterozygosity
Histologic findings*		
Well differentiated	2	1
Moderately differentiated	18	4
Poorly differentiated	1	4
T status†		
T1	0	4
T2	4	1
T3	9	2
T4	8	2
Smoking history‡		
Heavy (>20/d)	9	1
Moderate (<20/d)	4	0
Nonsmoker	2	5
Stopped smoking	3	1

**Poor vs well and moderately differentiated, Fisher's exact test, P=.02.*
†T1 and T2 vs T3 and T4, Fisher's exact test, P=.08; T1 vs T2, T3, and T4, Fisher's exact test, P=.005.
‡Nonsmoker vs smoker, Fisher's exact test, two tailed, P=.005.

p25.1 region. Tumors 192 and 224 showed well localized loss with D3S656. D3S656 is located at 3p25.1 and is approximately 3 cM from D3S1252 and approximately 10 cM from D3S1293. This indicates that the region showing LOH in these tumors is well localized to the 3p24-p25.1 region, with evidence in some tumors pointing to 3p25.1.

When the LOH results were analyzed in conjunction with the clinicopathologic data, LOH was noted most commonly in hypopharyngeal tumors, with 63% of hypopharyngeal carcinomas showing LOH with one of the markers used in the study (**Table 2**). Indeed, the first nine tumors shown in Figure 3 are hypopharyngeal tumors, and all showed LOH localized to the 3p24-p25.1 region, with tumors 192 and 224 offering strong evidence of localized LOH at 3p25.1 in particular. There was a significant association between LOH with D3S656 and poor histologic differentiation (Fisher's exact test, *P*=.02) (**Table 3**). Interestingly, all informative T1 tumors studied showed LOH with the use of D3S656, although the small numbers prevent any conclusions being drawn (Table 3). We investigated whether there was a relationship between LOH with these markers and a history of smoking and drinking. Loss of heterozygosity at the D3S656 locus was significantly more common in nonsmokers than smokers (*P*=.005) (Table 3). We found no relationship between history of drinking and LOH with these markers. Multivariate analysis showed no additional information, and we found no significant association with any other clinicopathologic factors. Similarly, we found no significant relationship between LOH with these markers and patient survival.

COMMENT

Allelic imbalance on the short arm of chromosome 3 (3p) has been implicated in the pathogenesis of several tumor types, including renal and lung tumors.[8,10] Specific interest has focused on a number of specific regions on this chromosome, usually within the region 3p14-p26.[20] The region 3p25-p26 is the site of the von Hippel–Lindau TSG, which is commonly deleted in von Hippel–Lindau–associated tumors.[21] We were particularly interested in investigating loss of genetic material on the short arm of chromosome 3 close to the region 3p24-p25, as frequent LOH in this region had been noted in previous studies on SCCHN.[14,15] This suggests the presence of a putative TSG in this region with a role in the development of SCCHN.[14,15] The region 3p24-p25 is large, containing many hundreds of genes, and not well enough defined to allow positional cloning techniques to iden-

tify the target gene involved in SCCHN. The aim of this study was to refine this region further and so more precisely localize the region of minimal loss. Of the 46 tumors analyzed in the present study, 21 (45%) showed LOH with one of the markers used. This study confirms previous reports that allele loss on the short arm of chromosome 3 is a frequent event in SCCHN. It also confirms reports indicating that the region 3p24-p25 may harbor a putative TSG. Our results with D3S656, D3S1252, and D3S1293 in particular offer evidence of a putative TSG in the 3p24-p25.1 region. Several of our results suggest that such a TSG located in this region may be located close to the location 3p25.1, as a number of tumors showed minimal loss in this region and the highest incidence of LOH occurred with the marker D3S656, which specifically maps this locus. This is important for future mapping studies, as it more precisely pinpoints the region concerned, thus making future mapping projects more practical by focusing closer to the site of any putative TSG.

Microsatellite instability (instability of simple repeated sequences) indicates genomic instability, the exact significance of which is poorly understood at the present time. Microsatellite instability has been noted in several tumor types, including colorectal tumors and, more recently, head and neck tumors.[22,23] In our laboratory, we have noted microsatellite instability for at least two markers in approximately 28% of head and neck tumors.[23] Seven of the tumors in this study showed evidence of mirosatellite instability (the tumors usually showing gain of an allelic band).

Interestingly, LOH was noted more commonly in hypopharyngeal carcinoma than in any of the other tumors studied. Loss of heterozygosity with at least one marker occurred in 63% of the hypopharyngeal tumors analyzed, and most of the tumors that showed highly localized LOH were hypopharyngeal tumors.

Loss of heterozygosity with D3S656 was also noted in all the T1 tumors analyzed; however, the numbers are small, preventing us from drawing any general conclusions. The LOH with D3S656 was also noted more commonly in poorly differentiated tumors than well or moderately differentiated tumors ($P=.02$), suggesting a significant association with poor histologic diagnosis.

In this study, we found that LOH with D3S656 occurred more commonly in nonsmokers than in smokers but found no significant relationship between LOH and alcohol intake.

In summary, we have identified frequent allelic imbalance on the short arm of chromosome 3 in SCCHN and a region of minimal loss bounded by the markers D3S656 and D3S1293. Our findings suggest that a putative TSG in head and neck cancer lies between these two markers in the 3p25.1 region. This is a relatively small region, and we suggest that future mapping and positional cloning studies focus attention close to this site.

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