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Advances in Brief

Allelotype of Head and Neck Squamous Cell Carcinoma[1]

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Abstract

To gain a better understanding of the molecular changes in head and neck squamous cell carcinoma, we tested every autosomal arm of 29 primary head and neck tumors for allelic loss. Fifty-eight microsatellite markers were used with at least two-thirds of patients informative for each chromosomal arm tested. A high frequency of allelic loss was found on chromosome 9p where 21 of 29 (72%) tumors had loss of heterozygosity for at least one polymorphic marker on this arm. Chromosomes 3, 11q, 13q, and 17p exhibited loss in over 50% of all informative cases, while chromosomes 4, 6p, 8, 14q, and 19q displayed loss in greater than 35% of all cases tested. Additionally, several other chromosomal arms exhibited loss of heterozygosity in 20 to 30% of tumors tested. This high frequency of allelic loss in these advanced stage neoplasms suggests multiple genetic steps in the progression of head and neck cancer and identifies several putative tumor suppressor loci on affected chromosomes.

Introduction

HNSCC[3] occurs in more than 50,000 (1) Americans each year yet little is known about the molecular changes in this often disfiguring and fatal disease (2). Few protooncogens have been found to be altered in primary HNSCC tumors, with the exception of an amplified locus on chromosome 11q13. This region contains at least three candidate genes and may be amplified in one-third of HNSCC tumors (3, 4). With regard to tumor suppressor genes, *p53* is now known to be involved in head and neck cancer progression. Initial immunohistochemical studies showed intense *p53* staining in many cell lines and tumors (5, 6). More recent evidence suggests the presence of *p53* mutations in approximately one-half of HNSCC tumors (7, 8). Additionally, *p53* mutations occur in preinvasive lesions such as carcinoma *in situ* in approximately 20% of cases (8).

Statistical analysis suggests that head and neck tumors arise following 6 to 10 independent genetic events (9, 10). Cytogenetic studies of cell lines have revealed occasional alterations of chromosomes 1, 3, 5, 8, 14, and 15 with substantial variations induced by different tissue culture conditions (11). To better define areas of chromosomal loss which may harbor putative tumor suppressor genes, we performed a comprehensive allelotype of primary HNSCC. The highest loss of genetic material was found on chromosome 9, with more than two-thirds of tumors exhibiting allelic loss on the 9p arm. Losses of 3p, 11q, 13q, and 17p were common and several other chromosomal arms also exhibited areas of allelic loss at a reduced frequency. The occurrence of multiple areas of allelic loss on several chromosomal arms in these tumors is consistent with statistical estimates implying multiple molecular steps in the progression of HNSCC.

Materials and Methods

Tissue and DNA Extraction. Twenty-nine primary HNSCC tumors were collected following surgical resection with prior consent from Johns Hopkins Hospital patients. These tumor specimens were fresh frozen and then meticulously microdissected on a cryostat to select for neoplastic cells. Tumors with less than 60% neoplastic cells were excluded from this study. More than fifty 12-μm sections from these tumors were cut and placed in sodium dodecyl sulfate/proteinase K at 60°C for 4 h. This was followed by phenol-chloroform extraction and ethanol precipitation as described previously (12). Blood was obtained by venipuncture from these patients and lymphocyte DNA was isolated as above for use as control DNA in allelotyping. Stage of tumors and clinical characteristics of the patients at surgical resection have been described (13).

Allelotyping. Microsatellite markers suitable for PCR analysis were obtained from Research Genetics (Huntsville, AL) and are listed in Table 1. One polymorphic marker was initially chosen from each chromosomal arm and then additional markers were added to ensure that at least two-thirds of all patients were informative in at least one locus on every chromosomal arm. Prior to amplification 50 ng of one primer from each pair was end labeled with [^{32}P]ATP (20 mCi; Amersham) and T4 kinase (New England BioLabs) in a total volume of 50 μl. PCR reactions were carried out in a total volume of 25 μl containing 50 ng of genomic DNA, 0.5 ng of labeled primer, and 75 ng of each unlabeled primer. The PCR buffer included 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, 1% dimethyl sulfoxide to which were added 1.5 mM deoxynucleotide triphosphates and 2.5 units of Taq DNA polymerase (Boehringer Mannheim). PCR amplifications of each primer set were performed for 25 to 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 50–58°C for 60 s, and extension at 70°C for 60 s as described (14). One-tenth of the PCR product was separated on 8% urea-formamide-polyacrylamide gels (15) and exposed to film from 4 to 48 h. For informative cases, allelic loss was scored if one allele was significantly decreased in tumor DNA when compared to the same allele in normal DNA.

Results

We screened 29 head and neck squamous cell carcinomas for LOH with a panel of 58 highly informative microsatellite markers representing every autosomal arm. Table 1 shows the primer pairs used to amplify each chromosomal locus by PCR and includes the number of informative cases for each marker tested. Sufficient polymorphic markers from each chromosomal arm were used to ensure that at least two-thirds (19 of 29) of all patients were informative for each arm. Representative results are shown in Fig. 1*a* where tumors H21 and H26 reveal loss of the larger and smaller allele, respectively, for anonymous marker D9S156 on chromosome 9p. This chromosomal arm was found to be lost in 22 of 29 (72%) cases. In contrast, markers (*GSN, D9S146*) on chromosome 9q rarely displayed LOH (Table 1).

In Fig. 1*b*, tumors H8 and H10 reveal loss of the larger and smaller allele, respectively, for marker *D3S1284* on chromosome 3p. This chromosomal arm was found to be lost in 18 of 27 (67%) cases (Table 1). Markers for chromosome 17p (data not shown) revealed LOH in approximately 50% of the cases. Loss of 13q was found in 56% of cases with the highest percentage of loss near the *RB* locus. A high percentage of loss was also discovered on chromosomes 4 (38%), 6p (42%), 8 (40%), and 14q (39%). The microsatellite marker *Int*-2 on

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; PCR, polymerase chain reaction.

Table 1 *Microsatellite markers used for each chromosomal arm with corresponding allelic loss divided by the number of informative cases and percentage of LOH in parentheses for the 29 tumors studied*

For arms in which more than one marker was used, data from each marker were combined showing any allelic loss on that arm divided by the total number of informative cases and resulting percentage of LOH in parentheses.

Chromosomal arm	Probe	Allelic loss/ informative cases	Total allelic loss/ informative cases
1p	D1s252	3/19 (16)	7/23 (30)
	D1s233	5/19 (26)	
1q	D1s259	5/22 (23)	
2p	D2s162	4/21 (19)	
2q	D2s111	3/20 (15)	
3p	THRB	6/23 (26)	18/27 (67)
	D3s1284	12/19 (63)	
3q	D3s196	9/19 (47)	14/28 (50)
	D3s1238	7/20 (35)	
4p	D4s404	8/21 (38)	
4q	D4s430	9/24 (37)	
5p	D5s392	5/26 (19)	
5q	D5s421	5/20 (25)	
6p	D6s265	8/19 (42)	9/24 (38)
	TCTE	2/14 (14)	
	D6s105	4/19 (21)	
6q	D6s264	5/15 (30)	6/26 (23)
	D6s255	2/23 (9)	
7p	D7s507	6/26 (23)	
7q	D7s495	7/24 (29)	
8p	D8s261	8/20 (40)	
8q	D8s273	3/19 (16)	10/26 (38)
	D8s257	8/20 (40)	
9p	D9s156	13/18 (72)	21/29 (72)
	D9s199	15/21 (71)	
	D9s200	13/25 (52)	
9q	GSN	3/17 (18)	3/23 (13)
	D9s53	3/19 (16)	
10p	D10s249	5/22 (23)	
10q	D10s221	5/24 (21)	
11p	D11s899	4/23 (17)	4/24 (17)
	D11s907	1/14 (7)	
11q	Int-2	6/13 (47)	14/23 (61)
	D11s490	9/19 (47)	
12p	D12s62	5/28 (18)	
12q	D12s60	6/24 (25)	
13q	D13s115	6/13 (46)	12/22 (54)
	D13s133	10/18 (56)	
14q	D14s51	9/23 (39)	
15q	D15s117	1/21 (5)	
16p	D16s404	2/20 (10)	
16q	D16s402	2/13 (15)	5/25 (20)
	SPN	3/22 (14)	
17p	CHRNB1	8/22 (36)	12/23 (52)
	D17s122	7/12 (58)	
	Tp53	7/14 (50)	
17q	D17s250	5/19 (26)	8/26 (31)
	D17s579	8/26 (31)	
18p	D18s40	6/22 (27)	
18q	D18s34	6/26 (23)	
19p	D19s221	6/19 (32)	
19q	D19s210	7/22 (32)	10/25 (40)
	D19s177	4/16 (25)	
20p	D20s95	6/20 (30)	
20q	D20s110	1/16 (6)	2/21 (9)
	D20s119	1/11 (9)	
21q	D21s59	5/19 (26)	
22q	IL2RB	7/24 (29)	

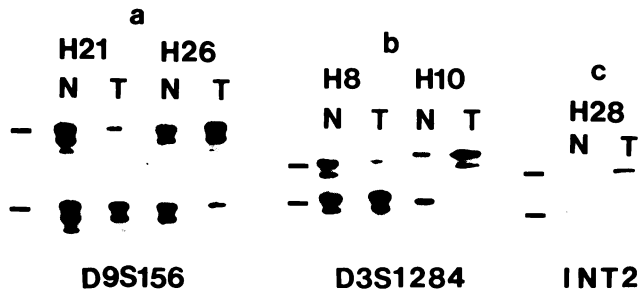

Fig. 1. Autoradiographs from loss of heterozygosity analysis with microsatellite markers. Representative HNSCC tumors (*T*) and corresponding normal tissue (*N*) are shown with microsatellite markers indicated on the *bottom*. In *a*, tumors H21 and H26 reveal loss of upper and lower allele with marker *D9s156* on 9p. In *b*, tumors H8 and H10 reveal loss of upper and lower allele, respectively, for marker *D3s1284* on 3p. In *c*, increased intensity is seen for top allele on tumor 28 with *int*-2 on 11q13 possibly suggesting amplification (see text).

chromosome 11q appeared to have LOH in 47% of cases. This marker lies within 11q13 and it is possible that some of this allelic imbalance may represent amplification rather than LOH (Fig. 1*c*).

LOH between 20 and 30% was found in several other chromosomal arms (Fig. 2). Because so many cases were informative, this represents between four and seven tumors displaying LOH on chromosomes 1, 5, 7, 10, 12, 20p, 21q, and 22q. Other chromosomes (including 15, 16, and 20) had less than 10% LOH consistent with rare losses of these chromosomes noted in other tumor types.

Discussion

Tumor suppressor genes appear to play a pivotal role in cancer progression (16). This has been clearly demonstrated in colon cancer where several areas of chromosomal loss correlate with histopathological progression (17, 18). In an effort to define similar progression models in other tumor types allelotypes have been completed recently for brain (19), bladder (20), lung (21), and breast cancer (22). We have completed a comprehensive allelotype for head and neck cancer as an initial step in the development of a molecular progression model for this tumor type. We found several areas of chromosomal loss common to other cancers, while several regions of loss appear unique to HNSCC progression.

LOH on chromosome 9p represents the most striking finding in the these tumors and is consistent with previous reports of a putative tumor suppressor gene near the interferon locus (23). Initially described in leukemia (24), chromosome 9 loss has now been well documented in cancer of the lung (25), bladder (26), and brain (27) and in familial melanoma (28). Fine mapping of this chromosomal arm in head and neck cancer should reveal an area of minimal loss shared by these tumors. A preliminary study appears to show that HNSCC tumors may target the same region of loss on 9p as the other cancers listed above (13). If the same area is targeted in all these tumor types, loss of this tumor suppressor locus may rival alterations of *p53* as the most common change in human cancer.

Chromosome 3p loss has been described previously in cell lines and our data confirm this finding in primary tumors (29). LOH and deletions of 3p have been implicated in different types of lung cancer (30–32). Mutations of *p53* are among the most common genetic changes in human tumors (33). Identification of 17p loss is consistent with previous estimates of *p53* mutations in a large number of primary HNSCC tumors and cell lines (7, 8). LOH on 13q was present in more than 50% of tumors and is commonly associated with inactivation of *RB* (16). Further mapping and direct testing for *RB* alterations should clarify the potential role of *RB* in HNSCC. Loss of chromosomes 3p, 9p, 13q, and 17p in HNSCC suggests a pathway of progression similar

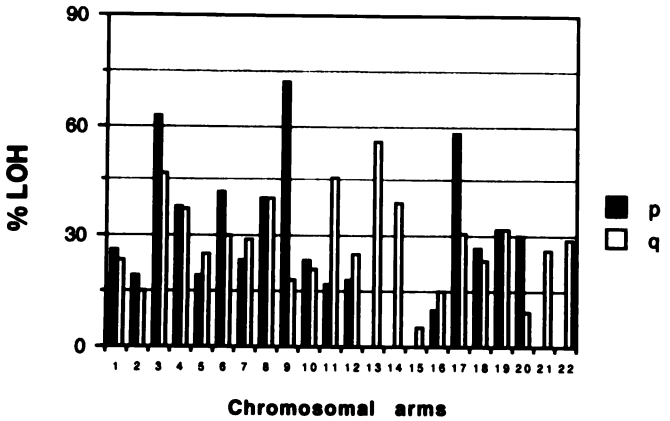

Fig. 2. Frequency of allelic loss for autosomes in HNSCC. Allelotyping was accomplished using polymorphic microsatellite analysis. The probes used are listed in Table 1. Markers with greatest LOH with respect to each chromosomal arm are shown.

to that of lung cancer (30). Inactivation of the same genes may be consistent with similar risk factors associated with both types of cancer.

Losses of 8p have been seen in several other tumor types (34–37) and also appear frequently in head and neck cancer progression. Likewise, frequent allelic loss on chromosome 6p has been reported in colon (17) and ovarian (38) cancer. Several chromosomes are lost in greater than 20% of cases (Table 1), and many of these have been implicated in the progression of other cancers. In contrast, losses on chromosome 4 and 14q have rarely been described previously. As with chromosome 9p, further mapping will have to be done to localize minimal areas of chromosomal loss that may harbor putative tumor suppressor genes.

The LOH on 11q may in fact represent amplification at this locus (3, 4). Because amplifications at this locus are usually not extensive (39), further mapping on either side of the *int*-2 marker may help assess the overall frequency of amplification *versus* loss. Southern blot analysis may also confirm amplification since small amounts of contaminating nonneoplastic tissue can significantly obscure the ability to distinguish between amplification and LOH by PCR analysis.[4] However, some tumors appear to have amplification of one allele (Fig. 1*c*), and the percentage of tumors with "loss" at this locus approximates the known percentage of 11q amplification in HNSCC (3, 4).

A completed allelotype of head and neck squamous cell carcinoma has revealed a distinctive pattern of LOH which is consistent with other molecular progression models. Loss of several chromosomal arms is shared with lung cancer while others appear to be unique to these tumors. In addition, HNSCC tumors appear to have a significant amount of loss on many chromosomal arms. The abundance of losses may be attributable to the fact that all of our tumors were of advanced stage and probably represent the most aggressive and genetically advanced lesions in HNSCC. However, previous statistical estimates based on the rate and age of tumor occurrence suggest that multiple events are involved in head and neck cancer progression. The number of estimated genetic events may be as high as 10 and rivals only prostate cancer for the highest number prior to clinical presentation (9, 10). Our data appear to be consistent with this hypothesis based on statistical models. Perhaps the significant and prolonged exposure to tobacco carcinogens may facilitate the accumulation of so many genetic events in HNSCC.

Further fine mapping of affected chromosomes should eventually reveal candidate tumor suppressor genes intimately involved in HNSCC progression. Detailed analysis of preinvasive lesions (8) should allow us to begin to develop a progression model where the specific timing of these events can be placed. Because these tumors have acquired so many genetic changes, identifying and targeting early events in progression may allow clinical intervention with novel chemotherapeutic agents (2) and diagnostic assays (40).

[4] Unpublished data.

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