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Microsatellite Instability in Preinvasive and Invasive Head and Neck Squamous Carcinoma

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To investigate the extent and significance of microsatellite instability in head and neck carcinogenesis we analyzed DNA extracted from normal squamous epithelium, severe dysplasia, and corresponding carcinoma specimens from 20 patients by multiplex polymerase chain reaction. Loci on chromosomes 3p, 5p, 5q, 8p, 9p, 9q, 11q, 17p, 17q, 18p, and 18q were selected for analysis. Our results show that three of the dysplasias (15.0%) and six of the invasive carcinomas (30.0%) manifested instability at multiple loci. Two of the dysplastic lesions had identical alterations in the corresponding carcinomas and one showed instability differences in only two of eight loci. Normal squamous epitheleum lacked microsatellite instability. No apparent association between smoking, alcohol use, or family history of cancer and instability was found in this small cohort. Invasive carcinomas with instability were relatively more poorly differentiated and had a higher stage and a high proliferative fraction. Our study indicates that microsatellite instability is 1) noted in a small subset of dysplastic lesions of head and neck squamous epithelium and 2) present in approximately one-third of invasive lesions, usually with aggressive characteristics, and may clinically be a late event associated with tumor progression. (Am J Pathol 1996, 148:2067–2072)

Head and neck squamous carcinoma tumorigenesis is characterized by early phenotypic lesions that can serve as a model for investigating the submicroscopic molecular changes associated with their development and progression.[1–3] This has been facilitated by the identification of microsatellite repeats for most chromosomal regions and their suitability for polymerase chain reaction (PCR) amplification, which allows for rapid and extensive molecular analysis from a small quantity of DNA.[4–8]

Studies in yeast and hereditary colon cancer have shown that instability at microsatellite loci occurs coordinately with mutations in mismatch repair genes, implicating impaired DNA repair mechanisms for the observed genome-wide mutations (mutator phenotype) and cancer development in these patients.[9–19] Evidence of DNA sequence instability in certain sporadic cancers has also been reported.[20–23] Alterations at the microsatellite sequence may, therefore, serve as sensitive markers for detecting replicative errors and assessing genomic mutations in cancer.[8]

The purpose of this study was to determine the extent and the biological implications of microsatellite instability (MI) in early tumorigenesis and in invasive head and neck squamous carcinoma.

Materials and Methods

Normal-appearing squamous epithelium, severely dysplastic/carcinoma *in situ* mucosa, and invasive carcinoma from 20 patients with head and neck squamous carcinoma, prospectively accessioned in the Frozen Section Unit of the Department of Pathology, University of Texas M. D. Anderson Cancer Center from 1991 to 1993, formed the materials for this study. Peripheral blood lymphocytes from each patient were also collected after Ficoll-Hypaque centrifugation of EDTA blood samples. Samples of normal squamous epithelium and epithelium with severe dysplastic/carcinoma *in situ*, exclusive of invasive carcinoma, were microdissected off the underlying submucosa after frozen section verification and localization. In each case, an initial frozen section was

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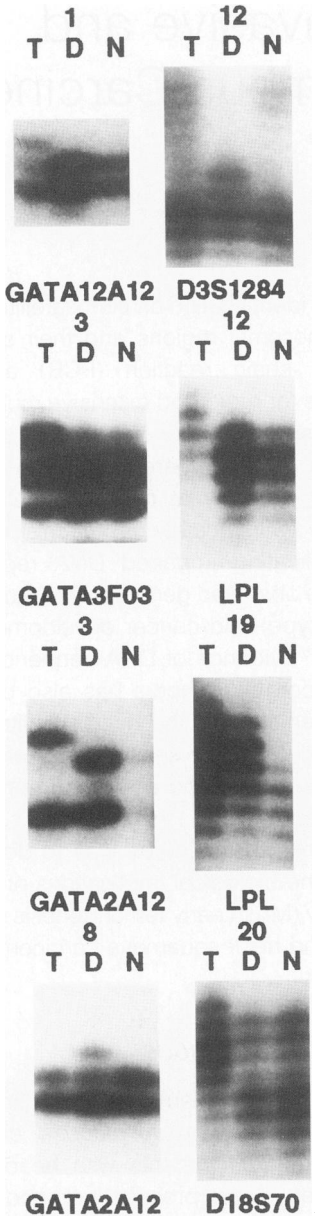

Figure 1. *Example of MI of different loci in selected cases. T, invasive tumor; D, dysplasia; N, normal mucosa.*

performed on grossly abnormal squamous mucosa, 1 to 1.5 cm from the invasive lesions, for diagnosis and localization of carcinoma *in situ*. Histologically normal squamous epithelium was obtained after frozen section verification from the farthest mucosal margin of the resected specimen. In most specimens, at least 3 cm separated the edge of the carcinoma from this microdissected margin. Invasive tumor samples were also carefully collected and verified by frozen section and cytospin preparation to contain <15% nontumor elements. Tissue samples from each specimen were immediately snap frozen and kept at −80°C until used.

DNA Extraction

For preparation of high molecular weight DNA, frozen tissues were ground in a lysing buffer containing 0.2 mol/L Tris-Cl, 1% sodium dodecyl sulfate, 0.25 mol/L NaCl, 25 mmol/L EDTA (pH 8.5). Proteinase K was then added to a final concentration of 200 μg/ml. DNA was then purified by extraction with phenol/chloroform and precipitated with ethanol. DNA was also extracted from peripheral blood lymphocytes.

Microsatellite Analysis

Twenty-five microsatellite loci (eighteen dinucleotides and seven tetranucleotides) of the most frequently altered chromosomal regions in head and neck squamous carcinoma were selected for analysis. Primers for PCR amplification of microsatellite markers were obtained from Research Genetics (Huntsville, AL) and constituted chromosomes 3p (D3S1307, GATA6F06, D3S1284), 5q (GATA3F03, GATA6E05, D5S408), 8p (LPL, D8S264), 9p (D9S, 126, 161, 162, 168, 171, and 199), 9q (D9S, 158, 301, and 302), 11q (D11S968), 17p (D17S799), 17q (D17S579, D17S795), 18p (D18S59, GATA11A06), and 18q (GATA2A12, D18S70).

We used multiplex PCR, in which two loci were amplified simultaneously in one reaction tube. One primer from each pair was end-labeled using T4 polynucleotide kinase (USB, Cleveland, OH) and [γ-^{32}P]ATP (10 mCi/ml; DuPont-NEN, Boston, MA). PCR was performed in a final volume of 25 μl, containing 200 ng of genomic DNA, 0.0125 μmol/L labeled primer, 0.5 μmol/L each unlabeled primer, 250 μmol/L deoxynucleotide triphosphate, 6.25% dimethlylsulfoxide, 0.25 mmol/L spermidine (Sigma Chemical Co., St. Louis, MO), 10 mmol/L Tris-Cl (pH 8.4), 40 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, and 0.5 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Twenty-five cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute were performed using an initial denaturation step and a final elongation step of 94°C for 5 minutes and 72°C for 3 minutes, respectively. After addition of 10 μl of loading dye, the PCR products were heat denatured and electrophoresed on 7% polyacrylamide gels containing 5.6 mol/L urea and 32% formamide at 80 W for 3 to 5 hours depending on the fragment size. The gels were dried and exposed to Kodak X-O-Mat-AR film (Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens.

Table 1. *Clinicopathological Factors, DNA Flow Cytometric Data, and Microsatellite Instability in Primary Head and Neck Squamous Carcinoma*

Case	Age (years)	Sex	Race	Site	Stage	Histology	DNA Index	%S-phase	MI
1	68	F	W	Larynx	II	MD	1.00	3	++
2	63	F	W	Larynx	II	MD	1.15	10	0
3	68	F	W	Tongue	IV	PD	1.78	12	++
4	58	M	M	Base of tongue	IV	PD	1.30	16	++
5	55	M	B	Larynx	III	MD	1.60	11	0
6	48	M	B	Tongue	IV	MD	1.65	13	+
7	39	F	B	Larynx	II	MD	1.00	13	0
8	68	M	B	Larynx	IV	PD	1.49	19	0
9	36	F	W	Tongue	II	MD	1.00	12	0
10	42	M	W	Tongue	II	MD	1.00	8	0
11	63	M	M	Tongue	III	PD	1.00	15	0
12	70	F	W	Tongue	III	PD	1.42	15	++
13	47	M	W	Tongue	IV	MD	1.00	9	0
14	68	F	W	Floor of mouth	II	WD	1.00	10	0
15	69	F	W	Tongue	III	MD	1.00	10	0
16	49	F	B	Tongue	II	MD	1.00	15	0
17	74	M	W	Tongue	III	PD	1.42	13	0
18	59	F	B	Larynx	III	MD	1.50	21	0
19	61	M	W	Larynx	II	MD	1.00	13	++
20	57	M	W	Larynx	IV	PD	1.00	23	++

F, female; M, male; W, White; B, Black; H, Hispanic; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated. MI was scored as follows: O, negative; +, less than two loci; ++, more than two loci.

Acridine Orange Flow Cytometry

Single-cell suspensions from solid tissues were prepared by mechanically mincing fresh tissue in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA). Acridine orange staining was performed using a two-step method previously described.[24] DNA/RNA analysis was performed with an EPICS Profile Cytometer (EPICS Division of Coulter Corp., Hialeah, FL) equipped with an argon ion laser emitting 488 nm (15 nW). A 525BP filter was used to collect green fluorescence (DNA) and a 610LP filter was used to collect red fluorescence (RNA). Peak *versus* integral signals were collected to discriminate doublets. Histograms were analyzed using the Histogram Analysis menu option of the Profile software.

Results

General Description

Table 1 presents the clinicopathological characteristics and the flow cytometric and MI findings in the invasive carcinomas from the study group. Ten females and ten males, who ranged in age between 36 and 74 years, with a mean of 59.6 years, formed the study population. Tumor sites included mobile tongue (ten), larynx (eight), floor of mouth (one), and mandible (one). Histologically, the group included one well differentiated, twelve moderately differentiated, and seven poorly differentiated carcinomas. Tumor stage was distributed as follows: eight stage II, six stage III, and six stage IV tumors. Flow cytometric DNA content data were available on seventeen tumors. Eleven tumors (62.5%) were DNA diploid and six (37.5%) were DNA aneuploid. The proliferative fractions of these tumors ranged from 3 to 23% with a mean of 14.9%. All patients but one gave a history of moderate to heavy smoking. Six patients had no occasional or mild drinking habit and fourteen admitted moderate to heavy drinking. A family history of carcinoma was elicited in four patients.

Microsatellite Instability

Instability of microsatellites was defined by the presence of extra band(s) and/or band shift in tumor DNA that was not present in the corresponding normal DNA. To exclude technical aberrations, differences in number and mobility of bands between carcinoma and corresponding normal samples were reproduced by separate, independent amplification of different DNA preparations. Specimen mismatching and tissue contamination were excluded by the presence of identical banding patterns of concordant size in normal and tumor DNA samples at different microsatellite loci. Instability was considered significant when more than two loci were noted.

Table 2 presents the MI in noninvasive and invasive carcinoma specimens at the chromosomal loci tested. None of the uninvolved (normal) squamous epithelium specimens showed microsatellite alter-

Table 2. *Microsatellite Instability in Head and Neck Squamous Carcinoma*

			1		3		4		6		8		12		13		19		20	
Chromosome	Location	Locus	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T
3	pter	D3S1307	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	p	GATA6F06	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−
	p13–14	D3S1284	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+
5	q21–22	GATA3F03	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
	q	GATA6E05	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−
	p35-ter	D5S408	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
8	p23-ter	D8S264	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
	p22	LPL	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−
9	p23	D9S199	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+
	p22–23	D9S168	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
	p21–22	D9S162	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	p21	D9S126	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−
	p21	D9S171	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−
	p21	D9S161	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	q13–21	D9S301	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
	q31–33	D9S302	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+
	q34-ter	D9S158	−	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−
11	qter	D11S968	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−
17	p11	D17S799	−	−	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−
	q11–12	D17S579	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	q23–24	D17S795	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
18	pter	D18S59	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
	p11	GATA11A06	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	q22–23	GATA2A12	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
	q23-ter	D18S70	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	

−, no instability; +, instability; D, dysplasia; T, tumor; numbers represent case numbers.

ations. Three (15%) of the noninvasive lesions showed instability at more than two loci at different chromosomal regions, and four showed instability at only one locus (Figure 1). Instability in dysplastic lesions (two) was found in the corresponding invasive carcinoma with additional alterations at other loci. In another instance (case 20), a matching instability between dysplasia and carcinoma was noted at six loci and a discrepancy at two loci was noted.

Of the seven invasive carcinoma specimens with instability, six (30%) manifested microsatellite alterations at multiple loci (more than two loci) of different chromosomal regions. There were no apparent differences in the incidence of MI between the chromosomal markers. Interestingly, a higher incidence of loss of heterozygosity was observed in the carcinomas with no instability than those with instability (data not shown). Of the six carcinomas with MI at more than two loci, four were stages III or IV and two were stage II; three were DNA diploid and three were DNA aneuploid; four were poorly differentiated and two were moderately differentiated; and four had high proliferative fractions. Two showed a low proliferation. There was a lack of apparent association between smoking, alcohol use, and a positive family history for malignancy and the presence or absence of instability at the microsatellite loci.

Discussion

Clonal evolution due to a progressive accumulation of genetic alterations in proto-oncogenes and tumor suppressor genes has been the principal hypothesis for neoplastic development. An alternative new mechanism, based on genomic instability at microsatellite repeats (mutation phenotype) caused by mutation in a gene(s) coding for a replication or repair factor(s) has recently been proposed.[8,11,25,26]

Invasive squamous cell carcinomas of the head and neck are preceded by histopathologically definable epithelial changes that constitute an excellent model for the investigation of neoplastic progression.[1] The genetic abnormalities associated with these lesions, however, have yet to be defined. To investigate the state of MI in early noninvasive lesions and invasive tumors, we analyzed microsatellite markers of certain chromosomal regions reported to be frequent targets of molecular alterations in head and neck squamous carcinoma.

Our results show that the incidence of MI at multiple loci in preinvasive lesions is less than 15%, but in related invasive lesions it is 30%. Such findings are comparable to those reported in head and neck and certain sporadic malignancies.[13,16,20,22,27–33] There are, however, relatively higher than those re-

ported by others for breast, skin and non-small-cell lung cancers.[34–36] These variations suggest that the incidence of MI varies between specific tumor types.

In our study, carcinomas manifested more loci alterations than preinvasive lesions, suggesting a progressive accumulation of MI during tumor development. These results corroborate previous studies of colorectal and other sporadic tumors,[12,16,22,30,31,38] but they are at variance with those of Wooster et al.[32] The type of microsatellite markers used and/or tissue/tumor phenotypes are likely one basis for the observed difference. Although most alterations in dysplastic lesions were manifested in the corresponding carcinoma samples, we observed a discordance at two of eight loci in one carcinoma. This could be attributed to either clonal or subclonal variability between dysplastic and invasive lesions.

Although the detection of sporadic microsatellite length variations in tumor samples may not necessarily indicate replication errors or pathogenetic involvement in these neoplasms, their presence in early neoplastic and invasive carcinoma may have practical applications in cancer diagnosis as markers of clonality. In that context, microsatellite alterations may be considered in prospective trials as a marker for early detection of clonal replicative errors and in monitoring additional accumulation of genetic alterations during tumor progression.[28]

We did not observe microsatellite alteration in peripheral blood lymphocytes or in phenotypically normal squamous epithelium within the field of the cancers. We also observed no apparent difference in the incidence of instability between different chromosomal loci. These results are in agreement with previous studies of familial and sporadic malignancies.[6,13,32,37,38]

PCR-induced alterations or differences in interpretation due to a lack of standardized criteria may also contribute to interstudy differences.[39] It is unlikely that this factor was at play in our study, as normal lymphocytes and uninvolved squamous epithelium lacked microsatellite abnormalities. Furthermore, the presence of an identical abnormality in independent PCR amplification from the same DNA extraction and the presence of length variations at different loci from the same sample render false positives unlikely.

Our results show that MI is slightly more noted in poorly differentiated, high stage, and highly proliferating tumors. As previously reported, our findings show an equal presence of MI in DNA diploid and DNA aneuploid neoplasms.[40] A definitive association between MI and aggressive behavior in head and neck squamous carcinomas, as shown in certain other tumors,[6,13,14,31] will require studies of larger patient populations with long follow-up.

In conclusion, our study shows that 1) a subset of dysplastic mucosal lesions and invasive carcinomas manifest MI and 2) MI in invasive carcinoma is more noted in tumors with aggressive phenotypic features. Additional studies of the hitherto known mismatch repair genes at 2p16, 3p21, 2q31, and 7q22 may determine their role in the initiation and progression of these neoplasms.

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