Research Article

Classical and molecular cytogenetic analysis in head and neck squamous cell carcinomas

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Abstract

Head and neck carcinomas represent the sixth most frequent type of cancer in the world, and 90% are derived from squamous cells (HNSCC). In this study of 15 HNSCC cases, extensive aneuploidy was detected by G banding in most tumors. The most frequently observed numerical changes involved gain of a chromosome 22, and loss of chromosomes Y, 10, 17, and 19. The most frequent structural alteration was del(22)(q13.1). As compared to G-banding, fluorescence *in situ* hybridization (FISH) proved to be an effective technique for detecting aneuploidy. Interphase FISH with a chromosome 17 centromere probe disclosed a high frequency of monosomy for chromosome 17, in contrast with G-banding, by which clonal monosomy 17 was detected in only three of the tumors. Painting probes for chromosomes 5 and 16 were used to evaluate a selected series of HNSCC in which G-banding analysis had shown marker chromosomes. FISH analysis failed to confirm the origin of the marker chromosomes, but four out of five cases showed a significant loss of chromosomes 5. This difference between FISH and G-banding results may reflect the smaller number of metaphase analyzed as well as the criteria adopted for sorting these metaphases. Therefore results obtained solely by G-banding analysis should be considered with caution. Our data confirmed the involvement of chromosome 17 in head and neck squamous cell carcinomas.

Key words: FISH, chromosomal aberrations, head and neck cancer, chromosome 17.

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Introduction

Head and neck carcinomas represent the sixth most frequent cancer in the world, with 90% of them being derived from squamous cells (Bockmühl *et al*., 1998). The overall 5-year-survival rate for patients diagnosed with head and neck cancer is estimated at 50%, and this percentage has not changed significantly over the past two decades (Reid *et al*., 2000). Tropical South America has one of the world's highest age-standardized rates of head and neck cancer (Foulkes *et al*., 1995). The consumption of tobacco and alcohol appear as the most important non-genetic risk factor associated with the development of head and neck squamous cell carcinomas (HNSCC) (Decker and Goldstein, 1982).

HNSCC develop through the stepwise accumulation of multiple somatic mutations (Nawroz *et al*., 1994; Califano *et al*.,1996, 2000). In agreement with this notion, a substantial proportion (40%) of the reported tumors show intratumoral heterogeneity in the form of cytogenetically related clones (Jin *et al*., 2002), reflecting the ongoing clonal evolution, through which new cell populations with an increased selective advantage emerge (Nowell, 1976).

Cytogenetic studies of HNSCC have revealed a complex pattern of non-random chromosomal abnormalities, and abnormal karyotypes were reported in more than 200 cultured HNSCC. The most frequent losses involved chromosomes 3p, 5q, 7q, 8p, 9p, 11q, 13p, 14p, 15p, and 18q, whereas chromosomal gains usually involved chromosomes 1q, 3q, 8q, and 15q, and band 11q13 (Mitelman *et al*., 2003). Among the recurrent structural abnormalities, the most common were 8q isochromosomes, 3p deletions, and homogeneously stained regions at 11q13 (Jin *et al*., 1990, 1993, 1995, 1997, 1998, 2002; Rao *et al*., 1994; van Dyke *et al*., 1994; Mertens *et al*., 1997). Losses involving 17p and gains at 17q have been frequently reported in HNSCC (Mitelman *et al*., 2003).

We performed cytogenetic analysis by G-banding in 15 HNSCC cases. Fluorescence *in situ* hybridization (FISH) with a centromere-specific probe for chromosome 17 was used on interphase nuclei to screen these tumors for aneuploidy. We also used painting probes for chromosomes 5 and 16 to evaluate some tumors, most of them having had marker chromosomes detected by the GTG-banding analysis.

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Material and Methods

Patients

Fifteen samples of primary head and neck squamous cell carcinomas (HNSCCs) were surgically removed at the Hospital A.C. Camargo, in São Paulo (Table 1). Informed consent was obtained from all patients prior to sampling. This work was performed with the authorization of the Brazilian Federal Ethics Committee-CONEP 813/2000. The medical records of all patients were examined to obtain clinical and histopathological data. For the family history of cancer, first- and second-degree relatives with cancer were considered as positive, and, whenever possible, the evidence of cancer was based on medical records. None of the patients had undergone radiotherapy or chemotherapy before surgery. Histopathological classification was based on the WHO International Classification of Diseases for Oncology (1990). The clinical staging was determined using the TNM Staging System (AJCC, 1998).

Cytogenetic studies

Fresh tumor samples were obtained under sterile conditions and immediately processed. Chromosome prepara-

Table 1- Features of HNSCC cases: age, sex, histopathological diagnosis, TNM status, clinical data and composite karyotype.

N case	Sex/age	Tumor localization	Tumor grade	TNM	Family history	Tobacco use	Alcohol use	Follow-up* (d,m)	Composite karyotype
SCP110	M/66	Floor of the mouth	I	$T_4N_0M_0$	-	+	+	DOC (2d)	41~45,XY,-Y,+17,-20,+mar [cp19]/46, XY[5]
SCP115	F/44	Floor of the mouth	II	$T_4N_2M_0$	Brother Ca gastric	+	-	ANR (67m)	42~50,X,-X,-2,-10,del(12) (p12.3),+15,-19,-20,+22,+mar [cp24]/46,XX[8]
SCP122	M/35	Tongue	II	$T_4N_0M_0$	Cousin Ca gastric	-	+	DOD (6 m)	43~48,XY,-9,+10,-17,+22 [cp15]/46, XY [21]
SCP123	M/40	Tongue	IV	$T_4N_2M_0$	Father Ca esophagus	+	+	DOD (31m)	44~48,XY,-Y,+10,+20,+22 [cp10]/46,XY[17]
SCP124	M/63	Oropharynx	I	$T_4N_0M_0$	-	+	+	Lost to follow-up (13,9m)	41~48,XY,-Y,+9,add(9)(p24), +13,-18,-19,+22[cp21]/46, X Y[8]
SCP125	M/58	Tongue	I	$T_4N_1M_0$	-	+	+	DOC (12m)	42~48,XY,-5,-13,+22[cp10]/ 46,XY[10]
SCP127	F/74	Floor of the mouth	I	$T_2N_0M_0$	Nephew Ca intestine	+	+	ANR (65.7m)	41~47,X,-X,del(6)(q23),+8, -10,-13,del(17)(p11.2),+22 [cp14]/46,XX[8]
SCP129	M/64	Larynx	II	$T_3N_0M_0$	-	+	+	ANR (65m)	44~48,XY,-Y,+7,-10,-21,+22, +del(22)(q13.1)[7][cp20]/ 46,XY[9]
SCP133	M/63	Floor of the mouth	II	$T_2N_0M_0$	Brother Ca?	-	-	ANR (71.6m)	42~48,X,-Y,-3,-9,-14,-16,-18, -19,+22,+mar[cp16]/46,XY [9]
SCP134	M/65	Retromolar	II	$T_4N_2M_0$	-	+	+	AWC (44.2m)	43~47, XY,-Y,del(1)(q21),-9, -10, inv(12)(p13.3q12),-17, -18,-21,+22,+mar1,+mar2 [cp21]/46, XY[17]
SCP135	M/57	Epiglotis	I	$T_3N_{2c}M_0$	-	+	+	DOC (21m)	41~48,XY,-Y,-4,+6,+del(6)(q 22), -11,-12,-15,-16,-19,-22, +?del(22)(q13.1),+mar[cp18]/ 46,XY[6]
SCP137	M/56	Tonsil	II	$T_3N_2M_0$	-	+	+	ANR (70.9m)	44~47,X,-Y,+7,-19,-20,+22, +mar[cp17]/46,XY[1]
SCP149	M/65	Larynx	I	$T_4N_0M_0$	-	+	+	ANR (54.1m)	44~49,XY,del(1)(q41),?add(4 q),+20,del(22)(q13.1),+mar [cp11]/46,XY[13]
SCP167	M/54	Larynx	II	$T_3N_0M_0$	-	+	+	ANR (37.9m)	43~47,XY,+1,-9,-10,+11,-11, -13,-16,-17,+22,+mar[cp14]
SCP172	F/40	Tongue	I	$T_1N_1M_0$	-	+	+	ANR (60.5m)	43~47,X,-X,-21,+del(22) (q13.1)[cp9]/46,XX[20]

*Since surgery until August 2002.

Abbreviations: Ca: carcinoma; (-): negative history; (+): positive history; d: days; m: months; DOC: died for other causes; DOD: died of disease; ANR: alive with non recurrence; AWC: alive with cancer. Survival time since the surgery is in parenthesis.

tion and cytogenetic analysis were carried out by standard techniques on primary cultures, as previously described (Rogatto *et al.*, 1993). The tumor fragments were dissociated with 0.4% type IV collagenase (Sigma), and the cells were grown in Ham's F-10 medium (Sigma) supplemented with 10% fetal calf serum. Cultures with an adequate number of mitotic cells were harvested with 0.1% trypsin and 0.2% EDTA, treated with a hypotonic solution (KCl 0.075M), and then fixed with methanol: glacial acetic acid (3:1). Metaphase chromosomes were GTG-banded (Scheres, 1972). The karyotype description and the requirements for clonality were based on ISCN (1995). A further requirement for clonality was that the changes had to be found in at least two culture flasks.

FISH was performed as described previously (Rogatto *et al.*, 1999; Dracopolli, 2000). In brief, the slides were treated with RNase 1x for 1 h at 37 °C. After rinsing in 2xSSC solution, the preparations were dehydrated in ethanol washes (70%, 85%, and 100%), air-dried, and then denatured in 70% formamide/2xSSC at 75 °C for 90 s to 120 s. The slides were dehydrated again going through a chilled ethanol series.

FISH was done with commercially available digoxigenin-labelled chromosome 17 centromere probe (D17Z1) and chromosome paintings (Coatasome 5 and Coatasome 16) from Oncor. Hybridization was done overnight at 37 °C, after which the slides were rinsed in 50% formamide/2xSSC at 45 °C and then in 2xSSC at 37 °C. The probes were detected with anti-digoxigenin-rhodamine, and chromosomes were counterstained with DAPI. On average, 100 interphase cells and 16 metaphase cells were analyzed from each sample. Normal controls were phytohemagglutinin-stimulated normal male lymphoblasts. The guidelines followed for FISH analysis were those described by Hopman *et al.* (1992). A case was considered to carry a numerical chromosome abnormality when the percentage of cells displaying an abnormal number of hybridization signals was higher than the mean value plus two standard deviations obtained for the same chromosome in the normal control. The results were verified independently by a second observer.

Results

The karyotypes of the 15 tumors based on GTG-banding analysis are shown in Table 1. Chromosome banding analysis showed complex karyotypes, with multiple numerical and structural alterations in almost all cases. Normal cells were detected at a low frequency, except in case SCP167. The most frequent numerical changes involved gain of chromosome 22 (11 cases) and loss of chromosomes Y (eight cases), 19 (five cases), 10 (five cases), and 17 (three cases). The most frequent structural alteration was del(22)(q13.1).

By G-banding analysis, total or partial loss of chromosome 17 was found to be clonal in four out of the 15

cases (SCP122, SCP127, SCP134, and SCP167). In addition, the loss of chromosome 17 was non-clonal in four cases (cases SCP133, SCP110, SCP137, and SCP149). Chromosome 17 gain was clonal in one case (SCP110) and non-clonal in three others (SCP127, SCP129, and SCP149). Interphase FISH analysis revealed monosomy 17 in all cases, at a frequency ranging from 50% to 78% (Figure 1, Table 2).

Five cases (SCP122, SCP129, SCP134, SCP135, SCP149) were analyzed using the painting probe Coatasome 5. In three of these cases a marker chromosome was present that could not be identified by the banding pattern (SCP134, SCP135, and SCP149). The selection of the other two cases was based on the quality of the preparations. On average, 13 metaphases were analyzed from each tumor. The probe did not hybridize to the markers, but in
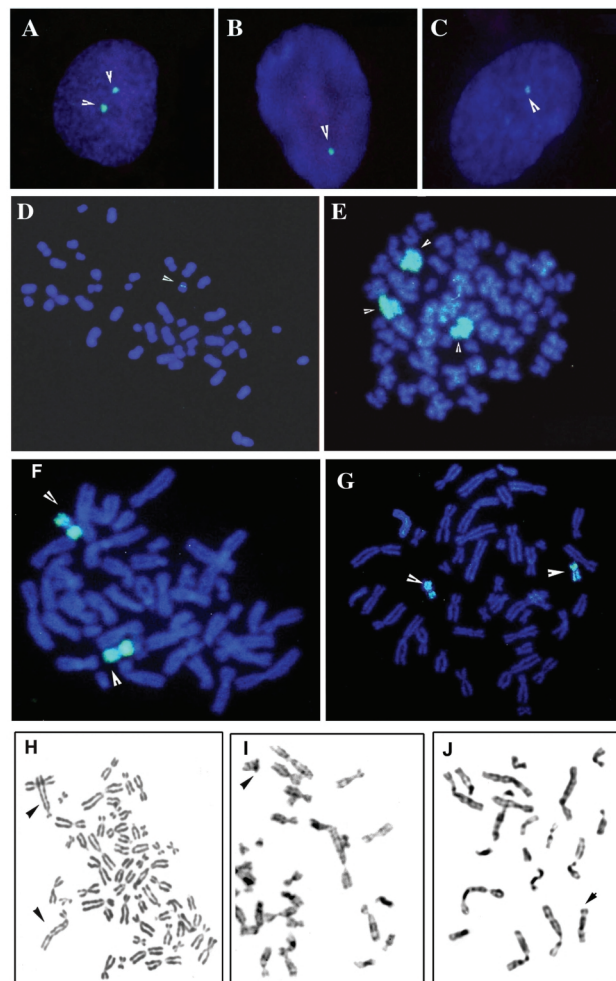

Figure 1 - FISH on metaphase chromosomes and interphase nuclei: (A - D) D17Z1 probe for chromosome 17 centromere hybridized to a control sample (A) and to tumor cases SCP122 (B), SCP135 (C), and SCP 134 (D), revealing monosomy 17 (only one hybridization signal) in all three tumors. (E) Trisomy of chromosome 5 observed in 1/13 metaphases after hybridization with chromosome 5 painting probe in case SCP122. (F - G) Disomy of chromosome 16 observed in cases SCP134 and SCP167. (H - J) The marker chromosomes (arrowheads) detected by G-band analysis in cases SCP134, SCP167, and SCP149.

Table 2- Summary of the chromosomal aberrations detected by FISH.

Chromosome 17 (D17Z1)					
Case	Number of signals				Total number of interphase cells
	1	2	3	4	
SCP 110	59	37	3	1	100
SCP 115	54	48	2	1	105
SCP 122	64	36	-	-	100
SCP 123	65	19	-	-	84
SCP 124	50	47	3	-	100
SCP 125	78	20	2	-	100
SCP 127	53	38	9	-	100
SCP 129	65	33	2	-	100
SCP 133	62	38	-	-	100
SCP 134	61	38	1	-	100
SCP 135	50	48	2	-	100
SCP 137	69	29	2	-	100
SCP 149	55	40	5	-	100
SCP 167	59	38	3	-	100
SCP172	72	28	-	-	100
Control	10	73	16	1	100

Chromosome 5 (Coatasome 5)					
Case	Number of signals				Total number of metaphase cells
	1	2	3	4	
SCP 122	3	9	1	-	13
SCP 129	8	8	-	-	16
SCP 134	4	11	-	-	15
SCP 135	1	14	-	-	15
SCP 149	1	5	-	-	6

Chromosome 16 (Coatasome 16)					
Case	Number of signals				Total number of metaphase cells
	1	2	3	4	
SCP 122	-	7	-	-	7
SCP 134	-	18	-	-	18
SCP 167	-	34	-	-	34

four cases (SCP122, SCP129, SCP134, and SCP149), loss of chromosome 5 was detected in more than 16% of the metaphases (Figure 1, Table 2).

A painting probe for chromosome 16 was used in three cases (SCP122, SCP134, and SCP167), two of them showing marker chromosomes (SCP122 and SCP134). In all cases the metaphases analyzed had disomy 16, and the marker did not show any hybridization signal (Figure 1, Table 2).

Discussion

Cytogenetic analysis of head and neck tumors has revealed extensive genetic heterogeneity and karyotype complexity (Oh and Mao, 1997; Scully and Field, 1997; Scully *et al.*, 2000; Gollin, 2001). The pattern of chromosome alterations observed in our study by G-banding, as well as the identification of the most frequently aberrant chromosomes, was consistent with other reports on the cytogenetics of head and neck carcinomas (Mertens *et al.*, 1997; Gollin, 2001; Jin *et al.*, 2002; Mitelman *et al.*, 2003).

In our study, FISH analysis showed a high frequency of monosomy 17 in all cases of head and neck carcinomas, regardless of the anatomical subsites (oral, laryngeal, and oropharyngeal tumors). In contrast, monosomy 17 was detected by G-banding in only three cases (20%). In her meta-analysis of a series of classical cytogenetic studies in HNSCC, Gollin (2001) estimated that 26% of these tumors had loss of chromosome 17. The discrepancy between the two methods (FISH and G-banding) in detecting this aneuploidy may result from the smaller number of metaphase analyzed as well as the criteria adopted for sorting these metaphases.

Soder *et al*. (1995), using a centromere probe for chromosome 17 in 51 HNSCC samples, demonstrated the gain and loss of chromosome 17 in six and thirteen cases, respectively. In a more recent study, Ai *et al*. (2001) used FISH in the study of paraffin-embedded tissue sections from 16 patients with HNSCC, including sites with normal mucosa, dysplasia, and invasive tumors. Loss of chromosome 17 was detected in a large fraction of cells in dysplasias, but its gain was prevalent in invasive carcinomas.

Comparative genomic hybridization (CGH) studies in HNSCC have identified non-random chromosomal gains and losses affecting chromosome 17, most often 17q gains and 17p losses (Brzoska *et al*., 1995; Speicher *et al*., 1995, Bockmühl *et al*., 1996; 2000; 2002; Hashimoto *et al*., 2001; Redon *et al*., 2001; Huang *et al*., 2002; Squire *et al*., 2002). The concomitant gain and loss of chromosome 17 segments suggest that cooperation exists between genes mapped at 17p and 17q, mediated by deletions of 17p and amplifications of segments of 17q. This phenomenon has been described in cell lung carcinoma (Varella-Garcia *et al*., 1998).

Loss of heterozygosity, deletions and other rearrangements involving chromosome 17, particularly the *TP53* gene, are the most common mutations investigated in cancer. The *TP53* gene is involved in many functions maintaining cellular integrity after DNA damage, and *TP53* mutations have been demonstrated in up to two-thirds of HNSCC, ranging from 12% to 100% in different series (Chang *et al*., 1992; Hainaut *et al*., 1998; Nagai *et al*., 1998; Kropveld *et al*., 1999; review in Scully *et al*., 2000, Beder *et al*., 2003). *TP53* mutations have been correlated with alcohol and tobacco exposure, and they appear to be associated with a short recurrence time (Shin *et al*., 1996). In addition, Blons *et al*. (1999) showed that loss of heterozygozity at 17p was predictive of low response to chemoterapy, indicating that *TP53* alterations could play a role in chemoterapy resistance in HNSCC.

Reports of one group showed an increased copy number of the *HER2* gene (17q11) in oral cavity HNSCC (Brandt *et al.*, 1995; Werkmeister *et al*., 1996; 2000). In addition, several studies have demonstrated increased *HER2/ERBB2* oncoprotein levels (Werkmeister *et al*., 1996; Ibrahim *et al*., 1999; Xia *et al*., 1999).

In our study, loss of chromosome 17 was not associated with clinical and histopathological parameters such as TNM staging, grade, familial or personal history of cancer, or tobacco and alcohol usage. Among the cases with the highest frequencies of loss, one (SCP125) died from other causes 12 months after surgery, while the other (SCP172) was alive without recurrence 60 months after surgery. HNSCC is thought to result from a multistep process (Nawroz *et al*., 1994; Califano *et al*., 1996), and a specific genetic event is expected to contribute only partially to the cancer phenotype (Hanahan and Weinberg, 2000).

We studied chromosome 5 by FISH in five cases, three of which showed markers that could not be identified by G-banding. The reasons for selecting this particular chromosome were that i(5p) is one of the most frequent structural rearrangements in HNSCC (10% of the cytogenetically abnormal cases), and chromosome 5 has been repeatedly reported to participate in whole-arm rearrangements with other chromosomes (El-Naggar *et al*.,1994; Ransom *et al*., 1998; Martins *et al*.,1999, Squire *et al*., 2002; Mitelman *et al*., 2003). Our FISH results showed significant loss of chromosome 5 in four cases, in which monosomy of chromosome 5 had been found to be nonclonal by G-banding analysis. The markers present in three of these cases could not be identified by the probe used.

Abnormalities of chromosome 16 were not detected in the three tumors studied after hybridization with chromosome 16 painting probe, two of them carrying marker chromosomes. A minority of HNSCC cases show loss of heterozygosity on 16p or 16q (Ah-See *et al*., 1994; Nawroz *et al*., 1994; Field, 1995), and some translocations have been found involving 16q22 (Owens *et al*., 1992). Loss of chromosome 16 has been detected by comparative genomic hybridization in a small number of HNSCC cases (Brzoska *et al*., 1995; Bockmühl *et al*., 1996; Komiyama *et al*., 1997; Wolff *et al*., 1998).

FISH strategies have enhanced the ability to detect subtle genomic changes and to diagnose complex karyotypes with marker chromosomes. However, standard FISH analysis requires the knowledge of the segments involved in the chromosome aberration, and, without a hint from the G-banding pattern, FISH analysis can be extremely laborious or even not feasible. The markers detected in our study could not be identified by G-banding, because they were either too small or showed poor band resolution. Recently, a new molecular cytogenetic technology, generically termed multi-fluorochrome FISH or M-FISH, has provided the means to directly examine the entire genome in one FISH experiment, thus allowing the elucidation of chromosomal rearrangements, including complex structural alterations that result in marker chromosomes (Bayani and Squire, 2001).

Multiple tumor-associated chromosome alterations occur in HNSCC. Our FISH results as well as other previously reported findings suggest that losses of genes mapped at chromosome 17 play a role in the pathogenesis of HNSCC.

Acknowledgments

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