BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 45 (2) 93-178 February 2012

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, February 2012, Volume 45(2) 172-178

doi: 10.1590/S0100-879X2012007500004

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Brazilian Journal of Medical and Biological Research (2012) 45: 172-178
ISSN 0100-879X

Loss of Y-chromosome does not correlate with age at onset of head and neck carcinoma: a case-control study

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Abstract

Loss of Y-chromosome has been correlated with older age in males. Furthermore, current evidence indicates that Y-chromosome loss also occurs in several human tumors, including head and neck carcinomas. However, the association between Y nullisomy and the occurrence of neoplasias in elderly men has not been well established. In the present study, the association between Y-chromosome loss and head and neck carcinomas was evaluated by comparison to cells from peripheral blood lymphocytes and normal mucosa of cancer-free individuals matched for age using dual-color fluorescence *in situ* hybridization. Twenty-one patients ranging in age from 28 to 68 years were divided into five-year groups for comparison with 16 cancer-free individuals matched for age. The medical records of all patients were examined to obtain clinical and histopathological data. None of the patients had undergone radiotherapy or chemotherapy before surgery. In all groups, the frequency of Y-chromosome loss was higher among patients than among normal reference subjects ($P < 0.0001$) and was not age-dependent. These data suggest that Y-chromosome loss is a tumor-specific alteration not associated with advanced age in head and neck carcinomas.

Key words: Fluorescence *in situ* hybridization; Y-chromosome; Chromosomal abnormalities; Head and neck cancer

Introduction

Functional genes mapped to chromosome Y can be divided into three distinct groups. The first consists of identical genes on X- and Y-chromosomes located within the pseudoautosomal region, including those that play a role in metabolic pathways for cell energy and cell surface antigen expression. Recent evidence has suggested the presence of two miRNA mapped to the pseudoautosomal region (1). The second group has homologous but not identical genes on the X-chromosome. They are located in the non-recombinant region of the Y-chromosome (NRY) or male-specific Y-chromosome (MSY). The third group includes genes from the non-recombinant region such as *SRY* (sex-determining region Y). The pseudoautosomal regions present 19 coding genes and the euchromatic region of MSY has 78 genes (1). Although a small number of genes mapped to the Y-chromosome has been described, recent studies have demonstrated their involvement in several diseases as well as in tumors affecting males such as gonadoblastomas (2) and prostate cancer (3). Therefore, the genes mapped to the Y-chromosome have the potential to influence cell proliferation and signaling transduction, or they can regulate gene expression by targeting specific mRNA. In general, Y-chromosome alterations show a great potential for involvement in human cancer (2).

Earlier studies have shown that Y-chromosome loss is a normal phenomenon observed in the bone marrow of elderly males (4-6). Pierre and Hoagland (7) and Sakurai and Sandberg (8) suggested that, although Y-chromosome

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Received September 14, 2011. Accepted December 20, 2011. Available online January 20, 2012. Published February 17, 2012.

loss should be recognized as a common age-related event, the percentage of Y loss is not dependent on age or disease. In 1992, the United Kingdom Cancer Cytogenetics Group (9) concluded that Y-chromosome loss in elderly males was not indicative of malignancy and should not be interpreted as a tumor marker. More recently, Zhang et al. (10) demonstrated that the frequency of Y-chromosome loss was significantly higher in patients with hematological disorders than in patients without such disorders, indicating that loss of the Y-chromosome is associated with a neoplastic process.

Current evidence indicates that Y-chromosome loss occurs in several human tumors, including prostate cancer (11-15), leukemia (16), esophageal carcinoma (17), gastric cancer (18), colorectal carcinoma (19), testicular germ cell tumor (2,20), renal carcinoma (21,22), as well as head and neck carcinomas (4). Recently, Singh et al. (23) demonstrated by large-scale epigenomic analysis the transcriptional potential of all protein-coding genes on the Y-chromosome including the *SRY* and the oncogene *TSPY*. According to the authors, the *GBY* locus may be hypomethylated in gonadoblastomas, resulting in overexpression of the *TSPY* and *TTTY* genes (24,25). However, the significance of Y-chromosome loss for the development or progression of these tumors is still unknown.

In head and neck carcinomas, Y-chromosome loss was initially detected by GTG-banding analysis (4,26). Using fluorescence *in situ* hybrization (FISH), Poetsch et al. (27) reported Y-chromosome loss in 53% of oropharyngeal, hypopharyngeal and laryngeal squamous cell carcinomas, except in poorly differentiated carcinomas. In laryngeal carcinomas, Szyfter et al. (28) found frequent Y-chromosome loss. Kujawski et al. (29) reported Y-chromosome loss as a common alteration in cell lines derived from primary and recurrent head and neck carcinomas, in primary larynx tumors and their corresponding metastases, and in multiple primary tumors from the same head and neck region.

In a previous study, our group reported Y-chromosome loss as the most frequent alteration in head and neck tumors analyzed by G-banding (27 of 59 cases). Fourteen of 27 cases showed lymph node involvement and 15 of 27 died from the disease. These data suggested an association between Y nullisomy and poor clinical outcome (30). Based on these findings and on the evidence that Y-chromosome loss might be correlated with aging, we used a case-control study involving age-matched individuals in order to investigate this hypothesis.

Material and Methods

Twenty-one samples of primary head and neck tumors were surgically removed and obtained from Hospital A.C. Camargo, São Paulo, Brazil (Table 1). The control sample consisted of 16 men randomly selected from the general population with no evidence of cancer or a family history of cancer matched for age with cases (ranging from 28 to 68 years). Peripheral blood samples were collected from 13 cases and a mucosa smear was obtained from three cases. The control individuals were not tobacco or alcohol consumers. Informed consent was obtained from all patients and controls prior to sampling. The Conselho Nacional de Ética em Pesquisa (CONEP #813/2000) approved this study. The medical records of all patients were examined to obtain clinical and histopathological data. First- and second-degree relatives with cancer were considered to be informative for family history. Whenever possible, the evidence for cancer was based on the medical records. None of the patients had undergone radiotherapy or chemotherapy before surgery. Histopathological classification was based on the International Classification of Diseases for Oncology of the World Health Organization (WHO) (31). Clinical stage was determined using the TNM (tumor, lymph nodes, metastasis) Staging System (32).

Cytogenetic study

Twenty-one fresh tumor samples were obtained under sterile conditions and immediately processed. Chromosome preparation and cytogenetic analysis were carried out using standard techniques following the direct harvesting of primary cultures. Metaphase chromosomes were processed for GTG-banding (33). The karyotype description and the requirements for clonality were based on the International System for Human Cytogenetic Nomenclature (ISCN) (34). A further requirement for clonality was the presence of chromosomal alterations in at least two culture flasks.

Normal cytogenetic controls were prepared from phytohemagglutinin-stimulated normal male lymphoblasts harvested for 72 h at 37°C, as described by Moorhead et al. (35).

Fluorescence *in situ* hybridization

Suspension cells stored at -20°C were used for FISH. Hybridization, suppression hybridization, detection, fluorescence microscopy, and microphotography were performed as previously described (36). Briefly, the slides were dehydrated in ethanol (70, 85, and 100%) and air-dried. FISH was performed with a commercially available biotin-labeled centromeric probe for the Y-chromosome (LPE00YcG, Cytocell, UK) and a digoxigenin-labeled centromeric probe for chromosome 1 (LPE001R, Cytocell). Chromosome 1 is one of the most stable in human tumors, since it is not frequently found altered in head and neck cancer (37). For hybridization, 10 µL of the probe was applied to each slide and denatured for 2 min at 75°C. Hybridization was performed for 1 h at 37°C in a dark chamber, after which the slides were rinsed in 0.25X SSC at 42°C followed by 2X SSC/Tween 20 at room temperature.

On average, 341 interphase nuclei with intact morphology, based on 4,6-diamino-2-phenylindole (DAPI) counterstaining, were scored to determine the number of hybridiza-

Table 1. Description of the 21 male patients studied according to age, histopathological diagnosis, TNM status, clinical data, and composite karyotype.

Patient	Age (years)	Anatomic site	Grade	TNM	Tobacco use	Alcohol use	Follow-up* (months)	Composite karyotype
1	63	Floor of the mouth	I	$T_3N_0M_0$	+	-	DOC (97)	nd
2	67	Glottic and infraglottic	I	$T_3N_0M_0$	+	+	ANR (200)	47,XY,+2[3]
3	58	Tongue	II	$T_3N_1M_0$	+	+	DOC (94)	nd
4	39	Retromolar	I	$T_4N_{2a}M_0$	+	+	DOD (15)	46,XY[5]
5	60	Palate	II	$T_4N_0M_0$	+	-	DOC (36)	nd
6	68	Tongue	I	$T_3N_0M_0$	+	+	DOC (99)	40~46,X,-Y,-22[cp9]/46,XY[4]
7	46	Floor of the mouth	I	$T_4N_0M_0$	+	+	DOD (18)	44~49,X,-Y,del(5)(q15q23),+8,-9,-13,-19, -21,+22,+mar[cp10]/46,XY[10]
8	58	Gingiva	II	$T_4N_0M_0$	+	+	DOD (52)	46,XY[7]
9	63	Retromolar	I	$T_4N_1M_0$	+	+	DOD (81.8)	nd
10	66	Floor of the mouth	I	$T_4N_0M_0$	+	+	DOC (2d)	41~45,X,-Y,+17,-20,+mar[cp19]/46,XY[5]
11	35	Tongue	II	$T_4N_0M_0$	-	+	DOD (6)	43~48,XY,-9,+10,-17,+22[cp15]/46,XY [21]
12	40	Tongue	IV	$T_4N_2M_0$	+	+	DOD (31)	44~48,X,-Y,+10,+20,+22[cp10]/46,XY[17]
13	63	Oropharynx	I	$T_4N_0M_0$	+	+	DOD (15)	41~48,X,-Y,+9,add(9)(p24),+13,-18,-19, +22[cp21]/46,XY[8]
14	64	Epiglottic	II	$T_3N_0M_0$	+	+	ANR (183)	44~48,X,-Y,+7,-10,-21,+22,+del(22) (q13.1)[7][cp20]/46,XY[9]
15	62	Tonsil	II	$T_4N_2M_0$	+	+	DOD (8)	42~47,X,-Y,-3,-15,+15,-17,-19,-22 [cp17]/46,XY[17]
16	63	Floor of the mouth	II	$T_2N_0M_0$	-	-	DOD (126)	42~48,X,-Y,-3,-9,-14,-16,-18,-19,+22, +mar[cp16]/46,XY[9]
17	65	Retromolar	II	$T_4N_{2b}M_0$	+	+	DOD (94)	43~47,X,-Y,del(1)(q21),-9,-10,inv(12) (p13.3q12),-17,-18,-21,+22, +mar[cp21]/46,XY [17]
18	57	Epiglottic	I	$T_3N_{2c}M_0$	+	+	DOC (21)	41~48,X,-Y,-4,+6,+del(6)(q22),-11,-12, -15,-16,-19,-22,+?del(22)(q13.1), +mar[cp18]/46,XY[6]
19	56	Tonsil	II	$T_3N_{2c}M_0$	+	+	ANR (183)	44~47,X,-Y,+7,-19,-20,+22,+mar[cp17]/ 46,XY[1]
20	65	Larynx/transglottic	I	$T_4N_0M_0$	+	+	ANR (182)	44~49,XY,del(1)(q41),?add(4q),+20, del(22)(q13.1),+mar[cp11]/46,XY[13]
21	32	Maxillary antrum	III	$T_4N_2M_0$	-	+	DOD (20)	43~47,-X,-Y,del(6)(q21q23),+10, +22[cp11]/46,XY[6]

*Months from surgery to July 2011. TNM = tumor, lymph nodes, metastasis; (-) = negative history; (+) = positive history; d = days; DOC = died from other causes; DOD = died of disease; ANR = alive with no recurrence; nd = not determined.

tion signals for the target probe and the frequencies of loss involving the Y-chromosome. A case was considered to have a numerical chromosome abnormality when the percentage of cells showing an abnormal number of hybridization signals was higher than the mean plus 2 standard deviations (2 SD) obtained for the same chromosome in the matched normal control. Image analysis was performed using an Olympus BX61 microscope connected to the FISHView EXPO 2.0 software (Applied Spectral Imaging, USA). The guidelines used for FISH evaluation were those described by Hopman et al. (38). Slides were assessed in a blinded fashin by two observers (LCVC and NAB). Any discrepancy in sample classification was addressed by immediate review and the final result was reached by consensus.

The Kruskal-Wallis or Mann-Whitney test was applied to compare Y loss frequencies between groups. Analysis of correlation between Y loss frequency and age was performed using the Spearman rank test.

Results

Twenty-one samples from male patients with head and neck carcinomas were investigated for Y-chromosome aneusomy in interphase nuclei. At least 100 interphase

nuclei were analyzed, except in three cases (samples 12, 14, and 15). A high frequency of Y-chromosome loss was observed, ranging from 11 to 85% (Table 2, Figures 1A,B and 2). Considering a cut-off rate of 25% as significant, Y-chromosome loss was detected in 18 of the 21 cases analyzed.

G-banding results were previously obtained in 17 cases (33), 12 of whom presented Y-chromosome loss as part of a more complex abnormal karyotype (Table 1). FISH analysis confirmed Y loss in all cases previously studied by G-banding, although 14 cases showed the alteration in more than 25% of cells.

To investigate the association between Y-chromosome loss and advanced age, 13 samples from normal individuals matched for age with the cases were evaluated. Y-chromosome loss was observed in 1 to 23% of the reference cells (Table 2). The normal mucosa from 3 healthy individuals revealed losses ranging from 14.7 to 30.8% of cells. When cases and the reference group were compared by age in periods of 5 years, a higher frequency of Y-chromosome loss was detected in head and neck patients compared to reference cases.

Tumor cases showed statistically higher frequencies of Y loss compared to control and buccal smear controls ($P < 0.0001$). Y-chromosome loss was more frequently detected in tumors than in peripheral blood samples from controls ($P < 0.0001$) and buccal smear from healthy individuals ($P = 0.0359$; Figure 1A). No correlation was observed between age and Y-chromosome loss frequency in controls (peripheral blood and buccal smear) and tumor cases ($P = 0.1090$, $r = 0.4160$, and $P = 0.3398$, $r = 0.2192$, respectively, Spearman test; Figure 1B).

Discussion

Abnormal karyotypes have been reported in about 400 primary head and neck squamous cell carcinomas including clonal loss of the Y-chromosome (37). Although the significance of this alteration in the biology of the disease

Table 2. Loss of Y-chromosome by head and neck cancer patients and controls grouped by age using FISH analysis.

Age groups	Age (years)	Case No.	Y loss/total No. cells	Frequency (%)
28-32 years	32	Case 21	31/184	17
	28	Control 1	5/534	1
	31	Control 2	71/307	23
		Control 1+2	76/841	9
	28	Smear 1	22/149	15
33-37 years	35	Case 11	48/101	47
	37	Control 3	84/684	12
38-42 years	39	Case 4	102/199	51
	40	Case 12	52/93	56
	39	Control 4	64/467	14
	41	Smear 2	25/153	16
43-47 years	46	Case 7	147/674	22
	46	Control 5	51/667	8
53-57 years	56	Case 19	55/216	25
	57	Case 18	219/784	28
	57	Control 6	42/529	8
58-62 years	58	Case 3	73/119	61
	58	Case 8	350/411	85
	60	Case 5	160/275	58
	62	Case 15	25/57	44
	59	Control 7	85/590	14
	64	Control 8	82/593	14
		Control 7 + 8	167/1183	14
63-67 years	63	Case 13	40/296	13
	63	Case 16	38/100	38
	63	Case 9	41/312	13
	64	Case 14	16/44	36
	65	Case 17	97/235	41
	65	Case 10	58/100	58
	66	Case 1	292/500	58
	66	Case 10	105/123	85
	67	Case 2	34/134	25
	64	Control 9	88/593	15
	65	Control 10	16/525	3
	68	Control 11	72/450	16
		Control 9+10+11	176/1568	11
68-72 years	68	Case 6	137/232	59
	68	Control 12	93/593	16
	68	Control 13	103/450	23
		Control 12+13	196/1043	19
	72	Smear 3	29/159	18

has remained elusive, recent studies have demonstrated that translation repression or mRNA degradation might also occur on the Y-chromosome. Recently two microRNAs (small RNAs that regulate gene expression) were identified on chromosomes X and Y (1). In an epigenetic profile of the euchromatic region of the human Y-chromosome, Singh et al. (23) observed in white blood cells that genes *TSPY* and *TTTY* mapped to the Y-chromosome appeared to be silenced by DNA methylation mechanisms. It is interesting to note that loss of methylation at this DNA locus has been

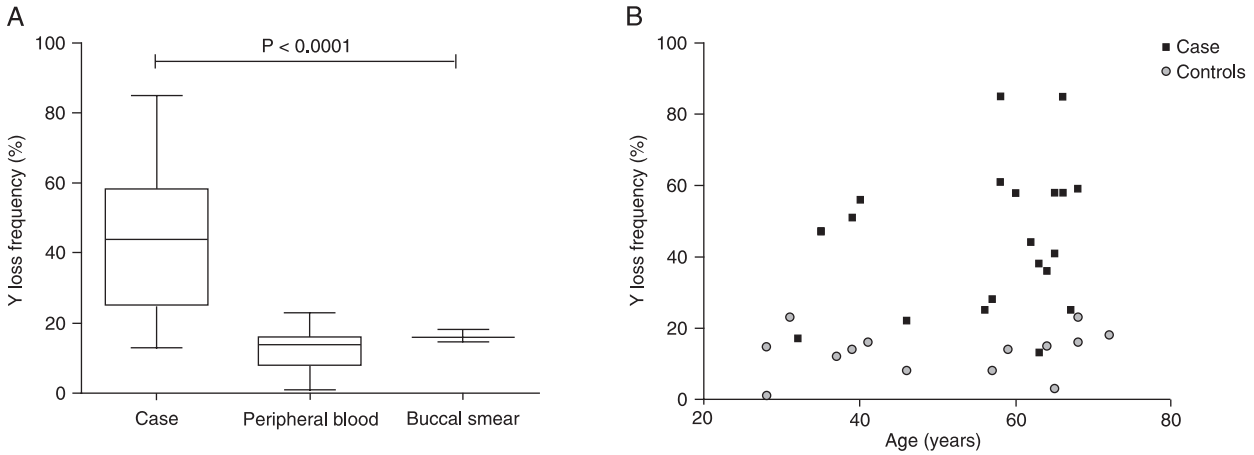

Figure 1. *A*, Y loss frequency in tumor cases, peripheral blood and buccal smear from controls. The Kruskal-Wallis or Mann-Whitney test was applied to compare the frequency of Y-chromosome loss between groups. *B*, Correlation analysis between age and frequency of Y-chromosome loss in tumor cases (P = 0.3398, r = 0.2192) and controls (P = 0.1090, r = 0.4160) was performed using the Spearman rank test.

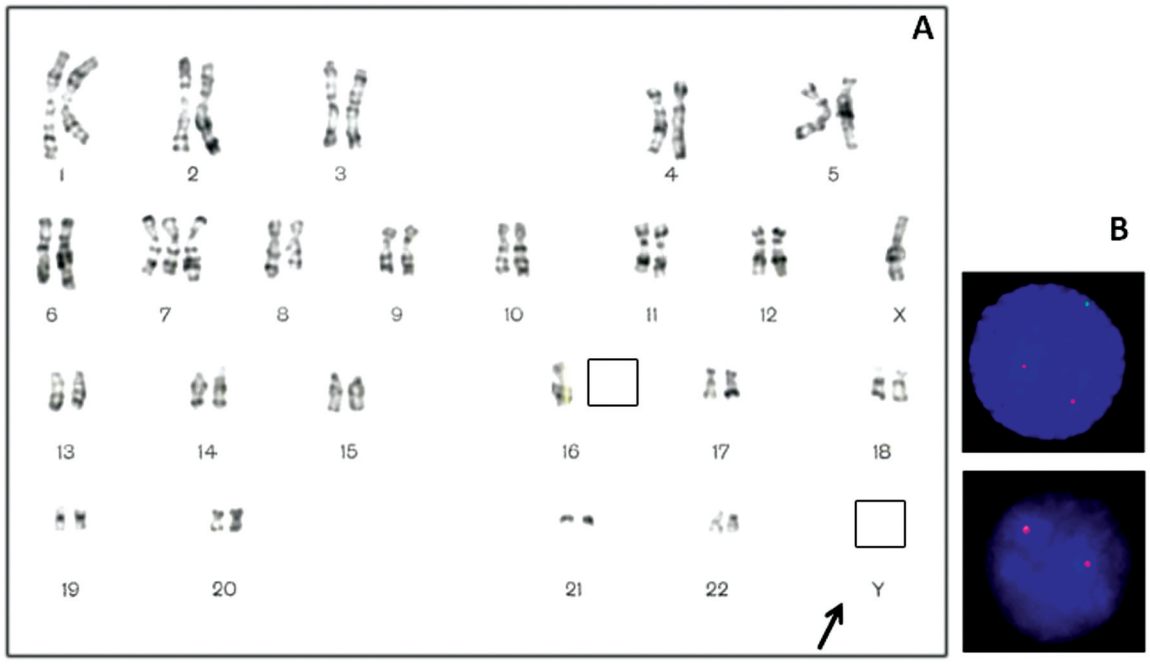

Figure 2. *A*, G-banded metaphase from a squamous cell carcinoma (case 14) with a 44,X,-Y,+7,-16 karyotype. *B*, Interphase normal cell (peripheral lymphocyte) with two red signals (chromosome 1), one green signal (Y-chromosome) and one tumor nucleus showing loss of the Y-chromosome.

observed in the promoters of oncogenes in cancer. These results revealed that genes mapped to the Y-chromosome could be considered as potential molecular markers in cancers.

In the present study, using dual-color FISH, it was demonstrated that Y-chromosome loss is age-independent and is a frequent chromosomal alteration in head and neck squamous cell carcinomas. As expected, older patients (>50 years old) were the most affected by the disease (17 of 21 male patients). All cases presented Y aneusomy; however, in two younger patients (7 and 21) and in two older patients (9 and 13) this alteration was non-significant (cut-off of 25% as significant). Four patients of this study were alive without recurrence after a follow-up ranging from 182 to 200 months; all of them were older (>50 years old) and presented a significant loss of the Y-chromosome. Previously, in a large series of head and neck carcinomas evaluated by GTG-banding, loss of Y-chromosome was detected in 26 of 58 (45%) head and neck carcinomas, 24 of which were T3-T4; 14 of 26 cases showed involvement of the lymph nodes and 16 of 26 died of the disease (30). Five patients aged <50 years showed loss of Y-chromosome; 4 of them were in stage T4 and two died of the disease. Although these data were not statistically significant, the authors showed a suggestive association between loss of the Y-chromosome and a poor outcome (30). Similarly, Kujawski et al. (29) were unable to find any correlation between Y-chromosome loss and disease progression and aggressiveness in head and neck carcinomas. However, the number of cases studied here is small for conclusions regarding the prognosis.

In addition, it was shown that the frequency of cells with Y loss did not increase with age and was significantly higher in cases than in cancer-free individuals matched for age. These data suggest that Y aneusomy is an event related to the disease. Regarding hematologic diseases, Wiktor et al. (6) reported an investigation of 185 male patients and 30 normal controls (aged 45-97 years) with Y loss as the only cytogenetic abnormality. They showed that Y loss was significantly greater in cases, with only 10% of the controls showing a >75% loss compared to 29% for those with disease.

In contrast, Jin et al. (26) reported that Y loss increased with advanced age in non-neoplastic upper aerodigestive tract mucosa. No Y-chromosome losses were detected in 15 samples from men aged more than 40 years, whereas 70% of the samples from men aged 60-79 years displayed clonal loss of chromosome Y. However, none of the samples analyzed were subjected to histopathological analysis. In the present study, Y-chromosome loss was not correlated with aging in peripheral blood cells or in normal mucosa from healthy individuals.

It has been suggested that chromosome Y has a tumor suppressor gene, the loss of which plays a role in the early steps of tumor development (39). Genes located on the Y-chromosome appear to be involved in cell cycle regulation, signal transduction, cellular growth, and protein degradation (40), as well as in the regulation of gene expression (1). Mutations involving these genes could lead to carcinogenesis (2). Vijayakumar et al. (15) used a PC-3 cell line, which shows Y aneusomy, to evaluate the effect of the addition of chromosome Y on the prostate tumorigenic phenotype in prostate cancer cells. The authors found tumorigenicity suppression suggesting the presence of a tumor suppressor gene mapped to the Y-chromosome.

The present findings suggest the involvement of the Y-chromosome as a tumor-associated abnormality not related to age at the onset of head and neck cancer. Recent literature findings demonstrating that genes mapped to the Y-chromosome may act as regulators of gene expression as well as being controlled by epigenetic events make this chromosome an interesting target for the study of tumor markers.

Acknowledgments

Research supported by CNPq, CAPES, and FAPESP.

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