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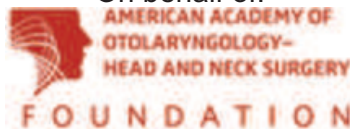
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Risk Factors for Multiple Malignancies in the Head and Neck

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Abstract

Objective. To define the prognostic role of multiple epidemiological, clinical, and biological factors for the development of multiple malignancies (MM) in patients with head and neck cancer (HNC).

Study Design. Historical cohort study. *p53* gene status, microsatellite instability (MSI) of the index tumor, and inherited chromosome fragility (CF) were studied.

Setting. Ninety-six consecutive patients affected by primary HNC, between January 1987 and October 1991, who were eligible for curative radiation therapy were followed up.

Subjects and Methods. *p53* gene status, MSI, and CF in 96 curative radiotherapy-treated patients were correlated with the risk for MM.

Results. Multiple malignancies occurred in 28.9%. Microsatellite instability ($P = 0.05$), CF ($P < 0.01$), and smoking after treatment of the index tumor ($P = 0.02$) were correlated with an increased risk of MM.

Conclusion. Genetic susceptibility may play a central role for MM development in patients with HNC.

Keywords

p53 gene status, microsatellite instability, inherited chromosome fragility, multiple malignancies, head and neck

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Multiple malignancies (MM) of the upper aerodigestive tract occur in approximately 10% to 30% of patients with head and neck cancer (HNC), and their appearance is the main cause of treatment failure and death. It is widely accepted that intrinsic susceptibility (ie, genetic factors) and exposure to carcinogens (ie, environmental factors) can act in concert to modulate cancer risk.^{1,2}

The literature reports a clear correlation between individual cancer susceptibility, expressed by mutagen-induced

chromosome fragility (CF), and an increased risk of developing a second primary tumor (SPT)¹⁻³; the same risk seems to be significantly higher among relatives of HNC patients with MM,⁴ indicating a genetic predisposition of cancer multiplicity. In MM patients, replication errors at microsatellite loci are also frequent,⁵ supporting the issue of a key role of genomic instability in the pathogenesis of MM. Finally, *p53* gene aberrations seem to be involved in the development of MM too, because of their role in regulating cell cycle and proliferation of normal cells.^{3,6}

In attempt to identify potential biomarkers predictive of a higher risk of MM, we investigated some inherited and environmental factors, previously reported as potential predictors of risk of SPT in HNC patients, in a series of 96 consecutive patients treated by curative radiotherapy at our institution.

Methods

Patients

The study was approved by the institutional review board at DPT Surgical Sciences, University of Florence, Florence, Italy.

Ninety-six consecutive patients were referred to our institution between January 1987 and October 1991 for previously untreated head and neck squamous cell carcinoma (SCC). Selection criteria were primary biopsy-proven SCC (excluding recurrent or multiple primary tumors and skin cancer), eligibility of the patients for curative radiotherapy, availability of the patients for a long-term follow-up, and adequate biopsy samples for genetic studies. Exclusion criteria were previous malignancy before HNC, previous chemotherapy, and/or previous radiotherapy treatment for noncancer disorders.

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Table 1. Series overview.

No. of patients	96
Sex	
Male	83 (86.5)
Female	13 (13.5)
Age at RT, y	
<60	31 (33.3)
>60	65 (67.7)
Cancer familiarity for HNC (82/96 patients)	
No	50 (61.0)
Yes	32 (39.0)
Smoking history before RT, packs/y	
No smokers	9 (9.4)
Moderate smokers (<30)	23 (24.0)
Heavy smokers (>30)	64 (66.7)
Smoking history after RT	
No smokers	11 (12.9)
Smokers	74 (87.1)
Alcohol consumption	
No drinkers	9 (9.4)
Moderate drinkers (<1 L wine/d)	74 (77.1)
Heavy drinkers (>1 L wine/d plus super-alcoholics)	13 (13.5)
Primary site	
Larynx	57 (59.4)
Oral cavity	18 (18.8)
Oropharynx	18 (18.8)
Hypopharynx	3 (4.0)
Primary tumor stage ^a	
T1	70 (72.9)
T2	22 (22.9)
T3	4 (4.2)
Primary nodal stage	
N0	79 (82.3)
N1	12 (12.5)
N2	5 (5.2)
Follow-up time, y	
Median	9.7
Range	10-18.7

Values are presented as No. (%) unless otherwise indicated. Abbreviations: HNC, head and neck cancer; RT, radiotherapy.

^aAccording to the TNM Union for International Cancer Control seventh edition staging system.

Clinical and epidemiological information are recorded in

Table 1.

After primary treatment, all 96 patients started with regular and strict follow-up for at least the first 6 years, which consisted of periodic ear, nose, and throat examination and diagnostic imaging. Suspicious lesions were analyzed by multiple biopsies. We defined an SPT as a biopsy-proven tumor that developed more than 2 cm from the index tumor or at least 3 years after the index tumor; a synchronous SPT was one that developed within 6 months of index tumor diagnosis, and metachronous carcinomas were those that developed more than 0.5 years after radiotherapy.

Chromosome Fragility Assessment (Bleomycin Test)

Cytogenetic analysis on peripheral blood lymphocytes obtained at the time of diagnosis of first HNC and prior to start with curative radiotherapy was performed on 70 of 96 patients (72.9%). Cytogenetic analysis was performed as previously reported.³ Among several parameters evaluated for breakage analysis,^{1,3} we focused on the number of breaks per cells (expressed as b/c value) as a representative parameter of chromosome fragility (CF) in our series. Accordingly, values of b/c >0.6 set bleomycin test positivity (CF+).

Genetic Analysis of HNC

Formalin-fixed, paraffin-embedded tissue sections, 7 to 8 mm, were placed on standard microscope slides. Specimens were deparaffinized with xylene, rehydrated in serial graded water-ethanol solutions (100%, 90%, 70%, 50%), and rinsed in deionized water. Hematoxylin and eosin stain was performed, and sections were examined under a microscope. Normal tissue was identified and scraped to obtain exclusively tumor cells, and DNA from paraffin-embedded tumor sections was extracted by overnight incubation at 55°C in an extraction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂, 0.1 mg/mL gelatin, 0.45% NP40, 0.45% Tween 20, 0.5 mg/mL proteinase K). The sample was boiled 8 minutes and centrifuged 15 minutes at 14,000 rpm. Then, 1 to 5 mL of the supernatant was used in the polymerase chain reaction (PCR) mixture.

Microsatellite Analysis

We collected 79 of 96 (82.3%) tissue samples from patients with HNC. This was mainly due to technical problems in DNA amplification in cases of small primary cancers.

Microsatellite status was assessed by examining 10 independent genomic sites, mapped on different chromosomal loci according to the literature.⁷ Lesions showing instability at 2 or more loci were defined as unstable (MSI+) and suggestive of a mutator phenotype.^{8,9} Standard PCR was performed in a final volume of 30 µL, containing 50 to 100 ng genomic DNA, 10 mM Tris-HCl (pH 8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 0.2% formamide, 2 to 8 pmol of each oligonucleotide primer, 200 µmol of each deoxynucleotide triphosphate, and 0.5 units of Taq polymerase. After an initial denaturing step at 94°C, 40 cycles were performed at 94°C for 1 minute each, at the annealing temperature (52°C-60°C, based on the primers employed) for 1 minute, and at 72°C for 1 minute. A final extension was done at 72°C for 7 minutes and separated by electrophoresis in an 8% to 12% polyacrylamide gel. Microsatellite instability (MSI) was scored when the PCR products from the tumor shifted up or down with respect to the band pattern of normal tissues or when there was evidence of loss of heterozygosity (LOH) greater than 50% visual reduction in the signal intensity of 1 allele. In the case of borderline allelic losses, the PCR reactions were repeated and quantified by densitometric analysis (GS-670 Imaging Densitometer; Bio-Rad, Hercules, California).¹⁰

Table 2. Distribution of the 27 second primary tumors: synchronous (#) and metachronous (+).

Site of Index Tumor	Site of Development of Second Primary Tumor							Total
	Larynx	Oral Cavity	Oro-pharynx	Hypopharynx	Lung	Gastrointestinal Tract	Prostate	
Larynx	+	+++	+		++	+	+	9
Oral cavity	+		#+	+	++	+	+	8
Oropharynx	+	#	+		+	#		5
Hypopharynx	+		#	+	+	+		5
Total	4	4	5	2	6	4	2	27

PCR and Single-Strand Conformation Polymorphism Analysis

Exons 5 to 8 of the *p53* gene were amplified.⁶ Amplification consisting of 32 cycles was carried out in 25 mL of 1.5 mM MgCl₂ buffer (PerkinElmer, Waltham, Massachusetts) with 1 mM of an exon-flanking primer set, 50 mM each dNTP, and 0.5 U AmpliTaq (PerkinElmer). Temperature and time for the reaction cycles were 95°C for 1 minute, 62°C for 1 minute, and 72°C for 30 seconds. The PCR products were heat denatured and subjected to single-strand conformation polymorphism (SSCP) analysis by electrophoresis on a 6% polyacrylamide gel with 5% to 10% glycerol. All samples were subjected to PCR and SSCP analysis at least twice to confirm the results obtained. To exclude the silent CGA/CGG dimorphism in codon 213, PCR products from samples showing SSCP abnormalities in exon 6 were subjected to restriction analysis with TaqI. The LOH was evaluated in those samples missing a mutation in relapsed tumors after radiotherapy. The polymorphism revealed by MspI digestion of a 107-bp fragment obtained from amplification of *p53* intron 6 was used. A further intragenic short tandem repeat polymorphism (AAAAT) was investigated.

Nucleotide Sequence Analysis

Abnormal bands detected by SSCP analysis were eluted from acrylamide gel and amplified by PCR using the same primers as for SSCP analysis with a modified 5' end to contain the M13(-20) sequence. These PCR products were purified on 2% NuSieve (FMC Corporation, Rockland, Maine) gels, phenol extracted, and subjected to automated sequencing with the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster Cit, California). Both strands were sequenced for confirmation of the mutations. *p53* wild-type samples were directly sequenced from the original PCR products.

Statistical Analysis

Statistical analysis was performed by Stata (StataCorp, College Station, Texas). Univariate and multivariate logistic regression analyses to assess the prognostic role of each factor were performed. Probability values less than .05 were considered statistically significant. The entry criterion for stepwise multivariate analysis was set at $P < .20$, and an exit criterion was set at $P < .05$. The significance of difference between mean values of

2 groups was evaluated using the Student *t* test (2-tailed). The same test was applied to assess significance of association between cigarette smoking, alcohol consumption, and *p53*.

The incidence rate of developing SPT per person per year was calculated by application of a nonparametric, locally weighted regression model and the use of Stata computer software.¹¹ Incidence rates were calculated at 3-month intervals from the time of definitive treatment up to death.

Results

Clinical Data

During follow-up, we documented an SPT in 27 of 96 patients (28.1%) with a median time of onset of 5.8 years (range, 2 months to 14 years). The SPT locations according to index cancer sites are summarized in **Table 2**. We documented 3 (11.1%) synchronous and 15 (55.6%) metachronous SPTs of the head and neck area. All patients with HNC with synchronous SPT had limited disease (stages I-II); 9 of 15 patients with HNC with metachronous lesions had limited carcinomas (stages I-II), and the remaining 6 presented advanced disease (stages III-IV). Twelve patients developed SPT in sites other than the head and neck.

Among relatives, we recorded 33 cases of HNC (10.9%) and 47 cases of malignancies in other areas (15.6%). Thus, we found 26.5% of relatives with a cancer history, and 39.7% of families had more than 1 member with a cancer history. Except for 9 nonsmoking subjects, the great majority of patients were moderate to heavy smokers.

Biological Factors

***p53* Status.** We documented a mutation on 5 to 8 *p53* gene exons in 49 of 96 (51%) tumor samples analyzed. Nineteen of 49 patients (38.8%) with a *p53* mutation developed an SPT. We found missense mutations in 30 (61.2%) samples and in-frame deletions in 4 (8.2%) samples, 14 (28.6%) frame shifts, and 5 (10.2%) nonsense mutations. Tumor stage ($P = .034$) and clinical lymph node metastases ($P = .001$) correlated with *p53* mutations, as well as alcohol ($P = .036$) and tobacco exposure ($P = .049$); conversely, CF+ and MSI+ did not.

Finally, *p53* gene status was not statistically significant in predicting the incidence of SPT ($P = 1.0$).

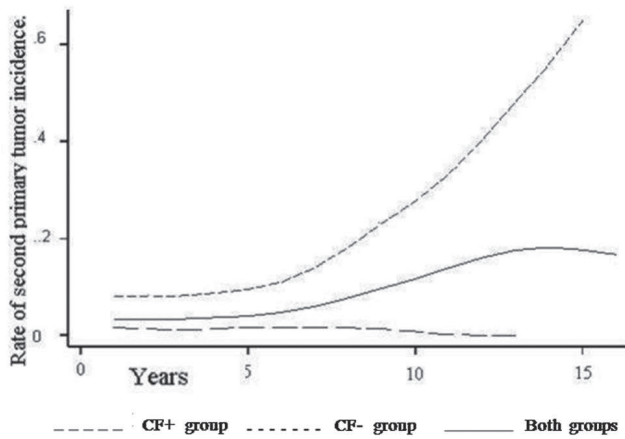


Figure 1. Univariate analysis of chromosome instability shows development rate of second primary tumors per person per year according to a nonparametric, locally weighted regression model. CF, chromosome fragility.

CF Assessment. CF+ was found in 33 of 70 (47.1%) patients with HNC. Twenty-three of 33 patients with CF+ (69.7%) developed an SPT. There were significant differences in mean values of breaks per cell between SPT patients and single primary patients ($P = .02$).

In the CF+ group, SPT frequency was about 10% in the 5th year, and it increased continuously to more than 60% in the 15th year (**Figure 1**); the rate of developing SPT in the

CF- group was lower: 5% in the 5th year and less than 20% in the 15th year.

MSI Assessment. Molecular analysis revealed MSI at more than 1 locus in 22 of 63 cancers analyzed (34.9%). Thirteen of 22 MSI+ patients developed SPT (**Table 3**). The microsatellite marker more frequently altered (17 of 22; 77.3%) was d2s123; in 6 of 22 (27.3%) cases, there was a mutation of D16S260. A high percentage of MSI was noted at loci D9S171 (10 of 22; 45.5%) and D9S156 (9 of 22; 40.9%). Samples from 2 synchronous lesions presented the same alterations according to a tumor clonal evolution. MSI+ was in at least 1 lesion in 11 (50%) HNC patients with a cancer history out of 22 HNC patients with MSI. A statistically significant correlation was between CF+ and MSI+ ($P = .03$).

Statistical Analysis of Risk Factors for SPT

We analyzed several variables according to SPT development with univariate and multivariate logistic regression analysis. At both tests, smoking persistence after treatment of the index tumor ($P = .01$ and $P = .02$, respectively) and T stage >2 ($P < .01$ and $P = .04$, respectively) were statistically significant risk factors for the development of SPT. CF+ ($P = .017$ and $P < .01$, respectively) and MSI+ ($P = .04$ and $P = .05$, respectively) were associated with a higher SPT risk (**Table 4**).

Discussion

The literature reports the mutation of the p53 gene in the cells of the index tumor, CF+, and MSI+ as potential

Table 3. Microsatellite instability loci mutations found in index tumor analysis.

Patients	d2s123	d3s27	Bat25	lfn α	d9s156	d9s171	d16s260	tp53	d17s261	ds1245
P1	■			■	■		■		■	
P2				■			■		■	
P3	■							■		
P4	■			■			■			
P5					■	■				■
P6		■				■			■	
P7			■		■			■		
P8	■					■			■	
P9	■			■		■				
P10	■				■		■	■	■	
P11	■						■	■		
P12	■							■		■
P13	■				■	■				
P14				■		■				
P15	■							■	■	■
P16					■			■	■	
P17	■				■			■		
P18	■						■	■	■	
P19	■	■			■	■				
P20	■							■		
P21					■	■	■			
P22	■				■		■			

Shaded cells represent loss of heterozygosis; black cells represent mutation. SAINT FRANCIS HOSPITAL on May 23, 2013

Table 4. Univariate and multivariate analysis results.

Variables	Univariate Analysis			Multivariate Analysis	
	Score	OR (95% CI)	P Value	OR (95% CI)	P Value
Sex					
Male	0				
Female	1	0.77 (0.27-3.42)	.69		
Age, y					
<60	0				
≥60	1	1.83 (0.60-5.45)	.31		
Family history for tumors					
No	0				
Yes	1	0.85 (0.23-3.52)	.74		
Smoking, packs/y					
No					
<30	0	0.69 (0.30-1.58)	.39		
≥30	1	1.51 (0.82-2.80)	.31		
Alcohol consumption					
No	0				
Moderate	1	1.33 (0.51-3.61)	.55		
Strong	2	2.01 (0.60-9.50)	.21		
Smoking after treatment					
No	0				
Yes	1	3.20 (1.30-7.62)	.01	4.35 (1.27-14.93)	.02
T stage					
T1	0				
T2	1	0.31 (0.59-1.61)	.16	0.20 (0.02-1.57)	.12
T3	2	14.0 (2.33-75.2)	.004	9.11 (1.09-75.97)	.04
N stage					
N1	0				
N2	1				
N3	2	0.92 (0.39-3.13)	.93		
p53 mutation					
No	0				
Yes	1	0.85 (0.23-3.52)	.74		
Chromosome fragility					
No	0				
Yes	1	4.09 (1.28-13.00)	.017	8.22 (1.90-35.63)	<.01
Microsatellite instability					
No	0				
Yes	1	3.12 (1.05-9.31)	.04	2.46 (0.96-6.25)	.05
p53 mutation and microsatellite instability					
No	0				
Yes	1	4.96 (1.31-18.66)	.015	5.25 (0.80-34.35)	.083
Microsatellite instability and chromosome fragility					
No	0				
Yes	1	4.95 (1.22;19.97)	.025		

Abbreviations: CI, confidence interval; OR, odds ratio.

predisposing factors of multiple cancerization; however, the great majority of these studies are retrospective case-control analyses evaluating the prognostic impact of a single biomarker in SPT in HNC patients. In this study, we tested the possible role of these 3 biomarkers in predicting the risk of

SPT, as well as their cumulative prognostic value, in a historical case series of HNC treated with radiotherapy.

Our group and other authors^{3,12,13} have previously reported p53 protein overexpression in the index tumor as a marker for individuals at high risk for SPT of the upper

aerodigestive tract. We documented that inherited cancer predisposition for SPT exists and correlates with aberrant inherited germline p53 mutations.¹⁰

However, in the current study, p53 gene analysis did not confirm this correlation.

We know from literature that, in response to environmental carcinogens, genetic damage accumulates more quickly in individuals with a genetic susceptibility to DNA aberration than in those without such instability.¹⁴ Moreover, DNA repair capacity is considered an independent susceptibility biomarker for HNC risk, and it could be useful to predict patients at highest risk of SPT.^{15,16} Our study confirms that CF is one of the most important nonenvironmental-related risk factors for cancer; however, we did not find any correlation between CF and epidemiological risk factors, including family history of cancer.

According to the genetic progression model, HNC progresses through a series of well-defined histopathologic stages, each one associated with progressive genetic alterations¹⁷; therefore, multiple DNA aberrations in a single clone may determine the acquisition of a “mutator phenotype” marker of genomic instability.¹⁸ Results of microsatellite analysis appear to be more controversial; in fact, although the role of genomic instability is clearly correlated with progression of benign lesions to cancer, as well as with a risk of SPT in the gastrointestinal tract,¹¹ this is less clear in HNC. Few studies on HNC have included MSI analysis or evaluated the association between MSI and hypermethylation in the promoter region of mismatch repair (MMR).¹⁹⁻²¹

In our series, we documented a high prevalence of HNC patients with stage I and II cancers; this is justified by the fact that the great majority of HNCs eligible for a curative radiotherapy are early staged tumors, which present also a better prognosis, a longer survival rate, and therefore a possible higher incidence of second primary malignancies.

In our study, we documented an MSI in 22 of 63 cancers analyzed (34.9%); 13 of 22 MSI+ (59.1%) patients developed an SPT. The microsatellite more frequently altered mapped near the *hMSH2* gene. There was no correlation between MI, locus involved, and site of first and/or SPT; however, MSI demonstrated a link with a family history of cancer. MSI+ was statistically related to CF+, suggesting that both might represent individual inherited factors useful in assessing the genetic risk of HNC. There was no correlation between CF+ and MSI+ and the status of the *p53* gene.

Multivariate analysis confirmed that CF+, MSI+, and persistent smoking habit after first HNC were independent prognostic factors in assessing the risk of SPT in our series ($P = .01$, $P = .05$, and $P = .02$, respectively). Altogether, these findings highlight the possibility of early identification of patients with a higher risk of SPT, which could lead to planning a different and personalized follow-up for these patients, with more frequent endoscopic controls and/or biopsies.²²

Our results suggest that inherited genetic factors, such as MSI of the index tumor and CF, may have a key role in determining multiple carcinogenesis of the head and neck.

These biomarkers could be helpful in identifying HNC patients with the highest risk of developing SPT.

Author Contributions

Alberto Deganello, design, analysis, and interpretation of data; **Gianni Gitti**, acquisition and analysis of data; **Giuditta Mannelli**, acquisition and analysis of data; **Giuseppe Meccariello**, acquisition and analysis of data; **Oreste Gallo**, drafting the article, revising it critically, and final approval of the revision to be published.

Disclosures

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