Mutagen Sensitivity as a Biomarker for Second Primary Tumors after Head and Neck Squamous Cell Carcinoma[1]

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Abstract

The occurrence of second primary tumors after curative treatment of early stage head and neck squamous cell carcinoma negatively influences the overall survival. Our aim was to prospectively evaluate whether mutagen sensitivity (mean number of chromatid breaks per cell in cultured lymphocytes exposed to bleomycin) could be used as a biomarker to predict which patients will develop second malignancies in the respiratory or upper digestive tract. Patients treated for head and neck squamous cell carcinoma ($n = 218$) were followed for approximately 6 years. Nineteen patients developed a second primary tumor, and each of these patients was matched on age, gender, cumulative smoking, tumor site, and tumor stage to two patients who did not develop any second malignancy. No difference between the groups was found with respect to mutagen sensitivity. Smoking at the time of the index tumor had a significant influence on the occurrence of second primary tumors (log-rank, $P = 0.019$). There was a significantly ($P = 0.005$) higher mean breaks-per-cell value in those patients who had developed their second primary tumor ≥3 years after the first tumor (0.97 ± 0.24; $n = 10$) compared with early second primary tumor patients (0.69 ± 0.09; $n = 9$). Conditional on a more than 3-year second primary tumor-free survival ($n = 38$), there is a significantly (log-rank, $P = 0.036$) higher probability of a second primary tumor for mutagen-sensitive patients [relative risk, 7.8 (95% confidence interval, 0.99- 61.74; $P = 0.05$)]. Mutagen sensitivity is a potentional biomarker for the occurrence of 'late' second malignancies (>3 years between tumors), and additional studies on the inclusion of this biomarker in chemoprevention trials is commendable because it would greatly improve their efficiency.

Introduction

HNSCC[3] accounts for 5% of all of the cancers in the western world. Well-known risk factors are tobacco smoking and alcohol abuse (1). It has been established that besides these exogenous risk factors, an intrinsic susceptibility to carcinogenic assaults also plays an important role. Mutagen sensitivity is one of those intrinsic biomarkers, which measures chromatid breaks in cultured peripheral blood lymphocytes after *in vitro* exposure to bleomycin. Several retrospective studies have revealed the importance of the inclusion of mutagen sensitivity for cancer risk estimation, especially in combination with exposure to tobacco smoke and alcohol use (2, 3). It has already been established that the mean number of chromatid b/c itself is not influenced by nonconstitutional risk factors such as age, gender, smoking, and alcohol intake of the subject. Moreover, we have provided evidence that mutagen sensitivity is an inherited characteristic (4).

HNSCC patients with small tumors can be curatively treated with surgery or radiotherapy. Although treatment strategies have greatly been improved, leading to better local tumor control, the overall survival of this patient group has not increased accordingly. This can partly be explained by the occurrence of a SPT which is not related to the treatment of the first tumor. HNSCC patients have a constant risk of about 3% each year to develop SPTs, whereas 87–100% of locoregional recurrences and distant metastases occur within 3 years (5). A SPT usually implies a poor prognosis because it often occurs at notoriously bad sites such as the esophagus or lungs or within previously treated areas within the head and neck, defying effective treatment.

Because early detection techniques are not available yet, it is required to focus on the population at the highest risk for SPTs and, therefore, investigate new methods to identify these high risk individuals. They can then be submitted to a more intense follow-up and enrolled in specific chemoprevention trials (6).

The risk of developing SPTs is not only related to the TNM stage of the first tumor (earlier stages are more prone for SPT) but also to the age and gender of the patient (7). Contradictory results on the effect of tobacco smoke exposure or the cessation of smoking after diagnosis of the first tumor have been reported (8, 9). These established risk factors are, however, not sufficient to explain all of the SPT cases. One prospective HNSCC patient trial has indicated that mutagen sensitivity may be a valuable biomarker of susceptibility to the development of multiple primary tumors (10, 11). Results of our previous retrospective study also suggested a particular role

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; %am, percentage aberrant metaphases; b/c, breaks per cell; SPT, second primary tumor; TNM, tumor-node-metastasis; CI, confidence interval.

of mutagen hypersensitivity in HNSCC patients who had developed two or more primary tumors (12).

In the current prospective study of HNSCC patients, we determined the predictive value of mutagen sensitivity for the development of SPTS in the respiratory and upper digestive tract.

Materials and Methods

Subjects. During 2.5 years (1991–1993), we collected heparinized blood samples from 218 consecutive patients who were referred to our hospital with a squamous cell carcinoma in the mucosal lining of the upper aerodigestive tract. Exclusion criteria were: any other malignancies besides head and neck or synchronous (double) tumors. In earlier studies, we and others have shown that treatment has no influence on the b/c value nor on the occurrence of SPTS (11). The only restriction is that patients should not have systemic treatment while the blood is collected because some additional damage can be expected. From 80% of both the SPT patients and the non-SPT patients, blood was drawn before radiotherapy. The patients who had had prior radiotherapy were analyzed 3 months after treatment, which has been shown not to influence the b/c score. In mid-July 1998 all of the patients' records were examined, and those patients who had developed a SPT in the head and neck region, esophagus, or lungs (which can be grouped together by using the term respiratory and upper digestive tract) during those years were selected ($n = 19$). For the classification of SPTS, the criteria of Warren and Gates (13), later modified by Hong *et al.* (14), were used. These criteria require that both tumors be histologically malignant and that they be geographically separate and distinct. The possibility of metastasis of the first tumor should be eliminated. In the present study, these criteria were strictly followed and verified by an experienced ear, nose, and throat physician. Person-year of follow-up were computed from the date of initial diagnosis to the date of death or the date of last follow-up, whichever came first.

From the cohort of HNSCC patients who did not develop any second malignancy, we matched two patients to one SPT patient. Matching was performed on gender, age, tumor site, TNM stage, and smoking history (pack-years). Cumulative smoking and alcohol intake were categorized as follows: smoking as <25 or ≥ 25 pack-years (all of the subjects were (ex)smokers and one pack contains 25 cigarettes); and alcohol intake as nondrinker, <100 or ≥ 100 unit-years. Pack-years were defined as the number-of-years-smoked multiplied by the number of cigarette-packs smoked daily (assuming that one pack contains 25 cigarettes). Unit-years were calculated similarly, i.e., the number of years multiplied by the number of drinks daily (one unit is defined as one alcoholic beverage per day). The cutoff points for high and low exposures originated from earlier mutagen sensitivity studies (2, 12). For the selected patient group ($n = 57$), the mutagen sensitivity was determined from the stored slides.

Mutagen Sensitivity Assay. At the time of blood collection, duplicate cultures were set up for each subject. Whole blood (0.5 ml) was diluted 10 times in RPMI 1640 (BioWhittaker, Walkersville, MD) with 2 mM L-glutamine (Life Technologies, Paisley, United Kingdom), 15% FCS (Hyclone, Logan, Utah), 1.5% phytohemagglutinin (Life Technologies), and 100 units/ml penicillin and streptomycin (BioWhittaker). After culturing the cells for 3 days at 37°C in 5% CO_2, they were incubated for 5 h with 30 mU/ml bleomycin (Lundbeck, Amsterdam, the Netherlands). To arrest the cells at metaphase, 100 μl of 50 μg/ml colcemid (Sigma, St. Louis, MO) was added to the cultures 1 h before harvesting. This yielded cells in metaphase that were damaged by the bleomycin in the late S-G_2 phase of the cell cycle. The RBCs were removed, and the lymphocytes were swollen in hypotonic solution (0.06 M KCl) and fixed in Carnoy's fixative (3:1 v/v methanol:acetic acid). After dropping the cells on wet slides, the metaphase spreads were air-dried and stained with Giemsa (Merck, Darmstadt, Germany). At this stage, the slides (two per person) were stored at room temperature until matching and scoring.

Before scoring 50 metaphase spreads for the presence of chromatid breaks, the slides were coded to assure objective "blinded" screening. The mean number of b/c of the two duplicate slides (a total of 100 metaphases) was used as a measure for the individual mutagen sensitivity. As has been published previously (15), the scoring of gaps did not influence the outcome of the assay and was omitted in investigations. Because DNA damage was introduced in late S-G_2 phase of the cell cycle, chromosome aberrations such as translocations were not present in the metaphases. Background levels of chromatid breaks without damage induction by bleomycin that had been determined in previous studies were very low (b/c values of about 0.06) and did not differ between patients and control persons. Therefore, data representing "spontaneous" breaks were not included. A second (more crude) measure of chromosomal damage was the %am calculated as the number of cells (of the 100 metaphases screened) that contained at least one break.

Statistical Analysis. For the analysis of the results, the matching was omitted. ANOVA was performed to determine differences in continuous variables (b/c; %am; age; follow-up time) between the two patient groups. For categorized variables (gender; smoking; alcohol use; tumor stage; site; treatment; occurrence of recurrences or metastasis) frequency tables were made and the Pearson χ^2 values were calculated. Multiple logistic regression was performed to reveal any interaction between variables. Overall survival, time to any secondary event (SPT, recurrence, or metastasis) and time to only SPT were investigated using the method of Kaplan-Meier (16). Comparisons were made (log-rank test) between mutagen sensitive [b/c, ≥ 0.8 (approximately the mean b/c value in this study)] and mutagen-insensitive patients (b/c, <0.8) and between smokers and nonsmokers at the time of the index tumor. In addition, relative risks were calculated using a Cox proportional hazards model. All of the *P*s are two-sided.

Results

Of the patients who were eligible for this study ($n = 218$), 19 (8.7%) developed a SPT. At the time of follow-up, 51.4% of the patients were still alive. On the basis of this number of patients, the incidence of SPT was 15.3% in 6 years. Each of these SPT patients was matched to two patients of the cohort who did not develop SPTs. The characteristics of these 19 clusters of matched patients are depicted in Table 1. The median person-years of follow-up was 4.5 years.

It was hypothesized that the mean b/c level could be used to predict which patients would develop SPTS. Table 1 shows the raw data of mean number of chromatid b/c and the percentage of metaphases in which damaged chromosomes (one or more) were present (%am). There was no difference ($P > 0.8$; Student's *t* test) in the b/c value or %am between SPT and matched non-SPT patients. The mean b/c value $\pm$ SD in non-SPT patients was 0.85 ± 0.30 and 0.84 ± 0.22 for SPT patients. For %am, these values were 44.9 ± 9.3 and 45.9 ± 7.5,

Table 1 Characteristics of 19 clusters of matched HNSCC patients

Cluster[a]	Sex	First tumor site	Mean no. of chromatid b/c			%am			SPT site	Interval[b] (yr)
			Non-SPT Patient I	Non-SPT Patient II	SPT	Non-SPT Patient I	Non-SPT Patient II	SPT		
1	M	Oral cavity	0.54	1.05	0.87	33	51	36	Oral cavity	5.4
2	M	Larynx	0.73	0.68	0.81	51	42	52	Lung	1.8
3	F	Oropharynx	1.24	1.61	1.18	66	65	53	Lung	3.7
4	M	Oral cavity	1.26	0.50	0.90	54	36	54	Oral cavity	4.2
5	M	Larynx	1.13	0.71	1.44	51	47	60	Lung	4.0
6	M	Oral cavity	0.60	0.89	1.04	35	45	60	Lung	3.4
7	M	Hypopharynx	0.90	0.95	0.73	46	46	43	Lung	1.3
8	F	Oral cavity	0.99	0.40	0.52	48	32	35	Oesophagus	3.5
9	M	Oropharynx	0.85	1.18	0.85	38	50	43	Oropharynx	3.4
10	F	Larynx	0.92	0.83	0.90	46	42	49	Lung	4.0
11	F	Oropharynx	0.74	0.60	0.65	43	39	40	Larynx	2.2
12	M	Larynx	1.25	1.01	0.59	63	39	32	Lung	2.0
13	M	Oral cavity	0.94	0.75	0.75	52	45	46	Oropharynx	2.7
14	F	Oropharynx	1.45	0.66	0.84	58	47	52	Lung	1.7
15	M	Oral cavity	0.63	0.45	0.63	40	35	41	Oral cavity	2.0
16	M	Oral cavity	0.52	0.48	0.58	30	38	43	Lung	1.8
17	M	Larynx	1.02	0.38	1.05	45	27	41	Larynx	4.3
18	M	Oropharynx	0.66	0.63	0.66	38	41	40	Lung	2.0
19	F	Larynx	0.96	1.32	0.91	52	44	46	Larynx	3.6

[a] In each cluster, two non-SPT cancer patients were matched to one SPT patient on the basis of age, gender, TNM stage, site, and cumulative smoking (pack-years).
[b] Time between the first and the second tumor.

Table 2 Distribution of several categorized variables of SPT and non-SPT HNSCC patients

	% of total no. per group		P^a
	Non-SPT (n = 38)	SPT (n = 19)	
Matched variables			
Gender: male	68.4	68.4	1.000
Smoking: >25 pack-years	68.4	78.9	0.404
TNM: T_1/T_2	55.2	57.9	0.563
Site			
Oral cavity	31.6	31.6	
Pharynx	34.2	36.8	
Larynx	34.2	31.6	0.926
Nonmatched variables			
Smoker at time of index tumor	55.3	84.2	**0.031**
Alcohol: >100 unit-years	42.1	63.2	0.308
Former[b] alcohol user	16.2	31.6	0.185
Alive at time of follow-up	68.4	26.3	**0.002**
Treatment			
Surgery	44.7	42.1	
Radiotherapy	26.3	15.8	0.410
Recurrences	28.9	15.8	0.277
Occurrence of metastasis	15.8	21.1	0.622

[a] P-values (2-sided) are given of the overall Pearson χ^2 analysis of each parameter. Bold figures indicate statistical significance.
[b] Quit before treatment of the first tumor.

respectively. Also dichotomized at a b/c level of >0.8 or 1.0, there was no difference between the groups.

No differences between SPT and control patients were found on gender, tumor site, tumor stage, and cumulative smoking (Table 2). Mean age of SPT patients was 69.5 ± 7.5 years compared with 69.0 ± 8.5 (P = 0.85) for non-SPT patients. All of the patients had smoked before the occurrence of the first primary tumor. In the SPT group, two patients (10.5%) were non-alcohol users; in the non-SPT group of three patients (7.9%), no drinking history could be determined, and three patients (7.9%) were nonusers. Tumor stage and site were similar in both groups.

Most of the parameters that were not used for matching did not differ significantly between the groups (Table 2). The number of patients that was still alive at the end of this study period was significantly different, and SPT patients (as could be expected) were the most likely to have died. Our data show that statistically significantly more patients in the non-SPT group had stopped smoking before the occurrence of the first tumor, which indicated a negative influence of the continuation of smoking after the first tumor on the development of SPT. Kaplan-Meier curves showed that smoking status at the time of the index tumor did not statistically significantly influence overall survival or the probability of any adverse event (log-rank: P = 0.11 and P = 0.10, respectively) but had a significant impact on the occurrence of SPT (log-rank: P = 0.019; Fig. 1). Cox proportional hazards analysis showed that the relative risk for smokers of developing SPT was 4.32 (95% CI, 1.25–14.86; P = 0.02) whereas the risk of death was 2.35 (95% CI, 0.86–6.44; P = 0.10). It has to be emphasized that information was not retrievable on how long and how much those patients continued to smoke after learning of the index tumor. Multiple logistic regression could not identify any other parameters or their interactions that could have influenced the occurrence of SPT.

Analysis of the time between the index tumor and the occurrence of the second tumor revealed that two groups could be identified. Patients with SPT that occurred within 3 years after the index tumor had a significantly (P = 0.005) lower b/c score (0.69 ± 0.09; n = 9) compared with SPT patients who developed the second malignancy after 3 years (0.97 ± 0.24; n = 10). For non-SPT patients, no difference was found in the groups who died before and those who died after 3 years (0.94 ± 0.34 and 0.83 ± 0.29, respectively; P = 0.377). The individual data of the SPT patients are visualized in Fig. 2, in which, also, a sensitivity borderline of b/c of 0.8 shows almost no overlap between groups. There was no difference in smoking at the time of the index tumor among STP patients of <3 years *versus* ≥3 years (Pearson χ^2, 0.532; P = 0.466). Further analyses of the data were performed on the set of patients

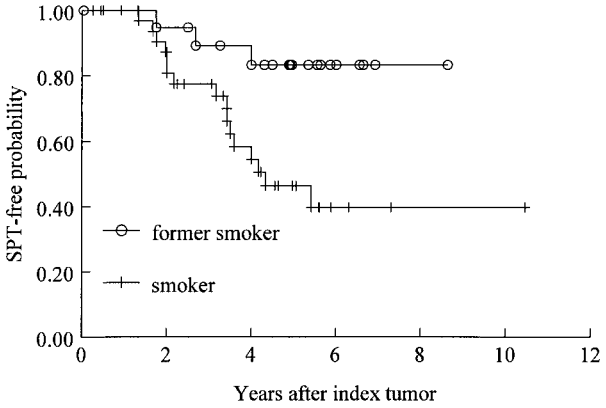

Fig. 1. Kaplan-Meier curves of probability to no development of SPT. *Former smoker*, those patients who stopped smoking before they were enrolled in this study at the time of the index tumor. It was not retrievable how long and how much the smokers continued to smoke after the index tumor. The difference between two groups was statistically significant (log-rank, $P < 0.02$).

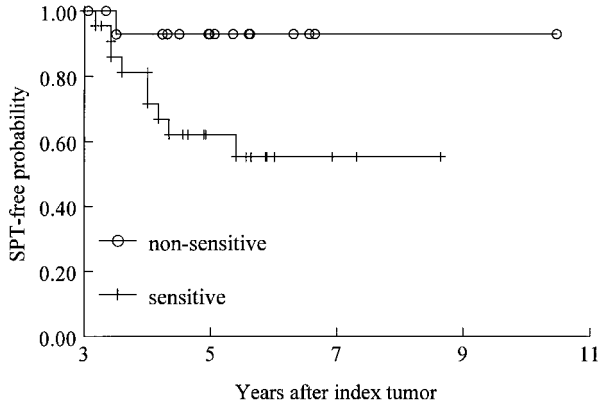

Fig. 3. Kaplan-Meier curves of probability to no development of SPT. Those patients were selected who were more then 3-year-SPT-free (and still living), and they were separated into hypersensitive (mean b/c ≥ 0.8; $n = 22$) and nonsensitive (mean b/c < 0.8; $n = 16$) groups. The difference between the groups was statistically significant (log-rank, $P < 0.05$).

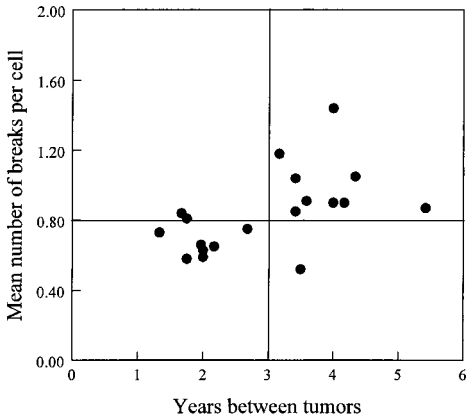

Fig. 2. The relationship between mutagen sensitivity and the time to develop SPT. The mean number of b/c was measured in peripheral blood lymphocytes as described in "Materials and Methods." A hypersensitivity border was used of a mean b/c value of 0.8.

among the 57 selected patients who had a SPT-free survival of 3 years ($n = 10$ for SPT patients and $n = 28$ for non-SPT patients). There were no differences in age ($P = 0.46$), cumulative smoking ($P = 0.76$), cumulative alcohol use ($P = 0.63$), site ($P = 0.89$), and stage ($P = 0.83$) of the index tumor and treatment ($P = 0.15$). The only two variables for which a difference remained were smoking at the time of the index tumor ($P = 0.017$) and the number of patients who had died in that time period after 3 years till follow-up ($P < 0.001$). Using the Kaplan-Meier method, we analyzed the patient group with a more-than-3 years SPT-free survival ($n = 38$); a clear difference in mean SPT-free survival time of nonsensitive patients (9.96 years; 95% CI, 9.02–10.90; $n = 16$) compared with mutagen-sensitive patients (6.57 years; 95% CI, 5.62–7.52; $n = 22$) was found. The Kaplan-Meier curves of this analysis are depicted in Fig. 3, and the two groups were statistically significantly different (log rank, $P = 0.0358$). Univariate analysis revealed a relative risk of 8.47 (95% CI, 1.07–66.97; $P = 0.04$) for smoking and 6.72 (95% CI, 0.85–53.04; $P = 0.07$) for mutagen sensitivity. In multivariate analysis, the relative risk for smoking was 9.70 (95% CI, 1.22–77.07; $P = 0.03$), and for mutagen sensitivity it was 7.8 (95% CI, 0.99- 61.74; $P = 0.05$).

Discussion

The objective of the present study was to evaluate whether mutagen sensitivity (sensitivity to bleomycin-induced chromatid breaks in peripheral blood lymphocytes) can predict the risk of developing a SPT after HNSCC. After matching two non-SPT patients to each SPT patient, we could not observe a difference in mutagen sensitivity between those two groups. Because matching was not performed on follow-up time the association between mutagen sensitivity and the occurrence of SPT may be underestimated. Some of the non-SPT patients could develop SPTs with longer follow-up times. In addition, it has to be emphasized that a sample size of 19 SPT patients is relatively small.

Interestingly, it was shown that "late-SPT" patients who developed their SPT at least 3 years after the development of the index tumor had a statistically significantly higher mean b/c value compared with those who developed their SPT within 3 years. Moreover, 9 of 10 had a b/c score of ≥ 0.8. This finding can also explain the relatively high b/c score in the multiple primary tumor group of our previous retrospective patient study (12), in which we found a significant difference compared with HNSCC patients with one tumor. That retrospective study, moreover, included patients who had had other malignancies besides SPT in the respiratory and upper digestive tract and patients who had synchronous tumors. When using the same criteria as in the current study, the SPT patients in the retrospective study had a mean number of b/c (1.06 ± 0.35; $n = 11$) and only one person had developed the SPT within 3 years (2.5 years), which again indicated that the late-SPT patients are the most hypersensitive.

Another interesting point is that the non-SPT patients who died before 3 years after treatment were relatively sensitive. This may indicate that some of these patients could have developed a SPT if they had lived longer.

Two theories have been proposed about the etiology of SPT: (*a*) a single cell is transformed and through migration (for instance, through the submucosa or lymphatic system) of daughter cells give rise to genetically related tumors with a common clonal origin (17, 18). Therefore, in this case, the SPT

can also be considered as metastasis or recurrence. It has been reported that 87–100% of all of the locoregional recurrences and distant metastases have occurred within 3 years (5); and, alternatively, (*b*) the new tumor has developed from independent foci that evolved from exposure to the same carcinogens, such as tobacco and alcohol (19, 20). These latter tumors will probably be of polyclonal origin and can develop any time after the index tumor (or be present at the same time; Ref. 21). The aspect of a possible difference in the time period after the index tumor between the two SPT types may give clues about the clonality of the first and second tumors. To exclude the possibility that a SPT is in fact a recurrence or a metastasis, a borderline of 3 years after the index tumor may be warranted. The importance of a borderline of 3 years between the tumors for the "real" SPTs of polyclonal origin is in line with the present study. It is concluded that, conditional on a SPT-free survival of at least 3 years, a high mutagen sensitivity greatly increases the risk of developing SPT.

Neither of the hypotheses on the etiology of SPT are in contradiction of the concept of field cancerization (22), which assumes that the whole mucous membrane is at risk for neoplasia. More studies to investigate clonal origins of SPT are now ongoing and will reveal whether there is a difference in the etiology of second malignancies (23, 24). It is possible that only a portion of the SPTs defined on the basis of Warren and Gates (13) are indeed independent second events, such as, probably, the late SPTs described in our present study.

On the basis of our current results, it is interesting to hypothesize that the hypersensitive patients develop a SPT by a second hit, possibly as a result of the continuation of smoking combined with their increased intrinsic susceptibility. Those patients who have a second tumor that has a different etiology or that might in fact be a recurrence or metastasis of the first tumor (early SPTs) are not mutagen-sensitive. Molecular analysis on the clonality of the tumor material, however, is needed to provide conclusive results. Further research on these molecular biomarkers and on the mechanisms underlying mutagen sensitivity should give more clues as to what brings us the best opportunity to give HNSCC patients individualized prevention strategies.

A lot of controversy exists as to the role of smoking cessation after the index tumor in regard to the risk of developing SPT (8, 9). Although it was not retrievable how long and how much the patients had smoked after the index tumor, it was clearly found in the present study that smoking at the time of the index tumor was a statistically significant factor for the development of SPT. This implies that primary intervention (smoking cessation) should be a major objective for preventive measures (25).

The low specificity (0.46) of the test, limits its use to predict which patients will develop SPT. Because of the high sensitivity (0.9) of the test, mutagen sensitivity can be used to select persons who are at the highest risk for a late SPT who can be enrolled in chemoprevention trials. This will clearly increase the efficiency of these trials because about 50% of the patients will not be treated who have a relatively low risk of developing SPT. So, for the design of head and neck cancer chemoprevention trials, the inclusion of mutagen sensitivity as a biomarker is commendable.

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