

Young Patients With Oral Squamous Cell Carcinoma

Study of the Involvement of *GSTP1* and Deregulation of the Fanconi Anemia Genes

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Objective: To investigate whether oral squamous cell carcinomas (OSCCs) from young (≤ 40 years) and older (≥ 60 years) patients have differential expression levels of *GSTP1*, *FANCA*, *FANCC*, *FANCD2*, and *FANCG*.

Design: Quantitative real-time reverse transcriptase-polymerase chain reaction and immunohistochemical analysis were used to assess gene and protein expression, respectively.

Setting: This study was performed in a research institute within a hospital setting.

Patients: Our study group consisted of 104 patients (42 young and 62 older). We collected RNA from 32 OSCC samples (10 young and 22 older patients) for gene expression analysis. Seventy-seven OSCC samples (37 from young and 40 from older patients) were used for protein expression analysis. Five patients were studied in both analyses.

Results: Lower expression of *GSTP1* ($P = .04$) and *FANCA* ($P = .01$) was observed in the tumors of young compared

with older patients. We also detected lower expression of *GSTP1* in the tumors of young patients compared with their nondysplastic mucosa ($P = .01$). *FANCA* was underexpressed in nondysplastic mucosa of young compared with older patients ($P = .01$). *GSTP1* protein showed negative or low expression in 41% ($n = 15$ of 37) of young vs 5% ($n = 2$ of 40) of older patient tumors ($P = .001$). *FANCG* protein expression was absent or low in 81% ($n = 30$ of 37) of young compared with 36% ($n = 15$ of 40) of older patient tumors ($P < .001$).

Conclusions: Differences in expression levels of *GSTP1*, *FANCA*, and *FANCG* in OSCC of young and older patients suggest that different mechanisms may be involved in tumor development through defective carcinogen metabolism and/or DNA repair capabilities. *GSTP1* plays a key role in detoxification; therefore, underexpression of this gene in tumors of young patients may cause deficient detoxification that could lead to an increased susceptibility to the development of oral carcinoma.

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ORAL SQUAMOUS CELL CARCINOMAS (OSCCs) represent 3% of all malignant tumors, with an estimated annual worldwide incidence of 170 000 cases and 81 000 deaths.¹ The most important behavioral risk factors associated with development of OSCCs are tobacco and alcohol consumption.² Oral squamous cell carcinomas generally occur in older men (> 50 years) with a history of smoking and/or alcohol abuse. These carcinomas occur less frequently in young individuals (< 40 years) and represent 3% to 6% of all OSCCs. However, for reasons not completely understood, the incidence of tongue OSCC in this particular group of patients has steadily increased since 1973 (from

0.06 to 0.32 per 100 000 for men and from 0.03 to 0.19 per 100 000 for women).³ In up to 72% of these younger patients, 1 or more behavioral risk factors are present,^{4,5} with similar rates as those observed in the older patient population.^{6,7} Young patients with OSCC and a history of carcinogen exposure may thus have a greater predisposition to disease development than their older counterparts in that they appear to develop disease despite a shorter exposure time to carcinogenic substances.^{8,9} Such a predisposition could be related to defective carcinogen metabolism and/or inadequate DNA repair.

A few reports have shown distinct molecular differences between young and older patients with OSCC as well as between non-smoking and smoking patients, support-

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ing the hypothesis that different subgroups of OSCC exist, especially with respect to exposure to tobacco carcinogens. A higher frequency of loss of heterozygosity at 3p, 4q, and 11q13 has been reported in OSCCs from patients with a history of smoking compared with non-smoking patients. *TP53* mutation is also more prevalent in OSCCs from smokers.¹⁰ In addition, single nucleotide polymorphisms of the *GSTP1* gene, which encodes an enzyme that functions in xenobiotic metabolism of polycyclic aromatic hydrocarbons,¹¹ have been associated with an increased risk of developing OSCC in nonsmokers and in light smokers.^{12,13} Our group has reported a high frequency (88%) of microsatellite instability at 2 or more loci in young, nonsmoking patients with head and neck squamous cell carcinomas compared with 36% in tumors from older patients with a history of smoking ($P < .001$).¹⁴

We hypothesize that a subgroup of individuals characterized by an early age of disease presentation and a shorter exposure time to behavioral risk factors develops squamous cell carcinoma, a histologically similar but genetically different OSCC compared with their older counterparts. This may be due to an increased susceptibility to the development of oral cancer as a result of lower expression of *GSTP1*, which is involved in the metabolism of carcinogens and/or DNA repair, as seen in other tumor types.^{15,16}

The *GSTP1* gene network includes *BRCA1*, *RAD51*, and the Fanconi anemia (FA) complex of genes. The FA genes are known to interact with *GSTP1* through binding with *FANCC*. Although the function of *FANCC* is not clearly understood, this protein has a role in redox regulation of *GSTP1*.¹⁷ Proteins encoded by *FANCC* and *GSTP1* are involved in the metabolism of reactive oxygen species, suggesting that interference with this interaction could play a role in cancer development.^{17,18} We thus also investigated whether any of the FA genes show deregulated expression in OSCC of young patients without FA.

Although other groups have studied alterations of *GSTP1* and FA genes in solid tumors, including head and neck carcinomas, none has examined deregulation of these genes exclusively in OSCC of young patients.^{19,20}

METHODS

PATIENTS

This study was approved by the University Health Network research ethics board, and informed consent was obtained from patients prior to sample collection. All patients were seen at the University Health Network, Toronto, Ontario, and only patients who had surgery between 1995 and 2004 as the primary treatment were included in this study. Two different groups of patients were studied: patients 40 years or younger (young patient group) and patients 60 years or older (older patient group). Only patients with primary OSCCs from sites in the oral cavity were included. All patients aged 41 to 59 years were excluded from the study. The same applied for patients whose primary treatment was not surgery and for all patients with FA syndrome. Furthermore, tumors from the lips, skin, pharynx, and larynx and metastatic tumor samples were excluded.

Medical records were examined to obtain the detailed clinical and histopathologic information for each patient, includ-

ing age, sex, tumor site, disease stage and grade, and history of tobacco and alcohol consumption. Our study group consisted of a total of 104 patient samples (42 from young and 62 from older patients). Of these 104 samples, 72 were available for immunohistochemical (IHC) analysis, 27 for quantitative real-time reverse transcriptase-polymerase chain reaction (QRT-PCR), and 5 for both analyses. Complete clinical information was not available for 26 of the 104 patients. Tumors were staged according to the current TNM classification as recommended by the American Joint Committee on Cancer (2002).²¹ Lingual mucosa samples from 2 young, healthy, nonsmoking volunteers (a man and a woman, ages 29 and 31 years, respectively) and 2 pooled samples of RNA from normal tongue mucosa (Stratagene, Vancouver, British Columbia) of older individuals (a man and a woman, ages 62 and 73 years, respectively) were also included to assess the messenger RNA (mRNA) expression levels of the genes in normal tongue tissue and to compare them with normal and tumor samples from young and older patients. This comparison was performed to exclude age-related gene expression changes.

TUMOR SAMPLES

Oral squamous cell carcinoma samples and adjacent nonmalignant mucosa were obtained at the time of surgery. Tissues were snap frozen in liquid nitrogen and stored at -80°C until use. Corresponding hematoxylin-eosin-stained tissue sections were examined, and histopathologic diagnosis of OSCC was confirmed. Tumor specimens consisted of at least 80% tumor cells, so the amount of stromal cells and nonmalignant keratinocytes present was limited. Formalin-fixed, paraffin-embedded tissue samples were obtained from the Faculty of Dentistry, University of Toronto, and from Princess Margaret Hospital, Toronto, Ontario.

GSTP1 GENE NETWORK

International databases (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/], PubMed, LocusLink, Unigene, Online Mendelian Inheritance in Man [OMIM], and UCSC Genome Bioinformatics [http://genome.ucsc.edu/]) were used to search for published data and to identify gene interactions involving *GSTP1* that could play a role in OSCC development and/or progression. *FANCC*, a member of the FA complex, is known to interact with *GSTP1*. Three other genes belonging to the same family (*FANCA*, *FANCD2*, and *FANCG*) are known to interact with *FANCC*. Proteins encoded by *FANCA*, *FANCC*, and *FANCG* participate in the formation of the FA complex. *FANCD2*, *BRCA1* (breast cancer 1), and *RAD51* (RecA homologue) are downstream targets of the FA complex. Protein products of these genes have putative roles in predisposition to, or development of, carcinomas in young individuals. We thus reasoned that it would be useful to evaluate whether deregulated expression of these genes was also observed in OSCCs of these patients.

RNA ISOLATION

Isolation of RNA was performed only on frozen samples. Tissues were homogenized in liquid nitrogen; total RNA was extracted using the Trizol reagent protocol²² and purified on a Qiagen mini-column (Qiagen, Valencia, Calif). We evaluated RNA quantity and quality by spectrophotometry and by electrophoresis on a 1.2% denaturing formaldehyde agarose gel. The RNA from all normal and tumor samples was found to be of high quality for QRT-PCR.

QUANTITATIVE REAL-TIME RT-PCR

The ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, Calif), with SYBR Green I fluorescent dye, was used for relative quantification of gene expression. Data were quantified and analyzed using the Sequence Detection System software, version 1.7 (PE Applied Biosystems).

PRIMERS

Complementary DNA (cDNA) sequences of *GSTP1*, *FANCA*, *FANCC*, *FANCD2*, and *FANCG* were selected (<http://www.ncbi.nlm.nih.gov/>), and primers were designed using Primer Express software, version 1.5 (PE Applied Biosystems). Primer sequences are available on request.

PCR AMPLIFICATION

Complementary DNA was reverse transcribed from 2 µg of total RNA from each sample or from Human Universal RNA (Stratagene). The 25-µL reaction mixtures contained 0.4 µM of each primer and 12.5 µL of 2X SYBR Green PCR Master Mix (PE Applied Biosystems), including SYBR Green I dye, 0.5 U of uracyl-N-glycosylase (UNG) enzyme, 1.25 U of DNA polymerase, and 200 µM of dideoxynucleotides. Amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. Each PCR assay included a human universal cDNA sample reverse transcribed from Human Universal RNA and a no-template control. Experiments were performed in duplicate for each sample in the same assay. The housekeeping gene *GAPDH* was used as an internal control and amplified in the same reaction plate. In all samples analyzed, *GAPDH* levels were comparable, permitting the use of this gene to normalize the amount of the amplified targets. Optimal hybridization kinetics of primers, and therefore amplification efficiency, was assessed using a standard curve consisting of serial dilutions of Human Universal cDNA (1000, 500, 250, 125, and 62.5 ng/µL). Primers were used only if correlation coefficients were 98% or higher.

ANALYSIS OF QRT-PCR RESULTS

Quantification of the expression of the target gene in unknown samples is accomplished by measuring the cycle number (Ct) at which the amount of PCR product reaches a set threshold and is directly related to the amount of starting template. The relative quantification was given by the Ct values, determined using duplicate reactions for test and reference samples for each target and for the internal control gene (*GAPDH*). This was performed following the Delta Delta Ct method of data analysis.²³ In this method, duplicate Ct values are averaged and the *GAPDH* Ct subtracted to obtain ΔCt [$\Delta Ct = Ct(\text{target gene}) - Ct(\text{GAPDH gene})$]. Values for ΔCt are calculated for each tumor sample and reference sample (Human Universal RNA). Relative expression level is determined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{target sample}) - \Delta Ct(\text{reference sample})$. For the reference sample, $\Delta\Delta Ct = 0$, and $2^0 = 1$, so the expression value in the reference sample equals 1, by definition. For the unknown samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the reference sample. The level of gene expression in tissue samples was measured relative to the Human Universal RNA.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections 4- to 5-µm thick were cut from paraffin-embedded tissue blocks, and IHC analysis was performed using the Avidin-

Biotin peroxidase method. Slides were deparaffinized in xylene, hydrated, and endogenous peroxidase activity was blocked by incubation in 3% (v/v) aqueous hydrogen peroxide (ID Laboratories Inc, London, Ontario) for 15 minutes. Slides were then washed in running water and heated for 15 minutes at 100°C in 10mM sodium citrate buffer (pH 6.0) for antigen retrieval. Sections were treated for 10 minutes with 10% normal serum from the species where the secondary linking antibody was obtained.

Antibody dilutions and incubation with the primary antibodies was performed as follows: BRCA1 (Oncogene, La Jolla, Calif) (1:200), overnight at room temperature; Glutathione S-Transferase Pi (MediCorp, Montreal, Quebec) (1:50), 1 hour at room temperature; RAD51 (MediCorp) (1:500) with casein, overnight at room temperature; FANCD2 (Novus Biologicals, Littleton, Colo) (1:1000) with bovine serum albumin, 1 hour at room temperature; and FANCG (Cedarlane Laboratories, Hornby, Ontario) (1:200), overnight at room temperature. Incubation with species-specific biotinylated secondary linking antibody (ID Laboratories Inc) was done for 30 minutes at room temperature. Sections were then incubated for 30 minutes at room temperature with Ultra Streptavidin-Horseradish Peroxidase Complex (Signet Level 2, Dedham, Mass) diluted by ¼ with Dako antibody diluting buffer.

The color was developed using NovaRed (Vector Laboratories, Burlington, Ontario) for 5 to 10 minutes and counterstained with Mayer hematoxylin. Finally, the slides were mounted with D.P.X. mountant (Fluka, Buchs, Switzerland). To ensure consistency, all samples were stained simultaneously in a single experiment. Controls included 3 separate normal mucosa samples from patients diagnosed as having conditions other than cancer. For negative controls, the appropriate antibody was omitted, and either the antibody diluent alone or isotype-matched IgG serum was used.

POSITIVE CRITERIA FOR IHC STAINING

Tissue sections were graded as immunopositive if 10% or more of the epithelial cells showed nuclear staining. Protein expression in the tissue sections was evaluated using a semiquantitative scoring system: 0 for absence of immunostaining or detectable immunostaining in fewer than 10% of cells; +1 for 10% to 30% positively stained cells; +2 for 31% to 60% positively stained cells; and +3 for more than 60% positively stained epithelial cells. The immunostaining of the proteins was evaluated in 5 different areas of the slide sections for confirmation of the IHC data. This analysis was performed independently by 2 of us (S.T. and G.B.) blinded to the identity of the samples.

STATISTICAL ANALYSES

Statistical analyses of the QRT-PCR and IHC data were performed using the Statistical Package of Social Sciences (SPSS for Windows, version 10.1; SPSS Inc, Chicago, Ill). The Wilcoxon signed-rank test was used to compare the mRNA levels of paired sample sets from the young and older patients with OSCC (young nondysplastic mucosa vs young tumor; older nondysplastic mucosa vs old tumor). The Mann-Whitney test was used to compare the mRNA levels of 2 independent sample sets from the same groups of patients (young nondysplastic vs old nondysplastic, young tumor vs old tumor). Adjustments for multiple comparisons were not performed. The χ^2 test was used to evaluate differences in the expression of genes and their protein products measured by IHC analysis (tumors from young vs older patients). It was also used to compare the clinical data between the 2 groups of patients and to evaluate the differ-

ences between smokers and nonsmokers. Statistical significance was defined as $P \leq .05$.

RESULTS

STATISTICAL CORRELATIONS WITH CLINICAL AND HISTOPATHOLOGIC DATA

Clinical and histopathologic data are listed in **Table 1**. For each variable (eg, age, sex, and tobacco use), the number of patients compared is similar between subgroups used for QRT-PCR and IHC analysis. Statistical analysis was carried out for all patients with available clinical information. We observed that tumors occurring in the young patient group were preferentially located on the oral tongue, representing 31 (74%) of 42 cases ($P < .001$) compared with older patients, who showed tumors of the tongue in 13 (21%) of 62 cases. In the older patients, the most common location of tumors was the floor of the mouth, seen in 19 (31%) of 62 cases. Alcohol consumption was reported in 10 (29%) of 34 younger patients in contrast to 25 (61%) of 41 older patients ($P = .01$). There was no significant difference in the proportion of smokers between the young and older patient groups, with 21 (62%) of 34 vs 30 (73%) of 41 patients smoking, respectively. Tobacco smoking showed no correlation with any of the other clinical parameters, including tumor site, stage, grade, and outcome.

QUANTITATIVE REAL-TIME RT-PCR

Relative expression levels of *GSTP1*, *FANCA*, *FANCC*, *FANCD2*, and *FANCG* were determined in 32 OSCC samples (10 from young patients and 22 from older patients), their paired nondysplastic mucosa, and 4 normal tongue mucosa samples, from which frozen tissue was available for RNA extraction. Human Universal RNA was used as baseline (expression = 1). Median expression levels were compared between tumor and nondysplastic adjacent mucosa, within and between the 2 groups of patients: young and older. The expression levels of the 2 healthy young individuals and of the pooled RNA samples from 2 healthy older individuals were as follows: *GSTP1* (11.06 vs 3.98), *FANCA* (0.044 vs 0.031), *FANCC* (1.602 vs 0.6), *FANCD2* (0.074 vs 0.075), and *FANCG* (1.321 vs 0.536). These expression levels were different, in some cases, from the paired nondysplastic mucosa of young and older patients. This is expected, however, because the number of patients in the healthy control group was small and also because nondysplastic mucosa may not be genetically normal.

When we compared tumors of young and older patients, a significantly lower expression of *GSTP1* (4.8 vs 11.78, 2.45-fold change) ($P = .04$) and *FANCA* (0.11 vs 0.23, 2.10-fold change) ($P = .01$) was observed in tumors of young patients. In young patients, *GSTP1* had a 1.5-fold decrease in expression in tumors (4.800) vs paired nondysplastic mucosa (7.327) ($P = .01$).

GSTP1 expression was increased in tumors of older patients (11.78) compared with their paired nondysplastic mucosa (8.35), although this was not a statistically significant change ($P = .15$). We also observed increased

Table 1. Clinical Characteristics of Oral Squamous Cell Carcinoma Samples From Young and Older Patients*

Characteristic	Young (n = 42)	Older (n = 62)
Age, mean (range), y	34.3 (21-40)	73.3 (60-95)
Sex		
Male	29 (69)	34 (55)
Female	13 (31)	28 (45)
Tobacco use		
Yes	21 (62)	30 (73)
No	13 (38)	11 (27)
N/A	8	21
Alcohol use		
Yes	10 (29)	25 (61)
No	24 (71)	16 (39)
N/A	8	21
Tumor site		
Oral tongue	31 (74)	13 (21)
FOM	3 (7)	19 (31)
Buccal mucosa	3 (7)	10 (16)
Alveolus	2 (5)	8 (13)
Tongue/FOM	1 (2)	5 (8)
Tongue base	1 (2)	3 (4)
Palate	1 (2)	2 (3)
Retromolar	0	2 (3)
Stage		
I	5 (15)	4 (10)
II	5 (15)	13 (32)
III	6 (18)	7 (17)
IV	17 (52)	17 (42)
N/A	9	21
Grade		
WD	0	14 (29)
MD	36 (92)	31 (65)
PD	3 (8)	3 (6)
N/A	3	14
QRT-PCR, No. of samples	10†	22
IHC, No. of samples	37†	40

Abbreviations: FOM, floor of the mouth; IHC, immunohistochemical analysis; MD, moderately differentiated; N/A, not available; PD, poorly differentiated; QRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction analysis; WD, well differentiated.

*Unless otherwise noted, data are presented as number (percentage) of patients except where N/A, for which number only is reported. Numbers and percentages shown were calculated based on number of patients from whom clinical information was available.

†In the young patient group, 5 samples were used for both QRT-PCR and IHC.

GSTP1 levels in tumors of older patients (11.78) compared with the pooled RNA samples from 2 healthy older individuals (3.98).

In the tumors of older patients, compared with their paired nondysplastic adjacent mucosa, we detected a significant increase in expression of *FANCA* (0.16 vs 0.23, 1.43-fold difference) ($P = .02$). The same was observed for *FANCC* (0.72 vs 0.97, 1.34-fold increase) ($P = .04$), *FANCD2* (0.52 vs 1.07, 2.06-fold increase) ($P = .002$), and *FANCG* (0.25 vs 0.47, 1.88-fold increase) ($P = .04$). Finally, *FANCA* expression was significantly lower in nondysplastic mucosa of young (0.043) compared with older patients (0.16) ($P = .01$).

These results are shown in **Figure 1**, including the median expression levels and the interquartile ranges, which contain 50% of values of *GSTP1*, *FANCA*, *FANCC*, *FANCD2*, and *FANCG* in the 2 groups of patients. **Table 2**

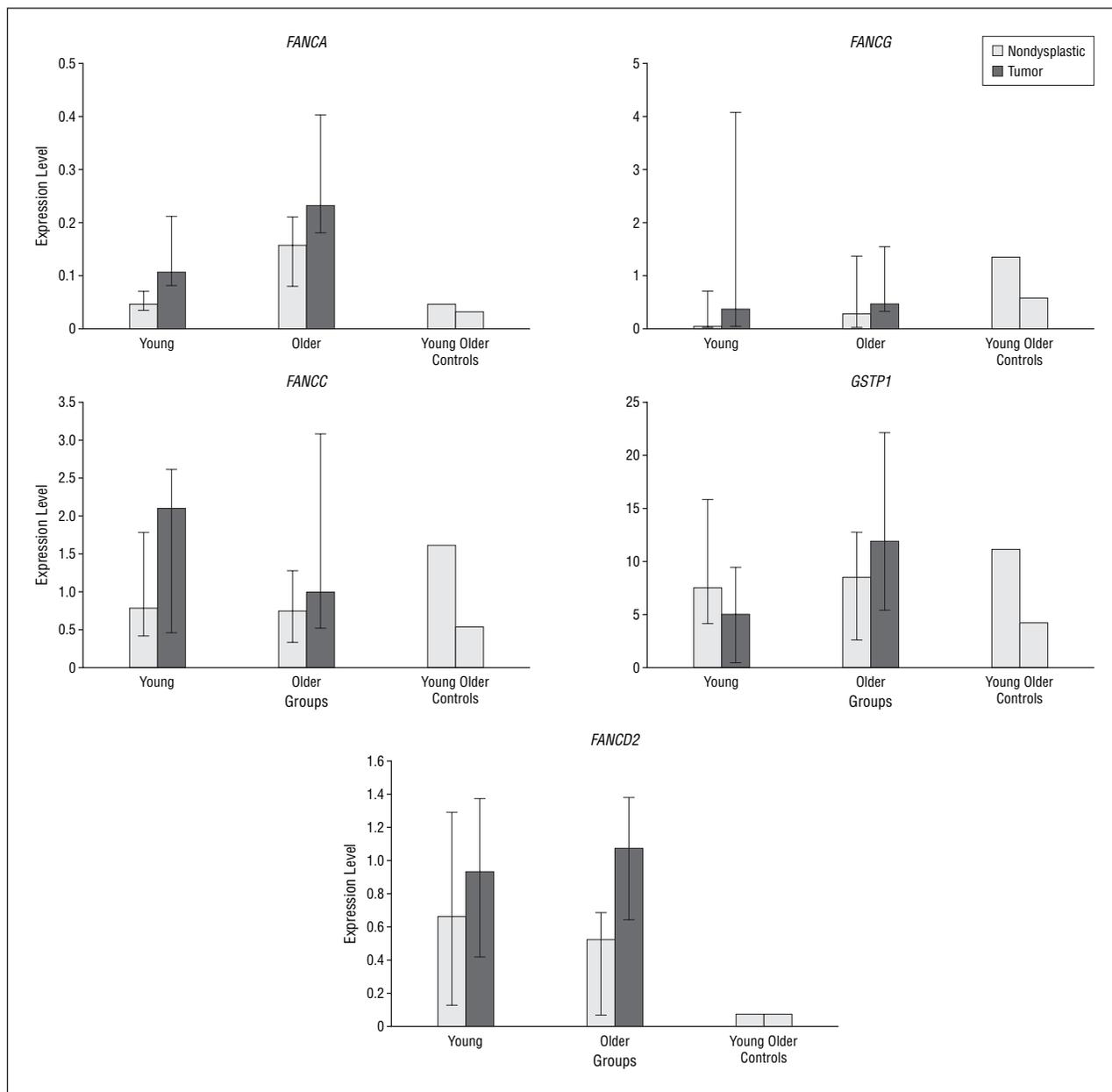


Figure 1. Relative expression levels of the genes investigated by real-time reverse transcriptase–polymerase chain reaction and calculated by the Delta Delta Ct method (see the “Methods” section) of data analysis, including median values (bars) and interquartile ranges (brackets), which contain 50% of values. Graphics show a comparison between the expression levels of each gene in the 2 different groups (young and older patients) for nondysplastic and tumor samples. Table 2 lists the significant *P* values.

lists the median values of gene expression for each group of samples (young nondysplastic, young tumor, older nondysplastic, and older tumor) and the statistical analysis results. Expression levels of *RAD51* and *BRCA1* were not measured by QRT-PCR, since IHC analysis results showed no significant differences between the 2 groups of patients and also owing to the limited amount of mRNA available.

IHC ANALYSIS

Immunohistochemical analysis was performed to evaluate the protein expression levels of *GSTP1*, *FANCG*, *RAD51*, *BRCA1*, and *FANCD2* in OSCCs from 37 young

and 40 older patients. *GSTP1*, *FANCD2*, *RAD51*, and *BRCA1* showed a diffuse nuclear staining pattern, whereas *FANCG* showed an intranuclear body staining pattern. **Figure 2** shows representative examples of absent and low or moderate and high expression of each protein tested. Immunohistochemical staining for *FANCA* and *FANCC* was not done owing to the lack of commercially available antibodies suitable for IHC analysis. *GSTP1* showed negative or low expression in 15 (41%) of 37 young patient tumors compared with 2 (5%) of 40 tumors from older patients ($P = .001$). We observed absent or low expression of *FANCG* in 30 (81%) of 37 young patient tumors compared with 15 (38%) of 40 tumors from older patients ($P < .001$) (**Table 3**). The same trend,

Table 2. Relative Median Expression Levels of the 5 Genes of Interest in the Young and Older Patient Samples by Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Gene	Relative Median Expression				P Value*			
	Young		Older		Young Nondysplastic vs Tumor	Older Nondysplastic vs Tumor	Young Nondysplastic vs Older Nondysplastic	Young Tumor vs Older Tumor
	Nondysplastic	Tumor	Nondysplastic	Tumor				
<i>GSTP1</i>	7.33	4.80	8.35	11.78	.01	.15	>.99	.04
<i>FANCA</i>	0.04	0.11	0.16	0.23	.06	.02	.01	.01
<i>FANCC</i>	0.77	2.10	0.72	0.97	.11	.04	>.99	>.99
<i>FANCD2</i>	0.66	0.92	0.52	1.07	.98	.002	.31	>.99
<i>FANCG</i>	0.02	0.36	0.25	0.47	.17	.04	.70	.65

*Wilcoxon test was used to compare paired sample sets from the young and older patients with oral squamous cell carcinoma (young nondysplastic vs young tumor and older nondysplastic vs older tumor groups); Mann-Whitney test was used to compare 2 independent sample sets (young nondysplastic vs older nondysplastic and young tumor vs older tumor groups).

although not statistically significant ($P=.33$), was observed for mRNA expression level of *FANCG*. No significant difference in staining was observed between tumors from the 2 groups of patients for *RAD51* ($P=.15$), *BRCA1* ($P=.08$), and *FANCD2* ($P=.88$) proteins. Because only 5 samples were available for both QRT-PCR and IHC analysis, no statistical correlation was performed.

In addition, to ensure that changes observed in the young patients were not the end result of human papillomavirus (HPV) infection, as suggested by another study,²⁴ we performed IHC analysis to evaluate the presence of the high-risk types of HPV, 16 and 18, in both groups of patients. Similar rates of infection were found in both groups of patients, with 31 young patient samples (83%) and 39 older patient samples (98%) harboring HPV. Similar levels of infection in OSCC samples have been previously reported.^{25,26} Therefore, in our study, the presence of HPV infection was not correlated with deregulated *FA/GSTP1* gene/protein expression.

COMMENT

The glutathione S-transferase superfamily of enzymes acts to conjugate or reduce exogenous or endogenous compounds, such as redox species, generated during oxidative metabolism and stress.^{27,28} This superfamily consists of several classes of glutathione S-transferases, including *GSTP1*, which is associated with genetic susceptibility to cancer and other diseases.²⁹

GSTP1 is commonly overexpressed in human tumors such as ovarian cancer, colorectal carcinoma, and melanoma.³⁰⁻³² This increased expression is believed to be associated with overexpression of *SPI1*, *API1*, and nuclear factor κ B, which regulate *GSTP1* transcriptional activity.³³ High expression levels of *GSTP1* have been reported in head and neck squamous cell carcinomas,^{27,34,35} in agreement with our results showing *GSTP1* overexpression in tumors of older patients. Increased expression levels of *API1* and nuclear factor κ B, known to occur in head and neck carcinomas, may help explain this finding.^{36,37}

In the present study, tumors of young patients showed a 1.5-fold decrease in expression of *GSTP1* compared with their nondysplastic mucosa, and a 2.3-fold decrease com-

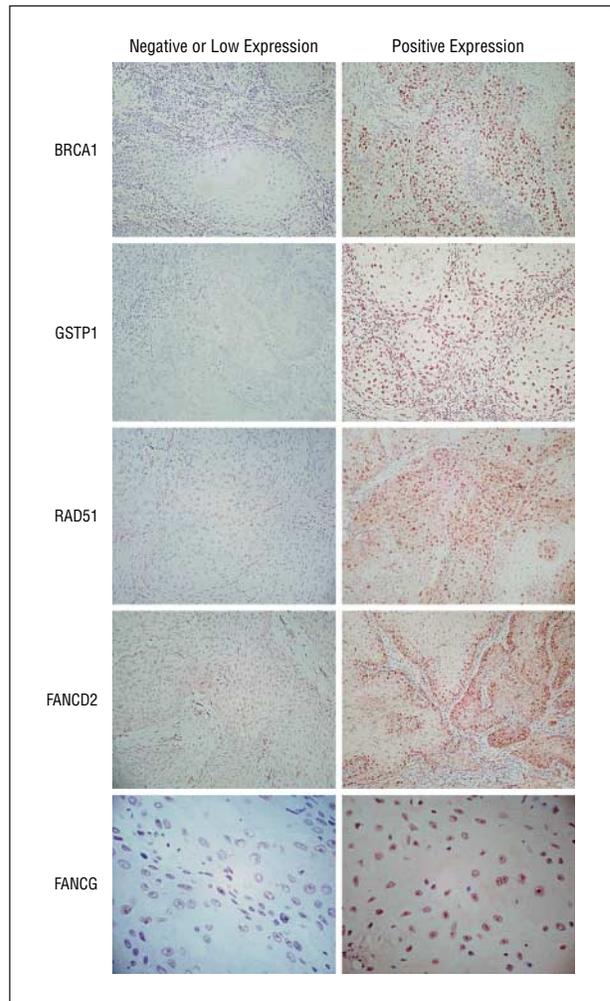


Figure 2. Representative examples of absent and low expression compared with positive expression of the proteins tested by immunohistochemical analysis. All proteins show total nuclear staining except *FANCG*, which shows a nuclear body staining pattern.

pared with the normal tongue mucosa from young controls. The low expression level of *GSTP1* in tumors of young patients was confirmed by IHC analysis. In other tumor types, such as endometrial carcinoma, hepatocellular carcinoma, and prostate adenocarcinoma, where *GSTP1* is underexpressed, hypermethylation of the pro-

Table 3. Immunohistochemical Analysis Results for *GSTP1* and *FANCG* in the Young and Older Patient Tumors

Protein Expression	Grading	<i>GSTP1</i>		<i>FANCG</i>	
		Young	Older	Young	Older
Absent	0	8	0	25	6
Low	+1	7	2	5	9
Moderate	+2	9	14	7	12
High	+3	13	24	0	13
Total		37	40	37	40
<i>P</i> value*		.001		<.001	

**P* value determined by χ^2 test.

motor region appears to be the main mechanism of deregulation.³⁸⁻⁴⁰ The same mechanism could be responsible for *GSTP1* underexpression in OSCC of young patients. We are currently investigating this possibility.

High expression of *GSTP1* has been associated with the development of antineoplastic drug resistance.⁴¹ In contrast, silencing of this gene has been shown to enhance tumor sensitivity to the same drugs.³² Low expression of *GSTP1* could thus be associated with a better response to chemotherapy and improved prognosis.

We hypothesize that *GSTP1* underexpression may contribute to the early steps of the oncogenic process through a decrease in the cellular ability to detoxify or inactivate carcinogens, thus permitting carcinogens to interact with DNA and initiate and/or promote cancer. In a *GSTP1*^{-/-} knockout mouse model, cutaneous papillomas developed earlier and at a much higher rate in the knockout than in wild-type mice when a carcinogen was applied to their skin.⁴² We suggest that decreased *GSTP1* expression may be associated with malignant transformation of oral mucosa in young individuals, although, admittedly, our results include only a limited number of young patients analyzed by QRT-PCR.

One of the FA complex genes, *FANCC*, binds and regulates *GSTP1*.¹⁷ Fanconi anemia is a cancer predisposition syndrome characterized by bone marrow failure with an increased incidence of aplastic anemia, myelodysplastic syndrome, and acute myeloid leukemia at a young age. Patients with FA also have a higher risk of developing solid malignant tumors, the most frequent involving the cervix, vulva, pharynx, esophagus, liver, and most immediately relevant, the oral cavity.^{43,44} The cumulative risk of developing OSCC in patients with FA is 14% by age 40 years,⁴⁵ representing an 1100-fold increase in the incidence of oral cancer compared with the general population.

Eight tumor suppressor genes have been associated with FA: *FANCA*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, and *FANCL*. Of these, 6 form the FA protein complex, which is involved in homologous recombination repair via *BRCA1*, *BRCA2*, and *RAD51*⁴⁶ and in repair through nonhomologous end joining.⁴⁷ Decreased expression or abnormal function of these proteins in patients with FA leads to genetic instability,⁴⁸⁻⁵⁰ which likely plays a role in the increased incidence of solid tumors.

Thus, deregulated expression of *GSTP1* and FA genes may lead to carcinogenesis through deficient detoxification and defective DNA repair capability.

In our study, *FANCA* expression was statistically significantly different in the nondysplastic mucosa from young and older patients. We also detected lower expression of *FANCA* in the 4 normal tongue mucosa samples compared with the nondysplastic mucosa from older patients. This higher expression of *FANCA* in older patient mucosa could be the result of a long-term carcinogen exposure and is unlikely age related, since mucosa from older healthy individuals shows an expression level similar to that of younger healthy individuals.

We also detected a significant difference in expression of *FANCA* between tumors from young and older patients. Although *GSTP1* is regulated by *FANCC*, which is, like *FANCA*, part of the FA complex, it is unknown if a lower expression of *FANCA* could directly trigger a decrease in expression of *GSTP1* and contribute to development of OSCC.

We observed significantly higher expression levels of *FANCA*, *FANCC*, *FANCD2*, and *FANCG* in tumors of older patients compared with their paired nondysplastic mucosa. These alterations are counterintuitive based on the tumor suppressor function of these genes and their role in DNA double-strand break repair.^{51,52} We therefore hypothesize that overexpression of these FA genes in older patients represents a consequence rather than cause of tumor development. To our knowledge, this is the first report showing overexpression of these FA genes in OSCC of older patients.

Similar patterns of *FANCC* expression, by QRT-PCR analysis, were observed in nondysplastic adjacent tissues and tumors from young and older patients; therefore, *FANCC* did not distinguish between these 2 groups of patients. In our study group, although not statistically significant (*P* = .15), tumors from older patients showed a slight increase in expression of *GSTP1* compared with their nondysplastic mucosa. This may be triggered by the increase in *FANCC* expression, which has been shown to up-regulate *GSTP1* expression.¹⁷ However, tumors from the young patient group showed a decrease in *GSTP1* expression despite *FANCC* levels similar to those of the older patient group. This finding suggests that different mechanisms of *GSTP1* deregulation may be involved in head and neck tumorigenesis of young vs older patients. Such mechanisms may involve proteins other than *FANCC* that may also regulate *GSTP1* expression, or they may involve epigenetic alterations such as hypermethylation of *GSTP1* in tumors of young patients, which is a mechanism known to result in *GSTP1* underexpression in other tumors.⁵³

To our knowledge, this is the first report showing deregulation of *GSTP1* exclusively in OSCC from young patients, and the findings suggest that these tumors are genetically different from those that develop in older patients. Aberrant expression of *GSTP1* and FA genes may lead to carcinogenesis through deficient detoxification and defective DNA repair capability. Further studies into the role of *GSTP1* and the FA gene complex in initiation or progression of OSCC in this unique patient group should be pursued. These findings may impact lifestyle and lead to improvement of treatment planning and more appropriate follow-up of young patients with OSCC.

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