Cytochrome P450 and Glutathione Transferase Expression in Squamous Cell Cancer

Shadan Ali, Basil F. El-Rayes, Lance K. Heilbrun, Fazlul H. Sarkar, John F. Ensley, Omar Kucuk, and Philip A. Philip

Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

ABSTRACT

Purpose: The cytochrome P-450 (CYP) and glutathione *S*-transferase (GST) enzyme systems modulate the carcinogenic effects of tobacco. Therefore, the expression of these enzymes may be in part responsible for the observed inter-individual and inter-racial differences in the risk of development of squamous cell carcinoma of the head and neck (SCCHN). The first aim of this study was to evaluate the feasibility of measuring the expression of the CYP and GST in target tissue from the head and neck. The second aim was to compare the expression of CYPs 1A1, 2E1, and 3A4 in squamous epithelium from African-American and Caucasian pediatric patients. The third aim was to compare the expression of CYPs 1A1, 2E1, 3A4, and GST-π on the p16 expression in patients with SCCHN.

Experimental Design: The expression of CYP 1A1, 2E1, 3A4, GST-π, and p16 was quantified by immunoblotting. Expression of CYPs 1A1, 2E1, and 3A4 was quantified in tissue from 160 pediatric patients undergoing tonsillectomy. Expression of CYPs 1A1, 2E1, 3A4, GST-π, and p16 was determined in 46 resected SCCHN patients.

Results: Large interindividual variability in the expression of these enzymes was observed in the pediatric and adult populations. No significant difference was observed in CYP 1A1, 2E1, and 3A4 expression of Caucasian and African-American patients. There was no correlation between p16 and enzyme expression in patients with SCCHN.

Conclusion: Evaluation of CYP expression in the target tissue of interest is feasible. The clinical significance of CYPs and GST-π alterations in the risk of developing SCCHN will need to be investigated in larger trials.

INTRODUCTION

Tobacco exposure is an established risk factor for several cancers including squamous cell cancer of the head and neck (SCCHN; Ref. 1). The development of tobacco related malignancies appears to depend on the duration and intensity of exposure to the carcinogen (2) as well as genetic susceptibility (1, 2). The presence of genetic susceptibilities is confirmed by the higher incidence of cancer in first-degree relatives of patients with SCCHN (3) and the inter-racial differences in the susceptibility to the carcinogenic effects of tobacco (4). Understanding the mechanisms of susceptibility will facilitate the prevention and early detection of SCCHN.

The carcinogen metabolizing enzymes are involved in the activation and deactivation of diverse chemical carcinogens. Interindividual and inter-racial variations in the expression of these enzymes in target tissues may explain the different susceptibilities observed in clinical and epidemiological studies (5). Cytochrome P450 (CYP) is a superfamily of heme-containing mono-oxygenases that is involved in the metabolism of a wide variety of endogenous and exogenous compounds (6). Enzymes of the CYP family may have different but overlapping substrate specificities (Table 1; Refs. 6, 7). The regulation of CYP expression is in part tissue-specific (8). Marked enzymatic activities of CYPs 1A1, 2E1, and 3A4 have been demonstrated in the human head and neck squamous epithelium (9). Glutathione *S*-transferases (GSTs) are involved in the Phase II metabolism that generally detoxifies carcinogens. GST-π has been shown to be expressed in the human squamous epithelium (10).

Carcinogens and their metabolites result in malignant transformation through mutations altering the function of critical proto-oncogenes or tumor suppressor genes. The inactivation of the *p16* gene is an early event in the development of SCCHN that occurs in up to 80% of patients (11). Therefore, the p16 expression may be used as a surrogate for the carcinogenic effects of tobacco in SCCHN.

The aim of this study was to evaluate the effects of the expression of CYP and GST-π enzymes on the development of SCCHN. We hypothesized that the expression of the CYPs 1A1, 3A4, and 2E1 would be higher in normal tissue from nonsmokers of African-American descent as compared with Caucasians. This might explain the observed increased susceptibility to the carcinogenic effects of tobacco in African Americans. The second hypothesis was that the expression of CYPs would be lower in the SCCHN tissue as compared with the normal adjacent tissue. The third hypothesis was that a higher expression of CYPs 1A1, 3A4, and 2E1 in normal tissue from patients with SCCHN would be associated with a lower p16 expression in the tumor tissue, and that a higher expression of GST-π in normal tissue from patients with SCCHN would be associated with a higher p16 expression.

Table 1 The biologic activity of CYP[a] enzymes assayed in this study

	Activity	Ref
CYP 1A1	Oxidative activation of polycyclic aromatic hydrocarbons and nitrosoamines forming keto-aldehydes	7
CYP 3A4	Oxidative activation of polycyclic aromatic hydrocarbons and nitrosoamines forming keto-aldehydes	7
CYP 2E1	Oxidative activation of polycyclic aromatic hydrocarbons and nitrosoamines forming keto-alcohols	7
	Metabolism of small molecules (alcohol)	6

[a] CYP, cytochrome P450.

MATERIALS AND METHODS

Study Population. Pediatric patients undergoing tonsillectomy and adult patients with SCCHN undergoing surgical excision were eligible for this study. All of the patients provided a signed informed consent in accordance with the Wayne State University Human Investigation Committee guidelines before enrolment on the study.

Tissue Collection. The pathological material was obtained fresh at time of surgery. The pathologist histologically examined tissue from patients with SCCHN. Tumor samples were obtained from unequivocal sites within the specimen. Normal tissue samples were obtained from morphologically normal adjacent tissue that was at least 1 cm away from the cancer site. All of the samples were covered with aluminum foil, sealed in plastic bags, coded, and stored at $-70°C$ until analysis. Assays were done in batches of 10–20 samples and without knowledge of the sample source.

Western Blot Analysis (Immunoblotting). The minimum tissue weights sufficient for all of the intended analyses were 200 mg. Tissue samples were suspended in 2 ml of homogenizing buffer (pH 7.4) containing 50 mM Tris, 0.25 M sucrose, and 1 mM EDTA. The samples were then homogenized twice followed by centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant was centrifuged at 45,000 rpm for 1 h at 4°C. The microsome pellet was suspended by sonication in 25 μl of microsomal storage buffer (50 mM Tris, 20% glycerol, and 1 mM EDTA). The concentration of proteins was determined by bicinchonic acid assay using spectrophotometer and measured at an absorbance of 562 nm.

Approximately 20 μg of protein from each sample were loaded onto a 10% SDS-polyacrylamide gel along with three different concentrations of each of the known enzyme standards. Gels were run at 120 V for 90 min and subsequently transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature by PBS/0.05 Tween 20 containing 5% powdered milk. It was then treated with the primary antibody 3A4, 1A1, or 2E1 (Gentest Corporation, Woburn, MA), GST-π (Oxford Biomedicals, Rochester Hills, MI), or p16 (Santa Cruz Biotechnology, Santa Cruz, CA) to the enzymes using optimized dilutions. Membranes were washed and treated with a secondary antibody (horseradish peroxidase conjugate). Protein bands were detected by enhanced chemiluminescent substrate. β-Actin expression was used to standardize the loading of the protein sample.

The intensity of the protein band was quantified by an image analysis system (Storm System; Molecular Dynamics, (Piscataway, NJ). The intensity of the bands from the patient samples was determined from the standard linear curve encompassing the actual experimental measurements and corrected for the expression of β-actin in each sample. Enzyme levels were expressed in relative arbitrary units.

Statistical Methods. The enzyme distributions were highly skewed and non-normal, so nonparametric methods of analysis were used (12). Comparisons of enzyme levels between subgroups of patients were performed using the Kruskal-Wallis rank sum test (for three groups) or the Wilcoxon rank sum test (for two groups).

The strength of association between enzymes was assessed via a Spearman's rank correlation. To control overall type I error, adjustment for multiple comparisons was performed using the Holm method (13).

RESULTS

Tonsillectomy Specimens. One-hundred and sixty pediatric patients undergoing tonsillectomy were enrolled on the study between June 1996 and August 1997 (Table 2). Fifty-three (34%) of the patients were African American. The distribution of patients by gender did not differ significantly over the race or the age groups. Similarly, the distribution of race did not differ significantly across the age groups. Wide interindividual variations in the expression of enzymes were noted. No significant relationship between the CYP 2E1, and either 1A1 ($r = 0.133$; $P = 0.214$) or 3A4 ($r = 0.160$; $P = 0.142$) enzymes was identified in this study indicating that the regulation of these isoenzymes is independent. A significant correlation was observed between CYP 1A1 and 3A4 ($r = 0.224$; $P = 0.008$) even after adjustment by the Holm procedure for the type I error of 6 correlations. Expression of CYP enzymes 1A1, 2E1, and 3A4 were compared by age group, gender, and race (Table 3). There were no significant differences by gender, age, or race in the level of CYP 1A1, 2E1, and 3A4.

Table 2 The characteristics of the 160 pediatric patients undergoing tonsillectomy and the 46 patients with SCCHN[a] enrolled on this study

	Number	Percentage
Tonsillectomy group	160	
Age		
<5 years	67	42
5–6 years	46	29
>7 years	43	28
Race		
African American	53	34
Caucasian	80	52
Other	21	14
Gender		
Male	99	62
Female	61	38
SCCHN group	46	
Age	60 years (mean)	
Gender		
Male	28	60
Female	18	40

[a] SCCHN, squamous cell carcinoma of the head and neck.

Table 3 A summary of enzyme expression levels in squamous epithelium from patients who underwent tonsillectomy

CYP[a] 1A1, 2E1, and 3A4 levels were determined by immunoblotting. Results are represented by arbitrary units from densitometric measurements of protein bands relative to β-actin expression in each sample.

Race		CYP 1A1	CYP 2E1	CYP 3A4
African American	*n*	46	31	47
	Median	1.915	0.320	1.820
	Mean	2.067	0.350	2.007
	SD	1.439	0.273	1.105
Caucasians	*n*	72	54	73
	Median	1.750	0.310	1.720
	Mean	1.874	0.350	1.777
	SD	1.306	0.254	1.046
Other	*n*	19	12	20
	Median	1.670	0.430	1.695
	Mean	1.813	0.522	2.243
	SD	1.161	0.420	1.580
P		0.788	0.226	0.446

[a] CYP, cytochrome P450.

Table 4 A summary of enzyme CYPs[a] 1A1, 2E1, 3A4, and GST-π expression levels in morphologically normal tissue adjacent to SCCHN tumor and expression of p16 in tumor tissue from SCCHN specimens

Expression levels were determined by immunoblotting. Results are represented by arbitrary units from densitometric measurements of protein bands relative to β-actin expression in each sample.

		Female	Male	*P*
CYP 1A1	*n*	16	27	0.359
	Median	0.291	0.298	
	Mean	0.264	0.390	
	SD	0.188	0.295	
CYP 2E1	*n*	16	27	0.297
	Median	0.092	0.130	
	Mean	0.132	0.223	
	SD	0.138	0.215	
CYP 3A4	*n*	16	28	0.121
	Median	0.467	0.650	
	Mean	0.554	0.800	
	SD	0.436	0.562	
GST-π	*n*	15	26	0.077
	Median	0.100	0.088	
	Mean	0.134	0.174	
	SD	0.052	0.190	
P16	*n*	18	21	0.044
	Median	0.658	0.144	
	Mean	0.589	0.322	
	SD	0.394	0.388	

[a] CYP, cytochrome P450; GST, glutathione *S*-transferase; SCCHN, squamous cell carcinoma of the head and neck.

The SCCHN Specimens. Forty-six patients were enrolled on the study. CYP 1A1, 2E1, 3A4, and GST-π levels of expression did not differ significantly by gender (Table 4). Fig. 1 demonstrates a representative immunoblot of the four enzymes that were studied. Fig. 2 demonstrates a representative immunoblot of p16 expression. Wide interindividual variations in the expression of enzymes were noted. No significant correlation between the CYP 2E1 and either 1A1 ($r = 0.14$) or 3A4 ($r = 0.21$) enzymes was identified in this study indicating that

the regulation of these isoenzymes is independent. The strongest correlation observed was between CYP 1A1 and 3A4 ($r = 0.406$). There were no significant correlations between p16 and the level of expression of CYP 1A1, 2E1, 3A4, and GST-π. The strongest correlation was a negative one between p16 and CYP 1A1 ($r = -0.164$).

A comparison of the expression of GST-π, CYP 3A4, 1A1, and 2E1 between histologically normal and malignant tissue from 16 patients with SCCHN is shown in Fig. 3. A significantly higher expression of CYP 3A4 and 1A1 was observed in normal tissue, even after adjustment for the type I error of a set of four comparisons. No difference in expression of CYP 2E1 and GST-π was present.

DISCUSSION

Polycyclic aromatic hydrocarbons, aromatic amines, and nitroso compounds are among the carcinogens that have been identified in tobacco smoke (14). The CYP enzymes oxidize these compounds yielding reactive epoxide intermediates, which can covalently bind and alter DNA structure (15). GST enzymes catalyze glutathione conjugation of these intermediates thereby decreasing their DNA damaging effects (16). Variations in the expression of CYPs and GST could potentially explain the observed difference in vulnerability to the carcinogenic effects
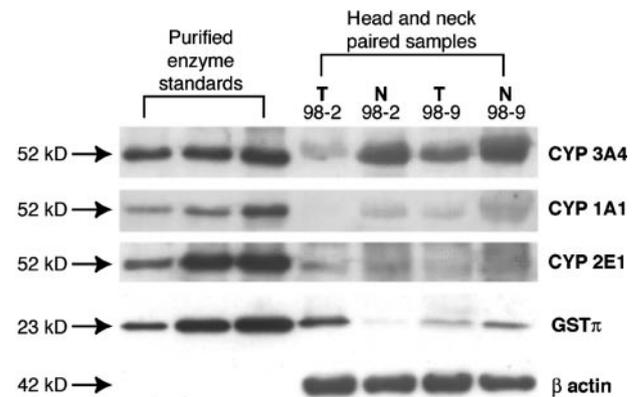
Fig. 1 A representative immunoblot of two paired tumor (*T*)/normal (*N*) tissue samples probed for CYP 1A1, 2E1, 3A4, glutathione *S*-transferase (*GST*)-π expression. A set of three loading concentrations of purified enzyme were used for each enzyme probed. β-Actin expression was performed for adequate loading of protein and to normalize the enzyme expression levels.
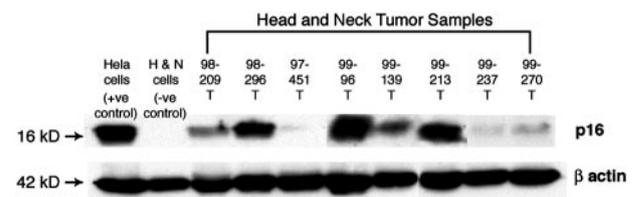
Fig. 2 A representative immunoblot of tumor tissue samples probed for p16 expression. Two controls (one positive and one negative) were used for p16 expression. β-Actin expression was performed to ensure adequate loading of protein loading for quantification.
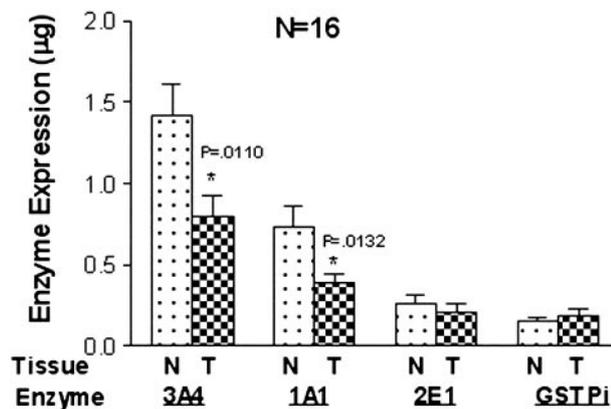
Fig. 3 Comparison of the mean enzyme expression levels in the tumor (*T*) *versus* adjacent morphologically normal (*N*) tissue from 16 patients with squamous cell cancer of the head and neck; *bars,* ±SE. A significant difference in the expression of CYP 1A1 and 3A4 was observed.

of tobacco. Previous studies have demonstrated the expression of CYPs and GST in normal squamous epithelium of the head and neck (9, 10, 17). CYP and GST expression varies in different tissues (18, 19). Tissue metabolism of carcinogens by these locally expressed enzymes may be a more important determinant of carcinogenesis than metabolism in the more distant organs such as the liver. Several studies assessed the risk of SCCHN in relation to CYP or GST genotypes (20–22). However, interindividual variations in the expression and activity of the CYPs and GST are dependent on genotypic as well as post-transcriptional factors, which maybe tissue specific (23–25). Therefore, the most direct method to evaluate the effects of CYPs and GST on the carcinogenic effects of tobacco is to evaluate enzyme expression in the tissue of interest.

The expression of CYP or GST in SCCHN can be determined by various methods. Immunoblotting, immunohistochemistry, or reverse transcription-PCR (26, 27) have been used to determine CYP or GST expression. Interpretation of the results from different studies may be influenced by the nonspecificity of the antibodies (28) and the semiquantitative nature of immunohistochemistry and reverse transcription-PCR. Another advantage of reverse transcription-PCR and immunoblots is the measurement of the expression of these enzymes in the tumor tissue as well as the nonmalignant epithelial and stromal cells admixed with tumor cells. The stromal cells could potentially contribute to the activation of carcinogens.

Epidemiological studies have identified an increased risk for tobacco-related cancer in the African-American population as compared with Caucasians (4). Garte *et al.* (29) reported on the frequency of the commonly studied CYP and GST genotypes by race. Information from >15,000 patients (including 996 African Americans and 12,525 Caucasians) was used. Significant differences in allele frequencies were noticed between the races. Therefore, we hypothesized that the expression of CYPs will be higher in the squamous epithelium tissue from African-American patients compared with Caucasians in concordance with the increased susceptibility to tobacco carcinogens and the differences in frequency of the genotypes of the

CYP alleles. In our study, no significant differences were seen in any of the three enzymes in the normal tissue from healthy African Americans and Caucasians. Several possible interpretations for the lack of difference in the enzyme expression exist. First, this study is underpowered to detect significant differences given the relatively large interindividual variability in CYP expression observed. Second, the observed difference in tobacco-related carcinogenesis between the races might be due to differences in the expression of other carcinogen-metabolizing enzymes or due to differences in DNA repair mechanisms. Finally, although the baseline expression of the CYP is similar in the two groups, the modulation of CYP expression with smoking might be different among the different races. Future studies may address the influence of the enzymatic activity of other carcinogen metabolism enzymes, gene repair mechanisms, or effect of smoking on CYP expression by race.

In this study the expression of CYPs 1A1 and 3A4 was significantly lower in the tumor tissue than in the adjacent normal tissue. Results of our study are consistent with results reported previously in other malignancies such as breast cancer (30, 31). Loss of heterozygosity at the chromosomal locus 9p21 is the most commonly observed alteration in SCCHN occurring in up to 80% of patients (11, 32). This abnormality is associated with an inactivation of the *p16* gene (11). In this study, p16 expression was used as a marker for the carcinogenic effect of tobacco. No significant association was observed between p16 expression and either CYP 1A1, 3A4, 2E1, or GST-π expression. The large interindividual variability in the expression of the CYP and GST-π enzymes observed might explain the lack of the correlation in this relatively small study. The association of p16 and p53 mutations with tobacco exposure, CYP 1A1, and GSTM1 genotypes in oral squamous cell cancer has been investigated previously (33). No significant association was observed between p16 mutations and tobacco exposure or CYP/GST genotypes. On the other hand, a strong association was reported for p53 mutations. This association of p53 mutations with tobacco exposure has been reported in other studies (34). Future trials may consider using p53 as a marker for the carcinogenic effects of tobacco.

The current study demonstrates the feasibility of designing trials evaluating CYP and GST expression in the target tissue of interest. This study also demonstrates the wide variability in enzyme expression in the SCCHN region. Incorporating such an approach in larger trials may help in elucidating the roles of these enzymes in carcinogenesis as well as in identifying potential targets for chemoprevention.

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