Phase I and II Carcinogen Metabolism Gene Expression in Human Lung Tissue and Tumors

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ABSTRACT

Purpose: **The regulation of carcinogen metabolism machinery may involve proximate tobacco smoke exposure, hormonal and other endogenous coregulatory factors, and an individual's underlying genetic responsiveness. The mRNA and protein expression patterns of known carcinogen metabolism genes encoding the aromatic hydrocarbon receptor Ahr; the cytochromes P450 CYP1A1 and CYP1B1; glutathione *S*-transferases GSTM1, GSTM3, GSTP1, and GSTT1; and NADPH quinone oxidoreductase NQO1 were examined.**

Experimental Design: **Paired tumor and nontumor lung tissue from 45 subjects was subject to a recently devised RNA-specific qualitative reverse transcription-PCR strategy, as well as Western immunoblotting. Tobacco exposure measured by plasma biomarkers nicotine and cotinine, plasma estradiol levels, α and β estrogen receptor (ER) expression in the lung, gender, age, and histological diagnosis were then analyzed using multivariate regression models.**

Results: **In nontumor lung tissue, multivariate models identified several correlates of mRNA expression: (*a*) CYP1B1 in females (positively: smoke status, $P = 0.024$; ER-β expression, $P = 0.024$); (*b*) GSTT1 in females (positively: cotinine, $P = 0.007$; negatively: age, $P = 0.001$; ER-β expression, $P = 0.005$) and in males (positively: plasma estradiol, $P = 0.015$; ER-β expression, $P = 0.025$); and (*c*) NQO1 in females (positively: smoke status, $P = 0.002$) and in males (positively: ER-β expression, $P = 0.001$). CYP1A1 (mRNA, 9.1%) and GSTM1 (mRNA, 17.5%) are uncommonly expressed in human lung. Confirmation by Western immunoassayed protein is described. The results in nontumor tissue differed from that in tumor tissue.**

Conclusions: **Regulation of carcinogen metabolism genes expressed in human lung seems impacted by hormonal and gender factors, as well as ongoing tobacco exposure. Expression differences between tumor and nontumor tissue in this pathway have both susceptibility and therapeutic implications.**

INTRODUCTION

One in 10 smokers develops lung cancer over a lifetime. Nonrandom clustering of cases in families indicates that some of this proclivity is inherited (1–9), possibly through low-penetrance, multigene loci (10). Because mainstream tobacco smoke is the primary cause of lung cancer, the carcinogen metabolism genes comprise a plausible lung cancer susceptibility pathway (1, 11).

Tobacco exposure measurement by self-reported smoking history can cause misclassification bias; an alternative approach has been to measure the exposure biomarker nicotine and its metabolite cotinine (12). Nicotine has a plasma half-life of ~2 h, whereas the half-life of plasma cotinine is ~17 h (13). Additionally, gender-specific factors may also be at play in lung carcinogenesis. The pack year-adjusted female to male lung cancer risk of 1.7 underscores a gender-associated risk (5, 14–24). Higher levels of PAH[3]-DNA adducts occur in women for any given level of smoking (20), and this effect may, in part, be mediated by higher Phase I enzyme expression and bioactivation (24). In addition, we have demonstrated gender-dependent ER-α and ER-β expression in human lung (25); the presence of these receptors may influence the extent of induction of Phase I and Phase II enzymes.

Among the carcinogens contained in inhaled mainstream tobacco smoke, the PAHs, such as benzo(*a*)pyrene, are minimally reactive on inhalation. After binding the Ahr, they induce Phase I and Phase II mRNAs and their corresponding proteins both *in vitro* and in experimental animals (26). The CYP superfamily (1, 27–32) members CYP1B1 and CYP1A1 have been reported to be expressed in human lung (33–35). The highly reactive bioactivated intermediates of inhaled PAH carcinogens, such as benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide, confer "hot spots" for benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide-induced mutations in the tumor suppressor *p53* gene *in vitro* and closely match the overall *p53* gene mutation spectra found in a wide array of epithelial cancers *in vivo* (36, 37). There is coordinate metabolism of estradiol and inhaled PAHs by CYP1B1 (38–44), suggesting a need to assay gender-associated factors impacting on carcinogen metabolism expression in human lung.

The families of GST and NQO coordinately subserve Phase II conjugation of reactive intermediates to less reactive,

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; ER, estrogen receptor; Ahr, aromatic hydrocarbon receptor; CYP, cytochrome P450; GST, glutathione *S*-transferase; NQO, NADPH-quinone oxidoreductase; RT-PCR, reverse transcription-PCR; HRP, horseradish peroxidase.

more hydrophilic compounds (45, 46). Lung expression in humans has been reported by RT-PCR (47, 48), immunoblot or immunohistochemistry (49–52), and enzyme activity (53, 54). These enzyme families are also Ahr-(xenobiotic response element) and estradiol regulated, among other factors (1, 55–58). Sensitivity to chemical carcinogenesis has been demonstrated experimentally to be dependent on such regulation of Phase II enzyme function (56, 59–62).

Many of the standard RT-PCR methods for measuring mRNA may be fundamentally confounded (63–68). We have circumvented a very common pitfall by developing a universal primer strategy that takes advantage of the unique, single-stranded features of mRNA sequence at typical reverse transcription temperatures. Genomic pseudogene amplification is avoided, and the universal approach allows for multiple different transcripts to be amplified from the same tissue-derived RNA sample in uniplex reactions on the same subject. We, therefore, have a mRNA-specific means for assay of human gene expression (68).

Tobacco-induced expression of carcinogen metabolism enzymes in human lung has been suggested to demonstrate wide interindividual variation (35, 69–75), over several orders of magnitude as assessed in endobronchial mucosal biopsies taken from active cigarette smokers (75). It is hypothesized that this variability may confer individual susceptibility to mutation and, therefore, carcinogenesis. To further understand factors impacting on human carcinogen metabolism phenotypes, we present RNA-specific gene expression and immunoblot protein expression data, on the carcinogen metabolism pathway enzymes in tumor and nontumor lung tissue from 45 individuals, and relate that expression in multivariate models to tobacco smoke exposure (measured levels of plasma nicotine and cotinine and self-reports), gender-related factors (gender, lung ER-α and ER-β expression, plasma estradiol), and clinical (age and histological diagnosis) factors.

MATERIALS AND METHODS

Subjects. The study comprised a group of 45 consenting individuals undergoing lung resectional surgery for clinically suspected carcinoma and had sufficient lung tissue for analysis (Table 1). Recruits came from the Albany, New York, region, and surgery was performed at either a tertiary care center (Albany Medical Center) or a large community hospital (St. Peter's Hospital), under the auspices of the respective institutional and New York State Department of Health review boards. Self-reported mainstream or sidestream tobacco exposure history, down to the cessation date if applicable, along with exposures to other inhaled toxicants and medications, were obtained preoperatively by direct interview by a trained research nurse. The subject's own medical history, including the presence of underlying lung disease and any family history of lung or other malignancies, were recorded as well.

Tissue Handling. Whole lung tissue was surgically resected for clinical indications, and if otherwise not needed for diagnostic purposes, the material was macroscopically divided by the pathologist into "involved" (usually tumor) *versus* "uninvolved" (nontumor) tissue, flash-frozen in liquid isopentane or nitrogen within 15 min of blood supply ligation, and placed into the −80°C tissue bank until analyzed. Speed and proper preservation for RNA analyses were preeminent considerations. Blood (30 ml) was collected preoperatively at the time of interview from each subject and stored briefly at room temperature, and the plasma fraction was frozen.

Table 1 Subject characteristics

Characteristic	Female (n = 19)	Male (n = 26)	P
Age (yr)	60.4 (±3.4)	63.2 (2.1)	NS[a]
Smoking history			NS
Current MS	7 (36.8%)	7 (26.9%)	
Current ETS	0 (0.0%)	1 (3.8%)	
Recent MS	1 (5.3%)	3 (11.5%)	
Former MS	9 (47.4%)	13 (50.0%)	
Never	2 (10.5%)	2 (7.7%)	
Plasma nicotine	2.6 (±1.3)	3.4 (±1.3)	NS
Plasma cotinine	60.0 (±27.7)	72.8 (±23.5)	NS
Plasma estradiol	48.1 (±11.6)	44.6 (±2.7)	NS
Histological diagnosis			NS
Adenocarcinoma, lung	6 (31.6%)	5 (19.2%)	
Squamous cell carcinoma, lung	5 (26.3%)	7 (26.9%)	
Mixed non-small cell carcinoma, lung	3 (15.8%)	7 (26.9%)	
Metastatic to lung	0 (0.0%)	3 (11.5%)	
Benign nodule	5 (26.3%)	4 (15.4%)	

[a] NS, nonsignificant, $p > 0.05$; current MS, mainstream smoke; current ETS, environmental tobacco smoke; recent MS, quit <3 weeks ago; former MS, quit before 3 weeks ago; never, never smoked tobacco.

RNA Extraction. RNA was extracted from ~100 mg of fresh-frozen human lung tissue using a standard thiocyanate guanidinium-based method (TRI Reagent protocol; Molecular Research Center, Inc., Cincinnati, OH). Great care was taken to keep lung tissue frozen throughout fractionation and pulverizing, via a liquid N_2-immersed mortar and pestle, until the moment of immersion in the guanidinium-containing solution. The yield was generally 1–5 mcg of total RNA/mg of lung tissue.

Standard RT-PCR. Standard-design qualitative RT-PCR was performed by oligo-dT isolation of mRNA and reverse transcription using Superscript II Reverse Transcriptase (Life Technologies, Inc., Gaithersburg, MD), according to manufacturer's instructions, except that dNTP concentration was augmented 8-fold over protocol. PCR of cDNA was performed using Ahr, ER-α, ER-β, CYP1A1, CYP1B1, GSTM3, GSTT1, and NQO1 primers selected to span at least one intron or one primer oligonucleotide of a pair that spans an exon/intron splice site for cDNA specificity and that produced PCR products of a size that allowed for kinetics favorable to the amplification of cDNA *versus* any contaminating genomic DNA. No reactions were multiplexed.

PCR was performed on a Perkin-Elmer Biosystems 9700 thermocycler, using Perkin-Elmer RT-PCR kit reagents (Roche, Branchburg, NJ) with the substitution of TaqDNA polymerase and Platinum Taq antibody (Hotstart; Life Technologies, Inc.) and Taq Extender buffer (Stratagene, La Jolla, CA), according to the manufacturer's protocols. cDNA PCR was accomplished in a single stage of 40 cycles, using the following protocol: 95°C for 1 min (Hotstart), then 40 cycles of 10-s denaturing at 95°C, 15-s annealing at 55–60°C (transcript dependent), then 1-min extension at 72°C, followed by a terminal 7-min extension at 72°C. β-actin cDNA amplification was performed for 30 cycles, using identical conditions to that for target gene cDNA amplification, but with the universal RNA-specific strategy described,

in separate parallel uniplex reactions on each cDNA sample. ER-α and -β cDNA-PCR primers and thermocycling reagents and conditions for RT-PCR were identical to that published previously (25).

Cross-reactivities of the PCR primers were checked with Genetics Computer Group-Wisconsin (Madison, WI) statistical software for sequence analysis. There was virtually no potential for primers to anneal to an alternate transcript with up to five mismatches in the primer oligo-sequence, a potential issue for subfamily members such as CYP1A1, CYP1B1, GSTM1, and GSTM3. Given the above precautions for avoiding contaminating genomic DNA amplification in the PCR, there was no experimental evidence of genomic DNA misamplification for the target transcripts.

RNA-specific Universal Reverse Transcription-coupled PCR. The existence of pseudogene sequences that have been experimentally determined to behave as confounding sequences for standard PCR primers in genomic DNA-contaminated RNA and cDNA samples prompted the use of an alternate RT-PCR strategy for samples intended for assay of β-actin, GSTM1, and GSTP1. For the reverse transcription, the universal reverse transcription primer was added to designated total RNA samples in place of oligo-dT in identical concentration (0.5 μg/μl). The unique tag sequence inserted into the cDNA at the 5′ end in the reverse transcription step by this universal reverse transcription primer was nonidentical and noncomplimentary to any known genomic DNA sequence. The unique tag integrated into the cDNA was then primed in the PCR by the universal reverse (antisense) primer. This was paired with a transcript-specific forward (sense) primer, and thermocycling occurring under identical conditions to standard RT-PCR yielded the PCR product. Given that the tag sequence is nonidentical and noncomplimentary to any known genomic DNA sequence, no DNA sequence that had not been reverse transcribed by the system was amplified in the PCR. No other alterations were made in the handling of these samples compared with those assayed by standard olig-dT-based RT-PCR. β-*Actin* was used as the reference housekeeper gene and assayed in duplicate by a similar universal RNA-specific reverse transcription and PCR primer set used in separate uniplex reactions on the same individual's cDNA, as a positive control of RNA integrity (68).

Samples from individual subjects were assayed in at least triplicate replicates, performed with positive specific control cDNA [dioxin-stimulated MCF-7 breast cancer cell line or commercially available human lung total RNA (Clontech), as appropriate] and water blanks. PCR product was displayed on ethidium bromide gel, photographed under UV light, and, if visually apparent, that experimental trial was recorded as "positive."

Western Immunoblotting. Microsomal preparation was performed from human lung tissue by a standard technique (35). Briefly, 100 mg of tissue were pulverized in a liquid N_2-immersed mortar and pestle apparatus and immersed in 1.0 ml of microsomal preparation buffer (0.2 mM phenylmethylsulfonyl fluoride, 1,0 mM DTT, 1.0 mM EDTA, 20 mM Tris acetate, and 0.14 M KCl). Samples were then sonicated for 15 s and centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was ultracentrifuged at 100,000 × *g* for 60 min at 4°C, and the pellet was resuspended in 0.5 ml of microsomal storage buffer (50 mM Tris acetate, 1.0 mM EDTA, 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and 1.0 mM DTT). Microsomal protein was quantified by BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). Multiple replicate protein expression trials for an individual were performed on the same microsomal isolation from a single sample.

For CYP1B1 and CYP1A1 Western immunoblotting, assays were performed in a Bio-Rad (Hercules, CA) assembly using standard running buffer (25 mM Tris HCl, 0.192 M glycine, 0.1% SDS, and 20% methanol). Each lane was loaded with 5 μg of microsomal protein, and the gel was run at 100 V for 1.5–2.5 h for maximum resolution. Positive control lanes containing 2.4, 10.2, or 51.0 fmol of lymphoblast expressed CYP1A1 and CYP1B1 (Gentest, Woburn, MA). Blocking was performed with 5% nonfat dry milk, 0.25% Tween 20, 1% BSA, and 5% goat or rabbit (depending on the primary antibody) serum in PBS for 2 h at room temperature and then in 5% nonfat dry milk, 0.25% Tween 20 at room temperature overnight. Primary rabbit antihuman CYP1B1 peptide IgG was initially provided by Dr. F. Kadlubar (National Center for Toxicologic Research, Little Rock, AK), and additional supplies were regenerated according to that group's published methods (76). Commercially available antihuman CYP1B1 IgG was insufficiently sensitive and specific. Primary goat antihuman CYP1A1 IgG was purchased from Gentest. Both anti-CYP1B1 and anti-CYP1A1 antibody titers were optimized at 1:400, applied in blocking solution for 2 h at 23°C, washed in PBS-buffered saline-Tween 20, and followed with an anti-IgG antibody appropriate to rabbit or goat primary IgG, respectively. The secondary antibodies were purchased pretagged with HRP (Sigma, St. Louis, MO) and applied in previously optimized titers of 1:40,000 (for CYP1B1) or 1:5,000 (for CYP1A1). Identical substrates were used in each assay for chemiluminescent detection (Pierce SuperSignal) per the manufacturer's protocol. Kodak X-Omat AR film was exposed from 10 s to 2 h, depending on signal strength, and visible target bands at the appropriate molecular weight were categorized as positive.

Ahr. Total cell protein was used because cytosolic and nuclear locations for Ahr are known. For positive control, an expression construct (N-terminal; a gift from Dr. Alvaro Puga, University of Cincinnati, Cincinnati, OH) was expressed in a bacterial system to serve as a positive control for immunoblotting, as did a second control from total cellular protein from dioxin-treated MCF-7 (Ahr-expressing) cells. Block overnight at 4°C was performed in a single step in the above-described blocking solution. The primary antibody was rabbit antihuman IgG (1:1600; Biomol, Plymouth Meeting, PA) in blocking solution for 45 min at room temperature. The secondary antibody was goat antirabbit IgG tagged 1:160,000 in blocking solution for 30 min at room temperature; the HRP substrate was FemtoSignal chemiluminescence (Pierce Chemical Co.).

GSTM1 and GSTP1. Lung tissue handling, electropheresis, blotting, and blocking procedures were identical to that described above for Ahr, with the following exceptions. Positive controls for GSTM1 (0.1 μg) and GSTP1 (0.05–0.1 μg) standard protein were commercially available (Accurate Chemical, Westbury, NY) and used reciprocally to assure primary antibody specificity. Primary antibody GSTM1- or GSTP1-specific rabbit antihuman IgG (1:1000; Accurate Chemical) was used in blocking solution for 30 min at room temperature. The secondary goat antirabbit IgG was used at 1:80,000 for 30 min at room temperature. SuperSignal chemiluminescence was the HRP substrate (Pierce Chemical Co.).

GST-T1 and NQO-1 were not tested for immunoreactive protein.

Nicotine and Cotinine Analysis. Plasma nicotine and cotinine levels were measured using a modification of the procedure by Davis (77). Plasma (500 μl) spiked at 100 ng/ml with deuterated nicotine and cotinine internal standards was diluted with 500 Öl 5 M sodium hydroxide and extracted with methylene chloride. The organic layer was concentrated to dryness, and the solvent changed to 2-propanol and was reduced to 10 Öl. Gas chromatography/mass spectrometry was used to separate and detect nicotine and cotinine using the deuterated internal standards to quantitate the amount of nicotine and cotinine present in plasma. The limits of detection for nicotine and cotinine were 0.27 and 4.45 ng/g plasma, respectively.

Statistical Analysis. All successive replicate experimental RT-PCR or Western immunoblotting trials on any given sample displayed appropriate negative and positive controls for that trial. The "ever-positive" analysis transformed to analyte presence if any of the repeated patient-specific assay trials was positive and transformed to absent otherwise. In the initial univariate analyses, endogenous variables (age, gender, histology, plasma estrogen, and ER-α and -β) and exposure variables (nicotine, cotinine, and smoking history) were assessed individually as nine independent variables for their effects on the eight mRNA expression variables (Ahr, CYP1A1, CYP1B1, GSTM1, GSTM3, GSTP1, GSTT1, and NQO1) and the five protein expression variables (Ahr, CYP1A1, CYP1B1, GSTM1, GSTP1). When univariate comparison of independent variable to the outcome variable (gene expression) displayed $P < 0.05$, the independent variable was taken to further explore variables in the multivariate models.

In the multivariate analysis, the nine independent variables were considered simultaneously as candidates for joint models explaining the variation in the expression of each gene (eight for mRNA and five for protein). To maximize the number of complete entries, missing values were imputed with the average of the remaining values. Regression parameters estimated by Best subsets regression (Sigma Stat; Jandel Scientific) were used to elucidate relationships in multivariate "ever-positive" analyses. Tumor and nontumor tissues were analyzed separately. The model chosen optimized r^2 adjusted to the number of variables in the model (adjusted r^2) while minimizing redundancy or collinearity in the regression parameter (variable inflation factor). Bonferroni adjustment, considering nine variables in the model for each gene, suggests $[\alpha = 0.05]/9 = 0.006$ is a more conservative threshold for statistical significance in judging which factors correlate with target gene expression.

RESULTS

The characteristics of the subjects are listed in Table 1. There were no statistical differences between the genders in any of the demographic, tobacco exposure, or histological parameters. Most women were of perimenopausal or postmenopausal age; as a result, no significant differences were apparent in plasma estradiol levels between the women and men.

Primers designed for the gene expression assays are listed in Table 2. Standard-design PCR primers (std) are listed alongside those specifically designed for the RNA-specific RT-PCR system (up), whereby the design of the reverse transcription primer and the PCR primers are coupled. The universal RNA-specific reverse transcription-coupled PCR strategy developed in this laboratory circumvents the false positive RT-PCR common from human tissue extracts, when assaying for common housekeeper (β-actin, glyceraldehyde-3-phosphate dehydrogenase, 36B4) or some target (GSTM1, GSTP1) transcripts (68). An example of RNA-specific RT-PCR for GSTM1 and GSTP1, whereby pseudogene sequence is otherwise found in the human genome and a potential confounder, is shown in Fig. 1 and is demonstrated as unconfounded.

Of the panel of genes assayed in this study, *Ahr*, *CYP1B1*, *GSTM3*, *GSTP1*, *GSTT1*, and *NQO1* were the most commonly expressed at a mRNA level (Table 3). GSTP1, in particular, was consistently expressed in 87–100% of samples from all subjects, depending on subgroup. CYP1A1 and GSTM1 were uncommonly expressed. For example, in nontumor extracts, 9.1% (CYP1A1) and 17.5% (GSTM1) of individuals had one or more replicate RT-PCR reactions that was positive. For tumor RNA

Table 2 PCR primers

Target	Forward (sense) primer	Reverse primer[a]	mRNA product size (bp)	Gene product size, bp (misses[b])
Ahr (std)[c]	cagaaaacagtaaagccaatcc	aatacaaagccattcagagcc	323	995 (7 misses)
CYP1A1 (std)	ttccgacactcttccttagt	atggttagcccatagatggg	368	705 (0 misses)
CYP1B1 (std)	gccactatcactgacatct	cttgcctcttgcttcttatt	684	3716 (0 misses)
GST-M1 (std)	actttcccaatctgccctac	ttctggattgtagcagatca	191	None[d]
GST-M3 (std)	actttcctaatctgccctacc	taacacacctgctctctcc	805	1866 (7 misses)
GST-P1 (std)	caccaactatgaggcgggcaa	atcagcagcaagtccagca	159	338 (8 misses)
GST-T1 (std)	tgccaagaagaacgacattcc	gccacactctccgtcaa	147	205 (6 misses)
NQO1 (std)	tgaagaagaaaggatgggagg	agggggaactggaatatcac	223	190 (7 misses)
B-Actin (std)	ccacgaaactaccttcaactcc	tcatactcctgctgcttgctgatcc	270	382 (6 misses)
GAPDH (std)	ggtcggagtcaacggatttggtcg	cctccgacgcctgcttcaccac	788	3016 (4 misses)
GST-M1 (up)	catgatctgctacaatccagaa	URP	807	None
GST-P1 (up)	tctccttcgctgactacaac	URP	282	None
B-Actin (up)	gccatcctaaaagccacc	URP	345	None
GAPDH (up)	gcacaagaggaagagagaga	URP	211	None

[a] The universal RT primer has the general formula 5′-XTnVVN-3′, where X is the sequence 5′-aacgagacgacgacagac-3′ ($n = 21$); V is A, C, or G; and N can be any nucleotide: A, C, G, or T. The universal reverse PCR primer (URP) used for these transcripts is 5′-aacgagacgacgacagac-3′ (Ref. 68, patent pending).

[b] Mismatches (misses) required to yield this size product from genomic DNA.

[c] (up), universal primary strategy target sequences; (std), other standard approach RT·PCR assays.

[d] One of the primers spans an exon/exon splice site and, therefore, yields no genomic product.

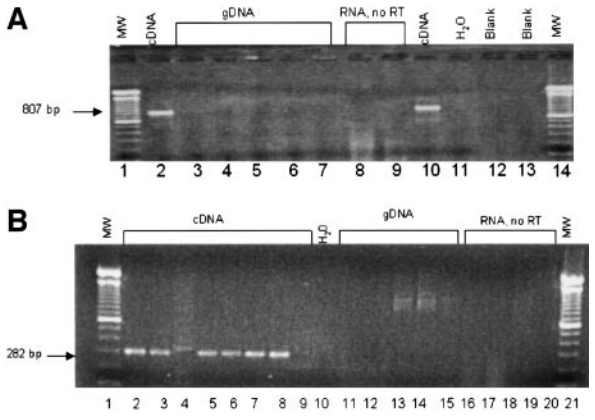

Fig. 1 *A*, GSTM1 mRNA expression by RNA-specific qualitative RT-PCR in human lung. *Lane 1*, molecular weight DNA ladder; *Lane 2*, total RNA extracted from nontumor lung tissue from subject A, treated with reverse transcriptase and the tagged universal reverse transcription (*RT*) primer used in the author's laboratory to yield cDNA, and then subject to PCR using the GSTM1-specific forward and universal reverse primer set listed in Table 2; *Lanes 3–7*, genomic DNA substituted for the RNA extract in the RT-PCR reaction from peripheral blood cells of five different subjects (M–Q); *Lanes 8* and *9*, total RNA samples from MCF-7 cells *(Lane 8*) and subject A *(Lane 9*) in which no reverse transcriptase enzyme was added during the RT step (negative control); *Lane 10*, identical to *Lane 2*; *Lane 11*, water substituted for the total RNA (negative control); *Lanes 12* and *13*, loading blanks; *Lane 14*, molecular weight DNA ladder. The RNA specificity of this RT-PCR strategy is apparent. GSTM1 is encoded by a pseudogene-like sequence in the human genome (see "Materials and Methods" and Ref. 68 for details). *B*, GSTP1 mRNA expression by RNA-specific qualitative RT-PCR in human lung. *Lane 1*, molecular weight DNA ladder; *Lane 2*, total RNA extract from subject A (nontumor), treated with the tagged universal reverse transcription (*RT*) primer developed in the authors' laboratory and reverse transcriptase to yield cDNA and then subjected to PCR using the GSTP1-specific forward and universal reverse primer set listed in Table 2; *Lane 3*, reverse transcribed RNA extract from a malignant lung tumor from subject B; *Lane 4*, reverse transcribed RNA extract from MCF-7 breast cancer cell line (no specific GSTP1 expression); *Lane 5*, reverse transcribed RNA extract from a benign human carcinoid lung tumor from subject C; *Lane 6* (malignant tumor) and *Lane 7* (nontumor), reverse transcribed RNA extracts from subject D; *Lane 8*, reverse transcribed RNA extract from malignant tumor and *Lane 9* (nontumor) from subject E; *Lane 10*, water RT-PCR blank (no RNA); *Lanes 11–15*, genomic DNA substituted for the RNA in RT-PCR from peripheral blood cells of five different subjects (M-Q); *Lanes 16–19*, RNA from subjects A (nontumor), D (tumor), and E (tumor), whereby no reverse transcriptase was added to the total RNA extract from these samples during the RT step (RT-negative control); *Lane 20*, water substituted for the total RNA and the absence of reverse transcriptase during the RT reaction (essentially identical to *Lanes 16–19*); *Lane 21*, molecular weight DNA ladder. The RNA specificity of this RT-PCR strategy is apparent. GSTP1 is encoded by a pseudogene-like sequence in the human genome (see Ref. 68 for details).

extracts, the fraction of individuals was 15.8% (CYP1A1) and 18.9% (GSTM1). Protein expression frequencies were comparable with those of RNA (Table 3).

Table 4 depicts the multivariate correlation of individual gene transcripts with tobacco exposure markers, estrogen exposure, histological diagnosis of the tumor, ER expression, and gender and age in tumor/involved and adjacent nontumor/uninvolved human lung samples. The data are derived from total RNA extracts from homogenized (nonmicrodissected) human lung, using our novel RNA-specific RT-PCR assay. Multivariate Best subsets regression (Sigma Stat) modeling analyses were performed of the exposure, hormonal, demographic, and clinical factors correlating with the frequency of expression of each transcript across 45 individual human subjects. Bonferroni adjustment, considering nine variables in the model for each gene, suggests $[\alpha = 0.05]/9 = 0.006$ is a more conservative threshold for statistical significance in judging which factors correlate with target gene expression.

In these multivariate models, several factors significantly correlated with nontumor mRNA expression. For CYP1B1 mRNA expression in females, smoke status ($P = 0.024$) and ER-β expression ($P = 0.024$) were positively associated with expression; in males, no factors emerged as explanatory across all subgroups. For GSTT1 mRNA expression in females, cotinine ($P = 0.007$) was positively associated, and age ($P = 0.001$) and ER-β expression ($P = 0.005$) were negatively associated; in males, plasma estradiol ($P = 0.015$) and ER-β expression ($P = 0.025$) were positively associated with expression. For NQO1 mRNA expression in females, smoke status ($P = 0.002$) was positively associated; in males, ER-β expression ($P = 0.001$) was positively associated with expression.

The protein assays on nontumor lung homogenates suggested that CYP1B1 may positively covary with smoke exposure status in males ($P = 0.005$); and although rarely detectable, CYP1A1 protein covaried with nicotine levels in males (positively; $P = 0.005$). Examples of Western immunoassays for GSTM1 and GSTP1 are shown in Fig. 2.

For both mRNA and protein, the presence or absence of qualitative expression of these genes in tumor tissue, and the factors associated with that expression, differed very substantially from that in nontumor tissue (Table 4).

DISCUSSION

The demonstration here by a novel, RNA-specific RT-PCR assay that multiple Phase I and II gene transcripts are expressed in human lung and that two of the most highly studied genes in the literature (*CYP1A1* and *GSTM1*) are uncommonly detected at both mRNA and protein levels has implications for which genes may be at play and, therefore, worthy of additional study in tobacco-induced lung carcinogenesis. Genotyping studies of candidate genes for lung cancer susceptibility and quantitative constitutive and inducible gene expression studies probing the biology of lung carcinogenesis should appropriately be aimed at those genes expressed in the nontumor human lung. The unusual expression of a carcinogen bioactivating Phase I enzyme (*e.g.*, CYP1A1) may confer risk to that minority of individuals who do express the gene. Coordinately, rare or absent expression of a potentially protective enzyme (GSTM1) may also confer risk. These hypotheses can be explored at the gene expression level in future case-control designs. The current study additionally suggests that hormonal status may, in addition to tobacco exposure, bear on the metabolism of inhaled carcinogens, in a gene- and tissue-specific manner.

The assertion that nontumor tissue gene expression is important in human studies of carcinogenesis presumes that nontumor tissue from a subject who has developed an adjacent lung malignancy is a plausible surrogate tissue for the premalignant lung, reflecting early events in lung carcinogenesis superimposed on an identical genomic blueprint. Because even the most ambitious human studies cannot otherwise identify and serially

Table 3 Gene expression frequency across subjects[a]

	Female				Male			
	Nontumor		Tumor		Nontumor		Tumor	
	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein
Gene								
Ahr	18/18 (100)[b]	15/16 (93.8)	15/15 (100)	13/14 (92.9)	23/26 (88.5)	22/24 (91.7)	23/23 (100)	19/19 (100)
CYP1A1	3/18 (16.7)	N/A[c]	3/15 (20.0)	N/A	1/26 (3.8)	3/11 (27.3)	3/23 (13.0)	2/10 (20.0)
CYP1B1	14/18 (77.8)	13/17 (76.4)	15/15 (100)	15/15 (100)	19/26 (73.1)	13/18 (72.2)	21/23 (91.3)	15/17 (88.2)
GSTM1	3/17 (17.6)	0/13 (0.0)	4/15 (26.7)	0/11 (0.0)	4/23 (17.4)	2/23 (8.7)	3/22 (13.6)	1/19 (5.3)
GSTM3	13/18 (72.2)	N/A	12/15 (80.0)	N/A	17/26 (65.4)	N/A	18/23 (78.3)	N/A
GSTP1	17/17 (100)	17/17 (100)	15/15 (100)	13/13 (100)	21/23 (91.3)	21/24 (87.5)	20/23 (87.0)	18/19 (94.7)
GSTT1	12/18 (66.7)	N/A	14/15 (93.3)	N/A	20/26 (76.9)	N/A	15/23 (65.2)	N/A
NQO1	11/18 (61.1)	N/A	10/15 (66.7)	N/A	8/22 (36.4)	N/A	12/23 (52.2)	N/A

[a] Fraction of individuals assayed demonstrating ≥1 positive replicate RT-PCR trial for individual genes, in nontumor/unaffected or tumor lung tissues.

[b] Numbers in parentheses are percentages (*e.g.*, 100%).

[c] N/A, data not available for immunoblot of protein because of insufficient tissue (females, CYP1A1) or specific antibody (GSTM3) availability.

biopsy target lung tissue destined to become a malignancy, and then follow these presymptomatic patients for decades of exposure up to and including the time of presentation with lung malignancy in that location, a valid and available surrogate for premalignant tissue is required. Given the original identity of genomic characteristics between lung epithelium before it does evolve into a malignancy and the surrounding macroscopically and microscopically spared nontumor lung tissue, along with the identical tobacco and other exposures of these two tissues within any given individual, nontumor tissue has been used as a plausible "virgin surrogate" for these early mutagenic events. The comparisons of nontumor tissue characteristics from an individual who develops a malignancy in adjacent tissue, with nontumor tissue from one who has not developed such an adjacent malignancy given identical exposure, may allow for considerable insight into predisposing individual characteristics. The current study had too few subjects without an adjacent primary lung malignancy submitting to surgical lung resection to allow this comparison, but ongoing studies hold promise for such a case-control analysis. Additionally, tumor-nontumor comparisons may allow for insights into the biological factors that distinguish the lung malignancy itself.

This study demonstrated that several transcripts are commonly expressed in human nontumor lung, including CYP1B1, GSTM3, GSTP1, GSTT1, and NQO1. Although tobacco exposure parameters often correlated with the expression of these genes, different tobacco exposure parameters (nicotine *versus* cotinine *versus* smoking history) seemed to uniquely correlate with expression of any given gene. For example, nicotine as a tobacco exposure surrogate with a half-life of several hours was more relevant to CYP1A1 expression (males only), consistent with the report of rapid turnover kinetics of the transient CYP1A1 transcript with a half-life of a few hours (78). Nicotine itself has recently been reported to induce CYP1A1 expression in human lung explants (79). Smoking history as a marker of longer-term exposure seemed to be more relevant to CYP1B1 expression at both mRNA and protein levels across both genders, consistent with reports of a longer half-life of that transcript (78), although these kinetics are controversial as studied in non-lung cells (80). GSTP1 expression was present almost uniformly across tissue and gender subgroups, and its presence was not obviously affected at a qualitative level by tobacco exposure or hormonal factors. This contrasts with GSTT1 mRNA expression in nontumor tissue, which was affected by both tobacco exposure (cotinine) and hormonal factors (ER-β expression and/or estradiol levels), depending on gender.

The variability as to which specific tobacco smoke exposure parameter is relevant to gene expression may be affected by the timing of plasma collection (performed days before planned lung surgery, at the time of interview), than with inherent features or reliability of the individual parameter. Closer temporal coupling of plasma and lung tissue harvest, not logistically possible in this study, would solve this interpretive dilemma. However, we interpret the data in this study with the assumption that the presence of self-reported tobacco exposure days before surgery by history, confirmed by plasma cotinine drawn at the time of interview, in most cases, implies a likelihood of ongoing tobacco exposure within hours before surgical harvest of lung tissue.

The prominence of hormonal factors such as ER-α and ER-β expression, as correlates of gene expression of these carcinogen pathway enzymes, is notable. A recent report from our group describes the common expression of the two isoforms in human lung, in a gender-dependent fashion (25). Of the commonly expressed transcripts in nontumor tissue in the current study, CYP1B1, GSTT1 and NQO1 seemed to correlate with ER expression, plasma estradiol levels, or both, with possible implications for gender-related proclivities in carcinogen metabolism and, therefore, cancer susceptibility. In tumor tissue, only one mRNA transcript (NQO1) displayed clear correlations with one or several of these hormonal factors.

Whereas interindividual variability in nontumor tissue expression of these genes is observed, the measured exposure, hormonal and clinical correlates modeled for the qualitative expression of any given transcript, represents only a small part of the statistical variance observed in the expression of that gene across individuals. Ongoing quantitative studies may reveal more discrimination between factors impacting on gene expression in humans.

No doubt, our study ignored many of the myriad transcription, genetic, and epigenetic factors that determine whether a gene is expressed, and to what degree, in any given individual. Clearly, more detailed studies using model systems, transfectants, reporter constructs, and other similar experimental studies are necessary to work out precisely what factors make one individual an expression outlier and another individual of aver-

Table 4 Qualitative expression by RNA-specific RT-PCR, Western immunoblot, and lung:multivariate regression analysis

	Model components[a]	mRNA or protein[b]	Patterns[c]	Direction of relationship[d]	β-Coefficient[e]	*P*
Transcript in tumor (T) or nontumor (NT)	(Covariate)		(Notable fractions of subjects on which analysis is based)	(+/−)		
Ahr-T	Insufficient variability to analyze (F)	RNA, protein	(13/14 subjects positive)			
	Insufficient variability to analyze (M)	RNA, protein	(22/23 subjects positive)			
Ahr-NT	Insufficient variability to analyze (F)	RNA, protein	(All subjects positive)			
	Insufficient variability to analyze (M)	RNA, protein	(All subjects positive)			
CYP1A1-T	No predictive variables (F)	RNA, protein	(12/15 subjects negative)			
	No predictive variables (M)	RNA, protein	(20/23 subjects negative)			
CYP1A1-NT	Histology (F)	RNA	(15/18 subjects negative)	(−)	−0.303	0.029
	Estradiol (F)	RNA	(15/18 subjects negative)	(+)	0.00535	0.041
	Insufficient variability to analyze (M)	RNA	(25/26 subjects negative)			
	No predictive variables (F)	Protein		(+)	0.169	
	Nicotine (M)	Protein	(20/23 subjects negative)			0.005
CYP1B1-T	Insufficient variability to analyze (F)	RNA, protein	(All subjects positive)			
	Insufficient variability to analyze (M)	RNA, protein	(22/23 subjects positive)			
CYP1B1-NT	Smoke history (F)	RNA		(+)	0.557	0.024
	Smoke history (F)	Protein		(+)	0.372	0.059
	ER-β (F)	RNA		(+)	0.667	0.024
	ER-β (F)	Protein		(+)	0.585	0.055
	Histology (F)	Protein		(−)	−0.434	0.049
	Smoke history (M)	RNA		(+)	0.0157	0.074
	Smoke history (M)	Protein		(+)	0.926	0.005
	Histology (M)	Protein		(−)	−0.607	0.016
GSTM1-T	Histology (F)	RNA	(11/15 subjects negative)	(+)	0.464	0.050
	ER-β (F)	RNA	(11/15 subjects negative)	(−)	−0.979	0.028
	Insufficient variability to analyze (F)	Protein	(All subjects negative)			
	Insufficient variability to analyze (M)	RNA	(19/23 subjects negative)			
	Insufficient variability to analyze (M)	Protein	(18/19 subjects negative)			
GSTM1-NT	No predictive variables (F)	RNA	(15/18 subjects negative)			
	No predictive variables (M)	RNA	(15/18 subjects negative)			
	Insufficient variability to analyze (F)	Protein	(All subjects negative)			
	Insufficient variability to analyze (M)	Protein	(All subjects negative)			
GSTM3-T	Age (F)	RNA		(−)	−0.0432	0.043
	Smoke history (F)	RNA		(+)	0.359	0.030
	Histology (F)	RNA		(+)	0.585	0.016
	ER-β (F)	RNA		(−)	−0.843	0.047
	Estradiol (M)	RNA		(−)	−0.0454	0.026
GSTM3-NT	No predictive variables (F)	RNA				
	Ahr-NT (M)	RNA		(+)	0.769	0.032
GSTP1-T	Insufficient variability to analyze (F)	RNA, protein	(All subjects positive)			
	No predictive variables (M)	RNA	(19/20 subjects positive)			
	Insufficient variability to analyze (M)	Protein	(All subjects positive)			
GSTP1-NT	Insufficient variability to analyze (F)	RNA, protein	(All subjects positive)			
	Insufficient variability to analyze (M)	RNA	(All subjects positive)			
	Insufficient variability to analyze (M)	Protein	(21/23 subjects positive)			
GSTT1-T	No predictive variables (F)	RNA, protein				
	No predictive variables (M)	RNA, protein				
GSTT1-NT	Age (F)	RNA		(−)	−0.0787	0.001
	Cotinine (F)	RNA		(+)	0.00431	0.007
	ER-β (F)	RNA		(−)	−0.662	0.015
	Estradiol (M)	RNA		(+)	0.0460	0.015
	ER-β (M)	RNA		(+)	0.235	0.025
NQO1-T	No predictive variables (F)	RNA				
	Ahr (M)	RNA		(−)	−0.999	0.006
	ER-α (M)	RNA		(+)	0.806	0.001
NQO1-NT	Age (F)	RNA		(−)	−0.0350	0.048
	Smoke history (F)	RNA		(+)	0.556	0.002
	ER-β (M)	RNA		(+)	0.848	0.001

[a] Model components contributing to explanation of the variance with $P > 0.05$. F, female; M, male.
[b] RNA by qualitative RNA-specific RT-PCR, and protein by Western immunoblot.
[c] Notable dominant patterns of expression, across all individuals (*e.g.*, all subjects were positive).
[d] Direction of relationship; +, direct (positive); −, inverse (negative).
[e] β-Coefficient indicates the relative strength of the influence of that factor on the expression of that gene.

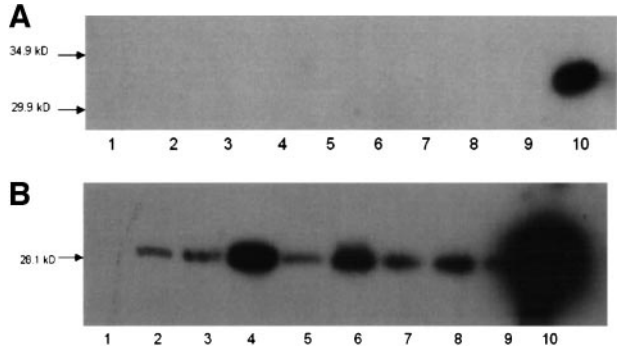

Fig. 2 *A*, GSTM1 Western blot of homogenized human lung, across several individuals. All subject samples were qualitatively positive. *Lane 1*, negative control (expressed GSTP1 protein); *Lane 2*, subject F (tumor); *Lane 3*, subject F (nontumor); *Lane 4*, subject G (tumor); *Lane 5*, subject G (nontumor); *Lane 6*, subject D (tumor); *Lane 7*, subject D (nontumor); *Lane 8*, subject H (tumor); *Lane 9*, subject H (nontumor); *Lane 10*, positive expressed GSTM1 protein control. There was no cross-reactivity with other GSTs (see "Materials and Methods" for Western immunoblotting procedure details). *B*, GSTP1 Western blot of homogenized human lung, across several individuals. All subject samples were qualitatively positive. *Lane 1*, negative control (expressed GSTM1 protein); *Lane 2*, subject F (tumor); *Lane 3* (nontumor); *Lane 4*, subject G (tumor); *Lane 5*, subject G (nontumor); *Lane 6*, subject D (tumor); *Lane 7*, subject D (nontumor); *Lane 8*, subject H (tumor); *Lane 9*, subject H (nontumor); *Lane 10*, positive expressed GSTP1 protein control. There was no cross-reactivity with other GSTs (see "Materials and Methods" for Western immunoblotting procedure details).

age constitutive or inducible expression, given the same exposure. Nonetheless, within the limits of the studies possible with observational human data such as those reported here, whereby temporal order of molecular events is simply not available, the types of multivariate correlations presented here may generate hypotheses on measurable and common factors that influence the expression of a gene. For example, for the commonly expressed GSTT1, this study might suggest fruitful future mechanistic studies by which proximate tobacco exposure, ER-β expression, and estradiol levels regulate the expression of this Phase II enzyme.

The current finding that the Phase I enzyme CYP1A1 is minimally expressed in human lung was recently reported by our group at both protein and message levels in a smaller series of subjects (35). The relatively short half-life of CYP1A1 transcript may underlie its low detection rate in our study, particularly if measured in surgical specimens procured hours to days after last tobacco exposure (78). For the Phase II enzymes, we are confident of the general paucity of GSTM1. We believe previous erroneous reports of common GSTM1 expression may have been attributable to the confounding of standard RT-PCR strategies by genomic DNA-encoded processed pseudogenes, in which standard PCR approaches cannot distinguish RNA-derived cDNA from contaminating genomic DNA sequence. This results in false positive RT-PCR results for these reference transcripts. Our RNA-specific RT-PCR strategy circumvents this problem (68). Similarly, we are confident that GSTP1 mRNA transcript expression is common, despite the existence of pseudogene sequences for this gene in the human genome. Multivariate models of CYP1A1 and GSTM1 expression were generally considered unstable, because they were based on a very few number of expressors in either tumor or nontumor tissue, despite some statistically significant correlations that are displayed.

The results of this study parallel some quantitative gene expression findings reported previously in brushed bronchial epithelial cells (48). Our qualitative RT-PCR results confirmed the common expression of GSTM3, GSTP1, and GSTT1 and clearly detected an influence of tobacco smoke exposure on the expression of many of these genes. The current study further implicates hormonal and gender influences on carcinogen metabolism enzyme expression.

Additionally, these mRNA studies were confirmed at the protein level, suggesting therapeutic advantage can potentially be taken of tumor-nontumor expression differences within the same individuals. Such differences could permit selection of model substrates for these xenobiotic-metabolizing genes that are preferentially activated in tumor tissue, possibly minimizing both the development of resistance in the lung tumor and toxicity to surrounding normal lung tissues.

In summary, we have demonstrated the presence of several measurable clinical factors that, on a gene-specific and lung tissue-specific basis, correlate with the expression of each of a panel of carcinogen-metabolizing enzymes at the mRNA level. Although several tobacco exposure factors predictably correlate with the expression of these genes, there are several hormonal and gender-related factors that were not predicted in advance. The results suggest that the accurate interpretation of future gene expression data in humans, either that from small panels of transcripts or from genome-wide expression arrays, requires assessment in sufficient numbers of individuals to allow for multivariate modeling on ongoing tobacco exposure, gender, hormonal, and other factors to fulfill the promise of insight into tobacco-induced human lung carcinogenesis. The limitations posed by qualitative gene expression profiling in homogenized human lung presented here are being addressed in ongoing real-time quantitative expression studies of microdissected human lung epithelium in this and other laboratories.

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