Journal of Cellular Biochemistry, Supplement 17F:129–137 (1993)

# Metabolic Activation, DNA Adducts, and H-*ras* Mutations in Human Neoplastic and Non-neoplastic Laryngeal Tissue

**Scott J. Stern, MD[1], Masakuni Degawa, PhD[3], Martha V. Martin, BS[2], F. Peter Guengerich, PhD[2], R. Keith Kaderlik, PhD[3], Kenneth F. Ilett, PhD[3,4], Randall Breau, MD[1], Michael McGhee, MS[1], Donna Montague, BS[1], Beverly Lyn-Cook, PhD[3], and Fred F. Kadlubar, PhD[3]**

[1] University of Arkansas for Medical Sciences, Department of Otolaryngology—Head and Neck Surgery, Little Rock, AR 72205
[2] Vanderbilt University, Nashville, TN 37232
[3] National Center for Toxicological Research, Jefferson, AR 72709
[4] Present Address: University of Western Australia, Perth, Australia

**Abstract** Metabolic activation, DNA adducts, and H-*ras* mutations were examined in human laryngeal tissue (n = 16) from both smoker and non/ex-smoker patients with laryngeal cancer. DNA adducts detected by $^{32}$P-postlabelling were evident only in smokers (n = 13); in fact, smoking cessation for as little as 10 months resulted in no DNA adducts detected (n = 3). Total DNA adduct levels in these samples were significantly correlated with levels of cytochromes P-4502C and 1A1 in laryngeal microsomes. Moreover, the P-4501A1 levels represent the highest yet found in human tissues. In contrast, laryngeal microsomes did not have detectable P-4501A2 activity, while laryngeal cytosols showed appreciable *N*-acetyltransferase activity for *p*-aminobenzoic acid (NAT1) but not sulfamethazine (NAT2).

DNA was extracted from laryngeal specimens and amplified by PCR. Nylon filter dot or slot blots were hybridized with $^{32}$P-labelled probes for codons 12, 13, and 61 of the H-*ras* gene. Sixty percent of specimens demonstrated mutations in either codon 12, 13, or 61; a single common and specific mutation was a Gln → Glu transversion in codon 61. This mutation appeared in 5 laryngeal specimens, all from smokers.

These results implicate cigarette smoke components, bioactivated by CYP1A1 and/or CYP2C, in DNA adduct formation. These results also demonstrate a probable smoking-related H-*ras* Gln → Glu transversion in codon 61. 

**Key words:** Cytochrome P-450, *N*-acetyltransferase, $^{32}$P-postlabelling, H-*ras* mutations, larynx

Carcinogenesis of the upper aerodigestive tract mucosa is poorly understood. Generalizations concerning the effects of cigarettes and/or smokeless tobacco and alcohol on these surfaces and the incidence of squamous cancer have certainly been reported; however, the characterization of genotypic and phenotypic changes in colon, lung, and bladder cancer have been elucidated to a much greater degree [1–3].

The mucosa of the larynx is particularly interesting from the standpoint of metabolic and genetic studies. The larynx is the portal to the lung, but the incidence of laryngeal cancer is much lower than that of lung cancer [4]. As alcohol rarely (if ever) reaches the mucosal surface of the larynx, it should not be a confounding factor from the standpoint of carcinogenesis. Dietary carcinogens, similarly, should

Address correspondence to Scott J. Stern, MD, Assistant Professor, Department of Otolaryngology—Head and Neck Surgery, 4301 W. Markham, Slot 543, Little Rock, AR 72205.

not be implicated in the development of laryngeal cancer for the same reasons. Conceptually, the surface of the larynx should only be susceptible to carcinogens in cigarette smoke. Tissue from this area is readily accessible and easily obtained and should therefore be a unique model for carcinogenesis of the upper aerodigestive tract.

## MATERIALS AND METHODS

### DNA Adduct and Metabolic Studies

Sixteen consecutive patients undergoing surgical procedures on the larynx (laryngectomy) were entered into the study. Two patients undergoing surgery for benign laryngeal disease were included as negative controls. After informed consent was obtained, a piece of normal-appearing laryngeal mucosa approximately 1 $cm^2$ was excised from a nonadjacent site, placed in saline and then on ice; the tissue was transported on ice and stored at –80°C until analysis.

Quantification of DNA adducts was performed by the $^{32}P$-postlabelling technique, which involves enzymatic hydrolysis of 3′ nonradioactive carcinogen-modified DNA, subsequent [5′-$^{32}P$]-phosphorylation with $^{32}P$-ATP (3,000–7,000 Ci $mmol^{-1}$) and polynucleotide kinase, followed by chromatographic separation of nucleotide-carcinogen adducts from unmodified nucleotides [5]. Briefly, DNA is digested from prepared tissues by the addition of 200 mu/ml micrococcal nuclease and 200 mu/ml phosphodiesterase (Sigma Chemical Co., St. Louis, MO) in 10 mM sodium succinate (pH 6.0) and 5 mM $CaCl_2$ at 38°C for 3.5 hours. Adducts are then isolated by adding 10 μl of the dilute digest to 5 μl each of 100 mM ammonium formate (pH 3.5), 10 mM tetrabutyl ammonium chloride (TBA), and 30 μl of water. Extractions are performed with 75 μl butanol or enhanced with nuclease P1. Reactions are neutralized with 1 μl of 200 mM Tris-HCl (pH 9.5). Labelling is terminated when 5 μl of the DNA digest is added to the labelling mix as previously described [5]; the labelled DNA adducts then undergo thin-layer chromatography and autoradiography for detection.

Laryngeal tissue cytosols were prepared from minced laryngeal mucosa. The tissue was placed in 2 volumes of buffer (20 mM Tris-HCl containing 1 mM EDTA, 2 mM dithiothreitol, and 50 uM phenylmethylsulfonyl fluoride) and ho-

**TABLE I. CYP1A2-Dependent 4-ABP N-oxidation of Human Larynx and Liver Microsomes**

| Tissue (n) | Rate (pmol/min/mg) ± s.d. |
|---|---|
| Larynx (11) | <1 |
| Liver (21) | 574 ± 416 |

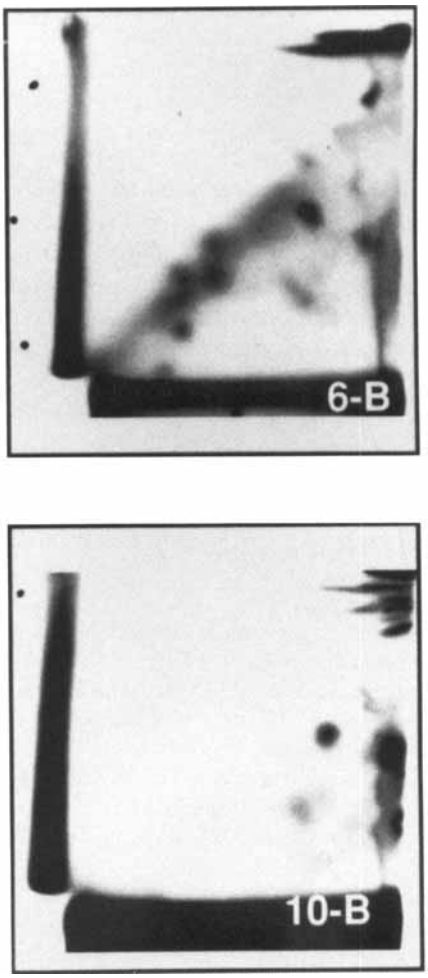



**Fig. 1.** A comparison of $^{32}P$-postlabelling of DNA adducts for a smoker (6-B) and a non-smoker (10-B). Multiple adducts are identified from laryngeal cytosols in smokers, while none are found in non-smokers. Butanol extraction versus nuclease P1 enhancement of DNA adducts was not appreciably different.

mogenized. Cytosols were cleared by centrifugation at 240,000 g for 26 minutes. Microsomes were prepared as described by Guengerich [6]. Antibodies to the cytochrome P-450s (CYPs) 1A1, 1A2, 2C, 2E1, 2E6, and 3A4 were used to create immunoblots of laryngeal microsomes as previously described [7–10]. Total DNA-adduct levels and the presence of various CYPs were correlated.

Cytosols of the laryngeal tissues were assayed for the presence of both monomorphic (NAT1) and polymorphic (NAT2) *N*-acetyltransferase activity. Assays for the metabolism of *p*-aminobenzoic acid (PABA; NAT1), sulfamethazine (SMZ; NAT2), and sulfotransferase (ST) were carried out using 0.5–1 mg cytosolic protein/ml in the reaction mixture and 2 mM AcCoA with either 0.5 mM SMZ or 0.1 mM PABA as substrate. Reactions were incubated at 37°C for 5 min and were terminated by the addition of trichloroacetic acid (final concentration 4%). After centrifugation (and neutralization with NaOH for PABA assays), 20 $\mu$l aliquots of the supernatant were analyzed by HPLC [12,22].

## IDENTIFICATION OF H-*RAS* MUTATIONS

Specimens from the same patients were histopathologically identified and examined. Both malignant and histologically normal tissues were obtained. From each cell block, 20 um sections were obtained; special care was taken to prevent contamination. Patient charts were also reviewed for smoking history and stage of disease.

DNA was extracted as follows: the histologic sections were deparaffinized and placed in 2 ml of xylene, vortexed and centrifuged at 2000 rpm for 3 minutes; this step was repeated 3 times. The specimens were precipitated with ethanol in decreasing concentrations (v/v) of 100, 90, and 70%. After the ethanol was removed, PCR buffer containing 0.5 mg/ml proteinase K was added and the mixture allowed to incubate overnight; phenol was then used to extract the DNA. Concentration of the DNA was determined from optical density measurements at 260 nm; purity was calculated from $A_{260}$:$A_{280}$ and $A_{260}$:$A_{230}$ ratios.

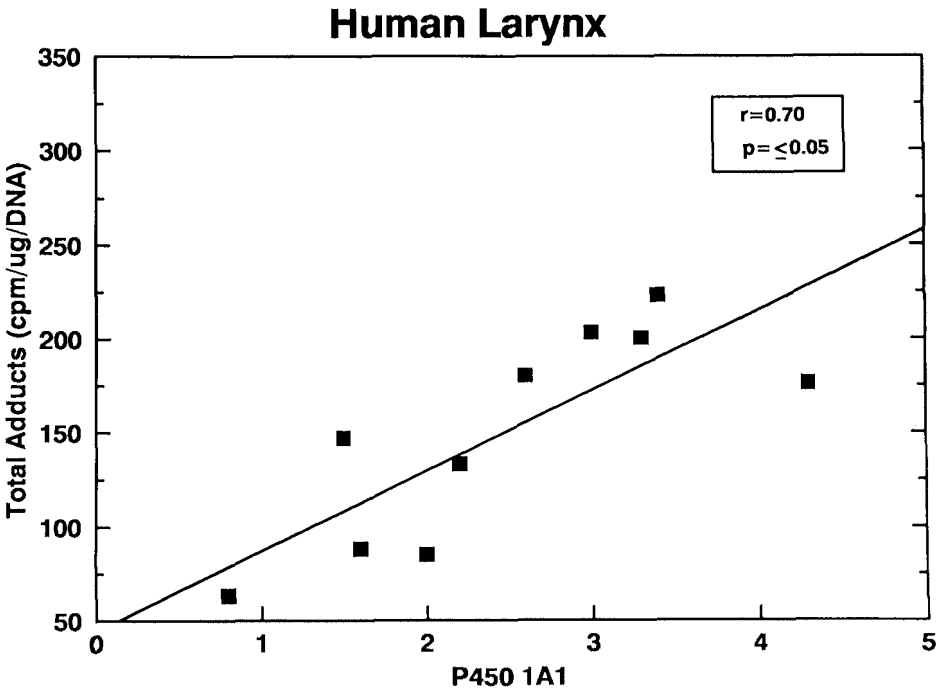



Fig. 2. Correlation between total adducts (cpm/ug/DNA) and cytochrome P-4501A1. Best-fit analysis demonstrates r = 0.70, p < 0.05.

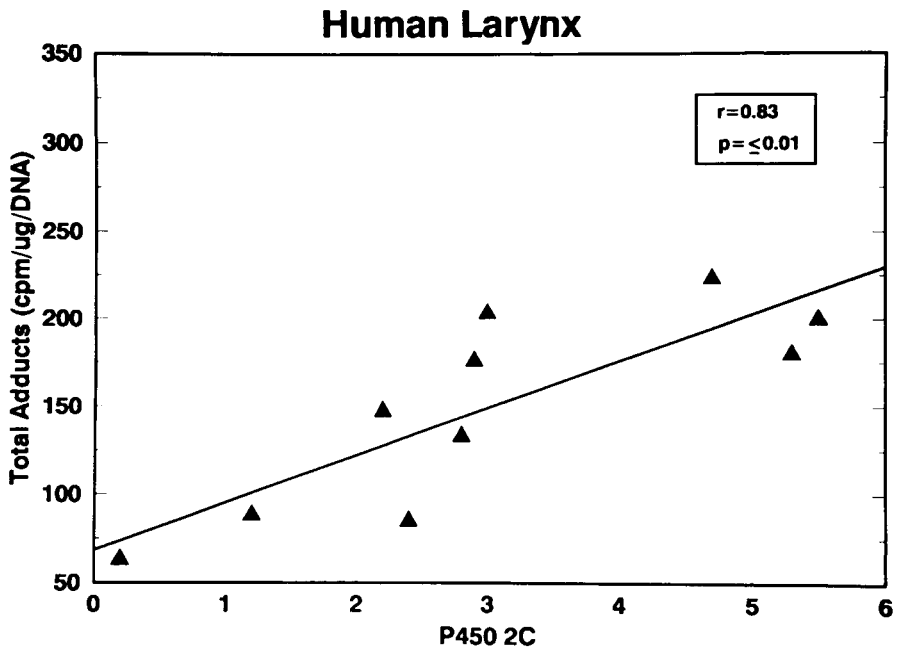



Fig. 3. Correlation between total adducts and P-4502C. r = 0.83, p < 0.01.

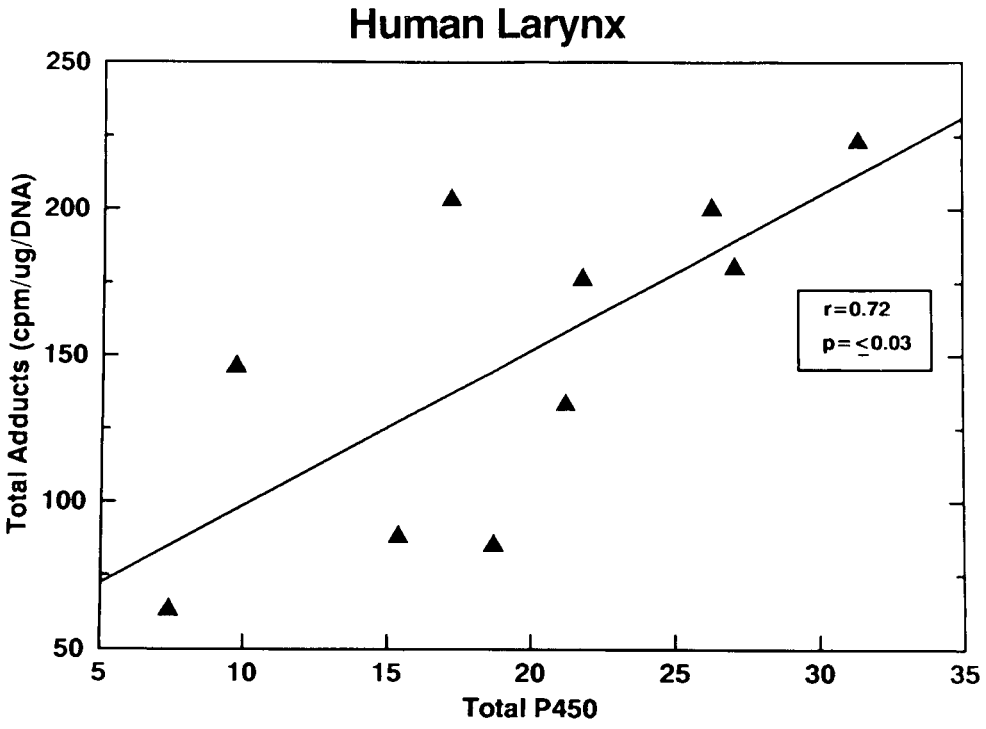



Fig. 4. Correlation between total adducts and total P-450. r = 0.72, p < 0.03.

**TABLE II. Acetyltransferase Activity of Human Larynx Cytosols**

| | Specific Activity (nmol/min/mg) ± s.d. | |
|---|---|---|
| **Samples (n)** | **PABA (NAT1)** | **SMZ (NAT2)** |
| Smokers (13) | 3.09 ± 1.39 | <0.01 |
| Non-smokers (3) | 3.06 ± 0.46 | <0.01 |

Sulfotransferase (ST) activity (α-naphthol) could not be detected (<0.01)

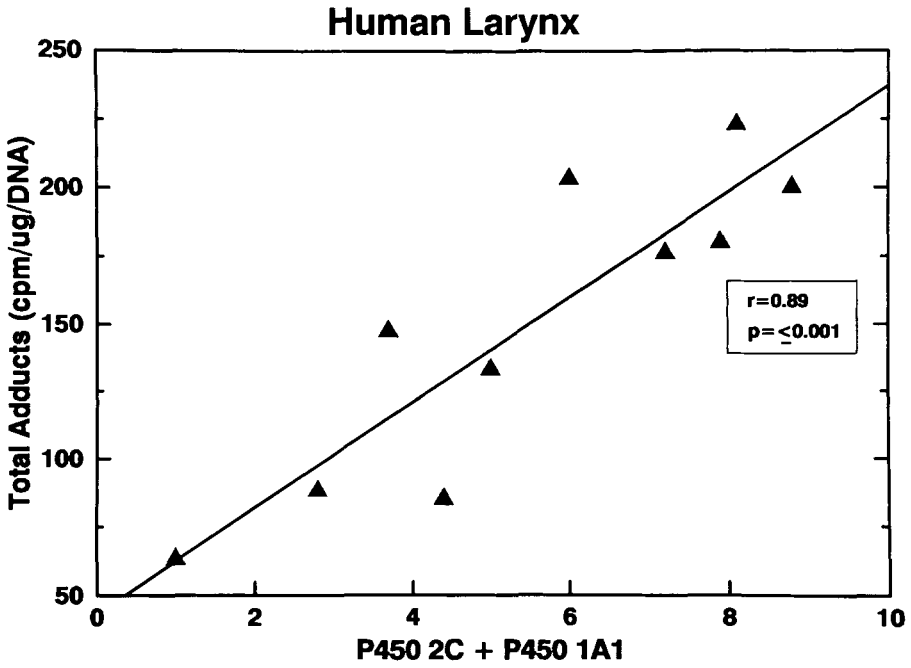


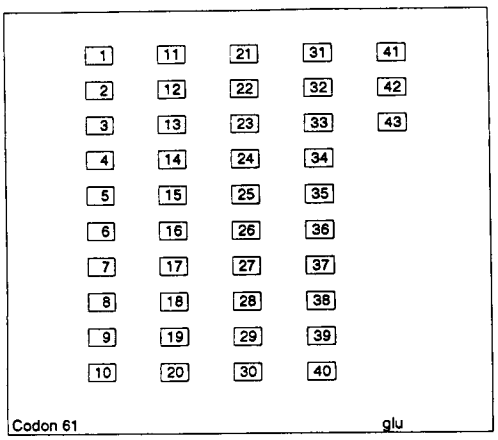

Fig. 5. Correlation between total adducts and P-4502C + P-4501A1. r = 0.89, p < 0.001.

After DNA purity was ascertained, point mutations in the *ras* gene were detected using PCR [11]. First, regions flanking codons 12, 13, and 61 were amplified. After cycling was complete, amplified fragments which had been denatured at 95°C for three minutes were applied to prefixed N-bond nylon filters. Ten μl of the PCR product were applied and cross-linked to the filter with UV light. The filters were prehybridized and then hybridized with fully degenerate $^{32}$P-labelled oligonucleotide probes (Clon Tech Oncolyzer Kit, Palo Alto, CA) specific for codons 12, 13, and 61. The filters were washed and subjected to autoradiography.

## RESULTS

Total DNA adduct levels were calculated after $^{32}$P-postlabelling. DNA adducts were evident only in smokers and the profiles were similar for both the butanol and nuclease P1 enhancement methods, indicating that aromatic amines are probably not contributing to the adduct pattern observed [5]. Patients who had stopped smoking for as little as 10 months prior to surgery did not demonstrate detectable adducts. A representative analysis is shown in Figure 1.

Immunoblots of laryngeal tissue microsomes



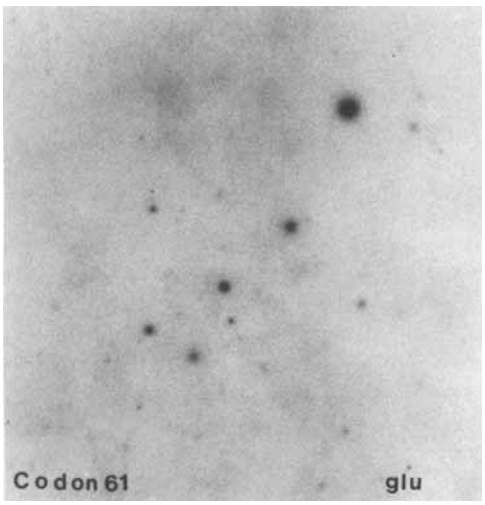




**Fig. 6.** Slot-blot analysis of mutations at codon 61 of the H-*ras* gene. Analysis was performed with specific oligonucleotide probes for possible mutations at codon 61. The blot shown in this figure demonstrates samples with a point mutation causing a glycine → glutamine transversion. Top: Schematic representation of 43 samples (tumor, non-tumor, controls) on the nylon filter. Bottom, positive mutations noted in samples 4, 7, 18, 26, 27, 34, and 41.

using antibodies directed against CYPs 1A1/1A2, 2C, 2E1, 2A6, and 3A4 demonstrated levels of microsomal proteins ranging from 1–10% of those found in human liver. No CYP1A2 activity could be detected (Table I). Thus, the CYP1A1 levels measured represent the highest yet detected in human (non-hepatic) tissues.

Figures 2–5 demonstrate correlations between total DNA adduct levels and CYPs.

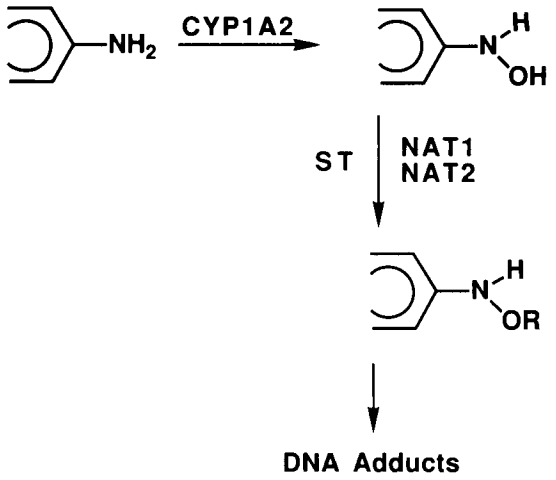



**Fig. 7.** Basic pathway for aromatic amine activation and conversion to ultimate carcinogen. The formation of the *N*-hydroxyarylamine is catalyzed by CYP1A2. CYP1A2 and NAT2 are felt to be important in human carcinogenesis. **ST:** sulfotransferase; **NAT1/NAT2:** *N*-acetyltransferases; **CYP:** cytochrome P-450.

Highly significant correlations between adducts and CYPs 1A1 ($r = 0.70$; $p < 0.05$, Fig. 2) and 2C ($r = 0.83$; $p < 0.01$, Fig. 3) were noted. Total CYP was significantly correlated with adduct levels ($r = 0.72$; $p < 0.03$, Fig. 4) and the addition of CYP1A1 and 2C was highly significant ($r = 0.89$; $p < 0.001$, Fig. 5).

*N*-Acetyltransferase activity in laryngeal cytosols was appreciable for *p*-aminobenzoic acid (NAT1) but not sulfamethazine (NAT2; Table II). No correlation between total DNA adducts and either NAT1 or NAT2 activity was seen.

H-*ras* mutations were noted in approximately 60% of specimens; both tumor and non-tumor specimens exhibited mutations in codons 12, 13, and 61. Specifically, a single common point mutation, a transversion in codon 61 (Gln → Glu) occurred in 5 patients, all of whom were smokers (Fig. 6).

## DISCUSSION

The metabolic phenotyping of laryngeal mucosa yields insight into the bioactivation of cigarette-related carcinogens. If putative carcinogens and/or their metabolic pathways can be elucidated, specific chemoprevention strategies can be designed.

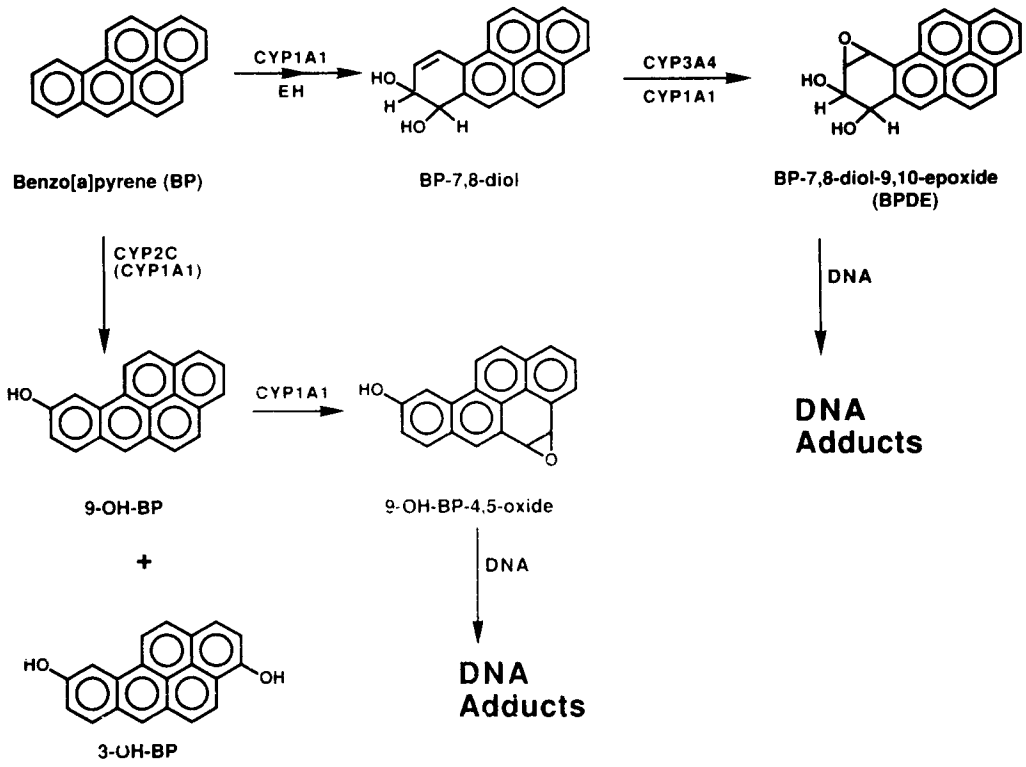



**Fig. 8.** Bioactivation pathway for a representative PAH, benzo(a)pyrene. As mentioned in the text, finding CYP1A1 and CYP2C activity may indicate that the ultimate carcinogen for larynx cancer is 9-OH-B(a)P-4, 5-oxide. **B(a)P:** Benzo(a)pyrene; **OH:** hydroxy; **CYP:** cytochrome P-450.

Our data demonstrate that the enzyme systems necessary for bioactivation of carcinogens exist within the laryngeal mucosa. The basic pathway for aromatic amine activation and conversion to ultimate carcinogens is based on the pathway demonstrated in Figure 7. The first step in this metabolic pathway is formation of the *N*-hydroxy arylamine intermediate which is catalyzed by CYP1A2. *O*-Acetylation of this *N*-hydroxy intermediate can be accomplished via the polymorphic NAT2 or by the monomorphic NAT1 enzyme. CYP1A2 and NAT2 are postulated to be important for aromatic amine bioactivation [1,12–14]. Our data indicate that neither CYP1A2 nor NAT2 are operative in laryngeal tissue, but that NAT1 activity (as measured using PABA as substrate) is appreciable.

The highly significant finding of CYP1A1 and 2C correlating with total DNA adduct levels suggests the involvement of polycyclic aromatic hydrocarbons (PAH). The bioactivation pathway for a representative PAH, benzo(*a*)pyrene [B(*a*)P], is shown in Figure 8. B(*a*)P has been implicated as a putative carcinogen in lung cancer [15,16]; the metabolic pathways for its initial activation involves CYPs 1A1, 3A4, and 2C almost exclusively. In view of the high correlation between adducts and CYPs 1A1 and 2C, ultimate carcinogenic metabolites such as 9-hydroxy-benzo(*a*)pyrene-4,5-oxide should be considered as the putative carcinogen for DNA adduct formation in laryngeal cancer.

That DNA adduct formation could be detected in laryngeal mucosa was not surprising; however, our initial hypothesis was that aromatic amine adducts would be significant. The comparison of butanol extraction and nuclease P1 enhancement in DNA adduct formation is important to determine adduct class. If aromatic amine adducts are detected by butanol extraction, they will be removed during nuclease P1 cleavage of the bulky adduct [17,18]. As there

was no difference between the butanol and nuclease P1 $^{32}$P-postlabelling autoradiographs, PAH must be considered strongly as the class of putative carcinogen.

The analysis of H-*ras* mutations in this report is preliminary and must be verified. However, a relatively specific and novel transversion appearing only in smokers, and not necessarily in tumor tissue, makes an interesting case for an early event in laryngeal carcinogenesis. Very specific K-*ras* mutations have been recently identified in lung cancer [19,20]; investigation of laryngeal tissue for similar genetic alterations may prove worthwhile.

Although these experiments have identified the appropriate CYPs involved in PAH activation, enzymatic activity of the specific CYPs has not been analyzed. Differential activity of these enzyme systems will yield further information concerning likely carcinogens; the actual analysis of specific carcinogens awaits these enzymatic studies.

Finally, it should be noted that as the clinical behavior of cancer in the head and neck differs according to site of origin, it is reasonable to expect that the metabolic profile of the various mucosal surfaces in the head and neck will be different. The correlation of biologic behavior to metabolic phenotype is an area of significant interest; these tissues await characterization and analysis. Determination of putative carcinogen class and agent could allow better planning for chemoprevention trials; the addition of information concerning *ras* mutations may provide insight into some of the events in carcinogenesis of the head and neck.

## REFERENCES


1. Turesky RJ, Lang NP, Butler MA, Teitel CH, Kadlubar FF: Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. Carcinogenesis 12:1839–1845, 1991.
2. Lang NP, Chu DZJ, Hunter CF, Kendall DC, Flammang TJ, Kadlubar FF: Role of aromatic amine acetyltransferase in human colorectal cancer. Arch Surg 121:1259–1261, 1986.
3. Kirlin WG, Trinidad A, Yerokun T, Ferguson RJ, Andrews AF, Brady PK, Hein DW: Polymorphic expression of acetyl coenzyme A-dependent arylamine *N*-acetyltransferase and acetyl coenzyme A-dependent *O*-acetyltransferase-mediated activation of *N*-hydroxyarylamines by human bladder cytosol. Cancer Res 49:2448–2454, 1989.
4. Menck HR, Garfinkel L, Dodd GD: Preliminary report of the National Cancer Data Base. CA 41: 8–9, 1991.
5. Gupta RC: Enhanced sensitivity of $^{32}$P-postlabelling analysis of aromatic carcinogen: DNA adducts. Cancer Res 45:5656–5662, 1985.
6. Guengerich FP: Microsomal enzymes involved in toxicology—analysis and separation. In Hayes AW (ed): "Principles and Methods of Toxicology." New York: Raven Press, Inc., 1982, pp 272–280.
7. Yun CH, Shimada T, Guengerich FP: Purification and characterization of human liver microsomal cytochrome P-450 2A6. Mol Phamacol 40:679–685, 1991.
8. Shimada T, Misono KS, Guenerich FP: Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism: Purification and characterization of two similar forms involved in the reaction. J Biol Chem 260:909–921, 1986.
9. Guenrich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ: Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. J Biol Chem 260:5051–5060, 1986.
10. Distlerath LM, Reilly PEB, Martin MV, Davis GG, Wilkinson GR, Guengerich FP: Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. J Biol Chem 260:9057–9067, 1985.
11. Innis MA, Gelfand DH, Sukilsky JJ, White TJ (eds): "PCR Protocols: A Guide to Methods and Applications." San Diego: Academic Press, Inc. 1992.
12. Ilett KF, Reeves PT, Minchin RF, Kinnear BF, Watson HF, Kadlubar FF: Distribution of acetyltransferase activities in the intestines of rapid and slow acetylator rabbits. Carcinogenesis 12:1465–1469, 1991.
13. Butler MA, Lang NP, Young JF, Caporaso NE, Vineis P, Hayes RB, Teitel CH, Massengill JP, Lawsen MF, Kadlubar FF: Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. Pharmacogenetics 2:116–127, 1992.
14. Talaska G, Al-Juburi AZSS, Kadlubar FF: Smoking related carcinogen-DNA adducts in biopsy samples of human urinary bladder: Identification of *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl as a major adduct. Proc Natl Acad Sci USA 88:5350–5354, 1991.
15. Van Schooten FJ, Hillebrand MJ, Van Leeuwen FE, Lutgerink JT, Van Zandwijk N, Jansen HM, Kriek E: Polycyclic aromatic hydrocarbon-DNA adducts in lung tissue from lung cancer patients. Carcinogenesis 11:1577–1681, 1990.
16. Alexandrov K, Rojas M, Geneste O, Castegnaro M,

Camus AM, Petruzzeli S, Giutini C, Bartsch H: An improved fluorometric assay for dosimetry of benzo-(*a*)pyrene diol-epoxide-DNA adducts in smokers' lung: Comparisons with total bulky adducts and aryl hydrocarbon hydroxylase activity. Cancer Res 52:1–6, 1992.

17. Reddy MV, Gupta RC, Randerath E, Randerath K: $^{32}$P-postlabelling test for covalent DNA binding of chemicals *in vivo*: Application to a variety of aromatic carcinogens and methylating agents. Carcinogenesis 5:231–243, 1984.
18. Reddy MV, Randerath K: Nuclease P1-mediated enhancement of sensitivity of $^{32}$P-postlabelling test for structurally diverse DNA adducts. Carcinogenesis 7:1567–1577, 1986.
19. You M, Candrian U, Maronpot RR, Stoner GD, Anderson MW: Activation of the Ki-*ras* protooncogene in spontaneously occuring and chemically induced lung tumors of the strain-A mouse. Proc Nat Acad Sci(USA) 86:3070–3074, 1989.
20. Rodenhuis S, Slebos R: The *ras* oncogenes in human lung cancer. Am Rev Respir Dis 142: 527–530, 1990.