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CARCINOGEN-DNA AND PROTEIN ADDUCTS-MARKERS OF EXPOSURE AND RISK

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Abstract

It is well established that the initiating event in chemical carcinogenesis is the binding of reactive carcinogens to DNA. Thus, a number of analytic methods have been developed for determining levels of carcinogen-DNA adducts in humans as a marker of individual exposure and, potentially, of risk for cancer development. In addition, reactive carcinogens also bind to protein suggesting protein adducts can be used as a surrogate for DNA adducts in some situations. We have developed monoclonal and polyclonal antibodies to carcinogen-DNA and protein adducts and highly sensitive ELISA and immunohistochemical assays for determining levels of adducts in human tissues. These studies have demonstrated higher levels of adducts in those with higher exposure as a result of workplace, dietary, chemotherapy, environmental or lifestyle (smoking) exposures. Elevated levels of adducts have been found in lung and liver cancer cases compared to controls. We have also used DNA adducts to determine efficacy of an antioxidant vitamin intervention. DNA adduct studies have demonstrated very different levels of damage in those with similar exposure levels. These interindividual differences are likely the result genetic differences in capacity to activate carcinogens, detoxify reactive intermediates and repair DNA adducts once formed. We are currently investigating the relationship between polymorphisms in a number of these genes to determine their relationship to adduct levels as well as their ability to confer increased risk for cancer development. The ability to identify high risk individuals will allow the targeting of screening and preventive strategies to those most likely to benefit.

Introduction

DNA damage is well established as the initiating event in chemical carcinogenesis; DNA

epidermal cells of treated patients compared to biopsies of untreated controls. Lymphocytes from individuals working in a Polish coke oven factory, living near it, or in a rural region were analyzed using a quantitative fluorescence method (13). In agreement with prior ELISA data, adducts were significantly higher in both the occupational and environmental groups compared to the rural control group. The immunofluorescence method was also applied to exfoliated oral cells of smokers and nonsmokers. However, autofluorescence of unstained oral cells necessitated the use of the immunoperoxidase method (14; 15). Quantitation of peroxidase staining indicated about a 2-fold increase in PAH-DNA in smokers compared to nonsmokers and similar data were obtained on exfoliated bladder cells of smokers and nonsmokers (16). The peroxidase method was also applied to the detection of damage in human arterial tissue using internal mammary arteries collected during coronary bypass surgery (17). Smoking related staining was observed in both endothelial and smooth muscle cells. More recently we applied this method to stored paraffin sections of breast tissue from breast cancer cases (18). While there was no significant difference in mean relative nuclear staining intensity in nonsmokers (444 90, n=75), ever smokers (435 91, n=72), and current smokers (456 98, n=35), these studies demonstrated that PAH, known animal mammary carcinogens, could reach breast tissue. The demonstration that paraffin sections could be used for adduct detection expands the types of studies that can be done.

4-Aminobiphenyl

4-Aminobiphenyl (4-ABP), an aromatic amine present in cigarette smoke, is an established animal carcinogen and, in humans, has been associated with urinary bladder cancer. Antibodies recognizing 4-ABP-DNA have been developed and characterized. An immunofluorescence method was validated by comparison of adduct levels measured in 4-ABP-treated mouse liver to those determined by alkaline hydrolysis release of 4-ABP from DNA isolated from the tissue, followed by gas chromatography-mass spectroscopy (GC/MS) (19). A good correlation ($r=0.98$, $p<0.0001$) was found between relative fluorescence intensity and adduct levels determined by GC/MS. Based on adduct levels determined by GC/MS, the immunohistochemical method has a limit of sensitivity

of approximately 1 adduct/10^7 nucleotides.

Immunohistochemical methods have been used for detection of adducts in exfoliated oral (16; 20) and bladder (16) cells of smokers and nonsmokers. Higher adducts were observed in smokers in both studies. Studies of stored paraffin blocks of bladder tumor tissue found dose (number of cigarettes smoked/day)-related increases in 4-ABP-DNA and an association with mutant p53 protein expression (21). Laryngeal tissues (tumor, polyp, and normal) also demonstrated smoking related adducts (22).

Oxidative DNA Damage

8-Hydroxy- or 8-oxo-deoxyguanosine (8-OHdG) is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals generated endogenously or exogenously. We developed antibodies and an immunoperoxidase method for quantification of 8-OHdG in human samples (23). Higher levels of specific nuclear staining were observed in oral mucosal cells of every smoker compared to their matched nonsmoker (24). The mean level of relative staining was elevated almost 2-fold in smokers compared to nonsmokers but there was no association between staining intensity and the number of cigarettes smoked/day. The method was more recently used on nasal biopsies from children living in Mexico City who are exposed to high levels of air pollutants and from children living in a much less polluted costal town (25). Higher levels (2-3-fold) of oxidative damage were observed in exposed compared to control children. We have been less successful using this method to detect oxidative damage in the same breast tumor tissues assayed for PAH-DNA, described above. There was no significant difference in mean relative nuclear staining intensity for 8OHdG between nonsmokers (213 86, n=67), ever smokers (190 84, n=78) and current smokers (193 75, n=36). In either current or ever smokers, 8OHdG levels were not related to the dose of exposure. While measurement of PAH-DNA may provide information about environmental exposure to this class of carcinogens, measurement of oxidative DNA damage in tumors probably reflects the enhanced oxidative stress of this tissue

Relationship between Polymorphisms in Carcinogen Metabolism Genes and Adduct Formation

In our studies and those by others, interindividual variability in adducts, given the same external exposure, was up to 100-fold. This variability suggests that individuals respond differently as a result of genetic susceptibility related activation or detoxification of carcinogens, DNA repair capacity, and other lifestyle or dietary factors. This is an area of active research in which correlations between adduct levels and genotype or phenotype for metabolism and DNA repair genes are determined. While data have not always been consistent, associations between high activity of enzymes involved in metabolic activation of carcinogens and decreased activity of detoxifying enzymes have been frequently observed. For example, in a study of smokers, subjects with the *CYP1A1*3* polymorphism, which has been associated with higher activity of this enzyme involved in the production reactive intermediates of PAH, had significantly higher (2-fold , $p<0.03$) levels of PAH-DNA than those without (26). In another study of smokers, an inverse relationship was found between serum levels of smoking-adjusted vitamin C ($r=-0.22$, $p<0.09$) and cholesterol-adjusted vitamin E ($r=-0.25$, $p<0.05$) and levels of mononuclear cell PAH-DNA (27). This protective effect was observed mainly in subjects who were deleted in the gene for *glutathione S-transferase M1 (GSTM1)* which is responsible for conjugation of the reactive intermediates of a number of carcinogens including BP.

Adducts as Markers of Risk

Two types of epidemiologic studies have been used to determine the relationship between adducts and risk for cancer development. The first method uses samples collected from subjects at the time of diagnosis in either hospital based or population based studies. Appropriate matched controls are also sampled. With this type of study there is concern that the disease may have influenced the biomarker. For example, in a study of PAH-DNA and lung cancer, cases may have recently quit smoking as a result of disease symptoms or diagnosis. For this reason, case-control studies nested in cohorts are considered to provide more reliable biomarker data. In

this type of study, samples are collected from large numbers of healthy subjects and banked for long periods of time until cases are identified and matched to healthy subjects. Stored samples are then retrieved and assayed. While more reliable, this type of study is also more costly since large number of subjects must be recruited and followed for long periods of time.

Polycyclic Aromatic Hydrocarbons and Lung Cancer

Our initial studies on adducts and risk for lung cancer collected blood samples from cases at the time of diagnosis. Elevated PAH-DNA was observed in white blood cells of lung cancer cases compared to controls after adjusting for smoking (10; 11). Genotype for *GSTM1* was also determined and when combined with PAH-DNA adducts, dicotomized into low and high adduct levels, demonstrated a greater than additive effect of null genotype and high adducts (28). These data suggest that risk for lung cancer development is affected by genetic susceptibility related to carcinogen metabolism and/or DNA repair which leads to higher adducts for a given level of smoking. Subjects with "more" susceptible genotypes form higher levels of adducts at the same level of cigarette smoking than those with "less" susceptible genotypes.

Aflatoxin B_1 and 4-Aminobiphenyl and Hepatocellular Carcinoma

Primary hepatocellular carcinoma (HCC), the leading cause of death in Taiwan is clearly related to chronic infection with hepatitis B virus (HBV). Aflatoxin B_1 (AFB_1), a contaminant of moldy food, is a well-established animal hepatocarcinogen. To determine the role of AFB_1 in human liver cancer, we developed antibodies to the DNA or protein adducts of AFB_1 or to the AFB_1 metabolites excreted in urine [reviewed in (29)]. These antibodies have been used in both standard and nested case-control studies. In one study, liver tissue was collected from 105 HCC patients at the time of surgery and from 37 control subjects undergoing abdominal surgery for other reasons. In cases and controls, chronic HBV infection was assessed by serum HBsAg, and AFB_1 exposure by immunohistochemical detection of AFB_1-DNA adduct in liver tissue. P53 protein mutations in tumor tissues were identified by immunohistochemistry and DNA mutations by single-stranded conformational polymorphism (SSCP) and sequencing analyses. We found both chronic

HBsAg carrier status and liver AFB_1-DNA adducts to be significantly higher in cases than in controls with odds ratios (OR) of 8.4, and 5.2, respectively. Moreover, HCC risk was greatest in individuals with both AFB_1-DNA adducts and HBsAg, suggesting a viral-chemical interaction. *P53* protein, DNA and codon 249 mutations were detected in 37%, 29%, and 13%, respectively, of the HCC cases. Most of the DNA mutations were transversions, and the only major clustering site for mutations was codon 249. AFB_1-DNA addicts were associated with *p53* protein (OR=2.9, p=0.054) and DNA mutations (OR=2.9,p=0.082) but with borderline significance.

Epidemiological studies have suggested that cigarette smoking is a risk factor for development of HCC. To further investigate this relationship, 4-ABP-DNA adducts were measured by an immunoperoxidase method on the same surgical liver tissues from HCC cases and controls (30). Mean relative staining intensity for 4-ABP-DNA was slightly higher in tumor than nontumor tissues from HCC patients. Both means were significantly higher than the mean of control tissues from non-HCC patients. However, no difference was found between smokers and nonsmokers in staining intensity in any tissues. After stratifying relative staining intensity of 4-ABP-DNA adduct levels into tertiles, according to the total control tissues analyzed, there was a monotonic increasing HCC risk. ORs were 4.14 (95%CI 1.15-15.50) and 9.71 (CI 2.82-34.86) for middle and high adduct levels compared to low adduct levels, respectively. The linear relationship between adduct levels in liver tissue and HCC risk was also significant after adjusting for covariates, including status of hepatitis B surface antigen (HBsAg). These results indicate that 4-ABP exposure plays a role in the development of HCC in humans.

Cytochrome P450 enzymes play a major role in the metabolism of both of these chemical carcinogens. To investigate interindividual differences in these enzymes, polyclonal antisera and immunoperoxidase staining were used to detect the expression of CYP1A1/2 and 3A4 in tissues from Taiwan (31). There was variability in the expression and staining pattern for both CYP1A1/2 and 3A4 in all tissue types. In tissues from controls, there was no correlation between P450 expression and smoking history or hepatitis B virus antigen status. Since these

samples had been previously analyzed for the DNA adducts AFB_1 and 4-ABP, we also investigated the relationship between P450 levels and DNA adducts. 4-ABP-DNA adducts were higher in tissues with elevated levels of CYP1A1/2 (p=0.02). There was no relationship between CYP1A1/2 or CYP3A4 and AFB_1-DNA adducts in control tissues. Staining intensity for CYP1A1/2 and 3A4 followed the order: tumor tissues < control tissues < adjacent nontumor tissues. CYP1A1/2 levels tended to be lower in tumor and adjacent nontumor tissues than for CYP3A4. In HCC cases, 4-ABP-DNA adducts were higher in subjects with higher levels of CYP1A1/2, stratified by tissue type, but these differences were not significant. For CYP3A4, in contrast to control tissues, there was a significant association with AFB_1-DNA adducts in tumor and adjacent nontumor tissue of HCC cases. These results suggest that one factor influencing carcinogen-DNA adducts is levels of specific P450 enzymes. However, adduct formation *in vivo* is a complex processes dependent upon numerous genetic and environmental factors.

Two case-control studies nested in cohorts recruited in Taiwan for cancer screening have been carried out. One cohort consists of 4841 male, asymptomatic, long-term HBV carriers 30-65 years of age who were recruited between August 1988 and June 1992 (32). At the time of initial follow up there were 32 patients with HCC who were matched with 73 controls on age (5 years) and time of sample collection. Aflatoxin-albumin was measured by competitive ELISA and subjects categorized into nondetectable, low, and high groups. Increasing risk was observed with higher adducts levels with OR of 1.6 [CI=0.6-4.0] and 3.8 (CI=1.0-14.5) in the low and high adduct level groups. Because this cohort consists of chronic HBsAg carriers, we could not investigate the interaction between carrier status and aflatoxin exposure.

The second study is nested in a community-based cancer screening project carried out in 7 townships in Taiwan between July 1990 and June 1992 (33). A total of 12,024 males and 13,594 females enrolled and provided blood and urine samples as well as questionnaire information. Between 1991 and 1995, 56 cases of HCC were identified and individually matched by age, sex, residence, and date of recruitment to 220 healthy controls from the same large cohort in Taiwan.

Blood samples were analyzed for HBV and HCV markers and for aflatoxin-albumin adducts; urine was tested for aflatoxin metabolites. As expected, the HBsAg carrier rate was higher in cases (48/56, 85.7%) than controls (31/219, 14.2%) resulting in a significantly increased risk for HCC [matched OR (OR_m)=45.5, CI=13.8-149.7). Anti-HCV was detected in 10.9% (6/56) of HCC cases and 7.4% (16/217) of controls. Compared to those positive for neither viral marker, the OR_m was 86.1 (CI=9.7-763.7) for subjects positive for both HBsAg and anti-HCV, 50.6 (CI=14.2-179.8) for subjects positive for HBsAg only, and 4.0 (CI=0.5-29.9) for subjects positive for anti-HCV only. Based on the Mantel extension test for increased risk with neither marker positive as referent, the odds ratio for developing HCC was found to increase in the presence of anti-HCV alone, HBsAg alone, and both anti-HCV and HBsAg (χ=10.32, $p<0.01$).

HCC cases had a higher percentage of detectable aflatoxin-albumin adducts than controls (31/52, 59.6% *vs.* 62/180, 34.4%; $p<0.01$). But the OR_m for detectable aflatoxin-albumin adducts was not significant after adjustment for HBsAg serostatus. The OR_m for those with urinary aflatoxin metabolites in the upper 50th percentile compared to those with levels in the lower 50th percentile was significantly different from unity irrespective of adjustment for HBsAg carrier status. Moreover, when metabolite levels were stratified into tertiles, there was a dose-response relationship between aflatoxin and HCC risk (test for trend, $p<0.05$), with an adjusted OR_m of 2.3 (CI=0.6-9.2) and 7.2 (CI=1.5-34.3) for subjects with medium and high levels, respectively, compared to those with a low level.

The combined effects of aflatoxin exposure and HBsAg carrier status on the development of HCC are shown in Table 2. OR_m for HBsAg seropositive subjects with high aflatoxin exposure were higher than for HBsAg seropositive subjects with low aflatoxin exposure or HBsAg seronegative subjects with high aflatoxin exposure. The increased risk ratio in HBsAg carriers was 4.02 (70.0/17.4) for detectable compared to nondetectable aflatoxin-albumin adducts and 4.91 (111.9/22.8) for high compared to low urinary aflatoxin metabolites.

Combined Effects of Exposure and Genotype on Risk

Genetic differences in carcinogen metabolizing enzymes are suspected to be partially responsible for individual differences in cancer risk. Polymorphism in both activating and detoxifying enzymes have been identified by phenotyping and genotyping methods (34). We are investigating the role of polymorphisms in *glutathione S-transferases* (*GST*) in risk for HCC in the nested cohorts (32). These enzymes are involved in the inactivation of reactive metabolites of a number of carcinogens including aflatoxin. Deletions in both *GSTM1* and *T1* are highly prevalent and can be easily determined by polymerase chain reaction amplification of the appropriate regions of the DNA. In the case-control study nested in the government workers cohort, we have examined the relationship between HCC risk and deletions in *GSTM1* and *T1* and aflatoxin-albumin adducts. The biological gradients between serum albumin adducts and HCC risk were observed among chronic HBsAg carriers who had null genotypes of *GSTM1* and *T1* but not among those who had the gene present. The multivariate-adjusted odds ratios of developing HCC for those who had low and high albumin adducts compared with those who had a nondetectable adduct level were 4.1 and 12.4, respectively, for carriers with null the *GSTM1* genotype ($p<0.01$) and 0.7 and 1.4 for those with the gene ($p=0.98$). For *GSTT1*, ORs were 1.8 and 10.2 for those with the null genotype (<0.05) and 1.3 and 0.8 for those with the gene present ($p=0.93$). Thus, those who lack *GSTM1* and *T1*, important detoxifying enzymes, are at greater risk of developing HCC once they are exposed to aflatoxin. We are continuing to investigate the gene-environment interaction between aflatoxin exposure and *GST* polymorphisms as well as polymorphisms in the cytochrome P450 which are responsible for the activation of aflatoxin to the reactive epoxide.

Intermediate markers in intervention studies

DNA adducts have potential as intermediate endpoints in chemoprevention studies [reviewed in (35). We recently carried out a randomized clinical trial in heavy smokers using DNA damage as the endpoint (36). A combination of antioxidant vitamins (vitamins C and E and β-carotene) or placebo was administered for 6 months and blood, oral cells and urine were collected

at various intervals. PAH-DNA adducts in mononuclear and oral cells and oxidative DNA damage (8-oxodeoxyguanosine) in oral cells were measured. Dropout rates were higher than anticipated and differed between the placebo and vitamin groups. Plasma levels of all three antioxidants rose significantly in the vitamin group but not in the placebo group. All three measures of DNA damage decreased in both groups; the between-group differences were not statistically significant. While these data do not provide clear evidence that antioxidant vitamin intake prevents DNA damage, the study demonstrates that DNA damage is a useful endpoint in chemoprevention trials.

Summary

The advantage of the immunologic approach is that once specific antibodies and assays have been developed they can be applied to large numbers of samples with relative ease. Those assays which use monoclonal antibodies can also be rapidly exported to other laboratories because of the ability to produce large amounts of antibody. A major disadvantage of the immunologic approach is the concern about cross-reactivity of the antibody with other material. For example, the antibody to BPDE-DNA cross-reacts with a number of other PAH diol epoxide adducts. Thus, this assay detects multiple adducts present in DNA and cannot give precise quantitative data on a specific adduct. This is not a problem in many studies in which relative levels of damage are of interest but must be considered in interpretation of data. Given these limitations, the immunologic approach can play an important role in molecular epidemiologic studies. Determination of polymorphisms in genes related to carcinogen metabolism and DNA repair are equally important. In combination, measurement of adducts and susceptibility may lead to the identification of high risk individuals. This should allow the targeting of preventive strategies to that part of the population at greatest risk.

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Reference

1. Poirier, M.C., Santella, R.M., and Weston, A. Carcinogen macromolecular adducts and their measurement. Carcinogenesis, *21*: 2000.

2. Santella, R.M. Immunologic methods for detection of carcinogen-DNA damage in humans. Cancer Epidemiology, Biomarkers & Prevention, *8*: 733-739, 1999.

3. Hemminki, K., Grzybowska, E., Chorazy, M., Twardowska-Saucha, K., Sroczynski, J.W., Putnam, K.L., Randerath, K., Phillips, D.H., Hewer, A., Santella, R.M., Young, T.L., and Perera, F.P. DNA adducts in humans environmentally exposed to aromatic compounds in an industrial area of Poland. Carcinogenesis, *11*: 1229-1231, 1990.

4. Perera, F.P., Hemminki, K., Young, T.L., Santella, R.M., Brenner, D., and Kelly, G. Detection of polycyclic aromatic hydrocarbon-DNA adducts in white blood cells of foundry workers. Cancer Res., *48*: 2288-2291, 1988.

5. Santella, R.M., Hemminki, K., Tang, D.L., Paik, M., Ottman, R., Young, T.L., Savela, K., Ludmilla, V., Dickey, C., Whyatt, R., and Perera, F.P. PAH-DNA adducts in white blood cells and urinary 1-hydroxypyrene in foundry workers. Cancer Epidemiology, Biomarkers & Prevention, *2*: 59-62, 1993.

6. Santella, R.M., Perera, F.P., Young, T.L., Zhang, Y.J., Chiamprasert, S., Tang, D.L., Wang, L.W., Beachman, A., Lin, J.H., and DeLeo, V.A. Polycyclic aromatic hydrocarbon-DNA and protein adducts in coal tar treated patients and controls and their relationship to glutathione S-transferase genotype. Mutat.Res., *5*: 241-247, 1994.

7. Perera, F.P., Hemminki, K., Grzybowska, E., Motykiewiez, G., Santella, R.M., Young, T.L., Dickey, C., Brandt-Rauf, P., DeVivo, I., Blaner, B., Tsai, W., and Chorazy, M. Molecular damage from environmental pollution in Poland. Nature, *360*: 256-258, 1992.

8. Fontana, R.J., Lown, K.S., Paine, M.F., Fortlage, L., Santella, R.M., Felton, J.S., Kinze, M.G., Greenberg, A., and Watkins, P.B. Effects of a chargrilled meat diet on expression of CYP3A4, CYP1A, and P-glycoprotein levels in healthy volunteers. Gastroent., *11*: 789-798, 1999.

9. Santella, R.M., Grinberg-Funes, R.A., Young, T.L., Dickey, C., Singh, V.N., Wang, L.W., and Perera, F.P. Cigarette smoking related polycyclic aromatic hydrocarbon-DNA adducts in peripheral mononuclear cells. Carcinogenesis, *13*: 2041-2045, 1992.

10. Tang, D.L., Santella, R.M., Blackwood, M.A., Young, T.L., Mayer, J., Tsai, W.Y., and Perera, F.P. A molecular epidemiological case-control study of lung cancer. Cancer Epidemiology, Biomarkers & Prevention, *4*: 341-346, 1995.

11. Perera, F.P., Mayer, J., Jaretzki, A., Hearne, S., Brenner, D., Young, T.L., Fischman,

H.K., Grimes, M., Grantham, S., Tang, M.X., Tsai, W.Y., and Santella, R.M. Comparison of DNA adducts and sister chromatid exchange in lung cancer cases and controls. Cancer Res., *49*: 4446-4451, 1989.

12. Zhang, Y.J., Li, Y., DeLeo, V.A., and Santella, R.M. Detection of DNA adducts in skin biopsies of coal tar-treated psoriasis patients: immunofluorescence and ^{32}P postlabeling. Skin Pharm., *3*: 171-179, 1990.

13. Motykiewicz, G., Malusecka, E., Grzybowska, E., Chorazy, M., Zhang, Y.J., Perera, F.P., and Santella, R.M. Immunohistochemical quantitation of polycyclic aromatic hydrocarbon-DNA adducts in human lymphocytes. Cancer Res., *55*: 1417-1422, 1995.

14. Zhang, Y.J., Hsu, T.M., and Santella, R.M. Immunoperoxidase detection of polycyclic aromatic hydrocarbon-DNA adducts in oral mucosa cells of smokers and nonsmokers. Cancer Epidemiol.Biomark.Prevent., *4*: 133-138, 1995.

15. Romano, G., Sgambato, A., Boninsegna, A., Flamini, G., Curigliano, G., Yang, Q., La Gioia, V., Signorelli, C., Ferro, A., Capelli, G., Santella, R.M., and Cittadini, A. Evaluation of polycyclic aromatic hydrocarbon-DNA adducts in exfoliated oral cells by an immunohistochemical assay. Cancer Epidemiology, Biomarkers & Prevention, *8*: 91-96, 1999.

16. Hsu, T.M., Zhang, Y.J., and Santella, R.M. Immunoperoxidase quantitation of 4-aminobiphenyl- and polycyclic aromatic hydrocarbon-DNA adducts in exfoliated oral and urothelial cells of smokers and nonsmokers. Cancer Epidemiology, Biomarkers & Prevention, *6*: 193-199, 1997.

17. Zhang, Y.J., Weksler, B.B., Wang, L.Y., Schwartz, J., and Santella, R.M. Immunohistochemical detection of polycyclic aromatic hydrocarbon-DNA damage in human blood vessels of smokers and non-smokers. Artherosclerosis., *140*: 325-331, 1998.

18. Santella, R.M., Gammon, M.D., Zhang, Y.J., Motykiewicz, G., Young, T.L., Hayes, S.C., Terry, M.B., Schoenberg, J.B., Brinton, L.A., Bose, S., Teitelbaum, S.L., and Hibshoosh, H. Immunohistochemical analysis of polycyclic aromatic hydrocarbon-DNA adducts in breast tumor tissue. Cancer Letters, 2000.

19. Al-Atrash, J., Zhang, Y.J., Lin, D., Kadlubar, F.F., and Santella, R.M. Quantitative immunohistochemical analysis of 4-aminobiphenyl-DNA in cultured cells and mice: Comparison to gas chromatography/mass spectroscopy analysis. Chem.Res.Toxicol., *8*: 747-752, 1995.

20. Romano, G., Mancini, R., Fedele, P., Curigliano, G., Flamini, G., Giovagnoli, M.R., Malara, N., Boninsegna, A., Vecchione, A., Santella, R.M., and Cittadini, A. Immunohistochemical analysis of 4-aminobiphenyl-DNA adducts in oral mucosal cells of smokers and nonsmokers. Anticancer Research, *17*: 2827-2830, 1997.

21. Curigliano, G., Zhang, Y.J., Wang, L.Y., Flamini, G., Alcini, A., Ratto, C., Giustacchini, M., Alcini, E., Cittadini, A., and Santella, R.M. Immunohistochemical quantitation of 4-aminobiphenyl-DNA adducts and p53 nuclear overexpression in T1 bladder cancer of smokers and nonsmokers. Carcinogenesis, *17*: 101-106, 1996.

22. Flamini, G., Romano, G., Curigliano, G., Chiominto, A., Capelli, G., Boninsegna, A., Signorelli, C., Ventura, L., Santella, R.M., Sgambato, A., and Cittadini, A. 4-Aminobiphenyl-DNA adducts in laryngeal tissue and smoking habits: an immunohistochemical study. Carcinogenesis, *19*: 353-357, 1998.

23. Yin, B., Whyatt, R.M., Perera, F.P., Randall, M.C., Jedrychowski, W., Cooper, Y., and Santella, R.M. Determination of 8-hydroxydeoxyguanosine by immunoaffinity chromatography-monoclonal antibody-based ELISA. Free Radical Biol.Med., *18*: 1023-1032, 1995.

24. Yarborough, A., Zhang, Y.J., Hsu, T.M., and Santella, R.M. Immunoperoxidase detection of 8-hydroxydeoxyguanosine in aflatoxin-B_1 treated rat liver and human oral mucosal cells. Cancer Res., *56*: 683-688, 1996.

25. Calderon-Garciduenas, L., Wang, L., Zhang, Y.J., Rodriguez-Alcaraz, A., Osnaya, N., Villarreal-Calderon, A., and Santella, R.M. 8-Hydroxy-2'deoxyguanosine, a major mutagenic oxidative DNA lesion and DNA strand breaks in nasal respiratory epithelium of children exposed to urban pollution. Environ.Health Perspect., *107*: 469-474, 1999.

26. Mooney, L.V., Bell, D.A., Santella, R.M., Van Bennekum, A.M., Ottman, R., Paik, M., Blaner, W.S., Lucier, G.W., Covey, L., Young, T.L., Cooper, T.B., Glassman, A.H., and Perera, F.P. Contribution of genetic and nutritional factors to DNA damage in heavy smokers. Carcinogenesis, *18*: 503-509, 1997.

27. Grinberg-Funes, R.A., Singh, V.N., Perera, F.P., Bell, D.A., Young, T.L., Dickey, C., Wang, L.W., and Santella, R.M. PAH-DNA adducts in smokers and their relationship to micronutrient intake and serum levels and glutathione *S*-transferase M1 genotype. Carcinogenesis, *15*: 2449-2454, 1994.

28. Tang, D.L., Rundle, A., Warburton, D., Santella, R.M., Tsai, W.Y., Chiamprasert, S., Zhou, J.Z., Shao, Y.Z., Hsu, Y.Z., and Perera, F.P. Associations between both genetic and environmental biomarkers and lung cancer: evidence of a greater risk of lung cancer in women smokers. Carcinogenesis, *19*: 1949-1954, 1998.

29. Santella, R.M., Chen, C.J., Zhang, Y.J., Yu, M.W., and Wang, L.Y. Biological markers of aflatoxin B_1 in hepatocellular cancer in Taiwan. In: M.L. Mendelsohn, L.C. Mohr and J.P. Peeters (eds.), Biomarkers medical and workplace applications, pp. 355-364, Washington, DC: Joseph Henry Press. 1998.

30. Wang, L.Y., Chen, C.J., Zhang, Y.J., Tsai, W.Y., Lee, P.H., Feitelson, M.A., Lee, C.S., and Santella, R.M. 4-Aminobiphenyl-DNA damage in liver tissue of

hepatocellular carcinoma patients and controls. Amer.J.Epidemiol., *147*: 315-323, 1998.

31. Zhang, Y.J., Chen, S.Y., Tsai, W.Y., Ahsan, H., Lunn, R., Wang, L.Y., Chen, C.J., and Santella, R.M. Expression of cytochrome P450 1A1/2 and 3A4 in liver tissues of hepatocellular carcinoma cases and controls from Taiwan and their relationship to hepatitis B virus and aflatoxin B_1- and 4-aminobiphenyl-DNA adducts. Biomarkers, 2000.

32. Chen, C.J., Yu, M.W., Liaw, Y.F., Wang, L.W., Chiamprasert, S., Matin, F., Hirvonen, A., Bell, D.A., and Santella, R.M. Chronic hepatitis B carriers with null genotypes of gluthione S-transferase M1 and T1 polymorphisms who are exposed to aflatoxin are at increased risk of hepatocellular carcinoma. Am.J.Hum.Genet., *59*: 128-134, 1996.

33. Wang, L.Y., Hatch, M., Chen, C.J., Levin, B., You, S.L., Lu, S.N., Wu, M.H., Wu, W.P., Wang, L.W., Wang, Q., Huang, G.T., Yang, P.M., Lee, H.S., and Santella, R.M. Aflatoxin exposure and the risk of hepatocellular carcinoma in Taiwan. Int.J.Cancer, *67*: 620-625, 1996.

34. Vineis, P., Malats, N., Lang, M., d'Errico, A., Caporaso, N., Cuzick, J., and Boffeta, P. Metabolic polymorphisms and susceptibility to cancer. Lyon: International Agency for Reseach on Cancer, 1999.

35. Santella, R.M. DNA damage as an intermediate biomarker in intervention studies. Proc.Soc.Exp.Biol.Med., *216*: 166-171, 1997.

36. Jacobson, J.S., Begg, M.D., Wang, L.W., Wang, Q., Agarwal, M., Norkus, E.P., Singh, V.N., Young, T.L., Yang, D., and Santella, R.M. Effects of a six-month intervention on DNA damage in heavy smokers. Submitted

37. Yang, X.Y. and Santella, R.M. Development of monoclonal antibodies to acetylaminofluorene modified DNA and poly(dG-dC)-Poly(dG-dC). In: C.M. King, L.J. Romano and D. Schuetzle (eds.), Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes, pp. 329-326, New York: Elsevier. 1987.

38. Hsieh, L.L., Hsu, S.W., Chen, D.S., and Santella, R.M. Immunological detection of aflatoxin B_1-DNA adducts formed in vivo. Cancer Res., *48*: 6328-6331, 1988.

39. Hsieh, L.L., Jeffrey, A.M., and Santella, R.M. Monoclonal antibodies to 1-aminopyrene-DNA. Carcinogenesis, *6*: 1289-1293, 1985.

40. Poirier, M.C., Santella, R.M., Weinstein, I.B., Grunberger, D., and Yuspa, S.H. Quantitation of benzo[a]pyrene-deoxyguanosine adducts by radioimmunoassay. Cancer Res., *40*: 412-416, 1980.

41. Santella, R.M., Lin, C.D., Cleveland, W.L., and Weinstein, I.B. Monoclonal antibodies to DNA modified by a benzo[a]pyrene diol epoxide. Carcinogenesis, *5*: 373-

377, 1984.

42. Zhang, Y.J., Geacintov, N.E., and Santella, R.M. Development of a monoclonal antibody recognizing benzo(c)pheananthrenediol epoxid-DNA adducts: application to immunohistochemical detection of DNA damage. Chem Res Toxicol, *10*: 948-952, 1997.

43. Young, T.L. and Santella, R.M. Development of techniques to monitor for exposure to vinyl chloride: monoclonal antibodies to ethenoadenosine and ethenocytidine. Carcinogenesis, *9*: 589-592, 1988.

44. Young, T.L., Habraken, Y., Ludlum, D.B., and Santella, R.M. Development of monoclonal antibodies recognizing 7-(2-hydroxyethyl) guanine and imidazole ring-opened 7-(2-hydroxyethyl)guanine. Carcinogenesis, *11*: 1685-1689, 1990.

45. Santella, R.M., Dharmaraja, N., Gasparro, F.P., and Edelson, R.L. Monoclonal Antibodies to DNA modified by 8-methoxypsoralen and ultraviolet A light. Nucleic.Acids.Res., *13*: 2533-2544, 1985.

46. Miolo, G., Stefanidis, M., Santella, R.M., Acqua, F., and Gasparro, F. 6,4,4'-Trimethylangelecin photoadduct formation in DNA: production and characterization of a specific monoclonal antibody. Photochem.Photobiol., *3*: 101-112, 1989.

Table 1 Polyclonal and monoclonal antibodies to carcinogen-DNA damage

Antigen	Ref
acetylaminofluorene-DNA	(37)
aflatoxinB_1-DNA	(38)
4-aminobiphenyl-DNA	(19)
4-aminobiphenyl-Guo	Unpublished
1-aminopyrene-DNA	(39)
benzo(a)pyrene diol epoxide-DNA	(40; 41)
benzo(a)pyrene diol epoxide-Guo	(41)
benzo(c)phenanthrene diol epoxide-DNA	(42)
1,N^6-etheno-Ado	(43)
3,N^4-etheno-Cyd	(43)
N^2,3-etheno-Guo	Unpublished
N^7-(2-hydroxyethyl)-Guo	(44)
5-hydroxymethyldeoxyuridine	Unpublished
8-methoxypsoralen-DNA (45)	
O^6-methylguanine	Unpublished
8-oxodeoxyguanosine	(23)
trimethylangelicine	(46)

Table 2 Combined effects of aflatoxin exposure measured in serum and urine and HBsAg carrier status on the development of HCC

Aflatoxin exposure index	HBsAg[a]	Cases	Controls		
Aflatoxin-albumin adducts					
Nondetectable	Negative	7	101	1.0[c]	
Detectable	Negative	1	52	0.3	0.0-3.6
Nondetectable	Positive	14	17	17.4*	3.7-81.9
Detectable	Positive	30	9	70.0*	11.8-415.4
Urinary aflatoxin metabolites					
Low	Negative	2	61	1.0[d]	
High	Negative	4	53	1.7	0.3-10.8
Low	Positive	10	14	22.8*	3.6-143.4
High	Positive	22	8	111.9*	13.8-905.0

[a] HBsAg: hepatitis B virus surface antigen

[b] OR_m : matched odds ratio adjusted for cigarette smoking and alcohol drinking

[c] Mantel extension test for increased risk: $\chi=9.29$ $p<0.01$

[d] Mantel extension test for increased risk: $\chi=8.01$ $p<0.01$

* $p<0.05$

From reference 33