Preferential DNA Damage and Poor Repair Determine ras Gene Mutational Hotspot in Human Cancer

Zhaohui Feng, Wenwei Hu, James X. Chen, Annie Pao, Haiying Li, William Rom, Mien-Chie Hung, Moon-shong Tang

Background: Mutations in ras genes are commonly found in human cancers and in animal models. Although mutations at codons 12, 13, and 61 of H-, N- and K-ras genes can activate their oncogenic function, mutations at codon 12 of K-ras are the most common mutations found among the three ras genes in human cancers. To investigate whether codon 12 of human K-ras is especially susceptible to carcinogens and/or whether carcinogen-DNA adducts at this codon are repaired less efficiently, we examined tobacco smoke carcinogen-induced DNA damage in normal human bronchial epithelial and fibroblast cells. Methods: We used the UvrABC nuclease incision method in combination with ligation-mediated polymerase chain reaction to map the distribution of DNA adducts induced by benzo[a]pyrene diol epoxide (BPDE) and other bulky carcinogens within exons 1 and 2 in H-ras, N-ras, and K-ras. We also analyzed BPDE-DNA adduct repair efficiency in these three genes using the same method. Results: Codons 12 and 14 of the K-ras gene were hotspots for carcinogen-DNA adduct formation, with little and no adduct formation at codons 13 and 61, respectively. The BPDE-DNA adducts formed at codon 14 were repaired almost twice as quickly as those formed at codon 12. There was some BPDE-DNA adduct formation at codons 12 of H-ras and N-ras, but this codon was not a hotspot. Furthermore, no substantial difference in repair rates between codon 12 and the other codons analyzed (codons 3 and 18) was observed in either the H-ras or N-ras genes. Conclusion: These findings link the human cancer mutational hotspot at codon 12 of K-ras to preferential DNA damage and poor repair.

Mutations in ras genes, which code for the p21 protein, a GTPase, are commonly found in human cancers and in mouse and rat models. These mutations occur almost exclusively at codons 12, 13, and 61 in both animal and human tumors (1,2). It has been shown that mutations at codons 12, 13, and 61 in any of the three ras genes can transform cells in tissue culture (2,3). Structural studies suggest that a mutation at codon 12, 13, or 61 of the H-ras gene reduces the intrinsic GTPase activity of the ras gene product, impairing its ability to bind to the GTPase-activating protein and thus confining p21 in a GTP-bound activated mode (4). Activated p21 is thought to trigger a kinase cascade, a signal transduction pathway that regulates growth and differentiation in many cell types (3,5).

In mouse models, it appears that occurrence of mutations at codons 12, 13, or 61 of the H-, K-, and N-ras genes in the tumors is dependent upon the tumor initiator and the tissue origin of the tumor (2,6). For example, 95% of papillomas induced by 7,12-dimethylbenz[a]anthracene in mouse skin have a CAA to CTA mutation at codon 61 of H-ras (2,6), whereas 57% and 16% of papillomas induced by 3-methylcholanthrene have mutations at either codon 13 or codon 61 of H-ras, respectively, and 42% of papillomas induced by nitrosomethylurea have mutations at codon 12 of H-ras (2). In contrast, 75% of radiation- or nitrosomethylurea-induced thymic lymphomas in mice have a mutation in K-ras (at codon 12) or N-ras (at codons 12, 13, and 61), but no mutation was seen in H-ras (2). These results suggest that, in animal models, susceptibility to carcinogen-induced DNA damage may determine in which ras gene a mutation occurs during tumorigenesis.

In human cancers, mutations at codon 12 of K-ras are the most common mutations found among the three ras genes. For example, 90% of human pancreatic cancers, 50% of colon cancers, and more than 30% of smoking-related lung cancers have a mutation at codon 12 of the K-ras gene (1,2,7–11). Interestingly, only 5% of lung cancers that are not smoking-related contain a mutation at codon 12 of the K-ras gene (7–11). These findings raise a long-answered question: why is it that mutations of ras genes occur preferentially at codon 12 of K-ras in human cancers, whereas mutations at codons 12, 13, and 61 of any of the three ras genes are capable of activating their oncogenic function? One possibility is that the K-ras gene with the mutation at codon 12 is more oncogenic than the K-ras gene with the mutation at codon 13 or 61 and also more oncogenic than the other ras genes with the mutation at codon 12, 13, or 61. Alternatively, codon 12 of K-ras may be more susceptible to damage by carcinogens than codons 13 or 61 of K-ras or codons 12, 13, or 61 of H- or N-ras. That is, it is possible that codon 12 of the K-ras gene in human pancreatic and colon cells may be particularly susceptible to damage by the etiologic agents of pancreatic and colon cancer. However, because the etiologic agents for these two types of cancers are not known, it is difficult to determine the role of DNA damage in forming the mutational hotspot at codon 12 of the K-ras gene in these cancers. In contrast, ample evidence from both epidemiologic and experimental studies has identified the etiologic agents of smoking-related...
Fig. 1. Benzo[a]pyrene diol epoxide (BPDE)-binding spectrum in exons 1 and 2 of ras genes in normal human bronchial epithelial (NHBE) cells. Panel A: Genomic DNA isolated from BPDE (2 μM)-treated (+) and untreated (−) NHBE cells was treated with UvrABC nuclease, followed by ligation-mediated polymerase chain reaction (LMPCR) (see “Materials and Methods” section). Typical autoradiographs showing the spectrum of BPDE binding in exon 1 of the K-ras (a), N-ras (b), and H-ras (c) genes and in exon 2 of K-ras (d). The sequences of codons 12, 14, and 61 (boxed) and their neighbors are indicated on the left of each autoradiograph; the guanines and the adenine bound by BPDE in these codons are indicated by G* and A* on the right of each autoradiograph. Lanes 1–3: Maxam–Gilbert sequencing controls of untreated genomic DNA. Lane 4: Genomic DNA isolated from BPDE-treated cells without UvrABC nuclease treatment. Lane 5: Genomic DNA isolated from BPDE-untreated cells with UvrABC nuclease treatment. Lane 6: Genomic DNA isolated from BPDE-treated cells with UvrABC nuclease treatment. Panel B: Quantitation of the relative levels of BPDE–DNA adducts formed at NHBE exon 1 sequences of the K-ras, N-ras, and H-ras genes. The intensity of UvrABC nuclease incision bands was quantified with the use of a Cyclone Phosphor-Imager. The relative levels of BPDE–DNA adduct formation at different codons of the different ras genes were calculated as described in the “Materials and Methods” section. Data are the mean ± 95% confidence intervals (error bars) from three independent experiments.
lung cancers as tobacco smoke carcinogens (12,13). Because the etiologic agents of smoking-induced lung cancer are known, it is possible to investigate whether these agents preferentially damage codon 12 of the K-ras gene.

In this study, we address the reason for the preferential mutation at codon 12 of the K-ras gene by mapping the distribution of DNA adducts induced by a major cigarette carcinogetic metabolite, benzo[a]pyrene diol epoxide (BPDE), and by other bulky chemical carcinogens at the nucleotide level in the three ras genes in normal human bronchial epithelial (NHBE) and normal human fibroblast (NHF) cells. We also determined the repair efficiency of carcinogen–DNA adducts in the three ras genes.

MATERIALS AND METHODS

Chemicals

BPDE, benzo[g]chrysene diol epoxide (BCDE), and N-acetoxy-2-acetylaminofluorene (NAAAF) were obtained from the National Cancer Institute Repository (Midwest Research Institute, Kansas City, MO). These compounds were dissolved in dimethyl sulfoxide. Aflatoxin B1 8,9-diol epoxide (AFB1-DE) was synthesized, as described in the literature (14), and dissolved in acetone.

Cell Culture and Carcinogen Treatment

NHFBE cells were cultured in medium provided by Clonetics (San Diego, CA). NHF cells (CCD-1064SK; American Type Culture Collection, Manassas, VA) were cultured in Iscove’s Modified Dulbecco’s Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were grown in Modified Dulbecco’s Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were grown until they were 50%–70% confluent, washed with phosphate-buffered saline (68 mM NaCl, 1.94 mM KCl, 1.07 mM KH2PO4 [pH 7.4]), and treated with carcinogens: BPDE for 30 minutes or BCDE, NAAAF, or AFB1-DE for 60 minutes at 37°C in the dark. Various concentrations of carcinogens were used in this study; however, we found that differing carcinogen concentrations did not qualitatively affect carcinogen–DNA adduct binding patterns. Thus, for the sake of clarity, we present data on only one concentration of each carcinogen (i.e., BPDE, 2 μM for NHBE and 1 μM for NHF; BCDE, 20 μM; NAAAF, 10 μM; and AFB1-DE, 50 μM). All of these carcinogens were prepared immediately before use. For kinetic analysis of DNA adduct repair, cells were treated with BPDE for 30 minutes, followed by incubation in fresh (BPDE-free) medium for varying periods of time (0, 4, 8, and 24 hours). The cells were then harvested for genomic DNA isolation.

DNA Isolation

Genomic DNA was isolated as previously described (15,16). In brief, cells were washed with phosphate-buffered saline and lysed with lysing buffer (0.5% sodium dodecyl sulfate [SDS], 10 mM Tris [pH 7.8], 10 mM EDTA, 10 mM NaCl, 100 μg/mL protease K) at room temperature for 1 hour. Protein was removed by repeated extraction with equal volumes of phenol and diethyl ether, and the DNA was then precipitated with sodium acetate (0.3 M, pH 7.0) and 75% ethanol and resuspended in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA). RNA was removed by treatment with RNase A (50 μg/mL) at 37°C for 2 hours, followed by repeated phenol and then diethyl ether extractions, and the DNA was precipitated by ethanol–sodium acetate and resuspended in TE buffer.

BPDE Modification of Genomic DNA

To determine the effect of chromatin structure on BPDE–DNA adduct formation, purified genomic DNA isolated from untreated NHBE cells was modified by treatment with 2 μM BPDE in TE buffer (pH 7.5) at room temperature for 2 hours (17,18). The unreacted BPDE was removed by repeated phenol and then

Fig. 2. Benzo[a]pyrene diol epoxide (BPDE)-binding spectrum in exon 1 of ras genes in normal human fibroblast (NHF) cells. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left of each autoradiograph; the guanines bound by BPDE in these codons are indicated by G* on the right of each autoradiograph. Methods for BPDE (1 μM) treatment and UvrABC nuclease mapping of the BPDE–DNA adduct distribution formed in NHF exon 1 of the K-ras (a), N-ras (b), and H-ras (c) genes were the same as those described in Fig. 1. Lane 1–3: Maxam–Gilbert sequencing controls of untreated genomic DNA. Lane 4: Genomic DNA isolated from BPDE-un-treated cells without UvrABC nuclease treatment. Lane 5: Genomic DNA isolated from BPDE-un-treated cells with UvrABC nuclease treatment. Lane 6: Genomic DNA isolated from BPDE-treated cells without UvrABC nuclease treatment. Lane 7: Genomic DNA isolated from BPDE-treated cells with UvrABC nuclease treatment.
diethyl ether extractions, and the genomic DNA was precipitated with ethanol–sodium acetate and resuspended in TE buffer.

**Cleavage of DNA Adducts by UvrABC Nuclease**

UvrABC nuclease, the DNA nucleotide excision repair enzyme complex isolated from *Escherichia coli*, was purified by the method of Sancar and Rupp (19). UvrABC nuclease makes a dual incision 5′ and 3′ to the adducted base; the 3′ incision occurs specifically at the fourth nucleotide position 3′ to a BPDE adduct (20,21). Purified genomic DNA was reacted with UvrABC nuclease to cleave the DNA adducts, as described previously (22). Briefly, a 10-fold molar excess of UvrABC nuclease was added to DNA (assuming an average size of 14 kb) in a reaction buffer containing 100 mM KCl, 1 mM ATP, 10 mM MgCl₂, 10 mM Tris–HCl (pH 7.5), and 1 mM EDTA at 37 °C for 1 hour. Additional increases in enzyme concentration (up to 40-fold excess) did not result in additional cleavage. The reactions were stopped by extracting with phenol and diethyl ether. The resultant DNA was precipitated with ethanol–sodium acetate and resuspended in TE buffer for further DNA adduct mapping.

**Mapping DNA Adducts at the Nucleotide Level With Ligation-Mediated Polymerase Chain Reaction**

To investigate the relationship between carcinogen adduct formation and mutations at codon 12 of the ras genes, we mapped the distribution of carcinogen adducts along exons 1 and 2 of K-ras, N-ras, and H-ras, using a modification of the ligation-mediated polymerase chain reaction (LMPCR) (23–25). Maxam–Gilbert sequencing reactions (26) were also performed to serve as a sequencing ladder—that is, the adduct-specific bands migrate four nucleotide positions faster than the corresponding bands in the sequencing ladder. A known quantity (1 μg) of DNA resulting from UvrABC nuclease incision or Maxam–Gilbert sequencing reactions was subjected to LMPCR (24,25). The resultant DNA was denatured at 90 °C for 5 minutes in 0.1 N NaOH and 90% formamide solution, separated by electrophoresis in 8% denaturing polyacrylamide gels, and electrotransferred to a GeneScreen nylon membrane (NEN, Boston, MA). Blots were hybridized with 32P-labeled DNA probes specific for exon 1 or 2 of the K-, H-, or N-ras genes (25) in hybridization buffer (0.25 M Na₂HPO₄ [pH 7.2], 1 mM EDTA, 7% SDS, 1% bovine serum albumin) at 60 °C for 12 hours. The membranes were then exposed to a Cyclone Phosphorimager (Packard, Meriden, CT), and the intensity of UvrABC nuclease incision bands and Maxam–Gilbert guanine reaction bands was determined. The blot hybridization method, LMPCR method, and primers used in this study were the same as those described previously (25), with slight modifications (23). Approximately 20 000 dpm of 32P-labeled linearized pBR322 plasmid DNA was added to each genomic DNA sample, including the Maxam–Gilbert sequencing reaction samples, at the beginning of the LMPCR reaction as an internal standard to monitor sample recovery. After LMPCR, equivalent counts of 32P (determined by a liquid scintillation counter [LKB-Wallac, Turku, Finland]) representing equivalent amounts of sample DNA were loaded into each lane of the sequencing gel to separate DNA fragments of different sizes.

**Quantitative Analysis of Adduct Formation**

Carcinogen–DNA adduct formation at different nucleotide sequences was determined as described previously (24,27–29), with slight modifications. In brief, the intensities of well-separated UvrABC nuclease incision bands and the Maxam–Gilbert guanine reaction bands (>15 bands) were quantified by using a Cyclone Phosphorimager. The intensity of each UvrABC nuclease incision band and Maxam–Gilbert guanine reaction band was subtracted from the control sample (samples without UvrABC nuclease treatment) band. The intensity per nucleotide was then calculated. The relative levels of carcinogen–DNA adduct formation at different codons of the ras genes were then calculated by normalization of the intensity per nucleotide of UvrABC nuclease incision bands to the intensity per nucleotide of the corresponding Maxam–Gilbert guanine bands.

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**Fig. 3.** Benzo[α]pyrene diol epoxide (BPDE)-binding spectrum in exon 1 of the K-ras gene in genomic DNA from untreated normal human bronchial epithelial (NHBE) cells treated directly with (+) or without (−) BPDE. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left of the autoradiograph; the guanines bound by BPDE in these codons are indicated by G* on the right of the autoradiograph. Genomic DNA was isolated from untreated NHBE cells and treated with BPDE (2 μM) in vitro, as described in “Materials and Methods.” Methods for BPDE (2 μM) treatment of NHBE cells and UvrABC nuclease mapping of the distribution of the BPDE adducts were the same as those described in Fig. 1. **Lanes 1–3:** Maxam–Gilbert sequencing controls of untreated genomic DNA. **Lane 4:** Genomic DNA isolated from untreated cells with UvrABC nuclease treatment. **Lane 5:** Genomic DNA isolated from untreated cells was modified with BPDE without UvrABC nuclease treatment. **Lane 6:** Genomic DNA isolated from untreated cells was modified with BPDE and then treated with UvrABC nuclease. **Lane 7:** Genomic DNA isolated from BPDE-treated cells without UvrABC nuclease treatment. **Lane 8:** Genomic DNA isolated from BPDE-treated cells with UvrABC nuclease treatment.
RESULTS

BPDE–DNA Adduct Formation in K-, N-, and H-ras Genes in NHBE and NHF Cells

To determine whether codon 12 of the K-ras gene may be more susceptible to damage by tobacco carcinogens than codons 13 or 61 of K-ras or codons 12, 13, or 61 of H- or N-ras, we measured the relative levels of DNA adducts induced by BPDE at the nucleotide level in the coding strand of exons 1 and 2 of the three ras genes in NHBE and NHF cells. Cultured human cells were treated with BPDE, and the locations of adducts formed in the three ras genes were determined using the UvrABC nuclease incision method in combination with the LMPCR technique (24,25). Previously (20,21), we have shown that UvrABC nuclease is able to incise BPDE–DNA adducts both specifically and quantitatively. Thus, this method allows quantification of BPDE–DNA adduct formation at the nucleotide level. The results shown in Figs. 1 and 2 demonstrate that BPDE–DNA adduct formation in both NHBE and NHF cells was highly selective in the coding strand of exons 1 and 2 of the K-ras gene: the first nucleotides of codons 12 and 14 of the K-ras gene were hotspots (i.e., these codons have the highest intensity of bands) for BPDE binding, with little and no BPDE–DNA adduct formation detected at codons 13 and 61, respectively (Fig. 1, A and B, and Fig. 2). In contrast, codons 12 and 14 of exon 1 in the coding strands of N-ras and H-ras were not hotspots for BPDE–DNA adduct formation, although the level of adduct formation at codon 12 of the K-ras gene is 4.4-fold (95% confidence interval [CI] = 4.0 to 4.8) higher than adduct formation at codon...
12 of the N-ras gene and 2.7-fold (95% CI = 2.2 to 3.2) higher than adduct formation at codon 12 of the H-ras gene (Fig. 1, A and B).

**Effect of Chromatin Structure on BPDE–DNA Adduct Formation in the K-ras Gene**

The selectivity of BPDE–DNA binding at codon 12 of the K-ras gene could be due to an unusual local chromatin structure in human cells resulting from protein–DNA associations, such as histone or nonhistone binding. To investigate this possibility, we treated genomic DNA isolated from NHBE cells directly with BPDE and then mapped the BPDE–DNA adduct distribution in the exon 1 sequence of the K-ras gene. Fig. 3 shows that treating genomic DNA directly with BPDE (lane 6) produced the same BPDE–DNA-binding spectrum in exon 1 of the K-ras gene as that obtained by treating NHBE cells with BPDE (lane 8). These results demonstrate that protein–DNA association does not contribute to the preferential BPDE–DNA binding at codon 12 of the K-ras gene and suggest that this preferential binding may be related to an intrinsic property of the genomic context in this region.

**Bulky Carcinogen Binding at Codon 12 of the K-ras Gene**

The reason for the high affinity of codon 12 of the K-ras gene for BPDE binding is unclear. Previously, we found that BPDE and other bulky chemical carcinogens, such as BCDE, NAAAF, AFB1-DE, and mitomycin C, bind preferentially to mutational hotspots with a CpG sequence in the p53 gene and that this preferential DNA binding is due to cytosine methylation at the CpG sequence (17,18,21,24,28). In the K-ras gene, however, neither of the two guanine residues of codon 12 (-TGGT) is within a CpG sequence (Fig. 1, B). It is possible that the first guanine residue at this sequence may be less strictly paired with the cytosine residue in the opposite strand; therefore, this guanine residue is more accessible to react with bulky electrophilic chemical carcinogens. If so, then codon 12 of the K-ras gene should have a high affinity for electrophilic chemical carcinogens, which bind to different moieties of the guanine residue, and not only for carcinogens that bind to exocyclic amino groups of the guanine residue, such as BPDE.

To test this possibility, we treated NHBE cells with either NAAAF, BCDE, or AFB1-DE and mapped carcinogen–DNA adduct distribution in exon 1 of the three ras genes. These carcinogens were chosen not only because of their carcinogenic potency but also because they bind to different moieties of the guanine residue: NAAAF binds to the C8 position; both BPDE and BCDE, two metabolically activated polycyclic aromatic hydrocarbons (PAHs), bind to the N2 position of the exocyclic amino group; and AFB1-DE binds to the N7 position of the guanine residue (18). Figs. 4 and 5 show that all of these bulky
chemical carcinogens bound preferentially at the first nucleotide of codon 12 of the K-ras gene in NHBE cells. DNA adducts induced by NAAAF were highly selective in the coding strand of exon 1 of the K-ras gene. Codon 12 was the hotspot for NAAAF binding, with only low levels of and no NAAAF–DNA adducts detected at codons 14 and 13, respectively (Fig. 4, A and B). Overall, NAAAF had a low affinity for the coding sequence of exon 1 of the H-ras gene, even though it bound strongly at the first two codons of exon 1 of N-ras. The level of NAAAF–DNA adduct formation at codon 12 of the K-ras gene is 4.7-fold (95% CI = 4.0 to 5.4) higher than at codon 12 of the N-ras gene and 5.8-fold (95% CI = 5.2 to 6.4) higher than at codon 12 of the H-ras gene (Fig. 4, A and B).

Both codons 12 and 14 of K-ras were preferential sites for AFB1-DE (Fig. 5, A and B) and BCDE (Fig. 5, C and D) binding. The BCDE–DNA-binding spectrum is similar to the BPDE-binding spectrum in exon 1 of the K-ras gene. The AFB1-DE–DNA-binding spectrum, however, is slightly different from the BPDE-binding spectrum, with one notable difference being that the level of AFB1-DE binding to codon 12 is higher than the binding to codon 14. These results suggest that codon 12 of the K-ras gene is, indeed, a preferential binding site for a variety of bulky chemical carcinogens and that this binding selectivity at codon 12 is higher for carcinogens that bind at the C8 and N7 positions of the guanine residue than for those that bind at the exocyclic N2 position.

**Repair Efficiency of Carcinogen–DNA Adducts at Codon 12 of the K-ras Gene**

In addition to preferential carcinogen–DNA binding, the higher mutation frequency observed at codon 12 of the K-ras gene compared with the other codons in that gene, or with codon 12 of the H- and N-ras genes could be due to poor repair efficiency of the DNA adducts formed at that site. To test this possibility, we determined the rate of BPDE–DNA adduct removal at different codons of exon 1 of the K-ras gene in both NHBE and NHF cells. Fig. 6, A and B, shows that BPDE–DNA adducts formed at codon 14 in NHBE cells were repaired almost twice as quickly as those formed at codon 12. At 8 hours after treatment, 65% (95% CI = 60% to 70%) of the adducts had been removed from codon 14, whereas only 30% (95% CI = 23% to 37%) had been removed from codon 12; at 24 hours after treatment, 90% (95% CI = 86% to 94%) of the adducts had been removed from codon 14, whereas only 50% (95% CI = 46% to 54%) had been removed from codon 12. Of note, the high level of BPDE–DNA adducts formed at codon 14 of K-ras was repaired as efficiently as the low level of adducts formed at codon 13. A difference in repair rates between codons 12 and 14 of K-ras was also observed in NHF cells. At 8 hours after treatment, 80% (95% CI = 70% to 90%) of the adducts at codon 14 had been removed, whereas only 35% (95% CI = 27% to 43%) of the adducts at codon 12 had been removed (Fig. 6, A and B).
No substantial difference in repair rates between codon 12 and the other codons (codons 3 and 18) analyzed was observed in either the N-ras or H-ras genes. The repair rates at codon 12 and at other codons of N-ras and H-ras were similar to those observed at codon 14 of K-ras (Fig. 6, A and B). Thus, not only is codon 12 of K-ras a preferential site for BPDE binding but also, as these results demonstrate, BPDE–DNA adducts formed at this codon are poorly repaired compared with adducts formed at the same codon in the H-ras and N-ras genes or at other codons of the K-ras gene.

**DISCUSSION**

Our results demonstrate that codon 12 of the K-ras gene is a preferential binding site for various bulky chemical carcinogens, including a major tobacco smoke carcinogen, BPDE, and that BPDE–DNA adducts formed at this codon are poorly repaired compared with adducts formed at codon 12 of the H-ras and N-ras genes or at other codons in the K-ras gene. The high susceptibility of this codon to carcinogen binding, combined with poor repair of the resulting adducts, may be a reason for the high mutation frequency at codon 12 of the K-ras gene observed in human cancers.

The high binding affinity of BPDE and other bulky chemical carcinogens for codon 12 of the K-ras gene suggests that tobacco smoke may induce a high frequency of DNA damage at this codon, increasing the probability of mutations at this codon and thereby contributing to lung carcinogenesis. It is already known that lung cancers in smokers have a high frequency of mutation at codon 12 of the K-ras gene (>30%) and that these mutations
bear the hallmark of bulky chemical carcinogen-induced mutations—that is, a G to T transversion at the first nucleotide of this codon (1,7–11). In contrast, although mutations at codons 13 and 61 of the K-ras gene also have oncogenic consequences, it appears that BPDE and possibly other carcinogens generated by tobacco smoke have low or no binding affinity for these codons. The etiology of lung cancers in nonsmokers is probably very different from that in smokers, and lung tumorigenesis in nonsmokers may or may not be initiated by mutations at the K-ras gene. Indeed, mutations at this gene are infrequent (about 5%) in lung cancers from nonsmokers (1,7–11). Interestingly, in a recent study (30), a high frequency of mutation at codon 12 of K-ras (30%) was observed in lung cancer from nonsmokers who had been exposed to PAH-rich coal combustion emissions; 86% of these mutations were G to T transversions, which is the hallmark of PAH-induced mutations.

The results from these studies raise the possibility that the high mutation frequency observed at codon 12 of the K-ras gene in human pancreatic and colon cancers may also be due, at least in part, to the etiologic agents of these cancers (whether generated endogenously or from exogenous sources) binding preferentially to this codon and to poor repair of adducts formed at this codon in these tissues.

It should be noted that knocking out the K-ras gene in mice results in lethality during embryonic development (31). In contrast, H-ras or N-ras knockout mice develop normally (31). These results indicate that K-ras, but not H- or N-ras, may play a crucial role in mouse embryonic development. Furthermore, the post-translation modifications and the route of trafficking of the three ras gene products to the plasma membrane are different (32). Thus, even though these three gene products have the same basic functions, the timing of their expression and their interactions with other factors crucial for development could be different. These differences may contribute to more favorable conditions for cancer development in individuals with a mutated K-ras gene. These recent findings support the notion that a mutation in the K-ras gene may be more oncogenic than mutations in the H- and/or N-ras genes. Indeed, if this is the case, then our finding that the mutational hotspot in the K-ras gene is a preferential binding site for several bulky carcinogens provides strong additional evidence as to why the K-ras gene is mutated far more frequently in most human cancers than the H- and/or N-ras genes. This preferential carcinogen binding also provides a plausible explanation as to why, even in the K-ras gene itself, mutations preferentially occur at codon 12 and not at codons 13 or 61 in human cancers.

The reasons for the high susceptibility of codon 12 of the K-ras gene to DNA damage by bulky carcinogens remain unclear. The same nucleotide sequences as codon 12 of K-ras and its nearest neighbors (-TGGTG-) are present not only in other regions of exon 1 in K-ras but also in both N- and H-ras genes (Fig. 1, B). However, none of these nucleotide sequences, except at codon 12 of the K-ras gene, has high affinity for bulky chemical carcinogens (Figs. 1, 4, and 5). Therefore, it is unlikely that this 5-base sequence determines the preferential binding of carcinogens at codon 12 of the K-ras gene. Moreover, the sensitivity of codon 12 of K-ras to DNA damaging agents is not limited to BPDE and BCDE, which bind to the exocyclic N2 position of the guanine residue, but is also observed with NAAAF, which binds to the C8 position of the guanine residue, and with AFB1-DE, which binds to the N7 position of the guanine residue (18). Thus, these results suggest that the first guanine residue in codon 12 may be present in a conformation that has relatively high affinity and freedom to interact with various bulky chemical carcinogens.

The high level of BPDE–DNA adduct formation at codon 12 of the K-ras gene may be due to methylation of C5 cytosine at the CpG site at this codon. Previously, we have shown that methylation of C5 cytosine at CpG sites greatly enhances the efficiency of DNA adduct formation at the guanine residue at CpG sites by various carcinogens, including BPDE (17,18,21,27). Because the first guanine residue of codon 14 in the K-ras gene (-CGTA-) is within a CpG sequence, the preferential BPDE binding at this codon in both NHBE and NHF cells may also be due to methylation of the CpG sequence. Although codon 14 of the K-ras gene is a preferential site for BPDE–DNA binding (Fig. 1), no mutation at this codon has ever been reported in human cancers, unlike the case for codon 12 (1,2). This lack of mutation at codon 14 in human cancers may be due to the K-ras protein that has this mutation retaining its wild-type function and, therefore, retaining normal regulation. Furthermore, our study demonstrates that DNA adducts formed at codon 14 are repaired more efficiently than adducts formed at codon 12. Moderate BPDE–DNA binding was also observed at codon 12 (-CGGC-) of the H-ras gene, possibly because this codon is also within a CpG sequence. However, mutations in H-ras are rarely found in lung and other solid tumors, except for breast and bladder cancers. We have found that DNA adducts formed at codon 12 of H-ras are repaired efficiently, which may contribute to the rarity of mutations at this codon in smoking-related lung cancer.

In summary, we have demonstrated that one of the major tobacco smoke carcinogens, BPDE, selectively binds to codon 12 of the K-ras gene, but not the H-ras or the N-ras gene. We have also demonstrated that DNA damage occurring at codon 12 of K-ras is poorly repaired compared with DNA damage occurring at other codons. We propose that preferential carcinogen binding and poor repair may explain why mutations at codon 12 of K-ras, but not at codons 13 or 61 of the K-ras gene or codons 12, 13, or 61 of the H- or N-ras genes are commonly found in human lung cancer and in humans in cancers in general. These results also raise the question as to why codon 12 of the K-ras gene has increased sensitivity to DNA damage because mutations at this codon are harmful.

REFERENCES


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NOTES

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