Mutagenesis of the H-ras Protooncogene and the \( p53 \) Tumor Suppressor Gene

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Abstract

Point mutations in ras protooncogenes and in the \( p53 \) tumor suppressor gene are common in many forms of human cancer. The identification of carcinogens which are responsible for their induction in humans is of great interest because it may suggest measures for disease prevention. Furthermore, the load of somatic mutations in cancer-related genes in premalignant tissues may become a useful parameter for risk assessment. For the measurement of such mutations, highly sensitive genotypic mutation systems are required which avoid the selection and clonal expansion of cells on the basis of a mutated phenotype.

We have developed the restriction fragment length polymorphism/polymerase chain reaction method for genotypic mutation analysis and applied it to the study of the mutability of hot-spot codons in c-H-ras1 and \( p53 \) genes with human carcinogens. In particular, we studied the mutability of codons 247–250 of \( p53 \) with the mycotoxin aflatoxin B\(_1\) (AFB\(_1\)) in human hepatocytes. AFB\(_1\) preferentially induced the transversion of guanosine to thymidine in the third position of codon 249, generating the same mutation which is found in a large fraction of hepatocellular carcinomas from regions of the world with AFB\(_1\)-contaminated food. Our results are in support of AFB\(_1\), as an etiological factor for hepatocellular carcinoma in AFB\(_1\)-contaminated areas.

In an ongoing study we are comparing the load of mutations in hot-spot codon 12 of c-H-ras1 in urinary bladder carcinoma and in normal tissue, by restriction fragment length polymorphism/polymerase chain reaction. We observed moderately elevated abundances of guanosine to thymidine transversions in the middle position of codon 12 in tumor DNA. These results may reflect a mutator phenotype of the tumor tissue or they could be the consequence of the heterogeneity of the biopsies which were analyzed.

Introduction

Somatic mutations have been detected in genes related to several forms of human disease and, in particular, in protooncogenes and tumor suppressor genes. The clonal character of human malignant tumors implies that the original mutation must be present at early stages in a minute minority of cells in essentially normal tissue. In most cases somatic mutations in disease-related genes do not give rise to a functional change of the mutated cell which would allow its isolation or expansion in vitro. Therefore, selection of mutated cells on the basis of an altered phenotype must be replaced by biochemical separation and detection of the altered sequence of the gene of interest. Such “genotypic” mutation analysis requires large numbers of cells at the outset, since the expansion of mutated cells is avoided. Genotypic mutation systems are required because the actual mutability of a particular nucleotide sequence is expected to vary substantially for different genetic loci. Therefore, phenotypic mutation model systems and model genes can give only general indications about the type of mutations which can be caused by a particular mutagen. Factors which affect the mutability are local chromatin structure and sequence context, the transcriptional state of the gene, its replication schedule, and the repairability of the mutagen-induced lesions (1, 2).

Genotypic mutation systems must possess analytical sensitivity which far exceeds the requirements for the detection of heterozygous or homozygous mutations in tissues from tumors or from patients with hereditary diseases. Average spontaneous mutation frequencies per base pair in human cells are estimated to be in the range of \( 10^{-8} \) to \( 10^{-10} \), and these frequencies increase only 10–1000-fold upon exposure to a mutagen. Therefore, methods are required which allow the separation and/or distinction of a few altered DNA sequences from \( 10^2 \) to \( 10^{10} \) copies of the corresponding wild-type sequence, in the presence of large quantities of cellular DNA. Ideally, genotypic mutation systems should allow the measurement of the type, frequency, and distribution of base pair changes, insertions, and deletions in any target gene. Several experimental approaches to genotypic mutation systems are being developed (1–9).

We have developed the RFLP3/PCR approach to genotypic mutation analysis (1, 2, 10, 11) and applied it to the study of the mutagenesis of the c-H-ras1 protooncogene and the \( p53 \) tumor suppressor gene in human cells by potential human carcinogens. The RFLP/PCR method measures base pair changes and, under certain conditions, insertions and small deletions which occur in restriction enzyme recognition sequences and render the mutated site resistant to cleavage by the corresponding endonuclease. A known number of copies of MS are added and a fragment of the gene of interest which contains the mutated restriction site is amplified by high-fidelity PCR and cloned into Agt10. Mutants are identified and quantitated by oligonucleotide plaque hybridization. Absolute mutation frequencies are obtained by calibration with MS.

Mutagenesis of Codons 247–250 of the \( p53 \) Tumor Suppressor Gene by Aflatoxin B\(_1\)

HCC represents a major cause of mortality in certain areas of the world. Liver cirrhosis and chronic infection with hepatitis viruses have been identified as important risk factors (12, 13). In addition, contamination of food with the mycotoxin AFB\(_1\) has been implicated as an etiological factor in certain regions of eastern Asia and sub-Saharan Africa (14, 15). Indeed, recent studies suggest that hepatitis B virus and AFB\(_1\) may exert synergistic effects (16). Approximately 50% of HCC in high-AFB\(_1\) regions (17, 18) but only 20% in low-AFB\(_1\) regions harbor mutations in the \( p53 \) tumor suppressor gene, and the spectrum of mutations is quite different (19, 20). More than half of the tumors from high-AFB\(_1\) regions contain guanosine to thymidine transversions in the third position of codon 249 (AGG), resulting in the replacement of arginine by serine (17–21). In contrast, mutations are distributed throughout the highly conserved domains IV and V of \( p53 \) in HCC from low-AFB\(_1\) regions and no prevalence of guanosine to thymidine transversions is observed (22, 23). These results indicate that the substitution of arginine-249 by serine in the \( p53 \) protein is not required for hepatocarcinogenesis in humans. While guanosine to thymidine transversions are in agreement with the mutational specificity of AFB\(_1\) (24, 25) and other carcinogens forming bulky DNA adducts (26, 27), there is no convincing explanation for the prevalence of mutations in codon 249, which almost never harbors mutations in other forms of human cancer. Therefore, we have directly evaluated the mutability with AFB\(_1\), of codons 247–250 in human hepatocarcinoma HepG2 cells, by genotypic analysis using MspI and HaeIII RFLP/PCR.

The abbreviations used are: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma(s); AFB\(_1\), aflatoxin B\(_1\); MS, mutant standard; ENU, N-ethyl-N-nitrosourea.

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2 To whom requests for reprints should be addressed.

1934s
and c-H-ras1.

As shown in Fig. 1 a MspI site (CCGG, residues 14,067–14,070) extends from the third position of codon 247 to the third position of codon 248. Msp1 RFLP/PCR analysis indicates that rat liver microsome-activated AFB1 strongly induced the transversions C14,067 to adenosine in the third position of codon 247 and of G14,069 to thymidine in the middle position of codon 248. The corresponding G14,070 to thymidine transversion at the third position of codon 248 occurred with approximately 3-fold lower frequency, and the C14,068 to adenosine transversion in the first position of codon 248 was not occurred with approximately 3-fold lower frequency, and the C14,069 to adenosine transversion, with a frequency of 5.9 X 10^-7, at the first position of codon 250. HaeIII RFLP/PCR analysis shows that the transversions G14,073 to thymidine transversion involving the third position of codon 249 was not observed. A HaeIII site (GGCC, residues 14,072–14,075) reaches from the middle position of codon 249 to the middle position of codon 250. HaeIII RFLP/PCR analysis shows that the transversions G14,073 to thymidine in the third base pair of codon 249 and C14,074 to adenosine in the first base pair of codon 250 occurred with highest relative mutation frequencies. No significant increases above background were observed for the remaining 18 possible base pair changes.

Absolute mutation frequencies were calculated from the MS content of the RFLP/PCR products, the initial number of MS copies, and the number of copies of the p53 gene present at the outset of the experiment. The data for the AFB1-induced guanosine to thymidine and cytidine to adenosine transversions in the 8 base pairs which have been analyzed are shown in Fig. 2. It is evident that the G14,073 to thymidine transversion involving the third position of codon 249 was induced most strongly, with a frequency of 8.4 X 10^-7. It was followed by the C14,074 to thymidine transversion, with a frequency of 5.9 X 10^-7, at the first position of codon 250.

Our results indicate that AFB1 induces the transversion of guanosine to thymidine in the third position of codon 249, in the p53 segment extending from codon 247 to codon 250, with highest absolute frequency. The transversion of cytidine to adenosine in the adjacent first position of codon 250 represents the second most frequent mutation. All observed base pair changes are transversions, in agreement with the mutagenic specificity of AFB1, (24, 25) and other carcinogens which form bulky DNA adducts (26–28). AFB1 is known to be metabolically activated to AFB2,3-oxide, which almost exclusively reacts with the N' -position of guanine in DNA (29–31). The reactions of AFB1-guanosine adducts in DNA have been studied in detail in vitro (29, 30) and in cellular DNA (31–33). Activated AFB1 forms chemically unstable 2,3-dihydro-2-(N'-guanyl)-3-hydroxy aflatoxin B1 adducts, which in secondary reactions either lose their substituent and revert to intact guanine, form apurinic sites, or ring-open to stable derivatives referred to as AFB1-triamino-Py or FAPYR (29, 31). It appears that the latter adducts are removed only slowly by cellular repair processes in mammalian cells (30), and they represent likely premutagenic lesions. Therefore, the observed guanosine to thymidine and cytidine to adenosine transversions most likely are a consequence of polymerase misincoding at guanine adducts in the coding and noncoding strands, respectively (34).

We conclude that AFB1 preferentially induces the same guanosine to thymidine transversion in the third position of codon 249 of p53 which is found in HCC from high-AFB1 regions of the world. This is in agreement with an etiological role of AFB1 in HCC from these areas. While we observed highest mutability of this G-C base pair of codon 249, the difference in mutation frequencies at neighboring base pairs was only moderate. It follows that mutability on the level of DNA, which is mostly a consequence of adduct distribution and repair, cannot fully explain the almost complete prevalence of this particular guanosine to thymidine transversion in codon 249 in HCC from high-AFB1 regions. Evidently, the altered function of the serine-

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Fig. 2. Absolute frequencies of aflatoxin B1-induced guanosine to thymidine and cytidine to adenosine transversions in codons 247–250 of p53 in hepatocarcinoma HepG2 cells (from Ref. 34). The data were calculated from the mutant composition of the RFLP/PCR products, the initial number of p53 gene copies, and the number of copies of MS added at the start of the experiments. The values for background mutations in untreated controls were subtracted.
249 mutant p53 protein must play a role in the development of HCC which harbor this mutation. However, the fact that this mutation is only rarely found in HCC from low-AFB_1 regions indicates that it is not a prerequisite for hepatocarcinogenesis. It is conceivable that hepatitis B virus and the mutant serine-249 p53 protein play synergistic roles (34).

**Mutagenesis of Codon 248 of the p53 Tumor Suppressor Gene by N-Ethyl-N-nitrosourea**

Mutations in codon 248 (arginine), CGG, have been reported in 114 of 1178 tumors with p53 mutations which have been sequenced so far. They occur in several types of tumors but are particularly frequent in colorectal carcinoma, where approximately one fourth of all mutations are located in this codon. The mutations consist almost exclusively of transitions at the CpG dinucleotide, and C-G and A-T and C-G to T-A transitions were observed with equal frequencies (35, 36). Deamination of 5-methylcytosine (or cytosine) at CpG (37) or aliphatic alkylation of guanine at the C' position (38, 39) is expected to generate the observed transitions. It should be noted that guanosine to adenine transitions represent the predominant spontaneous mutations in human T-lymphocytes from normal donors (40).

We studied the mutability of codon 248 of human p53 with the alkylating agent ENU (2 mm). Alkylating agents are implicated as etiological agents in carcinogenesis in the gut and other tissues in humans (41). As mentioned above and shown in Fig. 1, codon 248 of human p53 is part of a Mspl/HpaII site (CCGG, residues 14,067-14,070) and, therefore, accessible to RFLP/PCR analysis. Major ENU-induced mutations in codon 248 in the Mspl RFLP/PCR products could be quantitated by sequence analysis. Single-stranded copies of the transcribed strand prepared by asymmetric PCR were sequenced by primer extension. Two weak adenine bands at positions 14,069 and 14,070 were discernible only on the autoradiogram and tracing for the RFLP/PCR product from ENU-treated cells, indicating the presence of G14,069 to adenine and G14,070 to adenine transition mutations in the nontranscribed strand. A complete analysis of all possible base pair changes in the Mspl site at residues 14,067-14,070 was performed by selective hybridization of A plaques with 32P-labeled 19-mer oligonucleotide probes. In agreement with the sequencing data, the predominant mutation was G-C at residue 14,069 to A-T, which represented 13% of the total analyzed plaques, while the second most abundant ENU-induced mutation was G-C at residue 14,070 to A-T, which was present in 5% of the plaques. These transitions most likely are produced by the miscoding of O-ethylguanine lesions located in the nontranscribed coding strand. A transition of G-C at residue 1695 to T-A in codon 11 but strongly induced the transition of G-C at residue 1698 to A-T in codon 12. A relatively high background level of A-T mutations at residue 1698 in untreated controls coincided with a frequent polymerase-induced error. The absolute frequency of this ENU-induced transition was 5 x 10^-7/H-ras allele and was obtained in an experiment in which 10 copies of internal MS were added at the outset to DNA from ENU-treated cells, which contained 10^8 copies of the c-H-ras1 gene.

The observed guanosine to adenine transitions most likely are caused by miscoding of O-ethylguanine in the nontranscribed strand, and our data indicate strong strand bias (54, 55). As discussed for codon 248 of p53, this result may reflect poor repair of the coding nontranscribed strand by excision or, less likely, by alkytransferase. The predominant mutation, i.e., A1698, originates from the middle guanosine residue of codon 12 rather than from G1697, which is part of a CpG dinucleotide. Therefore, deamination of 5-methylcytidine (or cytidine) in the transcribed strand can be excluded as a mutagenic mechanism. While the reasons for the hypermutability of the middle G-C base pair with ENU in codon 12 remain unknown, it is interesting to note that the same base pair is almost exclusively mutated in human urinary carcinoma (56-59). Since RFLP/PCR analysis avoids the phenotypic selection of mutated cells, it follows that the middle position of c-H-ras1 codon 12 is hypermutable (with ENU) on the DNA level, independently of the structure and function of the p21 protein.

**Somatic Mutation Load in Codons 11 and 12 of the c-H-ras1 Protooncogene in Urinary Bladder Tumors**

While several studies reported mutations in codon 12 in a high fraction of aneuploid invasive urinary bladder carcinoma (56-59), these findings were not confirmed in a recent study on a large series of tumors (60). In all studies, regardless of the frequencies of mutations, the majority of the observed base pair changes were guanosine to thymidine transversions in the middle position of codon 12. The reasons for the striking specificity of H-ras mutations in bladder carcinogenesis are unknown.

In an ongoing study we determined the frequencies of mutations in codons 11 and 12 of H-ras1 in biopsies from urinary bladder papilloma, invasive carcinoma, and normal tissue. Interestingly, G to T
transversions in the middle position of codon 12 (GTC) were 4–10-fold more abundant in DNA from tumor tissue than from normal urothelium. The fact that the frequencies of these mutations are only moderately increased in tumors suggests that they have not been efficiently selected during bladder tumorigenesis. Rather, the increase relative to normal controls may reflect a general mutator phenotype of malignant urothelial cells. Alternatively, the biopsies which were analyzed may have contained an unusually large portion of normal tissue.

In the present vocabulary, “early” and “late” genetic events in tumorigenesis must be considered as operational terms. They do not strictly define when the event occurred. Rather, they describe the time when the altered phenotype has sufficiently expanded in the tissue for the underlying genetic event to become detectable with available technologies. Fig. 3 presents several scenarios for the timing of a (second) genetic event and its association with or dissociation from clonal expansion. The quantitation of somatic mutation loads in cancer-related genes at different stages of tumor progression, as well as improved cytological techniques, is required to distinguish between these alternatives. This information may prove to be valuable for risk assessment and prognosis.

References


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