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Molecular and cellular events associated with aflatoxin-induced hepatocarcinogenesis

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Abstract - Aflatoxin B_1 (AFB1) is a potent carcinogen for the liver of many experimental animals, and has recently been classified as a human carcinogen on the basis of experimental data and epidemiologic studies in exposed human populations. Covalent binding to DNA resulting from metabolic activation to the 9, 10 oxide is considered to be a critical initiating event in the carcinogenesis process. Total level of covalent binding in animal tissues is quantitatively related to carcinogenic potency and organ specificity of AFB1. Covalent binding to DNA is linearly related to AFB1 dose in animals over several orders of magnitude, and the effectiveness of protective agents is reflected in diminished DNA binding. AFB1 forms covalent adducts with DNA only at the N7 position of guanine following metabolic activation through the 9, 10 oxide, and a qualitatively similar adduct profile is found in all cells (mammalian, microbial, and plant) in which epoxidation takes place. However, formation of the N7 guanine adduct in genomic DNA is non-random, with frequency of binding showing neighboring base effects. Adduct formation is substantially higher in actively transcribed gene sequences compared to untranscribed regions.

The major AFB1-N7-guanine adduct in DNA is unstable, readily undergoing depurination or conversion to a stable formamido-pyrimdyl (FAPY) derivative. The kinetics of adduct formation and removal in rat liver have been thoroughly characterized following single and multiple exposures to AFB1. Adduct level rises rapidly to a maximum at two hours, then falls with a half-life of about 7.5 hours; within 24 hours after dosing only the FAPY-derivative remains. This derivative is stable over very long periods of time, and accumulates following multiple dosing. The potency with which AFB1 forms DNA adducts is also related to its mutagenicity for a very wide range of cell types, from prokaryotes of many types to mammalian, including human, cells. It also induces other genotoxic effects such as sister chromatid exchanges, recombination, chromosome aberrations as well as other clastogenic responses. In bacterial cells, the principal mutations generated by AFB1 have been characterized as G to T transversions or G to A transitions. These properties are relevant to the recent identification of activated Ki-*ras* oncogenes in rat liver tumors induced by AFB1. Investigation of the mechanisms of oncogene activation revealed that many of the tumors contain Ki-*ras* sequences with single base mutations in the 12th to 14th codons. The mutations characterized to date include mainly G to T transversions, with G to A transitions observed less frequently. These findings are consistent with the conjecture that the oncogenes may have been mutated as a result of AFB_1-DNA adduct formation, and may have been involved in initiation of tumor development. Attempts are being made to determine the time and frequency at which these mutant alleles appear in preneoplastic liver.

AFLATOXIN–DNA BINDING

An extensive body of epidemiological evidence has accumulated that suggests a possible role for certain environmental chemicals as etiologic agents for a number of human genetic diseases, including cancer (ref. 1). The mechanistic details of the means by which chemical transformation are still, for the most part, incompletely understood, but there is good evidence that the chemically-induced alteration of informational macromolecules is an early, and probably requisite event in the process. Most chemicals that are potent carcinogens bind effectively to DNA, RNA and proteins under *in vivo* conditions, usually following metabolic activation to reactive, electrophilic derivatives that form covalent adducts with cellular macromolecules.

DNA is considered to be the most critical macromolecular target for covalent modification by carcinogens. This conclusion is based in part on the fact that DNA is the cellular repository of genetic information, in part on the strong empirical relationship that has been established between mutagenicity and carcinogenicity of chemicals, and in part on the observation that people who have genetic defects in the capability for DNA repair are at higher risk for certain forms of cancer than the general population.

The formation of these carcinogen-DNA adducts is thought to induce heritable mutations in cells through mechanisms that are still, for the most part, incompletely understood. With respect to underlying mechanisms, mutations resulting from adduction of DNA could be consequences of misreplication of structurally altered DNA sequences, or might be due to infidelity of enzymatic repair processes whose function is to restore damaged DNA to its original nucleotide sequence. The types of mutation induced could be of multiple character, ranging from single base substitutions, to more complex rearrangements, deletions, or amplification of entire genetic elements. Regardless of how they arise, may such mutations represent permanent changes in the information content of affected cells. Importantly, they could also conceivably be the initiating events in carcinogenesis, as will be discussed below, if they cause the essential genetic or epigenetic changes that lead ultimately to transformation.

AFLATOXIN ADDUCT FORMATION *IN VIVO*

We have been studying the mechanisms underlying the carcinogenic properties of aflatoxin B_1, in particular its interactions with DNA, in a variety of experimental systems, and some of the major findings of our work can be summarized in the following manner (see ref. 2 for review). As is the case with most carcinogens, metabolism of AFB_1 is required in order for it to be converted into DNA-binding and other reactive forms that presumably are responsible for its potency as a carcinogen. An extensive body of data exists to indicate that the predominant form of AFB_1 that binds to DNA in human and animal tissues is the AFB_1-9, 10-oxide. This metabolite possesses such a high degree of reactivity in aqueous media that it has never been isolated despite many attempts to do so. However, its synthesis in a non-aqueous system has recently been reported (Harris, personal communication). Its existence was proven, however, on the basis of structural studies on DNA-bound forms of the carcinogen. These studies also established that the epoxide that reacts with DNA is exclusively the exo-isomer, probably reflecting a high level of specificity in the activity of the cellular mixed function oxidase system that biotransforms AFB_1 into the electrophilic form. AFB_1 can also be metabolically activated to DNA-binding forms through at least three other metabolic routes, all of which probably also involve epoxidation. Under *in vivo* conditions, such as in rat liver, a small proportion of the total DNA binding of AFB_1 is due to the activation of hydroxylated metabolites, including aflatoxins M_1, Q_1, and P_1. Formation of these primary metabolites does not alter the double bond in the terminal furan ring, leaving it available for secondary oxidative reaction, that results in the production of electrophiles.

In typical studies of the *in vivo* interactions of AFB_1 with DNA in the rat, animals were injected with ^{3}H-AFB_1, and after appropriate periods of time the animals were killed, organs of interest (e.g., liver and kidney) removed and DNA isolated from them. DNA was then acid hydrolyzed and the components of the resulting hydrolysate separated by HPLC. Major and some minor AFB_1-DNA adducts were separated, isolated, and identified structurally. The target in DNA for most, if not all of the activated AFB_1 is the N7 atom of guanine. This nucleophilic atom lies in the major groove of DNA, in a position that apparently is readily accessible to reaction with the carcinogen. The principal adduct that forms in liver and other tissues of animals, as well as *in vitro* when AFB_1 is activated in the presence of DNA, is 9, 10 dihydro-2-(N7-guanyl)-10-hydroxyaflatoxin B_1 (AFB_1-N7-Gua). In rat liver, two hours after dosing, this adduct makes up about 80% of the DNA adducts that are present. The formation of this adduct induces a positive charge in the imidazole ring of guanine. This in turn creates a situation in which the primary adduct can enter two competing pathways, one involving its removal from DNA, the other resulting in its structural alteration. The major reaction undergone by AFB_1-N7-Gua is its rapid removal from DNA. This is due at least in part as a consequence of the relatively weak glycosidic bond that is characterized by all 7-substituted deoxyribonucleosides. In addition, however, there is some evidence that removal of AFB_1-N7-Gua from DNA is facilitated enzymatically. In either event, removal of AFB_1-N7-Gua from DNA presumably leaves an apurinic site, which may be of great significance in terms of the induction of mutations, as will be discussed subsequently.

The second, alternative pathway for loss of the parent AFB_1-N7-Gua adduct is again the result of chemical reactions brought about by the positive charge on the imidazole ring of guanine. Following hydroxide ion attack at the C8 position with rearrangement, the AFB_1-formamidopyrimidine adduct (AFB_1-FAPY) is formed, which constitutes about 7% of the DNA-bound AFB_1 2 hours after dosing, making it the second most abundant DNA adduct. Formation of this adduct results in its stabilization, may also be of considerable biological significance in terms of induction of mutations.

The kinetics of formation and removal of AFB_1-DNA adducts following a single dose of the carcinogen to rats have been investigated extensively. Two hours after dosing at a level of 0.6 mg/kg body weight, the total DNA binding level was 1 AFB_1 residue per 11,000 nucleotides, and AFB_1-N7-Gua, the major adduct was present at 1 adduct per 14,000 nucleotides, with the FAPY derivative being the next most abundant, at a level of 1/160,000 nucleotides. Liver DNA of animals killed at later times revealed marked alterations in relative adduct levels. AFB_1-N7-Gua levels declined rapidly, with an apparent half-life of 7.5 hours, from an initial adduct level of 1/14,000 at two hours to 1 adduct in 1.2×10^7 nucleotides after 72 hours. These findings reflect the stability of the FAPY adduct as mentioned above, with the result that on multiple dosing, this adduct accumulates with each dose, and in chronic experiments represents the major form of DNA bound-AFB_1.

It is well established that a single AFB_1 dose is not an effective carcinogenic dosing regimen in rats, whereas a regimen of small, repeated doses can effectively induce hepatocellular carcinoma in every animal treated with an appropriate dose level. Profiles of AFB_1-DNA adducts were therefore determined following a regimen of repeated doses over a period of two weeks. Using the same dose as that used in the experiments summarized above, the kinetics of adduct formation and removal during the first 24 hours was as described above. Thereafter, the FAPY derivative adduct was present at the highest concentration in DNA, attaining a level of 1 adduct in 1.7×10^5nucleotides at the end of 5 days of dosing, with further accumulation over the remainder of the dosing period. Thus, in the Fischer 344 rat, which is very susceptible to induction of hepatocellular carcinoma by AFB_1, the FAPY derivative represents the major stable AFB_1 derivative, which accumulates with each exposure and thus may play an important role in the initiation of carcinogenesis. Comparison of the kinetics of formation and removal of AFB_1-DNA adducts in the susceptible rat with those of a completely resistant species, the Swiss mouse, revealed qualitative similarity in the adduct profile formed. However, there were large quantitative differences, with extremely low levels of formation of even the major adducts in the mouse liver and kidney.

Taken together, available data concerning adduct formation and removal support the general concept that differential ability to activate AFB_1 to its ultimate DNA-binding form, the 9, 10 epoxide, may play an important role in determining tissue specificity and species susceptibility to the toxic and carcinogenic actions of AFB_1. These data are of value in extrapolating data across species, in determining risk from aflatoxin exposure in man, since it has been shown that human cells and tissues are enzymatically competent in aflatoxin activation, and that similar DNA-adduct profiles are formed in human cells mutagenized by AFB_1.

INTRAGENOMIC LOCALIZATION OF AFB_1-DNA BINDING

Attempts to construct quantitative relationships between levels of adduction and biological endpoints such as mutation or transformation frequency have been constrained by the necessary assumption that adducts are randomly distributed along the genome. Several lines of evidence are available, however, indicating that in fact adduct distribution is non-random, and a number of factors associated with localized binding of carcinogens, including AFB_1, to DNA have thus far been identified. At the simplest level of organization, base composition of DNA sequences can cause preferential binding of carcinogens that form adducts only at specific nucleophilic sites. In the case of AFB_1, which forms only guanine adducts, they would obviously be concentrated in G-C rich regions of DNA. In addition, it has been demonstrated that guanine bases in DNA are not equally susceptible to adduction, but frequency of adduct formation is significantly affected by neighboring base sequences. Recent studies (ref. 3), for example, used molecular modeling in conjunction with molecular mechanical calculation to assess the binding modes available to the AFB_1-N7-Gua adduct, in attempting to define the structure of the adduct in double stranded DNA. The preferred mode entailed external binding of AFB_1 externally in the major groove of DNA, providing four potential binding sites on guanine residues. One particular external binding site was identified that appeared to have properties consistent with experimentally determined reactivity observed in different DNA sequences. The most favorable binding configuration involved the AFB_1-oxide interacting with 5-G<u>G</u>-3 sequences found in the A-DNA conformation, at least for short stretches of DNA.

Additional factors operative at higher levels of DNA organization in chromatin have been found to be important determinants of carcinogen-DNA

adduct localization in non-random fashion. In the specific case of AFB_1-DNA adduct formation, DNA conformation (e.g., B vs Z form) is strongly influenced by AFB_1-N7-Gua formation in *in vitro* experimental models (ref. 4). The impacts of chromatin structure on AFB_1-DNA binding are particularly evident in *in vivo* studies, in which fractionation of chromatin by partial hydrolysis with DNAse I or micrococcal nuclease has revealed a general tendency for carcinogen localization in regions of active transcription compared to bulk DNA, and preferential binding to internucleosomal over nucleosomal core DNA. Recently, preferential binding has also been demonstrated in specific sequences, such as repetitive elements and multicopy genes. We (refs. 5, 6) have studied the *in vivo* formation of covalent AFB_1-DNA adducts within the ribosomal RNA gene sequences (rDNA) in liver nuclear DNA of AFB_1-treated rats. The level of AFB_1-N7-Gua found in rDNA two hours after dosing was found to be 4- to 5-fold higher than that in nuclear DNA. These findings were not the result of guanine enrichment, but rather support the hypothesis that rDNA regions are preferentially accessible to carcinogen modificaton because of the diffuse conformation of chromatin maintained in actively transcribed genes. Adducts formed in rDNA sequences were also found to be removed preferentially, compared to bulk nuclear DNA over the 12 hour period following dosing.

AFLATOXIN MUTAGENICITY

The ability of AFB_1 to form covalent DNA adducts is reflected in many genotoxic properties, such as ability to induce chromosomal aberrations, sister chromatid exchanges, and other rearrangements. It is also a powerful mutagen for many prokaryotic and eukaryotic cells, including those of human origin (see ref. 7 for review). Aflatoxin mutagenesis has been described in many specific genes using a variety of selection systems, but available information concerning specific molecular changes involved in converting AFB_1-DNA adducts into stable mutations is still very limited. Knowledge of the mutational specificity of a mutagen, combined with an understanding of the biochemistry of its DNA reactions is critical for the identification of its important premutational lesions. One investigation in *E. coli* (ref. 8) has provided particularly valuable information in this regard. The spectrum of base substitution mutations generated by metabolically activated AFB_1 in the *lacI* gene of a *uvrB*$^-$ strain of *Escherichia coli* were determined. AFB_1 activated with rat liver microsomes induced a 50-fold increase in nonsense mutations in the gene, compared with spontaneous mutations in untreated cells. A total of 70 mutations were monitored, and the results showed that the majority were induced by G-C to T-A transversions. The base substitution mutations found in the study were: G-C to T-A (89%); G-C to A-T (6%); A-T to T-A (3%); A-T to C-G (0.5%); G-C to C-G (0%). The importance of the generation of apurinic sites by depurination of AFB_1-N7-Gua, with the preferential insertion of adenine opposite such lesions, as a determinant of the specificity of the observed G-C to T-A transversions was emphasized. However, a stable, bulky adduct such as the AFB_1-FAPY derivative may itself represent a noninformational site, opposite which adenine might be preferentially inserted during replication after DNA damage.

A role for mutations in the process of carcinogenesis has long been suspected, and has received support from recent observations that single base substitutions within cellular proto-oncogenes are sufficient to confer transforming ability to the mutated gene product. To date, activated oncogenes have been detected in a variety of human tumors, as well as those induced experimentally by chemical carcinogens, and many have been found to have been activated by single base substitution mutations at specific sites in the gene. Recent work by our group has addressed activation of oncogenes by AFB_1, and our findings to date are summarized in the following section.

ONCOGENE ACTIVATION IN AFLATOXIN-INDUCED TUMORS

Since much is known regarding the metabolism and binding of aflatoxin B_1 to DNA, we have sought to determine whether exposure of rats to this hepatocarcinogen would result in the mutagenic activation of oncogenes. Although activated oncogenes have been identified in a variety of chemically-induced tumors of animals, detection of oncogenes in liver tumors induced by treatment with several well characterized liver carcinogens has been elusive, and no oncogenes have previously been identified in spontaneous or chemically-induced rat liver tumors. In contrast, c-Ha-*ras* oncogenes have been identified in some spontaneous and chemically-induced liver tumors from the B6C3F1 mouse (ref. 9). Nonetheless, because of the sensitivity of the Fischer rat to AFB_1 we sought to determine whether rat liver tumors induced by exposure of the animal to it would result in the appearance of transforming genes.

As we have described previously (ref. 10), male Fischer rats were dosed with AFB_1 by 40 multiple intraperitoneal injections given over 8 weeks. All of the treated animals developed hepatocellular carcinomas within 16 months. The procedures used to detect oncogenes involved DNA-mediated transfer into NIH3T3 mouse fibroblasts using calcium phosphate co-precipitation followed by selection for transformed fibroblasts. One approach to identify oncogenes utilized DNA transfection of genomic DNA isolated from the primary liver tumor into mouse fibroblasts followed by selection of transformed foci. A second approach utilized the cotransfection of liver tumor DNA with a plasmid containing a selectable growth marker. Use of this latter method resulted in a population of G418-resistant fibroblast clones that could be pooled and injected subcutaneously into athymic Nu-Nu mice. In this case, tumor formation indicated the presence of a rat oncogene. Using both the focus formation and Nu-Nu assay, we were able to identify biologically-active oncogenes that were stable and effective in transforming NIH3T3 cells in subsequent transfections. Southern blot analysis of the transformant DNA led to the identification of these oncogenes as those of the c-Ki-ras gene family in 4/10 (40 %) liver tumors assayed by these methods.

The research of others strongly suggested that the activation of c-ras proto-oncogenes to oncogenic forms may be due to single-base mutations that result in single amino acid substitutions in the p21 protein. Moreover, it has been shown that the predominant sites for such subtle genetic changes occur in regions which include the 12th or 61st codon of the gene (ref.11). As discussed above, bacterial mutagenesis studies with AFB_1 (ref. 8) suggested that the AFB_1-induced mutations occur predominantly at G-C base pairs resulting in both G-C to T-A base transversions (89%) and G-C to A-T base transitions (6%). For these reasons, we chose to analyze the first exon of the rat c-Ki-ras gene which contains the 12th codon (GGT) in which two G-C base pairs could be sites of mutation by AFB_1. To accomplish this, we employed selective hybridization of synthetic oligonucleotides to identify particular mutated alleles resulting in amino acid substitutions at the 12th codon. Using this technique, mutant homoduplexes between the radiolabeled oligonucleotide and the mutated oncogene DNA are stable whereas heterduplexes containing the mutant oligonucleotide are unstable and melt at elevated temperature. Analysis of the transformants hybridized to particular mutant oligomers indicated the presence of activating mutations in the 12th codon resulting in TGT (CYS), GAT (ASP), and GTT (VAL) codons. In these cases, different mutated alleles were present in transformants from individual AFB_1-induced liver tumors. Importantly, the mutations in all the oncogenes resided at G-C base pairs and resulted in both G-C to T-A (3/4) and G-C to A-T (1/4) base changes. Such mutations are very consistent with the nature of AFB_1-induced mutations detected in bacterial mutation assays. Consequently, our results strongly suggest that the genetic changes in the liver oncogenes may have been a direct consequence of AFB_1-DNA interaction.

The analysis of such genetic changes in the oncogenes relied upon the stable transfer of the mutated gene into NIH3T3 cells followed by appropriate selection of transformed fibroblasts. We postulated that many genetic changes in this gene region may not have been detected, since obtaining a positive result in the transfection procedure was completely dependent upon a rare recombinant event. Consequently, we conjectured that the mutant alleles scored by this procedure may substantially underrepresent the full spectrum of genes containing somatic mutations actually present in liver tumor cells. In order to confirm the presence of these oncogenic mutations, and also to identify possible additional novel mutated alleles in this gene region, we therefore employed the polymerase chain reaction (PCR) DNA amplification method. This methodology utilizes sequential annealing of gene-specific oligonucleotide primers followed by extension with DNA polymerase, to amplify by enzymatic means, specific gene regions of tumor cell DNA. We have employed the PCR method to study the first exon of the rat c-Ki-ras gene from codons 1-35, as described previously (ref. 12). To quantitate mutation frequency and to detect novel mutant alleles in PCR amplified DNA, we have employed several methodologies. They include (i) allele-specific oligonucleotide hybridization (ASO), (ii) direct DNA sequencing of PCR DNA, and (iii) cloning and sequencing of PCR product into M13 phage. In order to characterize the gene region in detail, we cloned and sequenced PCR DNA product derived from the AFB_1-induced rat liver tumors. Amplimers for the PCR reaction were synthesized to include a gene-specific primer binding region and a restriction site linker domain. After sequential primer-directed DNA amplification, a PCR product was generated which contained a DNA region bounded by the gene-specific primer binding regions, with additional DNA segments containing novel restriction enzyme cutting sites. Restriction of the PCR DNA resulted in cohesive ends that were readily cloned into M13 bacteriophage. The resultant recombinants were then sequenced using conventional methods.

The major findings to date from these studies can be summarized as follows. Analysis of primary liver tumor DNA from AFB_1-treated animals revealed the presence of several different c-Ki-ras alleles that were not present in PCR amplified DNA from control liver samples. These tumor-specific mutations resulted in amino acid substitutions chiefly at codons 12 through 14. The oncogene alleles scored by either focus formation or the tumorigenicity assay represent a subset of the mutated genes present in the original tumor DNA and underestimate the AFB_1-induced mutational spectrum present in this gene region. These results strongly indicate that the mutations we have scored represent chemically-induced mutations as a result of direct chemical-DNA interaction. It is important to note that multiple mutated alleles have been found in individual liver tumor DNA preparations. In all cases, one such allele from each tumor DNA correlates with the 12th codon mutation detected by the transformation assays. However, additional alleles have also been found that were not scored in the transformation assays. For instance, one allele contained multiple mutations resulting in 3 amino acid substitutions, two of which resided in the 13th and 14th codons. Another mutated allele from a different tumor DNA resulted in a single amino acid substitution in the 14th codon. Analysis of hundreds of phage derived from Taq-amplified PCR DNA of control rat livers did not reveal the presence of any mutations.

The clustering of amino acid substitutions in the 12-14th codon region of the genes derived from primary liver tumors suggests that such a region in the protein may have an important role in the formation of the neoplasia. Importantly, this methodology enables us to estimate the relative allele distribution for a given tumor DNA preparation. As predicted, the oncogene alleles scored by either focus formation or the tumorigenicity assay represented a subset of the mutated genes present in the original template. In addition, it appears that the frequencies of the genetic changes indicated by oncogene identification are an underestimate of the AFB_1-induced mutational spectrum present in this gene region. Further studies are in progress to investigate the possible presence of additional mutations.

The experimental evidence summarized above would suggest that some aflatoxin-induced somatic mutations in biologically-relevant genetic loci may be operating in the emergent liver tumor cell. These findings are of particular interest in view of recent findings concerning oncogenes in human primary hepatic cancer. Gu et al. (13) recently reported that human N-ras oncogene sequences were present in the DNA from a high proportion of human liver tumors. Expression of N-ras was also markedly enhanced, as was the level of the gene product. Furthermore, c-myc was also highly expressed in most tumors, implying that the cooperating activities of the two oncogenes might be responsible for the malignant phenotypic alterations in some cases of human primary liver cancer. Identification of mutations in the N-ras oncogene associated with its activation was not reported. These findings are of particular interest in the present context, since the patients in whom the liver cancer occurred lived in a region in which aflatoxin intake is known to be high, through contamination of the diet. It is particularly noteworthy that the technical procedures employed in our experimental studies for the detection and identification of mutations are directly applicable to DNA samples from human tumors, and will greatly facilitate the further characterization of oncogenes in them. Such analyses will permit direct comparison of oncogene activation and other changes in DNA in human tumors with those induced in well characterized and defined experimental systems, and thus contribute to the further elucidation of the significance of aflatoxins as human carcinogens.

REFERENCES

1. R. Doll and R. Peto, J. Nat. Cancer Inst., 66, 1197- 1223 (1981).
2. J. M. Essigmann, C.L. Green, R.G. Croy, K.W. Fowler, G.H. Buchi, and G.N. Wogan, Cold Spring Harbor Symposia on Quantitative Biology, 47, 327-337, (1983).
3. E. L. Loechler, M. M. Teeter, and M. D. Whitlow, J. Biomed. Struct. & Dynamics, 5, 1237-1257, (1988).
4. A. Nordheim, W.M. Hao, G.N. Wogan and A. Rich, Science, 219, 1434-1436, (1983).
5. T. R. Irvin and G.N. Wogan, Proc. Natl. Acad. Sci. USA 81, 664-668, (1984).
6. T. R. Irvin and G.N. Wogan, Cancer Res. 45, 3427-3435, (1985).
7. W. F. Busby, Jr. and G.N. Wogan, Chemical Carcinogens, Second Edition, Volume 2, ACS Monograph 182, C.E. Searle, Ed., American Chemical Society, Washington, DC, pp. 945-1136, (1984).
8. P. L. Foster, E. Eisenstadt and J. H. Miller, Proc. Natl. Acad. Sci. USA 80, 2695-2698, (1983).
9. S. H. Reynolds, S. J. Stowers, R. M. Patterson, R. R. Maronpot, S. A. Aaronson and M. W. Anderson, Science, 237, 1309-1316, (1987).
10. G. McMahon, L. Hanson, J.-J. Lee and G.N. Wogan, Proc. Natl. Acad. Sci. USA 83, 9418-9422, (1986).
11. Barbacid, M., Carcinogenesis, 7, 1037-1042, (1986).
12. G. McMahon, E. Davis, and G.N. Wogan, Proc. Natl. Acad. Sci. USA 84, 4974-4978, (1987).
13. J.-R. Gu, L.-F. Hu, Y.-C. Cheng and D.-F. Wan, J. Cell. Physiol., Suppl. 4, 13-20 (1986).