

Analysis of Ki-ras Exon 2 Gene Mutations in 3-Methylcholanthrene and Butylated Hydroxytoluene-Induced Rat Lung Tissues

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Abstract: 3-Methylcholanthrene (MCA) is a polycyclic aromatic hydrocarbon and potent carcinogenic agent that is often used in experimental cancer studies. Butylated hydroxytoluene (BHT) has been widely used for many years as an antioxidant to preserve and stabilize the freshness, nutritional value, flavor, and color of foods. The aim of the present study was to investigate the role of the application of MCA and BHT in the development of lung cancer, and to detect any mutation in the Ki-ras gene exon 2. Rats were killed by cervical dislocation 26 weeks after the last BHT injection and lung tissues were surgically removed. Lung tissues of the control and experiment rat groups were examined for point mutations in the exon 2 of the Ki-ras by PCR based SSCP analysis. No point mutation was observed in the Ki-ras gene exon 2.

Key Words: Ki-ras gene, 3-methylcholanthrene, butylated hydroxytoluene, rat lung tissue, mutation

3-Metilkolantren ve Bütil Hidroksitoluenle Uyarılmış Rat Akciğer Dokularında Ki-ras Ekson 2 Geni Mutasyon Analizi

Özet: Bir polisiklik aromatik hidrokarbon ve potansiyel karsinojenik ajan olan MCA, deneysel kanser çalışmalarında sıklıkla kullanılır. BHT, yiyeceklerin tazeliğini, besin değerini, tadını ve rengini sabit tutmak ve korumak için, antioksidan olarak yıllardan beri yaygın olarak kullanılmaktadır. Bu çalışmanın amacı akciğer kanser gelişiminde, MCA ve BHT'nin rolünü araştırmak; Ki-ras ekson 2 geninde herhangi bir mutasyona neden olup olmadığını tespit etmektir. Ratlar son enjeksiyondan 26 hafta sonra servikal dislokasyon ile öldürüldü ve akciğer dokuları çıkarıldı. Kontrol ve deney grubu ratların akciğer dokuları Ki-ras ekson 2 geni nokta mutasyonları için PCR tabanlı SSCP analiziyle incelendi. Sonuç olarak Ki-ras ekson 2 geninde hiçbir nokta mutasyonu görülmüdü.

Anahtar Sözcükler: Ki-ras geni, 3-metilkolantren, bütil hidroksitoluen, rat akciğer dokusu, mutasyon

Introduction

3-Methylcholanthrene (MCA) is a polycyclic aromatic hydrocarbon (PAH) that is widely reported in polluted urban air. It is also a potential carcinogenic agent that is often used in experimental cancer studies. Butylated hydroxytoluene (BHT) has been widely used for many years as an antioxidant to preserve and stabilize the freshness, nutritional value, flavor, and color of foods and animal feed products. In some recent studies, it has been claimed that these agents may cause cancers in the human liver, lungs, colon, breast, stomach, and urinary tract (1,2).

PAHs are generally known as potential carcinogens. They constitute the active ingredient of the tar fraction of cigarette smoke, charbroiled foods, and atmospheric pollution. PAHs cross the placental membrane to induce fetal cytochrome P450 metabolizing enzymes (1,2). The formation of PAHs is related to the incomplete combustion of solid and fuel oils. For this reason, in daily life, heating systems and vehicle emissions are the most common sources of exposure to PAH. These sources are the most significant causes of air pollution in most cities (2,3). It is thought that 10% of cancer cases related to the respiratory system result from air pollution.

Additionally, there are some reports of an increased incidence of leukemia in some populations that are exposed to these substances (4).

Synthetic phenolic antioxidants are added to foods in order to retard the auto-oxidation of lipids. BHT is one of the major antioxidants and has been widely used in foods all over the world (5). BHT has played a role as a typical promoting agent for tumorigenesis in biological systems (6). Although BHT itself is not genotoxic or carcinogenic, it may modulate tumor formation in mice lung tumorigenesis within the concept of 2-stage carcinogenesis (7). In mice, BHT can prevent or promote lung tumor formation, depending on the inbred strain and age. The effect of BHT also depends on whether BHT is administered before or after carcinogen exposure. Tumor promotion by BHT application in rodents is achieved by repeated administration of this agent on a weekly basis after a single carcinogen exposure (8).

Coded protein products of proto-oncogenes generally have an important role in the regulation of cell signalization and cell growth. By being activated by mutation, chromosomal translocation, amplification, or transcriptional dysregulation, these genes cause abnormal protein synthesis or excessive protein production. The activated proto-oncogenes are called oncogenes while protein products are called oncoproteins.

The Ras family is composed of H-ras, Ki-ras, and N-ras. Ras proteins exhibit guanosine triphosphatase (GTPase) activity in the membrane and have a role in signal transduction from cell membrane to the nucleus and in cell growth. Since mutant Ras protein inhibits GTPase activity, the production of gene products turns into an uncontrolled process. In various tumors, including breast, lung, and colon cancer, amino acid substitutions were detected in Ras protein especially in the 12th, 13th, and 61st positions (9-11). In addition, according to Watanabe et al., Ki-ras is a very important gene for development in mice; mice, which do not carry this gene as a homozygote, are reported to have died on the 12th to 14th day of pregnancy (12). In a study carried out on mice using MCA, it was reported that there was an increase in transversion-type mutations in Ki-ras oncogenes in lung tumors and that these mutations occurred at a very early phase (13).

The purpose of the current study was to determine, experimentally, the combined mutagenic effects of MCA and BHT on the lung tissues of rats.

Materials and Methods

Animals

All procedures were approved and performed under the guidelines of the Animal Ethics Committee of the School of Medicine, Cumhuriyet University. Fifty non-transgenic adult male rats (Wistar albino strain), weighing 100 to 110 g, were received at 8 weeks of age and allowed 1 week for adjustment to their new environment. The rats were kept in optimal laboratory conditions, fed with standard rat food, and given tap water ad libitum. They were randomized into 1 control and 3 experimental agent administration groups. The rats were first injected with individual agents, and then together in various dose combinations. After 26 weeks of injection, the control and experimental group rats were killed by cervical dislocation and the lung tissues were used for genomic DNA isolation. The genotyping of the proto-oncogene Ki-ras exon 2 gene domain was performed using PCR based SSCP and direct sequencing techniques.

Experimental Design

The rats were exposed to potential carcinogens of MCA (as a cancer initiator) and BHT (as a progressive agent) in tissue tumorigenesis in the rat model. MCA (Aldrich-Chem) and BHT (Sigma –B1378) were dissolved in corn oil. Rats from the first experiment group received an intraperitoneal injection of 40 mg MCA per kilogram of body weight once a week (Group I, 15 male rats), 200 mg BHT per kilogram of body weight was administered after a single dose of MCA once a week to the second group (Group II, 14 male rats, dual agent administered group), and 200 mg of BHT per kilogram of body weight was administered to the third group once a week (Group III, 12 male rats). Control group animals were injected with corn oil (Group IV, 9 male rats of sham group that had been transferred to the laboratory under the same handling stresses as the treated rats). All administrations to the experiment groups were carried out in 6-week periods. Following 26 weeks of the experimental period, all rat groups were killed by cervical dislocation and lung tissues were surgically removed.

Isolation of Genomic DNA

Lung tissues of the animals in the control and experimental groups were taken and genomic DNA was isolated from the fresh tissues according to the basic DNA extraction method modified by Ozdemir et al. Briefly, the control and experimental rat tissues were digested with

proteinase K (25 µl of 10 mg/ml), the genomic DNA was extracted with PCI (phenol, chloroform, and isoamyl alcohol, at a ratio of 25:24:1 respectively), and DNA was precipitated with cold ethanol (14).

PCR Amplification

Lyophilized primers (MWG-Biotech CimbH Paris, France) were used to amplify the exon 2 of Ki-ras oncogene 170-bp amplicons, respectively (Ki-S, 5'-CTC CTA CAG GAA ACA AGT AG-3'; Ki-AS, 5'-GGT GAA TAT CTT CAA ATG ATT-3'). The thermocycling procedure consisted of 50 µl reaction mixtures containing 200 µM dNTPs (MBI, Fermentas), 0.5 µM primers, 30 ng template DNA, 2 µl Taq DNA polymerase buffer, 0.1 µl (5 U/µl) Taq DNA polymerase (Boehringer, Mannheim), and 38.8 µl distilled water in an Amplitron I DNA Thermal Cycler (Thermolyne) under the following conditions: initial template denaturation was programmed for 30'' at 96 °C and the cycle procedure was 30'' at 58 °C for annealing and 30'' at 72 °C for extension. Thirty-five cycles per sample were performed.

Genotyping

PCR products of the exon 2 of the Ki-ras were used for SSCP analysis. Five microliters of denaturing loading buffer (95% formamide, 100 mM NaOH, 0.25% bromophenol blue, 0.25% xylencyanol) were added to 5 µl of amplification product. Samples were denatured at 95 °C for 10 min and placed on ice for 10 min. Electrophoresis was carried out using a 10% vertical nondenaturing polyacrylamide gel (37.5-1, acrylamide to bis-acrylamide cross-linking) in the TBE buffer (0.089 M tris, 0.089 M boric acid, 0.001 M disodium EDTA) (Sigma). The mixture was loaded on to the gel and processed at 60 mA for 24 h. Using an ABI 310 DNA sequencer (Lontek Limited, İstanbul, Turkey) DNA sequencing analysis was performed on the samples that were suspected to have mutation.

Results

During the 26-week application period, 6 rats from the MCA group and 1 rat from each of the other "combined agent" groups were recorded. Gross tumor development was not observed in either the control group rats or in members of the experimental groups. There was also no change in tissue based on radiological and scintigraphic examinations. Regarding control group rats

and some of experimental group rats, lung tissues were normal in appearance and were a homogeneous pink color and crepitation was recorded in macroscopic and microscopic examinations. Alveoli, bronchia, and vessels were also normal in appearance histopathologically. Non-specific changes such as alveolar bleeding, a few inflammatory cells that were formed by lymphocytes around bronchial and interstitial area, and atelectasia were seen in tissue examinations of the experimental group rats. Tumoral developments were observed in 5 rats of the chronic MCA injected group. Those subcutaneous malignant mesenchymal tumors were 4 × 4 cm in diameter and localized at the injection sites. Fusiform cell proliferation and multinuclear tumoral giant cells were also detected at the injection site of the chronic MCA group rats after histopathologic examination (Figure 1). Immunohistochemically, desmin and vimentin were stained positively in those soft, malign tumoral tissue cells.

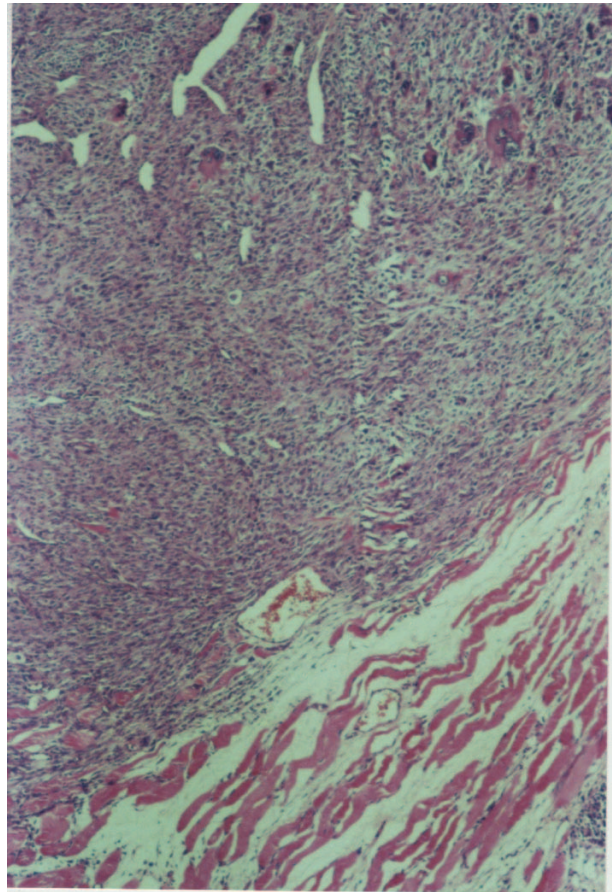


Figure 1. Malign soft tissue tumor with atypical fusiform cells, giant tumoral cells (H&E 25).

In the single strand conformational polymorphism analysis, the combined MCA/BHT applied gene domain showed a mutated profile that was not observed in the control or the other chronic MCA group rats (Figure 2). These suspected samples were identified by direct sequencing for the detection of possible point mutation and were compared to the control group rats. No point mutation was detected in the whole sequential gene domain that belonged to the control or experimental group rats (Figure 3).

Discussion

PAHs are among the most common environmental pollutants and are toxic substances that cause carcinogenesis, teratogenesis, and dysfunction of the immune system (15,16). MCA, which is also included in this group, has been used as a cancer inductive and stimulating agent in liver enzymes (17).

BHT, an antioxidant used as a promoting agent in carcinogenesis studies, has been used as an additive in various food substances in quantities of 25-400 mg/kg

(17). BHT can increase the stability of pharmaceuticals, fat-soluble vitamins, and cosmetics. The lifespan of rubber, elastomers, and plastics is increased via adding BHT (18). BHT, α -tocopherol, propyl gallate, and t-butylhydroquinone are all used as antioxidants throughout the world. These antioxidants modify the first or second stage of 2-step chemical carcinogenesis in various organs in rodents (6).

In this study we injected 40 mg/kg MCA and 200 mg/kg BHT both separately and in combination to the rat groups. After the injection period, PCR, SSCP, and DNA sequencing analysis techniques were applied to the DNA isolated from the stimulated lungs. The Ki-ras gene exon 2 domain was scanned for possible mutations.

In our study PCR/SSCP analyses were performed on Ki-ras exon 2 gene in rat lung tissues that were stimulated with 40 mg/kg MCA and were promoted with repetitive 200 mg/kg BHT doses (Figure 2). As a result of the PCR/SSCP analysis, an extra band in which mutation was thought to have occurred was detected in 3 rat lung tissues in the MCA+BHT group (Figure 3). PCR was re-

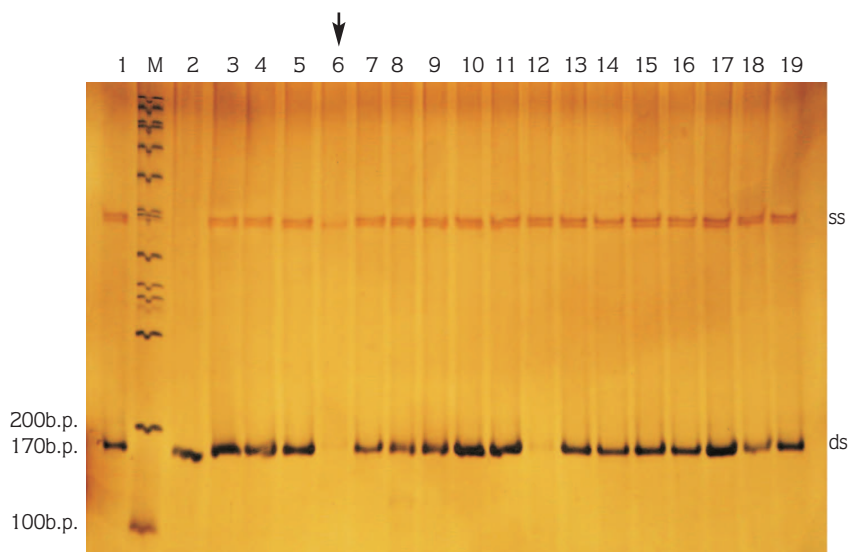


Figure 2. SSCP profiles belonging to the control and experimental group rat lung tissues that treated with MCA and BHT. ss: single strand, ds: double strand.

Lanes

Lane 1: Single and double strands (170 bp) DNA of normal appearance in control rat.

M:Marker, DNA ladder 100 bp.

Lanes 2-7: Non-denaturated (line 2) and denaturated PCR products from both MCA and BHT treated experimental group lung tissues. Arrow indicates mutated single strand in one rat (line 6).

Lanes 8-14: Denaturated products from only BHT treated experimental group rat tissues. No mutation was detected.

Lanes 15-19: Denaturated products from only MCA treated experimental group rat tissues. No mutation was detected.

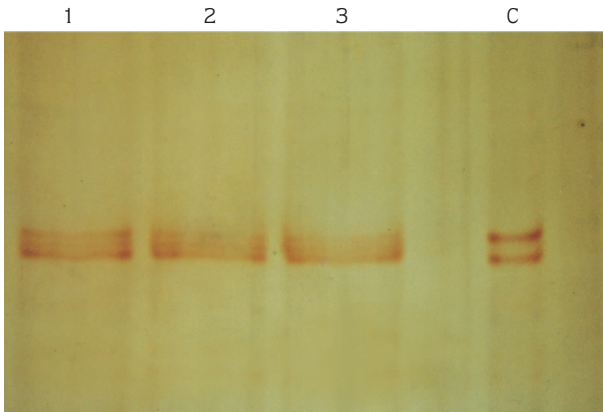


Figure 3. In a group to which MCA-BHT applied, SSCP images belonging to the samples carrying suspected mutation (Lanes 1-3) and control (C).

applied to those tissues thought to be mutant with the purpose of determining the possible point mutation type and localization; DNA sequence analyses were performed and compared with the wild type. As a result of the forward and reverse DNA sequence analysis, it was observed that the sequence of these samples matched that of control group samples and thus they were not mutations. As indicated in Figure 3, it is thought that this extra band in SSCP profiles of the samples may have appeared as a result of a possible contamination during PCR, a non-specific amplification, or a possible error caused by the present laboratory conditions or the polyacrylamide gel prepared during SSCP. The SSCP method has many advantages, including simplicity and a modest requirement in terms of laboratory equipment. However, various factors such as ionic strength, temperature, gel concentration, polymerization, glycerol amount, and tampon concentration affect the results obtained in polyacrylamide gel electrophoresis (19,20).

In the present study, no tumor formation was found and no mutation occurred in the pathological evaluation performed on lungs of the chronic BHT group. This may be because this substance is only a promoter in tumor formation or because the rats are resistant to (or developed a tolerance to) this substance. According to the studies conducted, when applied alone, BHT cannot form a tumor in mice; however, it causes severe pulmonary inflammation. Although BHT activates its tumor developing effect by blocking cellular communication channels when applied at high doses, it cannot form a tumor by activating such an effect when administered

alone (21). According to a study in which mice were repeatedly injected with BHT, only the first injection increased cell proliferation in the lung. After 2 or more injections no increase occurred in DNA synthesis. In their studies on A/J mice, Witschi and Morse confirmed that the mice developed a tolerance to repetitive BHT injections (22).

In a study conducted with Fisher 334 BigBlue® rats, MCA (80 mg/kg bw) was injected intraperitoneally, and it was found that transversion-type mutations developed in the Ki-ras oncogene in the lungs of the rats (13). Another experimental group in our study was injected intraperitoneally with 40 mg/kg MCA once a week for 6 weeks. In the pathologic examinations performed at the end of the experiment period, no tumor formation was detected, either at macroscopic or microscopic level, in the lungs. However, in 5 of 9 rats in this group only, a malign soft tissue tumor of mesenchymal origin weighing approximately 100-130 g was detected in the groin area. In the light of these findings, it can be stated that MCA is not only a cancer-inducing agent in the formation of soft tissue tumors, but can also be a promoter. However, the results of the SSCP conducted on both lung tissue and soft tissue tumors of this group showed no mutation in the Ki-ras exon 2 gene domain. In the studies conducted by Wessner et al. on pregnant rats using MCA, rats were killed with cervical dislocation 12 months after they were born and it was found that various tumors occurred in the lung and liver. They found that while Ki-ras mutations occurred in some of these tumors, in some of the tumors no mutation occurred in this gene (23).

In a study conducted by Witschi and Morse, one dose of MCA at a rate of 10 mg/kg was injected intraperitoneally into AJ mice. Observations made 2, 3, and 4 weeks after the injection showed that they were unsuccessful in producing cell hyperplasia in the lungs. They therefore concluded that MCA used at this dosage will influence the stimulation of lung tumors in mice. The same researchers injected AJ mice with 10 mg/kg MCA once a week for 6 weeks followed up with a 300 mg/kg BHT injection once a week for 5 weeks repeatedly. After 4 months, an increase was found in the number of tumors. They argued that BHT should be administered over a certain dosage that completely causes cell hyperplasia in the lungs; only under such a condition can BHT be effective as a tumor proliferator (22).

In conclusion, BHT, which is an antioxidant substance used in carcinogenesis studies, cannot form tumors in rats when injected alone and chronically. In our study, no tumors were formed in the lungs of the chronic MCA group; however, soft tissue tumors occurred in the groin area of 5 out of 9 rats. Another group was injected with a single dose of 40 mg/kg MCA and then 200 mg/kg of BHT once a week for 6 weeks. In this group, no tumor formation occurred in the lungs and no mutation was encountered in the Ki-ras exon 2 gene. In 2-stage carcinogenesis studies carried out on rats, we suggest that MCA, which is used as the inducing agent, and BHT, which is used as the promoting agent, should be injected at higher doses.

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