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MUTATION ANALYSIS OF THE PROTO-ONCOGENES Ki-RAS AND C-MYC IN THE SOFT TISSUE TUMORS OF THE RATS THAT WERE FORMED BY 3-METHYLCHOLANTHRENE *IN VIVO*

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ÖZET

Polisiklik aromatik hidrokarbonlar en potent karsinojenler olarak bilinmektedir. 3-Metilkolantren PAH'ların içinde yer alan karsinojenik bir ajandır. 3-Metilkolantren'in memeli sistemlerinde deneysel olarak toksik ve karsinojenik etkileri gösterilmiştir. Bu bileşiklere çevrede zift, kurum, sıvı ve katı yakıtlar nedeniyle havada, ekzos gazında, sigara dumanında, dumanlanmış yiyeceklerde sıklıkla rastlanmaktadır. Bu çalışmanın amacı 3-Metilkolantren' in 40 mg/kg'lık bir dozda kronik olarak ratlara verilmesiyle oluşan tümörlerde Ki-ras ekson 1, 2 ve C-myc ekson 2 genlerinde herhangi bir mutasyona neden olup olmadığını tespit etmektir. Ratlar son enjeksiyondan 26 hafta sonra servikal dislokasyon ile öldürüldü ve oluşan yumuşak doku tümörleri çıkarıldı. Dokular belirtilen genler için PCR tabanlı SSCP analiziyle incelendi. Sonuç olarak Ki-ras ekson 1, 2 ve C-myc ekson 2 genlerinde hiçbir nokta mutasyonu görülmedi.

Anahtar Kelimeler*: Ki-ras geni, C-myc geni, 3-Metilkolantren, rat*

ABSTRACT

Polycyclic aromatic hydrocarbon represents one of the most potent carcinogens known. 3-Methylcholanthrene is a carcinogenic agent included in PAH. In mammalian system toxic and carcinogenic effects of 3-Methylcholanthrene have demonstrated. These compounds are frequently encountered in the environment in tar, soot, in the air due to liquid or solid fuel, in the gas expelled from the exhausts, in cigarette fumes and in soot-coated food. The aim of this research has been to identify whether any mutation occurs in exons 1-2 of the Ki-ras gene and exon 2 of the C-myc gene in tumors formed in rats chronically treated with a dose of 40 mg/kg 3-Methylcholanthrene. Rats were killed by cervical dislocation 26 weeks after the last injection and soft tissue tumors were removed. Tissues were examined for given genes by PCR based SSCP analysis. As a result no point mutations were observed in any exons 1-2 of the Ki-ras gene and exon 2 of the C-myc gene.

Key Words: *Ki-ras gene, C-myc gene, 3-Methylcholanthrene, Rat*

1. INTRODUCTION

3-Methylcholanthrene (MCA) is both a polycyclic aromatic hydrocarbon (PAH) that is widely available in urban polluted air and also a potent carcinogenic agent that is often used in experimental cancer studies (1, 2).

PAHs as being potent carcinogens are considered to be the active ingredient of cigarette smoke, soot-coated food and polluted air. 3-MC is a potent inducer of cytochrome P4501A which catalyzes the bioactivation of food mutagenic compounds (1, 2). PAHs are formed in the incomplete combustion of solid and fuel oils. Thus, heating systems and vehicle motors are the sources of PAH exposure in the daily life. Air pollution in most cities is the result of the sources (2, 3, 4). According to the researchers air pollution is responsible for 10% of cancer phenomena related to the respiratory system. More over in some societies those resources are reported to be the reason of an increased leukemia incidence (5).

Generally protooncogens' coded protein products takes an important role in cellular signal and growth regulation. These genes results abnormal protein synthesis or producing extreme protein by activeting mutation,

chromosomal translocation, amplification or transcriptional disregulation. Activeted protooncogens are called oncogen, protein products are called oncoprotein. Ras family, consist of H-ras, Ki-ras, N-ras. Ras proteins have activation of GTPase that is found in membrane. They take an important role in signal transduction from cell membrane to nucleus and cell growth. Gene productions are produced uncontrolled because mutant Ras protein inhibits GTPase activation. Amino acids substitions are determined in Ras protein 12, 13 and 61 positions, in various tumors including breast, lung, colon cancer (6, 7, 8).
In addition, according to the statement of Watanabe and et al., Ki-ras gene is a very important gene for the development of the mouse; mice, which don't carry this gene as a homozygote, have died in the 12^{th} to 14^{th} day of their pregnancy (9).
In a research made with mice, it is pointed out that there is an increase in transversion type mutations in Ki-ras oncogens in lung tumors and it is understood that these mutations occur at very early times (10).
The C-myc is a proto-oncogene and is located on chromosome 8q24. The C-myc is expressed in many proliferating cells of the embryo and in some adult tissues. The C-myc proto-oncogene has a critical role in early embryogenesis, the control of cell growth, cellular differentiation, tissue repair processes, and apoptosis. C-myc following chromosomal translocation causes cancer in an abnormal way (11, 12, 13).
The aim of this study was to perform a mutation analysis by using SSCP, in the Ki-ras exon 1, 2 and C-myc exon 2 genes from the tumors that were formed by the injection of a polycyclic aromatic hydrocarbon, MCA, at a dose of 40 mg/kg to the rats.

2.MATERIALS AND METHODS

Animals

In the study, Wistar albino rats which are bred in Cumhuriyet University Experimental Animals Laboratory are used. Fifteen non-transgenic adult male rats, weighing 100 to 110 grams, were received at 8 weeks of age and allowed one week of 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 as one control and experimental agent administration group. The rats were injected with 3-Methylcholantrane (Aldrich-Chem) at a dose of 40 mg/kg/week intraperitoneally (i.p.) for 6 weeks. The rats in control group (n=10) were injected with placebo for the same duration of time. The study was terminated after 26 weeks from the last injections done. During this time 6 rats were lost from the study group. Before the termination of the study the rats were sent to the Nuclear Medicine Department of Cumhuriyet University, Faculty of Medicine for bone scintigraphy. The remaining rats (n=9) were then sacrificed by cervical dislocation and the abdomens were opened. In five of the rats, masses were observed close to the injection sites. 100mg of tissue were retrieved from these masses for DNA isolation and the remaining tissue was sent to the Department of Pathology, Cumhuriyet University, Faculty of Medicine for histopathologic investigation. In the pathology laboratory the specimens were fixed in 10% formaldehyde. After embedding the specimens into the paraffin blocks sections were taken and were dyed with hematoxylene-eosin. The specimens were evaluated with light microscopy.
DNA isolation was performed on the tissues and then Polymerase Chain Reaction was applied by using suitable primers for Ki-ras exon 1, 2 and C-myc exon 2 oncogenes. After PCR products were separated on a 2% agarose gel, Single Standard Conformation Polimorphism (SSCP) was applied for point mutation analysis.

Isolation of genomic DNA

Tumor tissues from the experimental group animals were taken and genomic DNAs were isolated from the fresh tissues according to the basic DNA extraction method modified by Ozdemir et al. Briefly, tumor tissues were digested with proteinase K (25 µl of 10 mg/ml), the genomic DNA was extracted with PCI (phenol, choloroform and isoamyl alcohol, in ratio of 25:24:1 respectively) and DNA was precipitated with cold ethanol (14).

PCR conditions

Amplification of Ki-ras exon 1; PCR amplifications were performed on the Amplitron Thermal Cycler. Primers for Ki-ras exon 1 were provided from MWG-Biotech Paris,France. PCR reaction mixture (50 µl final volume) contained 50-100 ng genomic DNA, 100µM of dNTP (Sigma), 0.5µM each of primers (sense: 5'-ATG ACT GAG TAT AAA CTT GT-3' and antisense: 5'-TCG TAC TCA TCC ACA AAG TG-3'), 5µl of 10x PCR buffer containing 1.5mM $MgCl_2$ and 0.5 units *Taq* polymerase (SigmaD-1806) (MBI fermentas). A 45-cycle PCR was performed to obtain amplified products. The PCR cycles were 2 min at 94ºC for predenaturation, 1 min at 94ºC

for denaturation, 2 min at 50ºC for annealing, 2 min at 74ºC for elongation and 7 min 74ºC for last elongation. Aliquots of each sample (20µl) were electrophoresed on a 1.5 % agarose gel.
Amplification of Ki-ras exon 2; Lyophilized primers with 170-bp amplicons (MWG-Biotech CimbH Paris/FRANCE) were used to amplify the Ki-ras exon 2 oncogene. PCR reaction mixture (50 µl final volume) contained 200µM dNTPs (MBI, Fermentas), 0.5 µM primers (Sense: 5'-CTC CTA CAG GAA ACA AGT AG-3' ; Antisense: 5'-GGT GAA TAT CTT CAA ATG ATT-3'), 30 ng template DNA, 2µl Taq DNA polymerase buffer, 0.1µl (5U/µl) Taq DNA polymerase (Boehringer, Mannheim), 38.8 µl distilled water. PCR amplifications were performed on the Amplitron I DNA Thermal Cycler (Thermolyne). A 35-cycle PCR was performed to obtain amplified products. PCR cycles were 30 sec at 96°C for predenaturation, 30 sec at 58°C for annealing, and 30 sec at 72°C for extension. Aliquots of each sample (20µl) were electrophoresed on a 1.5 % agarose gel.
Amplification of C-myc exon 2; Primers for exon 2 of C-myc oncogene were obtained from MWG-Biotech CimbH Paris/FRANCE. The sequences of primers were; Sense : 5'- CTC GGA AGG ACT ATC CTG CTG CCA A -3' , Antisense : 5'- GGC GCT CCA AGA CGT TGT GTG TTC G -3' . Amplifications of exon 2 of *c-myc* gene were performed in a volume of 50 µl reaction mixtures containing 200 µM dNTPs (MBI, Fermentas), 0.5 µM primers, 30 ng template DNA, 10x *Taq* DNA polymerase buffer, 1.5 U µl *Taq* DNA polymerase (Boehringer, Mannheim) for 35 cycles in Amplitron I DNA Thermal Cycler (Thermolyne) under the following conditions: denaturation at 96 ^{0}C for 30 sec, annealing at 55 ^{0}C for 30 sec and extention at 72 ^{0}C for 30 sec. PCR product was resolved in 1.5% agarose gel and stained with ethidium bromide (4µg/ml).

Genotyping
PCR products of the Ki-ras exon 1, *2* and C-myc exon 2 were used for SSCP analysis. Five microliters of denaturing loading buffer (95% formamide, 100mM NaOH, 0.25% bromophenol blue, 0.25% xylencyanol) were added to 5µl of amplification product and samples were denatured at 95ºC 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 (0.089M TRIS, 0.089M Boric Acid, 0.001M disodium EDTA) (Sigma). The mixture was loaded on to the gel and run at 60mA for 24 h.

2. FINDINGS
The findings obtained by the application of SSCP to the Ki-ras exon 1, 2 and C-myc exon 2 gene regions taken from the tumors that were developed by chronic administration of MCA to the rats, are given in figures 1 (A, B, C respectively).

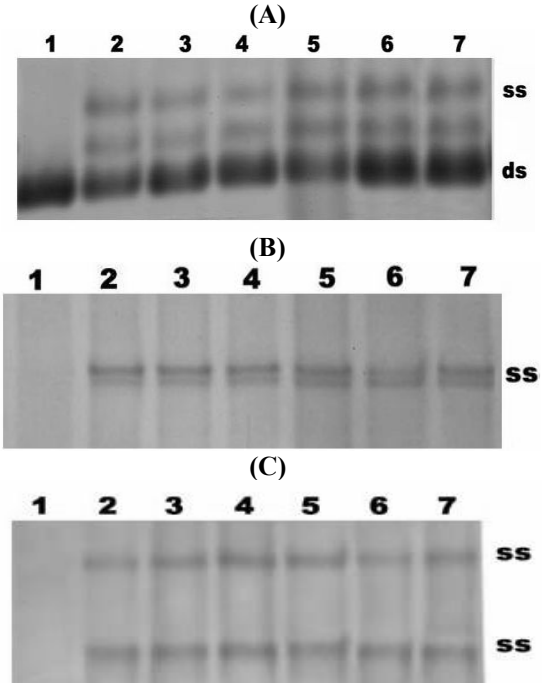

Figure 1: SSCP Image for the Ki-ras exon 1 (A), Ki-ras exon 2 (B) and C-myc exon 2 (C) in the soft tissue tumors developed by the chronic MCA application in rats (10% polyacrylamide gel).

Columns:
1: Non-denaturated control
2: Control
3-7: Group in which 40 mg/kg/week (6 doses) MCA was applied (chronic MCA group)
ss: Single strands
ds: Double strands

The histopathologic evaluation of the specimens taken from the rats that were left alive (5 of the 9) in which MCA was administered at a dose of 40 mg/kg/week and in those masses developed at the injection sites revealed atypical tumor cells characterized by the proliferation of the spindle shaped cells. Dispersed among the tumor cells hystiocytes and multinuclear giant tumor cells were driving attention. According to the histopathologic properties the tumor was defined as a malign soft tissue tumor with mesenchymal origin. A strongly positive result was obtained by the dying of the specimens with the immunohistochemical dyes vimentin and desmin. As a final diagnosis the tumor was thought to be a rhabdomyosarkoma, a malign soft tissue tumor of the skeletal muscle. The reason for not defining a definite diagnosis for the tumor is because the dyeing of the specimens with immune dyes specific for the myoglobin was not performed (Figure 2 and 3).

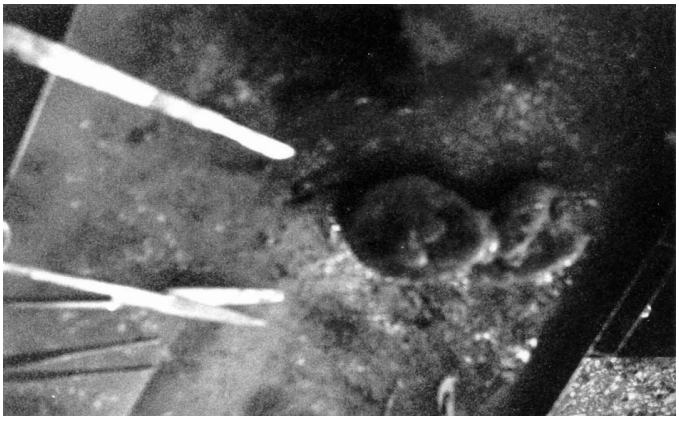

Figure 2: Macroscopic appearance of the masses developed close to the injection sites.

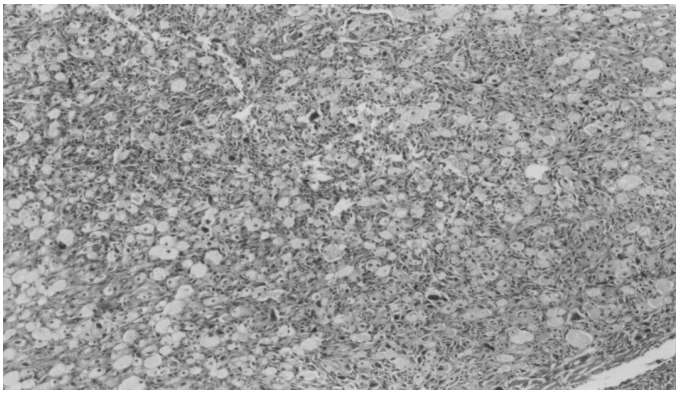

Figure 3: The malign soft tissue tumor in which atypical spindle shaped cells and giant tumor cells are observed.

3. DISCUSSION AND CONCLUSION

MCA, the polycyclic aromatic hydrocarbons found commonly in polluted air of the cities, cigarette fumes and soot-coated food, is a procarcinogen that requires a metabolic activation to perform its mutagenic effect. It is

widely used in the laboratories for chemical carcinogenesis studies (15, 16). In our study, malign soft tissue tumors with mesenchymal origin, weighing approximately 100-130 g, have been identified at the groins of 5 of the rats with chronic MCA administration (Figure 2 and 3). By the end of the experiment period PCR and SSCP analysis have been applied to the DNA extracted from the soft tissue tumors and the Ki-ras exon 1, 2 and C-myc exon 2 gene localizations have been scanned for possible mutations. Tumor tissue were compared by the non-mutant controls at the end of the SSCP study and the bands showed no profound differences, in other words, no mutations were observed at the gene zones of Ki-ras exon 1, 2 and C-myc exon 2 of the tumor tissues (Figure 1). In this context it is possible to predicate that in conducing soft tissue tumors MCA is not only an inducing agent but also is a promoting agent as different gene zones and different mechanisms are involved.

In a supporting study with MCA in pregnant rats, which were sacrificed 12 months after delivery by cervical dislocation, Wessner et.al. found various tumors that were present in the rats' lungs and livers but while in some there were Ki-ras mutations, genes in the others were observed to be normal (17). Similarly, Niwa et.al. found highly frequent mutations in mice minisatellite loci in the MCA induced mice sarcomas, amplification of the C-myc gene and absence of normal alleles in 35% of the sarcomas and mutations in the Ki-ras gene. The sarcomas were analyzed by PCR-SSCP method. From the 7 sarcomas carrying the C-myc gene amplification Ki-ras gene mutations were observed in 4 and no Ki-ras gene mutation was established in the tumors carrying normal C-myc genes. These investigators found the co-existence of Ki-ras gene mutation together with the amplification of the C-myc gene to be statistically significant (18).

In another study performed by Niwa et.al. MCA was injected subcutaneously to the mice and the developed sarcomas were investigated in terms of C-myc oncogene. This gene was found to be amplified in 16 of the 45 sarcomas (19).

The same investigators performed another study using the chemicals MCA, Alfa tocopherol and irradiation for induced primary tumors on mice. They established 4 amplified C-myc gene over 24 sarcomas induced by the mentioned method (20).

Watanabe et.al. applied PCR-SSCP analysis and performed subsequent direct sequencing to the sarcomas induced by MCA in mice and investigated the C-myc, ras and p53 genes. They found a strong correlation between C-myc and Ki-ras genes (9).

In our study, not seeing mutations in the Ki-ras exon 1, 2 and C-myc exon 2 gene regions in the soft tissue tumors induced by MCA in rats and also keeping in mind the results obtained from the studies on mice by Niwa et.al. and Watanabe et.al. seem to be supporting the idea that these genes are inherited together.

Keshava et.al. stimulated the Balb/c-3T3 cells with MCA and looked for the gene amplifications. They estimated an amplification in the Ki-ras and H-ras proto-oncogenes but failed to put forward a DNA amplification in the c-fos, c-jun and c-myc genes (21). In another investigation, MCA and crystalline nickel sulfide was injected intramuscularly to the mice and the induced rhabdomyosarcomas were assessed where the c-mos gene on the 4^{th} chromosome and c-myc oncogene on the 15^{th} chromosome showed no alteration or activation (22). A study performed on the dogs also supports data about no changes in the expression of c-myc proto-oncogenes' in the induced rhabdomyosarcomas (23).

In this study, we experimentally induced the Wistar albino rats with MCA and formed soft tissue tumors in which we assessed Ki-ras exon 1, 2 and C-myc exon 2 genes. In the SSCP profiles of these genes we did not observe any mutations. When this observation is considered together with the other investigators' results it strongly inculcates that these genes are inherited together and some other mechanisms are possibly effective in the formation of tumors. According to present study findings and in view of the previous study results we speculate that.

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