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RESTRICTION SITE MUTATION ANALYSIS IN TOBACCO SMOKE EXPOSED RATS

SİGARA DUMANINA MARUZ BIRAKILMIŞ SIÇANLARDA SINIRLI BÖLGE MUTASYON ANALİZİ

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Restriction site mutation (RSM) assay technique has been applied as a genotypic mutation analysis method in tobacco smoke exposed rats. In principle, the RSM assay technique may be used to analyze mutation induction in any organ, of any species, for which DNA sequence information is available. The RSM assay technique is a combination of two methods that of restriction enzyme digestion of DNA and amplification of resistant enzyme sequences using polymerase chain reaction (PCR). In this study, a number of parameters, which affect the two methods, were studied on p53 and ras genes. Several suitable restriction enzymes and primer pairs were identified for detecting mutations. The assay was carried out on 13 selected restriction enzyme recognition sequences located in the rat p53 and ras genes. No resistant amplification products have been observed in the selected restriction enzyme recognition sites in this study.

Sınırlı bölge mutasyon (RSM) tayin tekniği sigara dumanına maruz bırakılmış sıçanlarda bir genotipik mutasyon analiz metodu olarak uygulanmıştır. Prensip olarak RSM tayin tekniği, DNA dizilimi bilinen herhangi bir türde, herhangi bir organda indüklenmiş mutasyon analizi için kullanılabilir. RSM tayin tekniği, DNA'nın restriksiyon enzim ile kesilmesi ve rezistan enzim diziliminin polimeraz zincir reaksiyonu (PCR) kullanılarak çoğaltılmasından oluşan iki metodun kombinasyonudur. Bu çalışmada, iki metodu etkileyen parametrelerden p53 ve ras genleri üzerinde çalışıldı. Çeşitli uygun restriksiyon enzimleri ve primer çiftleri mutasyonların saptanması için aydınlatıldı. RSM tekniği sıçan p53 ve ras genlerinde lokalize olan 13 seçilmiş restriksiyon enzim tanıma bölgesinde gerçekleştirildi. Çalışmada seçilmiş olan restriksiyon enzim tanıma bölgelerinde rezistan amplifikasyon ürünü gözlenmemiştir.

Keywords: *Restriction site mutation assay technique; Tobacco smoke; Polymerase chain reaction*

Anahtar Kelimeler: *Sınırlı bölge mutasyon tayin tekniği; Sigara dumanı; Polimeraz zincir reaksiyonu*

Introduction

For four diseases, cancer of the lung, myocardial infarction, peripheral vascular disease, and chronic obstructive disease, the association with smoking has attracted a great deal of attention and has been investigated in many different ways.

Tobacco has been smoked for centuries and possibly been used for millennia. At first it was smoked only by the native populations of America; subsequently, after tobacco was brought to Europe in the middle of the sixteenth century, smoking became widespread throughout the world(1).

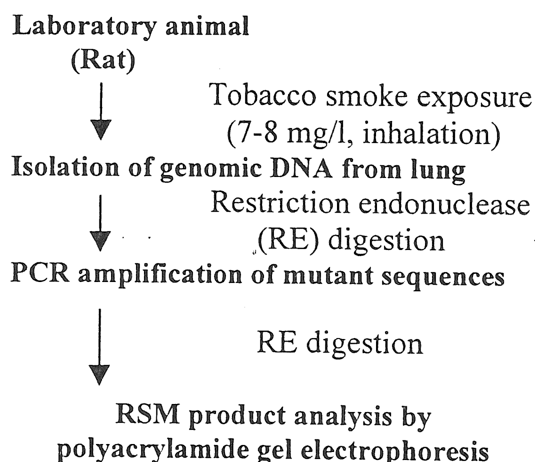
Epidemiological observations as well as experimental animal studies have provided evidence of a link between human lung cancer and tobacco smoke. More than 50 chemicals, from around 3800 compounds in the tobacco smoke, including polycylic aromatic hydrocarbons(PAHs), aromatic amines, heterocyclic amines and N-nitrosoamines have been demonstrated to be either complete carcinogens, initiators or promoters in various *in vitro* or animal bioassays(2).

Mutagenicity studies with *Salmonella typhimurium* have shown that the smoke

from four types of tobacco were mutagenic to *S. typhimurium* T.A. 1538(3). Exposure of rats to either side stream (SS) or mainstream smoke (MS) by an automatic smoking machine resulted in the excretion of frame shift mutagens in the urine as detected by *S. typhimurium* T.A. 1538(4). Putman *et al.* demonstrated dose-dependent increases in the frequency of sister chromatid exchanges (SCEs) in bone-marrow cells af B6C3F1 mice exposed to cigarette smoke on five days/week for two weeks(5).

Since mutations are largely responsible for activating protooncogenes and inactivating tumor suppressor genes in cancer development, methods for detecting DNA fragments carrying such mutations are of great interest to molecular toxicologist. Recently, new mutation detection methods, such as the restriction site mutation assay technique(6) and transgenic animal models (7), have improved the detection and evaluation of mutations *in vivo*. The Restriction Site Mutation (RMS) assay technique or Restriction Fragment Length Polymorphism/Polymerase Chain Reaction (RFLP/PCR) has been developed to detect base changes which occur within restriction enzyme recognition sequences (8-13). Base-pair substitutions and small insertions and deletions which render recognition sequences resistant to cleavage by restriction enzymes can all be recognized by this type of assay. Unlike most currently available mutation assays, the RSM assay technique does not rely upon the selection of a mutated phenotype and thus is not limited to mutational analysis in only a few genes. The need for phenotypic selection is avoided as the assay allows the isolation of rarely occurring mutated sequences from a vast excess of background unmutated wild-type sequences (for review see ref. 14).

Mutations are determined and identified as alterations (base changes and insertions or deletions) of the DNA sequences at a chosen restriction endonuclease recognition sequence. At the first step of the assay, genomic DNA that is exposed to physical or chemical mutagen is exhaustively digested with the restriction endonuclease without the selection of mutant phenotype. At the second step, resistance sequences containing the mutated target site are specifically amplified using the PCR. In this step, DNA without mutations (wild-type) will be cleaved at the selected restriction endonuclease site and can not be amplified by the PCR. Finally, at the last step, the RSM assay products are subjected to further restriction endonuclease digestion in order to remove any amplified products containing sensitive restriction endonuclease recognition sequences. Basic steps of the RSM assay are given in the Scheme.



Scheme. Outline of the RSM assay analysis in the tobacco smoke rats

Our previous results provide the RSM assay technique feasibility for direct acting genotoxin, N-methyl-*N*-nitrosourea (MNU), in the rat (8). In this study, tobacco smoke has been chosen as

a complex mixture that containes considerable number of carcinogenic compounds. The RSM assay was performed on the p53 and *ras* genes that are the most frequently detected genetic changes in human tumours (15,16). Several suitable restriction endonucleases, which are in the amplified target sequences and primers for the PCR, have been determined according to the rat p53 and *ras* genes. The assay was carried out on the lung of tobacco smoke exposed animals.

Materials and Methods

Experimental animals

The RSM assay technique was performed on 7 weeks old male (200-250 g) and female rats (170-190 g). The tobacco smoke treatment experiment was carried out in The British Industrial Biological Research Association (BIBRA), Surrey. Group of 9 animals (group 1) and group of 10 animals (group2) were exposed to tobacco smoke 7-8 mg per liter by inhalation for 15 days and for 25 days, respectively. Those and control rats (groups 3 and 4) were initially acclimatized to room conditions for a period of one week, then following procedure described below was applied on consecutive days, twice per day with a 4 hour interval between each procedure. Air control animals which consisted of 10 male (200-250 g) and 10 female (170-190 g) rats were restrained for the same time intervals as the tobacco smoke exposed groups.

Animals were exposed to :
Day 1: air alone for 8 minutes
Day 2:tobacco smoke for 1 minute
Day 3:tobacco smoke for 2 minutes
Day 4:tobacco smoke for 4 minutes
Day 5:tobacco smoke for 6 minutes
Day 6 to day 26:tobacco smoke for 8 minutes.

Groups 1 and 3, and groups 2 and 4 were sacrificed after 15 and 25 exposure days respectively. After the sacrifice rats were dissected and the lungs were removed and stored at -70°C until DNA extraction.

DNA extraction of the lungs

Genomic DNA was extracted using an Applied Biosystems 341 Nucleic Acid Purification System. After homogenisation of 0.5 g lung with phosphate buffered saline(PBS) on ice, a standard method including cell lysis, proteinase K, phenol extraction, chloroform extraction, isopropanol DNA precipitation and ethanol washing procedure was performed according to the manufacturer's instructions. Purified DNA was dissolved in sterilized water.

PCR primers for amplification of the RSM assay targets

PCR primers for DNA amplification and restriction enzymes present in the amplified target sequences were selected using the primer software package and restriction map program available on Seqnet (SERC Daresbury Laboratories). Oligonucleotides were synthesized on an automated synthesizer (Applied Biosystems Model 391) as instructed by the manufacturer using standard cynoethyl phosphoramidite chemistry.

RSM assay

The RSM technique assay consists of restriction enzyme digestion of genomic DNA and then amplification of unrestricted mutant target by PCR and further restriction enzyme digestion.

1μg of aliquots of genomic DNA extracted from the individual untreated and tobacco smoke treated rat tissues were digested overnight at the appropriate temperature, using 10 units or more of the selected restriction enzyme in 20 μl of 1x *Taq* buffer. The digested DNA was then heated to 95°C for 10 minutes to denature the restriction enzyme. The digested mixtures were amplified in a 50 μl PCR reaction using 1.25 U *Taq* polymerase, 200 μM of each dNTP and 20 pmol of each of the two appropriate primers. Negative control containing no DNA template was also analyzed with each batch of RSM assay performed. 17 μl of the PCR products were subjected to a second round of digestion with the appropriate enzyme overnight. 10 μl of the RSM products were mixed with 2 μl of loading buffer and electrophoresed on 6% polyacrylamide gels and then visualized by silver staining.

Results and Discussion

The RSM analyses of the restriction endonuclease recognition sequence/ amplification fragment combinations were performed on the genomic DNA extracted from lung of each of 20 controls and 19 tobacco

Table. Restriction enzymes, their recognition sites and fragment sizes(bp) employed in the RSM assay technique

Gene	Exon	Product length (bp)	Restriction endonuclease	Recognition site	Fragment size (bp)
H-ras	1	171	*Cfo*I	GCGC	62-109
			*Hind*III	AAGCTT	45-126
	2	261	*Hinf*I	GACTC	47-214
			*Cfo*I	GCGC	103-158
K-ras	2	170	*Nla*IV	GGTACC	78-92
P53	5	180	*Alu*I	AGCT	32-148
			*Dde*I	CTGAG	48-132
			*Nco*I	CCATGG	77-103
			*Nla*IV	GGTACC	85-95
	6	110	*Dde*I	CTGAG	49-61
			*Msp*I	CCGG	26-84
	7	94	*Bsl*I	CCN_7GG	42-52
	8	136	*Bsl*I	CCN_7GG	52-84

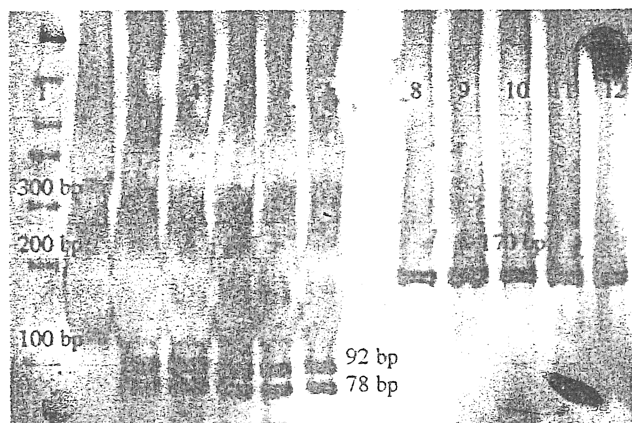


Fig.1. Silver stained 6% polyacrylamide gel shows RSM assay products (78-92 bp) of the K-*ras* gene of exon 2 segment from the lung of tobacco smoke treated rats using the restriction enzyme *Nla*IV (lanes 3-7). Lane 1 is gel marker. Lane 2 is negative control. No resistant restriction enzyme sequences were detected at the *Nla*IV recognition site (GGTACC), all samples completely digested by the enzyme. Lanes 8-12 contain amplification products (170 bp) for each sample.

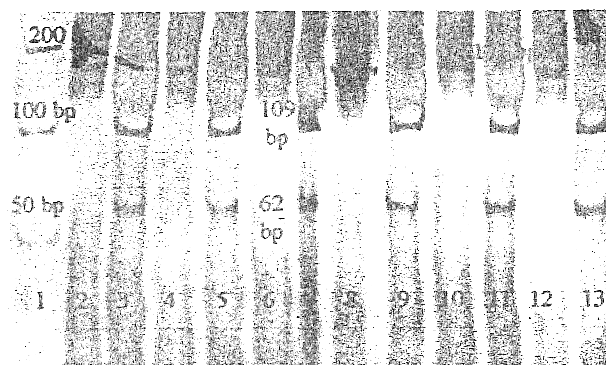


Fig.2. RSM assay products (62-109 bp) of the H-*ras* gene of exon 1 segment from the lung of tobacco smoke treated rats using the *Cfo*I restriction enzyme (lanes 3,5,7,9 and 13). No resistant restriction enzyme sequences were detected at the *Cfo*I recognition site, all samples were completely digested by the enzyme. Lane 1 is gel marker. Lanes 2,4,6,8,10 and 12 contain amplification products (171bp) of each sample.

smoke exposed animals. The assay was carried out on 13 selected restriction endonucleases recognition sequences located in the rat p53 and *ras* genes. The table shows the selected restriction enzymes that were used in the RSM assay.

Analyses were performed using *Alu*I, *Dde*I, *Nco*I and *Nla*IV restriction endonucleases sequences for in exon 5, *Msp*I and *Dde*I restriction endonucleases in exon 6 and *Bsl*I restriction endonuclease in exon 7 and 8 for the rat p53 gene. Similarly *Hind*III and *Cfo*I restriction endonucleases in the exon 1 of the H-*ras* gene and *Hinf*I and *Cfo*I restriction endonucleases in the exon 2 of H-*ras* gene and *Nla*IV restriction endonuclease in the exon 2 of K-*ras* gene were used to analyze mutation in the rats. Examples of the RSM assay analyzes are given in Figs 2 and 3.

A total of 507 RSM assays of which 260 assays were from control and 247 assays from treated animals were performed using 13 restriction endonucleases located in the p53 and *ras* genes on the total 39 control and tobacco smoke exposed rat lungs. No residual amplification products that were insensitive to restriction endonuclease digestion were observed.

Although resistant enzyme recognition sites were not observed in this study, *in vivo* mutations were detected in rodents and aquatic species using RSM assay in several studies. Mutations were analyzed in a variety of restriction enzyme recognition sequences of the adult α_1-globin gene and detected in the *Bsl*I recognition site (CCTGG) in toadlets(9). G to A transitions and G to T transversions were demonstrated in a number of restriction enzyme recognition sites (*BamH*I, *Pst*I, *Ban*I, *Msp*I, *Alu*I) in DNA extracted from a number of different tissues from 1-ethyl-1-nitrosourea treated mice using the RSM assay (10). In another RSM study, the induced mutations were determined in exon 4, exon 5 and intron 6 of the p53 gene in 1,2-dimethlylhydrazine treated mice (11). In our previous study, the RMS assay technique was applied to the MNU treated rats and induced mutations were detected and identified in a number of tissues (8). Resistant amplification products were detected in 5 of the 13 selected restriction enzyme recognition sequences tested (*Nco*I, *Bsl*I, *Cfo*I, *Dde*I, and *Hind*III).

Current mutational assays generally involve phenotypic detection. However, these systems are limited in their ability to detect the potential genotoxicity of chemicals due to their reliance on a small subset of genes which give rise to selectable phenotypes and the need for an expansion step *in vitro* (17). The developed technique in this investigation has considerable advantages over the cellular mutation systems. RSM has a number of conceptual benefits, since it does not require any form of phenotypic selection at the cellular level. It avoids the need for a large amount of tedious and time-consuming cell culture associated with conventional mutation assays. Since cellular selection is not required, there are no restrictions on the target site. Any locus can be used, in contrast to traditional mutagenesis experiments, in which, in mammalian cells, only a few selectable genes (e.g., *hprt*) can be used as mutation targets.

While there is sufficient evidence that the inhalation of tobacco smoke can cause cancer in experimental animals(18-21), several studies have demonstrated that tobacco smoke does not induce lung tumours. Davis

et al., shower that exposure of rats to tobacco smoke for 11 minutes, twice daily, for five days per week, for life, resulted in no lung tumours in exposed and control animals(22). Similarly Otto and Elmenhorst reported that, in mice exposed to tobacco smoke in a 200-1 chamber for 90 minutes daily until spontaneous death, no significant difference in adenoma incidence between control and treated groups could be detected(23). In addition, in a study in which rabbits were exposed daily to the smoke from 20 cigarettes for up to 66 months, no tumours that could be related to smoke exposure were found (24).

There have been a number of studies where exposure to cigarette smoke did not induce any detectable genetic effects in the animal subjects. Korte *et al.* reported no increase in chromosomal aberrations in Chinese hamster bone marrow cells exposed to cigarette smoke *in vivo* (25).In this study authors noted that the duration of exposure (1h per day for 12 weeks) was probably inadequate to induce chromosome aberrations. Although Schmid *et al.* increased the duration of the treatment with a low dose of mutagen, they also found no increase of aberrations in the bone marrow cells of Chinese hamsters(26). Furthermore, Basler also found that exposure of female rats to the smoke of 30 cigarettes/day for 28 days did not increase the frequency of SCEs in their cultured lymphocytes (27).

There are several possibilities that could explain the lack of detectable *in vivo* mutations in the exposed male and female rats. Animals which are being forced involuntarily to inhale smoke, can undergo avoidance reactions and change their breathing patterns to shallow, hesitant inspirations with reduced minute volumes(28) and this could affect the doses delivered to the different parts of the respiratory system. The differences in the respiratory systems of animals and humans are also important. As rodents are obligatory nose-breathers, they have more convoluted and intricate nasal turbinate patterns than humans, hence the dynamics of particle depositon in the upper respiratory tract might also be expected to be different (28,29).

Considering this potential difference in exposure and the failure of detecting mutations under the developed RSM assay conditions, increases in the tobacco smoke *dose* and *exposure time* could result in the detection of mutations with the RSM assay in tobacco smoke exposed animals. The major problems in experimental tobacco carcinogenesis studies have been that a suitable assay for the carcinogenesis of inhaled cigarette smoke has not yet been developed. Most of the carcinogenesis studies have been terminated at 1 or 2 years, instead of continuing for the lifetime of the animals(30). In spite of negative results, it cannot be concluded that cigarette smoke does not carry a genetic risk. Human smokers inhale cigarettes smoke for decades and it is difficult to reproduce this situation in experimental animals.

In contrast, the exposure conditions could well have induced mutations in the rat lung tissue but under the experimental conditions used, they were not detected. It is possible that any DNA damage induced by the

cigarette smoke could have been repaired before its fixation to a permanent mutation. It is also possible that mutations were induced but were not detected as they were not within the designated sequences covered by the restriction enzymes used. Only further research would determine if the aforementioned experimental conditions were responsible for the lack of detectable mutation induced by the 7-8 mg/l tobacco smoke inhalation in the rats.

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