

A GENOTYPIC MUTATION TECHNIQUE: RESTRICTION SITE MUTATION METHOD

BİR GENOTİPİK MUTASYON TEKNİĞİ: SINIRLI BÖLGE MUTASYONU YÖNTEMİ

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ABSTRACT

Although cellular mutation analysis techniques are still used for the identification of mutagens, they are unsuitable for the study of disease-related mutations. Restriction site mutation (RSM) is an alternative method to traditional mutation recognition techniques that have some limitations. RSM technique aims of selecting certain DNA target by restriction enzyme digestion and resistant restriction enzyme recognition sequences (mutant) are amplified using polymerase chain reaction (PCR). In this study, RSM has been developed as a genotypic mutation method in the rat. The method has been applied to rat p53 and ras genes. Since RSM is the combination of restriction enzyme digestion and PCR, a number of factors have been investigated for use in the RSM. Several suitable restriction enzymes and primer pairs have been defined for analyzing base changes in the rat p53 and ras genes.

Key words: Restriction site mutation, PCR, p53, ras.

ÖZET

Hücresel mutasyon analiz teknikleri mutajenlerin belirlenmesinde hala kullanılıyorsa da hastalıklarla ilgili mutasyonların çalışılmasına uygun değildir. Sınırlı bölge mutasyonu (RSM) bazı sınırlamaları olan klasik mutasyon tekniklerine alternatif bir yöntemdir. RSM yöntemi restriksiyon enzimi ile muamele ile belirli DNA hedefinin seçimini amaçlar ve rezistan restriksiyon enzim dizilimi (mutant) polimeraz zincir reaksiyonu (PCR) ile çoğaltılır. Bu çalışmada RSM bir genotipik mutasyon analizi olarak sıçanlarda geliştirilmiştir. Metod sıçan p53 ve ras genlerine uygulanmıştır. RSM, restriksiyon enzimi ile dijestiyon ve PCR'in kombinasyonu olduğu için, RSM'in kullanımına ilişkin birçok faktör incelenmiştir. Sıçan p53 ve ras genlerindeki baz değişikliklerinin analizi için çeşitli restriksiyon enzimleri ve primer çiftleri tanımlanmıştır.

Anahtar kelimeler: Sınırlı bölge mutasyonu, PCR, p53, ras.

INTRODUCTION

The frequent occurrence of single base pair changes in the activation of oncogenes (1) and in the inactivation of the tumour suppressor genes (2-4) documents the involvement of mutations in human carcinogenesis (5). 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*. The clonal character of human malignant tumours implies that the original mutation must be present in a small minority of cells in an essentially normal tissue (6). This small fraction of cells harbours a particular mutation in the earliest stages of the development of a disease and the mutation only rarely gives rise to a selectable altered phenotype (7). Selection of mutated cells on the basis of an altered phenotype has to be replaced by biochemical separation and detection of the altered sequence of the gene of interest (6). Evidently such genotypic mutation analysis requires large numbers of mutated cells is avoided. Genotypic mutation systems are required due to 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 only give general indications about the type of mutations which can be caused by a particular mutagen.

All classical mutation systems rely on the isolation of a few mutated cells from a large, usually dividing cell population. This limits mutation analysis to a few genes encoding proteins which produce a selectable cellular phenotype (5). Factors that 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 (7).

Genotypic mutation systems have to possess analytical sensitivity which far exceeds the requirements for the detection of heterozygous or homozygous mutations in tissues from tumours. Average spontaneous mutation frequencies per base pair in human cells are estimated to be in the range of 10^{-8} - 10^{-10} and these frequencies increase only 10 - 10^4 fold upon exposure to a mutagen (6). Therefore, methods are required which allow the separation of a few altered DNA sequences from 10^5 - 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. A number of experimental approaches to genotypic mutation systems are being developed (8-11). Several are based on Southern and Northern hybridisation often with sequence amplification by polymerase chain reaction. Other protocols take advantage of differences in electrophoretic mobility of heteroduplexes of mutated single-stranded nucleic

acids. The sensitivity of all these approaches is limited by backgrounds that originate from the large excess of wild-type DNA relative to mutated sequences. The Restriction Site Mutation (RSM) or Restriction Fragment Length Polymorphish/Polymerase Chain Reaction (RFLP/PCR) approach to genotypic mutation analysis greatly reduces this problem.

The RSM or RFLP/PCR approach has been developed to detect mutations which occur within restriction endonuclease recognition sequences (5, 7, 12-15). Base pair substitutions and small insertions and deletions which occur in restriction endonuclease recognition sequence can be detected by this type of assay. Unlike most currently available mutation systems the RSM assay does not depend on the isolation of a few mutated cells with selectable mutant phenotype. Thus, in principle the RSM assay is not limited only detecting mutations in a few genes. The assay allows the detection of specific mutated sequences from a vast excess of background wild-type sequences without need for the *ex vivo* or *in vitro* selection or expansion of phenotypically altered cells. The RSM assay can be applied to detection of mutations in any gene for which the DNA sequence is available.

The RSM approach for genotypic mutation analysis consists of three steps. In the first step, genomic DNA is subjected to restriction endonuclease digestion with the chosen restriction endonuclease which cleaves wild-type recognition sequences but leaves mutated molecules. The backgrounds, that due to the presence of a large excess of wild-type sequences selectively destroying by the appropriate endonuclease, greatly reduce in this step. In the second step, undigested molecules, which presumably contain a mutation in the restriction endonuclease recognition sequence, are amplified by polymerase chain reaction (PCR). This step allows the selective amplification of mutated restriction endonuclease recognition sequences. In the last step, the amplified mutant and wild-type targets are subjected to digestion with the chosen restriction endonuclease which cleaves residual amplified wild-type targets. The basic steps of the RSM assay are illustrated in Figure 1. Direct sequencing of the resistant RSM product ultimately allows the characterization of the predominant base pair changes in the mutated restriction endonuclease recognition sequence.

The aim of this study presented in this paper is to undertake the development of the Restriction Site Mutation (RSM) assay as a genotypic mutational analysis system in the laboratory rats. The optimization and validation of the assay have been carried out using major somatic tissues to determine their suitability for the assay. The RSM assay was performed on the p53 and *ras* genes mutations of which are the most frequently detected genetic changes in human tumours and in many animal tumour model systems (16, 17).

MATERIALS AND METHODS

Experimental animals

The RSM analyses were performed on the rats, 7 weeks old male SD rats (200-250 g) and female SD rats (170-190 g), obtained from The British Industrial Research Association (BIBRA), Surrey, UK.

DNA Extraction

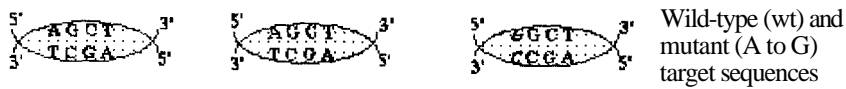
Genomic DNA was extracted from major somatic tissues including brain, kidney, liver, pancreas, spleen, testis and granuloma pouch tissue by a modified high salt method (18). The concentration of each DNA sample was quantified by UV spectroscopy.

PCR primers for amplification of the RSM assay targets

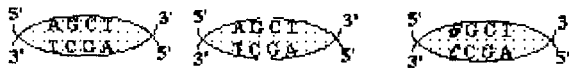
PCR primers for DNA amplification were selected using the primer software package available on Seqnet (SERC Daresbury Laboratories) or chosen on the basis of published DNA sequences of the rat p53 gene (19) and *H-ras*, *K-ras genes* (20, 21). Oligonucleotide sequences of the *ras* genes were designed to amplify sequences around either codons 12 and 13 or 61 of these genes. Synthetic oligonucleotides K2S (sense) and K2A (antisense) were used to amplify a region of exon 2 of the rat *K-ras* gene. PCR primers H1S and H1A, H2S and H2A were designed to amplify regions of exon 1 and exon 2 of the rat *H-ras* gene. Similarly p53.5S and p53.5A, p53.6S and p53.6A, p53.7S and p53.7A, p53.8S and p53.8A were used the amplification of regions of exons 5,6,7 and 8 of the rat p53 gene respectively.

The restriction endonucleases present in the amplified target sequences were selected using a restriction map program on Seqnet (SERC Daresbury Laboratories).

Extraction of DNA
from a variety of tissues



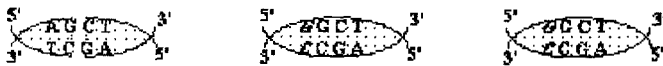
Restriction enzyme (RE)
digestion (AluI)



Amplification of unrestricted
mutant and wt targets by PCR



RE digestion of the
residual wt target



RSM Product Analysis
by PAGE

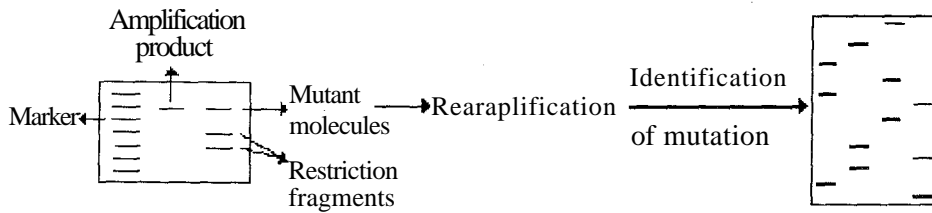


Figure 1. Schematic representation of the principal steps of the RSM assay.

The RSM assay

After selecting restriction endonucleases and determining the restriction enzyme parameters, the PCR cycle parameters and reaction conditions for each target, the RSM assay was performed on genomic DNA extracted from the rat brain, kidney, liver, lung, pancreas, testis and granuloma pouch tissue.

1 μ g aliquots of genomic DNA extracted from the individual untreated rat tissues were digested using 10 units or more of the selected 13 restriction endonucleases with *Taq* buffer in a final volume of 20 μ l overnight at the appropriate temperature. The digested mixtures were amplified in a 50 μ l PCR reaction using 1.25 U *Taq* polymerase, 200 μ M of each dNTP and 20 pmol of each of the two appropriate primers. After amplification, 17 μ l of the PCR products were subjected to a second round of the particular restriction enzyme digestion for overnight. 10 μ l of the reaction products were mixed with 2 μ l of loading buffer and electrophoresed on a 6% polyacrylamide gel and stained with silver. The each RSM assay target including the rat exons 5, 6, 7, and 8 of the p53 gene and exons 1, 2 of the *H-ras* gene and exon 1 of the *K-ras* gene products were investigated to ensure correct size of amplification and fragments and completely digestion of the amplification products using polyacrylamide gel electrophoresis (PAGE).

RESULTS AND DISCUSSION

The aim of the study in this paper was to develop a rapid, reliable, and convenient method to detect induced mutations *in vivo* in restriction endonuclease recognition sequence. Several factors were investigated for use in the RSM.

The RSM assay is a combination of two methods, that of restriction enzyme digestion of DNA and amplification of resistant enzyme sequences using PCR. A number of parameters which affecting the two methods were studied using the p53 and *K-ras* and *H-ras* genes of the rat.

Restriction enzyme digestion of DNA is an important part of the RSM assay because incomplete restriction of wild-type DNA sequences could lead to false positives. Complete restriction endonuclease digestion of the wild-type genomic DNA is necessary for the success of the RSM assay. The manufacturer's recommendation for the digestion of DNA with a restriction endonuclease is generally 1 hour for 1 μ g of substrate DNA (22). To achieve maximum digestion of the RSM assay targets the restriction endonucleases incubation time was carried out overnight (16 hours). In addition, two rounds of restriction endonuclease digestion were performed to ensure complete digestion of the wild-type recognition sequences. The primer sequences and selected restriction endonucleases used in the RSM assay are shown in Table 1.

Table 1. PCR primers employed for the amplification of the rat *H-ras*, *K-ras* and p53 genes and selected restriction enzymes in these targets.

Gene	Exon	Primer sequences (5' to 3')	Restriction Enzymes
<i>K-ras</i>	1	K2S: CTCCTACAGGAAACAAGTAG K2A: GGTGAATATCTTCAAATGATT	NlaIV
<i>H-ras</i>	1	HIS: GGCAGGAGACCCTGTAGGAG H1A: CCCACCTCTGGCAGGTAGTC	<i>CfoI</i> <i>HindIII</i>
	2	H2S: CTACCGGAAGCAGGTGGTCATT H2A: ATGCCATGGGCCACGGGCTAG	<i>HinfI</i> <i>CfoI</i>
p53	5	P53.5S: ACTCAATTTCCCTCAATAAG P53.5A: ATGAGCGTTGCTCTGATGGT	<i>AluI</i> , <i>DdeI</i> <i>NcoI</i> , <i>NlaIV</i>
	6	P53.6S: GGCCTGGCTCCTCCCAACA P53.6A: GTGGTACCGTATGAGCCACC	<i>DdeI</i> <i>MspI</i>
	7	P53.7S: ATACCACTATCCACTACAAG P53.7A: ATCATCACGCTGGAAGACTC	<i>BsII</i>
	8	P53.8S: TGGGAATCTTCTGGGACGGG P53.8A: CCCAGGGAGTGCAAAGAGA	<i>BsII</i>

The amplification and RSM products were detected by gel electrophoresis and visualised with a silver staining method. Polyacrylamide gels were used to produce a high degree of resolution of the products because of the amplification products (94-261 bp) and restriction enzyme fragments (26-158 bp) were relatively small in length. The advantages of the silver staining method were that the assay products could be detected 30 minutes after electrophoresis, the silver stained gels could be kept in polythene as a permanent record, and the detection of PCR and RSM products was more sensitive than ethidium bromide staining for small DNA fragments (23). Ethidium bromide is also a mutagenic chemical (24). For these reasons silver staining of DNA fragments resolved on the polyacrylamide gels was used as the detection method for the RSM assays.

It is important to optimise the amplification steps and reaction conditions for successful PCR (25). Several factors were adjusted to obtain maximum specificity, yield, and sensitivity for each gene segment during the PCR reaction in the assay. The dNTP concentrations used were 200 μ M of each dNTP in a 50 μ l reaction to obtain a specific product and minimize misincorporation. The magnesium chloride concentration is one of the key variables in the PCR and is relevant to both the specificity and yield. Excess magnesium chloride could stabilise spurious annealing of primer to produce incorrect template sites, resulting in larger amounts of undesired products and lower specificity. The magnesium

chloride concentrations used were 1.5 mM, as in a standard PCR buffer, in a 50 μ l reaction except for *H-ras* gene exons 1 and 2, and p53 gene exon 8 segments. Non-specific bands were observed in the amplification of these segments with the 1.5 mM magnesium chloride concentration. Therefore, the magnesium chloride concentrations were adjusted 1.0 mM for the *H-ras* gene exons 1 and 2, and 1.25 mM for the p53 gene exon 8 to obtain maximum specificity and yield. High primer concentrations may cause mispriming and accumulation of non-specific products. In the amplification of targets, the best balance between product yield and amplification specificity was achieved using 20 pmol of each primer for a 50 μ l reaction. The p53 gene exon 5 sequences could only not amplified with this concentration. 50 pmol of p53.5S and p53.5A primers concentrations were found to produce the desired product for this segment. 1.25 U of *Taq* polymerase in a 50 μ l PCR reaction was used for the amplification of the all RSM targets. This concentration gave the best balance between product yield and amplification specificity for each reaction. The annealing temperatures were determined experimentally for each reaction by varying the annealing temperature 2°C around the calculated temperature. Two concepts were considered to be of importance in the determination of the cycle number of PCR in the RSM assay. The first one was that the cycle number should be high enough to amplify a small number of mutated molecules. The second one was that it should provide complete digestion of control amplification products with restriction endonuclease. Table 2 shows the optimum annealing temperatures, MgCl₂ concentrations and cycle number for each gene segment. Example of the RSM assay analysis is shown in Figure 2.

Table 2. Optimum annealing temperatures, MgCl₂ concentrations and cycle number for each gene segment.

Gene	Exon	Annealing Temperature (°C)	MgCl ₂ Concentration (mM)	Cycle number	Product length (bp)
p53	5	59	1.50	30	180
	6	63	1.50	25	110
	7	53	1.50	25	94
	8	66	1.25	25	136
<i>H-ras</i>	1	54	1.00	32	171
	2	52	1.00	28	261
<i>K-ras</i>	2	58	1.50	28	170

PCR products require the stringent maintenance of the original DNA sequence content because during a PCR reaction misincorporation error could lead to false positive result in the assay. High fidelity amplification is necessary in the amplification step of the RSM assay by *Taq* DNA polymerase. Several experimental parameters were adjusted to achieve high fidelity amplification. It is very important that the concentrations of all deoxynucleotides are equal to

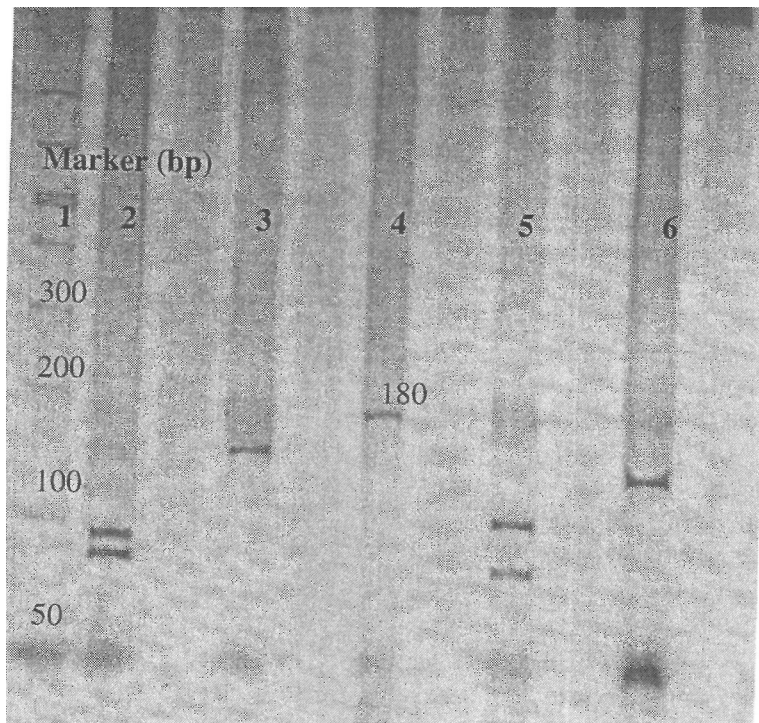


Figure 2. The restriction fragments and amplification product of the p53 gene exon 5 sequence. Lane 1 is the gel marker. Lane 4 is amplification product of the p53 gene exon 5 segment (180 bp). Lane 2, 3, 5, and 6 are the *NlaIV* (85-95), *AluI* (32-148), *NcoI* (103-77), and *DdeI* (132-48) enzymes fragments of the p53 gene exon 5 amplification product respectively.

prevent misincorporation errors. The four deoxynucleotide triphosphate precursors were used at equal concentrations, and the total dNTP concentration was at least necessary to support the desired amount of DNA synthesis in the assay. The magnesium chloride concentrations were adjusted as low as could support the desired amount of synthesis for the each gene segment and were not in large excess over the total dNTP concentration. The reaction times were adjusted as

to be as short as possible to obtain discrete bands, as this would minimise the time available for the polymerase to extend from misprimed termini. Therefore, annealing and extension times were limited to 20 seconds for the each target. The denaturation times were adjusted to 1 minute at 94°C to fully denature the DNA templates.

Cellular mutation systems are based on the selection of mutated cells which exhibit altered phenotypes. In forward mutation assays such as systems utilizing cultured mammalian cells or yeast, phenotypes which have acquired resistance to certain toxic compounds are selected (26). The cause of such a resistance is usually a mutation in a gene (reporter gene) coding for a carrier protein or another non-essential enzyme which has rendered the corresponding gene product inactive. As a result, the affected cells are able to survive in a toxic environment. The data yielded by cellular mutation assays have greatly aided our understanding of the processes underlying carcinogenesis. However, mutations in cancer-related genes such as cellular proto-oncogenes or tumour suppressor genes cannot be analysed using cellular mutation assays, because they do not give rise to readily selectable altered phenotypes (27).

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 (28).

In conclusion, these results of the RSM assay suggest that the assay can be used for detection of base changes in DNA from several tissues of the rat that were exposed with a mutagen or carcinogen. The assay developed allows the detection of base pair substitutions, small insertions or deletions within the restriction enzyme recognition sequence in any organ, in any gene for which the DNA sequence is known without selection of mutant phenotype.

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