Detection of minority point mutations by modified PCR technique: a new approach for a sensitive diagnosis of tumor-progression markers

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ABSTRACT

The detection of point mutations correlated with diseases, in enzymatically amplified DNA sequences (Polymerase Chain Reaction), is currently performed by digestion of PCR products when an existing restriction site disappears at least in one allele of the amplified mutated sequence or by allele specific radiolabeled probes in all other cases. These methods are the most sensitive but they cannot detect a mutation if it is present in less than 5% of the studied cells. We describe here a method based on the introduction of an artificial restriction site, using a modified primer during the PCR, which creates a RFLP indicative of the studied mutation. This RFLP is detected by a radiolabeled oligonucleotide probe which is not related to the mutation. Our approach multiplies the sensitivity by a factor of 1000 and it is practical for use in screening purposes and the detection, after treatment, of the residual disease in human malignancies. Using this method we detected 20% more mutations at codon 12 in the Ki ras oncogene in DNA from colorectal cancers that were undetectable with all the previous methods.

INTRODUCTION

Oncogenes are highly conserved genes in any living organism and have important functions in cellular proliferation and differentiation. Loss of regulation in this complex system, resulting from structural modification of a normal gene or of its controlling regions, is called oncogene activation. It is known to occur during the multiple step process of colonic carcinogenesis. However, no specific correlation has been demonstrated that was relevant to this process, until the detection of point mutations in total genomic DNA became feasible.

The Polymerase Chain Reaction (PCR) is an *in vitro* method for the primer directed enzymatic amplification of specific DNA sequences¹. It allows sensitive detection of point mutations in genes already sequenced. The amplified DNA product is analyzed by a dot blot hybridization, with synthetic radiolabeled oligodeoxynucleotide probes, under high stringency conditions. The probes are specific to normal DNA sequence or to each possible mutation of a given codon (ASO probes). The results of the hybridization are revealed by autoradiography². The sensitivity of this method is approximately 5% i.e. cells carrying a mutation can be detected, among a population of non carrying cells, if they represent at least 5% of the total cell population.

However tumors are composed of heterogeneous cell populations (neoplastic cells, infiltrated inflammatory cells, normal tissue cells). This heterogeneity increases the background 'noise' elevating the number of amplified non mutated sequences and thus decreases the sensitivity of the detection. Therefore, it is often important to detect point mutations at a level which is much lower than 5%, especially after a treatment, in order to evaluate the efficiency of the therapeutic action and the level (if any) of the residual

Table 1: Comparative results of the two methods for the detection of Ki ras codon 12 point mutations in 14 samples from 9 patients with colon cancer. The mutation was searched in parallel by 2 methods: (i) The modified PCR method, (ii) The ASO dot-blot hybridization method after PCR amplification. In addition to the wild type probe we used all the six possible versions of mutated codon 12 probes².

Sample N°	Patient code	Tissue type	Description & localization	Mutation with mod. PCR method	Mutation with ASO probes method
1	50A	lymphocytes	normal control	—	—
2	SG ₃	mucosa		—	—
3	"	polyp		+	—
4	"	adenocarcinoma	sigmoid	+	+
5	"	adenocarcinoma	ascending colon	+	+
6	SG ₆	mucosa		—	—
7	"	adenocarcinoma		+	—
8	SG ₇	mucosa		—	—
9	"	adenocarcinoma		+	—
10	SG ₈	mucosa		—	—
11	"	polyp		+	—
12	"	adenocarcinoma		+	NA
13	SG ₁₁	mucosa		—	—
14	"	adenocarcinoma		+	+
15	"	carcinoma	gastric (synchronous)	+	+
16	SG ₁₂	mucosa		—	—
17	"	mucosa		—	—
18	"	adenocarcinoma		—	—
19	SG ₁₃	mucosa		—	—
20	"	adenocarcinoma		—	—
21	SG ₁₈	mucosa		—	—
22	"	mucosa		—	—
23	"	adenocarcinoma		+	—
24	RIV	polyp		—	NA
25	"	adenocarcinoma		—	NA
26	"	adenocarcinoma		+	NA

NA = not available, + = Ki ras codon 12 mutated, — = Ki ras codon 12 not-mutated.

disease. The detection of a point mutation in the adjacent apparently 'normal' tissue provides a prognostic marker, relevant to the identification or the prevention of further tumor progression. These factors are of great importance in the understanding of the commitment of cells to neoplasia and malignancy. We describe here an approach 1000 times more sensitive than former methods and which can be used for screening analysis or detection of residual diseases.

PATIENTS AND METHODS

Patients:

Tissues were obtained from 9 patients operated for sporadic, non-familial, colon carcinoma. Specimens were removed immediately after colectomy, then frozen. They included fragments of the carcinomas and normal mucosas, as well as every other neoplasia found to be associated with the colon cancers. In three patients, synchronous benign adenomas were present; one patient had two synchronous colonic carcinomas; one patient had a synchronous gastric cancer (table 1).

Fig. 1: P.C.R. with modified primer creating artificial RFLP.

As a positive control, we used DNA from the SW 480 cell line, which is known² to carry 2 mutated alleles at codon 12 of Ki ras oncogene.

DNA from peripheral lymphocytes (50A), which are homozygous wild type at this same position as proved by ASO probe hybridization, was used as a negative control.

DNA extraction:

DNA from frozen or fresh colon carcinomas, adenomatous polyps and adjacent normal mucosas, were extracted either according to the classical method of phenol chloroform, or to a simplified guanidium method³.

Oligonucleotides primers and probes

The oligodeoxynucleotides were synthesised by the solid phase triester method. The primers were designed to introduce base substitution in the amplified fragments. The probe (Kx-1), which hybridized with the 3' region of these fragments, was end labeled using [γ ³²P]ATP and T4-Polynucleotide kinase.

Polymerase chain reaction

In vitro enzymatic DNA amplification (PCR) was performed on an automated apparatus (DNA thermal Cycler from Perkin Elmer Cetus).

We performed between 30 and 40 cycles of amplification. Each cycle includes 3 steps:

- denaturation of DNA at 94°C for 30 sec.
- annealing of the primers at 52°C for 40 sec.
- enzymatic extension at 72°C for 1 min.

Modified primers were designed to introduce a base substitution adjacent to the codon of interest in order to create an artificial restriction site with only one allelic form (wild type or mutated). We performed PCR with a modified primer creating a Msp I recognition site only if codon 12 was of the wild type (fig. 1). This approach allowed us to screen for point mutations at codon 12 of Kirsten ras oncogene⁴.

An aliquot of the PCR product was controlled in a 2–3% Nu Sieve gel for the presence of the amplified fragment (99 bp). The PCR product was digested by Msp I enzyme which gave two fragments of 21 and 78 bp in the case of a wild type sequence or left the product undigested in the case of a mutated sequence. The fragments were analyzed by electrophoresis on Nu Sieve agarose gels.

Detection of the minority fragments:

After the electrophoresis, the fragments were transferred onto a nylon filter according to the standard method of Southern⁵. These filters were hybridized with the probe Kx-1 in a solution containing tetramethylammonium according to Verlaan de Vries *et al.*² at 50°C

Figure 2: Electrophoresis of PCR products :

1 μ g of DNA from 50A (A and B), SW80 (C and D) and SG3d1 (F and G) were amplified by PCR (40 cycles) using the primers described in figure 1.

20 μ l of the amplified samples were digested with Msp I (B, D and G) and electrophoresed on a 3% NuSieve agarose gel.

Lane E: 2 μ g of the Φ X 174/Hae III molecular weight marker.

for an hour. The filters were washed twice at 52°C in 0.1 SDS, 5 $\times$ SSPE solution for 10 min. and autoradiographed for an hour at -70°C using intensifying screens.

RESULTS:

The validity of our modified primer method is presented in figure 2: 50A and SW 480 samples have been already described, SG3d DNA has a wild and a mutated allele as confirmed by allele specific radiolabeled probes. Lanes A and B show the PCR products of 50A DNA before and after Msp I digestion respectively; lanes C and D, those of SW480; lanes F and G, those of SG3d. The results of the electrophoresis correspond to the known genotype of these DNAs. The visualization of the results in the gel under U.V. light, without the use of radioactive probes, gives a sensitivity analogous to the previous method with the allele specific radiolabeled probes (i.e. $\geq 5\%$). However, we can increase its sensitivity using an internal (i.e. non mutation specific) radiolabeled oligonucleotide probe (Kx-1), which reveals the presence of minority amplified and non digested fragments.

An example of this strategy is shown in figure 3. SG3c DNA is negative for the mutation with the ASO probe method. In the stained gel after the PCR with the modified primer,

Figure 3: Hybridization of P.C.R. products with Kx-1 probe. (Kx-1: 5'-GCTAATTCAGAATCATTTTG-3')

Lane A: 50A DNA (2 wild alleles)

Lane B: SG3d1 DNA (1 wild & 1 Ki-ras 12 mutated allele)

Lane C: SG3c DNA (1 wild & 1 Ki-ras 12 mutated allele)

Figure 4: Dilutions of SG3b1 (heterozygous mutated-normal DNA) in 50A (homozygous wild type DNA)

LANE A:	1:1	50A/SG3d1
LANE B:	1:0	50A/SG3d1
LANE C:	10:1	50A/SG3d1
LANE D:	100:1	50A/SG3d1
LANE E:	1000:1	50A/SG3d1
LANE F:	5000:1	50A/SG3d1
LANE G:	1:0	50A/SG3d1
LANE H:	25000:1	50A/SG3d1

we visualize only the 78bp fragment (corresponding to the wild type allele). But the hybridization with Kx-1 radiolabeled probe reveals, after autoradiography, another band (99 bp, corresponding to the presence of a minority mutation among the cells of the SG3c sample).

To establish the detection threshold of our method we performed a series of dilutions containing a decreased number of mutated alleles in a medium containing wild type DNA. Our dilution scale extended from 1/1 to 1/25000 of mutated alleles in normal ones. This allelic dilution corresponds to cell dilution, as mutated cells usually carry a normal and a mutated allele⁶. In figure 4 the spot corresponding to the large (non digested) fragment (1) represents the mutated alleles of the heterozygous SG3d1 cells and the spot corresponding to the light (digested) fragment (2) represents the wild type alleles of the heterozygous SG3d1 and of the normal homozygous 50A cells. To interpret these results we must compare the intensities of spots (2) to those of spots (1) on the same lane and to those of spots (2) on the negative control lanes (B and G). This strategy leads to the detection of the mutated allele until the 1:25000 dilution.

Using this protocol we studied 9 patients and the results are shown in table 1 in comparison with a previous study⁷ of the same patients with the ASO probes approach. In 5 cases (samples N° 3, 7, 9, 11, 23) we found a mutation that was undetectable with the previous method. In four cases (polyp 24 and adenocarcinomas 18, 20, 23) the results remained negative for the mutation. In all cases the normal adjacent mucosa (2, 6, 8, 10, 13, 16, 17, 19, 21, 23) do not present mutations with either method.

DISCUSSION

A practical way to detect point mutations, not affecting natural restriction sites in oncogenes, is the use of PCR combined with a dot blot hybridization with ASO probes. This method has a sensitivity of about 5% and is easier to perform than former approaches such as NIH 3T3 cell line DNA transfection⁸ or mismatch detection by RNase A cleaving⁹. However, the obligation to use many radiolabelled probes, the poor sensitivity and the requirement of critical wash conditions which are difficult to control¹⁰, prompted us to develop the method described above.

During PCR with modified primer the introduction of base substitution adjacent to the studied codon is possible because Taq polymerase lacks exonucleasic activity and therefore

does not correct the sequence of this primer. Under our PCR conditions, the enzymatic extension of the modified primer, which has a one base mismatch with the genomic template, is always possible and independent of the adjacent point mutation. Under different PCR conditions, it was described¹¹ that a mismatch at the 3' end of a primer stops the amplification process. It is therefore possible to detect a point mutation by selective amplification of the mutated allele, if it is present in the studied sample, using a primer that is complementary to the mutated sequence. This primer generates a mismatch with the wild type sequence and stops the amplification process. However, according to the same paper, a non selective amplification of the normal sequence can be obtained under slightly different PCR conditions. This side effect is a limiting factor in the use of the selective amplification method which is based on very stringent PCR conditions. Our method does not differentiate the mutated and the wild type sequences during the PCR and it introduces in both sequences the same base substitution, but the restriction site is formed in only one allele (wild or mutated). The product of PCR with this homogeneity and increased concentration of small DNA fragments with only one possible restriction site, is suitable for digestion even with low quantities of enzyme. To avoid any possibility of partial digestion we use a large excess of the restriction enzyme and we test always the efficiency of this step by running in parallel a positive (SW80) and a negative (50A) control.

If a unique mutation has to be detected, it is possible to design a primer generating a restriction site only in the amplified mutated allele^{12,13}. When several different mutations have to be screened in a given codon it is better to use the reverse approach: artificial restriction site is created only in the wild type allele⁴, and is absent in any other mutated one. The later situation applies to the mutations of codon 12 occurring in the ras gene family². We therefore used the second strategy for this study.

We also applied this approach to the detection of point mutations at codon 13 of the Ki ras oncogenes using a modified primer with 2 base substitution at its 3' end. The corresponding modified primer K13Nm (5' ACTTGTTGGTAGTTGGAGCTGCC 3'), with primer KB12, amplifies a fragment of 96 bp with a Msp I restriction site only if codon 13 is a wild type one. Preliminary results indicate that no mutations are present at this codon in our samples.

The decreased efficiency of PCR with a modified primer is compensated by a decrease in the annealing temperature and an increase in the number of PCR cycles. In all cases our conditions lead to the production of only one amplified fragment without non specific amplifications. Therefore, our approach is sensitive, selective and practical for use in small and large scale studies and screening purposes.

Without using radioactivity our method has a sensitivity which is equivalent to that of the ASO probes technique, but it is very easy to perform and leads to results within 24 hours, especially if it is coupled with boiling extraction of DNA samples¹⁴. Combined with a Southern blot hybridization with a non mutation specific radiolabeled probe under non stringent conditions, it can detect minority point mutations (1 mutated cell among 25000 wild type cells) in a week. Actually the detection of point mutations at such a level could be very important, especially after the treatment of a tumor known to harbor a mutated cellular component, in order to evaluate the efficiency of the therapeutic action and the level of the residual disease. The simultaneous analysis of the adjacent tissue could help the understanding of tumorigenesis in an organ at risk.

In the case of colonic tumorigenesis, point mutations of Ki ras oncogene at codon 12 have been demonstrated to be an early event during tumor progression. The classical

methods for the detection of the mutations failed to demonstrate any correlation with the available histological criteria in adenomas. It is of a major importance to test a more sensitive method, in order to identify a possible prognostic significance of Ki ras mutations. As a matter of fact, cancer prevention policies are based upon the removal of adenomas, but specific criteria for the actual premalignant conditions are still unidentified. Attempts should be made to determine the prevalence of ras mutations in adenomas of different sizes, ages, degrees of dysplasia, villous components, and in synchronous adenomas from polyposis coli patients.

In table 1, we see that polyps N°3 and 11, which were classified as non mutated by the ASO method, carry a mutation of small proportion as proved by our approach. The same is true for adenocarcinomas N°7,9 and 23. Otherwise negative control (1) and normal mucosas (2,6,8,10,13,16,17,19,21,22) remain negative for mutation with both methods. This confirms the somatic origin of the involved mutations and excludes false positive artefacts during the manipulations. Among all our samples, polyp N°24, adenocarcinomas N°18,20,25 still remain negative for mutations at codon 12 of Ki ras oncogene and are worth studying at codons 13 and 61.

In addition, our approach allows a fast detection of specific polymorphisms or point mutations in a given gene, and the analysis can be performed within a day. With an automated device for PCR we can test simultaneously many samples and screen for various single locus anomalies like the susceptibility for HLA correlated disorders (i.e. HLA and insulin dependent diabetes mellitus) or known defective gene diseases (i.e. β -thalassaemia). Moreover, one could detect with this method asymptomatic heterozygous carriers of recessive genetic diseases even in the absence of a natural restriction site affected by the mutation.

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