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A METHOD TO INCREASE THE SENSITIVITY OF MUTATION SPECIFIC OLIGONUCLEOTIDE HYBRIDIZATION USING ASYMMETRIC POLYMERASE-CHAIN REACTION (PCR)

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Summary We propose a simple and reliable method to increase the sensitivity of mutation specific oligonucleotide hybridization (MSOH) at least 2.5 times, when it is used to detect mutations in samples of DNA from tumor tissues. The method is based on using single stranded (ss) DNA, amplified by asymmetric PCR, as a target for MSOH analysis. During the first step, genomic DNA, isolated from tissue samples, has to be amplified by "standard", symmetric PCR, with sense and antisense primers in equimolar concentration. This amplification can be performed in a diminished volume of reaction mixture. In the second step obtained double stranded (ds) PCR DNA-product can be used as a template for asymmetric PCR, using only a single primer. The ss DNA must be complementary to the set of mutation specific oligonucleotides. By this innovation we have been able to clarify questionable results of MSOH using ds DNA as a target. Comparing MSOH from ss DNA to that from ds DNA, the observed rate of Gs-alpha mutations in thyroid tumor tissue samples increased to 16.7 % (14/66) from 6% (4/66).

Various assays have been used to detect somatic mutations in *gsp*, *ras*, *p53* and other oncogenes in tumor tissues (1-6). The most of these methods are based on PCR- amplification of target genes and allow rapid analysis of multiple specimens.Several types of PCR are possible by varying the reaction components, principally the oligonucleotide primers. A "standard" or "symmetric" PCR contains equal amounts of sense and antisense primers to amplify a ds DNA product. Asymmetric PCR relies on using unequal concentrations of primers, or only one primer, to allow accumulation of single stranded copies of one of the DNA template strands (sense or antisense, depending on which primer is used). The most important application of asymmetric PCR is direct sequencing, but this method has been used to join immunoglobulin heavy and light chain variable region genes (7) and for gene analysis (8). We were not able, however, to find any published data about using asymmetric PCR to increase the sensitivity of

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mutation specific oligonucleotide hybridization (MSOH). This method is extremely rapid, but presumes that a high percentage of the DNA sample analysed contains mutant alleles (not less, than 10% (3, 4). Despite the lack of sensitivity MSOH is wide-spread method for detecting mutations in eucaryotic genes, principally since to the advent of non-radioactive labelling. In order to provide a more sensitive means for detecting mutations by MSOH, we have introduced the second step - asymmetric PCR, producing predominantly ssDNA target. Thyroid tumor tissue samples were used as the subject for detection of mutations in codon 201 of the Gs-alpha subunit gene.

MATERIALS AND METHODS

Materials Ampli Taq DNA-polymerase was obtained from Perkin Elmer Cetus Co. Primers for Gs-alpha (sense: 5'GTGATCAAGCAGGCTGACTATGTG 3' and antisense 5'TAACAGTT GGCTTACTGGAA 3') and recently described mutation specific oligonucleotides for codon 201(9) were synthesized in the Inst.for Biol.Physics, Univ.of Düsseldorf. dNTPs were purchased from "Sigma", nylon membranes ("Hybond-N^+ ") and E.C.L. (enchanced chemiluminescent) 3'-end DNA labelling and detection kit from "Amersham". Glass MAX columns were obtained from "Gibco-BRL". DNA- size marker (ϕx174 / HinfI fragments) was received from "Stratagene".

DNA extraction and amplification followed our recently published protocol (9), excluding some details which are described in the Results. The PCR-amplified samples were analyzed by 8% polyacrylamide gel-electrophoresis (PAGE) . DNA concentration was measured by absorption at 260 nm ("Pelkin-Elmer" Lambda 2 UV/ VIS spectrometer).

MSOH experiments were performed using a heat-denaturation procedure (95^0 C, 5 min.) followed by immediately chilling on ice. Probes (5 µl) were spotted on nylon membrane and fixed by UV-crosslinking ("Biometra", 2,0 j /cm^2). Oligonucleotides were labelled and detection of MSOH was by E.C.L. according to the manufacturer ("Amersham"). Discrimination between wild type and mutant alleles was performed by using high stringency washes (5).

RESULTS AND DISCUSSION

At the beginning of our investigation, we examined a total of 66 thyroid tumor samples, histologically classified as follicular carcinomas (20), papillary carcinomas (20), C-cell carcinomas (21) or follicular adenomas (5). The analysis was focused on detection of mutations in codon 201 of the Gs-alpha-gene using DNA isolation, amplification by PCR of a 258 fragment of the Gs-alpha-gene and "standard" MSOH with a set of mutation specific oligonucleotides (Table 1). After duplicate hybridization experiments, we received clear results for 42 samples: four (6 %) were classified as mutation positive and 39 (59 %) as mutation negative (wild-type). However, the results obtained with 23 tissue samples (35%) were unclear.

In order to clarify this latter data, we produced antisense ssDNA, which was directly complementary to the sense oligonucleotide probes. We used 1 µl of the first (symmetric) PCR product as a template for generation of antisense ss-DNA. Only antisense primer was used to prime this assymmetric PCR in concentration of 50 pMol per reaction (100 µl each). The resulting PCR products were checked by PAGE in comparison to original double stranded

Table 1. Detection of Gs-alpha codon 201 mutations in tissue from differentiated thyroid carcinomas and follicular adenomas using MSOH

	Mutations in Gs-alpha codon 201		
	Positive (n)	**Negative (n)**	**Questionable (n)**
Symmetric PCR	4	39	23
Asymmetric PCR	14	44	8

products (Fig.1) and 5 µl of each was spotted onto nylon membrane and hybridization was done as before.Using this modified MSOH analysis we retested 19 DNA-samples with ambigous results (4 samples were not retested), 9 with negative and 3 positive for control. All of previously negative or positive results were confirmed. 10 of 19 previously ambiguous samples revealed strongly positive signals with corresponding mutation specific nucleotide, 5 were negative and 4 were still uncertain. Thus, along with the previously identified mutations in samples, we have proved the presence of a mutation in codon 201 in 10 additional samples of the 66 analyzed. Consequently, by using single stranded antisense DNA as a target, we increased the observed rate of mutated alleles by MSOH 2.8 times, from 6% until 16,7%.

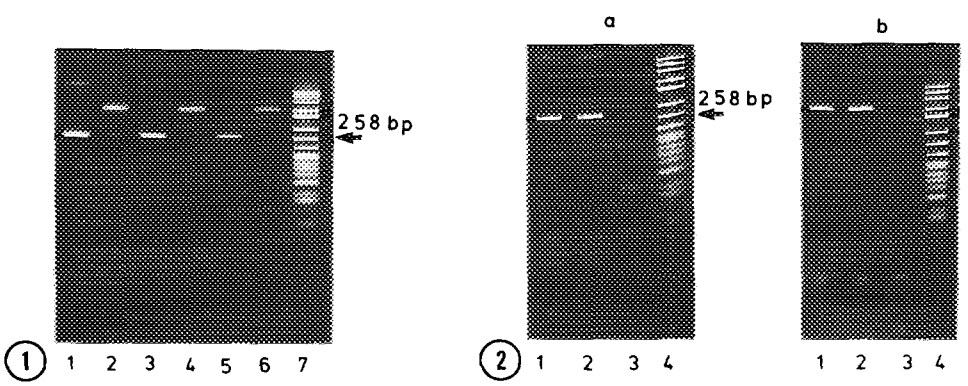


Figure 1. PAGE of DNA samples amplified by symmetric and asymmetric PCR. Lanes: 1, 3, 5 - ds DNA (258 bp) obtained using different genomic templates; 2,4,6 - corresponding ss DNA after asymmetric PCR using ds PCR product as template; 7 - ds DNA size marker.

Figure 2. PAGE of DNA samples, obtained using subcloned wild- and mutant types of Gs-alpha gene fragments as templates.
a. Lanes: 1 - wt (wild type) ds DNA; 2 - mut (mutated) ds DNA; 3 - negative control; 4 - ds DNA marker.
b. Lanes: 1 - wt ss SNA; 2 - mut ss DNA; 3 - negative control; 4 - ds DNA marker.

Furthermore, using semiquantitative assay , we have attempted to determine the sensitivity of MSOH directed by assymetric PCR. Plasmid DNA of two of subclones,(one of them containing mutated and other - wild type Gs-alpha codon 201), were used as a templates for the generation double- and single-stranded PCR products (Fig.2). DNA sample solutions were purified using Glass MAX columns, then equilibrated to an equal volume of 70 µl. The concentration of DNA in the samples was estimated by spectrometry (Table 2).Wild type and mutant DNA were then mixed in differing proportions as follows: 50%, 25%, 10% and 5% mutant DNA by volume, in wild type DNA solution (Vmut / Vwt x 100%). Then, we have performed hybridization experiments according to our standard procedure using wild type and the mutation specific oligonucleotide probes. As shown in Fig.3 the positive signal of hybridization against mutation specific oligonucleotide practically disappeared, when double-stranded mutated DNA represented only 5% in wild type of ds DNA. Notably, a 5% concentration of mutated single-stranded antisense DNA, gave a hybridization signal of the same intensity as 10% mutated ds DNA. Taken together, data about DNA concentration (DS >> SS=1:1,5) and hybridization analysis demonstrate that the sensitivity of MSOH can be increased at least 2.5 times using asymmetric (ss) PCR- products.

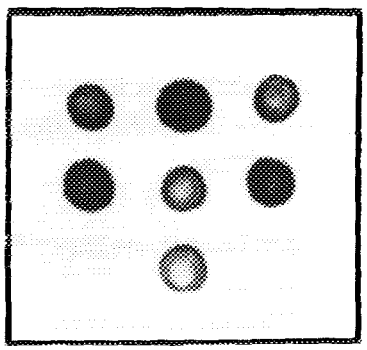
It has been known that MSOH works best when a high percentage of tumor tissue bears the clone / clones with mutant genetic alteration, due to a preferential expansion of these clone / clones.

We have compared preliminary data on subcloning (unpublished) with that obtained by MSOH-analysis of corresponding original tumor samples, using a standard protocol (ds PCR-amplified DNA as a target). We found that distinctly positive signals of mutant hybridization against oligonucleotide could be seen when the content of the mutant alleles in the original sample was not less than 15%. At the same time using ss DNA as a target for hybridization against mutation specific oligonucleotides makes detection possible, when tumor tissues contain not more than 5% mutant cell.

Thus, the harnessing of complementary ssDNA amplified by asymmetric PCR as a target essentially (at least 2,5 times) increases the sensitivity of MSOH to detect mutations. This

Table 2. Concentration of DNA in the samples, obtained by symmetric and asymmetric PCR (µg / ml)

Type of DNA in sample	Wild	Mutant
ds	54,6	55,02
ss	36,04	35,08



mutant DNA (ds or ss)

50% ds	50% ss	10% ds
25% ss	10% ds	10% ss
5% ds	5% ss	0% ds

KEY

Fig.3 . MSOH analysis of wild and mutant types DNA mixtures hybridized against oligonucleotide probe, harboring the mutation (Arg → Leu) in codon 201 of Gs-alpha gene.

means that a large number of tissue or cell culture samples can be analyzed for preliminary selection of mutant variants. Moreover, the same samples of ss DNA, suspected of harboring a mutation, can be used for direct sequencing.

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