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Rapid detection of loss of heterozygosity of chromosome 17p by polymerase chain reaction-based variable number of tandem repeat analysis and detection of single-strand conformation polymorphism of intragenic *p53* polymorphisms

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Abstract Intragenic restriction site polymorphisms in amino acid residue 72 in exon 4 and a *Mspl* polymorphism in intron 6 of the *p53* tumour suppressor gene can both serve as polymorphic markers. Probe YNZ22 (D17S5) is a highly polymorphic, variable number of tandem repeat (VNTR) marker which maps to chromosome 17p13.1 where the *p53* gene is located. Locus specific amplification by polymerase chain reaction (PCR) technique and subsequent non-isotopic single-strand conformation polymorphism analysis of the PCR fragments was used for the detection of loss of heterozygosity (LOH) of 17p including the *p53* gene locus. In combination with a PCR-based method for the analysis of the VNTR locus D17S5 using unique sequences flanking the polymorphic region of YNZ22 we investigated tumour DNA and corresponding constitutional DNA from 69 patients, including 39 patients with gastric cancer, 21 patients with osteosarcomas and 9 patients with Ewing's sarcomas. Using all three methods, 49/69 (71%) patients were informative for LOH, which revealed allelic loss in 5/39 (12.8%) gastric cancers, 1/9 (11.1%) Ewing's sarcoma, and 4/20 (20%) osteosarcomas.

Key words *p53* tumour-suppressor gene
Loss of heterozygosity (LOH)
Single-strand conformation polymorphism (SSCP)
Gastric cancer · Soft tissue tumour

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Introduction

Allelic deletions of chromosome 17p13 where the *p53* gene is located occur frequently in a variety of human cancers including carcinomas of the breast, colon, ovary, lung, brain and urinary bladder [5, 11, 23, 25, 27, 38, 41, 46, 49, 50]. Several studies have suggested that the *p53* gene, which is commonly deleted in these tumours, functions as a tumour suppressor gene [16, 17, 21]. Knudson [24] originally postulated that initiation of tumorigenesis may involve chromosomal deletions unmasking recessive mutations in tumour suppressor genes. A steadily growing number of studies provide evidence that missense mutation in the *p53* gene are a frequent feature of human neoplasms [5, 20, 26, 38]. A mutation occurring in one of the parental alleles of the *p53* gene, and subsequent loss of the normal allele is assumed to direct the cells toward the malignant phenotype. Therefore detecting loss of heterozygosity (LOH) at the *p53* gene locus would be a potential diagnostic marker for progression of these cancers.

In the present study we demonstrate the combination of two polymerase chain reaction (PCR)-based techniques for the detection of LOH of chromosome 17p. Detection of an intragenic *p53 Msp*l enzyme restriction site polymorphism in intron 6 [33] and an *Acc*II polymorphism in amino acid residue 72 in exon 4 [4, 10] was established by non-isotopic single-strand conformation polymorphism (SSCP). Analysis of the variable number of tandem repeat (VNTR) region according to YNZ22 at chromosome 17p was performed by a single PCR amplification [51]. Our approach can be applied routinely to the detection of LOH of the *p53* gene and can be used for the investigation of small tumour biopsies processed for histological examination.

Material and methods

Tumour material was obtained at the time of surgery, snap frozen in liquid nitrogen and stored at −70° C. Tumours were classified

Table 1 Oligonucleotides used for polymerase chain reaction amplification

P1	5'TTGCCGTCCCAAGCAAATGGATG 3'
P2	5'ATACGGCCAGGCATTGAAGT 3'
P3	5'CCGAGTGGAAGGAAATTTGC 3'
P4	5'GAGGTCAAATAAGCAGCAAGG 3'
P5	5'AAACTGCAGAGAGAAAGGTCGAAGAGTGAAGTG 3'
P6	5'AAAGGATCCCCCACATCCGCTCCCCAAGTT 3'

according to the WHO criteria. Corresponding sections of the specimen used for DNA extraction were checked for the presence of normal cells to indicate the amount of contamination by normal DNA in the extracted sample.

High molecular weight DNA was prepared from tumour and normal tissues or blood samples by proteinase K digestion and phenol/chloroform extraction as described [28]. From 20 gastric cancer patients formalin fixed and paraffin embedded tissues were available for DNA extraction, which was carried out as follows. Excess paraffin was removed from 5 µm paraffin sections prior to proteinase K digestion, which was performed overnight at 55° C. After digestion residual paraffin could be removed from the top of the digestion mixture with a pipette tip. DNA was subsequently prepared by the standard phenol/chloroform method.

The oligonucleotide primers used for PCR amplification are listed in Table 1. PCR was performed according to the standard protocol by Mullis and Faloona [36] which was modified so that a 20 µl final volume contained 4 pmol of each primer (P1 and P2 for ACCII; P3 and P4 for MspI), 200 µM of each dNTP, 10 mM TRIS-hydrochloric acid (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 U Ampli-*Taq* DNA-polymerase (Perkin-Elmer Cetus), and 40 ng of the appropriate template.

Amplification of 36 cycles was performed by means of an automated thermocycler (Biomed, Bachhofer, Germany). Following a first denaturation step at 94° C for 4 min, cycles consisted of denaturation at 94° C for 30 s, annealing for 60 s starting at 61° C and a stepwise decrease of 1° C in each second cycle within the first 6 cycles to finally 59° C, and extension at 72° C for 90 s.

Amplification of the VNTR region YNZ.22 as previously described [51] was carried out using the modification of a 20 µl final volume with the same buffer conditions as described above and 10 pmol of each of the respective primers P5 and P6 (Table 1), 0.5 U Ampli-*Taq* polymerase (Perkin Elmer Cetus) and 40 ng of template. Initial denaturation at 94° C for 4 min was followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 63° C for 1 min, and extension at 72° C for 5 min.

Two microlitres of the reaction mixtures were analysed on native 12% polyacrylamid gels without further purification. Polyacrylamidgel electrophoresis (PAGE) was carried out on a horizontal electrophoresis system (Multiphor, Pharmacia, Biotech) with ultrathin gels baked on GelBond Pag (FMC, Rockland, Me., USA). Electrophoresis was performed under thermostatic control (Multitemps, Pharmacia, Biotech) at 15° C and 10 mA for 1 h using a discontinous buffer system with 35 mM sulfate-borate (pH 9.0) as the leading-trailing ion and 141 mM TRIS-borate (pH 9.0) as the trailing ion as previously described [3]. Bands were visualised by silver staining according to standard protocols [19]. For SSCP analysis 1 µl of the reaction mixture was added to 1.5 µl 95% deionized formamid heated to 94° C for 2 min and kept on ice until loading. The samples were applied to a 8% native ultrathin PAGE prepared for the Multiphor system. Gels were run with the same buffer systems as decribed above at 15° C and 2 mA for 5 h and subsequently silver stained.

After DNA amplification the PCR products containing the respective polymorphic sites in exon 4 and intron 6 were digested with the restriction endonucleases *Acc*II and *Msp*I (Gibco, BRL) according to standard protocols given by the supplier. After digestion DNA fragments were separated on a 12% PAGE as described above.

Results

We examined whether normal tissues or blood samples from 69 patients were informative (heterozygous) for the polymorphic sites in exon 4 and intron 6 of the *p53* gene. From all patients with constitutional heterozygosity the respective tumour samples were analysed. Typical results of SSCP analysis of a 190 base pair (bp) PCR fragment containing the *Msp*I restriction site polymorphism in intron 6 is given in Figure 1. Cases c and d were not suitable for the detection of LOH due to constitutional homozygosity for the *Msp*I enzyme restriction site polymorphism demonstrated by two bands in SSCP analysis. Constitutional heterozygosity was found in the normal tissue of cases a and b with two polymorphic bands. In the corresponding tumour material one of the two polymorphic bands was lost indicating reduction to homozygosity and LOH of the *p53* gene locus. To demonstrate the validity of our approach these eaxamples have been analysed with the respective restriction site enzyme. The

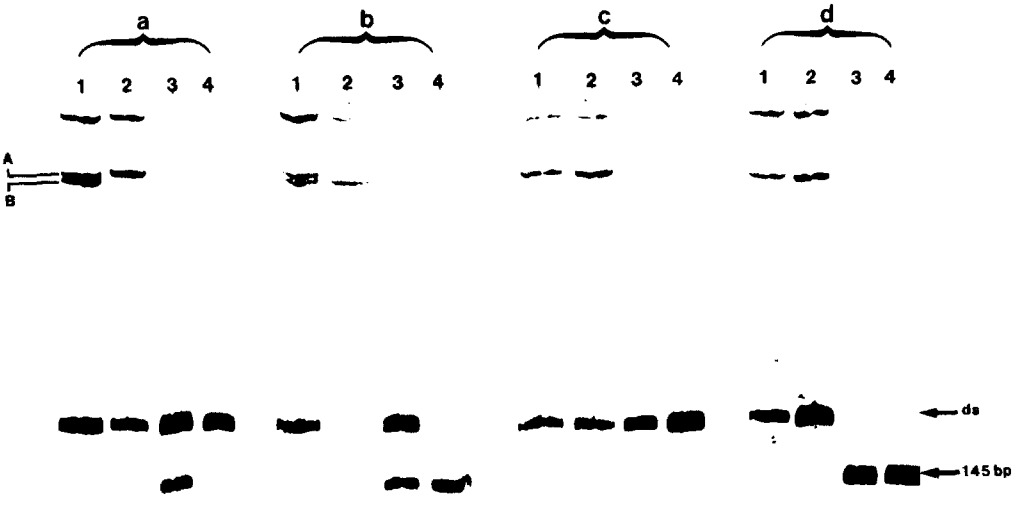

Fig. 1 Single-strand conformation polymorphism (SSCP) analysis of the MspI restriction site polymorphism in intron 6 of the *p53* gene. *A,B* denote the polymorphic single strands. Unmarked bands are due to non-polymorphic single strands (top), undenatured double-stranded DNA (*ds*), or fragments obtained from enzyme digestion (145 bp). Lanes 1 (normal tissue) and 2 (tumour) represent SSCP analysis; lanes 3 (normal) and 4 (tumour) show results obtained from the enzyme digestion, respectively. Loss of heterozygosity (LOH) is shown in the cases a and b, whereas the cases c and d were not informative

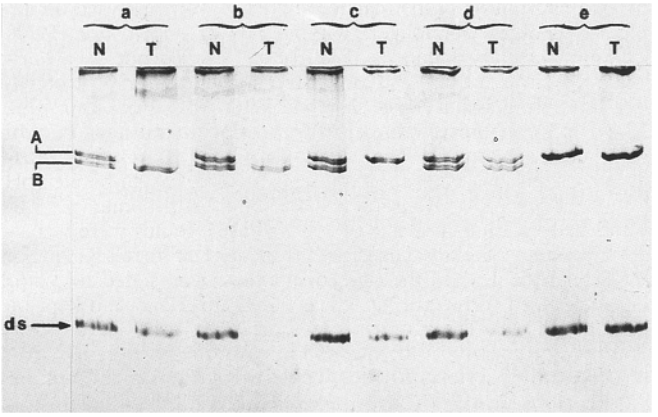

Fig. 2 Detection of LOH at the AccII restriction site polymorphism in exon 4 of the *p53* gene. *A, B* denote the polymorphic bands. LOH was detected in cases a, b, and c. Cased d was informative (heterozgous) without LOH, case d was homozygous. *T* tumour, *N* normal tissues

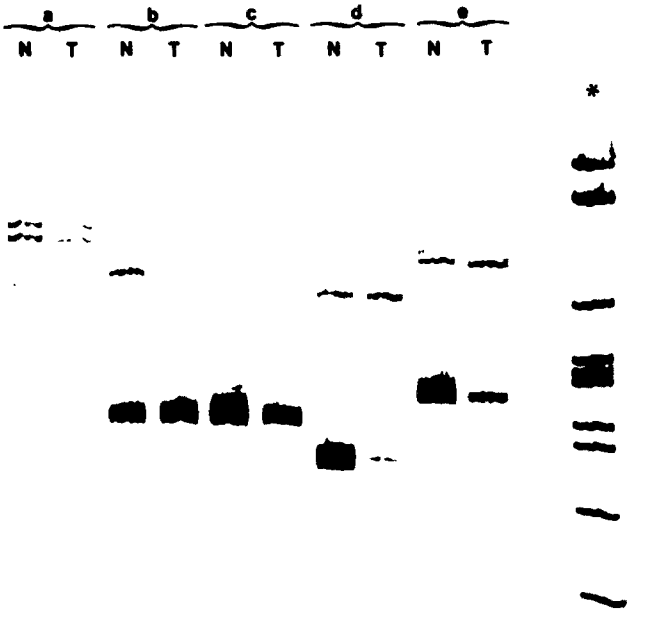

Fig. 3 Polymerase chain reaction-based variable number of tandem repeats analysis of the YNZ22 locus. Heterozygosity was detected in cases a, b, d, and e. LOH has occurred in cases b, d and e. The weak band obtained from the tumour material in d and e was due to contaminating normal cells. Case c was not informative due to consitutional homozygosity. *Molecular weight standard. *T* tumour, *N* normal tissues

Table 2 Frequency of heterozygosity detected for the intragenic *p53* polymorphisms and the YNZ22 locus

Polymorphic marker	Number tested	Informative (heterozygous)
*Msp*I	69	14 (20.3%)
*Acc*II	69	19 (27.5%)
YNZ22	69	41 (59.4%)
Total	69	49 (71.0%)

Table 3 Frequency of allele loss at the *p53* and YNZ22 locus

Tumour	Polymorphic marker	Number tested	Informative (heterozygous)	Loss of heterozygosity
Gastric cancer	*Msp*I	39	9 (23%)	3
	*Acc*II	39	12 (30.7%)	3
	YNZ.22	39	11 (28%)	3
	Total	39	23 (59%)	6 (26.1%)
Ewing's sarcoma	*Msp*I	9	0	0
	*Acc*II	9	1 (11.1%)	0
	YNZ.22	9	8 (88.9%)	1
	Total	9	8 (88.9%)	1 (12.5%)
Osteosarcoma	*Msp*I	21	5 (23.8%)	3
	*Acc*II	21	6 (28.5%)	4
	YNZ.22	21	16 (76.2%)	4
	Total	21	18 (85.7%)	6 (33.3%)

results are included in Figure 1. Digestion of the PCR fragments of 190 bp in length resulted in two fragments of 145 bp and 45 bp when the sequence corresponds to the enzyme recognition site (Fig. 1c). Without the respective sequence no digestion of the 190 bp fragment was obtained (Fig. 1d). Heterozygosity revealed two bands of 190 bp and 145 bp (Fig. 1a, b).

The results of the SSCP analysis of the *Acc*II restriction site polymorphisms are given in Figure 2. The SSCP results were completely confirmed by the respective restriction enzyme digestion (data not shown).

Of 69 patients, 14 (20.3%) individuals were informative (heterozygous) for the *MspI* restriction site and 19 (27.5%) patients for the *Acc*II polymorphism. Due to this relatively low frequency of heterozygosity we included a PCR-based analysis of the D17S5 locus on chromosome 17 in our study. Using primers flanking the VNTR marker corresponding to the probe YNZ22 we detected heterozygosity in 40/69 (58%) of the constitutional DNA of the patients. An example of the VNTR specific amplification is given in Figure 3. Informative cases are shown in a, b, d, and e. LOH could be clearly demonstrated in case b. In d and e, a weaker lower band in the tumour material than in the constitutional DNA was detected indicating that LOH has occured. Amplification of the smaller alleles in the tumour material was due to con-

tamination of normal cells. Employing these three different markers heterozygosity was found in 49 out of 69 patients (71%; Table 2). When compared with the tumour type, 23 (59%) of the gastric cancer patients were informative, revealing LOH in 6 (26.1%) tumours. Eighteen (85.7%) of 21 osteosarcomas were heterozygous either for the intragenic polymorphisms and/or the VNTR region. LOH was found in 6 (33.3%) of the tumours. Eight (88.9%) of 9 Ewing's sarcoma were informative exhibiting LOH in 1 (12.5%) sample. Table 3 summarizes the results.

Discussion

Allelic deletions of the *p53* gene have been previously shown by restriction fragment length polymorphism (RFLP) analysis with a wide variety of DNA probes [5, 6] and more recently with PCR based detection of VNTR [44, 51] and *p53* intragenic polymorphic markers [39]. Two reports describe the use of SSCP technique for the detection of allelic loss of the *p53* gene in brain tumours and hepatocellular carcinomas with identifiable *p53* mutations [29, 37]. RFLP analysis by classical Southern blotting is time-consuming and cumbersome, due to the use of radiolabelled probes. Furthermore, there are several reports demonstrating that the use of RFLP probes for chromosome 17p can fail to detect deletion of the *p53* gene locus [8, 9, 44]. To gain more insight into the allele loss pattern at 17p13 and the putative involvment of the *p53* gene we have employed intragenic *p53* gene polymorphisms which can be analysed by SSCP technique. This technical approach has been recently described as a non-isotopic method for the detection of subtle structural changes within a gene [1, 2, 12, 13, 14, 22, 45, 52]. However, the application of intragenic *p53* markers for the detection of LOH is limited by the frequency of constitutional heterozygosity. In our study, 20.3% of the individuals tested were informative for the *Msp*I polymorphism in intron 6, and 27.5% for the *Acc*II polymorphic restriction site in exon 4. Data on intragenic *p53* polymorphisms and in particular on heterozygous deletion of the *p53* gene locus itself are scarce. Analysis of the polymorphic enzyme restriction site of exon 4 for the detection of LOH in 62 patients with renal and bladder carcinomas revealed constitutional heterozygosity in 34.5% [39], whereas in another study of 115 independent individuals the frequency of heterozygosity in their constitutional DNA was determined to 45%. In the same study 71 independent patients with colorectal cancer were examined and heterozygosity was found in 43 (48%) individuals [40]. However, our findings revealed heterozygosity in 27.5% of the individuals but are not statistically significant due to the low number of cases and may represent a normal variation among population. It is unlikely that this discrepancies are due to the SSCP analysis because restriction enzyme digestion confirmed this low frequency of informative cases in the collective of our patients. To our knowledge precise data on constitutional heterozygosity of the *Msp*I polymorphism in intron 6 of the *p53* gene are not available. The frequency of informative cases for the *Msp*I restriction site polymorphism in the course of our study was low. Information for LOH using this intragenic polymorphism can be obtained as an adjunct from examinations of *p53* exon 6 mutations since the polymorphism is included in the PCR fragment amplified for this purpose.

The use of PCR amplification of the VNTR region YNZ22 for evaluating loss of heterozygosity revealed 58% of the individuals to be informative for this marker. Similar studies have reported constitutional heterozygosity of this VNTR region up to 80%. One possible technical problem in the PCR analysis of VNTR type polymorphisms is the relatively poor amplification of larger alleles in the presence of smaller allelels. Efficiency of PCR amplification can be increased by decreasing the concentration of genomic DNA in the reaction. Furthermore, highly sensitive gel electrophoresis, as used in our approach, visualizes even weak PCR products, which are undetectable in agarose gel electrophoresis. Probably it is necessary to include a control DNA from a known heterozygote with a large allele in each PCR run for amplification control.

However, with all three methods constitutional heterozygosity was detected in 71% of all individuals. In 13 out of the 49 informative cases LOH has occured. In 10 cases it was possible to evaluate LOH by emloying the intragenic polymorphisms. The remaining three samples were not informative for the intragenic polymorphisms but for the YNZ22 region.

When comparing our findings of the different tumour types with previously published data, LOH of 17p in osteosarcomas was found in 33% of the tumours representing a significant lower frequency in the course of our study. Previous reports have suggested gross rearrangments of the p53 gene and loss of heterozygosity in up to 72% of human osteosarcomas [30, 34, 35, 48]. These discrepancies are most likely due to the different technical approaches used for the detection of LOH. While Southern blot technique with polymorphic DNA markers detect chromosomal regions which may be different from the *p53* gene locus, the use of intragenic *p53* gene polymorphisms is restricted to the gene itself. Several studies have shown deletion of 17p without any mutational alteration in the *p53* gene, suggesting the presence of another tumour suppressor gene telomeric to the *p53* gene locus [7, 8, 9, 18, 42, 44].

Deletion of 17p has been reported in up to 37% of gastric cancers [31, 43]. Empolying intragenic *p53* gene polymorphisms we detected LOH in 26.1% of the gastric cancers which is slightly lower but not significantly different.

Little is known about LOH of chromosome 17p in Ewing's sarcomas. In one of nine Ewing's sarcomas, LOH of 17p was detected by YNZ22 analysis. As previously reported, *p53* alterations are not very common in Ewing' sarcomas [47, 48]. However, the number of Ewing's sarcomas studied is not representative.

In summary, our findings suggest that the analysis of intragenic *p53* polymorphisms in combination with the VNTR type YNZ22 provide a useful tool for the evaluation of loss of heterozygosity of the *p53* gene itself and could be used as an adjunct to surgical pathology in diagnosis of cancer, as well as for linkage analysis. More recently assessment of LOH by microsatellite polymorphism analysis has been shown to be at least as sensitive as detection of RFLPs by Southern blotting and may compete with the employment of SSCP analysis of intragenic *p53* polymorphisms [15]. Compared to Southern blot analysis of tumour DNAs with probes that detect RFLPs, both procedures can generate LOH data in a manner that is more accurate, quicker, and less expensive. Only small amounts of template DNA are required which can be obtained from biopsy material processed for routine morphological examination by formalin fixation and paraffin embedding. Thus, this technical approach is feasable for routine laboratory setting in diagnostic molecular pathology.

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