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# The *p53* status in juvenile chronic arthritis and rheumatoid arthritis

H. TAUBERT*, B. THAMM†, A. MEYE*, F. BARTEL*, A.-K. ROST†, D. HEIDENREICH*, V. JOHN‡, J. BRANDT§, M. BACHE¶, P. WÜRL**, H. SCHMIDT* & D. RIEMANN†† *Institute of Pathology, Martin Luther University Halle-Wittenberg, Halle/Saale, †Institute of Human Genetics, University of Leipzig, Leipzig, ‡Department of Children's Rheumatology, Martin Luther University Halle-Wittenberg, Halle/Saale, §Clinic of Orthopedic Surgery, Martin Luther University Halle-Wittenberg, Halle/Saale, ¶Department of Radiotherapy, Martin Luther University Halle-Wittenberg, Halle/Saale, **Surgical Clinic 1, University of Leipzig, Leipzig and ††Institute of Medical Immunology, Martin Luther University Halle-Wittenberg, Halle/Saale, Germany

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## SUMMARY

The aim of this study was to investigate the *p53* status in two autoimmune diseases; juvenile chronic arthritis (JCA) and rheumatoid arthritis (RA). In a PCR-sequencing analysis of exons 4–9 of the *p53* gene, no mutation was identified, except for the case of an RA synovectomy sample with two mutations of intron 7. *p53* gene polymorphisms for codons 36, 47, and 213 were not detected. Codon 72 polymorphism showed an indication of an increased occurrence of the Pro/Pro allelotype in JCA. Expression of P53 protein was comparable for JCA and RA synovectomy samples. For all RA samples P53 protein was detectable, whereas one sample of a JCA patient failed to express P53 protein.

**Keywords** juvenile chronic arthritis rheumatoid arthritis fibroblast-like synoviocytes *p53* tumour suppressor gene

## INTRODUCTION

Juvenile chronic arthritis (JCA) and rheumatoid arthritis (RA), are both chronic inflammatory systemic diseases. They belong to the autoimmune diseases and are characterized by a pathological immune response that attacks synovial tissue, cartilage and bone cells resulting in an irrespective damage to joints, vasculature and back bone. JCA is the most common autoimmune rheumatic disease in childhood and presents different clinical subtypes. It is considered to be of a polygenic nature and its genetic background is still under investigation.

Besides several models describing pathogenesis [1,2], the invasive behaviour of transformed-appearing synovial fibroblasts encouraged several authors to see paralleles to tumourigenesis [3,4]. Synovial fibroblasts from RA patients can (i) grow independently of anchorage [4]; (ii) overexpress oncogenes as, e.g. H-ras, c-myc, c-fos [5,6]; (iii) grow in nude mice [7,8]; (iv) and express matrix degenerating enzymes such as cathepsins B and L and metalloproteinases [5,9]. Mice transgenic for c-*fos* are able to develop a destructive arthritis [10]. Additionally, the findings from inadequate apoptosis of synovial fibroblasts from RA patients [11] support this hypothesis.

Recently, alterations in the *p53* status of RA patients have come into focus. The tumour suppressor *p53* is a major regulator of apoptosis inducing pro-apoptotic genes as *bax* or *Fas and* suppressing antiapoptotic genes as bcl-2 [12,13]. Interestingly, both an overexpression of P53 protein and occurrence of *p53* mutations were described for RA but not osteoarthritis patients [11,14–16]. However, Kullmann *et al.* [17] did not detect *p53* mutations in synovial fibroblasts of RA-patients from Germany, but *p53* mutations were found in clones from three additional RA synovial fibroblast cell lines from the USA. In short, the *p53* mutational and expression status in inflammatory autoimmune diseases has not been comprehensively investigated to date. This is especially true for JCA; therefore we are studying it for the first time.

Correspondence: Helge Taubert, Institute of Pathology, Faculty of Medicine, University Halle-Wittenberg, Magdeburger Strasse 14, D-06097 Halle/Saale, Germany.

E-mail: helge.taubert@medizin.uni-halle.de

## MATERIALS AND METHODS

*Patients, tissues and cell lines*

Between 1992 and 1998, synovial pannus tissues were taken from 16 patients with JCA (17 samples) and from 15 patients with RA (17 samples) these samples were collected from the Department of Paediatric Rheumatology and the Clinic of Orthopedics of the Martin Luther University Halle-Wittenberg, Germany. For RA patients, the time between diagnosis and operation was on average 6·25 years (range 1–22 years). Table 1 summarizes the clinical features of the patients.

Postsurgical specimens were placed on ice and were snap frozen. In four cases, cell lines of synovial fibroblasts were established from pannus, by dissociating the minced tissues enzymatically with HBSS containing 0·5 mg/ml, collagenase type II (Sigma, Deisenhofen, Germany), 0·15 mg/ml DNAse I (Boehringer Mannheim, Mannhein, Germany) and 5 mM $Ca^{2+}$, as described earlier [18]. The cells were

**Table 1.** Clinical features of JCA and RA patients

| Samples | Age at operation in years | Sex | RF | Diagnosis | Medication | Synovial pannus cells |
|---|---|---|---|---|---|---|
| JCA | | | | | | |
| G 12 | 13 | m | 0 | JCA (systemic) | CyA, Pred,SSZ, NSAID | fr |
| G 16 | 3 | m | 0 | JCA (systemic) | Pred, SSZ, NSAID | fr |
| G/Z 26 | 12 | m | 0 | JCA (systemic) | Pred, SSZ | fr/c |
| G 1 | 2 | f | 0 | JCA (poly) | MTX, NSAID | fr |
| G 10 | 9 | f | 0 | JCA (poly) | Pred, Gold | fr |
| G 11 | 12 | m | 0 | JCA (poly) | MTX, Pred, SSZ | fr |
| G 20 | 13 | f | 0 | JCA (poly) | Pred, NSAID | fr |
| G 22 | 7 | m | + | JCA (poly) | Pred, SSZ | fr |
| G 2 | 14 | m | 0 | JCA (pauci), iridocyclitis | Pred, SSZ | fr |
| G 7 | 20 | m | + | JCA(pauci) | Pred, SSZ, NSAID | fr |
| G 14 | 15 | f | (+) | JCA (pauci) | SSZ, NSAID | fr |
| G 23 | 11 | f | + | JCA (pauci) | Pred, SSZ, NSAID | fr |
| G 25 | 7 | m | 0 | JCA (pauci) | SSZ | fr |
| G 44 | 14 | m | + | JCA/psoriasis | Pred, SSZ | fr |
| G 48 | 10 | f | 0 | JCA/tendovaginitis | NSAID | c |
| G 24 | 6 | m | 0 | JCA/sarcoidosis | Leukeran, NSAID, Pred | fr |
| RA | | | | | | |
| G/Z 4 | 61 | f | 0 | RA + SLE | Pred, Azathioprin | fr/c |
| G/Z 5 | 70 | f | + | RA | Pred, Tauredon, NSAID | fr/c |
| G 9 | 71 | f | + | RA | MTX, Pred, NSAID | fr |
| G 13 | 40 | f | + | RA | Azathioprin | fr |
| G 15 | 41 | f | + | RA | SSZ | fr |
| G 17 | 61 | f | + | RA | Azathioprin, NSAID | fr |
| G 21 | 27 | f | 0 | RA | SSZ, Tauredon, NSAID | fr |
| G 28 | 58 | f | 0 | RA | Chloroquine | fr |
| G 31 | 34 | f | (+) | RA | MTX, Pred, NSAID | fr |
| G 32 | 62 | f | + | RA | Pred | fr |
| G 36 | 64 | f | 0 | RA | MTX, Pred | fr |
| G 37 | 63 | f | + | RA | MTX, Pred | fr |
| G 46 | 76 | f | + | RA | Predni, Azathioprin | fr |
| G 47 | 65 | f | (+) | RA | Predni, Endoxan | fr |
| G 49 | 53 | m | 0 | RA | MTX, Pred, NSAID | fr |

Poly, polyarticular onset; pauci, pauciarticular course; systemic, systemic course; SLE, systemic lupus erythematosus; RF, rheumatic factor; Pred, prednison; MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drugs; SSZ, sulphasalazine; fr, frozen tissue; c, culture of synovial fibroblast cells; +, positive; (+), weak positive; 0, negative.

cultured in RPMI 1640 containing 10% foetal calf serum, antibiotics and glutamin. Cells were used at confluence at the third to fifth passage.

*DNA preparation and PCR*

Genomic DNA was isolated from 10 to 15 cryo-sections of 50 μm of synovial pannus tissue as previously described [19,20]. Exons 4–9 of the *p53* gene were amplified in five separate PCR reactions. We used 20-mer oligonucleotide primers described by Mashiyama *et al*. [21], PCR conditions were as following: predenaturation at 92°C for 5 min and 35 cycles of denaturation at 92°C for 1 min, annealing for 30 s at 56°C (exons 8–9), 58°C (exon 5), 62°C (exons 4,6,7) and DNA synthesis at 72°C for 1 min, postsynthesis at 72°C for 5 min and storage of the PCR products at 4°C [19].

*Sequencing and allelotyping*

Using the same protocol and primers as for the PCRs in a cycle sequencing method (TSII sequenase kit/Pharmacia, Freiburg, Germany) amplification products were sequenced in both directions and subsequently analysed on an ABI 373 (Perkin Elmer, Weiterstadt, Germany).

*p53*-allelotyping for codon 72 was performed after PCR by an RFLP analysis as described by Klaes *et al*. [22]. Briefly, PCR products were digested with two restriction endonucleases having a restriction site either in the proline coding allele (*Bsa*JI, New England Biolabs, Schwalbach, Germany) or in the arginine coding allele (*Bst*UI, New England Biolabs). Digestion with *Bsa*JI resulted for Arg/Arg allelotypes in four bands (138 bp, 75 bp, 49 bp and 34 bp), whereas in Pro/Pro allelotypes the 138 bp band was further separated in bands of 94 bp and 44 bp. Digestion with *Bst*UI delivered for Pro/Pro allelotypes a 269-bp band that was further digested in Arg/Arg allelotypes to 167 bp and 127 bp bands.

*Western blot analysis*

Thirty μg of total protein was separated on a 12·5% polyacrylamide/SDS gel (Minigel system; Biometra, Göttingen, Germany).

Afterwards, the proteins were transferred to a PVDF Immobilon membrane (Millipore; Eschborn, Germany) at 200 mA for 90 min (Miniblotter; Biometra). The membrane was blocked with 0·1% Tween 20 containing 3% BSA and incubated with anti-p53 or antiactin antibodies (DO-7; 1 : 500; Dianova, Hamburg, Germany or AL-40; 1 : 500; Sigma) for 1 h and with horseradish peroxidase-conjugated anti-mouse IgG antibody (1 : 1000; Dako, Gostrup, Denmark) at room temperature for 1 h. For protein detection the membrane was placed in ECL-substrate for 1 min (Amersham, Braunschweig, Germany) and exposed to Biomax film (Kodak, Braunschweig, Germany). As positive control for P53 protein expression, RD cells/ATCC CCL 136, carrying a p53 mutation and overexpressing P53 protein were used. Furthermore, for comparison of P53 expression, a human myeloid leukaemia cell line (HL-60; Serva, Heidelberg, Germany) and HUVEC cells were tested for P53 expression. First, for evaluation purposes, the band intensities were equalized to the positive control running on each blot and, second, the amount of P53 protein was related to the actin band which was determined in the same sample and expressed as the ratio p53 to actin signal intensity. The ratios were considered as week ($\leq$ 0·3), moderate ($>$ 0·3–0·9) and strong ($>$ 0·9).

## RESULTS

*Sequencing of p53 gene (exons 4–9)*

The sequences of exons 4–9 of the *p53* gene were determined from the 34 samples taken from 31 patients including 16 JCA-patients and 15 RA-patients. With the exception of one sample from a RA patient with two point mutations in intron 7, no *p53* mutation could be detected. The two point mutations (nucleotides 14201 and 14121 according to the GenBank-Accension No. X54156, Table 2) do not concern splicing sites and may not influence synthesis of a correct P53 protein.

*Analysis of polymorphisms in the p53 gene*

Four polymorphisms for codon 36, codon 47, codon 72 (all three in exon 4) and codon 213 (in exon 6) of the *p53* gene were investigated. Codons 36, 47 and 213 revealed no polymorphism in our study group. This result is not unexpected at reported frequencies of 98%, 95·3% and 96·8%, respectively [23]. The genotypes of codon 72 were determined by sequencing and additional RFLP analysis was subsequently performed (Fig. 1). The distribution of the three allelic types Pro/Pro, Pro/Arg and Arg/Arg was 12·9%, 38·7% and 48·4% and is comparable to the normal Caucasian controls from published research (10·2%, 44·2% and 45·6%, respectively) (Table 3). Pro alleles were identified in 32% of our JCA/RA patients and in the control group (Table 3), in accordance with results previously reported [24]. Considering the small number of JCA patients, a predominance of Pro/Pro (18·8%) is noticeable (Table 3), but requires further investigation.

*Western blot analysis*

P53 protein expression detected by Western hybridization did not differ on average between JCA (1·00) and RA synovectomy tissue (0·98). In both groups of diseases, cases with weak (0–0·3), moderate ($>$ 0·3–0·9) or strong ($>$ 0·9) P53 expression were found (Fig. 2, Table 2). On closer examination, samples of nine JCA and RA patients each showed a strong expression ($>$ 0·9) of P53 protein. Two samples of JCA patients and four samples of RA-patients possessed a moderate P53 expression ($>$ 0·3–0·9). For three samples of JCA patients and for one sample of an RA patient, a weak P53 expression (0–0·3) was detected. One sample from a patient with JCA was completely negative, whereas in RA synovectomy tissue, P53 protein was always detectable. In comparison to JCA and RA synovectomy tissue, a human myeloid leukaemia cell line (HL-60) and HUVEC cells showed values between 0 and 0·3 (data not shown).

## DISCUSSION

In this study, we investigated the gene status and protein expression of tumour suppressor *p53* in JCA and RA patients. We did not detect *p53* mutations in synovial pannus in these patients, with the exception of two point mutations in intron 7, identified in a sample of an RA patient. Although, we can not exclude the fact that we may have missed mutations occurring in just a few cells, our result is in accordance with a recent study describing the failure to detect *p53* mutations in RA synovial fibroblasts using three different techniques [17]. A reason for the detection of *p53* mutations in synovial cells found in US RA patients but not found in German patients might be that the latter represent longstanding destructive diseases with chronic inflammation, oxydative stress and permanent genetic changes (including *p53* mutations), resulting from synovectomies which were performed rather late in the USA [25]. To date, three groups could identify *p53*-mutations in RA tissue [14,15,26] with two mutations possessing a dominant negative effect suppressing wild-type *p53* [27]. However, taken together, these few findings strengthen the opinion of Reme and colleagues [15], who stated that *p53* abnormalities may not play a fundamental role in the aetiology of RA, which might also be true for JCA. Furthermore, in patients with another autoimmune disease, Sjogren's syndrome, there was no *p53* mutation either, but in combination with non-Hodgkin's lymphoma, it was detectable [28].

Besides *p53* mutations, we analysed the status of five known *p53* polymorphisms (four in exon 4 and one in exon 6). According to the expected frequencies, only for the codon 72 (exon 4) polymorphism could different haplotypes be identified. Taken together, the samples did not differ from the distribution in a normal Caucasian population. However, when considering only the JCA cases, a conspicuous occurrence of the Pro/Pro haplotype appeared. Based on the common features between autoimmune diseases and cancer, findings of codon 72 polymorphisms in cancer seem interesting. An early onset of lung cancer may correlate with occurrence of the Pro/Pro allele, suggesting that the Pro/Pro genotype predisposes individuals to an early outbreak of this disease [29]. For different cancers, the influence of the codon 72 polymorphism on prognosis may vary. On one hand, it can be associated with a higher risk for lung, breast, ovarian and colorectal cancer [24 29–32]; but this finding was not shared by other authors [23,33]. On the other hand, different allelotypes were found to have no effect on cervical cancer [22,34]. However, further investigations are necessary to find out if different allelotypes of the codon 72 polymorphism may increase susceptibility for autoimmune diseases.

Investigating P53 expression by Western hybridization, we agree with the findings of Firestein *et al*. [11] and Tak *et al*. [16], who all investigated RA synoviocytic cell lines and RA synovectomy samples and demonstrated P53 protein expression. In contrast to the suggestion of Tak and colleagues [16], in our

**Table 2.** Sequencing of p53 gene and Western blot analysis of P53 protein

| Samples | Diagnosis | Sequencing of *p53* gene | | | Western blot P53/actin |
|---|---|---|---|---|---|
| | | Exon 4–9 | bp cod 72 | aa cod 72 | |
| JCA | | | | | |
| G 12 | JCA (systemic) | wt *p53* | CGC/CGC | Arg/Arg | 1·37 |
| G 16 | JCA (systemic) | wt *p53* | CGC/CGC | Arg/Arg | 0·3 |
| G 26 | JCA (systemic) | wt *p53* | CGC/CGC | Arg/Arg | 0 |
| Z 26 | JCA (systemic) | wt *p53* | CGC/CGC | Arg/Arg | 0 |
| G 1 | JCA (poly) | wt *p53* | CGC/CCC | Arg/Pro | 1·51 |
| G 10 | JCA (poly) | wt *p53* | CGC/CCC | Arg/Pro | 1·45 |
| G 11 | JCA (poly) | wt *p53* | CGC/CGC | Arg/Arg | 0·93 |
| G 20 | JCA (poly) | wt *p53* | CGC/CCC | Arg/Pro | 1·01 |
| G 2 | JCA (pauci), iridocyclitis | wt *p53* | CCC/CCC | Pro/Pro | 1·43 |
| G 7 | JCA(pauci) | wt *p53* | CCC/CCC | Pro/Pro | 1·71 |
| G 14 | JCA (pauci) | wt *p53* | CGC/CGC | Arg/Arg | 0·77 |
| G 22 | JCA (poly) | wt *p53* | CGC/CCC | Arg/Pro | 0·51 |
| G 23 | JCA (pauci) | wt *p53* | CGC/CCC | Arg/Pro | 1·88 |
| G 25 | JCA (pauci) | wt *p53* | CGC/CGC | Arg/Arg | ND |
| G 44 | JCA/psoriasis | wt *p53* | CCC/CCC | Pro/Pro | 0·08 |
| G 48 | JCA/tendovaginitis | wt *p53* | CGC/CGC | Arg/Arg | ND |
| G 24 | JCA/sarcoidosis | wt *p53* | CGC/CGC | Arg/Arg | 1·16 |
| RA | | | | | |
| G 4 | RA + SLE | wt *p53* | CCC/CCC | Pro/Pro | 1·28 |
| Z 4 | RA + SLE | wt *p53* | CCC/CCC | Pro/Pro | ND |
| G 5 | RA | wt *p53* | CGC/CGC | Arg/Arg | 1·32 |
| Z 5 | RA+ | wt *p53* | CGC/CGC | Arg/Arg | ND |
| G 9 | RA | wt *p53* | CGC/CGC | Arg/Arg | 1·06 |
| G 13 | RA + SLE | wt *p53* | CGC/CGC | Arg/Arg | ND |
| G 15 | RA | wt *p53* | CGC/CGC | Arg/Arg | 1·07 |
| G 17 | RA | wt *p53* | CGC/CCC | Arg/Pro | 0·54 |
| G 21 | RA | wt *p53* | CGC/CCC | Arg/Pro | 1·65 |
| G 28 | RA | wt *p53* | CGC/CCC | Arg/Pro | 1·04 |
| G 31 | RA | wt *p53* | CGC/CCC | Arg/Pro | 1·00 |
| G 32 | RA | wt *p53* | CGC/CCC | Arg/Pro | 1·48 |
| G 36 | RA | wt *p53* | CGC/CGC | Arg/Arg | 1·51 |
| G 37 | RA | wt *p53* | CGC/CGC | Arg/Arg | 0·24 |
| G 46 | RA | wt *p53* | CGC/CCC | Arg/Pro | 0·50 |
| G 47 | RA | Intron 7* | CGC/CCC | Arg/Pro | 0·43 |
| G 49 | RA | wt *p53* | CGC/CGC | Arg/Arg | 0·65 |

*nt 14121 C to T, nt 14201 T to G.

Poly, polyarticular onset; pauci, pauciarticular course; systemic, systemic course; SLE, systemic lupus erythematosus; +, positive; (+), weak positive; 0, negative; bp, basepairs; aa, amino acids; Z-cell line samples; G-frozen samples.

**Table 3.** Frequency of codon 72 alleles of the *p53* gene in JCA, RA and control Caucasians

| | | Pro/Pro | | Pro/Arg | | Arg/Arg | | |
|---|---|---|---|---|---|---|---|---|
| | *n* | *n* | % | *n* | % | *n* | % | Pro alleles |
| Normal control Caucasians worldwide* | 885 | 90 | 10·2 | 391 | 44·2 | 404 | 45·6 | 0·32 |
| JCA and RA patients† | 31 | 4 | 12·9 | 12 | 38·7 | 15 | 48·4 | 0·32 |
| RA patients† | 15 | 1 | 6·7 | 7 | 46·7 | 7 | 46·7 | 0·30 |
| JCA patients† | 16 | 3 | 18·8 | 5 | 31·2 | 8 | 50·0 | 0·34 |

*Calculated from references [22,24,32,36–40].

†This study.

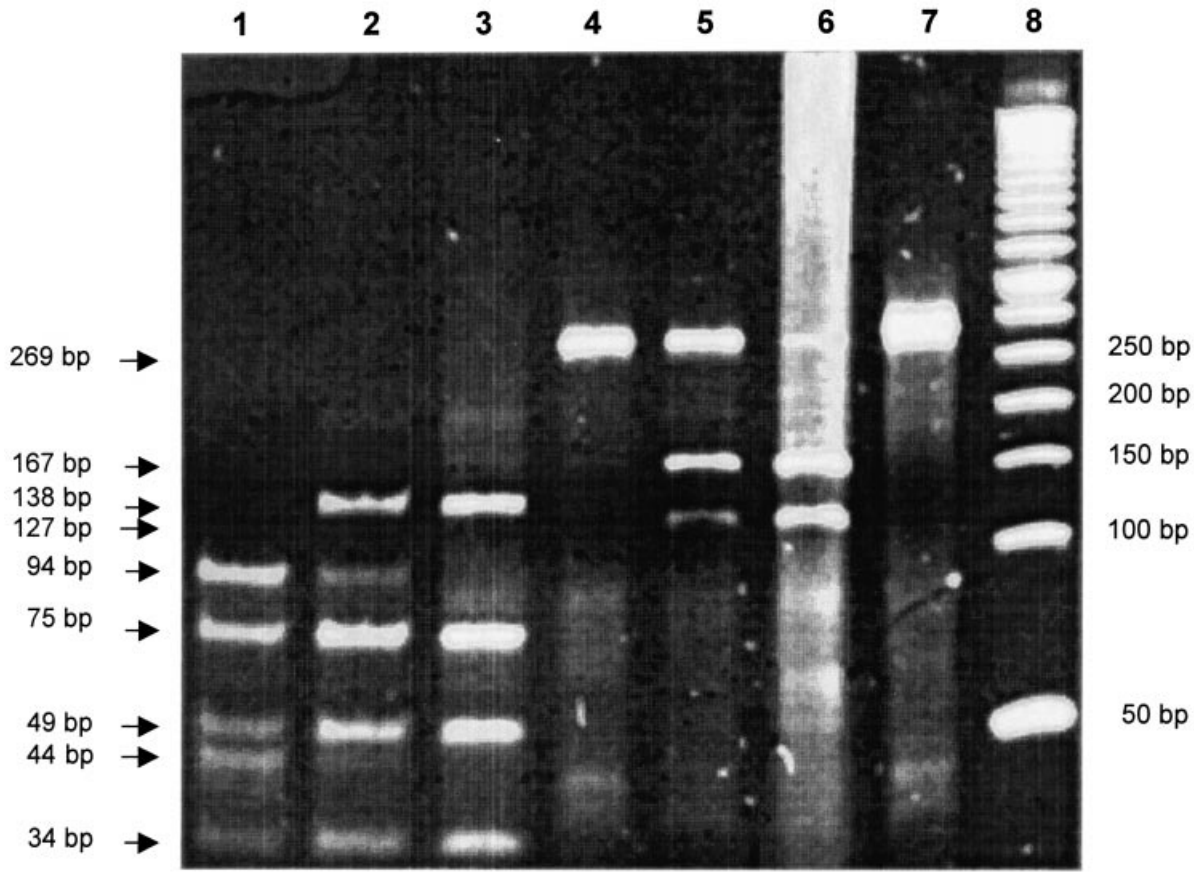


**Fig. 1.** Determination of codon 72 polymorphisms by restriction endonuclease digests. PCR products of exon 4 were digested either with *Bsa*JI (lane 1–3) or with *Bst*UI (lane 4–6); undigested DNA (lane 7) and a 50-bp marker (lane 8). The samples were homozygously Pro/Pro (lane 1 and 4), heterozygously Arg/Pro (lane 2 and 5) and homozygously Arg/Arg (lane 3 and 6). In the heterozygous condition, note the somewhat weaker expression of bands (i.e. lane 2, bands at 94 bp and 44 bp; lane 5, band at 127 bp.

samples of RA and JCA patients, P53 overexpression is not secondary to a *p53* mutation. We observed that the expression level of P53 protein can widely differ between individual JCA and RA patients. Comparable differences in the expression level of P53 for RA samples were also found by Nickels *et al*. [35], who considered Western blot analysis to be the gold standard for demonstration of P53 expression in their study of inflammatory tissues. However, an increased P53 expression detected either immunohistochemically or by Western hybridization has been found by several authors, related to an increased presence of DNA strand breaks, a disturbance of apoptosis or a putative higher potential of aggressiveness of synovial fibroblasts [11,15,16,35]. In conclusion, we would like to emphasize that P53 protein expression is a characteristic feature of RA and JCA. Furthermore, for JCA patients, we found a conspicuous occurrence of the Pro/Pro haplotype for codon 72 of the *p53* gene. This finding is still preliminary but could be remarkable, since this haplotype is suspected of possessing an increased cancer susceptibility.

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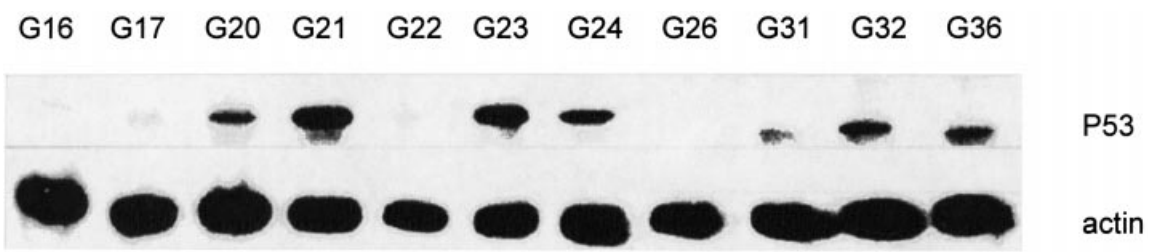


**Fig. 2.** P53 protein expression in JCA and RA patient samples in a Western blot hybridization.

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