Use of specific ELISA for the detection of antibodies directed against p53 protein in patients with hepatocellular carcinoma

S D Ryder, P M Rizzi, M Volkmann, E Metivier, L M M B Pereira, P R Galle, N V Naoumov, H Zentgraf, R Williams

Abstract

***Aims*—To analyse the significance of antibodies to p53 protein as a serological marker for changes in p53 gene expression in patients with hepatocellular carcinoma.**

***Methods*—Thirty eight patients with hepatocellular carcinoma, 19 showing accumulation of p53 protein by immunohistochemistry and 19 having no accumulation, were studied. The presence of anti-p53 was tested using a novel ELISA utilising a recombinant p53 protein as a capture system and verified by western blotting. p53 gene mutations were sought by single strand conformational polymorphism and DNA sequencing analyses.**

***Results*—Of 19 patients with p53 protein accumulation in tumour tissue, 10 (52%) had antibodies to p53 in serum by ELISA. Four patients with p53 negative immunohistochemistry also had detectable anti-p53. Western blot analysis confirmed the specificity of the ELISA positive serum samples. The presence of anti-p53 was independent of serum α-fetoprotein and was detected in 50% of small tumours while only 8% were α-fetoprotein positive. Mutations affecting exons 5 and 6 seem to be more frequently associated with development of anti-p53, than mutations in exons 7 or 8.**

***Conclusions*—The ELISA for anti-p53 is a convenient and specific test for the detection of humoral response to alterations in p53 gene expression and could be of value in the diagnosis and characterisation of patients with hepatocellular carcinoma.**

(*J Clin Pathol* 1996;49:295-299)

Keywords: hepatocellular carcinoma, p53, anti-p53, ELISA.

Institute of Liver Studies, King's College Hospital, London SE5 9PJ
S D Ryder
P M Rizzi
E Metivier
L M M B Pereira
N V Naoumov
R Williams

Applied Tumour Virology, German Cancer Research Centre, 69009 Heidelberg, Germany
H Zentgraf

Department of Internal Medicine, University of Heidelberg, 69115 Heidelberg, Germany
M Volkmann
P R Galle

Correspondence to:
Dr N Naoumov,
Institute of Liver Studies, King's College School of Medicine and Dentistry, Bessemer Road, London SE5 9PJ.

Accepted for publication 12 December 1995

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases in the world with an annual estimated incidence of 1 million cases. The poor survival after diagnosis has led to the introduction of screening programmes using α-fetoprotein (AFP) estimation and ultrasound scanning with some evidence that tumours may be detected at an earlier, presymptomatic stage.[1] Unfortunately, AFP concentrations are frequently normal in patients with small HCC,[2] the accuracy of real-time ultrasound scanning and computed tomography (CT) in the detection of small HCCs is relatively poor[3] and there is clearly a need for other markers of malignant change that can be used to screen cirrhotic patients.

Mutations in the p53 gene are the most common genetic changes in human cancers[4] and experimental evidence suggests that mutations in the p53 gene are involved in hepatocarcinogenesis, with a substantial proportion of patients from high risk areas having mutations within the p53 gene.[5] In European populations the rate of p53 mutations found has varied in different studies but p53 mutations are still found in 25–40% of cases.[6] Mutations detected in tumours usually result in a p53 protein with an increased half-life and altered immunological properties, such as changes in the binding of specific monoclonal antibodies.[7] The accumulation of mutant p53 protein and its conformational change render it potentially immunogenic and antibodies to p53 protein have been detected in HCC[8] and in other human cancers.[9] Using an immunoblot assay, circulating antibodies to p53 protein were found in 25% of serum samples from patients with HCC.[8] Western blot assay is not suitable for large scale diagnostic testing, whereas ELISA techniques can be applied more widely in different laboratories.

The aim of this study was to analyse the relation between the presence of antibodies to p53, as detected by a novel ELISA method with hepatic accumulation of p53 protein, and the presence of mutations in the p53 gene in tumour tissue from patients with HCC.

Methods

Thirty eight patients presenting with HCC between 1985 and 1993 were chosen for the study. These patients were purposely selected on the basis of the immunohistochemical detection of p53 protein accumulation in the tumour cells in 19 (50%), while the remaining patients had no detectable p53 protein in the HCC tissue. In order to ensure that true overexpression of p53 protein was present, all samples selected as positive by immunohistochemistry had p53 protein detectable in 80% or more of the tumour cell nuclei. Thirty four patients had underlying cirrhosis and none of the patients had the fibrolamellar variant of HCC. Aetiology of underlying liver disease was hepatitis B in 19, hepatitis C in 10, cryptogenic in five (both hepatitis B and C virus serology negative), alcoholic cirrhosis in four, and primary biliary cirrhosis in one. Tumour size and

number were determined at the time of presentation by ultrasound, CT scanning and angiography. Serum AFP concentrations were measured using a standard ELISA technique (Abbott Diagnostics, Maidenhead, UK) in all serum samples used for detection of anti-p53 antibodies. No patient in this series was known to have had previous malignant disease or had clinical evidence of a second primary tumour elsewhere. Formalin fixed, paraffin wax embedded sections from all 38 hepatocellular carcinomas were studied. Tumour tissue was obtained from total hepatectomy at the time of transplantation in 13 cases, resection specimens in 10 cases and by needle biopsy in 15 cases. Serum samples were taken at the time of diagnosis and stored at −20°C.

Serum samples from six healthy laboratory staff and stored serum samples from five patients with chronic hepatitis B virus infection were also studied. Two of these patients had cirrhosis but none had clinical or serological evidence of HCC development during a minimum two year follow up.

IMMUNOHISTOCHEMICAL DETECTION OF p53 PROTEIN

Tissue sections were dewaxed in xylene, followed by graded alcohols. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol. Slides were pretreated by being heated in a microwave oven for 15 minutes in citrate buffer (citric acid 2·1 g, sodium hydroxide pellets 10, pH 6·0). Monoclonal antibody directed against p53 protein (DO1, Cambridge BioScience, Cambridge, UK) was used as a primary antibody at a dilution of 1 in 100 in 0·05 M Tris buffered saline (pH 7·4) and incubated for one hour at 37°C. The second antibody (biotinylated rabbit anti-mouse, dilution 1 in 400) (Dako Ltd, High Wycombe, UK) was applied for one hour at 37°C, followed by Streptavidin biotinylated peroxidase complexes (Dako) and colour developed with diaminobenzidine. Positivity for the purposes of this study was defined as nuclear staining in 80% or more of nuclei and negative samples had no staining of the tumour tissue at all.

DETECTION OF p53 GENE MUTATIONS

Tumour samples from all 38 cases with HCC were studied for the presence of mutations affecting exons 5, 6, 7, and 8 using the polymerase chain reaction (PCR) and single strand conformational polymorphism analysis.[10] Primers were selected as described previously[11] and ^{32}P labelled PCR product was electrophoresed into standard polyacrylamide sequencing gels. Samples with different mobility from control, wild-type p53 were then sequenced using the dideoxynucleotide chain termination method.

ELISA FOR DETECTION OF ANTIBODIES TO p53

To obtain p53 protein we amplified the p53 gene from Hep G2 cell line using PCR. The amplified product was cloned in the vector pQE-8 (Quiagen, Hilden, Germany) and expressed in *Escherichia coli*. This resulted in production of a 53 kilodalton protein which was immunoreactive with a monoclonal antibody directed against p53 protein-1801 (Dianova, Hamburg, Germany) and the polyclonal antiserum to p53 HZp53R.[8] This protein was then used to coat the wells in 96 well plates using 200 µl per well. Serum samples (2 µl) were diluted 1 in 50 to a final volume of 100 µl, added to precoated wells and incubated for one hour at 37°C. The plates were washed extensively in buffer. Detection-antibody solution (anti-human IgG Fc specific antibody labelled with horseradish peroxidase) was added in a total volume of 100 µl and incubated for 30 minutes at 37°C, washed and peroxidase/detector solution added (100 µl). Following a 30 minute incubation in the dark at room temperature, 50 µl 2 N HCl was added per well and the optical density (OD) measured in an ELISA plate reader at 450 nm with a 620 nm filter. The dilution and washing buffers, as well as the detection system for the ELISA were supplied by Dianova (Hamburg, Germany). As positive controls we used serum samples containing known levels of anti-p53 antibody,[8] which were included in each plate. Negative controls comprised: omission of serum samples (phosphate buffered saline control), and serum samples from six healthy controls and from five patients with chronic hepatitis B. All samples were run in duplicate and results used are the mean values of the two readings. To confirm the results for anti-p53 detection by ELISA further, we tested all serum samples by western blotting using p53 protein absorbed onto nitrocellulose filters, as described previously.[8]

Results

DETECTION OF ANTI-p53 ANTIBODIES IN SERUM SAMPLES IN RELATION TO p53 PROTEIN EXPRESSION

A positive test for antibodies directed against p53 using the ELISA assay was defined as an OD greater than 2 SD above the mean value of all negative controls. The cut off for positivity was defined as 0·39 which was equal to the OD obtained for the lowest positive control sample used. Duplicate samples showed good concordance with no paired samples having a different classification (high, medium, low levels) relative to the positive controls. The coefficient of variation in a single serum sample with eight duplicate measurements was 4·5%. Fourteen of the 38 serum samples from patients with HCC showed positive results. The comparison between the serological data for antibodies directed against p53 and the expression of p53 protein in tumour tissue showed that 10 (52%) of 19 patients with positive immunohistochemistry for p53 protein had positive results in the ELISA and four of 19 with negative histochemistry were also positive (fig 1).

All samples positive in the ELISA were confirmed as positive when tested by western blotting for the presence of p53 antibodies. In addition, two serum samples (with positive

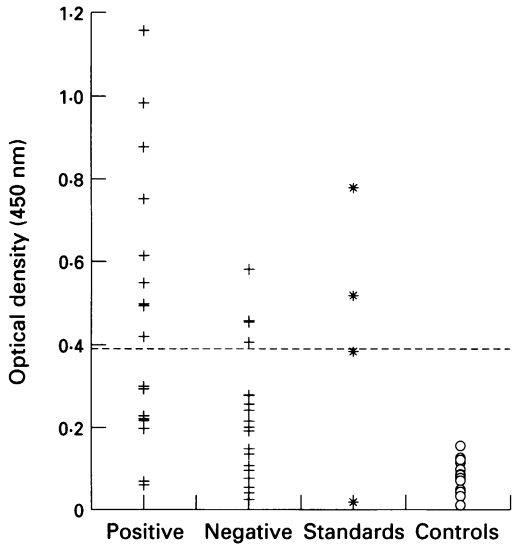

Figure 1 Results of anti-p53 ELISA in serum samples from patients with HCC and controls. The dotted line represents the cut off value for positivity in the ELISA.

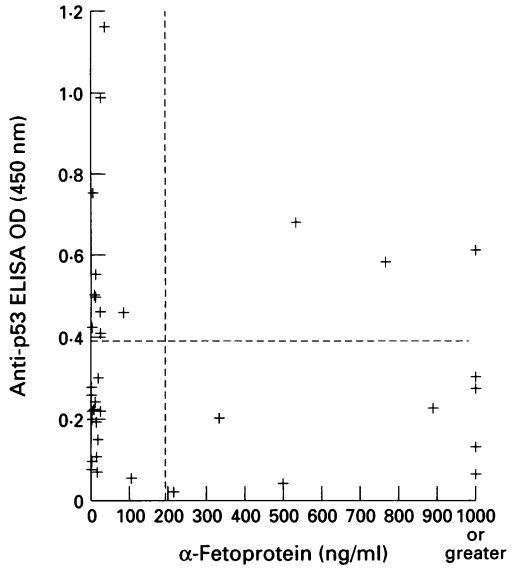

Figure 2 Correlation between AFP concentrations and anti-p53 detection in patients with HCC.

immunohistochemistry) showed weak but definite positive reaction on western blot. These samples had OD results in the ELISA of 0·33 and 0·29, well below the cut off level of 0·39.

CORRELATION OF ANTI-p53 WITH AFP CONCENTRATIONS AND TUMOUR SIZE

No significant relation was observed between the presence of anti-p53 antibodies and AFP positivity (fig 2). It seems that the two tests detect different patients with HCC as eight cases showed a positive reaction for AFP alone, 12 positive for anti-p53 alone, three cases positive for both markers, and 15 negative for both. p53 antibodies were detectable in 50% of small (<4 cm in diameter) tumours (table 1) in contrast to AFP, where only 8% of small HCC samples were positive.

RELATION BETWEEN ANTI-p53 AND THE SITE OF p53 MUTATIONS

A PCR product was not detected in one of the 19 histochemically positive tumours. In the remaining 18, mutations were detected in exons 5–8 in 15 cases (table 2). Full DNA sequencing of the amplicons was possible in nine patients (table 3). Of the 19 histochemically negative tumours, p53 mutations were found in two (11%), both of which had detectable antibody levels (table 3). The other two patients with negative histochemistry but with detectable anti-p53 had wild-type gene sequence in exons 5–8. There was a tendency for more frequent development of antibodies directed against p53 in HCC with mutations affecting exons 5 and 6 (seven of nine patients) in comparison with HCC with mutations in exons 7 or 8 (three of six patients).

Discussion

In patients with breast cancer a humoral immune response to p53 has been shown to be associated with accumulation of mutant p53 protein,[12] suggesting that anti-p53 antibodies can be used as a serological marker for genomic variations in this tumour suppressor gene. The only previous study of anti-p53 antibodies in hepatocellular carcinoma utilised western blotting[8] and of the 80 cases tested serologically, only eight were shown immunohistochemically to have p53 accumulation in the tumour. Western blotting is not easily applicable for use as a clinical diagnostic test. In the present study we used a new ELISA technique based on recombinant p53 protein, which was easier to carry out and gave highly reproducible results. The sensitivity was only slightly lower than western blotting, as the latter identified only two additional cases of anti-p53 positivity.

In this study we have shown that if the HCC has accumulated p53 protein (and is therefore likely to have a p53 mutation), 52% of cancers have a significant humoral response with detectable antibody levels. Thus, the presence of anti-p53 in the serum is usually indicative of abnormal p53 protein expression in a high proportion of tumour cells. The aim of this study was not to evaluate the prevalence of anti-p53 in HCC in general and accordingly, the study was designed to test a selected group of patients with HCC. This explains the large proportion of cases seropositive for anti-p53 in comparison with previously reported series on tumours from other sites.[13,14] In breast and lung cancer between 10 and 15% of patients have detectable antibodies to p53 protein.[15,16] In Northern Europe, about 25% of patients with HCC will have over-expression of p53 protein[6]; hence, if half of these mount a humoral response then a similar 10–15% of the total patient population would be expected to have p53 antibodies. It is therefore unlikely that this test in isolation would be suitable as a primary diagnostic or screening test for HCC. On the other hand, the humoral response to mutant p53 is clearly independent of AFP status. In contrast to AFP, which is more likely to be positive in large tumours, antibodies to p53

Table 1 Relation between tumour size and serological detection of AFP and anti-p53 antibodies

Tumour size	Total number	AFP positive	Anti-p53 positive
<4 cm	12	1 (8%)	6 (50%)
4–8 cm	10	3 (33%)	4 (40%)
Over 8 cm or multiple	14	7 (50%)	5 (30%)

Table 2 Exon containing p53 mutation (by single strand conformational polymorphism analysis) in 13 histochemically positive HCCs and base substitution where sequencing available

Case number	ELISA (+/−)	Western blot (+/−)	Exon of mutation	Base substitution
1	+	+	7	T→A (257)
2	+	+	No mutation	N/A
3	−	+	No data*	N/A
4	+	+	5	A→G (163)
5	−	−	7	G→T (249)
6	−	+	5	T→A (176)
7	−	−	6	N/A
8	+	+	8	C→T (273)
9	−	−	5	N/A
10	+	+	6	N/A
11	+	+	7	G→T (248)
12	+	+	5	N/A
13	+	+	6	A→C (205)
14	−	−	No mutation	N/A
15	−	−	8	C→G (278)
16	+	+	5	T→A (163)
17	−	−	8	N/A
18	−	−	No mutation	N/A
19	+	+	6	N/A

N/A = no result available; * no PCR product could be obtained.

Table 3 Exon containing p53 mutation (by single strand conformational polymorphism analysis) in four histochemically negative HCC with positive results for detection of anti-p53 and base substitution where sequencing available

Case number	ELISA (+/−)	Western blot (+/−)	Exon of mutation	Base substitution
24	+	+	7	G→T (249)
29	+	+	5	N/A
32	+	+	None found	N/A
35	+	+	None found	N/A

N/A = not available.

protein are detectable in a substantial proportion of small HCCs and there is no relation with tumour size, but instead is highly dependent on the presence of mutations in the p53 tumour suppressor gene. If AFP and anti-p53 results are combined this would increase sensitivity of detection; in our series of 38 cases of HCC, 23 (60%) of 38 were positive for AFP, anti-p53 or both tests. This suggests that anti-p53 antibodies may be a valuable addition to current screening tests with the potential to detect tumours at an early and therefore more treatable stage. The ELISA detection of anti-p53 could be useful as a screening to identify a subgroup of patients with HCC with abnormal expression of the p53 gene, thus selecting potential candidates who may benefit from a delivery of the wild-type p53 sequences using gene therapy. Alternatively, antibodies to p53 could be useful as carriers for targeting tumour cells with p53 protein accumulation.

The presence of anti-p53 did not correlate with vascular metastasis or tumour aetiology. As all the histochemically positive tumours used in this study had very high concentrations of p53 protein, it is unlikely that the amount of p53 protein is the only factor determining the immune response. It is also possible that certain p53 mutations may produce a more immunogenic protein. Evidence from patients with breast cancer suggests that the immune response to p53 is strongly related to the presence of missense mutations rather than stop, splice/stop, splice, or frameshift mutations.[15] It has been postulated that the development of an immune response to mutant p53 protein may depend on mutant p53 complexes with a 70 kilodalton heat shock protein (HSP 70)[12] on the basis that anti-p53 was associated with specific mutations in exons 5 and 6 which produce proteins known to associate with HSP 70[17] and to have a greater transforming potential. Our data shows that mutations in exon 5 or 6 are more likely to produce an immunogenic protein than those in exons 7 and 8. The finding of high levels of anti-p53 activity in mutations affecting codons 257 (exon 7) and 273 (exon 8) is strong evidence against the hypothesis of HSP 70 being the only required complexing protein as both of these mutants are unlikely to bind HSP 70.[12,18] It is possible that the immunogenicity of p53 protein may be enhanced by complexing with other oncogene proteins, as has been demonstrated in mice with the Simian virus 40 large T antigen[19] and such alternative protein/p53 immune presentation mechanisms may explain these results.

Four samples with no detectable p53 protein accumulation in the tumour tissue were p53 antibody positive. In two of these a p53 mutation was detected by molecular analysis and it is clear that these represent immunohistochemically false negative results. The antibody used, DO1, binds to an epitope of p53 protein which is known to be present in an available form in wild-type and most mutant p53 protein but it is possible that the mutations present in these tumours may produce a protein lacking this determinant. An alternative, and perhaps more likely, possibility is sampling error in the tumour as accumulation of p53 protein is often variable in different areas of the tumour. Three out of four histochemically negative samples were needle biopsy specimens and it is well described that p53 accumulation is not universally seen throughout the tumour. In the other two cases no mutation was found in exons 5 to 8.

In conclusion, the ELISA for anti-p53 is a convenient and specific test for the detection of humoral response to alterations in the p53 gene expression and could be of value in the diagnosis and characterisation of patients with HCC.

This work was partially supported by a travel grant from the British-German Academic Research Collaboration Programme (ARC Project 371) of the British Council.

1 Oka H, Kurioka N, Kim K, Kanno T, Mizoguchi Y, Kobayashi K. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. *Hepatology* 1990;**12**:680–7.
2 Wesic HT, Kirkpatric A. Alpha-fetoprotein and its relevance to human disease. *Gastroenterology* 1984;**86**:1404–9.
3 Colombo M, de Franchis R, Del-Ninno E, Sangiovanni A, De Faxio C, Tommasini M, *et al.* Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991;**325**:675–80.
4 Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991;**351**:453–6.
5 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NY, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinoma. *Nature* 1991;**350**:427–8.
6 Volkmann M, Hofmann WJ, Muller M, Rath U, Otto G, Zentgraf H, *et al.* p53 over-expression is frequent in

European hepatocellular carcinoma and largely independent of the codon 249 hot spot mutation. *Oncogene* 1994;**9**:195–204.
7 Stephen CW, Lane DP. Mutant conformation of p53: precise epitope mapping using a filamentous phage epitope library. *J Mol Biol* 1992;**225**:577–83.
8 Volkmann M, Muller M, Hofmann WJ, Meyer M, Hagelstein J, Rath U, *et al.* The humoral immune response to p53 in patients with hepatocellular carcinoma is specific for malignancy and independent of alpha-fetoprotein status. *Hepatology* 1993;**18**:559–65.
9 Crawford LV, Pim DC, Bulbrook RD. Detection of antibodies against cellular protein p53 in sera from patients with breast cancer. *Int J Cancer* 1992;**30**:403–8.
10 Oda T, Tsuda H, Scarpa A, Sakamoto M, Hirohashi S. Mutation pattern of the p53 gene as a diagnostic marker for multiple hepatocellular carcinoma. *Cancer Res* 1992; **231**:3674–8.
11 Buchman VL, Chumakov PM, Ninkina N, Samarina OP, Georgiev GP. A variation in the structure of the protein-coding region of the human p53 gene. *Gene* 1988;**70**: 245–52.
12 Davidoff AM, Iglehart JD, Marks JR. Immune response to p53 is dependent on p53/HSP70 complexes in breast cancers. *Proc Natl Acad Sci USA* 1992;**89**:3439–42.
13 Crawford LV, Pim DC, Lamb P. The cellular protein p53 in human tumours. *Mol Biol Med* 1984;**2**:261–72.
14 Labreque S, Naor N, Thomson D, Matlashewski G. Analysis of the anti-p53 antibody response in cancer patients. *Cancer Res* 1993;**53**:3468–71.
15 Winter SF, Minna JD, Johnson BE, Takahashi T, Gazdar AF, Carbone DP. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res* 1992; **52**:4168–74.
16 Schlicholz B, Legros Y, Gillet D, Gaillard C, Marty M, Lane D, *et al.* The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. *Cancer Res* 1992;**52**: 6380–4.
17 Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, *et al.* p53 mutations in human lymphoid malignancies—associations with Burkitt lymphoma and chronic lymphocytic leukaemia. *Proc Natl Acad Sci USA* 1991;**88**:5413–17.
18 Finlay CA, Hinds PW, Tan T-H, Eliyahu D, Oren M, Levine AJ. Activating mutations for transformation by p53 produce a gene product that forms a hsp70-p53 complex with an altered half life. *Mol Cell Biol* 1988;**8**: 531–9.
19 Dong X, Hamilton KJ, Satoh M, Wang J, Reeves WH. Initiation of auto-immunity to the p53 tumour suppressor protein by complexes of p53 and SV 40 large T antigen. *J Exp Med* 1994;**179**:1243–52.