Expression of mutant p53 protein in hepatocellular carcinoma

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Abstract
p53 mutations are a common genetic finding in hepatocellular carcinoma from areas of high aflatoxin exposure. Recent small studies have shown that p53 gene mutations may be less common in areas with a low prevalence of hepatocellular carcinoma such as Great Britain. The protein product of mutant p53 can be detected immunohistochemically because of its longer half life in comparison with native protein. This study used a novel monoclonal antibody DO-7, raised against recombinant p53 and effective in routinely processed biopsy specimen tissue, to detect the mutant protein in a series of 45 cases of hepatocellular carcinoma occurring in white subjects resident in the United Kingdom. Focal nuclear labeling was seen in four cases (9%); surrounding cirrhotic tissue in one of these was negative for p53 expression. This study shows that p53 mutations are a rare event in hepatocarcinogenesis in Great Britain, an area of low aflatoxin exposure, and supports the concept of geographical variations in the cause and pathogenesis of hepatocellular carcinoma.

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The tumour suppressor gene p53 is located on the short arm of chromosome 17. It encodes a 53 kD nuclear phosphoprotein, which is expressed in low values in normal cells and has the ability to complex with viral and cellular antigens. Its precise function remains to be fully elucidated but it probably participates in controlling cell proliferation as values are influenced by the cell cycle and microinjection of antibodies to p53 prevent cells entering S phase.

Initial reports suggested that p53 values were raised in many human tumour derived cell lines in contrast with normal cells, but further work showed the protein to be a mutant form. The ability to detect mutant p53 in malignant cells is now related to its established increased half life, as a result of changed conformation. Mutant p53 has changed cell regulatory properties; it can transform cells in culture in the presence of low values of wild type p53 possibly by binding to and inhibiting the native (wild type) protein, and in the absence of wild type p53, it has been shown to stimulate cell growth.

The genetic basis for mutant protein has received intense investigation and mutations of the p53 gene have been discovered to be a common change found in human cancer. In hepatocellular carcinoma a mutational hot spot at codon 249 has been reported in a substantial proportion of tumours from areas of aflatoxin exposure, and this contrasts with the less specific pattern of mutations reported in other tumours. These mutations are G to T transversions and it is postulated that they are related to aflatoxin damage as this carcinogen is known to bind to guanine in DNA causing G to T mutations. This contrasts with the infrequent finding of p53 mutations in hepatocellular carcinoma from areas of low prevalence; these mutations are neither confined to codon 249 nor to GT transversions.

The biological significance of these various point mutations is, however, far from clear. Not all point mutations have the same functional effect on p53 and this includes the inability of some mutations to stabilise the protein. As it is the mutant protein that ultimately affects cell regulation and thus the malignant phenotype it is important that the presence of mutant protein is assessed together with genetic changes in the p53 gene.

We have therefore examined a series of cases of hepatocellular carcinoma in Great Britain immunohistochemically for the presence of mutant p53 protein.

Patients and methods

PATIENTS
Formalin fixed paraffin wax embedded archival material was available for study from 45 cases of hepatocellular carcinoma seen at the Freeman Hospital. Clinical details were available from the medical records on all patients and survival data in 39 cases. The mean age of the patients was 70 (range 43–84) with a male:female ratio of 2:1. Seventy seven per cent of patients had biopsy proved cirrhosis and serological markers of hepatitis B infection were present in 12 (27%) cases, three patients had HBsAg and in nine cases HBeAb alone was present.

METHODS
Sections of 3 μm were cut from paraffin blocks and endogenous peroxidase was blocked with 0·5% hydrogen peroxide in methanol. p53 was identified with two primary antibodies raised against recombinant p53 protein CM-1, and DO-7. CM-1 is a rabbit polyclonal antibody, raised against human wild type p53 produced in a bacterial expression system using a full length p53 cDNA, and used at a dilution of 1/1000. The monoclonal antibody DO-7, used at a dilution of 1/100, is also raised against recombinant human wild type p53 and has been shown to be specific for the denaturation resistant epitope at the N terminus of the protein. Incubation with primary antibody was performed overnight at
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Figure 1A: intense staining with monoclonal antibody DO-7 within nuclei of a well differentiated hepatocellular carcinoma; B: positive nuclear staining with anti-p53 polyclonal antibody CM-1 in malignant cells; note greater background cytoplasmic immunoreactivity with this antibody compared with DO-7.

Figure 2: Expression of mutant p53 in malignant hepatocytes as shown by nuclear immunoreactivity with monoclonal antibody DO-7 but not in hepatocytes of adjacent cirrhotic tissue (arrowed).

4°C. Immunostaining was detected with an avidin-peroxidase system; 3′3′ dianinobenzidine was used as the chromagen.

A malignant fibrohistiosarcoma of the liver, shown to have a mutation at codon 248 of the p53 gene, was used as a positive control (data not shown). Negative controls consisted of replacement of the primary antibody by buffer alone.

Results

With the monoclonal antibody DO-7 immunolabelling was detected in 4/45 (9%) tumours (Fig 1A). The staining was focal, affecting less than 10% of the malignant cells, but of uniform intensity and was confined to the nucleus except in those cells undergoing mitosis. Immunostaining with the polyclonal antibody CM-1 was also only seen in the four DO-7 positive tumours with a similar focal nuclear distribution. Background staining was higher, however, using CM-1 compared with DO-7 (Fig 1B). In the four positive hepatocellular carcinoma tissue blocks surrounding cirrhotic tissue was present in one and was negative for p53 (Fig 2). In 10 p53 negative tumours surrounding cirrhotic tissue was also negative.

The Table shows the clinical features of the four cases expressing mutant p53. The median survival of these cases was six weeks (range 1-60), which compares with a median survival of 10 weeks (range 1-96) for those patients whose tumours did not express mutant p53.

Discussion

Although the frequency of p53 gene mutations in hepatocellular carcinoma varies geographically, from 10% in low incidence areas to 50% in high incidence areas, it is still unknown whether this variation is also reflected in expression of mutant protein and cell growth. Native (wild type) p53 protein cannot normally be detected immunohistochemically because of its short half life. Many p53 gene mutations result in expression of protein with a prolonged half life and resulting cellular accumulation.

The ability to detect p53 expression immunohistochemically thus usually occurs when the protein is mutant, but also has been shown to sometimes reflect stabilisation of native protein by interaction with cellular and viral (for example SV40) proteins. More recently abnormal expression of wild type p53 protein has been found in normal cells of a cancer family patient, implying impaired degradation.

In this study expression of p53 was shown in 9% of tumours. Other studies have shown there is overall a good correlation between p53 gene mutations and detectable protein immunohistochemically. In a previous study we reported 19 cases of hepatocellular carcinoma, two of which (16%) had p53 gene mutations, one of which was detected immunohistochemically with the polyclonal antibody CM-1. Combining our two studies five out of 64 (8%) of cases of hepatocellular carcinoma in Great Britain express mutant p53 protein.

Not only was expression of mutant protein uncommon in our series but in those cases where it did occur the distribution was focal. Values of native p53 protein are known to vary throughout the cell cycle, accumulating during G1 and falling after cell division. Mutant p53 protein may also be regulated by the cell cycle and focal distribution may reflect this. A heterogeneous pattern of p53 gene mutations, however, has also been shown in nodules from multiple hepatocellular carcinoma in Japanese patients, suggesting a multifocal rather than clonal origin for the tumour in most patients. The absence of staining in cirrhotic tissue adjacent to tumour expressing mutant p53 is consistent with a role for the protein in hepatocarcinogenesis. It is of interest that in gastric cancer expression of p53 mutant protein has been considered to be a poor prognostic indicator. In this retrospective study it was not possible to evaluate the prognostic significance of p53 expression in hepatocellular carcinoma but preliminary evidence...

Clinical features of patients with hepatocellular carcinoma expressing immunoreactive p53

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Antiology</th>
<th>HBSAg</th>
<th>αFP (IU/L)</th>
<th>Survival (weeks)</th>
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<tr>
<td>60</td>
<td>M</td>
<td>Cryptogenic cirrhosis</td>
<td>Negative</td>
<td>&lt;10</td>
<td>60</td>
</tr>
<tr>
<td>76</td>
<td>M</td>
<td>No cirrhosis</td>
<td>Negative</td>
<td>670</td>
<td>6</td>
</tr>
<tr>
<td>77</td>
<td>M</td>
<td>Cryptogenic cirrhosis</td>
<td>Negative</td>
<td>90,000</td>
<td>92,000</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>Hepatitis B cirrhosis</td>
<td>Positive</td>
<td>91,000</td>
<td>?</td>
</tr>
</tbody>
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HBSAg = hepatitis B antigen, αFP = α fetoprotein.
suggests that it may contribute to an aggressive tumour phenotype. A prospective study is now necessary to investigate the value of p53 expression as a prognostic indicator.

In this study we have shown the feasibility of detecting mutant p53 in paraffin wax embedded material. Immunoreactivity with the novel reagent DO-7 was comparable with that seen with CM-1 but with the advantage of significantly less background staining. A large prospective analysis of p53 expression with DO-7 is therefore possible with routinely processed paraffin wax embedded biopsy material rather than frozen tissue.

This study has confirmed that the low frequency of p53 gene mutations seen in hepatocellular carcinoma in Great Britain is reflected in a similar frequency of mutant protein expression. The geographical difference in aberrant p53 expression suggests differences in the molecular mechanisms of hepatocarcinogenesis with p53 being less important in low incidence areas such as Great Britain.

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