

# p53 Protein overexpression in cholangiocarcinoma arising in primary sclerosing cholangitis

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## Abstract

The protein encoded by the p53 tumour suppressor gene plays an important part in the regulation of cell growth. Abnormalities of this gene represent one of the most common genetic changes in the development of human cancers. This study investigated the expression of p53 protein in cholangiocarcinoma arising in association with primary sclerosing cholangitis (PSC). Of the 14 patients with cholangiocarcinoma studied, 13 had underlying PSC. The expression of p53 protein was detected immunohistochemically in paraffin wax embedded liver specimens, after microwave pre-treatment. The expression of p53 protein was shown in the cholangiocarcinoma tissue of 11 of 14 (78.5%) patients. In five of 10 patients, the accumulation of p53 protein highlighted the presence of neoplastic cells in biliary tissue separate from the main tumour. These cells were mainly located in the septal bile ducts or in the accessory glands, or both, but occasionally also in large portal areas at the periphery of nerves and lymphatics, and in one case in the mucosa of an extrahepatic bile duct. No p53 protein was detectable in liver tissue obtained at the time of transplantation in 15 patients with PSC but not cholangiocarcinoma. These results show that cholangiocarcinoma development in PSC is commonly associated with abnormalities of p53 and that these occur at a late stage in the development of the malignant process. Staining for p53 protein could represent an additional criterion for the diagnosis of cholangiocarcinoma development in patients with PSC.

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Inactivation of the p53 tumour suppressor gene is a frequent step in the development of human cancer.<sup>1</sup> Two essential functions of the protein encoded by this gene are now recognised: to arrest the cell cycle in the G<sub>1</sub> phase, permitting the cells exposed to mutagenic factors to repair their DNA, or to induce apoptosis, programmed cell death.<sup>2</sup> Mutations in the p53 gene may block these normal functions and result in prolonged survival of cells carrying DNA mutations. Mutations in the gene lead to a synthesis of an abnormal p53 protein, which has a longer half life and accumulates in

cell nuclei. The half life of this mutated protein (between four to eight hours) is longer than that of the wild type (five to 20 minutes), permitting detection by immunohistochemistry.<sup>3</sup>

Mutations of the p53 tumour suppressor gene and overexpression of p53 protein have been described in liver tumours originating from hepatocytes, such as hepatocellular carcinoma<sup>4,5</sup> and hepatoblastoma.<sup>6</sup> A comparatively high incidence of p53 mutations has also been shown recently in gall bladder carcinoma.<sup>7</sup> In addition, patients with the Li-Fraumeni syndrome, a condition with an inherited germ line mutation affecting the p53 gene, develop cholangiocarcinoma.<sup>8</sup> Therefore, changes in the function of this gene may participate in biliary tract carcinogenesis.

Development of cholangiocarcinoma in primary sclerosing cholangitis (PSC) is common, the prevalence of the tumour varying from 7% in asymptomatic patients to 42% of necropsy series.<sup>9</sup>

In this study we have investigated the expression of p53 protein in cholangiocarcinoma and PSC.

## Methods

Fourteen patients with cholangiocarcinoma were studied, 13 of whom had underlying PSC. Nine patients were male and five female, median age 40 years. Ten patients underwent orthotopic liver transplantation, and four partial liver resection.

Non-neoplastic biliary tissue surrounding the tumour area was available for study in the specimens examined from 10 of 14 patients. In one patient with PSC a specimen from an extrahepatic bile duct was obtained by endoscopic retrograde cholangiopancreatography (ERCP) biopsy. Liver tissue from 15 patients transplanted for PSC with no evidence of cholangiocarcinoma in the explanted liver was also studied.

The expression of p53 protein was compared with the serum biochemistry tests (albumin, bilirubin, and alkaline phosphatase), the serum tumour markers for cholangiocarcinoma (carcinoembryonic antigen and carbohydrate antigen 19-9),<sup>10</sup> and the histological classification of cholangiocarcinoma.

## Detection of p53 protein expression

Cellular expression of p53 protein was detected in formalin fixed, paraffin wax embedded tissue specimens using the avidin-biotin-peroxidase complex method. Sections were deparaffinised, rehydrated through graded alcohols, and the endogenous peroxidase activity was blocked

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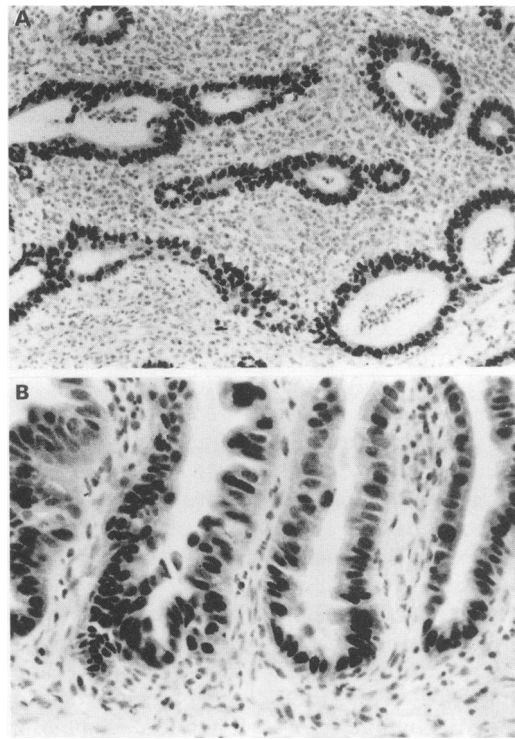


Figure 1: Intense nuclear staining for p53 protein in cholangiocarcinoma tissue. (A): Well differentiated adenocarcinoma with extensive inflammatory infiltrate in the stroma. (B): Malignant glandular epithelium lying in the lumen of a large intrahepatic duct.

with 1% hydrogen peroxide in methanol for 15 minutes. The sections were transferred into 0.01 M citrate buffer (pH 6.0) and microwaved for 15–20 minutes at maximum power using Proline microwave oven (Proline Power Wave 700) rated at 700 W.<sup>11 12</sup> In preliminary experiments these conditions have been optimised and we have shown that microwave pretreatment is an essential step for adequate detection of p53 protein in formalin fixed liver specimens. The sections were subsequently incubated with TRIS buffered saline (TBS, 0.05 M TRIS-HCL and 0.15 M sodium chloride, pH 7.4) containing 10% normal serum from the species in which the secondary, biotinylated antibody was raised. p53 Protein was detected with a monoclonal antibody to p53 protein D01 (Cambridge Bioscience, UK) diluted 1:100 in TBS, for 60 minutes at 37°C. The sections were washed and then incubated

for 30 minutes with a biotinylated rabbit anti-mouse antibody (DAKO Ltd, High Wycombe, UK) diluted 1:400 in TBS, followed by Streptavidin ABC (DAKO Ltd) diluted in TBS 1:200 for 30 minutes. The peroxidase substrate was 3',3'-diaminobenzidine-tetrahydrochloride (0.5 mg/ml) in TBS plus 0.01% H<sub>2</sub>O<sub>2</sub> and was applied to the tissue for five minutes. The sections were then counterstained with haematoxylin, dehydrated, and mounted in distrene-tricresyl phosphate-xylene. Nuclear staining for p53 protein was defined as intense if a positive reaction was present in more than 80% of the nuclei and weak if less than 20% were positive. The immunostaining was assessed by two independent observers (PMR and BP). As positive controls for immunodetection of mutant p53 protein we used two hepatoma cell lines (PLC/PRF/5 and HUH-7), which have different mutations in the p53 gene and are known to produce mutant p53 proteins.<sup>13</sup> Both cell lines were cultured under standard conditions, the cells were trypsinised, washed, and the cell pellets were fixed in buffered formalin and embedded in paraffin wax. The positive staining with the monoclonal D01 antibody was also compared with a polyclonal rabbit antibody to p53 protein (kindly donated by Dr H Zentgraf, DKFZ, Heidelberg, Germany), diluted 1:500, and detected with a biotinylated goat antirabbit antibody and Streptavidin ABC (DAKO Ltd). The negative controls included replacement of the primary antibody to p53 with buffer only or a monoclonal antibody with different specificity, as well as immunostaining of normal liver tissue with anti-p53.

## Results

Accumulation of p53 protein was shown as dense nuclear staining in the neoplastic tissue of 11 of 14 cholangiocarcinoma samples (78.5%). In seven cases, the staining was intense and present in more than 80% of the cells (Fig 1), while in the remaining four cases it was seen in some 20% of the nuclei. The proportion of cells with overexpression of p53 protein did not correlate with serum biochemistry and the serum tumour markers or the histological classification of cholangiocarcinoma (Table).

### Characteristics of patients with cholangiocarcinoma and p53 protein expression in the neoplastic biliary epithelium

| No | Biochemistry and tumour markers                 |   |                                |                           |                                 | Neoplastic biliary epithelium  |                         |
|----|---|---|--------------------------------|---------------------------|---------------------------------|--------------------------------|-------------------------|
|    | Bilirubin<br>( $\mu\text{mol/l}$ )<br>(NR=3–20) | Alkaline phosphatase<br>(IU/l)<br>(NR=30–120) | Albumin<br>(g/l)<br>(NR=35–50) | Cea<br>(ng/ml)<br>(NR=<5) | Ca 19-9<br>(U/ml)<br>(NR=<200)* | Histological type<br>(adenoca) | p53 Nuclear<br>staining |
| 1  | 339   | 1849  | 19                             | 10                        | 81 362                          | Muco-secreting                 | >80% +ve                |
| 2  | 328   | 1925  | 37                             | 56                        | 20 219                          | Papillary                      | >80% +ve                |
| 3  | 206   | 1083  | 28                             | 9                         | 334                             | Muco-secreting                 | <50% +ve                |
| 4  | 306   | 786   | 33                             | 1                         | 254                             | Muco-secreting                 | -ve                     |
| 5  | 150   | 1084  | 44                             | 3                         | 26                              | Tubular                        | 100% +ve                |
| 6  | 201   | 1587  | 37                             | 1                         | 346                             | Muco-secreting                 | <10% +ve                |
| 7  | 324   | 1155  | 35                             | 16                        | 24                              | Papillary-muco secreting       | >80% +ve                |
| 8  | 358   | 998   | 32                             | 61                        | 51                              | Tubular                        | <10% +ve                |
| 9  | 346   | 1932  | 34                             | 2                         | 44                              | Papillary-muco secreting       | -ve                     |
| 10 | NA  | NA  | NA                             | NA                        | NA                              | Muco-secreting                 | -ve                     |
| 11 | 911   | 641   | 28                             | 1                         | 126                             | Tubular                        | >80% +ve                |
| 12 | NA  | NA  | NA                             | NA                        | NA                              | Tubulo-papillary               | 10% +ve                 |
| 13 | 135   | 866   | 22                             | 1                         | 3081                            | Muco-secreting                 | >80% +ve                |
| 14 | NA  | NA  | NA                             | NA                        | NA                              | Muco-secreting                 | >80% +ve                |

NA=data not available; NR=normal range. Cea=carcinoembryonic antigen, Ca 19-9=carbohydrate antigen 19-9.

\*A normal range has not been firmly established in PSC patients and for the present analysis a value of <200 U/ml has been taken (see reference 10).



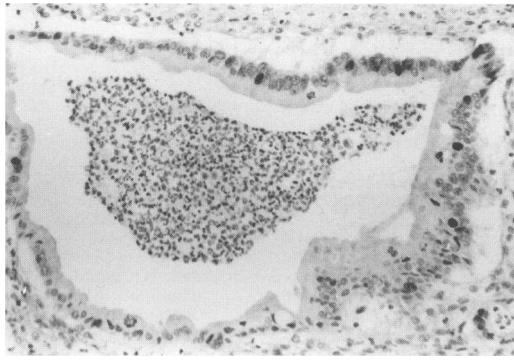


Figure 2: Dysplastic and focally malignant epithelium mimicking a septal bile duct with pus in the lumen.

In five of 10 patients with PSC and cholangiocarcinoma, screening of large tissue blocks including perihilar tissue showed, away from the main tumour area, small islands of biliary epithelium whose nuclei stained strongly for p53 protein (Fig 2). These glandular elements were not noticed on the haematoxylin preparation because of their high degree of differentiation, which mimicked bile duct branches or islands of accessory seromucinous glands. In retrospect they appeared more consistent with highly differentiated tumour glands within thin walled lymphatic channels or distant foci of well differentiated, tubular adenocarcinoma, or both. In addition, minute nests of carcinoma cells were similarly highlighted within perineural tissue and lymphatics by nuclear p53 staining (Fig 3). In a single case a sample of bile duct epithelium obtained by ERCP and diagnosed as dysplastic showed a focus of p53 nuclear staining; this potentially neoplastic area was not recognised as such by conventional histology.

In the 15 patients with PSC without cholangiocarcinoma there was no nuclear staining for p53 protein in the liver tissue.

### Discussion

This study shows that p53 protein is overexpressed in a high proportion (78.5%) of cholangiocarcinoma developed in patients with PSC. This tumour usually originates above the junction between the cystic and the hepatic duct, and often spreads along the nerves and the nearby vascular structures invading the liver parenchyma.<sup>14</sup> This mode of metastatic spread is further highlighted by

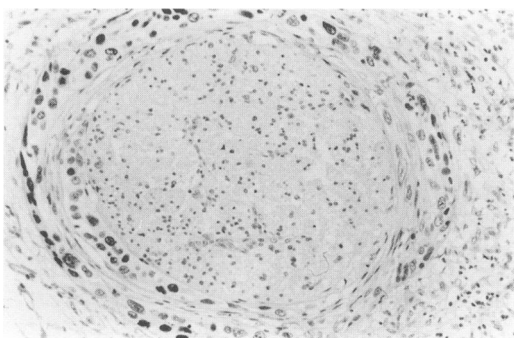


Figure 3: Group of tumour cells in perineural tissue highlighted by p53 staining.

p53 protein staining, which allowed the recognition of minute tumour deposits.

The detection of mutant p53 protein has important implications for the clinical practice.<sup>15 16</sup> It has recently been shown that p53 dependent apoptosis modulates the cytotoxic effects of ionising radiation and chemotherapy agents,<sup>17</sup> raising the possibility that the p53 gene participates in the mechanism of multi-drug resistance.<sup>18</sup> In patients with solid tumours both poor prognosis and failure of chemotherapy may be associated with the abnormalities of p53 mutations.<sup>15</sup> The finding of a high prevalence of mutant p53 accumulation in cholangiocarcinoma may be linked with the rapid progression of this tumour and its poor response to different therapeutic approaches.

In a recent series of cholangiocarcinoma samples from Japan, overexpression of the p53 protein was detected in 22% of surgical tumour specimens.<sup>19</sup> In that study the underlying state of the liver is not described. In our series, the cholangiocarcinoma arose on the background of PSC, and the prevalence of p53 staining in the neoplastic tissue was very high when compared with that obtained by Terada *et al.*<sup>19</sup> In our study there was no convincing evidence that normal or inflamed, but non-neoplastic biliary epithelium outside the tumour bulk stained for p53 protein and all 15 cases with longstanding PSC without associated cholangiocarcinoma were negative in that respect. This would suggest that the accumulation of p53 is a late stage in the development of the malignant process.

A very close correlation between immunohistochemical overexpression of p53 protein and mutations in the p53 gene has been established,<sup>20</sup> and positive tissue staining for p53 protein has been proposed as a marker of malignancies.<sup>16 21</sup> Detection of mutant p53 protein in tissue biopsy material could be of value in the follow up of patients with PSC. In these patients it may be very difficult to distinguish between benign and malignant bile duct strictures. Immunohistochemical staining for intracellular components such as carcinoembryonic antigen<sup>22</sup> have been used to identify cells with cholangiocarcinoma phenotype. Our finding of positive staining for p53 protein in malignant cells outside the main tumour mass suggests that immunohistochemical detection of p53 protein could represent an additional criterion for establishing a diagnosis of cholangiocarcinoma in PSC.

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