The diagnostic and prognostic value of genetic aberrations in resectable distal bile duct cancer
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Citation for published version (APA):

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Chapter 6

P53 Expression as a Prognostic Determinant in Resected Distal Bile Duct Carcinoma

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Eur J Surg Onc; Accepted for Publication
ABSTRACT

Background. P53 encodes a protein with known tumour-suppressor activity. Mutations of this gene are the most common genetic alterations in human cancers and result mostly in the stabilization of the protein product, which is then detectable by immunohistochemical staining. Positivity for p53 immunohistochemistry is a useful prognostic marker in a variety of tumours. The aim of this study was to determine the incidence and prognostic value of p53 immunopositivity in resectable distal bile duct carcinoma (DBDC).

Methods. Forty-seven archival tumour samples of patients with DBDC, who underwent subtotal pancreatoduodenectomy from 1985 to 1996, were immunohistochemically examined for p53 positivity, using the anti-p53 antibody D07.

Results. Nineteen (40%) of the 47 tumours demonstrated positive (>30%) p53 protein immunostaining. Focally positive or negative staining was seen in the remaining 28 (60%) cases. Patients in this low p53 category survived significantly longer than those in the high p53 category, with median survival duration of 29, and 13 months respectively (p=0.039). P53 positivity was independent of age, sex, tumour size, radicality of resection, histopathological grading, lymph-node status, perineural invasion and vasoinvasive growth.

Conclusion. This study indicates that low (0-30%) p53 expression is a favorable prognostic factor in patients with resected DBDC.

INTRODUCTION

Distal bile duct carcinomas (DBDCs) have one of the worst prognosis among various cancers, caused partly by the difficulty in making an early diagnosis. Small lesions confined to the distal bile duct are potentially curable but are rarely detected, resulting in a low resectibility rate (13%) and dismal 5-year survival (24%) of resected DBDC. Although in clinical practice negative microscopic margins provide the most important prognostic information, it does not account for the observed heterogeneity in tumour behaviour. Factors influencing the final outcome of patients with DBDC may lie beyond the scope of histological examination, at the molecular genetic level. It has been shown in various neoplasms, that some tumours with more extensive genetic damage, have a considerably worse prognosis compared to tumours containing fewer aberrations.

The most common genetic alteration in human cancers is mutation of p53. The p53 gene, located on chromosome 17p, produces a protein that plays an important role in the regulation of the cell cycle process, cell growth and apoptosis. The normal (wild-type) p53 protein has tumour suppressor properties and mutation of the p53 gene results in the synthesis of a mutant protein, which disrupts critical growth-regulating mechanisms. As a result, p53 alterations are thought to play a crucial role in the carcinogenesis of many types of human neoplasia. Detection of p53 mutations at the molecular genetic
level, however, is cumbersome and time consuming, therefore not feasible in routine diagnosis\textsuperscript{12}. Since mutant p53 protein has a longer intracellular half-life than that of the wild-type protein\textsuperscript{7,13,14}, a large amount of stabilised mutant protein accumulates in tumours and can be detected rapidly by routine immunohistochemical staining. At present, immunohistochemistry (IHC) is the most widely used approach for assessing p53 alterations in human cancers\textsuperscript{15}. Currently, a number of studies have been published, which have found that p53 overexpression is a useful prognostic marker in a variety of tumours\textsuperscript{16-18}. To date, no data have been published on the prognostic significance of p53 protein in DBDC.

In view of the importance of prognostication of DBDC, the purpose of this investigation was to determine the frequency and prognostic value of mutant p53 protein overexpression in this type of tumour using IHC. We report here on 47 patients with this rare malignancy whose tumours were resected with curative intention.

MATERIALS AND METHODS

Patients and histopathological criteria
The study group consists of 47 consecutive patients with DBDC, who had undergone subtotal pancreateoduodenectomy (Whipple procedure) at the Academic Medical Center between 1985 and 1996. Of these patients, 37 were male and 10 female, with a mean age of 60 years (range 37-77 years).

DBDCs were defined as malignant neoplasms arising from the distal portion of the bile duct, located between the superior border of the pancreas and the ampulla\textsuperscript{19}. The tumours were reviewed by one pathologist (GJAO) and classified into three categories\textsuperscript{20}, as follows: 9 patients had a well differentiated tumour, 30 patients had a moderately differentiated tumour and the remaining 8 patients a poorly differentiated tumour at the time of diagnosis. Complete information on surgical resection margins, degree of tumour differentiation, lymph node involvement, perineural invasion and vaso-invasive growth was available. Unfortunately, the size was not reported for 8 tumours. Postoperative follow-up data were obtained by reviewing the medical records or autopsy reports.

Immunohistochemistry for p53
Paraffin-embedded sections of all DBDC specimens were stained for the p53 tumour suppressor gene product using the anti-p53 antibody D07, a mouse monoclonal IgG antibody. Five \textmu m sections of tissue were mounted on 3-aminopropylthrietoxysilane (Sigma, U.S.A.) coated glass slides and dried overnight at 56°C. Adjacent sections were stained with hematoxylin and eosin to confirm the presence of carcinoma tissue. After rehydration, endogenous peroxidase activity was blocked in 0.3% H\textsubscript{2}O\textsubscript{2} solution in methanol for 20 min. Citrate buffer was used for antigen enhancement. Slides were submerged in citrate buffer (0.01 M, pH 6.0) and heated in a temperature-probe controlled microwave oven for 10 minutes at 100°C. After cooling for 15 min, 10%
normal goat serum in phosphate-buffered saline (PBS) was applied for 20 min. The sections were subsequently incubated with the primary antibody D07 (Dakopatts, Denmark) at a concentration of 1:200 in PBS and biotinylated rabbit anti mouse IgG antibody (1:200 in 10% normal human AB serum in PBS) (Dakopatts, Denmark) for 30 min. Sections were immunostained using streptavidin-biotin peroxidase complex (1:200 in 10% normal human AB serum in PBS) (Dakopatts, Denmark) for 30 min. The peroxidase was visualized using diaminobenzidine (DAB) as the chromagen and counterstaining of the nuclei was done with hematoxylin. Positive controls were sections from colonic adenocarcinoma previously shown to express high levels of p53. As a negative control, omission of the primary antibody (PBS) was used in each IHC run.

Stained slides were evaluated by 2 independent investigators who were blinded to the other features of these patients. P53 expression was considered positive when more than 30% of the tumour cells showed nuclear staining. This quantitation is based on our previous experience that a cut-off point of 30% made p53 IHC virtually 100% specific for underlying mutations in the gene.

Statistics
Differences in frequency distribution of p53 were determined using the Pearson chi-square test or Fisher's exact test. Survival curves were constructed using the Kaplan-Meier method, and univariate survival comparisons were made using the log-rank test (SPSS 6.0 statistical software). P<0.05 was regarded as statistically significant.

RESULTS
In 19 (40%) of the 47 DBDCs examined, there was more than 30% p53 protein immunostaining (Figure 1), whereas in the remaining 28 samples a less than 30% positivity was encountered. In all cases a specific nuclear staining was seen and cytoplasmic positivity was not observed; adjacent areas of normal or reactive bile duct tissue also failed to exhibit p53 immunostaining.

Three patients died post-operatively (hospital mortality 6%) and thus were excluded from the survival analysis. Histopathological assessment showed tumour positive margins in 21 (45%) of the 47 specimens. Survival data were available on all cases, but one patient was lost to follow-up after 2 years, and treated as a censored event at that time. In addition to surgical resection, two patients received external beam radiation therapy and one patient received both chemotherapy and radiation therapy. The overall median survival of patients with a resected DBDC was 20 months. The medical records or autopsies revealed that all the patients who died during the follow-up period, had died of recurrent tumour.
Figure 1. High expression (>30%) of p53 immunohistochemistry was found in this well differentiated adenocarcinoma of the distal bile duct. Inset. Staining pattern in p53 immunohistochemistry with antibody D07 shown in more detail.

Figure 2. Cumulative survival (Kaplan-Meier curves) of patients whose tumours were positive for p53 overexpression (>30% staining) compared with those showing less than 30% staining (Log rank, p=0.039). Three patients died post-operatively and were excluded from this survival analysis.
A significant correlation was observed between p53 tumour staining and survival ($p=0.039$) (Figure 2); this significance slightly increased after stratification for postoperative adjuvant therapy ($p=0.024$). The median survival of patients with evidence of p53 protein accumulation was 13 months compared to 29 months for those patients without p53 protein accumulation. P53 overexpression was independent of age ($p=0.41$), sex ($p=0.07$), positive microscopy of resection margins ($p=0.37$), histopathological grading ($p=0.76$), lymph-node status ($p=0.37$), perineural invasion ($p=0.66$) and vaso-invasive growth ($p=0.86$). The prevalence of p53 protein accumulation in larger tumours (>2 cm) was higher than that found in smaller tumours (<2 cm), but this did not reach statistical significance ($p=0.07$).

**DISCUSSION**

This study concerns the immunohistochemical results of p53 protein overexpression in resected DBDCs, and demonstrates for the first time that overexpression of p53 nuclear protein is an unfavorable prognostic factor in patients with this rare malignancy. In this series of 47 patients, the detection of p53 overexpression showed a clear association with survival ($p=0.039$). Due to the relatively small number of patients included in this study, multivariate analysis was not possible and the independence to other prognostic variables is therefore uncertain. However, the fact that in a previous study $^{21}$ of the same 47 adenocarcinomas negative surgical resection margins was the only significant prognostic histopathological parameter, suggests that p53 has additional prognostic value. Recent IHC studies on a variety of cancers have analyzed p53 overexpression and its impact on prognosis. In several tumours, including pancreatic cancer $^{17}$, bladder cancer $^{18}$, gastric $^{22,23}$ and colorectal cancer $^{22}$, the accumulation of nuclear p53 correlated with shorter patient survival time. Washington et al. $^{24}$ observed no correlation between p53 overexpression and survival in 21 carcinomas of the extrahepatic bile duct, but the results of this study should be interpreted cautiously in view of the small sample size and incomplete survival data.

The frequency of p53 overexpression detected in this series of DBDC was 40% (19/47), a frequency lower than that observed by Diamantis et al. $^{25}$. By using a cut-off point of 10%, Diamantis et al. $^{25}$ found that 18 (62%) of the 29 stained DBDCs were D07 positive. It is our experience that high expression (>30%) of p53 protein in IHC is more specific for the presence of mutations in the p53 gene $^{12}$; below the 30% false-positive IHC results will be encountered, and this could explain the discrepancy between our results and those of Diamantis. In fact, when the 10% cut-off point was used, survival differences also became non-significant in our study group.

Significantly more p53 protein expression has been demonstrated in bile duct carcinomas derived from the lower-mid region (62%) than in those arising in the proximal region (30%) $^{25}$, supporting the view that hilar tumours and distal bile duct tumours form distinct entities with probably different tumourigenic mechanisms. The fact
that most studies do not distinguish between proximal and distal tumours, may be another reason why the reported incidence of p53 mutations varies in the literature\textsuperscript{24,25,26,27} (Table 1). The small number of patients in each study and differences in the sensitivity of IHC techniques also contribute to the wide variety of results. To be efficient, IHC studies of tumour cells require specific antibodies with high affinity for the antigen and no cross-reactivity with other cellular proteins\textsuperscript{15}. In our experience D07 has shown to give the most sensitive and specific IHC results for the p53 tumour suppressor gene product\textsuperscript{12}.

Table 1. P53 overexpression in extrahepatic bile duct tumours as determined by immunohistochemistry (IHC).

<table>
<thead>
<tr>
<th>Tumour location</th>
<th>Number of patients</th>
<th>Used antibody (cut-off point)</th>
<th>P53 over-expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>Distal*</td>
<td>47</td>
<td>D07 (30%)</td>
</tr>
<tr>
<td>Diamantis, 1995\textsuperscript{25}</td>
<td>Distal</td>
<td>29</td>
<td>D07 (10%)</td>
</tr>
<tr>
<td>Teh, 1994\textsuperscript{26}</td>
<td>Proximal†</td>
<td>10</td>
<td>D07 (10%)</td>
</tr>
<tr>
<td>Washington, 1996\textsuperscript{24}</td>
<td>CBD</td>
<td>18</td>
<td>D07 (10%)</td>
</tr>
<tr>
<td>Soon Lee, 1995\textsuperscript{27}</td>
<td>Proximal</td>
<td>13</td>
<td>Ab-2 (5%)</td>
</tr>
<tr>
<td></td>
<td>CBD</td>
<td>8</td>
<td>Ab-2 (5%)</td>
</tr>
<tr>
<td></td>
<td>CBD</td>
<td>7</td>
<td>CM1/D07(§)</td>
</tr>
</tbody>
</table>

* Distal = Intra-pancreatic location of the tumours, † Proximal = Tumours involving the hepatic duct bifurcation, CBD = Common bile duct, § Staining of cells was scored on a 4-point scale according to both the intensity and proportion of cells staining.

It is tempting to speculate that the negative prognostic value of p53 overexpression and the tendency for increased p53 expression in large tumours (>2 cm) in the present study, support the hypothesis that p53 gene alterations occur late in carcinogenesis and therefore are involved in the more advanced stages of carcinogenesis\textsuperscript{11,28}. In this regard, the timing of p53 mutation in DBDC progression may be similar to pancreatic head carcinomas\textsuperscript{26}. Although resection remains the primary treatment of DBDC, the response to adjuvant chemotherapy and radiation therapy may depend on the integrity of the p53 tumour suppressor pathway\textsuperscript{30}. P53 status could thus serve as a guide for more selective differential therapy in these patients. In addition, patients with p53 mutated tumours may in the near future benefit from new adjuvant gene therapy and these patients are likely to be identified by IHC staining techniques.

In conclusion, in the present study p53 protein overexpression was detected in 40% of the DBDCs examined and correlated with decreased patient survival. The median survival of the patients more than doubled when less than 30% p53 positivity was present. These results suggest that aberrant p53 expression is associated with the biologically more aggressive DBDC.
REFERENCES


