The diagnostic and prognostic value of genetic aberrations in resectable distal bile duct cancer
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Chapter 1

General introduction and outline of the thesis
Chapter 1

GENERAL INTRODUCTION

The age adjusted incidence of extrahepatic bile duct carcinoma is approximately 1 per 100,000 person-years. The extrahepatic bile duct has been subdivided anatomically into: upper third (proximal), middle third, and lower third (distal). Carcinoma of the distal (lower third) bile duct (DBDC) is defined as a tumor that involves the intrapancreatic portion of the extrahepatic bile duct. These tumors represent 10 to 20 per cent of the extrahepatic bile duct carcinomas and belong to the group of periampullary carcinomas, which also include carcinomas arising from the head of the pancreas and from the papil of Vater. The large majority of DBDCs are adenocarcinomas, other histologic types are rare. According to the WHO, adenocarcinomas are subdivided into three grades: (1) well differentiated, (2) moderately differentiated, and (3) poorly differentiated.

DBDCs show a slight male predominance and the average age of presentation is 60 years. Jaundice, as a result of progressive biliary obstruction, is the most common presenting symptom. Weight loss, pruritus, and abdominal pain are other less frequent symptoms. The widespread availability of either endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC), followed by the use of ultrasonography combined with Doppler and spiral computed tomography, have resulted in earlier diagnosis of DBDC. Correctly predicting operability depends upon assessment of locoregional invasion and identifying small peritoneal and intrahepatic metastases. Allema et al. reported 70% resectability in 188 consecutive patients with a tumor of the pancreatic head region, after combined use of these preoperative procedures. Unfortunately, small metastatic lesions (<1 cm in diameter) are easily missed on CT scan. Therefore, laparoscopy with ultrasonography has been introduced in the Academic Medical Centre of Amsterdam for the evaluation of operability in patients with periampullary carcinoma. It remains, nevertheless, difficult to differentiate preoperatively an adenocarcinoma from a benign stricture, leading to at least a 5% chance of resecting a benign lesion.

In the past decades remarkable advances have been made in the management of patients with DBDC, and when resection is possible, (pylorus preserving) pancreatoduodenectomy (Whipple's procedure) is considered to be the procedure of choice. It has a less than 5 per cent operative mortality and 33 per cent morbidity in experienced centers. The 5 year survival of patients with DBDC who have undergone resection varies from 18 to 39 per cent. Over 50 per cent 5 year survival rates have been reported, but this constitutes only those cases with radical resection (negative resection margins) as assessed by light microscopy.

Most patients with DBDC, unfortunately, do not manifest early symptoms and in the majority of patients the diagnosis is made when the disease is already too advanced for potentially curative therapy to be considered. The proximity to vital structures and the aggressive growth often precludes curative resection, leaving palliative treatment as the only option left. For two decades adjuvant radiotherapy and chemotherapy, either
separately or in combination, have been tested in patients with DBDC. Up to date the beneficial effect of adjuvant therapy remains, however, controversial. Integration of this modality within the multidisciplinary management of patients with DBDC needs further prospective evaluation and its routine use in DBDC is therefore not recommended.

A central issue in the management of patients with DBDC is to predict, for any given individual, the natural course of this malignancy. For many years, surgical resection and negative microscopic margins have been the most important prognostic factors for survival in DBDC. In addition, poor tumour differentiation and lymph node status have been considered as prognostic factors, but these have not added much to the value of microscopically tumour negative resection margins. The literature is rather conflicting about the prognostic value of tumour volume and perineural invasion. However, also microscopically tumour negative resection margins cannot predict outcome for a specific patient. In fact, even after curative operation, the recurrence at the surgical margins is not infrequent. Factors influencing the final outcome of patients with DBDC may lie beyond the scope of histological examination, at the molecular genetic level. Currently, a number of studies have been published, which have found that certain molecular markers are useful prognosticators in a variety of tumors, such as: DNA ploidy in pancreatic carcinoma, resectable cancer of the ampulla of Vater and carcinoma of the proximal bile duct; Ki67 antigen in gallbladder cancer, non-Hodkin lymphoma and breast cancer; K-ras codon 12 point mutation in curative resected adenocarcinoma of the lung; and p53 protein overexpression in pancreatic cancer, bladder cancer, gastric cancer and colorectal cancer. Thus there maybe an additive value of these molecular markers as an adjunct for histopathological examination of DBDCs, and quantitation of these markers could potentially enhance the prognostic information. Archival material can be used for most of these molecular techniques and it enables to retrospectively study a relatively large group with well documented outcome of the disease.

Furthermore, only limited information is available concerning molecular genetic alterations underlying distal bile duct tumourigenesis. Identification of these genetic alterations would highlight locations of potential important (new) oncogenes and tumour suppressor genes. A major drawback of diagnosis of DBDC is that it usually identifies only advanced tumours, precursor lesions are inaccessible, and a discrimination between oncogenic factors leading to tumour initiation or progression is difficult.

**AIMS AND OUTLINE OF THE THESIS**

Since early detection (i.e. in a stage that resection can be radical and curable) and correct prognostication are considered critical in the proper management and treatment of DBDC, the aim of our research was to study cellular tumour characteristics that could explain the diversity in the clinical outcome of patients with DBDC. Features of potential biological and clinical importance that were studied in this thesis concern DNA ploidy, proliferation...
(Ki67 antigen), K-ras, and p53 protein overexpression. We also performed a survey of the whole genome of DBDC to detect the changes in DNA copy number. It was expected that the study on the occurrence of these molecular events and the detection of genome regions that are altered in DBDC would increase further understanding of the pathogenesis and provide insight into tumour progression of this carcinoma with so far almost uniformly fatal outcome.

In contrast to normal tissue, neoplastic lesions often undergo chromosomal aberrations resulting in the appearance of nondiploid (aneuploid) populations within the tumour cell population. The instrumentation for clinical cytometric measurements currently includes flow cytometry (FCM) and image cytometry (ICM), which have made the measurement of DNA content in dissociated cells of fresh and archival material more readily available. In both techniques the cells are stained with fluorescent dyes that bind specifically to DNA and by measuring the amount of fluorescence, the total amount of DNA in each cell can be determined. In a malignant specimen clones of tumour cells with aberrant (other than 2N) DNA content can give rise to aneuploid peaks. In Chapter 2 therefore a non-concurrent prospective study is presented of 126 peritoneal, pleural and pericardial effusion samples, from 102 patients with a variety of malignancies which were examined by cytology and FCM. In addition, we performed ICM on the malignant cytologic cases with a diploid flow pattern. The samples were collected and examined at the Presbyterian-University Hospital, Pittsburgh, USA. The aim of this study was to determine the diagnostic role of FCM combined with ICM in malignancies in conjunction with the more conventional, but time-proven, cytologic technique and to determine its utility.

Retrospective FCM and ICM studies of archival tumours have provided the basis for much of our knowledge of the prognostic value of DNA-ploidy in a wide variety of tumours. Tumour aggressiveness may correspond to presence of aneuploidy and in several types of human malignant tumours there is in fact considerable evidence suggesting that DNA content correlates with long-term prognosis. In periampullary carcinomas, however, the literature is rather conflicting about the value of ploidy status.

In addition to DNA-ploidy, the investigation of the proliferative fraction (cells in S-phase) of tumours has received much attention. Cell proliferation characteristics may also reflect the aggressiveness of a tumour and have been implicated as important predictors of biological behaviour in various tumour types. MIB-1, a monoclonal antibody that recognises a nuclear antigen associated with the cell cycle (Ki-67), can be used for the assessment of cell proliferation and its prognostic value in paraffin-embedded material by immunohistochemistry. So far only two studies on a rather small number of patients have been carried out to investigate the prognostic value of Ki-67 in DBDC. In Chapter 3 the prognostic value of cell proliferation (Ki-67 antigen) and nuclear DNA content in resected DBDC is investigated in formalin-fixed tumour specimens from 35 patients.
patients, who were treated by sub-total pancreateoduodenectomy (Whipple procedure) at the Academic Medical Centre, Amsterdam, The Netherlands, between 1985 and 1992. Long term clinical follow-up data from all patients were available.

The methodical aspects and applications of FCM and ICM have been reviewed extensively in the literature\(^ {43,70-78}\). Guidelines have been published on the interpretation of histograms\(^ {79,80}\), as well as quality assurance prerequisites\(^ {81-89}\), to improve comparison between the two methods and between laboratories. Theoretically, FCM has a strong statistical basis and ICM is more specific in identifying smaller abnormal cell populations. Because DBDCs often contain relatively abundant non-neoplastic stroma, ICM could be the procedure of choice in this particular tumour type. Reports of flow cytometric or image cytometric analysis of DBDCs are rare\(^ {63,90-91}\), and show an aneuploidy rate of 25%-40%. These studies, on limited number of patients, suggest that the majority of DBDCs are diploid tumours. In Chapter 4 we have applied FCM and ICM to 44 resected (Whipple procedure) DBDCs to compare both methods, to evaluate the ploidy incidence, and to investigate the relation between DNA-ploidy and survival in these tumours. The Whipple procedure was performed at the Academic Medical Centre, Amsterdam, The Netherlands, between 1985 and 1996.

There is increasing evidence that the accumulation of activating mutations in proto-oncogenes and inactivating alterations of tumour-suppressor genes underlies the multistage process of tumour genesis and progression of malignancy\(^ {92,93}\). This has been best established in colorectal tumour genesis, largely as the result of work by Vogelstein and coworkers\(^ {94-96}\). Under normal circumstances, proto-oncogenes encode proteins that stimulate cell growth and proliferation. Mutation within proto-oncogenes results in an increased activity of the gene product and enhanced cell growth\(^ {97}\). These activated proto-oncogenes are called oncogenes and the \textit{K-ras} gene is one of the most extensively investigated oncogenes in a wide variety of human tumours. The incidence varies strongly among the different tumour types. The highest incidence is found in pancreatic cancer (75-100\%)\(^ {98-102}\). Most studies have concentrated on the extrahepatic biliary system without distinction between tumours of the proximal, middle or distal third of the common bile duct and so far, only 9 mutations were reported in 25 DBDCs overall\(^ {33,98-101}\). The purpose of the study described in Chapter 5 was to address the incidence of mutations in codon 12 of the \textit{K-ras} oncogene in 47 patients who had undergone a resection for DBDC at the Academic Medical Centre, Amsterdam, The Netherlands between 1985 and 1996, and to investigate its prognostic and diagnostic value.

\textit{P53}, located on the short arm of chromosome 17, encodes a protein with known tumour-suppressor activity\(^ {103-106}\). Normal (wild-type) p53 protein mediates a pathway that either permits damaged DNA to be repaired before DNA replication or pushes cells into apoptosis thereby eliminating substantially damaged cells\(^ {103-105,107,108}\). P53 is therefore also regarded as the guardian of the genome. Mutations of this gene are the most common
Chapter 1

Genetic alterations in human cancers resulting in a protein with altered conformation and prolonged half-life\(^{103,105,109,111}\). Mutant p53 protein, which is therefore detectable by immunohistochemical staining, loses its function as cell cycle checkpoint, thereby allowing considerable genetic damage. Since the dysfunction of mutated p53 is apparently reflected by its end product, examination of the end product might provide biological relevant information. In terms of clinical correlation, the incidence of mutant p53 protein is cancer type- and tissue-specific\(^{112-114}\) and some studies have reported a correlation between p53 protein overexpression and poor prognosis\(^{40,41,65,115}\), while others found no correlation\(^{37,113,116}\). Up till now, such study has not been performed in DBDC. The clinical significance of p53 expression in resected DBDC is investigated in Chapter 6. Forty-seven archival tumour samples of patients with DBDC, who underwent subtotal pancreatoduodenectomy from 1985 to 1996 at the Academic Medical Centre, Amsterdam, The Netherlands, were immunohistochemically examined for p53 protein overexpression, using the anti-p53 antibody D07.

Comparative genomic hybridization (CGH)\(^{117-121}\) is a technique that provides a global view of the relative gains and losses of chromosomal DNA that have occurred during tumour development and progression. Chromosomal regions that are consistently lost may highlight locations of putative tumour suppressor genes, whereas gains or amplifications may identify chromosomal regions were oncogenes are activated\(^{122}\). This is the case because tumour suppressor genes act as recessive genes and only when both allelic copies of the gene are disrupted, the tumour suppressor gene will lose its function\(^{103,105,123-125}\). The most common mechanism is loss of one allele accompanied by mutation of the gene on the other allele. Amplification of an oncogene, which acts dominant, is a common mechanism for their activation\(^{124-126}\). In the recent literature there are indeed some examples of the utility of CGH in identifying the locus of a candidate oncogene or tumour suppressor gene\(^{127}\). Because structural rearrangements such as inversions and translocations are not detected by CGH\(^{118}\), this technique is complementary to other techniques such as karyotyping for detection of genetic changes in cancer cells.

Recently, CGH has been applied increasingly, contributing significantly to our understanding of cancer genetic mechanisms and the analysis of the biological basis of the tumour progression process. The genetic background of sporadic DBDC, however, is poorly characterized. The aim of the study described in Chapter 7 was to identify candidate regions for genes that are involved in the tumour genesis of DBDC and its progression. Genetic aberrations were determined by comparative genomic hybridization in seven xenografts and one fresh frozen sample of distal bile duct carcinomas. In addition, 13 DBDCs were characterized by karyotype analysis. All the primary adenocarcinomas of the distal bile duct were obtained from surgical pancreatoduodenal resections (Whipple procedure) performed at The Johns Hopkins Hospital, Baltimore, USA, between September 1992 and May 1996.
Chapter 8 offers a summary of the main results of this thesis and a general discussion with respect to clinical implications and future studies.

REFERENCES


