Chapter 8

Summary and General Discussion
SUMMARY

This study of molecular events in distal bile duct tumour genesis gives further insight into the pathogenesis and progression of one of the most fatal human cancers. In the present thesis we have investigated parameters that contribute to defining the biological profile of DBDC.

Chapter 1 provides an overview of the epidemiology, symptoms and therapeutic options of distal bile duct carcinoma (DBDC). Different, currently available prognostic factors in DBDC were reviewed.

Most human neoplasms contain detectable chromosome abnormalities and this, of itself, is strong evidence for neoplasia. Flow cytometry (FCM) offers the possibility to measure the DNA content of a large population of cells in solid tumours and in dispersed cells, whereas image cytometry (ICM) has the capability to analyse the DNA content of individual cells rather than large populations. The aim of the study described in Chapter 2 was to determine the role of FCM combined with ICM in effusions of a variety of patients in conjunction with the more conventional, but time proven, cytologic technique and to determine its utility. One hundred twenty-six effusion samples from 102 patients were examined by cytology and FCM. FCM revealed an aneuploid peak in 20 (56%) of the 36 malignant cases determined by cytologic examination. ICM performed on the malignant cytologic cases with a diploid flow pattern detected two additional aneuploid peaks. In addition, FCM indicated three aneuploid cases in which cytologic characteristics were initially interpreted as benign (false negative). Aneuploidy was therefore detected in 64% of the malignant effusion specimens by FCM and ICM. Twenty-three of the total of 24 aneuploid cases detected by FCM were associated with malignancy (predictive value=96%), there was one false-positive case with FCM. Aneuploidy by itself is therefore not a conclusive sign of malignancy. FCM is an excellent tool when moderate to large numbers of tumour cells are present, whereas use of ICM is advantageous for specimens containing smaller numbers of malignant cells because these can be directly analysed. When an aneuploid peak is present, a diagnosis of malignancy must be suspected, and, if the initial cytological screen is negative, a critical review of the cytology slides is justified. In some malignancies the tumour cells will be diploid (in this study 36%) and neither FCM nor ICM will add to tumour detection.

DNA content has gained importance as an independent prognostic variable in a number of tumours and can be assessed by FCM. Proliferation is another feature of tumours that has been used to determine their malignant potential. Ki-67 antigen expression provides a direct measure of the growth fraction of tissue and is an accurate indicator of cell proliferation. In Chapter 3, the prognostic value of cell proliferation (Ki-67 antigen) and DNA content was investigated in 35 patients with DBDC who underwent a
pancreatoduodenectomy between 1985 and 1992. Microscopically radical resection margins were achieved in only 54% of patients reflecting the aggressive nature of this malignancy. A significant correlation was found between decreased survival time and the extent of nuclear reactivity with Ki-67 (p=0.035), using a cut-off point of 20%. Flow cytometry revealed a 35% aneuploidy rate, but, no correlation of DNA-ploidy with survival time could be found (p=0.62). Thus, Ki-67 antigen proved to be an additional parameter to predict more reliably the behaviour of this kind of tumour in the individual patient, confirming the hypothesis that fast growing cancers are more rapidly fatal than those that are slow-growing.

In Chapter 4 we have shown in a comparative study that approximately 59% of DNA histograms in DBDC contained aneuploid cell populations as documented by flow cytometry (FCM) and image cytometry (ICM). Both, FCM and ICM, were complementary methods in this study to identify aneuploid tumour populations. When results could be compared, a moderate strength of agreement (Kappa=0.45) was demonstrated. We detected more cases of aneuploidy with ICM than FCM in formalin-fixed, paraffin-embedded DBDCs, 62% versus 33%, respectively. Most discrepancies between FCM and ICM were due to the dilution of aneuploid populations by non-neoplastic diploid cells, which are common in DBDC. ICM showed a higher sensitivity for detection of minor DNA-aneuploid cell population, and FCM for detection of near-diploid tumours. DNA-ploidy assessment in DBDC, unfortunately, did not offer the possibility to improve the ability to predict survival. Attempts to correlate DNA-ploidy with decreased patient survival in DBDC have not been successful in our studies. We therefore think that routine determination of ploidy values in DBDC will not alter current clinical practice.

K-ras codon 12 is the predominant type of oncogene mutation in various tumours. Thus far, only limited data are available precluding a systematic correlation between the incidence of K-ras mutations and prognosis in DBDCs. In Chapter 5 we describe a study to address the incidence of K-ras codon 12 and to investigate its prognostic and diagnostic value in resected DBDC. A polymerase chain reaction (PCR) was used to detect mutations in 47 patients who had undergone resection for DBDC. The PCR mismatch amplification technique demonstrated that 35 (75%) of the 47 tumours harboured a point mutation in codon 12 of the K-ras oncogene. Patients with mutated tumours had no statistically different survival time compared to those patients without a mutation in the tumor (p=0.34). If negative microscopic margins were obtained after resection, survival was significantly improved (p=0.005). The finding that a high incidence of the tumors in this study harbor a K-ras codon 12 mutation indicates that activation of this oncogene must be an important event in distal biliary tract carcinogenesis. Although K-ras codon 12 mutations have no value as a prognosticator, it may prove to be useful in the diagnosis and early detection of these tumors. Because of the limited amount of patients studied, the results of this analysis must be viewed with
caution. The results of this study also suggest that the state of the surgical resection margins remains the mainstay of prognostication in resectable DBDC.

The most common genetic alteration in human cancers is mutation of p53. The product of p53 mutations, mutant p53 protein, disrupts critical growth-regulating mechanisms and can be detected rapidly by routine immunohistochemical staining. Chapter 6 focuses on the incidence and prognostic value of mutant P53 expression in resected DBDC. Nineteen (40%) of the 47 tumours investigated demonstrated positive (>30%) p53 protein immunostaining. Focal or negative staining was seen in the remaining 28 (60%) cases. Overall, the detection of P53 overexpression showed a clear association with survival (p=0.039). In fact, the median survival more than doubled when less than 30% p53 positivity was present. P53 overexpression was independent of sex, tumour size, radicality of resection, histopathological grading, lymph-node status, perineural invasion and vasoinvasive growth. This study indicates that low (<30%) or negative p53 overexpression is a favorable prognostic factor in patients with resected DBDC. Aberrant p53 expression (>30%), on the other hand, is associated with the biologically more aggressive DBDC.

Comparative genomic hybridization (CGH) is based on hybridization of differentially labelled tumour and normal DNA on normal chromosome spreads. This technique has the advantage of providing a global view of DNA increases (amplifications) and DNA decreases (deletions) that have occurred anywhere in the genome, in stead of targeting only one locus at a time.

Because the somatic genetic changes in sporadic DBDC are poorly characterized, we used CGH and cytogenetics to identify genes that are involved in the tumourigenesis of DBDC and its progression (Chapter 7). Genetic aberrations were determined by comparative genomic hybridization (CGH) in seven xenografts of DBDCs and one fresh frozen DBDC. In addition, 13 primary DBDC were analysed using cytogenetics. When the results of both methods were combined, the gains were most prevalent on chromosome regions (in decreasing order) 8q and 20q (6 tumors each), 12p, 17q and Xp (5 tumors each), 2q, 6p, 7p, 11q, 13q, and 19q (4 tumors each). The most frequently lost regions were 18q (8 tumors), 6q and 10p (7 tumors each), 8p, 12q and 17p (6 tumors each), 7q, 12p, and 22q (4 tumors each). In conclusion, this study represents the first analysis of DBDC by CGH and cytogenetics, and illustrates the large genetic variability of DBDC and the large amount of changes per tumor. Gain of chromosome 8q and 20q and loss of 18q were the most common amplifications and deletions respectively and are likely involved in the pathogenesis of DBDC.
Today, the state of the surgical margins is used as the main postoperative indicator of prognosis, but the addition of genetic markers could further help to characterise the tumour phenotype and thereby guide the treatment. Prognostic factors will only become clinically significant when they can be applied in clinical decision-making. Although it is encouraging that Ki67 and p53 protein overexpression in this study give a guide to prognosis, its use to predict the outcome for individual patients should be used with caution. Ideally, the clinical use of prognostic factors should be assessed in prospective studies, before application to distinguish between major surgery or medical treatment. The presence of an aneuploid population or K-ras mutation did not predict for an increased stage or reduced survival of patients with DBDC in our series. However, identification of K-ras mutation as a crucial genetic alteration in this study could permit development of molecular-based diagnostic tools.

Other gene regions also undergo mutation in DBDC, but additional studies are needed before definitive conclusions can be made with respect to their importance in the pathogenesis of DBDC. Among the wide spectrum of genetic aberrations we found in advanced DBDCs, using CGH and karyotype analysis, the most interesting ones are presumably those that occur in premalignant and pre-invasive lesions. A major limitation in the analysis of genetic changes in the early stage of tumour genesis is that premalignant lesions usually constitute a small fraction of cells. In the premalignant phase of DBDC, these cells can only be obtained during endoscopic retrograde cholangiopancreatography. By using PCR based methods it may be possible to detect the premalignant genetic aberrations in the near future, which might be helpful in evaluating the malignant potential of the DBDC in the individual patient.

Our results shed some light on the sequence of carcinogenic events involving K-ras and p53 protein overexpression. Purely based on the incidence rate, it can be argued that K-ras mutations may happen earlier during distal bile duct carcinogenesis than p53 protein overexpression. It is tempting to speculate that this alteration of a growth-promoting gene is presumably essential in strongly stimulating the proliferation of the distal bile duct tumour cells, increasing the risk of subsequent genetic errors. Subsequent overexpression of p53 oncoprotein might be an additional trigger in progression to genomic instability and a more aggressive phenotype.

The results from this study indicate a striking biological analogy between DBDC and pancreatic carcinoma and support the hypothesis from Longnecker that the initiation and progression of both tumours may involve the same mechanisms.

To improve outcome, the development of new therapeutic strategies is of major importance in the treatment of these patients. In contrast to colorectal adenoma, the endoscopic identification and treatment of early neoplastic lesions of the distal bile duct is not a realistic option in the near future. Theoretically, a genetic approach to defects in tumor-suppressor genes is more feasible and the mutated p53 gene provides excellent targets for new adjuvant therapy. The development of drugs to mimic or restore the
tumour suppressor function of p53 or drugs to inhibit the acquired activity of mutant p53 (protein) are novel strategies that could have a therapeutic benefit\textsuperscript{2,4,5}. In this respect, the selective killing of cancer cells expressing high levels of mutant p53 protein and the inhibitory effect of wild type p53 gene on clonal growth of carcinoma cells are encouraging results from previous cell culture studies\textsuperscript{6-7}. In addition, blocking K-ras activity by inhibition of an enzyme called farnesyl transferase may be another treatment strategy\textsuperscript{8}, especially because K-ras codon 12 mutation is probably an important early event in DBDC carcinogenesis. Therefore, gene therapy or pharmacological therapy could be a promising first step towards a new therapeutic approach for treating patients with DBDC but its efficacy has to be determined in studies designed to address this issue.

REFERENCES