K-ras and p53 in cancer of the pancreas and extrahepatic biliary tract

Sturm, P.D.J.

Citation for published version (APA):
Summary

Chapter 1

Profound insight is an alternative source of information. It can be obtained through a thorough and detailed analysis, which together with data from the literature and practical experience, can lead to new understanding in the field of science.
The incidence of cancer of the pancreas and extrahepatic biliary tract is relatively low. In contrast, the mortality of these cancers is high. Pancreatic cancer is the fifth leading cause of cancer-related death in the United States.

Cancer and benign disease of the pancreas and extrahepatic biliary tract often lead to a similar symptomatology and overlapping results of imaging studies which can make their distinction difficult. To determine an appropriate therapeutic approach it is important to distinguish malignant from non-malignant disease. Therefore, in certain cases a tissue diagnosis is sought.

Histological sampling from the pancreatic head region is mostly not practical. To arrive at a definitive diagnosis, the emphasis lies on biliary cytology that can be easily collected during endoscopic retrograde cholangiopancreaticography (ERCP). However, although specificity of conventional light microscopic cytology is high, the sensitivity is only 30-40%.

The recent research on cancer genetics has revealed the importance of genetic alterations in the development of cancer. Clinical applications of this knowledge concerning diagnosis, therapy, prognosis, epidemiology and prevention are currently investigated.

In chapter 1 through 5, the value of the detection of alterations in the K-ras oncogene and p53 tumor suppressor gene for the diagnosis of cancer of the pancreas and extrahepatic biliary tract was evaluated.

Alterations in the K-ras and p53 genes are the most common genetic alterations in human cancer. Both can be detected relatively easy and are frequent in pancreatic carcinoma and cholangiocarcinoma, the 2 most important carcinomas of the region of the head of the pancreas. In all studies K-ras oncogene mutations were detected with a sensitive polymerase chain reaction method. Standard immunochemical procedures were used to demonstrate alterations in the p53 tumor suppressor gene.

Chapter 1 and 2 describe studies in which cytology specimens collected from post-surgical resection specimens were investigated. This gave us the opportunity to directly compare K-ras and p53 results of cytology and the primary lesion, and thus to gain insight in potential diagnostic use and possible determinants of sensitivity and specificity when used in a clinical setting. In chapter 3 through 5 the K-ras and p53 analyses were evaluated in clinical situations.

**Chapter 1**

Duodenal fluid is an attractive source to investigate as a substrate for diagnostic tests. It can be collected relatively easy and duodenal fluid is the forerunner of stool, which together with urine and blood, are the most accessible ‘human sources’ one can investigate. Bowel fluids affect morphology of cells, which makes duodenal fluid not suitable for conventional cytology. DNA, however, is robust and resistant to enzyme activity present in the bowel. Therefore, it is possible to detect cancer-specific alterations in the DNA of cells shedded from the primary tumor.

Despite the high frequency of K-ras mutations in the primary tumors, mutations were detected in the duodenal fluid of a relatively small proportion of the resection specimens with carcinoma. The sensitivity of K-ras mutational analysis was 25%, which does not exceed the sensitivity of conventional cytology. In all cases except one, mutations in the primary tumor and duodenal fluid were identical, indicating the tumor as origin of the alterations found in the duodenal fluid. K-ras mutations were not detected in duodenal fluid collected from cases without cancer, but the number of resection specimens without malignant pathology was too small to determine specificity.

Importantly, in some resection specimens without cancer, K-ras mutations were detected in non-malignant ductal hyperplasias, which has been described previously in other studies. The role of mucinous ductal hyperplasia in the development of pancreatic cancer is not well established. It is clear that not all
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these lesions necessarily progress to carcinoma, and the significance of finding ductal hyperplasias needs as yet to be determined.

The study at least demonstrates the potential of detecting genetic alterations in the duodenal fluid of patients with malignancy in the pancreatic head region harboring such genetic alterations and future developments may ultimately lead to their use in diagnosis.

Chapter 2

ERCP is an important diagnostic tool in patients with malignant biliary stenosis. During ERCP cells can be collected to provide a definitive cytopathological diagnosis.

Compared to conventional cytology (sensitivity 30-40%), K-ras mutational analysis of endobiliary brush cytology specimens promised to have a better potential for the diagnosis of cancer of the region of the head of the pancreas. K-ras mutations were detected in the brush cytology specimens in 63% of the resection specimens with carcinoma. The cytology specimens were also tested for the presence of p53 alterations, 56% showed positive p53 immunostaining. The combination of both methods raised the diagnostic yield to 81%. No K-ras or p53 alterations were found in the surgical specimens without cancer or in their corresponding brush cytology.

To determine factors with possible influence on sensitivity and specificity, the emphasis of this study was on the comparison of the findings in the cytology specimens and the primary tumors. In the majority of the cases the results of both surgical and cytology specimens were identical (88%). However, a substantial number of cases showed discrepant results. Discrepancy analysis indicated that intratumor heterogeneity and sampling error could lead to false negative results.

Although no mutations were found in the cytology specimens from cases without malignancy, K-ras mutations could be detected in mucinous ductal hyperplasia. Therefore, the presence of K-ras mutations in these lesions with undefined neoplastic properties was considered a potential source of false positive results.

Absence of alterations in K-ras or p53 in the primary tumor would be the most important determinant of sensitivity.

It was concluded that K-ras mutations as well as p53 alterations detected in endobiliary cytology were suitable diagnostic markers for the presence of malignant disease in the pancreatic head region. Their actual diagnostic value needed to be assessed in a 'real life' clinical setting in which their specificity could also be better evaluated.

Chapter 3

Endobiliary brush cytology specimens obtained during ERCP from 312 patients with extrahepatic bile duct stenosis were studied.

The sensitivities of conventional light microscopic cytology (36%) and K-ras mutational analysis (42%) for the diagnosis of extrahepatic bile duct stenosis were almost identical. However, conventional cytology was most sensitive for bile duct carcinoma, while the mutational analysis was most sensitive for pancreatic carcinoma. This is the result of the in many cases more direct brushing of carcinomas arising from the biliary tract as compared with pancreatic carcinomas, and the higher frequency of K-ras mutations in pancreatic carcinomas. As a result K-ras mutational analysis had a strong additive effect on the sensitivity of conventional cytology and the combination of both tests was clearly supplementary (sensitivity 62%).

Although K-ras mutations have been demonstrated in ductal hyperplasias, a lesion with unknown biology that can be present in the pancreas without malignant disease, the specificity was 89%. This is probably because cells from these ductal hyperplasias are rarely shedded and thus these cells are not captured with bile duct brushing during ERCP.

Thus, the specificity of K-ras mutational analysis was slightly lower than the specificity of conventional cytology of bile
duct brushings (98%), but the study showed that K-ras mutational analysis can be used as a diagnostic adjunct in patients with malignant extrahepatic biliary stenosis, especially in patients with pancreatic carcinoma.

Chapter 4

P53 immunocytoLOGY was performed on the endobiliary brush cytology specimens of 53 patients with extrahepatic bile duct stenosis. In all cases the immunocytoLOGY results were compared with immunohistochemistry of the primary stenotic lesion and with the conventional cytology outcomes.

Sensitivities of both p53 immunocytoLOGY (24%) and conventional cytology (29%) were low, but there was an increase of the sensitivity when the two tests were combined (43%). P53 immunocytoLOGY is more dependent on the number and quality of cells collected compared with PCR-based techniques in which a small amount of DNA is sufficient. This became apparent through the higher sensitivity of p53 immunocytoLOGY and conventional cytology for the diagnosis of bile duct carcinomas (40% and 46%) which are more directly brushed than pancreatic carcinomas (9% and 13%), whereas the frequency of p53 alterations in the 2 carcinomas was similar (53% vs. 48%).

Comparison of the results from immunocytochemistry of the cytology specimens with the histological specimens and the conventional cytology determined absence of p53 alterations in the primary tumor, sampling error, false negative staining, and possible intratumor heterogeneity as factors with a negative effect on the sensitivity of p53 immunocytoLOGY.

None of the cytology specimens from patients with benign disease showed positive immunostaining, but the actual specificity could not be assessed because of the small number of patients with benign disease. Important is the fact that there are no reports on p53 mutations in non-malignant lesions.

On the other hand, false positive p53 immunostaining of tissue does occur, mainly as a result of technical problems.

From this study we conclude that in individual cases p53 immunostaining of endobiliary brush cytology specimens, in addition to conventional cytology, may certainly be helpful in the diagnosis of patients with a malignant bile duct stenosis, in particular in those patients with cholangiocarcinoma.

Chapter 5

During preoperative laparoscopic staging for pancreatic head tumors or preoperatively, the liver is inspected for the presence of metastases. This is important to determine the therapeutic approach. In the presence of metastases, palliative treatment is generally the treatment of choice, while radical resection is intended in the absence of metastases. It is not always possible to distinguish liver metastases from benign bile duct proliferations on clinical grounds or even histologically.

It was investigated whether K-ras codon 12 mutational analysis can help to make this distinction by examining paired primary carcinomas and liver lesions from 48 patients. An additional 45 mostly incidental benign bile duct proliferations were examined to determine the prevalence of K-ras mutations in these lesions.

All metastases harbored K-ras mutations and more than 90% of the bile duct proliferations were wild-type. It was concluded that the K-ras mutational analysis might have additional value in the diagnosis of liver metastases. However, the presence of K-ras codon 12 mutations in some of the apparently benign liver lesions means that one cannot solely rely on this mutational analysis to determine further therapy. More specific molecular markers would therefore be needed.

In chapter 6 and 7 alterations in the K-ras oncogene and p53 tumor suppressor gene are used to address questions concerning epidemiology and pathogenesis.
Chapter 6

The frequency and type of alterations present in p53 and K-ras were compared in 2 groups of cholangiocarcinomas, one group coming from an area with a recently established high incidence of these carcinomas and one group existed of 'conventional' cholangiocarcinomas. Exogenous factors may play a role in the clustering of malignancies and carcinogens can lead to specific mutational patterns in cancer-causing genes. The purpose was to determine whether there was a molecular basis for the high incidence, which could indicate a different etiology and a common causative agent for the carcinomas from this high-incidence area.

Significantly more carcinomas from the high-incidence area were p53 immunohistochemistry positive which may reflect a different etiology. However, mutational analysis of p53 and K-ras did not show differences in type or frequency. The high incidence may be the result of a difference in carcinogenic dose or a different etiology that is not reflected in the alterations present in p53 and K-ras. However, the study provided no unequivocal molecular explanation for the carcinomas from this high-incidence area.

Chapter 7

Osteoclast-like giant cell tumors in the pancreas and liver are composed of infiltrating mononuclear cells and multinucleated giant cells. Their histologic origin is unclear. Conflicting data suggest both an epithelial and a mesenchymal origin. Also, the giant cells have been interpreted as an intrinsic tumor component as well as bone-marrow-derived monocytes recruited in the tumor.

To determine the origin of osteoclast-like giant cell tumors and its components, we examined 5 tumors in which atypical ductal proliferations were present, which is a precursor lesion of pancreatic duct adenocarcinoma. The 3 components (atypical ductal proliferations, infiltrating mononuclear cells, and giant cells) were separately analyzed for the presence of K-ras mutations and immunoreactivity to p53, epithelial markers, and histiocyte markers.

The high prevalence of K-ras codon 12 mutations in the osteoclast-like giant cell tumors as seen in pancreatic duct adenocarcinoma, and identical mutations in the atypical ductal proliferations and corresponding infiltrating mononuclear cells suggested an epithelial origin.

The same mutations in codon 12 of the K-ras oncogene were detected in the corresponding giant cells. Although these findings suggest an epithelial origin of the multinucleated giant cells, this is contradicted by a uniformly strong staining with the histiocyte/macrophage marker. The obvious explanation for this is that the giant cells are cells of the monocye lineage that have phagocytosed tumor cells of epithelial nature. This hypothesis was supported by morphologic observations and could have been supported by the absence of p53 overexpression in the nuclei of the giant cells, while present in the mononuclear epithelial component of the tumor. Unfortunately none of the five tumors analyzed showed any p53 accumulation.

In conclusion, osteoclast-like giant cell tumors must be considered of epithelial origin with recruited bone-marrow-derived multinucleated giant cells.

In this thesis, clinical applications of K-ras and p53 mutations, two common genetic alterations in cancer of the pancreas and extrahepatic biliary tract, were examined.

The studies suggest that alterations in the K-ras oncogene and the p53 tumor suppressor gene can be used in the diagnosis of cancer of the pancreas and extrahepatic biliary tract in addition to conventional light microscopic examination of cytological and histological specimens. In case of doubt about the etiology of an extrahepatic bile duct stenosis, the detection of K-ras or p53 alterations in endobiliary brush cytology is indicative for malignant disease in the region of the...
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head of the pancreas. It should lead to therapeutic consequences or to more rigid diagnostics and close follow-up of the patients. Unfortunately, the absence of K-ras mutations or p53 overexpression has no unequivocal meaning. Whether these molecular analyses are useful as routine diagnostic tests still has to be determined. It depends on the number of patients in which a diagnosis of malignancy based on these tests leads to an earlier diagnosis and subsequently to a better prognosis.

Because there is some overlap in the K-ras mutational spectrum of benign bile duct proliferations and pancreatic carcinomas, the diagnosis of a liver metastasis can not solely be based on the presence of a K-ras mutation. Additional markers are therefore needed before molecular diagnosis of liver metastases can be applied clinically.

Molecular biology could not provide evidence for a different etiology of the cholangiocarcinomas in the high-incidence area. However, the study in this thesis illustrates the possible use of molecular biology in epidemiology and in the identification of carcinogenic events in the future.

The debated epithelial origin of osteoclast-like giant cell tumors of the pancreas was supported by K-ras mutational analysis of the different components of these neoplasms. Genetic analysis of tumors can lead to insight into tumor pathogenesis and in certain cases this may have clinical implications.

The clinical significance of cancer genetics will be improved by the following. 1. New (molecular) techniques, such as quantitative PCR methods with a mutation threshold, will increase the objectivity and accuracy of molecular analyses [1]. 2. The identification of patient populations who will benefit from these sensitive techniques is important. 3. Molecular analysis of different patient materials must be explored. The detection of genetic alterations in plasma or serum DNA is promising in the diagnosis of patients with cancer, to establish their prognosis, and evaluate treatment [2-5]. 4. New insights into oncogenesis and the underlying genetic processes could identify more specific and more universal molecular markers and may lead to new therapeutic modalities [6-7].

The importance of molecular biology will increase in modern oncology with the development of new molecular techniques and the growing knowledge of cancer genetics.

References
