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

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CHAPTER 2

The Potential Diagnostic Use of K-ras Codon 12 and p53 Alterations in Brush Cytology from the Pancreatic Head Region


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ABSTRACT

It can be difficult to distinguish between malignant and benign disease of the region of the head of the pancreas using conventional methods. K-ras and p53 alterations occur frequently in malignancies in this region and are therefore candidate tumor markers. To define the utility of these alterations in interpreting pancreatic head cytology, the present study investigated first, to what extent alterations in the carcinomas were detectable in the cytology, and secondly, whether the alterations found in the cytology came from the carcinomas.

Fifty-seven consecutive pancreaticoduodenectomy resection specimens (52 with a malignancy and 5 without) and the ductal brush cytology specimens collected post-operatively from these resection specimens were compared for the presence of K-ras and p53 alterations. K-ras mutations were detected using the polymerase chain reaction and allele-specific oligonucleotide hybridization, p53 alterations using immunochemical staining for the p53 gene product.

After discrepancy analysis the results from the resection specimens and corresponding brush cytology specimens were identical in 88% for the K-ras analysis, and 84% for the p53 analysis. In 2 cases K-ras mutations found in the brush cytology specimens were not derived from the carcinoma but from pancreatic ductal hyperplasias. Intratumor heterogeneity and sampling error were also identified as causes for discrepant results. The 5 resection specimens without a malignancy and the corresponding brush cytology specimens were negative for both genetic alterations.

In conclusion, the detection of K-ras and p53 alterations in cells obtained from the pancreatic head region might be a valuable adjunct to conventional cytology for the diagnosis of malignancies in the pancreatic head region. However, intratumor heterogeneity, mucinous pancreatic duct hyperplasia harboring K-ras mutations and sampling error will hinder their diagnostic accuracy in routine clinical use.
Introduction

Both benign and malignant diseases of the region of the head of the pancreas may give rise to a similar symptomatology and it can be difficult to distinguish between them on clinical grounds, including diagnostic imaging techniques [1-3]. A firm diagnosis can be established with cytology collected from the pancreatic head region. Although the specificity of conventional cytology is high, the sensitivity is low [3,4]. This is primarily because it is difficult to distinguish malignant cells from reactive epithelial cells, but also because often only small numbers of cells are collected. More sensitive methodologies that could be applied to these cytology specimens would therefore be of use.

The high prevalence of K-ras oncogene and p53 tumor suppressor gene alterations in malignancies in the pancreatic head region, and the relative simple techniques by which they can be detected, make them attractive candidate tumor markers for the diagnosis of these malignancies [5-12]. Previous studies have shown K-ras and p53 alterations in material collected in various ways from the pancreatic head region, and found that these genetic alterations can be used as tumor markers with high sensitivity and specificity [13-20]. However, most of these studies were performed on small study groups, a definitive tissue diagnosis was often not available, and comparison of findings in the cytology specimens and in the corresponding carcinomas concerning these genetic alterations was often not included. These limitations make it impossible to determine the rate and causes of false positive and false negative outcomes. The absence of genetic alterations in the tumor, technical problems, intratumor heterogeneity, the presence of K-ras mutations in non-malignant lesions such as pancreatic duct hyperplasia, and sampling error all need consideration.

The possible use of tests based on the detection of K-ras and p53 alterations in cytology collected from the distal common bile duct for the diagnosis of a malignancy in the pancreatic head region depends on two critical questions: 1. To what extent are the genetic alterations present in the carcinomas detectable in these cytology specimens? 2. Are the genetic alterations detected in these cytology specimens derived from the carcinomas?

In this study, brush cytology specimens were collected under controlled conditions from a large series of consecutive pancreaticoduodenectomy resection specimens. This allowed us to compare directly the findings in the brush cytology with the findings in the corresponding resection specimens, thereby enabling us to specifically address the two above questions and ascertain possible causes of inaccurate outcomes.

Materials and Methods

Study Group and Tissue Collection

The study group consisted of resection specimens from 57 consecutive patients who underwent pancreaticoduodenectomy at the Academic Medical Center in Amsterdam between January 1994 and December 1995. Six patients who underwent pancreaticoduodenectomy in the same period were not included because sampling of the study material was insufficient. The mean age of the patients was 62 years (range 45-76). Twenty-seven were males. The histological diagnoses according to the World Health Organization classification were as follows: 34 ductal adenocarcinoma of the pancreas, 1 mucinous cystadenocarcinoma of the pancreas, 1 clear cell carcinoma of the pancreas, 10 adenocarcinoma of the ampulla Vateri, 1 signet-ring cell carcinoma of the ampulla Vateri, 5 adenocarcinoma of the distal common bile duct [21-23]. Five patients had no malignancy in the pancreatic head region (3 patients with chronic pancreatitis, 1 with gastrinoma, and 1 without histological alterations).

The distal common bile duct was brushed via the ampulla Vateri with an
endocervical brush (Cervibrush ™, CellPath, Hemel Hempstead, UK) immediately after arrival of the resection specimens at the pathology laboratory. Brush cytology smears were made for light microscopy and immunocytochemistry. The remaining cells were then suspended in 10 ml of a buffered saline solution (pH 7-8). The cell suspensions were fixed by adding 10 ml 96% ethanol and used for K-ras analysis. The resection specimens were fixed in buffered formalin overnight and tissue sections were paraffin-embedded for routine diagnostic histology, K-ras analysis, and immunohistochemistry.

Isolation of DNA
DNA was isolated from representative tissue sections, as well as from 1 ml of the brush cytology cell suspensions, using proteinase K [24]. Areas defined as malignant by light microscopy were microdissected from H&E-stained paraffin sections. Random areas of ductal epithelium were dissected when no malignancy was present.

Detection of K-ras Codon 12 Point Mutations
A two step, semi-nested PCR with mutant-enrichment was used to detect K-ras codon 12 mutations [6]. The PCR products are visualized by hybridization with wild-type-specific and mutant-specific radioactive labeled oligonucleotides followed by autoradiography [24]. Controls for amplification, contamination and specific and aspecific hybridization are included. Figure 2 is an example of an autoradiogram from this study. All analyses were performed in duplicate to exclude technical artifacts. Autoradiograms were evaluated without knowledge of diagnosis and other test results. DNA isolates from the brush cytology specimens were classified as K-ras mutation-positive if there was a mutant signal in the mutant-enriched PCR product and if the mutation was confirmed in the duplicate analysis. When the results from the first and second analysis were discrepant, a third analysis was performed to resolve the discrepancy. A similar classification was employed for DNA isolates from the resected carcinomas, but in that event a mutant signal also had to be visible in the non-enriched PCR product; in these cases, microdissection forms an enrichment step in favor of tumor tissue.

Demonstration of the p53 Gene Product
Immunocytochemical staining was performed using standard methodologies and the anti-p53 rabbit polyclonal antibody CM 1 (Novocastra Laboratories, Newcastle upon Tyne, UK). A representative 5 μm tissue section and a fixed brush cytology smear (Pro-Fixx, Lerner-Laboratories, Pittsburgh PA, USA) prepared from each resection specimen were immunostained. These were considered p53 immunocytochemistry (IC)-positive when there was specific nuclear staining of approximately 10% or more of the epithelial cells [11]. Immunocytochemical staining was evaluated by 2 independent observers without knowledge of any of the other results. A representative p53 IC-positive brush cytology smear is shown in figure 3.

Discrepancy Analysis
In various cases the findings in the brush cytology specimens and corresponding resection specimens were discrepant after initial analysis. Specific causes of the discrepancies were evaluated as follows.

ALTERATIONS IN THE BRUSH CYTOLOGY SPECIMEN BUT NOT IN THE PRIMARY CARCINOMA OR DIFFERENT FROM THOSE FOUND IN THE PRIMARY CARCINOMA
- Intratumor heterogeneity
  All additionally available tissue blocks of the primary carcinoma were analyzed for K-ras or p53 alterations. Intratumor heterogeneity was considered present when 1 or more of the additionally tested tissue blocks showed the previously unexplained alteration found in the corresponding brush cytology specimen.
- Pancreatic duct hyperplasia harboring K-ras mutations
  If intratumor heterogeneity could not be demonstrated for K-ras mutations and
K-ras and p53 in endobiliary brush cytology

Table 1 shows the histological diagnosis and the results of the analyses for K-ras and p53 alterations in the resection specimens with malignancies. K-ras codon 12 point mutations were found in 37 of the 52 carcinomas (71%), and 31 of 52 were p53 IC-positive (60%). K-ras mutations were most frequently found in pancreatic carcinomas. The results of p53 IC were comparable in the three types of carcinoma.

Tables 2 and 3 show the results of the K-ras and p53 analyses of the brush cytology specimens in comparison with the results of the corresponding carcinomas. Thirty-three of 52 (63%) brush cytology specimens were K-ras mutation-positive and 29 (56%) were p53 IC-positive.

All 5 resection specimens without malignancy and the corresponding brush cytology specimens were K-ras mutation-negative and p53 IC-negative.

Concordance of the results from the brush cytology specimens and corresponding resection specimens, including the resection specimens without malignancy, was found in 49 of 57 cases (86%) for the K-ras analysis and in 47 of 57 cases (82%) for the p53 analysis.

Table 2 also shows the K-ras mutational spectrum found in the primary carcinomas and the brush cytology specimens. Five of the 6 possible permutations in codon 12 were detected. One primary carcinoma and 1 brush cytology specimen contained 2 different mutations. The most frequent mutation was a GGT to GAT mutation (glycine to aspartic acid).

K-ras and/or p53 alterations were present in the brush cytology specimens of 42 of 52 resection specimens with malignancy: 20 were positive for both K-ras mutation and p53 IC, 13 for K-ras mutation only, and 9 for p53 IC only.

Of all resection specimens with malignancy except 3 with pancreatic cancer, the brush cytology smears contained malignant cells. These results were used for the discrepancy analysis.
TABLE 2
K-ras analysis results from resection specimens with malignancy

<table>
<thead>
<tr>
<th>Mutations in brush cytology</th>
<th>Cys</th>
<th>Ser</th>
<th>Arg</th>
<th>Val</th>
<th>Asp</th>
<th>Ala</th>
<th>Gly1</th>
<th>Concordance</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>Arg</td>
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<td></td>
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<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Val</td>
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<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
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<td>17</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td></td>
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<tr>
<td>Ala</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gly1</td>
<td>2</td>
<td>3</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Concordance</td>
<td>1</td>
<td>8</td>
<td>7</td>
<td>20</td>
<td>1</td>
<td>15</td>
<td></td>
<td>44</td>
<td>52</td>
</tr>
</tbody>
</table>

1 Wild-type codon 12 (GGT) codes for glycine.
2 One resection specimen with glycine to cysteine and glycine to valine mutation; only mutation concordant with the one found in the brush cytology specimen is noted.
3 One brush cytology specimen with glycine to valine and glycine to aspartic acid; only glycine to aspartic acid mutation is noted.

TABLE 3
p53 IC results from resection specimens with malignancy

<table>
<thead>
<tr>
<th>Carcinoma</th>
<th>p53 IC-positive</th>
<th>p53 IC-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 IC-positive</td>
<td>25</td>
<td>6</td>
<td>31</td>
</tr>
<tr>
<td>p53 IC-negative</td>
<td>4</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>23</td>
<td>52</td>
</tr>
</tbody>
</table>

The results of the discrepancy analysis are summarized in Table 4. Discrepancies between the results of the brush cytology specimens and corresponding resection specimens were found in 8 cases for the K-ras analysis and in 10 cases for the p53 analysis.

Intratumor heterogeneity was determined as the source of the discrepancy in 8 cases. In 2 cases the source of the discrepancy was the presence of mucinous pancreatic duct hyperplasia harboring K-ras mutations. Sampling error appeared to be the cause in 3 cases. In the remaining 5 cases we were unable to establish the cause of the discrepant results.

Discussion

In more than 80% the findings in the brush cytology specimens and corresponding resection specimens were identical. Discrepant results were found in 8 and 10 cases for the K-ras and the p53 analysis, respectively.

Technical errors as a cause for discrepant findings were unlikely because all DNA analyses were performed in duplicate.

Intratumor heterogeneity is a well-recognized phenomenon in carcinomas [25]. It has been described at a variety of levels, including genetic alterations such as K-ras and p53 mutations [26-31], and we determined intratumor heterogeneity as the cause for discrepant results in 8 cases. Intratumor heterogeneity was identified as the source of discrepancy in 1 case with a K-ras mutation-positive brush cytology specimen and in 1 case with a p53 IC-positive brush cytology specimen from resection specimens with carcinomas initially negative for the alterations found in the brush cytology. In 1 ampullary carcinoma with a GGT to GAT mutation but K-ras mutation-negative brush cytology that contained malignant cells, the resection specimen consisted of invasive carcinoma next to
TABLE 4
Discrepancy analysis

<table>
<thead>
<tr>
<th>Cause of discrepancy</th>
<th>Alterations present in brush cytology, but not or different in carcinoma</th>
<th>Alterations present in carcinoma, but not in brush cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K-ras</td>
<td>p53</td>
</tr>
<tr>
<td>Intratumor heterogeneity</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K-ras mutations in hyperplasia</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Sampling error</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unknown</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

adematous mucosa. Separate micro-dissected parts of both carcinoma and adjacent adenoma were found to be K-ras mutation-negative. The 5 p53 IC-positive carcinomas with p53 IC-negative brush cytology specimens but unequivocal malignant cells in the light microscopy all contained distinct p53 IC-negative parts. The latter 6 cases illustrate that intratumor heterogeneity may cause false negative results.

In 2 cases, ductal hyperplasia harboring K-ras mutations was the cause of different K-ras mutations in the brush cytology specimen and the primary carcinoma [21, 32-36]. Mucinous pancreatic duct hyperplasia is frequently seen in a pancreas with a carcinoma. There is substantial evidence for a sequential change from hyperplasia to carcinoma [36-39], but the high frequency of hyperplastic epithelium in the pancreatic duct of autopsy cases without pancreatic neoplasms suggests that only a fraction of these lesions progresses to invasive carcinoma during life [32]. Therefore, the detection of hyperplastic lesions is not (yet) an indication for therapeutic intervention i.e. a surgical procedure, and the detection of K-ras mutations from such lesions should be considered as a false positive result. Although there was malignant disease present in the above 2 cases, it does show the potential of hyperplasias to give rise to false positive outcomes.

Sampling error can be caused by technical problems or by absence of tumor cells in the distal common bile duct. The latter may be the case because the tumor only compresses the bile duct without transmural infiltration or shedding of cells into the bile, as can be encountered in pancreatic carcinoma. Sampling error could be an explanation for discrepant results in cases with K-ras mutation-positive or p53 IC-positive carcinoma but with a negative brush cytology specimen. In 3 such cases, all pancreatic carcinoma, no malignant cells were detected by light microscopy and thus sampling error following the above mechanism may have been the cause.

In 5 cases, a certain cause for the discrepant results between the brush cytology specimen and the corresponding primary carcinoma could not be determined. Nevertheless, intratumor heterogeneity remains the most likely cause in 2 cases in which the brush cytology specimens from the resection specimens with K-ras mutation-positive carcinomas were negative for these alterations, because unequivocal malignant cells were seen with light microscopy. The same accounts for the 3 resection specimens with p53 IC-negative carcinomas and p53 IC-positive brush cytology specimens in which the p53 IC-positive cells could be identified as malignant cells by light microscopy. It is of course impossible to analyze every single cell of the whole tumor to rule out or establish intratumor heterogeneity as the underlying cause.
After discrepancy analysis, concordance rates increased to 88% (50/57) for the K-ras analysis and to 84% (48/57) for the p53 analysis. These high percentages support the conclusions of previous studies that the genetic alterations found in material collected from the distal common bile duct reflect the alterations present in malignancies in the head region of the pancreas, and thus might be useful as tumor markers [13-20].

However, because this study was performed on pancreaticoduodenectomy resection specimens, our results may not apply completely to brush cytology collected during ERCP. Brushing has more limitations during ERCP, a smaller brush is used and the manner in which the brushing is performed is less robust. This will lead to a lower yield of cells. Also the surgical manipulation of the resection specimens may have contributed to the larger number of malignant cells in the brush cytology specimens in this study. Indeed the brush cytology smears of 94% of the resection specimens with malignancy contained malignant cells, and this yield will not be acquired through ERCP brush cytology in the clinical situation. Therefore, the readings of the cytology in this study formed a check on the adequacy of sampling rather than a diagnostic test.

One would expect that at least the accuracy of the K-ras analysis would not be very dependent on the number of cells collected, because the PCR with mutant-enrichment enables the detection of at least 1 heterozygous mutant cell among 500 homozygous wild-type cells [39]. This assumption was supported by findings in ERCP brush cytology specimens that were available for K-ras mutational analysis from 14 patients included in this study. In all 14 cases the ERCP brush cytology showed identical results as the brush cytology specimens obtained from the pancreaticoduodenectomy specimens (7 were K-ras mutation-positive and 7 were negative).

Our results suggest that intratumor heterogeneity and sampling error will be of importance when routine clinical brush cytology is used for the detection of K-ras or p53 alterations for the diagnosis of malignancies in the region of the head of the pancreas. These two will lead to false negative outcomes. More importantly, pancreatic ductal hyperplasias containing K-ras mutations are also potential confounders and will diminish the specificity of K-ras mutations.

Our study does not allow an estimation of the occurrence and detection of K-ras or p53 alterations in brush cytology specimens from patients without malignant disease, because only 5 resection specimens without malignancy were included. Such data, however, are essential before these tests can be applied clinically. Therefore, in order to estimate the specificity, in particular of K-ras mutations, one needs first to determine the prevalence of these alterations in brush cytology specimens coming from a larger group of persons without malignant disease in the region of the head of the pancreas [40]. In contrast to K-ras mutations that can be found in pancreatic ductal hyperplasias, it is our experience that precursor lesions are rarely p53 IC-positive, and we have only observed such positivity in carcinoma in situ at the earliest [11]. Thus, whereas K-ras maybe (overly) sensitive, p53 seems a more specific marker. On the other hand, in colonic tumors with a proven homozygous absence of a functional p53 gene, we have seen IC positivity on rare occasions due to the employed procedures [41]. Careful attention to methodology is thus warranted.

Unfortunately, the absence of K-ras and p53 alterations in a substantial number of the carcinomas will limit their sensitivity but both tests were of additional value to one another. A similar percentage of resection specimens with malignancy had brush cytology positive for K-ras mutation or p53 IC, 63% and 56% respectively, but with both tests combined this percentage raised to 81%.

In conclusion, K-ras codon 12 point mutations and the overexpression of the p53 gene product were demonstrated in the ductal brush cytology specimens in a large percentage of pancreaticoduodenectomy resection specimens with car-
References


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