Clinical significance of molecular markers in pancreatic cancer
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Pancreatic Cancer after Remote Peptic Ulcer Surgery

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ABSTRACT

Background
Peptic ulcer surgery may carry an increased risk for pancreatic cancer development. Molecular analysis of K-ras codon 12, frequently mutated in conventional pancreatic cancers, might provide insight into etiologic mechanisms.

Methods
The relative risk of pancreatic cancer was computed by multivariate and person-year analysis in a cohort of 2633 postgastrectomy patients. Lung cancer risk was analyzed to indirectly assess smoking behaviour. K-ras codon 12 mutational analysis was performed on 15 postgastrectomy pancreatic cancers.

Results
There was an overall increased risk of pancreatic carcinoma of 1.8 (95% C.I. 1.3-2.6) 5-59 yrs postoperatively that gradually increased to 3.6 after 35 years or more postoperatively (Chi-square test for trend, p<0.05). Multivariate analysis indicated that parameters other than postoperative interval did not influence the risk. Lung cancer risk was significantly increased after surgery, but no time trend was observed. The spectrum and prevalence of K-ras codon 12 mutations were comparable to conventional pancreatic cancer.

Conclusions
Remote partial gastrectomy is associated with an increased risk of pancreatic cancer. Postgastrectomy and non-postgastrectomy pancreatic cancers may share similar etiologic factors, like smoking. However, the neoplastic process in operated patients appears to be accelerated by factors related to the surgery itself.

Key words: pancreas cancer, postgastrectomy, peptic ulcer, epidemiology, K-ras codon 12
INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer-related death. This is mainly due to an extremely poor prognosis, because most cases are detected late in the course of the disease when the neoplasm has spread and curable resection is no longer possible. The identification of patients with an increased risk of pancreatic cancer may lead to a higher index of suspicion and to early detection when curative resection is still possible. Patients with a remote partial gastrectomy for benign ulcer disease may constitute a high-risk group for pancreatic cancer, although reports are conflicting. The mechanism by which partial gastrectomy may contribute to the development of pancreatic cancer is unclear, but an increased production of N-nitroso compounds and nitrosamines in the operated stomach is believed to play a role. These carcinogens may not only act locally, but also at sites distant from the stomach, and N-nitroso compounds and nitrosamines have been shown to produce adenocarcinomas of the pancreatic duct in animal models. In addition, increased levels of cholecystokinin after partial gastrectomy may also mediate pancreatic carcinogenesis. Finally, smoking is a major risk factor for both the development of pancreatic adenocarcinoma and for peptic ulcer disease, and peptic ulcer patients are smokers who continue to smoke after surgery.

Activating point mutations in codon 12 of the K-ras oncogene are among the most frequent genetic alterations in pancreatic carcinoma. Interestingly, K-ras codon 12 mutations appear more common in pancreatic carcinomas from smokers than in pancreatic carcinomas from patients who never smoked. Tobacco-specific nitrosamines induce carcinomas of the pancreas in rat models and generate G to A transitions at the second G of a GG pair of the ras oncogene, which is the predominant type of mutation found in pancreatic cancers in humans. Thus, analysis of the prevalence and type of K-ras codon 12 mutations in pancreatic carcinoma occurring after remote peptic ulcer surgery may provide etiologic clues.

We have been following a cohort of 2633 postgastrectomy patients who underwent surgery between 1931-1960, and in a preliminary analysis we observed an almost twofold increased risk for pancreatic cancer in these patients 20 years or more after their surgery.
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this cohort is still complete and 82% of the cohort has now died. Therefore, the present analysis was performed to investigate whether the previously suggested increasing trend for pancreatic cancer risk after peptic ulcer surgery could be further evaluated and definitively established. Specifically, we were interested in seeing if the longest survivors had a particularly high risk for pancreatic cancer. Furthermore, we performed analysis of codon 12 of the K-ras oncogene in 15 postgastrectomy pancreatic carcinomas in order to compare these molecular genetic alterations with the mutations encountered in conventional pancreatic cancer in non-operated patients.

MATERIALS AND METHODS

Study population:
The characteristics of the study population have been described previously 8. Briefly, the study population consisted of 2633 postgastrectomy patients who underwent surgery for benign conditions in the Academic Medical Center of the University of Amsterdam between 1931 and 1960. There were 2300 men and 333 women; 207 patients had a Billroth I, and 2343 a Billroth II and in 83 patients the type of gastrectomy was not specified. The indication for operation was a duodenal ulcer in 1683 patients, a gastric ulcer in 807 patients and was not specified in 143 patients. Patient data were collected in 1975 by a review of records of the departments of Pathology and Surgery. Surgical specimens of all partial gastrectomies were routinely examined by the pathologist. Therefore, this dual department review of records is thought to have yielded a virtually complete data set. Patients were traced using the Dutch population register system, in which every citizen has a unique registration card. This allows one to determine the underlying cause of death (in International Classification of Diseases codes) as registered on the official death certificate from the Netherlands Central Bureau of Vital Statistics 29.

Statistical analysis:
The computation of person-years at risk for pancreatic cancer started on January 1, 1935 because population mortality rates for pancreatic cancer were only reliably available after that date. The observation time was time from initial surgery to date of death, date of emigration, date of loss to follow-up, or to December 31, 1995, the closing date of the study. Person-years at risk according to sex and 5-years age categories from 10 years of age onwards were
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calculated using a Fortran computer program for cohort analysis. At 85 years of age patients were censored from the study because causes of death above this age were considered too inaccurate for comparison. Expected deaths were calculated by multiplying the number of person-years for each 5-year age and sex by the corresponding age-, sex-, and calendar time-specific death rates. These death rates were obtained from the Netherlands Central Bureau of Vital Statistics, the same source that provided the official cause of death for the deceased in the cohort. Pancreatic cancer deaths during the first 5 postoperative years were excluded because they might have included pancreatic cancer cases missed at the time of surgery. Standardized mortality ratios of observed over expected pancreatic cancer deaths, and 95% Confidence Intervals were calculated for each postoperative interval. One person less than 10 years of age and 5 persons who died at the time of operation were excluded. Multivariate analysis of the risk of pancreatic cancer in the cohort according to postoperative interval, sex, diagnosis and age at the time of surgery, and type of surgery, was performed assuming a Poisson distribution as described previously.

Since smoking is related to peptic ulcer (surgery) and pancreatic carcinoma, it is a potential confounding variable. Patient data of the postgastrectomy cohort were collected by review of the reports from the departments of Pathology and Surgery and information on smoking behavior was not reliably available from these sources. Therefore, the effect of smoking was indirectly assessed in the study population by computing the observed over expected mortality for lung cancer, as described above for pancreatic carcinoma.

A chi square test for trend was performed to estimate the change in relative risk for lung cancer and pancreatic cancer during the observation time since initial peptic ulcer surgery.

Molecular analysis:

Fifteen postgastrectomy pancreatic carcinomas were available for molecular analysis. These carcinomas were obtained from the archives of the Academic Medical Center and other hospitals in The Netherlands; some of the cases were obtained from The Johns Hopkins Hospital in Baltimore, USA. All tumors were primary cancers, the diagnosis was histologically proven in all cases. Tumor tissue was carefully microdissected from archival 5 μm H&E stained sections providing a sample in which at least 50% of the cells comprised the tissue of interest. DNA was extracted as described previously.
The protocol for the K-ras codon 12 mutational analysis was described earlier. DNA is subjected to PCR amplification using primers centered around codon 12. One of the primers introduces a restriction site in the PCR products derived from wild-type codon 12 alleles but not in those derived from mutant codon 12 alleles. Digestion of the PCR products is followed by a second round of PCR amplification, which then yields a PCR product enriched for K-ras codon 12 mutations. The resulting DNA products are denatured and dot-blotted onto nylon membranes and subjected to allele-specific oligonucleotide (ASO) hybridization with radioactive labeled probes, specific for each possible K-ras codon 12 mutation, followed by autoradiography.

Cell suspensions with mutant:wild type ratios of 1:100 and 1:1000 were used as positive controls in every PCR procedure. The suspensions were made of the human colon cancer cell line SW 480 with a homozygous GGT to GTT mutation at codon 12 of K-ras and the human colon cancer cell line HT 29 with wild type K-ras. Water was used as a control for contamination, placental DNA for non-specific hybridization. All PCR products were hybridized with oligonucleotides with the wild type sequence to control for amplification of the DNA samples. Enriched and non-enriched PCR products were dot-blotted next to each other to check for the digestion and the mutant-enrichment. We have previously validated the above mutant enriched PCR with ASO hybridization through comparison with sequence analysis.

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RESULTS

During the eight additional years of follow up since the previous analysis, another 361 patients died, two more patients emigrated (treated as censored data), while four other formerly emigrated patients returned to the Netherlands (included in the present analysis). At the closing date of the study (January 1, 1996) 462 patients were still alive. The official cause of death was obtained from all the 361 deceased patients. Eight additional patients died of pancreatic cancer totaling 39 pancreatic cancer deaths. Importantly, follow-up was complete for all but 8 patients who were excluded from the analysis. Detailed results from the person-year analysis are listed in Table 1.

Tabel 1. Observed and expected numbers of pancreatic cancer deaths in an Amsterdam cohort of 2633 postgastrectomy patients in consecutive postoperative intervals compared with the general Dutch population.
The period of follow up is 1935-1995, the age range of the patients is 10-84 years.

<table>
<thead>
<tr>
<th>years since gastrectomy</th>
<th>Number in group</th>
<th>observed pancreatic cancer deaths (o)</th>
<th>expected pancreatic cancer deaths (e)</th>
<th>ratio o/e</th>
<th>95% Confidence Interval</th>
<th>p value (2 sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>men and women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>2619</td>
<td>4</td>
<td>0.90</td>
<td>4.4</td>
<td>1.2-11.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5-14</td>
<td>2511</td>
<td>5</td>
<td>3.9</td>
<td>1.3</td>
<td>0.42-3.0</td>
<td>0.68</td>
</tr>
<tr>
<td>15-24</td>
<td>2087</td>
<td>8</td>
<td>6.1</td>
<td>1.3</td>
<td>0.56-2.6</td>
<td>0.54</td>
</tr>
<tr>
<td>25-34</td>
<td>1474</td>
<td>11</td>
<td>5.9</td>
<td>1.9</td>
<td>0.93-3.3</td>
<td>0.08</td>
</tr>
<tr>
<td>35-59</td>
<td>825</td>
<td>11</td>
<td>3.1</td>
<td>3.6</td>
<td>1.8-6.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5-59</td>
<td>2511</td>
<td>35</td>
<td>19.0</td>
<td>1.8</td>
<td>1.3-2.6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

A total of 35 pancreatic cancer deaths were observed 5 years or more after peptic ulcer surgery. The overall risk was statistically significantly increased for the complete 5-59 years postoperative interval (RR 1.8; 95% C.I. 1.3-2.6). The risk of developing pancreatic cancer increased continuously with increasing time since surgery, reaching a statistically significant level 35-59 years postoperatively. The trend test indicated a statistically significant increasing
trend (Chi square, 1 df=4.80 p<0.05). Data for both sexes were combined, because no
interactions by sex were noted, and the number of women was too small to make a
meaningful statement about them separately. The multivariate analysis indicated that
parameters other than postoperative interval, such as gender, type of surgical procedure, and
indication for the operation, did not contribute to the risk of pancreatic cancer. The observed
over expected mortality for lung cancer was increased in all post-operative intervals and the
overall mortality was also statistically significantly increased (Table 2).

**Table 2. Observed and expected numbers of lung cancer deaths in an Amsterdam cohort of 2633
postgastrectomy patients in consecutive postoperative intervals compared with the general Dutch
population.**

The period of follow up is 1935-1995, the age range of the patients is 10-84 years.

<table>
<thead>
<tr>
<th>years since gastrectomy</th>
<th>number in group</th>
<th>observed lung cancer deaths (o)</th>
<th>expected lung cancer deaths (e)</th>
<th>ratio o/e</th>
<th>95% Confidence Interval</th>
<th>p value (2 sided)</th>
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<td>men and women</td>
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<td></td>
<td></td>
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<tr>
<td>0-4</td>
<td>2619</td>
<td>8</td>
<td>7.3</td>
<td>1.1</td>
<td>0.5-2.2</td>
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<td>29.5</td>
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<td>1.2-2.1</td>
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<tr>
<td>15-24</td>
<td>2087</td>
<td>78</td>
<td>47.4</td>
<td>1.6</td>
<td>1.3-2.1</td>
<td>&lt;0.001</td>
</tr>
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<td>1474</td>
<td>80</td>
<td>48.9</td>
<td>1.6</td>
<td>1.3-2.1</td>
<td>&lt;0.001</td>
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<tr>
<td>35-59</td>
<td>825</td>
<td>49</td>
<td>27.2</td>
<td>1.8</td>
<td>1.3-2.4</td>
<td>&lt;0.001</td>
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<tr>
<td>5-59</td>
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<td>152.9</td>
<td>1.7</td>
<td>1.5-1.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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The trend test, however, was not significant (Chi square 1 df= 0.31 p>0.25). Figure 1 visualizes the pattern of change of the relative risk of lung cancer and pancreatic cancer during the follow-up period.

Figure 1.
Bar-diagram of the relative risks for pancreatic and lung cancer among postgastrectomy patients for different postoperative intervals. Trend test for pancreatic cancer is significant (Chi square, 1 df 4.80; p<0.05), whereas trend test for lung cancer is not (Chi square, 1 df 0.31; p>0.25).

Fig.1 Relative risks of postgastrectomy pancreatic cancer and lung cancer

<table>
<thead>
<tr>
<th>postoperative intervals</th>
<th>relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-14 yrs</td>
<td>1.5</td>
</tr>
<tr>
<td>15-24 yrs</td>
<td>1.5</td>
</tr>
<tr>
<td>25-34 yrs</td>
<td>2</td>
</tr>
<tr>
<td>35-59 yrs</td>
<td>3.5</td>
</tr>
</tbody>
</table>

P<0.05
Molecular analysis:

Of the fifteen postgastrectomy pancreatic carcinomas analyzed 10 (66%) showed a mutation in codon 12 of the K-ras oncogene, four were wild type and one could not be amplified. In 6 (60%) of the 10 mutations the normal GGT sequence (glycine) was mutated to GAT (aspartic acid). In 3 (30%) of the 10 mutations, it was mutated to GTT (valine) and in 1 (10%) it was mutated to CGT (arginine). (Figure 2).

Figure 2.
Representative autoradiograph of the K-ras codon 12 point mutation analysis.
Four nylon membranes, each hybridized with a different radioactive labelled oligonucleotide specific for the sequence of the wild type codon 12 (left) and the six possible mutations (3 of them depicted). On each membrane in the left lane the non-enriched PCR products are represented and in the right lane the mutant-enriched PCR products. co: hybridization controls, on each membrane cloned DNA fragments with a known codon 12 sequence complementary to the labelled oligonucleotides are used for the hybridization of that membrane. H2O: negative water control. pla: placenta DNA. 1 to 5: DNA isolated from postgastrectomy pancreatic cancers with mutations resulting in an amino acid change; sample 2: glycine to valine, samples 1, 3 and 4: glycine to aspartic acid, sample 5 has a wild type K-ras codon 12 sequence.
DISCUSSION

The increase in pancreatic cancer risk after gastric surgery for benign conditions is not well established. Several investigators have observed an increased risk of pancreatic cancer after gastrectomy for benign gastroduodenal disease 3-9, whereas others have not 10-14. An observation time of at least 20 years since initial peptic ulcer surgery appears to be the discriminating factor between these positive and negative studies.

In the present analysis the overall (5-59 years postoperative) pancreatic cancer risk of 1.8 (95% C.I. 1.3 to 2.6) confirms the prior preliminary results observed in our cohort 8. Furthermore, the present analysis provides insight into the pancreatic cancer risk after a longterm postoperative interval, i.e. more than 25 years after the initial surgery. The additional 8 pancreatic cancer deaths observed since our previous analysis all occurred 30 years or more after peptic ulcer surgery.

There is a trend of increasing pancreatic cancer risk with increasing time since surgery; in our cohort the risk gradually increases to a statistically significantly 3.6 fold of the expected rate after 35 years or more postoperatively. Multivariate analysis indicated that variables other than postoperative interval had no significant influence on the pancreatic cancer risk.

There are several putative mechanisms through which gastric surgery might enhance pancreatic cancer risk. The hypochlorhydric postoperative stomach provides an environment in which nitrate reducing bacteria can proliferate. This leads to an increased formation of carcinogens such as nitrosamines and N-nitroso compounds, which can produce adenocarcinomas of the pancreatic duct in animal models 16 17. Interestingly, an increased pancreatic cancer risk was also observed in a cohort of patients with pernicious anemia, and atrophic gastritis, both conditions are accompanied by high gastric pH 35. Hepatic excretion of carcinogens into the bile and subsequent bile reflux into the pancreatic duct may lead to topical exposure to the carcinogens 36. Duodenogastrectomy reflux appears to enhance pancreatic carcinogenesis in animal models 9 18 37 38. An increased production of the polypeptide hormone cholecystokinin (CCK) may have a promoting effect under these circumstances. In the rat-azaserine model, CCK stimulates pancreatic carcinogenesis 39. In man, the CCK response to oral fat is increased in postgastrectomy patients compared to normal controls 40.
Although *H. Pylori* infection as a cause for peptic ulcer disease is firmly established \(^4^1\), it is unlikely that this microorganism plays a direct causative role in the carcinogenesis of the pancreas after peptic ulcer surgery. Recently, the presence of Helicobacter DNA in the bile of humans obtained by percutaneous transhepatic bile drainage was demonstrated \(^4^2\). In another study comparable seroprevalence rates for *H. Pylori* were found in both pancreatic cancer and gastric cancer patients, and were significantly higher than the seroprevalence in the control groups (colorectal cancer patients and healthy individuals) \(^4^3\). However, in a large proportion, at least 50%, of peptic ulcer patients *H. Pylori* is eradicated after peptic ulcer surgery, due to the bile reflux \(^4^4\) \(^4^5\). Alternatively, factors other than those directly related to the gastric surgery itself could play a role in the increased pancreatic cancer risk. Peptic ulcer patients in general are smokers and few quit smoking after surgery \(^7\). Smoking of cigarettes is the strongest established risk factor for conventional pancreatic cancer \(^4^5\) \(^1^9\) \(^2^4\). Unfortunately, detailed information on smoking behaviour in our study population is not available. Nevertheless, the observed increased lung cancer risk indicates that smoking in our cohort was more common than in the general Dutch population. The pattern in lung cancer risk after gastrectomy is, however, markedly different from the steadily increasing trend observed for pancreatic cancer risk (fig.1).

For lung cancer, the risk is increased after surgery from the beginning and remains constant throughout the postoperative observation period. Thus, although smoking may contribute to the increased pancreatic cancer risk after gastric surgery, the findings in our study suggest that factors related to the surgery itself may accelerate the neoplastic process subsequent to the surgical procedure. Indeed, Mills et al. (1988) observed a significant association between risk for pancreatic cancer and a history of peptic ulcer surgery among adventists in a study that controlled for smoking. In a previous autopsy study with adjustment for smoking, we also observed a statistically significant association between pancreatic cancer risk and remote gastric surgery \(^6\).

The results of the molecular analysis in this study support the role of smoking as a contributing factor to postgastrectomy pancreatic cancer \(^2^8\). The frequency and specific types of K-ras codon 12 mutations in the 15 postgastrectomy pancreatic cancer samples is comparable with the frequency and types of mutations found in
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non-postgastrectomy pancreatic cancer 25-26, 46-52. The similarities in prevalence and types of mutation in the two groups suggest that similar carcinogens are etiologically important in both. Further analysis would be required to investigate whether the other genes involved in the development of pancreatic cancer also play a role in pancreatic cancers which develop after remote partial gastrectomy for ulcer disease. Pancreatic intraepithelial neoplastic lesions were also observed in the tissue surrounding the pancreatic carcinomas in the postgastrectomy cases (figure 3).

Figure 3.
Example of a pancreatic intraepithelial neoplastic lesion, in this case papillary hyperplasia without atypia, observed in the tissue surrounding a pancreatic carcinoma in one of the postgastrectomy cases.

This indirectly supports a similar pathway for the development of infiltrating pancreatic adenocarcinoma in the postgastrectomy patients as the one postulated for conventional pancreatic cancer 53. An increased index of suspicion in the long-term postgastrectomy patient is therefore important.
In conclusion, an increased risk for pancreatic cancer exists in the postgastrectomy patients, particularly after a long-term postoperative interval. The underlying etiologic factors may be similar to those which play a role in the development of non-postgastrectomy pancreatic cancer, but the neoplastic process apparently takes place at an accelerated rate.

ACKNOWLEDGEMENTS

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