Mutation Status of K-ras, p53 and Allelic Losses at 9p and 18q Are Not Prognostic Markers in Patients with Pancreatic Cancer

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Abstract. Background: K-ras mutations and allelic losses of tumor suppressors p16 and DPC4 are perceived as potential markers for screening of pancreatic malignancy. In this study, molecular data is compared with survival statistics of the patients and whether they correlate with patients’ prognosis is questioned. Patients and Methods: Fifty three consecutive patients with advanced pancreatic cancer (stage III and IV) who underwent EUS-guided fine needle aspiration (FNA) were enrolled into the study (28 males, 25 females, 63±10.5 years). Samples were evaluated on-site for presence of malignant cells. DNA was extracted from Giemsa stained smears using laser microdissection, and mutation status of K-ras and p53 was tested by cycling-gradient capillary electrophoresis (CGCE). In addition, allelic losses of tumor suppressor genes p16 (INK4, CDKN2A) and DPC4 (MADH4, SMAD4) were detected by monitoring the loss of heterozygosity (LOH) at 9p and 18q loci. Molecular data were compared with survival statistics using Kaplan-Meier method. Results: The median survival in K-ras positive group was 7.0±2.4 months (95% CI 2.3-11.7) and in K-ras negative group was 10.0±0.6 months (95% CI 8.7-11.3). The median survival in p53 positive group was 10.0±2.2 months (95% CI 5.6-14.4) and in p53 negative group was 6.0±2.5 months (95% CI 1.1-10.9). The median survival in LOH 9p positive group was 9.0±5.1 months (95% CI 0-18.9), in LOH 9p negatives was 10.0±5.0 months (95% CI 0.2-19.8). The median survival in LOH 18q positive group was 10.0±4.2 months (95% CI 1.8-18.2) and in LOH 18q negative group was 3.0±1.3 months (95% CI 0.5-5.5). After the adjustment for age using Cox proportional hazards model, none of the evaluated molecular markers was shown to be an independent prognostic marker for survival of patients with pancreatic cancer. Conclusion: None of the studied molecular markers was identified as an independent factor determining survival prognosis.

Pancreatic cancer (PC) is considered one of the most lethal tumors of the human digestive system. Despite considerable progress in understanding the molecular background of pancreatic carcinogenesis in the last decade as well as novel diagnostic and therapeutic approaches, survival rate of patients remains generally poor (1). Mortality of the disease is almost at the level of its incidence as the majority of cases are diagnosed in an advanced, unresectable stage (2).

The development of pancreatic cancer follows a distinct path from normal ductal epithelia, pancreatic intraepithelial neoplasia (PanIN I-III) up to the carcinoma (3, 4). This path is accompanied by sequential accumulation of genetic changes (mostly point mutations, gene amplifications and allelic deletions). An early event in pancreatic carcinogenesis is activation of K-ras oncogene by somatic point substitution (5). This alteration can be detected already in PanIN-1A lesions as well as in chronic pancreatitis and therefore represents an independent risk factor for pancreatic cancer. In advanced pancreatic cancer, K-ras mutation is found in close to 90% of cases, and is therefore considered as a potential molecular marker for early detection of PC. Following the initial K-ras activation, a number of other genetic abnormalities take place. PanIN-1A and PanIN-1B phases are characterized by overexpression of Her-2/neu oncogene, which is found in 50% of pancreatic neoplasms. Increased Her-2/neu expression, however, is a result of higher transcription rate rather than gene amplification,
rendering Her-2/neu as an unsuitable therapeutic target (6). Aside from the above oncogenes, there is a number of tumor suppressor genes affected by genetic alterations during the PC transformation process. Among them, p16 tumor-suppressor (also referred to as CDKN2 or INK4), located at chromosome 9p21 is already inactivated in the transition from PanIN-1B to PanIN-2 phases (7). Furthermore deletion of another important tumor-suppressor gene, SMAD4 (known also as deleted in pancreatic carcinoma, DPC4), located at chromosome 18q21 has also been observed (8). Consequently, the p16 and DPC4 are inactivated in almost 95% (55% respectively) of cases of invasive PC, and are therefore suitable molecular markers.

The above mentioned genetic changes substantially affect control of the cell cycle, thus enabling defected cells to proliferate. Oncogene K-ras encodes for GTP-binding protein responsible for signaling in MAP-kinase pathway of intracellular signal transduction (9). Tumor suppressor gene p53 is translated into a product that regulates transcription of other regulatory proteins, such as p21, that are potent inducers of apoptosis in response to severe DNA damage. It exerts its regulatory activity at the G1/S interface of the cell cycle (10). The product p16 tumor suppressor binds to the complex of cyclinD/CDK4 or CDK6, and thus regulates cell cycle (10). The product p16 tumor suppressor binds to the complex of cyclinD/CDK4 or CDK6, and thus regulates progression of cell cycle at the G1 control point (11). Finally, the DPC4 tumor suppressor, a member of the SMAD protein family, exerts its function via the TGF-beta signalling pathway and inhibits cell cycle progression by inducing G1 arrest (12); it also regulates angiogenesis (13).

From the above progression model it is clear that the pancreatic malignant conversion comes from a combination of multiple genetic events rather than originating from a single mutation (3). Accordingly, no single molecular marker suitable for diagnosis of early stage of the disease has been identified. Given the inherent heterogeneity of the carcinogenic pathways, simultaneous examination of multiple markers should lead to improved testing efficacy. Therefore, gene microarray and protein profiling techniques hope to unveil significant genetic keys for early diagnosis, prediction of prognosis and estimate of the responsiveness to chemotherapy (14).

In a recent study (15), there has been an attempt to determine the diagnostic potential of molecular changes in K-ras, p53, p16 and DPC4. Patients with chronic pancreatitis and pancreatic cancer (n=101) who underwent endoscopic ultrasound guided fine needle aspiration biopsy (FNA) were examined. Sensitivity and specificity of separate molecular markers was 70% and 100% for K-ras (p<0.001), 24% and 90% for p53 (NS), 85% and 64% for LOH at 9p (p<0.001), and 78% and 57% for LOH at 18q (p<0.05), respectively. In search for an optimum combination of molecular markers of pancreatic malignancy it was found that screening for K-ras mutations along with loss of heterozygosity (LOH) at chromosomes 9p and 18q (sites of tumor suppressor genes p16 and DPC4) is a sensitive test of PC in patients with inconclusive finding on FNA cytology.

In this paper, the scope is further extended to question the relevance of K-ras and p53 mutation status as well as LOH at 9p and 18q as prognostic factors for disease survival. In addition, the correlation of the above molecular factors to the appearance of the tumor in endoscopic ultrasound (EUS) and/or serum levels of tumor markers CA 19-9 and CEA is evaluated.

### Patients and Methods

**Patients.** A total of 57 consecutive patients with advanced pancreatic cancer (stage III and IV according to WHO classification (16)) who underwent EUS-guided fine needle aspiration (FNA) were enrolled into the study. The diagnosis was proven by FNA cytology and histology of surgical specimens and/or long term follow-up. Five patients were excluded due to other diagnosis (adenoma, malignant fibrous histiocytoma, endocrine tumor, cholangiogenic carcinoma), or for malignant duplication (a patient with breast carcinoma).

Of the 53 patients there was a total of 28 (53%) males and 25 (47%) females. The mean age in the group was 63±10.5 years, range 40-84 years (+1.95/-2.25 standard deviation). All patients signed informed consent with participation in the study as well as with genetic analysis of their tissue material.

**Methods.** Evaluation of CA 19-9 and CEA serum levels was undertaken prior to invasive endoscopy (Table I). EUS was performed by a single experienced endoscopist using GFUM-20 radial and GFUCT-140 linear array scanning echoendoscopes (Olympus Europe) (Table II). FNA samples were stained using the rapid Hematoxylin and Eosin (H&E) staining technique, and evaluated by on-site cytologist to ensure that the potential tumor cells were present in the smear. Definitive FNA diagnosis was stated by a single pathologist, blinded to the EUS, after staining remaining samples by Giemsa. The same samples were subsequently submitted for genetic analysis. Laser microdissection of Giemsa-positive cells was performed on P.A.L.M. Microlaser instrument (Carl Zeiss, Germany). Normally, between 100 and 200 cells were dissected from each slide. Genomic DNA was extracted from the dissected cells by a spin-column extraction protocol using JetQuick Tissue DNA Isolation Kit (GENOMED GmbH, Loehne, Germany).

### Table I. Serum tumor markers

<table>
<thead>
<tr>
<th>Tumor marker</th>
<th>Normal</th>
<th>High</th>
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</thead>
<tbody>
<tr>
<td>CA 19-9</td>
<td>27 (82%)</td>
<td>6 (18%)</td>
</tr>
<tr>
<td>CEA</td>
<td>27 (82%)</td>
<td>6 (18%)</td>
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Note: normal serum level for CA 19-9 is 0-37 U/ml; normal serum level for CEA is 0-5.1 μg/l.
Presence of somatic point mutations in codons 12 and 13 of K-ras and in exons 5-8 of p53 was detected by GenoScan (Genomac, Prague, CZ), a high-sensitivity mutation detection technique based on heteroduplex analysis in temperature gradient (Table III, Figure 1) (17). Briefly, a PCR amplification of the target sequence containing the mutation hotspots was performed with one of the primers fluorescently labeled and the other primer extended by a 40 bp artificial high-melting domain (GC-clamp). Following PCR, the 100-140 bp fragment was heated and slowly cooled to allow formation of homo- and heteroduplex forms upon re-annealing of wildtype and mutant sequences. The resulting double-stranded fragments were subjected to capillary-electrophoretic separation at a cycling temporal temperature gradient.

Allelic losses at chromosomal positions 9p and 18q were uncovered by the loss of heterozygosity (LOH) analysis using a total of 3 microsatellite (STR) markers for 9q (D9S157, D9S171 and D9S1748) and 2 markers for 18q (D18S363 and D18S474) (Table III, Figure 2) (18, 19).

All capillary electrophoretic experiments including previously described temperature-gradient and fragment analysis were performed on a capillary-array DNA sequencer (MegaBACE™ 1000, GE Healthcare, Piscataway, NJ, USA) equipped with Caddy™ 1000 robotic sample loader (Watrex Praha, Prague, Czech Republic) for unattended overnight operation.

Statistical analysis. Survival statistics were calculated by estimation of Kaplan-Meier survival function and Mantel-Cox test for testing the equality of survival curves. Molecular data were compared with survival statistics by chi-square test in two-way frequency tables with Yate’s correction, where appropriate.

**Results**

The mean survival in the K-ras positive and negative groups was 8.4±1.3 months (95% CI 5.9-10.9) and 8.3±1.2 months (95% CI 5.9-10.7), respectively. The median survival was 7.0±2.4 months (95% CI 2.3-11.7) and 10.0±0.6 months (95% CI 8.7-11.3) in K-ras positive and negative groups, respectively. There was no statistically significant difference in survival between the two groups (p=0.636).
The mean survival in the p53 positive and negative groups was 11.5±2.6 months (95% CI 6.4-16.7) and 7.5±0.9 months (95% CI 5.6-9.3), respectively. The median survival was 10.0±2.2 months (95% CI 5.6-14.4) and 6.0±2.5 months (95% CI 1.1-10.9) in p53 positive and negative groups, respectively. The difference was statistically significant (p<0.05).

The mean survival in LOH 9p positive and negative groups was 8.8±1.5 months (95% CI 5.9-11.8) and 7.4±2.4 months (95% CI 2.8-12.1), respectively. The median survival was 9.0±5.1 months (95% CI 0-18.9) and 10.0±5.0 months (95% CI 0.2-19.8) in LOH 9p positive and negative groups, respectively. There was no statistically significant difference in survival of the two groups (p=0.509).

The mean survival in LOH 18q positive and negative groups was 9.6±1.6 months (95% CI 6.4-12.7) and 6.6±2.2 months (95% CI 2.2-10.9), respectively. The median survival was 10.0±4.2 months (95% CI 1.8-18.2) and 3.0±1.3 months (95% CI 0.5-5.5) in LOH 18q positive and negative groups, respectively. There was no statistically significant difference in survival of the two groups (p=0.311).

After the adjustment for age using Cox proportional hazards model, none of the evaluated molecular markers was shown to be an independent prognostic marker for survival of patients suffering from pancreatic cancer (Table IV, Figures 3-6).

As a secondary aim, the possible correlation between occurrence of gene mutations and EUS image of tumors and/or levels of serum tumor markers CA 19-9 and CEA was questioned (Table V). There was no difference in overall survival of patients with tumors <30 mm and ≥30 mm. No correlation between tumor size and frequency of gene mutations and/or serum levels of CA 19-9 and CEA was found. EUS signs of angioinvasion, infiltrative growth pattern and cystic component were not independent prognostic
factors regarding overall survival; neither did they correlate with frequency of gene mutations and/or serum levels of CA 19-9 and CEA. No difference in overall survival of patients with and without EUS signs of chronic pancreatitis was observed. However, absence of chronic inflammatory changes in EUS correlated with high frequency of LOH at 18q (site of DPC4) \((p<0.05)\). A statistically significant correlation between EUS signs of chronic pancreatitis and high serum CEA was detected \((p<0.05)\). All patients with EUS signs of chronic pancreatitis deceased within 10 months (NS).

**Discussion**

K-ras is a specific marker of PC with prevalence estimated to reach 90%-95% \((3, 5)\). Its activation by somatic point mutation occurs early in pancreatic carcinogenesis, however, most papers published in recent years have failed to prove its association with patient prognosis. Kawesha *et al.* did not find any overall association with survival in the group of 157 patients following resection, however, there was a significant difference in survival according to the type of K-ras mutation.
The clinical potential of K-ras consists in differentiating benign from malignant lesions in EUS-FNA samples rather than in estimating patients’ prognosis (21). Although the majority of studies proved that there is no association between p53 mutation status and patient survival, some scholars found a significant correlation of p53 mutation with decreased survival (22-26). Surprisingly, the presented results initially indicated an opposite correlation when the group with mutated p53 demonstrated significantly higher survival than the non-mutated group. After adjustment for age, however, no significant correlation between p53 mutation status and survival was found.

Loss of p16 expression appears to be an early event in pancreatic carcinogenesis; it is described in up to 98% of PC (7). Genetic mechanisms underlying defected expression of p16 are not only point mutations and allelic deletions, but also aberrant promoter methylation by which epigenetic mechanisms may contribute to pancreatic oncogenesis (27, 28). The majority of published papers have proven its association with decreased survival as well as advanced stage of the disease (29). In the present study, however, in none of the cases did LOH at a particular chromosomal site correlate with survival, nor did additional testing of p16 somatic mutations (data not shown). It appears that other mechanisms, e.g. epigenetic inactivation, may play a more significant role in the case of the p16 gene.

Aberrant expression of DPC4 gene was described in 55% of PC. As noted above, it is a late event in the process of PC development. Results published on DPC4 as a...
potential prognostic marker in PC are conflicting. It seems logical that preserved DPC4 expression would correlate with longer survival, as Tascilar has shown in patients after surgical resection (30), however, other papers failed to confirm this. Yatsuoka studied LOH at selected chromosomal loci in association with clinicopathological features of patients; LOH 18q was identified as a marker of poor prognosis (31). In the present cohort, no correlation between LOH at site of DPC4 gene and patient survival was found. It has been shown that LOH at the DPC4 locus may not be an obligatory event in SMAD4-dependent tumorigenesis (32). The prognostic relevance of loss of DPC4 function is equivocal; on the other hand, preserved DPC4 expression is correlated with better prognosis of the patients with PC (33).

Serum glycosylation marker CA 19-9 is a commonly accepted antigen to assess prognosis and monitor response to therapy (34). However, it is not recommended for diagnostic purposes (35). In the present cohort, no correlation between value of CA 19-9 at time of diagnosis and survival was observed. The fact that elevation of CA 19-9 is dependant on tumor differentiation and extent of PC, makes this marker unsuitable for detecting small and early tumors (36-38). Its prognostic significance was examined by the following cut of values: preoperative value of less than 200 U/mL, decrease in CA 19-9 level after surgery, or significant reduction in CA 19-9 levels after chemotherapy (39, 40). The problematic use of CA 19-9 in clinical praxis is also potentiated by the fact that 5-14% of the population is unable to synthetise CA 19-9 as they lack the Lewis antigen glycosyltransferase (41).

The spotlight in biomarkers is now directed onto other molecules such as the previously known mucines. Using novel immunoassays, MUC-1 was shown to be superior to CA 19-9 in distinguishing cancer from normal tissue and even from pancreatitis (42). MUC-4, abnormally glycosylated tumor-associated mucin, was detected in tumor samples and cell lines, whereas it was negative in chronic pancreatitis and normal pancreas tissues (43). Its value in the clinical setting is, however, to be questioned.

In conclusion, none of the studied molecular markers was identified as a prognostic marker in this study. In light of conflicting results reported in the literature, such finding only further highlights the complexity of pancreatic carcinogenesis, its interaction with cytostatic therapy agents as well as other exogenous effects yet to be uncovered. The genetic predetermination of the pancreatic cancer susceptibility on the one hand, and the therapy response on the other, define the patients’ overall survival. In the era of expression profiling techniques and whole genome scans, new biomarkers complementary to the existing DNA variations are to be unveiled. Only reliable markers (or multiple marker combinations) based on interactions between various signaling pathways will enable development of sensitive diagnostic tests for patients with suspicious pancreatic malignancy. In addition, such tools may also be applied to accurately estimate prognosis, and to identify candidates for chemotherapeutic agents as well as targeted biological or gene therapies.

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References


