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*RAPID COMMUNICATION*

# Differential c-erbB-1 and c-erbB-2 mRNA expression in cancer of the pancreas compared with cancer of the papilla of Vater

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## Abstract

**AIM:** We examined quantitative mRNA expression of growth factor receptors (c-erbB-1, c-erbB-2) and the anti-apoptosis gene survivin known to be regulated in pancreatic adenocarcinomas and compared the expression pattern with that in carcinomas of the papilla of Vater.

**METHODS:** Quantitative real-time reverse transcriptase-PCR (QRT-PCR, Taqman™) was performed to analyze mRNA expression levels of c-erbB-1, c-erbB-2 and survivin in normal and corresponding tumor samples of 31 pancreatic adenocarcinomas and 8 cancers of the papilla of Vater.

**RESULTS:** The overall median mRNA expression of survivin was significantly increased in both adenocarcinoma of the pancreas ($P<0.01$) and papilla of Vater ($P<0.008$) compared with uninvolved normal control tissue. In pancreatic cancer, expression of c-erbB-1 was significantly decreased compared with the normal pancreatic tissue ($P<0.03$), whereas in the cancer of the papilla of Vater expression of c-erbB-2 was significantly downregulated ($P<0.05$) compared with the paired normal samples. Gene expression was not associated with tumor stage, differentiation or prognosis.

**CONCLUSION:** The common anti-apoptosis gene survivin is overexpressed both in the cancer of the papilla of Vater and pancreas. In contrast, the growth factor receptor genes c-erbB-1 and c-erbB-2 are differentially regulated in both tumor entities adding further evidence that pancreatic cancer is biologically different from the cancer of papilla of Vater.

## INTRODUCTION

Adenocarcinoma of the pancreas has the worst prognosis of all cancers, with a 5-year survival rate less than 3%, accounting for the fifth highest number of cancer related deaths in North America and Europe with a rising incidence[1-3]. The only curative treatment for pancreatic cancer (PC) is complete resection which is possible in only 10-20% of patients[4] and associated with relatively constant 5-year survival rates of approximately 20% for the last 30 years[5,6,7]. The dismal prognosis and the lack of effective therapeutic regimens for this disease are attributed to several causes: (a) PC shows an aggressive biological phenotype, which is characterized by the early invasion of surrounding structures and rapid metastatic spread[8,9]; (b) radiation therapy and chemotherapy are reported to be effective only in selected patients after tumor resection[9-11]; (c) although various genetic alterations were identified, which have improved our understanding of the carcinogenesis of this disease, the responsible molecular mechanisms are largely unknown. New treatment regimens based on molecular classifications of the individual tumor may improve the outcome for patients with PC.

In contrast, cancer of the papilla of Vater (CPV) has a better prognosis with a 5-year survival rate of over 40% after curative resection[12,13]. CPV is a rare disease representing 6-12% of all periampullary malignancies[14] with an estimated incidence of 2.9 per million[15]. Because of its anatomical location, CPV becomes symptomatic at an earlier stage than PC. Therefore, the majority of the patients with CPV are candidates for surgical therapy. Evidence is increasing that differences in the tumor

biology of these two entities contribute to the different prognosis[16].

Survivin is a member of the inhibitor of apoptosis gene family, which is unique for its expression in a wide range of embryonic and fetal tissues, whereas almost no transcripts are detected in terminally differentiated normal adult tissues. In human pancreatic islets survivin expression is also developmentally regulated[17] . Survivin expression in human fetal islets was identified immunohistochemically in alpha and beta islet cells whereas adult pancreases showed a staining only in the alpha cells. However, it is re-expressed in several human cancers[18] and has an additional function in the regulation of cellular proliferation[19] and angiogenesis[20] of cancers. Survivin expression has been reported to be associated with poor survival in patients with colorectal cancer[21,22] and several other human cancers[23]. In a recent immunohistochemical study on 52 PC patients, increased survivin expression was strongly correlated to a higher proliferative index, nevertheless no correlation between survivin expression and survival of the patients was shown[24].

The epidermal growth factor receptor (EGF-R) family contains four structurally homologous transmembrane proteins with intracellular tyrosine kinase activity. The best-described growth factor receptors of this family are EGF-R also known as c-erbB-1[25] and the c-erbBs-2, 3, and 4[26]. They share a significant sequence homology and are frequently overexpressed in PC. Activation of the receptors leads to increased DNA synthesis, and changes in cell motility and cell metabolism[27]. EGF-R is activated by a family of peptide ligands that includes EGF, TGF-α, heparin binding EGF-like growth factor, betacellulin, and amphiregulin[28]. The EGF-R is encoded by the c-erbB-1 proto-oncogene and is a transmembrane growth factor with tyrosin kinase activity. In normal pancreas, c-erbB-1 is expressed only in the islets of Langerhans. Nevertheless the c-erbB-1 gene is overexpressed in human pancreatic cell lines and in 95% of ductal adenocarcinomas, due to an increase in gene transcription[29]. Overexpression of c-erbB-1 detected by immunohistochemistry is correlated with reduced survival rates in several studies[30,31] .

The c-erbB-2 (also called HER2/neu) gene encodes a 185-ku transmembrane glycoprotein with tyrosine kinase activity, and acts as a receptor for a class of ligands that includes the heregulins, gp30, and NEU-differentiation factor. C-erbB-2 is overexpressed in the bladder, breast, esophageal, and gastric cancer, where it appears to have a role in lymphatic tumor spread[32,33]. In ductal adenocarcinomas of the pancreas and in ampullary tumors, c-erbB-2 is overexpressed in 20%, which is usually caused by gene amplification[34]. Reports about the prognostic significance of c-erbB-2 are not uniform throughout the literature. One study showed a survival advantage for patients without the overexpression of c-erbB-2 in their tumors[35] , while another study showed a correlation of c-erbB-2 serum levels and survival of the patients [36]. In the majority of studies no prognostic significance for the expression of c-erbB-2 in PC was revealed[37,38].

In the present study we have determined gene expression levels of survivin, c-erbB-1 and c-erbB-2 in PC and CPV applying quantitative real-time RT-PCR (TaqMan ™) in order to look for gene profiles that might account for the different biological behavior.

## MATERIALS AND METHODS

### Patients and specimens

Between July 1997 and December 2003 fresh frozen tumor and corresponding normal tissue from 63 patients, who underwent curative resection of suspected or proven pancreatic or ampullary tumors were collected. Informed consent was obtained from all the patients. Data and tissue collection were in accordance with the regulation of the local ethic committee. Tissues were frozen immediately in liquid nitrogen and stored at -80 °C until further analysis.

The definitive histology of the tissue used for RNA isolation was confirmed in serial sections by a staff pathologist. Only tumor specimens with at least 75% malignant cells were used for RNA isolation.

Finally, 31 specimens from ductal adenocarcinomas of the pancreas and 8 from adenocarcinomas of the papilla of Vater with paired normal control tissues were available for gene expression analysis. This study population consisted of 24 (62%) men and 15 (38%) women with a median age of 59.4 years (range, 33-81 years). Tumor staging was performed according to the International Union Against Cancer (UICC) tumor-node-metastasis classification. In patients with PC, 1 (3.2%) patient had a stage I tumor, 3 (9.6%) patients had a stage II tumor, 24 (77.4%) patients had a stage III, and 3 (9.6%) had a stage IVa tumor. In patients with CVP, 5 patients presented with a stage II tumor, 2 patients had stage III, and 1 patient a stage IV tumor.

Twenty four patients underwent a Kausch-Whipple procedure, and in 10 patients the pylorus was preserved. In four patients a left-sided pancreatic resection and in one patient a pancreatectomy was performed. In patients with cancer of the papilla ($n$=8) of Vater, five received a pylorus preserving partial pancreaticoduodenectomy, whereas three patients underwent a Kausch-Whipple procedure. The median follow-up for surviving patients was 9.5 mo and no patient was lost to follow-up.

### RNA isolation

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden) adding proteinase K (0.2 mg/mL) and quantified at $A_{260/280\ nm}$ (SmartSpec; Bio-Rad, Hercules, CA, USA).

### Quantitative RT-PCR

Total cellular RNA (0.5 μg) was reverse-transcribed as described previously[39]. An amount of 25 ng of cDNA was taken for real-time PCR using the TaqMan ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). To normalize the amount of total RNA present in each reaction, the housekeeping gene β-actin was amplified.

Primers and probes were designed to encompass intron between exon sequences using the Primer Express Software (Applied Biosystems, Darmstadt, Germany). The β-actin probe was labeled with 5′-VIC and 3′-Minor Groove Binder/Non-Fluorescent Quencher (Applied

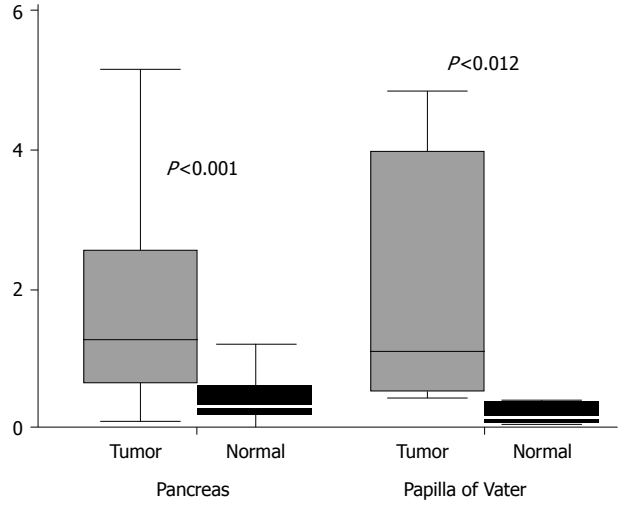


**Figure 1** Expression of survivin in PC ($n$=31) and CPV ($n$=8).

Biosystems, Darmstadt, Germany). The survivin, c-erb-B-1 and c-erbB-2 probes were labeled at the 5′ end with FAM and at the 3′ end with the quencher TAMRA (Eurogentec, Seraing, Belgium). The sequences for the primers and probes were as follows: survivin described by Warnecke-Eberz *et al* 2005. C-erbB-2 sense 5′ CCA GGA CCT GCT GAA CTG GT 3′, anti-sense 5′ TGT ACG AGC CGC ACA TCC 3′, probe 5′ CAG TTG CCA AGG GGA TGA GCT ACC TG 3′. C-erbB-1 sense 5′ CGC AAG TGT AAG AGT GCG AA 3′, anti-sense 5′ CGT AGC ATT TAT GGA GAG TGA GTC T 3′, probe 5′ CCT TGC CGC AAA GTG TGT AAC GGA AT 3′. The reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs prior to analyses of patient samples. To ensure that no genomic DNA was amplified the assays were checked with RNA samples minus reverse transcription control as well as with genomic DNA as template.

The PCR reaction was performed as described previously[39]. All analyses were done in triplicates. Gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Laboratories, Palo Alto, CA, USA).

### Statistical analysis

The gene expression analyses yielded values, which were expressed as ratios between two absolute measurements: the gene of interest and the internal reference gene β-actin. Gene expression levels were described using the median as point estimator and the range of values. Cut-off values for discrimination of dichotomized mRNA expression levels and clinicopathologic parameters were derived from receiver operating curve data (area under the curve and the 95% confidence interval) according to Metz *et al*[40]. Associations between gene expression levels and clinicopathological parameters were evaluated using the $\chi^2$ test for dichotomized variables, Wilcoxon's rank test for paired variables and the Mann-Whitney test for independent variables applying Fisher's exact testing for significance.

Partitioning of gene expression levels to construct prognostic groups was performed according to LeBlanc *et al*[41]. Briefly, the best cut-off value for a supposed prognostic variable is determined by simulating the log-rank test for all observed covariate values within the entire data set. The minimal log-rank $P$-value determines the best cut-off value for dichotomization of the covariate. Kaplan-Meier[42] plots were used to describe the survival distribution and the log-rank test was used to evaluate survival differences[43].

The level of significance was set at $P<0.05$ in all statistical testing. Unless otherwise specified, $P$-values were given for two-sided testing.

**Table 1 Results of non-parametric paired samples Wilcoxon's test. mRNA expression of c-erbB-1 and c-erbB-2 are compared between tumor (T) and normal (N) samples**

| Tumor | | c-erbB-1 | *P* | c-erbB-2 | *P* |
|---|---|---|---|---|---|
| Pancreatic cancer | T<N | 21 | 0.03 | 14 | 0.99 |
| | T>N | 9 | | 17 | |
| Cancer of the papilla of Vater | T<N | 5 | 0.38 | 6 | <0.05 |
| | T>N | 3 | | 2 | |

## RESULTS

### Survivin mRNA expression

Survivin mRNA expression was detected in 30 of 31 (97%) PC in all tumor specimens of the papilla of Vater and in all paired non-malignant control specimens. In 26/31 (83%) PC expression of survivin mRNA was higher in tumor compared to normal control tissue and this difference was statistically significant (Wilcoxon's test: $P<0.001$). In CVP, all tumor specimens ($n$=8) had a higher expression than the normal tissue (Wilcoxon's test: $P<0.01$). Expression of survivin mRNA was not significantly different between PC and CPV (Mann-Whitney test: $P=0.195$). Representative box-plots are shown in Figure 1.

### C-erbB-1 mRNA expression

C-erbB-1 mRNA expression could be detected in 30 of 31 (97%) pancreatic tumors, all tumors of the papilla and all normal tissues. Expression of c-erbB-1 was lower in the malignant than in the normal pancreatic tissue in 21 out of 31 (68%) individual cases. This difference was statistically significant (Wilcoxon's test: $P=0.028$), whereas in CPV no statistically significant difference ($P=0.38$) in c-erbB-1 expression between tumor and corresponding normal tissue was revealed (Table 1).

### C-erbB-2 mRNA expression

The expression of c-erbB-2 was detectable in all tumor and non-malignant specimens. In PC paired non-parametric testing revealed no statistically significant difference in c-erbB-2 expression between tumor and normal tissue. In contrast, c-erbB-2 expression was significantly (Wilcoxon's test: $P<0.05$) downregulated in 6/8 cases of CPV (Table 1).

No significant association between gene expression levels and clinical parameters such as sex ($\chi^2$ test), tumor stage, lymph node stage, and grade of differentiation (Mann-Whitney test) was observed.

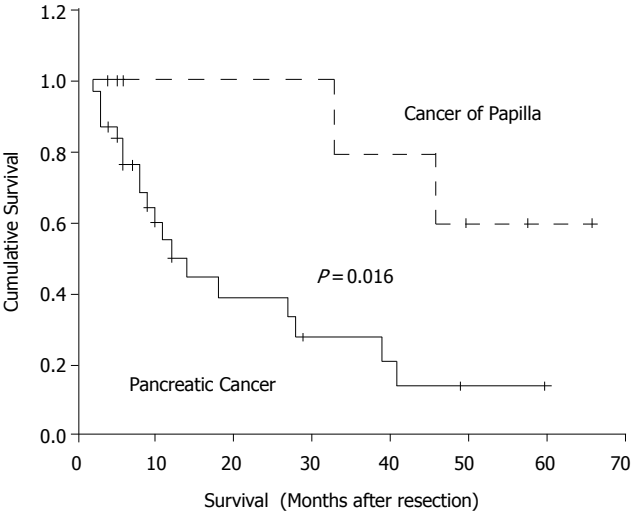


**Figure 2** Kaplan–Meier analysis of survival of patients with pancreatic cancer ($n = 31$) and CPV ($n = 8$) after resection.

### *mRNA expression and survival of patients with pancreatic cancer and cancer of the ampulla of Vater*

At a median follow-up of 56 months (range 7-89 mo) for surviving patients, median survival was 14 mo (range 2-60 mo) for patients with pancreatic carcinoma and 39.5 months (range 4-66 mo) for patients with carcinoma of the papilla of Vater (log-rank: $P<0.016$). Kaplan-Meier survival curves are shown in Figure 2.

Partitioning of gene expression levels to construct prognostic groups according to LeBlanc *et al*[41] did not reveal any correlation between gene expression (c-erbB-1, c-erbB-2, and survivin) and survival of patients with pancreatic or papillary cancer.

## DISCUSSION

The surgical therapy of carcinoma of the papilla of Vater and adenocarcinoma of the pancreas consists of a pylorus preserving or a classical pancreaticoduodenectomy (Whipple procedure). Despite this identical surgical approach, the long-term prognosis is different. Five-year survival rates from 0% to 25% for PC and from 15% to 56% for CPV have been reported[1,12]. It was shown that different tumor biology, represented by higher expression of members of the EGF-R family (c-erbB-1, c-erbB-2, and c-erbB-3) contributes to the different behavior[16,44]. C-erbB-1 and c-erbB-2 were also differentially expressed in our study. In contrast to Friess *et al*[16], our results showed a significant downregulation of c-erbB-1 expression in PC tissue and of c-erbB-2 expression in the malignant tissue of the papilla of Vater compared with the adjacent normal tissues. These results were based on a smaller number of patients, but were obtained by quantitative real time RT-PCR. Whereas the above mentioned study[16] applied immunohistochemical staining and Northern blot analysis, quantitative real time RT-PCR provides more accurate and reproducible quantitation of gene expression and has a large range of results[45,46].

The downregulation of c-erbB-1 in malignant pancreatic tissues compared with the adjacent normal tissue is in contrast to the existing literature that shows an overexpression of c-erbB-1 in pancreatic ductal adenocarcinoma and a worse prognosis for patients with high expression of c-erbB-1 in their tumors[29,30,47,48]. As mentioned above, these studies, which were mainly published about 10 years ago, were based on immunohistochemical techniques showing c-erbB-1 expression in only 30-70% of the investigated PC cases. Importantly, the results were not compared with the matching normal pancreatic tissue from the same patients. In our investigation using real time RT-PCR, c-erbB-1 expression was detected in 97% of PCs and in all matching normal pancreatic samples.

The reported expression of c-erbB-2 in PC has varied widely (7-82%) in different studies due to the differences in methodology and/or patient selection[35-38]. Our study, which is the first in using quantitative real-time RT-PCR to assess the expression of c-erbB-2 in pancreatic and CPV, did not show a different expression of c-erbB-2 between tumor and normal pancreatic tissue. This is in agreement with two recent studies[49,50] using immunohistochemical methods, which found no correlation between c-erbB-2 expression and stage or survival of patients with PC. However, a series of other publications[45-48,51] reported overexpression of c-erbB-2 in a subset of patients with PC and its prognostic relevance. For other malignancies such as gastric cancer[52], breast cancer[53], and ovarian cancer[54] an increased expression of the c-erbB-2 oncogene has been reported, applying PCR based methods. According to our results and the recent literature one must consider, that the role of c-erbB-2 in the tumorigenesis and its value as a prognostic marker in PC has not been finally elucidated.

In concordance with other authors[24,55-57], we found an increased expression of survivin in the samples of PC and CPV compared with the adjacent non-malignant pancreas. One study[24] also found an overexpression of survivin in a series of 12 ampullary carcinomas; expression was not different from PC, which is comparable with our result. Kami *et al*[58] reported a significant correlation between poor survival and increased expression of survivin in a study with 47 patients with PC using immunohistochemistry. Whereas Sarela *et al*[24], as well as our study revealed no association between survival and expression of survivin. The comparable results of survivin expression in our patients and other studies in the literature strengthen our data regarding the expression of c-erbB-1 and c-erbB-2 in PC and CPV. For a variety of other malignancies it was shown that survivin expression is a reliable marker for an unfavorable disease with poor overall survival[59,60]. The high number of locally advanced tumors, on which our and other studies are based, could explain the difference to PC. The significantly increased expression of survivin in PC, obtained by different methodologies, opens possibilities for new diagnostic and therapeutic strategies, such as new targets for gene therapy, differentiation between malignant and benign pancreatic lesions before surgical resection and to monitor response to neoadjuvant treatment regimens.

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