Gene Expression of *Col11A1* Is a Marker Not only for Pancreas Carcinoma But also for Adenocarcinoma of the Papilla of Vater, Discriminating Between Carcinoma and Chronic Pancreatitis

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Abstract. *Aim: For tumors of the periampullary region clinical differentiation between primary and tumor-associated pancreatitis might be difficult. Early diagnosis of these malignancies is essential, as they present with early invasion of surrounding tissue thus limiting therapeutic options. Using molecular markers, the preoperative diagnosis (EUS-guided needle biopsy, brush biopsy pancreatic duct) could be optimized and surgical therapy potentially adapted. Alpha1 (XI) collagen Col11A1 is essential for the extracellular matrix and normal skeletal development and has been associated with carcinogenesis. Materials and Methods: Forty-three patients with adenocarcinoma of the pancreas, 11 with adenocarcinoma of the papilla of Vater and 23 patients with chronic pancreatitis were included in the study. For all patients mRNA expression of Col11A1 was quantified by TaqMan RT-PCR in tumor or pancreatitis specimen, as well as in the corresponding normal uninvolved tissue and correlated with diagnosis of cancer and chronic pancreatitis. Results: Col11A1 mRNA expression was 5.25-fold higher in adenocarcinoma of the pancreas (p=0.006) and 8.25-fold in the papilla of Vater (p=0.002) compared to that of chronic pancreatitis specimen. Conclusion: Differential mRNA expression of Col11A1 may be applied to preoperatively differentiate between tumors of the periampullary region and chronic pancreatitis and this may potentially have a positive effect on patient survival.*

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Adenocarcinoma of the periampullary region are a heterogeneous group of tumors including neoplasms of the distal bile duct and the ampulla of Vater and need to be distinguished from Adenocarcinoma of the pancreas (PC). PC is currently the fifth leading cause of cancer-related deaths in North America and Europe with rising incidence and has a dismal prognosis, with a five-year survival rate <3% (1).The dismal prognosis for this disease are attributed to an aggressive biological phenotype that is characterized by early invasion of surrounding structures and rapid metastatic spread (2, 3). The lack of early symptoms and early detection tests often result in a late diagnosis that limits the therapeutical options, as only 15-20% of the tumors are resectable at presentation (1).

Cancer of the papilla of Vater (CPV) is a rare disease representing 6-12% of all periampullary malignancies (4) with an estimated incidence of 2.9 per million (5). In contrast to PC, CPV has a better prognosis with a 5-year survival rate of >40% when curative resection can be achieved (6). Preoperative differentiation between chronic pancreatitis, PC and CPV is sometimes difficult. Early differentiation between inflammation and malignancy is essential for treatment strategies and therefore might improve patient survival.

Collagen is the major component of the interstitial extracellular matrix (ECM). ECM plays an active role in biological processes like cell shape, proliferation, migration, differentiation, apoptosis, as well as carcinogenesis (7). Collagen type XI alpha I (Col11A1) is essential for normal skeletal development. Mutations in Col11A1 cause Marshall and Stickler syndromes, both of which are characterized by craniofacial abnormalities, nearsightedness and hearing

deficiencies. *Col11A1* promoter is activated by the lymphocyte enhancer-binding factor 1 (Lef1) dependent upon the DNA binding domain Lef1. Col11A1 suppresses terminal osteoblast differentiation (8). The aim of the present study was to analyze the potential role of *Col11A1* mRNA expression in tumors of the periampullary region and in pancreatitis.

Materials and Methods

Patients. Tumor and corresponding normal tissues from 54 consecutive patients, who underwent curative resection of periampullary malignancies (PC n=43, CPV n=11) and of 22 patients who underwent surgery for chronic pancreatitis were collected. Informed consent was obtained from all patients. Data and tissue collection were in accordance with the regulations of the local Ethics Committee.

For 43 patients with ductal adenocarcinoma of the pancreas, for 11 patients with tumors of the papilla of Vater and for 22 patients with chronic pancreatitis matched tissue was available for gene analysis. The study population consisted of 47 (62%) men and 29 (38%) woman, with a median age of 59.4 years (range, 33-81 years). All patients with malignancies of the periampullary region had adenocarcinomas. Tumor staging was performed according to the International Union against Cancer (UICC) tumor-node-metastasis (TNM) classification. In patients with pancreatic cancer one (3.2%) patient had a stage I, four (9.6%) patients had a stage II, 34 (77.4%) patients had a stage III, and four (9.6%) were stage IVa. In patients with cancer of the papilla of Vater seven patients presented with a stage II, three patients had stage III and one patient was in stage IV.

In patients with periampullary cancer 19 underwent a Whipple's procedure, in 30 patients the pylorus was preserved. In four patients a left-sided pancreatic resection and in one patient a total pancreatectomy was performed. In 6 patients with chronic pancreatitis a pylorus preserving partial duodenopancreatectomy was performed, 16 patients underwent duodenal preserving pancreatic head resection. The mean follow-up for surviving patients was 9.5 months (range=62 months) with no patient lost to follow-up. The definitive histology of tissues used for RNA isolation was confirmed in serial sections analyzed by a staff pathologist.

Sample collection and RNA isolation. For the evaluation of gene expression, samples were obtained immediately after resection, snap-frozen in liquid nitrogen, and stored at -80˚C. Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) adding proteinase K (0.2 mg/ml) according to the manufacturer's recommendation, quantity was estimated at A260/280 nm (Smart Spec; Biorad, Hercules, CA).

Real-time RT-PCR by TaqMan. Total cellular mRNA (0.5 μg) was reverse transcribed using oligo(dT)18 primer and Moloney murine leukemia virus reverse-transcriptase Advantage RT-PCR kit (Clontech Takara, Palo Alto, Calif.) as described previously (9). Real-time PCR was performed by applying 25 ng of cDNA by TaqMan ABI PRISM7900HT Sequence Detection System (Life Technologies, Darmstadt, Germany).

By means of fluorescence emission, this technique allows the cycling point to be found when PCR product is detectable (threshold cycle). To normalize the amount of total RNA present in each reaction, the housekeeping gene β-actin was amplified, as described before (10).

The PCR reaction mixture contained 300 nM of each primer and 200 nM probe in a final volume of 20 μl. PCR conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min. Gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Takara). Reliability of PCR amplification and detection was verified by serial dilutions of standard cDNAs before analyses of patient samples. All reactions were performed in triplicates. Primers and probes were obtained from Life Technologies, Col11A1 Assay on demand: Hs00266273_m1.

Statistical analysis. Gene expression analyses yield values that are 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 clinico-pathologic parameters were derived from receiver operating curve (ROC) data (area under the curve and the 95% confidence interval). Associations between gene expression levels and clinico- pathological parameters were evaluated using the χ^2 test for dichotomized variables, Wilcoxon rank test for paired variables and the Mann-Whitney-Test for independent variables applying Fisher's exact testing for significance.

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 plots were used to describe the survival distribution and the log-rank test was used to evaluate for survival differences. The level of significance was set to $p<0.05$ in all statistical tests. Unless otherwise specified, *p*-values were given for 2-sided testing.

The relative expression quantities of *Col11A1* were compared between the tumor and corresponding normal tissue using Wilcoxon test for paired samples. Results are graphically presented in Box- and Whisker-plots with a line for the median. The upper end of the box represents the 75% value, the lower end the 25% value. All statistical tests were performed using the Software Package SPSS for Windows, Version 20, Chicago, IL, USA.

Results

Expression of *Col11A1* mRNA was detected in all PC, CPV, chronic pancreatitis and normal pancreatic specimens. *Col11A1* expression was up-regulated in tumor specimens compared to corresponding normal tissue in patients with PC (0.8 *vs*. 0.3; $p<0.0001$, Figure1) and CPV (CPV: 1.3 *vs*. 0.2; $p=0.015$, Figure 2). In chronic pancreatitis there was a trend towards a lower *Col11a1* mRNA expression in pancreatitis tissue compared to the corresponding normal tissue (0.2 *vs*. 0.5; $p=0.24$). *Col11A1* mRNA expression was similar in PC compared to CPV although there was a trend towards a higher median mRNA expression of Col11a1 in CPV (1.32 *vs*. 0.84; $p=0.84$). Median *Col11a1* mRNA expression was significantly

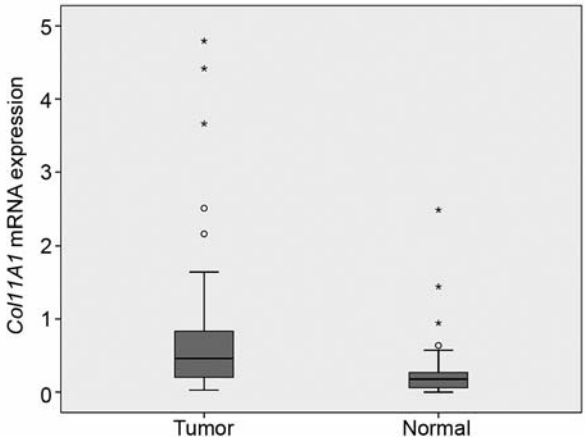

Figure 1. *Col11A1 overexpression in pancreatic cancer compared to corresponding normal tissue. Relative mRNA expression in tumor and corresponding normal tissue were compared using the Wilcoxon test for paired samples, Box- and Whisker-plots with a line for the median, upper end of the box represents the 75% value, the lower end the 25% value, p<0.0001.*

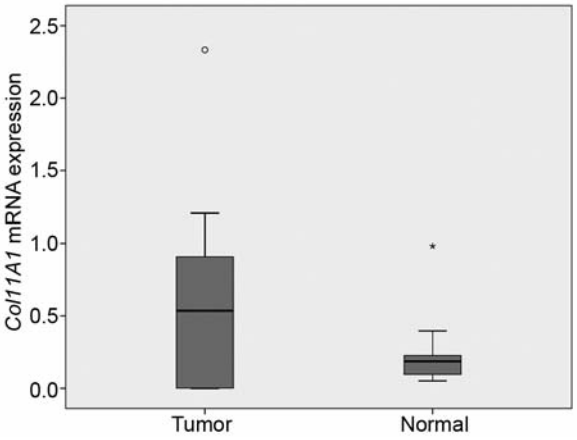

Figure 2. *Col11A1 overexpression in cancer of the papilla of Vater compared to corresponding normal tissue. Relative mRNA expression of tumor and corresponding normal tissue were compared using the Wilcoxon test for paired samples, Box- and Whisker-plots with a line for the median, upper end of the box represents the 75% value, the lower end the 25% value, p<0.015.*

elevated in periampullary cancers compared to chronic pancreatitis (PC 0.84, CPV 1.32 *vs*. 0.16, p=0.006), (Figure 3A). Comparison of the matching normal uninvolved tissue of these three patient groups resulted in a *Col11A1* mRNA increase in the PC and CPV group compared to uninvolved normal tissue of the pancreatitis group (Figure 3B).

No significant associations between gene expression levels and clinico-pathological parameters as UICC tumor stage, pT and pN-categories, or grading of the primary tumor were observed.

Discussion

Our results showed a significant (5.25-fold) up-regulation of *Col11A1* mRNA expression in pancreatic cancer compared to pancreatitis cells, that verifies data published by Garcia-Parva *et al*. (11). In addition our study showed an overexpression of *Col11A1* mRNA in patients with carcinoma of the papilla of Vater compared to pancreatic cells. Moreover there was also an up-regulation of *Col11A1* mRNA in tumors of PC and also CPV compared to chronical pancreatitis.

This is an important finding, since in tumors of the periampullary region, differentiation between chronic pancreatitis and adenocarcinoma of the papilla of Vater is of major clinical importance as treatment strategies are very different for these two diseases. When possible, CPV requires a radical oncological resection (12), whereas pancreatitis can be treated conservatively. Therefore, markers distinguishing between these two diseases can be helpful for clinical decision making (13).

However, clinical therapy of these two diseases is completely different: In contrast, PC is an aggressive tumor with a reported median survival of 5 to 8 months (14). So far, surgical resection is the only potential curative option (15) if a R0 resection can be achieved (16). For the majority of the patients, resectability is limited by early local invasion of the surrounding anatomic structures or distant metastasis. Hence an earlier diagnosis of PC may increase the number of potentially resectable patients and improve the overall survival. In primary non-operable patients, novel neoadjuvant concepts promise a down-sizing of the tumor to achieve respectability and more favorite prognosis. Early identification of these patients might accelerate the start of therapy and improve survival (12).

The high frequency of KRAS mutations (>90%) in pancreatic duct adenocarcinoma suggests a search for KRAS status to improve the diagnosis in suspected cases. The different KRAS mutations correlated with the frequency of cancer recurrence and had no prognostic significance (17). The combination of a *K-ras* mutation and ≥2 loss of heterozygosity has been described as highly specific (96%) but insensitive for malignant behaviour (50%) (18). Thus, further biomarkers are needed in order to complement this successful approach.

Col11A1 expression in tumor cells, PC and CPV, was overexpressed compared to the corresponding normal tissue. Regarding the delayed diagnosis of pancreatic cancer a clinical biomarker might be an important contribution to better prognosis. In global gene expression analyses Col11A1 has been described to be up-regulated in different

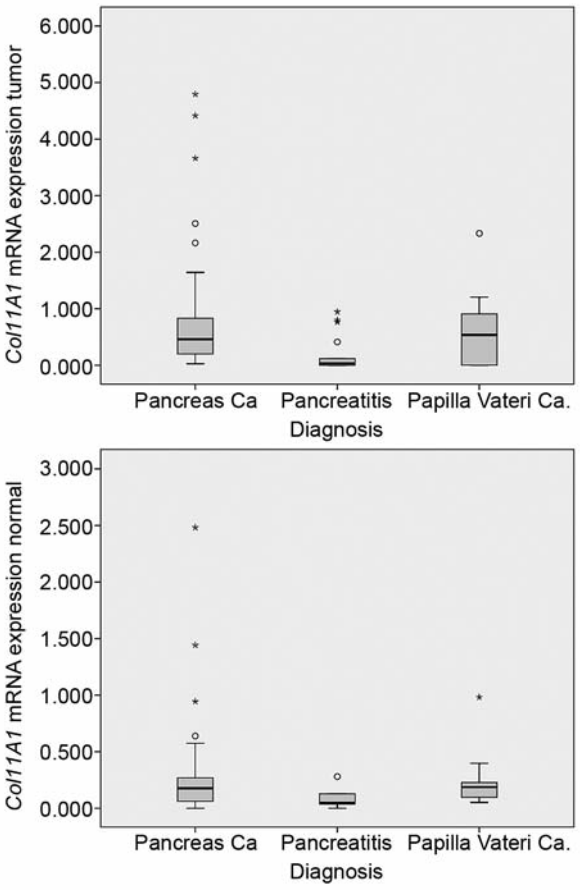

Figure 3. *(A) Col11A1 expression in pancreatitis, pancreatic cancer and cancer of the papilla of Vater and (B) in paired uninvolved normal tissue. Relative mRNA expression (A) comparison in tissue from patients with pancreatitis, pancreatic cancer and cancer of the papilla of Vater (B) in paired normal uninvolved tissue were compared using using Wilcoxon test for paired samples, Box- and Whisker-plots with a line for the median, upper end of the box represents the 75% value, the lower end the 25% value.*

tumors including pancreatic cancer located in stroma by immunohistochemical analysis (15). The potential role of collagen expression as a clinical biomarker distinguishing between pre-malignant and malignant lesions has been also discussed for gastric cancer (19), as well as for a variety of other cancers including lung (20), colorectal (7) and breast cancer (21). *Col11A1* mRNA expression might be a useful diagnostic tumor marker applicable for a variety of cancer entities.

However, *Col11A1* has not been applied in clinical practice for any tumor entity. This might be due to the invasive application of immunohistochemical or mRNA analysis of tumor biopsies. Therefore, the liquid-biopsy approach detecting Col11A1 protein by ELISA quantification, from patients' serum or mRNA originating from tumor cells circulating in blood, might be an important step. The translation of results generated by tumor marker research to clinical application will be essential to prove the impact of this marker in improving tumor detection.

Col11A1 might be a metastatic marker. In a multi-cancer computational analysis Col11A1 was described as invasion-associated (22). Overexpression of *Col11A1* mRNA in NSCLC was identified as a diagnostic marker correlating with pathological stage, lymph node metastasis and poor prognosis (23). Molecular profiling of genes associated with metastatic oral cavity/pharynx squamous cell carcinoma revealed Col11A1 as the most differentially expressed gene between metastatic and non-metastatic OC/OP SCC tumors (24). In our study we were not able to associate *Col11A1* mRNA expression with tumor stage or metastases, however, this might be due to the limited number of patients.

The tumor microenvironment is extremely complex and depends on the interaction between tumor cells and responding host cells. The interaction between cancer and stroma, plays a key role in pancreatic cancer development, invasion and, metastasis. The prominent stromal reaction in the pancreatic tumor local microenvironment is one of the remarkable characteristics of pancreatic cancers. Thus, the potential approach for targeting the components of this pancreatic tumor microenvironment might represent therapeutic approach to advanced pancreatic carcinoma (25). Collagens belong to the main ingredients of the extracellular matrix. A more complete understanding of these interactions will contribute to targeting and treatment of pancreatic cancer. Our data support the role of Col11A1 in tumor environment, since we detected an increase of *Col11A1* mRNA expression in normal pancreas tissue of tumor patients compared to normal pancreas tissue of patients with pancreatitis. This indicates a first increase already in normal "uninvolved" microenvironment.

Since Col11A1 knock-down decreases *in vitro* cell migration, invasion, and tumor progression in mice (26), Col11A1 may be useful as a therapeutic target.

The *Col11A1* gene is localized on chromosome 1p21 and encodes the pro-alpha1 (XI) chain of type XI collagen. Type XI collagen adds structure and strength to connective tissues that support the muscles, joints, organs, and skin. Type XI collagen is normally found in cartilage, a tough but flexible tissue that makes up much of the skeleton during early development. There is an inverse correlation of 5'-UTR methylation and gene expression of Col11A1 in lung cancer (20) indicating at least a partially regulation by DNA methylation.

Within a colorectal cancer screening by cytogenetic tests using biopsies and stool samples mutations in Col11A1 gene (exon 54) were discovered (27), another potential reason for alteration of mRNA expression. Col11A1 furthermore was predicted to be targeted by miR-29 (28).

Conclusion

Due to the significantly differential mRNA expression of *Col11A1* this marker may be applicable for preoperative differentiation between patients with primary pancreatitis from patients with pancreatitis due to carcinoma of the periampullary region. *Col11A1* might help identify potentially resectable patients and improve patient survival. *Col11A1* expression in tissue obtained by needle or brush biopsy might be taken into consideration when developing biomarkers for the early detection of carcinoma of the periampullary region. Since it is possibly associated with tumor stage and metastases, it might also be a prognostic marker. Therefore, it should be included for developing highly specific biomarkers sensing cancer invasion. Additionally, Col11A1 might be a potential target for an anticancer therapy.

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