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Prognostic significance of AGR2 in pancreatic ductal adenocarcinoma

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Summary. Background/Aims: The human Anterior Gradient-2 (AGR2) is strongly upregulated in various human cancers, including pancreatic ductal adenocarcinomas (PDAC), but its prognostic value in PDAC has not yet been studied. Methods: We analysed 19 microdissected PDAC cases at the mRNA level, and also 148 cases at the protein level by immunohistochemistry based on tissue microarray, using a monoclonal AGR2 antibody, and statistical analyses were applied to test for prognostic associations. Results: Overexpression of AGR2 mRNA was found to be elevated in most pancreatic cell lines and in microdissected pancreatic cancer compared to microdissected normal ductal cells. AGR2 protein was expressed in 109/148 (73.7%) of PDAC, with a higher expression in female patients (p=0.040), whereas no significant associations with other clinical-pathological parameters were found. A prognostic value of AGR2 could not be demonstrated in univariate analyses. Conclusion: Although a prognostic value of AGR2 seems unlikely, further studies are warranted to investigate the biological role of AGR2 in pancreatic adenocarcinomas.

Key words: Pancreatic adenocarcinoma, AGR2, mRNA, Prognosis, Immunohistochemistry

Introduction

In the United States pancreatic carcinoma is, with 37,680 estimated new cases, ranked number 10 (male) and number 11 (female) of the most common cancer types in 2008. Still, because of its high lethality (34,290 estimated deaths for 2008) it is the fourth most common cause of cancer mortality in the United States in both sexes (Jemal et al., 2008). Despite improving therapies in surgery and oncology the 5-year survival rates for pancreatic carcinomas remains very low (Jemal et al., 2008). The vast majority (85-90%) of pancreatic carcinomas are ductal adenocarcinomas (PDAC). Conventional tumour parameters of the TNM-classification are valuable prognostic markers (Ridwelski et al., 2001). Complete surgical resection of the tumour still remains the most important curative therapeutic option (Wagner et al., 2004) but nevertheless additional therapy is necessary (Butturini et al., 2008). As even with systemic chemotherapy the mortality rates are high (Murakami et al., 2008), there is a need for novel therapeutic options and therapy targets for this extremely lethal disease (Willett et al., 2007). S100A2 (Ohuchida et al., 2007), Netrin-1 (Link et al., 2007), SPARC (Infante et al., 2007), ADAM9 (Grutzmann et al., 2003) and PPAR-γ (Kristiansen et al., 2006), among others, are prognostic markers in PDAC, but none of them have been validated in larger prospective trials.

The Anterior Gradient protein XAG-2 of *Xenopus laevis* (Komiya et al., 1999) is the homologue to the human AGR2 (synonyms: hAG-2 (Fletcher et al., 2003), Gob-4 (Komiya et al., 1999)). Ectodermal patterning of the frog embryo is the main role of XAG-2. In a fibroblast growth factor dependent way, XAG-2 influences neural marker genes and induces cement gland differentiation (Aberger et al., 1998). We and others described recently up-regulation of the AGR2 gene in prostate cancer, breast cancer and non-small cell lung cancer (Kristiansen et al., 2005; Zhang et al., 2005; Fritzsche et al., 2006). In patients with advanced cancers, AGR2 was proposed for the detection of circulating tumour cells in the peripheral blood (Smirnov et al., 2005). In human breast cancer tissue and cell lines AGR2 expression has been described by several research

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groups and its expression was associated with a positive estrogen receptor status of the tumour cells (Fritzsche et al., 2006, Innes et al., 2006). AGR2 is located on chromosome 7p21, a region commonly deleted in Wilms tumours (Sossey-Alaoui et al., 2003). cDNA microarray studies have identified AGR2 to be overexpressed in pancreatic carcinomas (Iacobuzio-Donahue et al., 2003 Missiaglia et al., 2004). Furthermore, AGR2 protein is expressed in the majority of pancreatic carcinomas (Missiaglia et al., 2004, Ramachandran et al., 2008). However, little is known about its function in PDAC. Since in oesophageal adenocarcinoma cell lines AGR2 was found to promote tumour growth, cell migration and cellular transformation (Wang et al., 2008), it can be assumed that it confers these pro-tumorigenic functions also to pancreatic carcinomas, and might be annotated with a worse prognosis in this disease.

First, we analysed AGR2 mRNA expression in pancreatic cancer tissues and pancreatic cancer cell lines. Secondly, we evaluated AGR2 protein expression in 148 PDAC using immunohistochemistry on a clinically well characterised tissue micro array, and correlated our findings with clinical-pathologic parameters, including overall survival.

Materials and methods

Cell lines

Pancreatic cancer cell lines were obtained from ATCC: ASPC-1 was originally isolated from ascites of a patient with a G2 PDAC. MiaPaCa-2 and PANC1 stemmed from weakly differentiated (G3) primary PDACs. Capan-1 was isolated from a lymph node metastasis of a PDAC patient.

AGR2 expression profiling

Within the study we analysed the expression value for AGR2 in 13 microdissected normal ductal epithelia and 19 microdissected PDAC, as well as pancreatic cancer cell lines using the Affymetrix U133 A/B GeneChip Set (Pilarsky et al., 2008). The complete data set is available at ArrayExpress E-MEXP-950 and E-MEXP-1121. The procedure of microdissection of these samples were described elsewhere (Pilarsky et al., 2008). In brief, frozen tissue specimens were cut into 10 µm-thick sections and immediately fixed on slides in 70% ethanol. The sections were briefly stained with hematoxylin and eosin (H&E), and coverslipped. Suitable areas for microdissection were marked on these slides serving as a template. The tissue blocks were serially cut to 5 µm-thin sections, briefly fixed in 70% RNase-free ethanol, and stained with H&E. PDAC cells and normal ductal cells were microdissected manually using a sterile injection needle. The estimated cellularity was 10,000 to 11,000 cells per microdissected sample. The purity of the dissections was approximately 95%. These cells were pooled in ice-cooled guanidine thiocyanate (GTC) buffer (Promega, Heidelberg, Germany) for further RNA preparation. We reanalyzed the expression of the two AGR2 probesets 228969_at and 209173_at using t-Test statistics from the SPSS package.

Western Blot of AGR2

Pancreatic cell lines were cultivated in RPMI1640, 10% FCS (Invitrogen, Karlsruhe, Germany), lysed using Novex LDS sample buffer and the resultant lysate was subjected to gel electrophoresis using the Novex Nupage system (Invitrogen, Karlsruhe, Germany). After completion, the proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biosciences, Freiburg, Germany). AGR2 was detected by using a commercially available monoclonal Antibody (clone 1C3, Abnova Corp., Taipeh, Taiwan) diluted 1:500 and the ECLplus western blot detection system (Amersham Pharmacia Biosciences, Freiburg, Germany). After detection the blot was stripped and beta-actin was detected using a mouse monoclonal antibody (clone AC-15, #ab6276, Abcam) diluted 1:5000. The secondary antibody was diluted 1:25.000 in both cases.

Patients

Our study included 148 patients with PDAC, diagnosed at the Institute of Pathology, Charité – Universitätsmedizin Berlin, between 1991 and 2005. Patient age at the time of diagnosis ranged from 31 to 89 with a median of 65 years. Clinical follow-up data was available for 100 patients who where further treated at the Charité – Universitätsmedizin Berlin. The median postoperative observation time was 13 months and ranged from less than 1 month to 113 months. 80 patients (80%) died during follow-up. The selection of cases for the creation of the micro array was based on availability of tissue and these were not stratified for any known preoperative or pathological prognostic factors. Four tissue microarrays (TMA) were constructed as previously described (Kristiansen et al., 2002, 2003a,b). Briefly, suitable areas for tissue retrieval were marked on standard haematoxylin–eosin (HE) sections, punched out of the paraffin block and inserted into a recipient block. Three cores per tumour were punched with a diameter of 0.6 mm. The tissue arrayer was purchased from Beecher Instruments (Woodland, USA). After sectioning and staining at least two cores per tumour case were evaluable. Seven patients had systemic disease (pM1) at the time of diagnosis. Histological typing of tumours was carried out according to the criteria of the World Health Organization (Hamilton and Aaltonen, 2000). Clinical-pathologic data were gathered from the archival pathology reports and are described in Table 1.

Immunohistochemistry

The arrays were freshly cut (3µm), mounted on prefabricated coated glass slides (Superfrost Plus®,

Menzel Gläser, Mannheim, Germany), deparaffinised with xylene and gradually rehydrated. Antigen retrieval was achieved by pressure cooking in 0.01M citrate buffer for 5 minutes. In contrast to our previous studies, a commercially available monoclonal AGR2 antibody was used (clone 1C3, Abnova Corp., Taipeh, Taiwan). The primary antibody was diluted 1:200 using a background blocking buffer (DAKO, Hamburg, Germany) and incubated at 4°C overnight. As a negative control, one array slide was processed without primary antibody. As a positive control we used whole tissue slides of AGR2 positive breast cancers from previous studies. Detection took place by the labelled streptavidin-biotin method (kit K5005, DAKO, Hamburg, Germany) with alkaline phosphatase as the reporting enzyme according to the manufacturer´s instructions. Fast-Red (Sigma-Aldrich, Munich, Germany) served as chromogen. Afterwards the slides were briefly counterstained with haematoxylin and aquaeously mounted.

Table 1. Clinical-pathological parameters and association with AGR2 expression of the tumour set.

Variable	Patients	No. of patients (%) AGR2 neg.	AGR2 pos.	p value
Patient age				
<65 years	74	19 (25.7)	55 (74.3)	1
≥ 65 years	74	20 (27.0)	54 (73.0)	
Gender				
male	81	27 (33.3)	54 (66.7)	0.040
female	67	12 (17.9)	55 (82.1)	
pT-status°				
pT1	0	0	0	0.611
pT2	23	7 (30.4)	16 (69.6)	
pT3	113	31 (27.4)	82 (72.6)	
pT4	9	1 (11.1)	8 (88.9)	
pN-status°				
pN0	34	10 (29.4)	24 (70.6)	0.825
pN1	111	29 (26.1)	82 (73.9)	
pM-status°				
pM0	137	36 (26.3)	101 (73.7)	0.389
pM1	7	3 (42.9)	4 (57.1)	
pl-status°				
pl0	21	6 (28.6)	15 (71.4)	0.796
pl1	125	33 (26.4)	92 (73.6)	
pv-status°				
pv0	123	30 (24.4)	93 (75.6)	0.197
pv1	23	9 (39.1)	14 (60.9)	
Histological grade°				
G1	9	3 (33.3)	6 (66.6)	0.391
G2	73	16 (21.9)	57 (78.1)	
G3	64	20 (31.3)	44 (68.7)	
Residual status°				
negative R0	90	21 (23.3)	69 (76.7)	0.387
positive R1	39	12 (30.8)	27 (69.2)	

* Chi square test for trends, ° data not available for all 148 patients.

Evaluation of the immunohistochemical stainings

The immunostainings were examined by two pathologists who were blinded to patient outcome. We used a simple scoring system classifying the staining intensity into these categories: negative (0), weakly (1), moderately (2) and strongly positive (3). Tumours were only considered positive when at least 10% of tumour cells on the spot showed expression of AGR2.

Statistical analysis

Statistical analysis was performed using SPSS, version 15.0. Correlations were calculated according to Spearman. Fisher's exact and chi-square tests for trends were applied to assess the statistical significance of the associations between expression of AGR2 and various clinical-pathological parameters. Univariate survival analysis was carried out according to Kaplan-Meier, differences in survival curves were assessed with the Log rank test. P values <0.05 were considered significant.

Results

AGR2 mRNA expression in pancreatic cancer

AGR2 mRNA expression levels are interrogated by two probesets on the Affymetrix U133A/B GeneChips. One probeset (228969_at) is located near the 3'terminus of the mRNA, the other (209173_at) covers the terminal part of the coding region of AGR2 (Fig. 1A). In our comparison between microdissected tumour and normal ductal cells we could observe a higher expression of AGR2 mRNA in the pancreatic cancer cells, which was statistically significant. However the expression levels were highly heterogenous in the different tissue samples, as indicated by the sizes of the boxes (Fig. 1B). Such heterogenous expression is also evident in the pancreatic cancer cell lines, where arbitrary expression levels range from below 100 (MiaPACa-2) to over 1,800 (CAPAN-1; Fig. 1C).

AGR2 immunostaining in pancreatic ductal adenocarcinoma

Western blot analyses of the cell lines showed a strong AGR2 expression for CAPAN-1, CAPAN-2 and HPAF-II. A weak to moderate expression was found in AsPC-1, Panc1 and BxPC-3, while MiaPaCa-2 was negative (Fig. 2).

Immunohistochemistry showed a diffuse cytoplasmic immunoreactivity for AGR2 in positive cases, while no membranous or nuclear staining was noted. 8 of the 148 PDAC (5.4%) were negative for AGR2 (Fig. 3). 31 (20.9%) showed a weak staining, 71 (48.0%) showed a moderate and 38 (25.7%) a strong expression of AGR2 (Fig. 3). For cross-tables and survival analyses tumours were divided into AGR2 negative (score 0 and 1) and AGR2 positive (score 2 and 3) ones. AGR2

expression was significantly higher in female patients (p=0.040). Other associations of AGR2 with clinical-pathological parameters were not noted (Tables 1, 2).

AGR2 expression and survival times

We analyzed the impact of AGR2, age, gender, pT-status, pN-status, pM-status, pl-status, pv-status, histologic tumour grade and Residual status on overall survival time. The conventional histo-pathological parameter pT-status reached significance, while AGR2 like all other parameters tested, remained insignificant (Table 3, Fig. 4). To evaluate the prognostic value of AGR2 expression in selective patient groups, we repeated the univariate survival analyses in subgroups stratified according to pT-status, pN-status, tumour grade

Table 2. Correlation of AGR2 expression in pancreatic ductal adenocarcinoma with conventional clinical or tumour parameters.

AGR2	pT-status°	pN-status°	Grading°	R-status°	Age
correlation coefficient	.073	.031	-.072	-.078	-0.015
significance (two-sided)	0.383	0.708	0.387	0.378	0.853
N	145	145	146	129	148

R-status = residual status, ° data not available for all 148 patients.

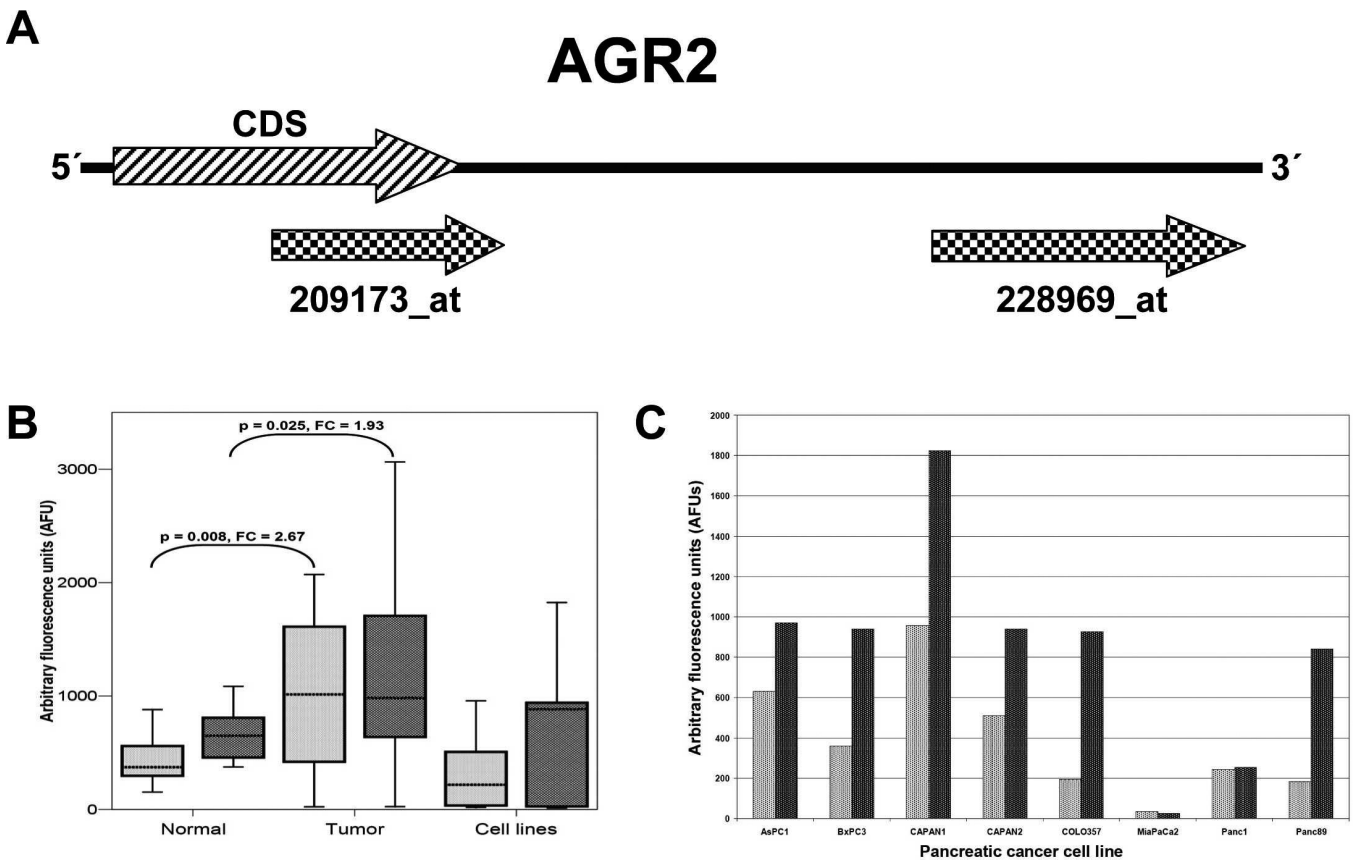

Fig. 1. A. Distrubution of probesets along the AGR2 mRNA. Striped arrow: AGR2 coding sequence, hatched arrows: target sequence of the two probesets of the Affymetrix U133A/B GeneChip set. **B.** Boxplot of AGR2 expression in microdissected primary pancreatic tissues and pancreatic cancer cell lines. AGR2 expression is highly variable in tumour tissues, which is mirrored in the cell lines. However, the median of AGR2 expression in cell lines is equal to the median of AGR2 expression in tumourous tissue. Using T-test statistics a differential expression of AGR2 between tumour and normal tissue could be observed for both probesets, whereas the fold change (FC T/N) was higher in probeset 228969_at. This might be due to the fact that this probeset interrogates the outmost 3' end of the AGR2 mRNA. **C.** AGR2 expression in different pancreatic cancer cell lines in detail. Light hatched: probeset 228969_at; dark hatched: probeset 209173_at.

Table 3. Univariate disease specific survival analyses (median survival time in months).

Variable	Disease specific survival			
	No. of cases	No. of events	Median survival time (±95%CI)	p-value
AGR2				0.434
weakly (0/1).	29	20	12.4 (4.4-20.5)	
strongly (2/3)	71	60	13.1 (11.3-15.5)	
Age				0.277
<65 years	47	35	13.3 (6.2-20.5)	
≥ 65 years	53	45	12.4 (6.6-18.2)	
Gender				0.826
male	55	44	12.9 (9.9-15.9)	
female	45	36	15.9 (8.5-23.3)	
pT-status°				<0.001
pT1	0	0		
pT2	21	17	15.9 (10.3-21.5)	
pT3	71	55	13.8 (11.1-16.5)	
pT4	6	6	4.1 (1.1-7.1)	
Nodal status°				0.055
pN0	28	18	16.3 (1.6-31.1)	
pN1	70	60	12.4 (8.7-16.1)	
pM-status°				0.637
pM0	89	69	13.1 (10.9-15.4)	
pM1	4	4	6.5 (1.0-21.5)	
pl-status°				0.190
pl0	21	14	16.3 (3.6-29.0)	
pl1	78	65	12.5 (9.0-15.9)	
pv-status°				0.565
pv0	84	66	13.1 (10.7-15.6)	
pv1	15	13	9.6 (1.0-18.9)	
Histological grade°				0.059
G1	6	5	17.7 (13.8-21.6)	
G2	47	34	16.3 (9.4-23.2)	
G3	45	39	10.1 (6.1-14.2)	
Residual status°				0.322
negative (R0)	56	43	11.9 (8.3-15.7)	
positive (R1)	22	20	9.2 (3.9-14.4)	

° data not available for all patients.

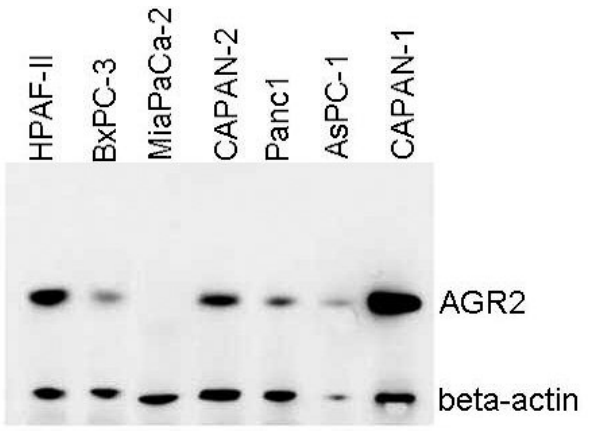

Fig. 2. Western blot analysis of AGR2 expression in human pancreatic cancer cell lines showing a single band. ß-Actin was used as loading control.

and residual tumour (R0/1) status, respectively. AGR2 expression did not reach significance in any of the subgroups analysed.

Discussion

AGR2 is the human homologue of the *Xenopus* Anterior Gradient-2 (XAG-2) protein (Liu et al., 2005), which has been shown to be expressed in several human tissues. In breast tumour tissue we were able to show that AGR2 expression is up-regulated (on mRNA and protein level) in carcinomas, and that upregulation is associated with estrogen receptor status, better tumour differentiation and a lower proliferative fraction (Fritzsche et al., 2006). Innes et al. validated the former two associations recently (Innes et al., 2006). Our study on breast cancer expression of AGR2 revealed that its overexpression was significantly associated with longer overall survival time. This is in contrast to Innes et al. (Innes et al., 2006) results, as they found a decrease in patient survival in the subgroup of estrogen receptor and AGR2 positive carcinomas. AGR2 over-expression has been found in Barrett´s epithelium, a metaplastic change of the esophageal squamous epithelium with a potential to advance to dysplasia and carcinoma (Pohler et al., 2004). Lung adenocarcinoma cell lines were analysed with array comparative genomic hybridisation and quantitative real time polymerase chain reaction by Zhu et al. As a strong up-regulation of AGR2 (Zhu et al., 2007) was found in their study they concluded that AGR2 is a candidate oncogene in lung cancer. We analysed AGR2 expression in non-small cell lung carcinomas (NSCLC) and found an upregulation, particularly in adenocarcinomas, although in this tumour entity no prognostic value of AGR2 could be demonstrated (Fritzsche et al., 2007). The previously published results of AGR2 in human cancers lead to the conclusion that it might play a role in the biology of certain cancers, especially in adenocarcinomas.

Using cDNA microarrays AGR2 has been found to be highly expressed in pancreatic carcinoma tissues and in pancreatic cancer (Iacobuzio-Donahue et al., 2003, Missiaglia et al., 2004). Missiaglia et al. confirmed their data by using immunohistochemistry and tissue microarrays containing 290 ductal pancreatic adenocarcinomas, and found that the majority (63%) of the carcinomas had a strong AGR2 expression (Missiaglia et al., 2004). Using a polyclonal antibody Ramachandran et al. found AGR2 in 56/57 (98%) of PDAC analysed (60% strong, 22% moderate and 18% weak (Ramachandran et al., 2008). These findings are in good concordance with our results, as we found AGR2 protein expression using a monoclonal antibody with a high frequency (73.7%) in PDAC. In our study group AGR2 expression was significantly higher in female patients (82.1% vs. 66.7% in male patients, p=0.040). This is of potential interest, since epidemiological data has shown that pancreatic carcinomas are at least partly estrogen driven (Kreiger et al., 2001) and AGR2

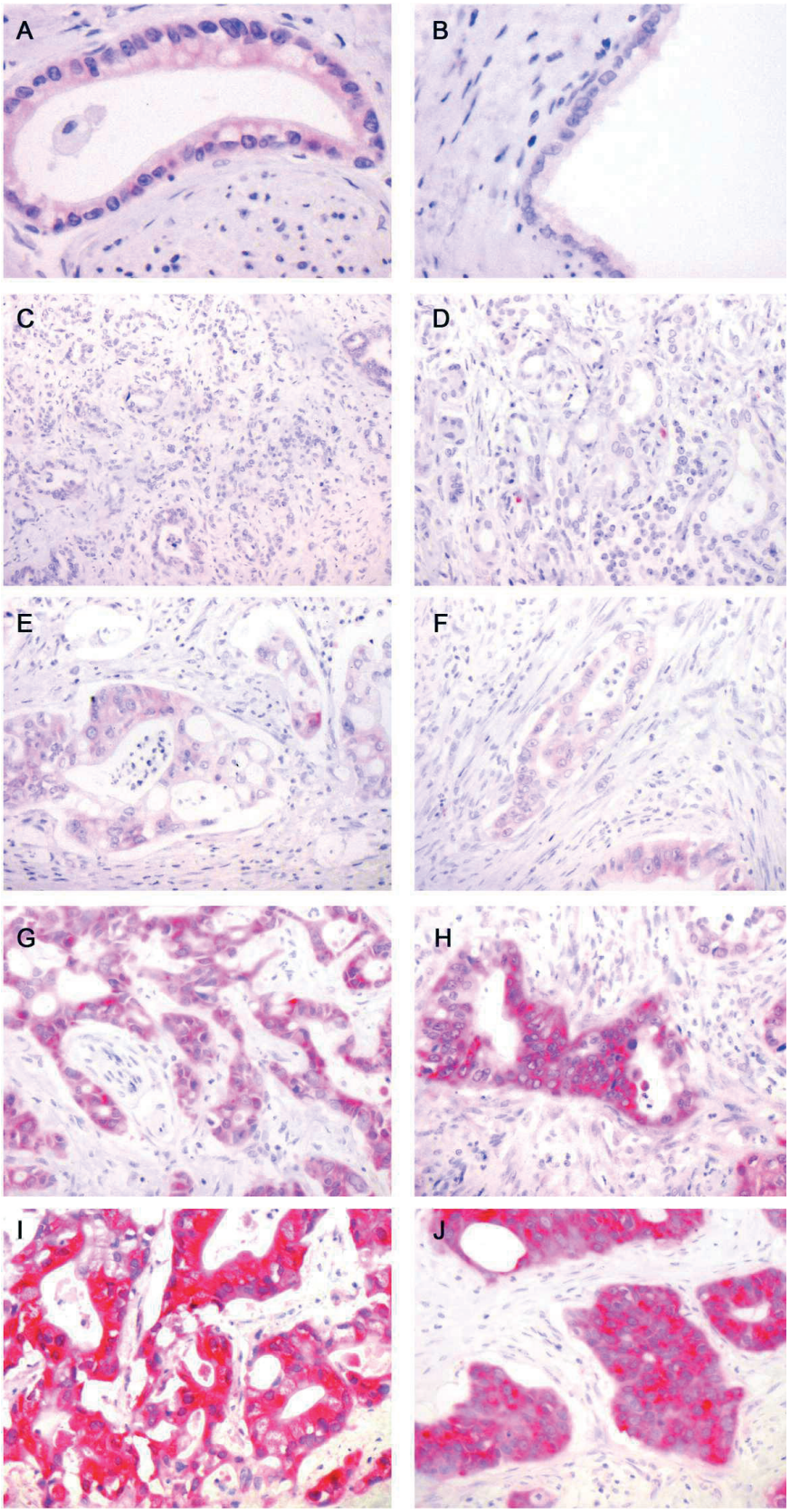

Fig. 3. AGR2 Expression in normal pancreas and pancreatic ductal adenocarcinoma. **A.** Normal pancreas with weak expression of AGR2. **B.** Normal pancreas negative for AGR2. **C/D.** AGR2 negative PDAC. **E/F.** PDAC weakly positive for AGR2; Score=1. **G/H.** Moderate AGR2 expression in PDAC; Score=2. **I/J.** PDAC with strong AGR2 expression; Score=3. x 400

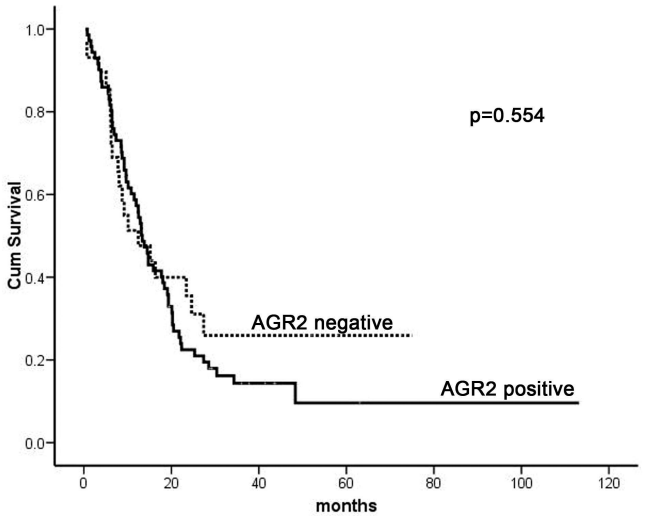

Fig. 4. Kaplan-Meier curve for Disease Specific Survival: AGR2 positive vs. negative pancreatic adenocarcinomas (n=100).

expression was found to be associated with a positive estrogen receptor status in breast carcinoma (Fritzsche et al., 2006). It can be speculated,, that AGR2 could be involved in the responsiveness of PDAC to hormonal stimulation in females.

Additionally, we found that AGR2 mRNA is over expressed in several pancreatic cancer cell lines, where it is secreted during pancreatic cancer development and plays an important role in cancer cell growth and survival (Ramachandran et al., 2008). In esophageal adenocarcinoma cell lines AGR2 promotes the classical features of malignant tumours, such as: tumour growth, cell migration and cellular transformation (Wang et al., 2008). Although our results did not show a correlation between AGR2 expression and overall survival, the high expression rate of AGR2 might contribute to the high mortality of PDAC.

In conclusion we can confirm that AGR2 mRNA is over expressed in PDAC cell lines and the AGR2 protein in the vast majority of PDAC tissue samples. Functional studies to investigate the biological role of AGR2 in PDAC and a possible use as a target of therapy are clearly warranted.

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