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Desmogleins as prognostic biomarkers in resected pancreatic ductal adenocarcinoma

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Background: Frequent disease relapse and a lack of effective therapies result in a very poor outcome in pancreatic ductal adenocarcinoma (PDAC) patients. Thus, identification of prognostic biomarkers and possible therapeutic targets is essential. Besides their function in cell–cell adhesion, desmogleins may play a role in tumour progression and invasion that has not been investigated in PDAC to date. This study evaluated desmoglein expression as a biomarker in PDAC.

Methods: Using immunohistochemistry, we examined desmoglein 1 (DSG1), desmoglein 2 (DSG2) and desmoglein 3 (DSG3) expression in the tumour tissue of 165 resected PDAC cases. Expression levels were correlated to the patients' clinicopathological parameters and postoperative survival times. We confirmed these results in two independent gene expression data sets.

Results: A total of 36% of the tumours showed high DSG3 expression that correlated significantly with shorter patient survival ($P=0.011$) and poor tumour differentiation ($P<0.001$), whereas no such association was detected for DSG1 or DSG2. In RNA-Seq data and in microarray expression data, high DSG3 expression correlated significantly with poor survival ($P=0.000356$ and $P=0.00499$).

Conclusions: We identify DSG3 as a negative prognostic biomarker in resected PDAC, as high DSG3 expression is associated with poor overall survival and poor tumour-specific survival. These findings suggest DSG3 and its downstream signalling pathways as possible therapeutic targets in DSG3-expressing PDAC.

Despite significant efforts during the past decades in basic and clinical research, pancreatic ductal adenocarcinoma (PDAC) remains a devastating disease with ~46 000 estimated cases and 40 000 estimated deaths in 2014 in the United States alone (DeSantis *et al*, 2014). In contrast to other solid malignancies, these efforts did not result in a substantial increase in overall survival times of PDAC patients. Still, the overall 5-year survival rate is 6% only (Siegel *et al*, 2014). The majority of PDAC cases are diagnosed with advanced, that is, metastatic disease, with palliative chemotherapy being the only treatment option in these cases (Ryan *et al*, 2014). A minority of 10–20% of cases present with primarily resectable disease or resectability can be achieved by downstaging through neoadjuvant chemotherapy (Ghaneh *et al*, 2007). Resection with curative intent and additional adjuvant chemotherapy results in an increase in 5-year survival rates up to 20% (Ryan *et al*, 2014). Nevertheless, disease recurrence rates approach 80% because of local recurrence or development of distant metastases (Arvold *et al*, 2012). In addition, PDAC incidence rates are rising in western societies (Hidalgo *et al*, 2015), stressing the socioeconomic impact of the disease in the future. Thus, there is an urgent need to identify prognostic factors and potential therapeutic targets to improve the outcome of PDAC patients.

The desmoglein (DSG) glycoproteins (DSG1, DSG2, DSG3 and DSG4) are a group of essential cadherins in desmosomal intercellular junctions that establish a link between adjacent cells by both homophilic interactions with desmoglein molecules and

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heterophilic interactions with desmocollin molecules (DSC1, DSC2 and DSC3) on opposing cells in the extracellular space (Desai *et al*, 2009; Berika and Garrod, 2014). Their intracellular ends then bind via several intermediate proteins to the filaments of the cytoskeleton, thus helping to maintain the integrity of epithelial tissues (Desai *et al*, 2009). Hence, desmogleins are widely expressed throughout stratified epithelia and a variety of desmosome-forming tissues (Holthofer *et al*, 2007). Besides their pivotal function in cell–cell adhesion, desmosomes have been discovered to be involved in extracellular to cytoplasmic signal transduction processes that may play a role in the development of malignancy (Chidgey and Dawson, 2007; Kolegraff *et al*, 2011). Accordingly, abnormal expression of desmosomal proteins has been detected in several types of cancer, for example, downregulation of DSC2 in colorectal cancer or downregulation of DSC3 in breast and oral cavity cancer (Oshiro *et al*, 2005; Khan *et al*, 2006; Wang *et al*, 2007). In contrast, other desmosomal proteins have been found to be upregulated in other tumours, for example, DSG2 and DSG3 in oesophageal squamous carcinoma, prostate, lung as well as head and neck cancer (Kurzen *et al*, 2003; Biedermann *et al*, 2005; Trojan *et al*, 2005; Yashiro *et al*, 2006; Fukuoka *et al*, 2007). These findings indicate that a dysregulation of desmosomal proteins plays a role in carcinogenesis. Particularly, desmogleins have been reported to be involved in cancer progression by contributing to tumour cell growth and tissue invasion through activation of oncogenic signalling pathways (Brennan *et al*, 2007; Mannan *et al*, 2011). For example, DSG3 expression is the most sensitive parameter for lymph node metastasis in head and neck cancer (Ferris *et al*, 2011; Patel *et al*, 2013). Recent data indicate that especially DSG3 is directly or indirectly involved in the activation of the canonical WNT signalling cascade and the Ezrin/protein kinase C (PKC) pathway, thus conferring tumourigenic properties and an invasive phenotype (Chen *et al*, 2013; Brown *et al*, 2014).

Despite their obvious importance in many types of cancer, expression profiles and functional data on desmogleins in PDAC are limited to date and their role in PDAC tumourigenesis and progression remains poorly understood. In the present study, we therefore examined desmoglein expression in resected PDAC tissue and a potential link to the clinical outcome of PDAC patients. We show that high DSG3 expression in PDAC tumour tissue is significantly associated with poor patient survival and establish DSG3 as a prognostic biomarker in pancreatic ductal adenocarcinoma.

MATERIALS AND METHODS

Study population and tumour samples. The study population consisted of 165 PDAC patients who underwent resection with curative intent at the Department of Surgery at the Friedrich-Schiller University Jena (Jena, Germany; $n=102$) and the Department of Surgery at the University of Munich (Munich, Germany; $n=63$) between 1995 and 2012. Median age was 66 years (range 33–87 years; female $n=82$, male $n=83$). Study exclusion criteria were perioperative mortality within 90 days, distant metastases at the time of surgery and the presence of microscopic or macroscopic residual disease after surgical resection (R1 or R2 status). Patients with ampullary carcinomas or carcinomas of the distal bile duct were excluded from analysis. Data on clinical parameters, including survival time, sex, age and adjuvant chemotherapy, were obtained from the prospective tumour registry of the Department of Surgery at the Friedrich-Schiller University Jena and the Munich Cancer Registry (MKR). Histopathological findings (tumour location, T-classification and lymph node status) were obtained from the pathologists' original reports. In addition, histopathological parameters such as tumour type and degree of differentiation (grading) were reassessed by two pathologists (SO and TK) on whole mount tissue sections.

Formalin-fixed, paraffin-embedded tumour tissue of the 165 PDAC cases was acquired from the archives of each University's Institute of Pathology. A tissue microarray (TMA) consisting of two cores of PDAC tumour tissue, each 0.6 mm diameter, was constructed using a semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Exemplary samples of normal pancreatic epithelium were included into the staining runs.

This retrospective study was carried out according to the recommendations of the local ethics committee of the Medical Faculty of the University of Munich.

Immunohistochemistry. Immunohistochemical staining of 4 μm sections was done strictly following the manufacturer's instructions on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ, USA) using an anti-DSG1 rabbit monoclonal primary antibody (clone EPR6766(B), Abcam, Cambridge, UK; dilution 1 : 400), an anti-DSG2 rabbit polyclonal primary antibody (Atlas Antibodies, Stockholm, Sweden; dilution 1 : 350) and an anti-DSG3 mouse monoclonal primary antibody (clone 7B9, Abcam; dilution 1 : 150). For signal detection, the ultraView diaminobenzidine kit (Ventana Medical Systems) was employed. Appropriate positive controls (normal skin for DSG1, normal colon mucosa for DSG2 and normal squamous esophageal mucosa for DSG3) and negative control tissue (skeletal muscle) were included in the staining runs (Supplementary Figure S1). The expression level of each factor examined was scored semiquantitatively using a four-tier scale (0 = negative; 1 = weak; 2 = moderate; 3 = strong). Finally, staining intensities were analysed as dichotomous variables, defining scores 0–1 as low and 2–3 as high expression levels. The staining intensities of each marker were independently evaluated by two experienced pathologists (SO and TK), blinded to the patient's clinical outcome and clinicopathological parameters. Concordance between the two pathologists was as follows (all $P<0.001$): for DSG1: 0.83, for DSG2; 0.82 and for DSG3: 0.90. Discrepant cases were discussed until agreement was reached.

Pictures of immunohistochemically stained slides were acquired on a camera-equipped Zeiss Axioskop microscope (Zeiss, Wetzlar, Germany) at 200-fold magnification using proprietary Zeiss Axiovision software.

Statistical analysis. Overall survival time was calculated from the date of pancreatic surgery to death, irrespective of its cause. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were calculated by the Kaplan–Meier method and the log-rank test was used to assess differences in survival. Significant and independent predictors of disease-specific survival and recurrence were identified by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. Statistical significance was defined as P-value <0.05.

TCGA data set analysis. Publicly available RNA-Seq expression data and corresponding clinical data of 135 PDAC samples were retrieved from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga/). Tumour-specific survival (TSS) was defined as patient death with tumour. For TSS prediction, receiver operator curve analyses (ROC) were used to determine optimal cutoff values for DSG1, DSG2 and DSG3. The expression of each biomarker was then correlated with patient survival times in univariate analyses and Kaplan–Meier curves were plotted. Significant differences in survival were detected by the log-rank test.

Computational expression data analysis. *SurvExpress* (Aguirre-Gamboa *et al*, 2013) and *Oncomine* (Rhodes *et al*, 2007) online tools, both analysing publically available microarray gene expression data, were used to explore potential alterations of DSG1, DSG2 and DSG3 in normal and disease states. *SurvExpress* was utilised to compare

survival profiles for individuals segregated based on high and low DSG1, DSG2 and DSG3 expression with risk groups maximised and censored for survival in months ((Aguirre-Gamboa *et al*, 2013) GSE21501, (Stratford *et al*, 2010)). *Oncomine* was used to compare studies in which both cancer and adjacent normal samples were present to determine whether patterns in DSG1, DSG2 and DSG3 expression existed (Logsdon *et al*, 2003).

RESULTS

Patient characteristics and survival data. The age of the patients ranged from 33 to 87 years (median age 66 years, female $n=82$, male $n=83$). At 5 years after surgery, 138 patients (84%) had deceased and 27 patients (16%) had survived. The median survival time for all 165 patients was 19 months (95% CI 16–22 months) with a 3- and 5-year survival rate of 25% and 8%, respectively. A total of 46 patients (27.9%) showed no lymph node metastasis at the time of surgery and were thus classified as UICC stage I or IIA, whereas in surgical specimens of 119 patients (72.1%) lymph node metastases were detected and thus were classified as UICC stage IIB. For statistical reasons, patients were grouped according to age (<70 years and ⩾70 years), differentiation grade (G1/G2 = low grade, G3 = high grade), stage (stage I or stage IIA, stage IIB or stage III) and pT category (pT1/pT2 or pT3/pT4).

As expected, positive lymph node status and corresponding UICC stage had a marked, although not statistically significant, effect on overall survival in this patient cohort (25 months *vs* 18 months, $P=0.071$, Table 1 and Figure 1A). No statistically significant differences between age groups (22 months *vs* 19 months, $P=0.506$, Table 1 and Supplementary Figure S2), sexes (20 months *vs* 19 months, $P=0.559$) or pT category (20 months *vs* 19 months, $P=0.258$) could be detected (Table 1). Of note, the majority of patients had high-grade tumours ($n=106$; 64.2%) with significantly shorter survival than patients with low-grade tumours (28 months *vs* 16 months, $P=0.001$, Table 1 and Figure 1B).

Table 1. Correlation of clinicopathological parameters and median survival times

	N	Median survival (months)	*P*, log rank (Mantel–Cox)
Total patients	165	19	
Age			
<70 Years	111	19	0.506
⩾70 Years	54	22	
Sex			
Female	82	20	0.559
Male	83	19	
pT category			
pT1/2	38	20	0.258
pT3/4	127	19	
UICC stage			
I/IIA	46	25	0.071
IIB/III	119	18	
Grade			
Low	59	28	0.001
High	106	16	
DSG1			
Low	82	21	0.339
High	83	19	
DSG2			
Low	57	21	0.880
High	108	19	
DSG3			
Low	106	23	0.011
High	59	15	

Abbreviations: DSG1 = desmoglein 1; DSG2 = desmoglein; DSG3 = desmoglein 3; UICC = Union for International Cancer Control. Frequency of clinicopathological patient characteristics (age, sex, pT category, UICC stage, tumour differentiation grade) and expression of DSG1, DSG2 and DSG3 in the pancreatic ductal adenocarcinoma (PDAC) patient collection.

Desmoglein expression and clinicopathological parameters. In immunohistochemical stainings of representative tumour and normal pancreatic tissue as well as in *Oncomine* analysis (Rhodes *et al*, 2007) (Supplementary Figure S3), DSG3 was significantly overexpressed in PDAC tissue as compared with normal pancreatic epithelium, where we found very low or absent expression (Figure 2). In contrast, we found no such clear differences for DSG1 and DSG2, where we detected varying degrees of expression in normal tissue already (Figure 2 and Supplementary Figure S3). In PDAC tumour tissue, we observed variable expression of the three desmogleins, showing a strong membranous staining reaction for all three desmogleins in the positive cases (score ⩾1). To preclude sampling errors because of intratumoural expression heterogeneity, we additionally examined the expression pattern of each marker in an exemplary subset on whole mount tumour tissue sections, showing no significant differences between central

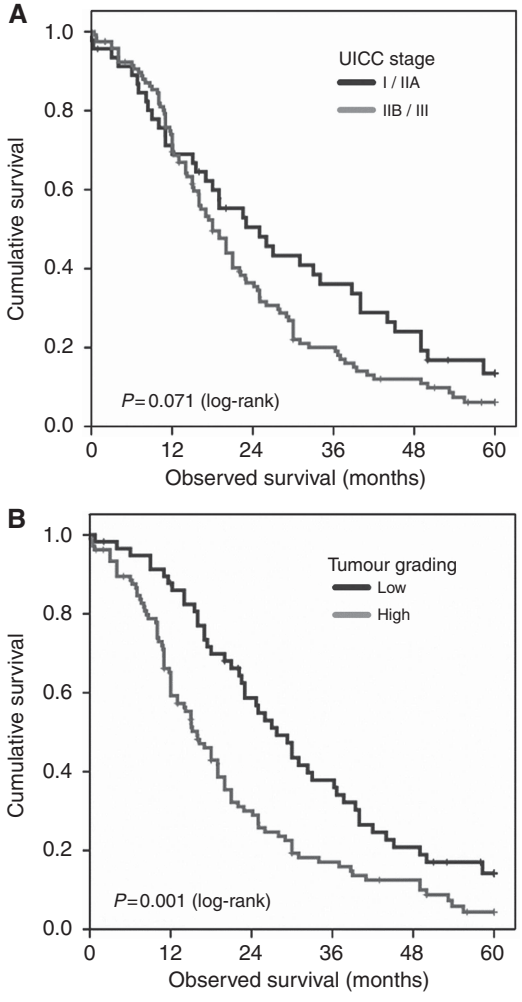

Figure 1. Poor tumour differentiation grade correlates with inferior survival of PDAC patients. Univariate analysis (Kaplan–Meier curves and log-rank tests) of (**A**) UICC stage and (**B**) tumour differentiation grade as prognostic parameters in resected PDAC patients. Crossed lines indicate censored cases.

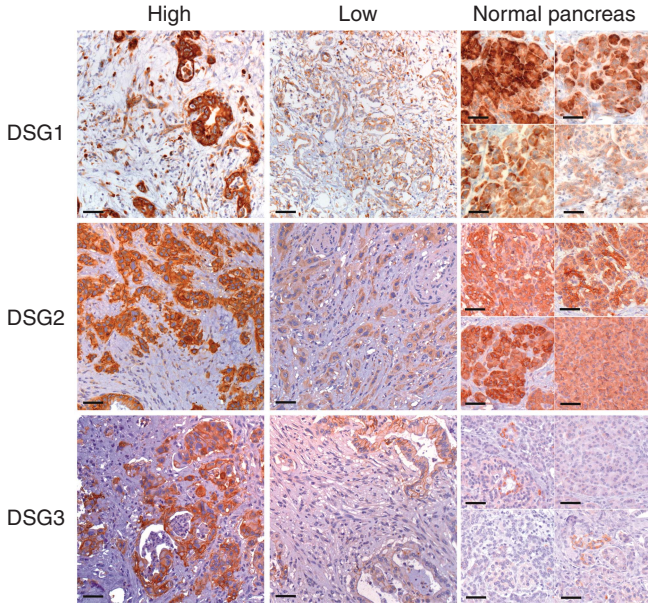

Figure 2. Variable expression of DSG1, DSG2 and DSG3 in normal pancreatic and PDAC tissue. Immunohistochemical staining of DSG1, DSG2 and DSG3 in exemplary normal pancreatic tissue as well as in exemplary PDAC cases classified as high or low expression, according to the staining intensity. It shows a variable expression pattern of DSG1 and DSG2 in normal pancreatic and PDAC tissue, whereas strong DSG3 expression is confined to PDAC tissue. 200-fold magnification. Scale bars, 50 μm.

Table 2. Correlation of clinicopathological parameters and DSG1, DSG2 and DSG3 expression

	N	DSG1 high	*P*	DSG2 high	*P*	DSG3 high	*P*
Age							
<70 Years	111	49%	NS	58%	0.003	37%	NS
⩾70 Years	54	54%		82%		33%	
Sex							
Male	83	49%	NS	63%	NS	36%	NS
Female	82	51%		68%		35%	
Grade							
Low grade	59	44%	NS	71%	NS	15%	<0.001
High grade	106	54%		62%		47%	
pT category							
pT1/2	38	63%	NS	63%	NS	24%	NS
pT2/3	127	47%		66%		39%	
pN category							
pN 0	46	54%	NS	61%	NS	39%	NS
pN 1	119	49%		67%		35%	
UICC stage							
Stage I/IIA	46	54%	NS	61%	NS	39%	NS
Stage IIB/III	119	49%		67%		35%	
L							
L0	71	55%	NS	68%	NS	35%	NS
L1	91	47%		65%		36%	
V							
V0	137	50%	NS	68%	NS	35%	NS
V1	21	52%		52%		38%	
Pn							
Pn0	58	59%	NS	62%	NS	28%	NS
Pn1	82	44%		66%		37%	

Abbreviations: DSG1 = desmoglein 1; DSG2 = desmoglein; DSG3 = desmoglein 3; NS = not significant; UICC = Union for International Cancer Control. Correlation of patients' clinicopathological characteristics (age, sex, differentiation grade, pT category, pN category, UICC stage, lymphatic vessel invasion (L -status), blood vessel invasion (V status), perineural invasion (Pn status)) with DSG1, DSG2 and DSG3 expression.

and marginal tumour areas (Supplementary Figure S4). High expression was observed in 50.3% for DSG1, in 65.5% for DSG2 and in 35.7% for DSG3 of all cases. Subsequently, we analysed the correlation of DSG1, DSG2 and DSG3 expression levels with patients' clinicopathological parameters age, sex, tumour grade, tumour stage and lymph node metastasis as well as lymphatic and blood vessel invasion or perineural invasion. High DSG2 expression was significantly associated with patient age ($P = 0.003$, Table 2). Interestingly, high DSG3 expression was associated with poor tumour differentiation ($P < 0.001$), whereas no such association was detected for DSG1 and DSG2. We found no further statistically significant association of tumoural desmoglein expression to other clinicopathological traits that is, lymph node metastasis, lymphatic or blood vessel invasion or perineural invasion (Table 2).

High DSG3 expression is a negative prognostic factor in PDAC. We conducted a univariate analysis to correlate the outcome of patients monitored during the 5-year period with the expression of the desmogleins examined within the tumour tissue. No statistically significant differences in survival times of patients were observed for DSG1 or DSG2 expression levels (21 months *vs* 19 months, $P = 0.339$ and 21 months *vs* 19 months, $P = 0.880$, Figure 3A and B and Table 2). Interestingly, high DSG3 expression within the tumour tissue significantly correlated with a shorter post-interventional survival time (23 months *vs* 15 months, $P = 0.011$, Figure 3C and Table 2). As we found a significant correlation between DSG3 expression and tumour differentiation (Table 2), we additionally calculated the postoperative patient survival times according to tumoural DSG3 expression in the low-grade tumour and high-grade tumour subgroups. Here, the prognostic impact of tumoural DSG3 expression was still detectable, although not statistically significant because of the small subgroup sample size (low-grade group 33.2 *vs* 24.5 months, $P = 0.192$; high-grade group 22.2 *vs* 18.8 months; $P = 0.233$, Supplementary Figure S5A and B). To finally test whether DSG3 expression could serve as independent prognostic biomarker, we subsequently examined the previously identified factors in a multivariate analysis. In a Cox regression model, which included age, stage, tumour grade, lymphatic and blood vessel invasion, perineural invasion and expression level of DSG1, DSG2 and DSG3, high tumour grade and tumour stage were the only statistically independent variables (hazard ratio 2.040 (1.305–3.188), $P = 0.002$ and 1.878 (1.167–3.024), $P = 0.009$, respectively, Supplementary Table S1).

To test whether DSG expression has an effect on TSS and to verify our findings, we examined the association of DSG1, DSG2 and DSG3 expression and patient TSS in an independent, publicly available data set of the TCGA database, containing 135 PDAC samples. After defining a cutoff value for each biomarker using ROC analysis, cases were divided into low and high expression for all three markers, and patient survival was analysed by Kaplan–Meier plots. For DSG1, 72 tumours were classified as high expression and 63 as low expression. The ratio (high *vs* low expression) for DSG2 was 39 to 96. In all, 81 tumours showed a high DSG3 expression, whereas 54 tumour samples were classified as low DSG3 expression. Using the log-rank test to calculate statistical differences between the patient groups (high *vs* low expression for each desmoglein), no effect of DSG1 expression on patient survival was detected (median survival time 22.7 months *vs* 19.8 months; $P = 0.366$, Supplementary Figure S6). Surprisingly, we also detected a significantly shorter survival for patients with a

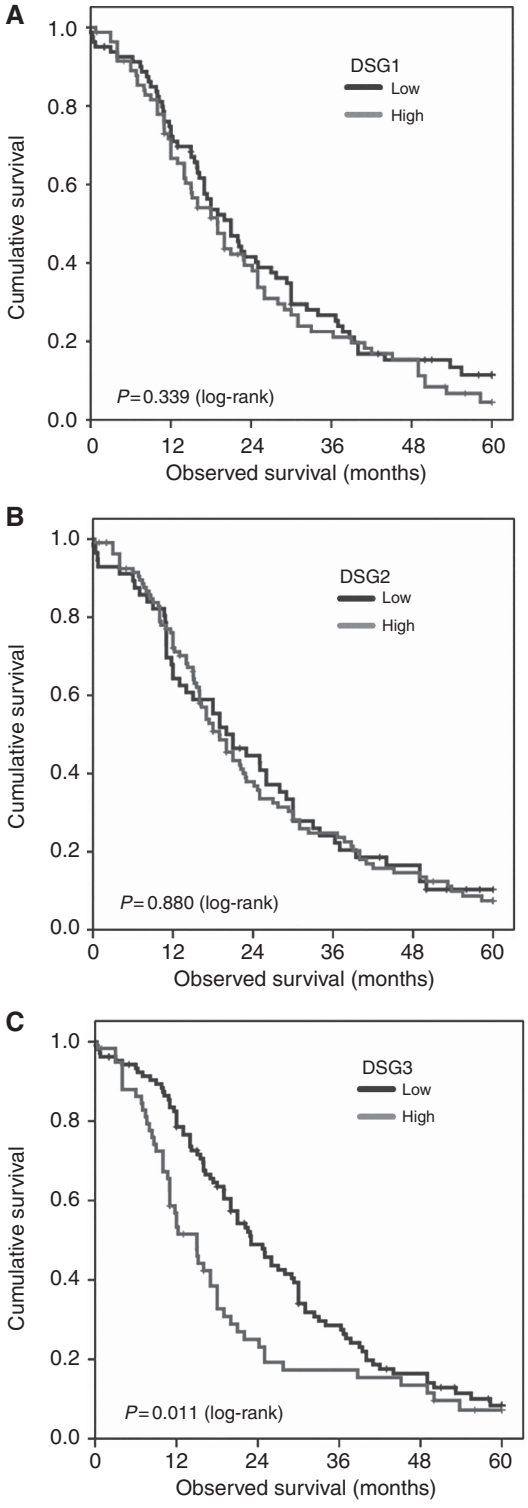

Figure 3. The DSG3 expression correlates with inferior survival of PDAC patients. Univariate correlation analysis (Kaplan–Meier curves and log-rank tests) of (**A**) DSG1, (**B**) DSG2 and (**C**) DSG3 expression and postoperative survival in resected PDAC patients. Crossed lines indicate censored cases.

high DSG2 expressing tumour (median survival time 19.7 months *vs* 22.5 months; $P=0.029$, Figure 4A). However, the statistically strongest inverse association between desmoglein expression and patient survival was found for DSG3 (median survival time 12.9 months *vs* 22.8 months; $P=0.000356$, Figure 4B and Supplementary Table S2). For additional external validation of

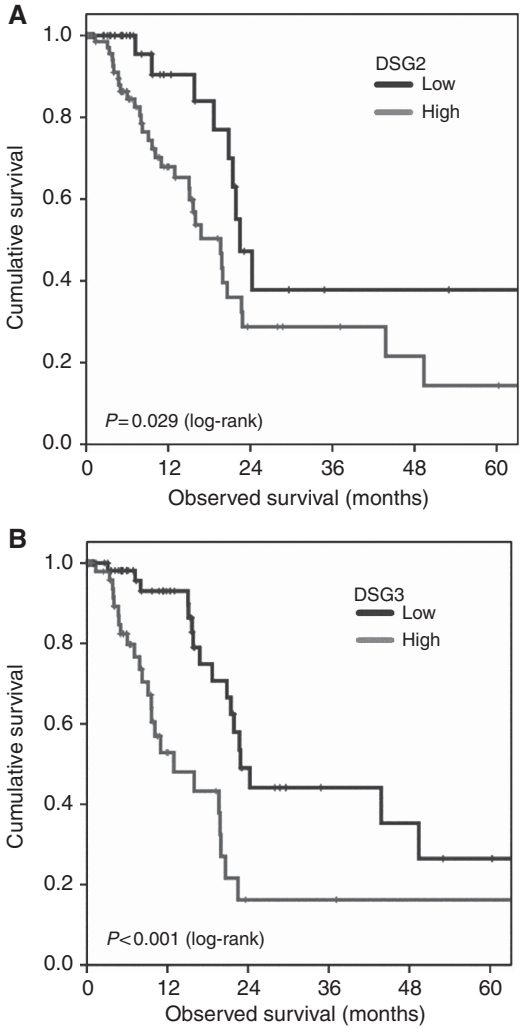

Figure 4. DSG2 and DSG3 expression correlate with inferior survival of PDAC patients. Univariate analysis (Kaplan–Meier curve and log-rank test) in a TCGA RNA-Seq data set of PDAC tissue samples examining (**A**) DSG2 and (**B**) DSG3 expression levels as dichotomous variable after defining a cutoff via ROC analysis. Crossed lines indicate censored cases.

these findings, we compared the survival profiles of 102 PDAC patients based on high or low expression of DSG1, DSG2 and DSG3 using publically available microarray expression data (GSE21501 (Stratford *et al*, 2010)) applying the online analysis tool *SurvExpress* (Aguirre-Gamboa *et al*, 2013). Again, high DSG3 expression was significantly associated with poor patient survival (median survival time 21 *vs* 15 months; $P=0.00499$, Supplementary Figure S7A), whereas no such association was detected for DSG1 (median survival time 17 *vs* 19 months; $P=0.607$, Supplementary Figure S7B) or DSG2 (median survival time 19 *vs* 17 months; $P=0.592$, Supplementary Figure S7C).

DISCUSSION

In this study, we investigated the value of desmoglein expression as prognostic biomarkers in resected PDAC. Using a well-defined collection of R0-resected PDAC specimens, we show that high DSG3 expression is significantly associated with shorter postoperative patient survival, whereas no such association was detected for DSG1 or DSG2 expression. Moreover, we tested these findings in two independent, publicly available gene expression data sets. In the RNA-Seq gene expression data from the TCGA

consortium, as well as in microarray gene expression data, DSG3 expression was confirmed as strong negative prognostic factor, adding further evidence to our data. In addition, these findings revealed the significance of DSG3 expression not only for overall survival of patients but also for TSS. Surprisingly, in the TCGA data set also high DSG2 expression correlated with poor patient survival, although with a much lower statistical power. As we readily detected variable DSG1 and DSG2 expression in normal pancreatic tissue but no prognostic effect of their expression, neither in our patient collection nor in the microarray data set, the prognostic role of DSG2 expression in PDAC remains at least questionable. In contrast, high DSG3 levels were detected in tumour tissue only and correlated with poor tumour differentiation grade, though not with lymphatic or blood vessel invasion or perineural invasion. However, the association with poor clinical outcome reflects a more aggressive and invasive tumour biology. The poor prognosis in PDAC is mainly due to disease recurrence as distant metastasis or local recurrence after curative resection (Iacobuzio-Donahue *et al*, 2009), in which invasive growth and tumour cell motility play a major role (Hanahan and Weinberg, 2011). This could be mediated by an increased activation of oncogenic and invasiveness promoting signal transduction pathways in which DSG3 is involved (Brown and Wan, 2015). In fact, DSG3 expression has been reported to activate the transcriptional factor activator protein 1 (AP-1) and the PKC/Ezrin pathway, inducing migratory and invasive properties in tumour cells and thus increasing their metastatic potential (Brown *et al*, 2014). Another possible mechanism through which DSG3 could mediate the development of an invasive phenotype is the upregulation of the WNT-target genes c-myc, cyclin-D1 and matrix metalloproteinase 7 (MMP7) via plakoglobin that has been shown to increase cell migration and tissue invasion (Chen *et al*, 2013). In line with these findings, high DSG3 expression has been associated with increased metastasis formation (Trojan *et al*, 2005; Wang *et al*, 2007). The fact that DSG3 is overexpressed in PDAC, whereas DSG1 and DSG2 are not, may explain its prognostic impact in PDAC and supports its possible role in oncogenic and invasiveness promoting signalling. Moreover, these previously published data on DSG3 function together with our findings offer potential therapeutic strategies by targeting DSG3 or inhibiting downstream pathways in tumours with high DSG3 expression (Brown *et al*, 2014). As DSG3-targeted therapy has been shown to be feasible and effective in DSG3-expressing tumours (Kawai *et al*, 2009), this could prove to be especially valuable in PDAC where effective therapies are still desperately searched for (Hidalgo *et al*, 2015). Further research is necessary to elucidate the mechanisms of DSG3 function in PDAC progression and to validate its significance as a possible therapeutic target.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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