Preservation of *HUGL-1* Expression as a Favourable Prognostic Factor in Pancreatic Carcinoma

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Abstract. *Aim: The expression of the human homologue of Drosophila tumour suppressor gene lgl (HUGL-1) in pancreatic cancer was retrospectively assessed in 97 patients with surgically treated pancreatic cancer in order to correlate the HUGL-1 profile with patients' survival. Materials and Methods: Immunohistochemistry was performed on 4-μm-thick paraffin sections from representative tumour blocks using a standard protocol. The expression of HUGL-1 was evaluated semiquantitatively as negative (0), weak (1), medium (2) or strong (3). The results were correlated with clinicopathological parameters and with patients' survival, considering an observation period of 17 (mean) ± 16 (SD) months. Results: In normal and inflammatory tissue, a uniform and relatively strong staining was observed in ductal epithelium, ganglion cells and some acinar epithelia. The endocrine islets exhibited a weak positivity. Human pancreatic cancer revealed variable intensities of HUGL-1 expression. A total of 69 tumour specimens were classified as negative and 28 as positive. The HUGL-1 expression was not correlated with clinical variables (age, gender), staging or tumour grading. HUGL-1 positivity proved to be prognostically favourable (p=0.0241) conferring a higher survival rate, especially for patients who had survived more than 12 months. The presence of distant metastases (M1) at diagnosis had a weak significant influence on survival (p=0.0474). The other staging parameters (T, N, UICC stage), tumour grading and clinical variables (age, gender) gave no significant prognostic information. In a multivariate Cox model, only HUGL-1 expression passed the entry limits. Conclusion: Preservation of HUGL-1 expression in pancreatic adenocarcinoma is a good prognostic factor that contributes to a better overall survival.*

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Pancreatic adenocarcinoma, which accounts for >85% of all pancreatic malignancies, represents an extremely unfavourable tumour entity, with an almost 95% mortality, a median survival time of <6 months after diagnosis and a 5-year survival rate that has increased only from 3% to 5% within the last 30 years (1-2). In Germany, currently more than 13,000 cases are diagnosed per year, with a still increasing incidence, at least in women (2). Worldwide, the number of deaths per year by pancreatic adenocarcinoma has been estimated at 227,000 (3). The main reason for the negative clinical course is the advanced stage of the tumour at diagnosis, which also limits the resectability of many cases (resection rates: 2.6%-9%) (4-6).

However, even in the group of surgically treated patients, the majority develop disease recurrence within two years after resection in spite of tumour-free margins (R0 resection), indicating the presence of clinically undetectable micrometastases at the time of surgery in lymphatic vessels or nerve sheaths. Adjuvant therapy has also only resulted in very slow progress, and a significant improvement of prognosis, for example, by targeted therapy against key pathways or tumour-associated proteins, has not yet been achieved, following the report of the 2011 Annual Meeting of the American Society of Clinical Oncology (ASCO) (7). Even after extended pancreatic resection with R0 status, the 5-year survival rate is still <20% (8-13).

Considering the fatal prognosis of most of the cases, it is not surprising that molecular or morphological prognostic factors are rarely identified. To date, no molecular variable has been acknowledged as being generally useful for the prognosis of pancreatic cancer (14): in the current nationwide German S3 guideline for diagnosis and treatment of exocrine pancreatic cancer, published in 2007 (15), such factors are not even mentioned. Thus, findings of genetic abnormalities in pancreatic

cancer such as mutational activation of the K-ras oncogene, inactivation of tumour suppressor genes such as CDKN2A, p53, SMAD4, and BRCA2, or findings of chromosomal losses, gene amplifications, and telomere shortening (16-18) are looked upon as being important for the explanation of evolutionary steps of pancreatic cancer, but not as being clinically relevant. The same is true for immunohistochemistry. In a worthy recent review (19), it was stated that none of the 76 immunohistochemical variables considered can really be recommended for routine clinical use at present due to inconsistent results between various studies and the mostly small cohorts investigated. This statement was also valid for markers which are well established in other tumour entities such as MIB-1, BCL-2, VEGF, Cox2, CD34, p53, and p27. Moreover, the results of the immunohistochemical analysis of HER-2neu overexpression remain inconclusive, ranging from strong multivariate prognostic influence (20) to lacking univariate prognostic significance (21), or even to contradictory results with a shorter survival in the HER2-neu-positive group (22). The application of the UroVysion test, a fluorescence-*in situ*-hybridization (FisH) technique, has been evaluated as being very useful for diagnostics (23), but is of only limited prognostic influence as the copy number of chromosome 17, which is part of the UroVysion test, revealed prognostically significant results in only one study (24).

As a new prognostic approach for pancreatic carcinoma, we investigated the influence of *HUGL-1* immunohistochemistry. *HUGL-1* is the human homologue of the Drosophila tumour suppressor gene *lgl* that encodes for a cortical cytoskeleton protein and thus plays an important role in preserving epithelial integrity and cellular polarity (25-27). The *HUGL-1* gene is located in a pericentromeric region on the short arm of chromosome 17 (17p11.2–12), which may contain a potential cancer susceptibility gene for primitive neuroectodermal tumours (PNETs) (28-29). Molecular characterization of this region showed the presence of a cluster of deletion breakpoints in PNETs, but to date, the role of *HUGL-1* in human cancer remained largely unknown (30). In molecular or immunohistochemical studies on colorectal carcinoma (31), malignant melanoma (32), endometrial carcinoma (33) and liver cell carcinoma (34), a reduced or even loss of expression of *HUGL-1* was correlated with tumour progression. However, no data on pancreatic tissue have been presented yet. In this study, we evaluated the immunohistochemical expression of *HUGL-1* in a series of patients with surgically treated pancreatic adenocarcinoma and correlated the results with the patients' clinicopathological data and survival.

Materials and Methods

Patient characteristics and tumour material. Ninety-seven cases of surgically treated exocrine pancreatic adenocarcinoma were enrolled in the study (Table I). The tumour diagnoses were made at the Institute of Pathology of the Johannes Gutenberg University, Mainz, investigating hematoxylin and eosin (H&E-) and periodic acid-Schiff (PAS–) stained paraffin sections by conventional light microscopy.

The clinicopathological data represent a typical cohort of patients with pancreatic carcinoma, *i.e.* patients of higher age presenting with mostly advanced tumours at the date of diagnosis. Overall, 56 patients (57.7%) were male, and 41 patients (42.3%) were female. The mean age of the patients was 64.6 years ± 8.8 (SD) years and did not significantly differ between men and women ($p>0.05$). According to the TNM classification, most of the tumours were of an advanced stage (T3/T4=68.0%) and revealed lymph node metastases (N1/N2=72.2%). The rate of distant metastases (M1) was 9.3%. Several tumours were classified as being high grade (G3/G4=42.3%).

The observation period ranged between two and 92 months (mean 17±16 (SD) months). Eighty-six patients had died after a mean survival time of 14±12 (SD) months, while 11 patients were alive with survival periods of 44±22 (SD) months.

Immunohistochemistry. Four-micrometre-thick paraffin sections from representative tumour blocks were screened for *HUGL-1* protein expression using the LSAB+ System-HRP Kit (DAKO, Glostrup, Denmark). In brief, the sections were deparaffinized, rehydrated and subsequently incubated with the primary polyclonal rabbit *HUGL-1* antibody (dilution: 1:300; courtesy of D. Strand, Ph.D., First Department of Internal Medicine, Johannes Gutenberg University Mainz) for 3 h after previous blocking of endogenenous peroxidase by H_2O_2. The secondary antibody (biotin-labelled anti-rabbit; DAKO) was incubated for 15 min, followed by incubation with streptavidin-labelled peroxidase for 15 min. Antibody binding was visualized using a 3.3-diaminobenzidine (DAB) solution for 15 min. Finally, the tissues were counterstained by a conventional H&E staining and mounted using a conventional mounting medium with all steps of the immunohistochemical reaction performed at room temperature. Per case, an additional section was used as a case-specific negative control by omitting the primary antibody. As a case-specific positive control, normal pancreatic tissue surrounding the tumour tissue was investigated. The evaluation of *HUGL-1* immunohistochemistry was performed semiquantitatively by light microscopy and the intensity of staining was graded as negative (score 0), weak (score 1), medium (score 2) or strong (score 3).

Statistics. For statistical purposes, the BMDP package (Statistical Software Inc., Los Angeles, CA, USA) was used. For the analysis of correlation between categorized variables, multi-field tables were calculated and interpreted using the Pearson Chi^2-test of independence. For the comparison of continuously scaled variables, the Mann Whitney *U*-test was used. Univariate survival analysis was performed according to Kaplan and Meier, and the respective curves were tested for significant differences by a pairwise Mantel Cox test. For multivariate survival analysis, Cox regression models were calculated. Statistical significance was accepted for $p<0.05$ (Cox models: $p<0.10$).

Results

Tumour-free pancreatic tissue. As HUGL-1 immunohistochemistry was applied to pancreatic tissue for the first time, it appeared to be useful to analyze its staining pattern in microscopically normal tissue. The reaction revealed

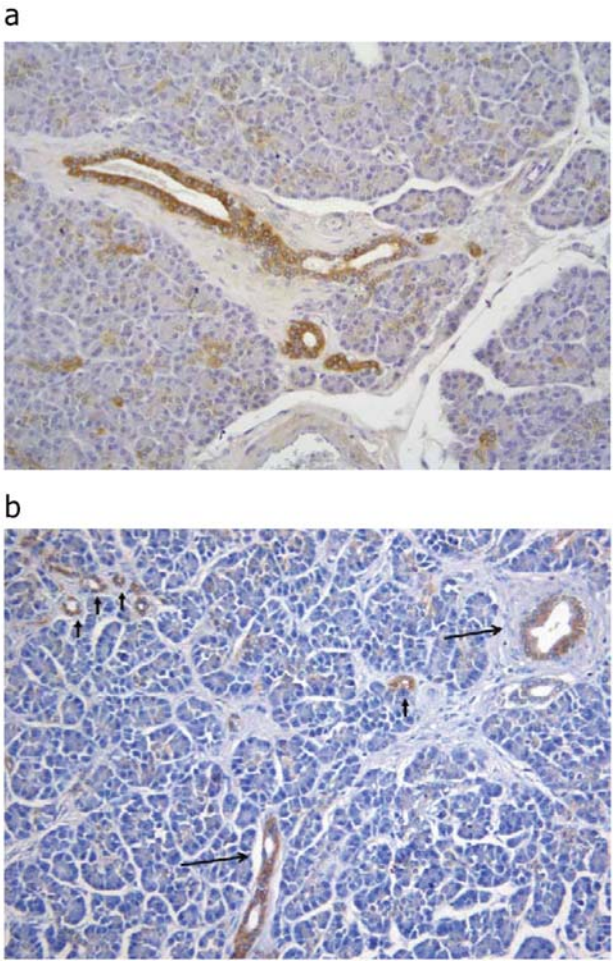

Figure 1. *In normal pancreatic tissue, strong HUGL-1 expression is present in ductal epithelium: a: large ducts, b: small ducts, indicated by arrows. Magnification, ×10.*

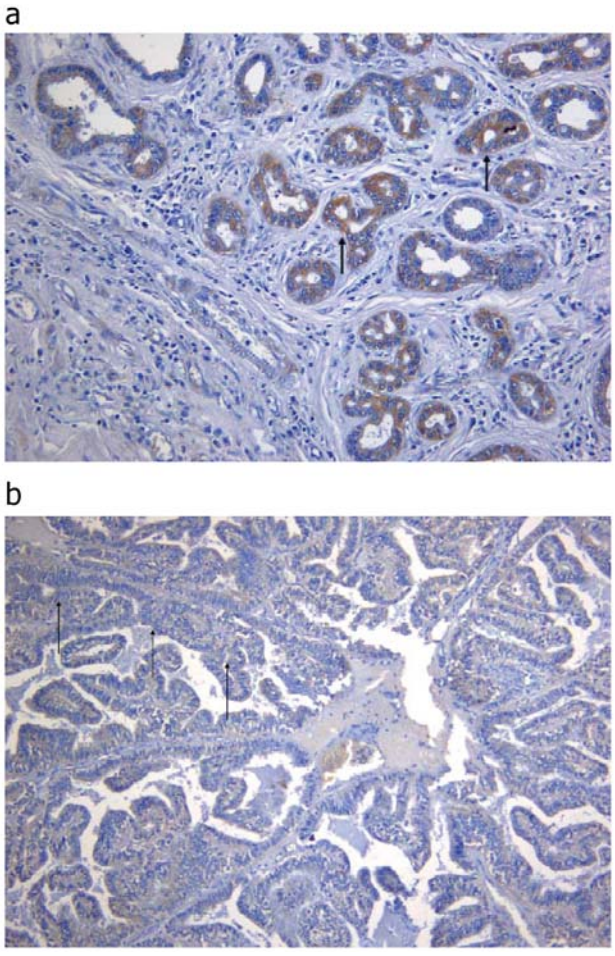

Figure 2. *In pancreatic carcinoma, HUGL-1 expression varies between clearly positive (a) and negative (b). Representative tumor ducts indicated by arrows. Magnification, ×10.*

cytoplasmatic staining of pancreatic ducts of all sizes, consistently to be evaluated as medium or strong (Figure 1). Only few epithelial cells of the exocrine parenchyma reacted positively. The endocrine islands exhibited a weak, but almost uniform cytoplasmatic positivity. As to be expected from previous studies on brain tissue (28), the peripancreatic parasympathetic ganglia exhibited a strong staining of the perikaryon of the ganglion cells. Staining of vessel walls or fibrous tissue was not observed. In cases of chronic pancreatitis or with acute exacerbation of a pre-existing chronic inflammation, very similar staining patterns compared to those of normal pancreatic tissue were present.

Pancreatic adenocarcinoma. Of the 97 tumour specimens, 36 showed no *HUGL-1* expression (37.1%) and 33 (34.0%) revealed an only weak staining. These 69 cases were classified as negative for clinical interpretation. As positive, we defined those 28 cases with a staining intensity evaluated as medium (n=19) or even as strong (n=9) (Figure 2). The HUGL-1 expression was not correlated with clinical variables (age, gender), staging or tumour grading ($p>0.05$, Table I). *HUGL-1* positivity proved to be prognostically favourable ($p=0.0241$, Table II); the respective Kaplan Meier curve revealed a better survival for patients who survived longer than 12 months in particular (Figure 3). Not unexpectedly, the presence of distant metastases (M1) had an at least weak significant influence on patients' survival ($p=0.0474$). Neither the other staging parameters (T, N, UICC stage), nor grading (G), nor clinical variables such as age at diagnosis or gender gave significant prognostic information ($p>0.05$). Only the lymph node status (N) showed a trend in the respective Kaplan Meier curves (data not presented). In a multivariate

Table I. *Patient and tumour characteristics of HUGL-1-negative (score 0-1) and HUGL-1-positive (score 2-3) pancreatic cancer specimens (n=97). In a few cases, data for N status, M status and UICC classification were not available. Threshold for age grouping: median value. There were no significant differences between the two groups.*

Variable		*Hugl*1 expression	
		Negative	Positive
No. of tumours		69	28
Age, years	≤65	34	15
	>65	35	13
Gender	Male	41	15
	Female	28	13
Tumour size	T1/T2	21	10
	T3/T4	48	18
Lymph node status	N0	19	6
	N1/N2	48	22
Distant metastasis	M0	60	25
	M1	6	3
UICC classification	I/II	58	24
	III/IV	8	4
Tumour grading	G1/G2	38	18
	G3/G4	31	10

UICC: Union Internationale contre le Cancer.

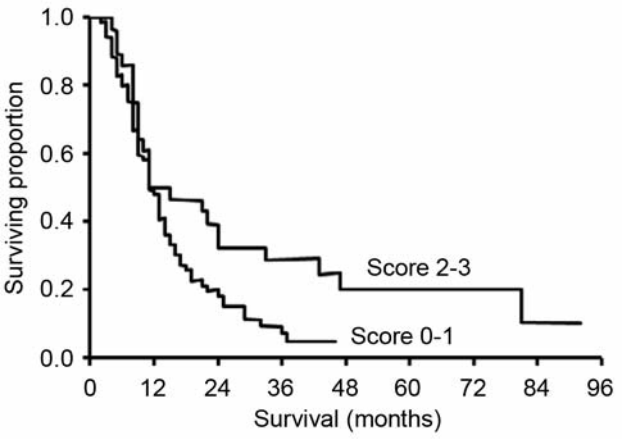

Figure 3. *Kaplan Meier survival curve for HUGL-1 expression in pancreatic carcinoma (p=0.0241, Mantel Cox test).*

Cox model, the *HUGL-1* expression passed the entry limits (Chi²-entry value 5.82, p=0.0158), while the other variables failed (Chi²-entry values ≤2.19, p>0.10).

Discussion

In various tumour entities, the down-regulation or loss of *HUGL-1* expression was correlated with tumour dissemination and prognosis. Current knowledge can be briefly summed up as follows. In a polymerase chain reaction (PCR) based and immunohistochemical study on colorectal cancer, loss of *HUGL-1* expression was associated with advanced stage, in particular with lymph node metastasis (31). Thus, down-regulation of *HUGL-1* had a significant association with lymphatic dissemination and tumour progression and appeared to be an early genetic event during the adenoma-carcinoma sequence of colorectal epithelial cells. In melanocytic skin lesions, *HUGL-1* was investigated by PCR assays and by immunohistochemistry in parallel (32). A reduced or even loss of expression was found in malignant melanoma and its metastases compared to nevi and normal skin, indicating a correlation with tumour progression from nevus to metastatic melanoma. In functional assays, *HUGL-1* down-regulation contributed to dissemination of tumour cells. Thus, the results of the study were interpreted as the first indication of a tumour suppressor role of *HUGL-1* also in malignant melanoma. In a solely PCR-based study on endometrial carcinoma, the loss of *HUGL-1* expression was positively correlated with lymph nodal metastasis and was identified as a prognostic factor, with statistically significant poorer survival for patients with a HUGL-1-negative tumour during a 6-year follow-up period (p=0.012) (33). Sequence alterations of *HUGL-1* were investigated in specimens from hepatocellular carcinoma (HCC), a tumour entity with a prognosis as fatal as that for pancreatic carcinoma (34). Abnormal expression of *HUGL-1* was significantly correlated with poor differentiation (p=0.002) and tumour size >3 cm (p=0.031), most probably indicating a role of this phenomenon in HCC progression. However, survival data were not provided.

The results of our study tally with those of the previous investigations: We analyzed the immunohistochemical expression profile of *HUGL-1* in a series of patients with surgically treated pancreatic adenocarcinoma with a follow-up period of up to >7 years. A preserved expression of *HUGL-1* was significantly associated with a better survival probability and was the only variable with multivariate prognostic meaning in a Cox regression model. Hence, our results imply a relevant influence on the patients' outcome independent of staging and grading parameters. Although it was not possible to perform molecular analysis within this project, our prognostic data underline that loss of *HUGL-1* expression may have an impact on tumour progression. As far as tumour recurrence is explained by undetectable dissemination of single tumour cells or small tumour cell clusters beyond the resection line in

Table II. *Univariate survival analysis concerning clinical data and HUGL-1 data (n=97). In a few cases, data for N status, M status and UICC classification were not available. Threshold for age grouping: median value.*

Variable	Threshold	No. of patients	Survival analysis			*p*-Value
			Median survival (months)	Mean survival (months)	Survival rate (%)	
All patients		97	11	21	11.3	---
Age, years	≤65	49	14	22	12.2	0.4787
	>65	48	11	20	10.4	
Gender	Male	56	11	24	16.1	0.5075
	Female	41	12	18	4.9	
Tumour size	T1/T2	31	11	21	9.7	0.6729
	T3/T4	66	11	20	12.1	
Lymph node status	N0	25	13	26	20.0	0.5123
	N1/N2	70	11	19	7.1	
Distant metastasis	M0	85	13	22	11.8	0.0474
	M1	9	9	10	0.0	
UICC classification	I/II	82	11	21	9.8	0.7251
	III/IV	12	11	14	16.7	
Tumour grading	G1/G2	56	13	22	10.7	0.7616
	G3/G4	41	9	20	12.2	
HUGL-1 expression	2-3	28	11	30	17.9	0.0241
	0-1	69	11	15	8.7	

UICC: Union Internationale contre le Cancer.

pancreatic carcinoma, loss of *HUGL-1* could also be interpreted as a key event for an increase in cell migration, reduced cell-to-cell contact and reduced maintenance of epithelial integrity through its cytoskeletal interactions, as shown in previous studies (31-34).

Several immunohistochemical and molecular variables were investigated in the past, and none of them were accepted as being important for clinical decision making (19), mainly due to the fatal prognosis of pancreatic cancer as a whole. Furthermore, in the subgroup of surgically operable patients, high perioperative mortality and a long postoperative convalescence period must be considered; their influence on survival may be derived from Figure 3: only after the first postoperative year did an influence of *HUGL-1* on survival become obvious. Similar survival curves have been demonstrated in several previous studies for other prognostic variables, for example, for HER2-neu (20), carbonic anhydrase IX 8 (35), the Hu protein antigen (36), and vimentin (37), respectively.

Finally, the very low survival rates of patients with pancreatic carcinoma raise the question if searching for prognostic factors is useful at all. On the one hand, we should be aware that prognostic studies have not really helped any patient with pancreatic carcinoma to date. However, on the other hand, we should be motivated to continue further investigations to identify hopeful variables for the development of targeted therapeutic agents for patients in the future; the inhibition of pancreatic cancer progression by *HUGL-1* antagonists might become a promising therapeutic option within this new and rapidly growing therapeutic field. A current overview of the major signalling pathways and tumour-stroma interactions involved in pancreatic cancer which could be influenced by new therapeutic developments has been published recently (38).

For future perspectives concerning *HUGL-1*, an immunohistochemical or molecular analysis of marker expression could be also useful in tumour entities with a broader prognostic spectrum and with a well-known tendency for dissemination, for example, lobular breast cancer, gastric cancer, or bile duct carcinoma. For diagnostic purposes, it should be further analyzed if loss of *HUGL-1* in endosonography-guided fine-needle aspirates could serve as diagnostic marker for malignancy in diagnostic cytopathology.

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