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Expression of Death Associated Proteins DAP1 and DAP3 in Human Pancreatic Cancer

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Abstract. *Background: Death associated proteins (DAPs) are involved in the apoptosis of various cell types in response to interferon gamma, including cancer cells. The present study assessed both DAP1 and DAP3 in human pancreatic cancer. Materials and Methods: DAP1 and DAP3 transcripts were quantitatively analysed in pancreatic tumour tissues and paired adjacent normal tissues using real time PCR followed by statistical analyses for their clinical implications. Results: Levels of DAP3 transcripts in pancreatic cancer were markedly higher than in normal tissues, whereas DAP1 had lower levels in cancer versus normal tissues. Adenocarcinomas showed higher levels of DAP3 than other histological types. Patients with high levels of DAP3 had a significantly shorter overall survival than those with low levels (p=0.012). The status of DAP3 and lymph node involvement identified patients with poor survival (p<0.00001). Conclusion: DAP3 was highly expressed in pancreatic tumour tissues and was significantly associated with shorter survival.*

Death associated proteins (DAPs) are a small group of proteins that have been implicated in the programmed death process of various cell types including cancer cells. There are two known members, DAP1 (or DAP) and DAP3. DAP was initially discovered to be a factor in interferon-gamma (IFNγ) induced apoptosis in HeLa cells and is a 15kDa protein (1). The other member, DAP3, was discovered in the same year, again as an IFNγ inducible cell death related gene in the same cell type, encoding a 46kDa protein (2). DAP1 has since been identified as a substrate of the mammalian target of rapamycin (mTOR) and is a negative regulator of autophagy (3); it is a responsive gene to type II protein arginine methyltransferase PRMT5 in various cell types (4). DAP3 has also been shown to be a regulator of *anoikis*, cell adhesion dependent apoptosis in HEK293 cells (5).

DAPs have been linked to cell death and cell migration. DAP1 has been reported to be reduced or lost in glioblastoma and medulloblastoma cells (6). In contrast, DAP3 was found to be overexpressed in glioblastoma multiforme and knocking down DAP3 in glioblastoma cells resulted in a more mobile phenotype (7). Loss of DAP1 by knockdown resulted in cells with rapid growth and migration (8) in breast cancer. However, the opposite was seen in the ovarian cancer cell line SKOV3; knockdown of DAP1 reduced cell growth (9).

Since their discovery, there have been continued studies to explore the role of DAP1 and DAP3 in cancer cells and in some of the solid cancers. In human cancers, expression of DAP1 has been found to be reduced or lost in late stage tumours and tumours with recurrence or metastasis in breast cancer (10), and the reduction in colorectal cancer was associated with shorter survival of the patients (11). In contrast, higher levels of DAP1 have been reported in oral squamous cell carcinoma, in thyroid cancer (12), and in patients with lymph node metastasis of oral squamous cell carcinoma (13). In addition, DAP3 has been shown to be present at higher levels in glioblastoma multiforme tumours and in late stage thymomas (7, 14). In breast cancer, DAP3 expression was found to be reduced compared to normal tissues. Reduced DAP3 was seen in aggressive and late stage tumours and in patients who developed metastasis and recurrence (15). The same trends were observed in gastric cancer (16, 17). Reduced expression of DAP3 in patients with gastric cancer also resulted in chemoresistance (16).

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DAP3 is also linked to resistance to radiotherapies in lung cancer (18). In non-epithelial derived osteosarcoma cells, high levels of DAP3 have been reported to induce apoptosis of the tumour cells (19). Despite its importance in cancers, no significant mutations of the DAP3 gene, at least in its P-loop region, have been reported in multiple cancer tissues including gastric, liver, colorectal and lung (20).

Collectively, DAP1 and DAP3 have been shown to play important roles in the living and death of cancer cells and in the development and progression of certain solid tumours. However, their pro- or anti-apoptotic properties and their tumour suppressive or oncogenic properties remain unclear; they seem to primarily depend on the cell and cancer type.

Pancreatic cancer remains one of the most fatal malignancies worldwide accounting for 466,000 cancer deaths in 2020. The number of the patients who died from the this cancer type per year is almost the same as the number of newly diagnosed cases which was 496,000 for the same period (21). In contrast to the advances and improvement in the diagnosis and treatment of other malignancies, demand for early detection and effective therapeutic approaches for pancreatic cancer requires a better understanding of the corresponding molecular and cellular machinery. The present study investigated the expression profile of both DAP1 and DAP3 in a large cohort of human pancreatic cancer, together with a small public database. We report a clinical and survival benefit of reduced levels of DAP3 expression, and to a lesser degree DAP1, in patients with pancreatic cancer.

Materials and Methods

Clinical cohort and collection of tumour tissues. 223 patients entered the study. Tumour tissues and matched normal tissues were immediately collected after pancreatomy and stored in liquid nitrogen until further use. The study was supported by the Ethics Committee of Peking University Cancer Hospital and is in full accordance with the Helsinki declaration. Consents were obtained from the patients. Clinical and pathological information as well as follow up data were collected retrospectively. The median patient follow up was 12 months.

Tissue processing and quantitative assessment of DAP1 and DAP3 gene transcript. Tissues were homogenised in an RNA isolation buffer. RNAs were extracted, purified and quantified by a UV spectrophotometer. The RNA concentration was then standardised before reverse transcription was carried out. Reverse transcription was carried out using a reverse transcription kit (Promega, Southampton, UK). The primers used for DAP1 were ATGGACAAGCATCCTTCC and ACTGAACCTGACCGTACACTCTGTCAGGGAAATACCAA, for DAP3 AAAGCACTGAGAAAGGGAGT and ACTGAACCT GACCGTACACCTCTTTAGCTCTTTCAGCA, and for GAPDH AAGGTCATCCATGACAACTT and ACTGAACCTGACCGTACA GCCATCCACAGTCTTCTG, in a 5 to 3' direction. The detection of amplicons was with a molecular beacon based Uniprimer^{tm} system in which the FAM tagged Uniprimer^{tm} worked with the reverse primer *via* a unique sequence, present in each of the reverse primers for the specific genes (the z sequence, underlined above). Quantitative PCR was carried out on a StepOne Plus qPCR detection system (Fisher Scientific, Loughborough, UK).

Statistical analysis. Pairwise comparisons were made using the Student's *t*-test. Survival analysis was carried out with the Kaplan Meier's method. Multivariate analysis and logistic regression of clinical/pathological factors and levels of DAPs were carried out against clinical outcome. Comparison of number of deaths in the respective groups were carried out by Fisher Exact test. All the analyses were carried out using SPSS version 26 (IBM UK, Portsmouth, England, UK).

Results

Expression of DAP1 and DAP3 in pancreatic cancer. Expression levels of DAP1 and DAP3 in normal and tumour tissues showed a different pattern. DAP3 showed higher levels in tumour tissues whereas DAP1 showed lower levels in tumour tissues compared with normal tissues, but these differences did not reach statistical significance (Table I). Adenocarcinoma had significantly higher levels of DAP3 than and other histological types ($p<0.0001$). Overall, high levels of DAP3 are seen in node negative groups ($p=0.14$) but the same was not seen with DAP1 ($p=0.75$). There was no overall association between the degree of differentiation. Tumours from different anatomical locations tend to have similar levels of DAP3, although tumours from the body ($p=0.029$) and tail ($p=0.0021$) region of the pancreas had significantly lower levels of DAP1 than tumours from the head of pancreas (Table I). However, T-stage 3 and TNM2 tumours seem to have significantly higher levels of DAP1 ($p=0.0021$ and 0.01 *vs*. stage TNM1, respectively). Although other groups had higher levels than TNM1 and stage 1 tumours, the difference was not significant. A similar trend was seen with DAP3 but neither comparisons were significant.

DAP3 and DAP1 were not correlated in pancreatic cancer tissue. To explore if the two death associated proteins are indeed correlated in tissues in the pancreas, we undertook correlation analysis for the two DAPs in the whole cohort, in normal and tumour tissues independently. Within the entire cohort, normal and tumour combined, a significant inverse correlation was seen between DAP3 and DAP1 ($r=–0.097$, $p=0.019$). However, when the tissues were separated into normal and tumour groups, no significant correlations were seen ($r=–0.073$, $p=0.307$ for tumour tissues and $r=–0.054$, $p=0.388$ for normal tissues). Overall, this indicates that the expression levels of DAP1 and DAP3 in pancreatic cancer tissues has little correlation.

DAP3 and DAP1 expression and patient clinical outcome. In our cohort, we found that there was a significant relationship between DAP3 and the patients' overall survival. Patients with

Table I. *Pathological information of pancreatic tumours and the expression profiles of DAPs.*

Category	Variables	N	DAP3		DAP1	
			Mean±SD	*p*-Value*	Mean±SD	*p*-Value*
Tissue types	Tumour tissues	223	103.9±22.4	0.065	725±154	0.064
	Normal tissues	223	58±10.6		1574±429	
Gender	Male	132	96.7±27.5	0.7	872±235	0.18
	Female	91	114.9±38.18		502±1478	
Histological types	Adenocarcinoma	192	114.6±25.6		529±121	
	All other types	29	33.98±9.27	<0.0001	2198±886	0.15
Differentiation	High	15	51.2±14.2		2764±1644	
	High/Moderate	20	70.7±23.41	0.49[a]	11.63±8.041	0.12[a]
	Moderate	77	76.8±11.9	0.18[a]	775±220	0.26[a]
	Moderate/Low	78	138.5±46.94	0.079[a]	380±1854	0.18[a]
	Low	15	235±211	0.4[a]	966±460	0.31[a]
Anatomical sites	Head	73	89.4±33.2		1188±376	
	Body	16	71.1±23.2	0.65[b]	227±213	0.029[b]
	Body and Tail	32	58.8±15.4	0.41[b]	880±370	0.56[b]
	Tail	5	46.7±14.5	0.24[b]	15.6±15.2	0.0027[b]
	Other locations	5	52.9±36.5	0.48[b]	1885±1884	0.74[b]
T staging	T1	6	55.2±30.55		127±1055	
	T2	27	69.1±16.1	0.7[c]	169.9±93.4	0.77[c]
	T3	122	107±30.2	0.25[c]	833±191	0.0021[c]
	T4	23	77±21.32	0.59[c]	426±4142	0.49[c]
Nodal status	Node negative	89	153.1±53.41	0.14	797±290	0.75
	Node positive	107	72.2±11.3		685±195	
TNM staging	TNM-1	21	64.4±20.6		197±116	
	TNM-2	137	89.5±21.9[d]	0.4[d]	704±155	0.01[d]
	TNM-3	19	74±24.83[d]	0.64[d]	521±506	0.54[d]
	TNM-4	15	77.6±23.8[d]	0.71[d]	890±884	0.46[d]
Microvessel embolism	Without embolism	126	126.4±37.8	0.3	623±213	0.3
	With embolism	58	82.7±18.53		998±2881	

[a]*versus* highly differentiated; [b]*versus* tumours of head of pancreas; [c]*versus* T1 tumours; [d]*versus* TNM1 tumours. *by Student *t*-test.

high levels of DAP3 had significantly shorter survival compared to those with low levels (21.6±3.2 months *vs*. 35.1±6.7 months respectively, $p=0.012$) (Figure 1A). In contrast, patients with high levels of DAP1 showed a marginally longer survival than those with lower levels (28.9±5.8 months *vs*. 24.4±3.6 months respectively, $p=0.74$) (Figure 1C). This finding was supported by the TCGA dataset from a smaller cohort (n=178), where $p=0.028$ and 0.41 for DAP3 and DAP1, respectively (Figure 1B and 1D, respectively).

DAP3 and patient clinical outcome stratified by DAP1. We attempted to stratify the cohort by DAP1 expression and explore the value of DAP3 in patient survival. As shown in Figure 2, when the patients were segregated based on high and low DAP1 expression, DAP3 levels showed a greatly improved survival prognosis (Figure 2).

Combined expression of DAP3 with nodal status dramatically enhances its value as independent prognostic survival indicator. As shown in Table I, tumours with or without lymph node involvement had different levels of expression of DAP3. We cross analysed if lymph node status and DAP3 status had an impact on the survival of the patients. As seen in Table II, it is clear that patients who had high DAP3 and node positive tumours had the poorest outcome, with a survival rate at 5.8% during the follow up period, where the remaining patients have significantly higher survival rate, namely 26.2% ($p=0.0036$), 34.8% ($p=0.0013$) and 32.0% ($p=0.0022$) for DAP3 high/node (–), DAP3 Low/Node (–) and DAP3 low/node (+), respectively. In this regard, the combination of DAP3 and nodal status can be a significant independent prognostic indicator for survival (HR 10.06, $p=0.006$) (Table III) and patients who were node positive and possessed high levels of DAP3 had poor rates of survival (13.6±2.8 months *vs*. 29.4±3.7 months for the remaining patients, $p<0.00001$) (Figure 3).

Discussion

Although DAP proteins have been identified for their role in regulating apoptosis, *anoikis* and other cellular functions

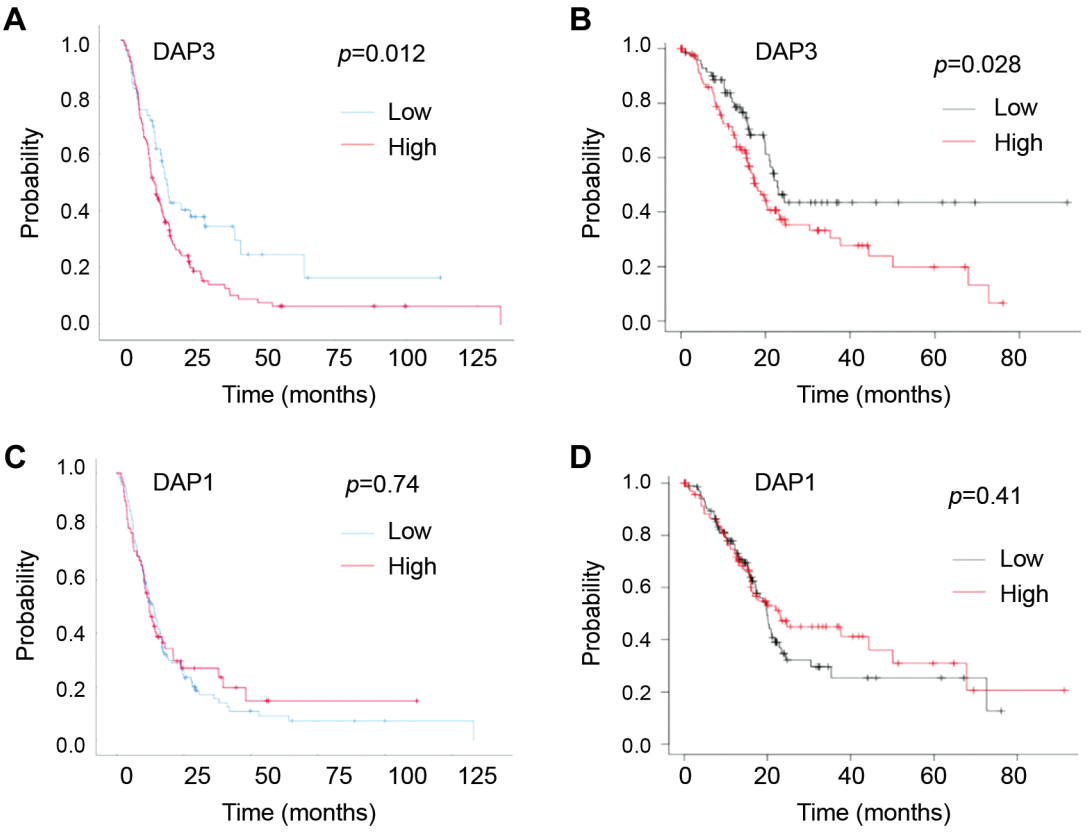

Figure 1. *DAP3 and DAP1 expression and the overall survival of patients in the host cohort (A, DAP3; C, DAP1) and TCGA cohort (B, DAP3; D, DAP1). p-Value as indicated in the figure by Kaplan Meier survival analysis.*

Table II. *DAP3 expression and nodal status in relation to clinical outcomes.*

DAP3 expression	Nodal status		Clinical outcomes		
		Total	Died of pancreatic cancer	Alive	Survival (%)
DAP3 low	(–)	23	15	8	34.8%[a]
	(+)	35	17	8	32.0%[b]
DAP3 high	(–)	42	31	11	26.2%[c]
	(+)	69	65	4	5.8%

[a]p=0.0013, [b]p=0.0022, [c]p=0.0036 *versus* DAP3 high/node (+), by Fisher's exact test.

related to cancer cells including cell migration, DAP1 and DAP3 have been shown to play a tumour suppressive or stimulating role, depending on the cell and tumour type (7, 10-14). Clinically, the pattern of expression of DAP1 and DAP3 differs and it is sometimes contrasting within the same tumour type and dependent on the cancer histological type.

The present study, by employing a sizeable cohort of pancreatic cancers, has provided evidence that in pancreatic cancer, DAP1 and DAP3 express a contrasting pattern. This was seen with high levels of DAP3 but low levels of DAP1 in tumour tissues compared to normal tissues, in adenocarcinoma compared to other histological malignancies of the pancreas, as well as contrasting patterns in tumours with microvessel embolism when compared with tumours without embolism. This clearly shows that in the same cohort and the same cancer type, the two DAPs have different and rather contrasting patterns. This contrast is unexpected as both DAP1 and DAP3 were discovered as apoptosis related genes and we anticipated

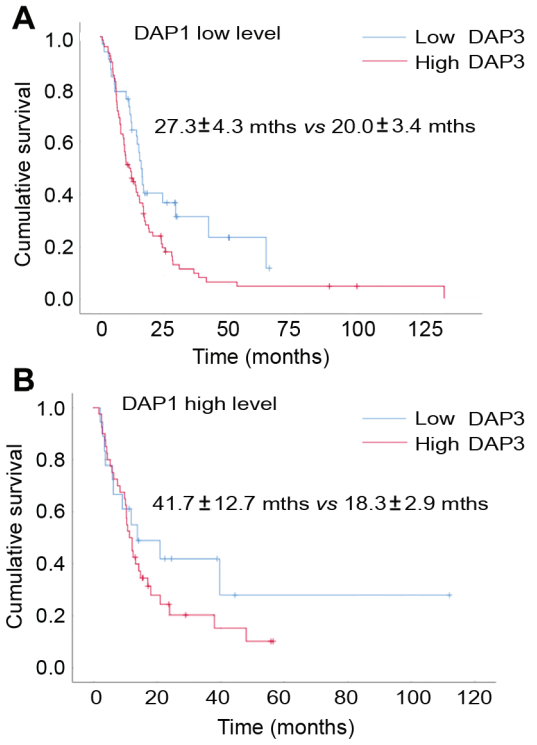

Figure 2. *Overall survival and DAP3 expression as stratified by DAP1 (A, DAP1 low levels; B, DAP1 high levels). The grouping has showed a significant relationship between DAP3 and survival in both groups by Kaplan-Meier survival analysis (p=0.024). mths: Months.*

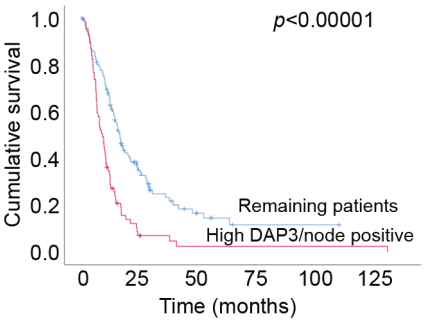

Figure 3. *DAP3 expression and nodal status strengthened the predictive power of overall patient survival, as calculated by Kaplan-Meier survival analysis (p<0.00001).*

Table III. *Clinical, pathological and DAP3 expression in predicting the mortality of patients*.*

Factors	*p*-Value*	HR
Gender	0.664	0.734
Age	0.293	1.035
Histological type	0.598	0.537
Differentiation	0.825	0.918
Location of tumours	0.92	1.042
Local invasion	0.631	1.846
Nodal status	0.18	0.256
TNM staging	0.769	0.624
Tumour vascular embolism	0.644	0.657
DAP3	0.296	0.372
DAP3/Nodal status	0.006	10.036

*Multivariate analysis against death of patients.

a similar pattern of changes in the same tumour type. The reasons underlying this contrast remain unclear. It is interesting to note that the two DAPs are located on different chromosomes, namely DAP1 on 5p15.2 and DAP3 on 1q22. It is plausible that gene transcription regulations are very different. The contrasting patterns of expression of both DAPs seen here, and in the literature, strongly argue that their expression is indeed regulated differently. Further work is required to fully understand these observations. Additionally, immunohistochemical analyses of both DAP1 and DAP3 would be important to evaluate if the differential pattern between the two molecules in pancreatic cancer and other tumour types also holds true at the protein level.

The most interesting finding of the study is the significant correlation between the levels of DAP3 expression and patient clinical outcome and that high levels of DAP3 are seen with significantly shorter survival. This correlation is in contrast to that of DAP1, in that high levels of DAP1 are seen with a marginally longer survival. DAP3 expression and survival in pancreatic cancer has been similarly reported in glioblastoma multiforme tumours (7) and in late stage thymomas (14), but is opposite to those reported in breast (15) and gastric cancer (16, 17). Collectively, it is clear that DAP3 has prognostic value and is predictive of poor patient outcome. This relationship seems to be tumour type dependent.

DAP3 expression and lymph node involvement has been recently reported in squamous cell carcinoma of the oral cavity (13). The present study has also demonstrated that the status of DAP3 expression and lymph node involvement can together provide a powerful and independent indicator for patient survival. This collectively argues for a pivotal role of DAP3 in lymph node spread in pancreatic cancer and likely in other cancer types.

Overall, DAP3 and DAP1 have a contrasting expression pattern in human pancreatic cancer with DAP3 highly expressed in tumour tissues. Patients with high levels of DAP3 in their tumour tissues have significantly shortened survival indicating an independent prognostic value.

Conflicts of Interest

None to be declared.

Authors' Contributions

Experimental design: CH and WGJ; Data collection: LY, AJS, CH and WGJ; Data analysis: LS, YM, AJS, JY and WGJ; Manuscript preparation: all.

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