FULL PAPER

BJC

British Journal of Cancer (2014) 110, 421–429 | doi: 10.1038/bjc.2013.712

Keywords: gastric cancer; neoadjuvant chemotherapy; apoptosis; DAP3; effectiveness

Death-associated protein-3, DAP-3, correlates with preoperative chemotherapy effectiveness and prognosis of gastric cancer patients following perioperative chemotherapy and radical gastrectomy

Y Jia[1,2,3], L Ye[1,2], K Ji[1,2], L Zhang[1,3], R Hargest[1,2], J Ji*[,1,3] and W G Jiang*[,1,2]

[1]Cardiff University–Peking University School of Oncology Joint Institute, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK; [2]Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK and [3]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Gastrointestinal Surgery, Peking University Cancer Hospital and Institute, No. 52, Haidian District, Beijing 100142, China

Background: DAP3 is a member of the death-associated protein (DAP) family and is characterised by proapoptotic function. It is involved in both exogenous and endogenous apoptotic pathways. In our previous studies, apoptotic level was found to be correlated with the effectiveness of preoperative chemotherapy. The effectiveness of preoperative chemotherapy was also associated with the overall effectiveness of the combined therapy and prognosis. The present study aimed to investigate the role of DAP3 in the evaluation of preoperative chemotherapy effectiveness and its ability to predict prognosis in gastric cancer.

Methods: Quantitative PCR and immunohistochemistry staining were performed in 87 patients who received combined therapy. Knockdown of DAP3 was conducted in gastric cancer cell lines to investigate its impact on cell growth, migration, adhesion and invasion. Tolerance to chemotherapy agents was determined by assessing apoptosis and caspase-3.

Results: Higher DAP3 expression in gastric tumours was correlated with better prognosis. Knockdown of DAP3 expression promoted cell migration and enhanced resistance to chemotherapy by inhibiting apoptosis.

Conclusion: DAP3 is a potential molecular marker for response to preoperative chemotherapy and for predicting prognosis in gastric cancer patients treated with neoadjuvant chemotherapy and gastrectomy.

Combined therapy has been widely accepted as a standard strategy in the management of many malignancies, including gastric cancer. However the combined therapeutic model varies across Europe, America and Japan, depending on the extent of lymphadenectomy, the use of endoscopic screening for the detection of early lesions and the use of adjuvant/neoadjuvant treatment. In China, most gastric cancer patients are diagnosed at a late stage, which leads to a low R0 resection rate and increasing use of chemotherapy. Where possible, combined therapy using perioperative chemotherapy and curative gastrectomy with D2 lymphadenectomy are widely accepted as the standard therapeutic models in China. According to current clinical data from other medical centres and our own institute, gastric cancer patients who exhibit a positive response to neoadjuvant chemotherapy can also benefit from perioperative chemotherapy and obtain a better prognosis than non-responders (Lowy *et al*, 1999).

*Correspondence: Professor J Ji or Professor WG Jiang; E-mail: jiafuj@hotmail.com or jiangw@cf.ac.uk

Received 5 August 2013; revised 18 October 2013; accepted 21 October 2013; published online 3 December 2013

If it was possible to identify potential responders at an early stage and make adjustment to the postoperative chemotherapy regimen of non-responders, the effectiveness of combined therapy could be enhanced, and some patients may be spared unnecessary treatment. Our previous study indicated that the apoptotic level of gastric tumour cells significantly correlated with the effectiveness of preoperative chemotherapy and also with prognosis (Jia *et al*, 2012).

DAP3 belongs to the death-associated protein (DAP) family, which includes DAP1, DAP2 (DAP kinase), DAP3, DAP4 and DAP5. This protein family was initially isolated through a technical knockout strategy, which generated resistance to IFN-γ-induced apoptosis through random inactivation of genes with antisense cDNA libraries (Kissil *et al*, 1995). The DAPs share some common feature domains, which confer proapoptotic function. DAPs are also involved in Fas-, TNF-α- and Fas receptor-mediated cell death, which indicates that DAPs are downstream effectors of various apoptotic-stimulating signals (Inbal *et al*, 1997; Cohen *et al*, 1999).

DAP3 has been shown to be localised in the mitochondria. Its highly conserved 17 amino-acid sequence is crucial for this localisation and consequently affects its activity in the mitochondrial fragmentation process (Berger *et al*, 2000; Morgan *et al*, 2001). Proteomic analysis also revealed DAP3 as a protein component of mitochondrial ribosome 28S small subunit in mammals and yeast cells (Cavdar Koc *et al*, 2001; Saveanu *et al*, 2001; Suzuki *et al*, 2001). It is retained in the mitochondria during apoptosis (Mukamel and Kimchi, 2004). These findings suggest that DAP3 may exert its proapoptotic function in mitochondria through regulating mitochondrial fragmentation.

It is reported that DAP3 overexpression could promote apoptosis, the downstream mechanism of which may include enhanced mitochondrial fragmentation, decreased mitochondrial membrane potential and increased permeability via opening of transition pores and release of cytochrome *c* (Berger *et al*, 2000). It is usually under stress conditions that DAP3 exerts its proapoptotic function.

The DNA damage-induced apoptosis is reported to be mainly through mitochondrial pathways (Green and Reed, 1998; Korsmeyer *et al*, 2000; Kroemer and Reed, 2000; Martinou and Green, 2001), which indicates that DAP3 may be involved in the process of chemotherapy-induced apoptosis via mitochondrial pathways and may be correlated with the effectiveness of chemotherapy. The present study aimed to investigate DAP3 expression in a cohort of gastric cancer patients following neoadjuvant chemotherapy and analyse the correlation of its expression level with clinicopathological parameters and response to chemotherapy.

MATERIALS AND METHODS

Materials. AGS and HGC27cell lines were obtained from the European Collection of Cell Cultures, (ECACC, Salisbury, UK). Reagents and kits were obtained from Promega Corporation (Promega, WI, USA), Bio-Rad Life Science company (Bio-Rad, CA, USA), Gibco Invitrogen Corporation (Gibco BRC, Paisley, Scotland, UK). Fresh-frozen gastric adenocarcinoma or Siewert Type III gastroesophageal junction (GEJ) adenocarcinoma tissues ($n = 85$), along with matched normal tissue from the same patients, were collected immediately after surgical resection at the Beijing Cancer Hospital and were stored in the Tissue Bank of Peking University Oncology School. Clinicopathological factors, including age, sex, histological type, TNM stage and lymph node metastasis, were recorded and stored in the patients' database. All protocols were reviewed and approved by the local ethics committee, and informed consent was obtained from the patients before therapy.

Patients and the therapy. This study comprised 85 gastric cancer patients with cT2-4N0M0 or cT1-4N1-3M0 treated between January 2006 and December 2007. A total of 12 cycles of perioperative chemotherapy with FOLFOX7 was recommended for all patients. The regimen used was modified FOLFOX7: oxaliplatin 100 mg m^{-2} and folinic acid 200 mg m^{-2} intravenous infusion for 2 h on the first day, followed by 5-fluorouracial (5-FU) 2400 mg m^{-2} continuous infusion for 46 h. This was repeated every 2 weeks, usually two or four cycles before surgery. Gastrectomy with D2 lymphadenectomy was performed. Primary tumour site, grade, depth of tumour invasion, status of lymph node metastasis, distant metastasis and TNM stage were recorded in histopathology reports. Pathological stage was determined according to the seventh edition of the TNM staging system recommended by the International Union against Cancer.

Preoperative chemotherapy effectiveness evaluation and follow-up. A complete series of paraffin sections, including primary lesions and resected lymph nodes, were sent to at least two pathologists for evaluation. If a complete pathological response was suspected, slicing of the entire tissue and careful re-evaluation were undertaken for confirmation.

Pathological evaluation was conducted according to the Japanese system of histological evaluation of tumour response. This includes: grade 0, no effect; grade 1, slight effect (1a: viable tumour cells occupy $>2/3$ of the tumour area, 1b: viable tumour cells remain in $<2/3$ of the tumour area); grade 2, considerable effect (viable tumour cells remain in $<1/3$ of the tumour area); and grade 3, complete response (no viable tumour cells remain and additional resection is recommended to confirm this finding). Viable tumour cells are defined as cells capable of proliferation (Japanese Gastric Cancer Association, 2011). According to our previous study, we assigned grade 0, 1a and 1b as non-response to chemotherapy, while grades 2 and 3 are considered as response to chemotherapy (in the present study, grade 3 patients who exhibit complete response were not enrolled, because no tumour cells remained and DAP3 expression in tumour tissue could not be determined).

Patients were followed up every 3 months for 2 years, then biannually for 3 years and then annually. Overall survival and disease-free survival were the primary and secondary end points, respectively. The follow-up was done through telephone and mail by the statistical department of our hospital.

RNA isolation and reverse transcription PCR. Total RNA was isolated from the homogenised gastric tissues and cell lines using the Total RNA Isolation Reagent (ABgene, Surrey, UK). Synthesis of cDNA and subsequent PCR was performed using the standard methods. DAP3 primers were as following, sense: 5′-AAAGCACT GAGAAAGGGAGT-3′; antisense: 5′-ACTGAACCTGACCGTA CACTCTGTCAGGGAAATACCAA-3′ (exons 5–10).

Quantitative analysis of DAP3 expression. The level of DAP3 transcripts was determined on the Icycler IQ5 system (Bio-Rad, HammelHemstead, UK) with QPCR master mix (Bio-Rad) as previously described (Jiang *et al*, 2001).

DAP3 primers designed using the Beacon Design software (PREMIER Biosoft, Palo Alto, CA, USA) are as follows: sense, 5′-ATGGACAAGCATCCCTTCC-3′; antisense, 5′-ACTGAACCT GACCGTACACTCTGTCAGGGAAATACCAA3-3′. The underlined sequence in the reverse primers was the additional Z sequence, which is complementary to the universal Z probe (TCS Biologicals Ltd., Oxford, UK). Internal standard GAPDH primer sequences were: sense, 5′-CTGAGTACGTCGTGGAGTCc-3′; antisense, 5′-ACTGAACCTGACCGTACAGAGATGATGACCCTTTTG-3′. To exclude the effect of tissue heterogeneity, the DAP3 quantification was normalised against the corresponding CK19 (an epithelial marker) of each individual sample. The primers used

for CK19 were: sense, 5′-CAGGTCCGAGGTTACTGAC-3′; antisense, 5′-ACTGAACCTGACCGTACACCGTTTCTGCCAGT GTGTCTTC-3′.

Immunohistochemical staining of DAP3. Sections of 4-μm thickness from formalin-fixed, paraffin-mbedded tissues were mounted on poly-L-lysine -coated slides and then deparaffinised in xylene and rehydrated through alcohol to distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. After pressure cooking the slides in 10 mmol l^{-1} EDTA (pH 8.0) for 3 min, the sections were incubated with 5% goat serum, then incubated overnight at 4 °C with DAP3 antibody (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and also without primary antibody as a negative control. Primary antibodies were detected using a two-step EnVision System (Dako, Glostrup, Denmark).

Horseradish peroxidase and diaminobenzedene hydrochloride were the enzyme and chromogen used, respectively. Staining was independently assessed by two pathologists.

The percentage of positive cells and the intensity of cytoplasmic staining were analysed. Thus all final scoring estimations were stratified into four categories: −, 0% of stained cells; +, <20% weakly to moderately stained cells; ++, 10–20% intensively stained cells and 20–50% weakly stained cells; and +++, 20–50% positive cells with moderate-to-marked staining or >50% positive cells. There was a low level of discrepancy (<5% cases) among the pathologists in terms of scoring, but a consensus was reached after joint review (Supplementary Figure S2).

Construction of DAP3 ribozyme transgenes and transfection. Anti-DAP3 ribozyme transgenes were used to knockdown the expression of DAP3 in the AGS and HGC27 gastric cancer cells and were generated using the methods previously described (Parr and Jiang, 2009). Briefly, an anti-DAP3 hammerhead ribozyme was designed based on the secondary structure of DAP3 mRNA and generated using the Zuker's RNA mFold program. Then the ribozymes that specifically target DAP3 were generated using the touchdown PCR with the appropriate primers (sense: 5′-CTGCAG TTCAACCACTTCTCCCAGAGCTGATGAGTCCGTGAGGA-3′; antisense: 5′-ACTAGTAGAGAAAGCACTGAGAAAGGGAGTT TCGTCCTCACGGACT-3′). The amplified ribozymes were cloned into the pEF6/V5-His TOPO TA plasmid vector (Invitrogen, Paisley, UK) in accordance with the protocol provided. Ribozyme transgenes and control plasmids were transfected into HGC27 and AGS cells individually using an Easyjet Plus electroporator (EquiBio, Kent, United Kingdom). After up to 5 days of selection with blasticidin, the transfectants were verified for knockdown of DAP3.

Western blotting. Wild-type, DAP3 knockdown and empty vector PEF-transfected cells of both HGC27 and AGS were planted into small flask at a density of 25×10^5 cells per well and incubated overnight and then received combined treatment of 5-FU and oxaliplatin at a concentration of 2×10^{-6} M for 6 h. Cell lysates along with that of the negative control (without treatment) underwent western blotting as follows. The protein concentration in cell lysates was determined using the DC Protein Assay kit (Bio-Rad) and an ELx800 spectrophotometer (Bio-Tek, York, UK). Proteins were probed with the anti- DAP3-antibody (1:200), anti-Caspase 9-antibody (1:200), anti-Caspase 3-antibody (1:200) and anti-GAPDH-antibody (1:500) (Santa-Cruz Biotechnologies, Santa Cruz, CA, USA) as an internal control, followed by a peroxidase-conjugated secondary antibody (1:1000). Protein bands were visualised and photographed using an UVITech imager (UVITech, Inc., Cambridge, UK).

***In vitro* cell growth assay for cells under normal culture conditions or exposed to concentration gradients of 5-FU and oxaliplatin.** The concentration of 5-FU and oxaplatin in the blood

Table 1. Immunohistochemical staining of DAP3 in gastric cancer patients with combined therapy

	DAP3 IHC staining		
	Low expression	High expression	*P* value
Age, years			
<40	3	5	0.743
>40	24	31	
Sex			
Male	22	26	0.393
Female	5	10	
Location			
Proximal	6	16	0.076
Distal	19	15	
Total gastric	2	3	
Bormann type			
Bormann1	2	0	0.533
Bormann2	4	7	
Bormann3	10	14	
Bormann4	4	4	
Differentiation			
Differentiated	8	8	0.204
Undifferentiated	19	21	
Others	0	5	
Tumour size			
T>50	11	13	0.265
T<50	10	22	
Vascular invasion			
No	13	18	0.92
Yes	13	16	
T stage			
T1–2	2	5	0.396
T3–4	25	30	
N stage			
Negative	7	6	0.369
Positive	20	30	
TNM stage			
I	1	2	0.477
II	5	6	
III	12	21	
IV	9	6	
D2 lymph node resection			
Yes	19	31	0.127
No	8	5	
Response to neoadjuvant chemotherapy			
Response	5	15	0.051
No response	22	21	
Incidence			
No incidence	15	26	0.122
Incidence	12	9	

Abbreviations: DAP3 = death-associated protein 3; IHC = immunohistochemistry; TNM = Tumour, Node, Metastatis. Correlation of DAP3 immunohistochemical staining with clinical pathological characteristics in gastric cancer tissues.

and tissue fluid was calculated as 5×10^{-5} M and 1×10^{-4} M, respectively, according to the dosage used in clinical chemotherapy regimens. Wild-type HGC27 and AGS cell were planted under concentration gradients starting from five times blood concentration. The threshold dose was set as the concentration with obvious inhibitory effect on cell growth.

Cells were planted into 96-well plates at 2500 cells per well, with a concentration gradient of a combination of 5-FU and oxaliplatin at 1:5 dilution, the dilution started from 10 times the threshold concentration (2×10^{-6} M). Cell growth rates under normal conditions and under treatment were assessed after 1, 3 and 5 days. Crystal violet was used to stain cells, and the absorbance was determined at a wavelength of 540 nm using a spectrophotometer (Bio-Tek, ELx800).

Cell matrix adhesion assay. The cell matrix-adhesion assay was performed as previously described (Jiang *et al*, 1995). Cells were added to a 96-well plate precoated with Matrigel (5 μg per well). After 40 min of incubation, non-adherent cells were washed off using BSS buffer. The remaining cells were fixed, stained and counted.

Wounding/migration assay. The wounding assay was performed as previously described (Jiang *et al*, 1999). The monolayer of cells was scraped with a fine gauge needle. The movement of cells to close the wound was recorded on a time lapse video recorder and analysed using Optimas 6.0 motion analysis (Meyer Instruments, Houston, TX, USA).

***In vitro* invasion assay.** The *in vitro* invasion assay was done as previously described (Jiang *et al*, 1999). Transwell inserts with 8-μm pore size were coated with 50 μg of Matrigel (Collaborative Research Products, Bedford, MA, USA) and air-dried. Following rehydration, 40 000 cells were added to each well. After 3 days of incubation, cells that had migrated through the matrix to the other side of the insert were fixed, stained and counted.

Apoptosis assay using flow cytometry. DAP3-Rib and empty vector PEF-transfected cells of both HGC27 and AGS were planted into a six-well plate at a density of 3×10^5 cells per well. Each cell line was planted into three wells at the same time, the first well was used as control with no treatment, the second and third wells received combined treatment of 5-FU and oxaliplatin at a concentration of 2×10^{-6} M and 1×10^{-5} M, respectively. All cells, including those floating in the culture medium, were harvested after 6 h of incubation. The apoptotic population of the cell was determined using a Vybrant apoptosis Assay Kit (Millipore, Billerica, MA, USA) and flow cytometry and FlowMax software package (Portec, Münster, Germany) as previously described (Ye *et al*, 2008).

Statistical analysis. Statistical analysis was performed using the SPSS software (SPSS Standard version 13.0; SPSS Inc., Chicago, IL, USA). The relationship between DAP3 expression and tumour grade, TNM staging and nodal status was assessed using the Mann–Whitney *U*-test and Kruskal–Wallis test. The error bar shown in the graph represents the s.e.m. Survival was analysed using the Kaplan–Meier survival analysis. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Patient characteristics. In total, 85 gastric cancer patients were included in this study. The cohort consisted of 61 men (71.76%)

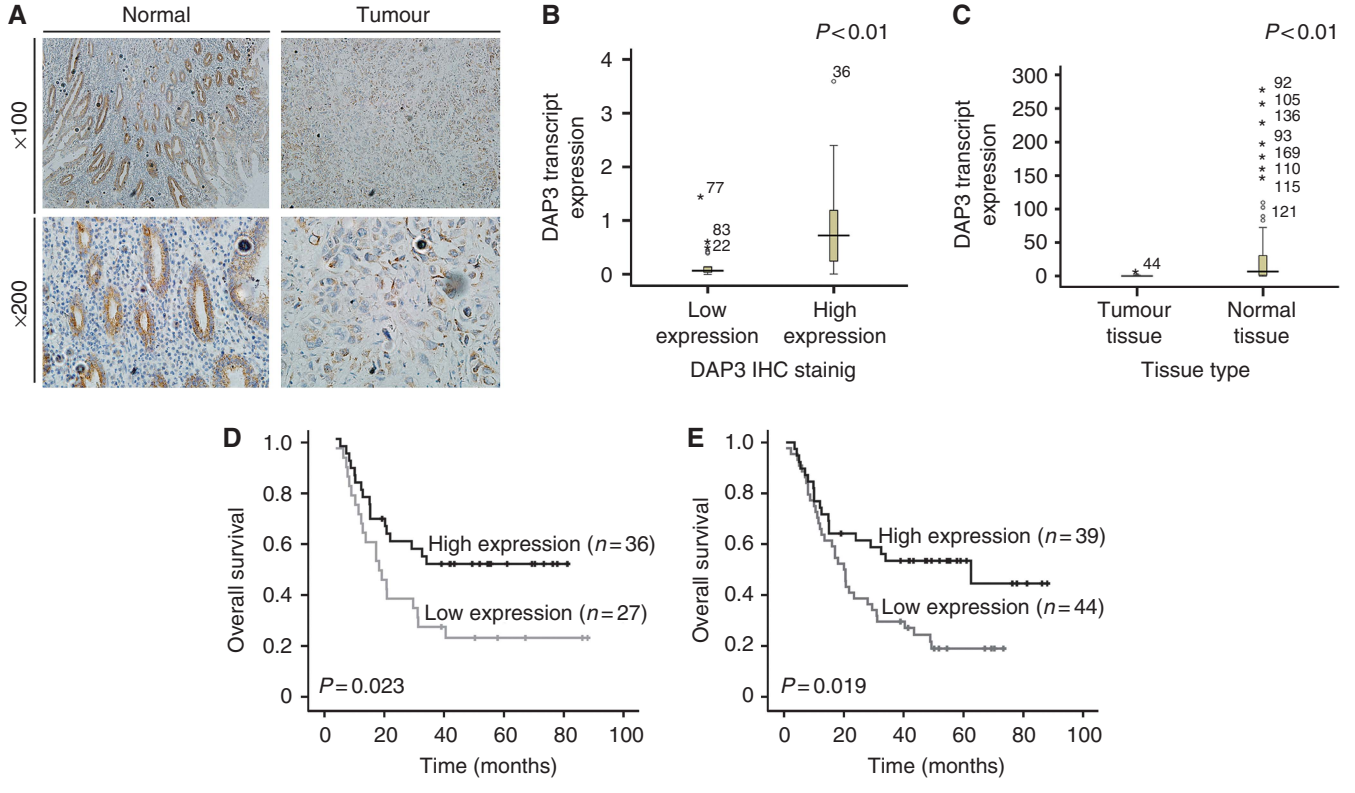

Figure 1. DAP3 expression in gastric cancer patients and correlation with prognosis. (**A**) Immunohistochemical staining of DAP3 was less intensive in tumour tissue than in background normal tissues; (**B**) correlation of DAP3 transcript expression with DAP3 immunohistochemical staining result; IHC high expression correspond to higher level of DAP3 transcript (Mann–Whitney, $P < 0.01$); (**C**) DAP3 transcript expression was lower in tumour tissue than in background normal tissues (Mann–Whitney, $P < 0.01$); (**D**) correlation of DAP3 expression with overall survival at protein level; DAP3 IHC high expression was related to longer overall survival; and (**E**) correlation of DAP3 expression with overall survival at mRNA level; higher DAP3 transcript expression was related to longer overall survival.

and 24 women (28.24%). The median age was 60 (25–78) years. The course of preoperative chemotherapy ranged from two cycles to six cycles with an average of 4. No obvious difference was observed in the response rate between patients receiving more or less than four cycles of preoperative chemotherapy (Chi-squared test, $P=0.388$). No significant difference was seen in the survival between patients receiving either the complete (12) or <12 cycles perioperative chemotherapy (log rank test, $P=0.191$). No patients gave up chemotherapy due to chemotherapy-related three or four toxicities, such as obstruction, gastrorrhagia and gastric perforation. The median follow-up time was 21.7 (1–88) months, 25 patients had recurrence, 52 patients died of gastric cancer, 2 patients died of other causes (heart attack and cerebral hemorrhage), 12 were lost in follow-up and 16 remained alive and disease free.

Immunohistochemical staining of DAP3 in human gastric specimens. To confirm the qRT-PCR analysis result, we also performed immunohistochemical staining analysis of DAP3 in 63 corresponding gastric cancer patients (Table 1). DAP3 was mainly detected in cytoplasm, and the staining was obviously stronger in normal tissues (Figure 1A). We assigned staining results of negative and '+' as low expression and '++' and '+++' as high expression. In line with the finding from real-time RT-PCR, IHC intensive staining corresponded to higher levels of DAP3 transcript expression (Mann–Whitney, $P<0.001$; Figure 1B). Kaplan–Meier analysis showed that patients with high DAP3 expression according to the DAP3 IHC had better prognosis ($P=0.026$; Figure 1D).

Quantitative PCR analysis of the DAP3 transcripts in gastric and GEJ adenocarcinoma tissues. Real-time RT-PCR was used to examine the expression of DAP3 in 85 gastric and GEJ adenocarcinoma specimens. Figure 1C shows mean DAP3 transcript copies per μl of RNA from 50 ng total RNA and standardised with GAPDH. DAP3 mRNA expression in tumour tissues (median, 0.24; Q1, 0.024; Q3, 0.684) was significantly lower than that of the normal background tissues (median, 6.92; Q1, 1.32; Q3, 31.63) ($P<0.001$). The expression level of DAP3 was higher in well or moderately differentiated tumours, and a decreasing trend of DAP3 expression was observed in more advanced tumours according to the T and TNM staging. However, these differences were not statistically significant (Table 2). There was significant correlation ($P=0.0005$) between the level of DAP3 expression and the incidence of recurrences, including local recurrence and metastasis. Expression of DAP3 in patients without recurrence was higher than in patients with either local recurrence or metastasis. Using 0.25 as the cut off value to define high and low DAP3 transcript expression, Kaplan–Meier analysis revealed that patients with higher DAP3 expression also have significantly better overall survival than those with lower DAP3 expression ($P=0.013$; Figure 1E). In addition, high levels of DAP3 expression were significantly correlated with longer disease-free survival ($P<0.001$).

Correlation of DAP3 expression with response to chemotherapy. Immunohistochemical staining showed that the intensity of DAP3 staining correlated with the stage of pathological response (Figure 2A). A similar correlation could be observed in the tumours in relation to the level of DAP3 transcript expression (Figure 2B). Pathological evaluation grades 2 and 3 were assigned as response to chemotherapy and grades 0, 1a and 1b as non-response. DAP3 transcript expression in the responders appeared to be higher than that of the non-responders ($P=0.0881$; Figure 2C). A further Chi-squared test showed that although most patients with stronger DAP3 IHC staining were in the responder group, the P value was only $P=0.051$ (Figure 2D).

Table 2. Quantatitive analysis of DAP3 in gastric cancer patients with combined therapy

	DAP3 transcript expression				
	N	**DAP3 median**	***P* value**	**Q1**	**Q3**
Tissue					
Tumour	85	0.24	<0.001	0.024	0.684
Normal	87	6.92		1.32	31.63
Sex					
Male	61	0.228	0.5289	0.02	0.639
Female	24	0.3033		0.025	0.854
Location					
Proximal	30	0.2477	0.744	0.007	0.564
Distal	45	0.0745		0.025	0.819
Total gastric	7	0.3048		0.0681	0.3881
Bormann type					
Bormann1	3	0.0253	0.244	0.00076	0.0291
Bormann2	13	0.3854		0.033	0.641
Bormann3	34	0.2443		0.02	0.682
Bormann4	8	0.1595		0.018	0.3673
Differentiation					
Differentiated	19	0.251	0.301	0.029	1.172
Undifferentiated	55	0.069		0.021	0.5445
Others	8	0.3489		0.026	0.664
Tumour size					
T>50	41	0.2673	0.2111	0.025	0.732
T<50	34	0.0715		0.019	0.512
Vascular invasion					
No	40	0.3467	0.1402	0.033	0.773
Yes	42	0.0683		0.018	0.473
T stage					
T1–2	8	0.747	0.081	0.115	1.182
T3–4	76	0.08		0.021	0.588
N stage					
Negative	16	0.1655	0.8971	0.027	0.686
Positive	69	0.2443		0.022	0.677
TNM stage					
I	4	0.747	0.588	0.144	2.922
II	14	0.259		0.0264	0.5781
III	43	0.228		0.016	0.617
IV	23	0.068		0.029	0.738
D2 lymph node resection					
Yes	64	0.2592	0.2689	0.025	0.686
No	21	0.0593		0.013	0.562
Response to neoadjuvant chemotherapy					
Response	29	0.3881	0.0881	0.034	0.745
No response	56	0.0771		0.016	0.588
Incidence					
No incidence	59	0.3311	0.0005	0.043	0.871
Incidence	25	0.0247		0.006	0.0739

Abbreviation: DAP3 = death-associated protein 3; TNM = Tumour, Node, Metastatis. Correlation of DAP3 transcript expression with clinical pathological characters in gastric cancer tissues.

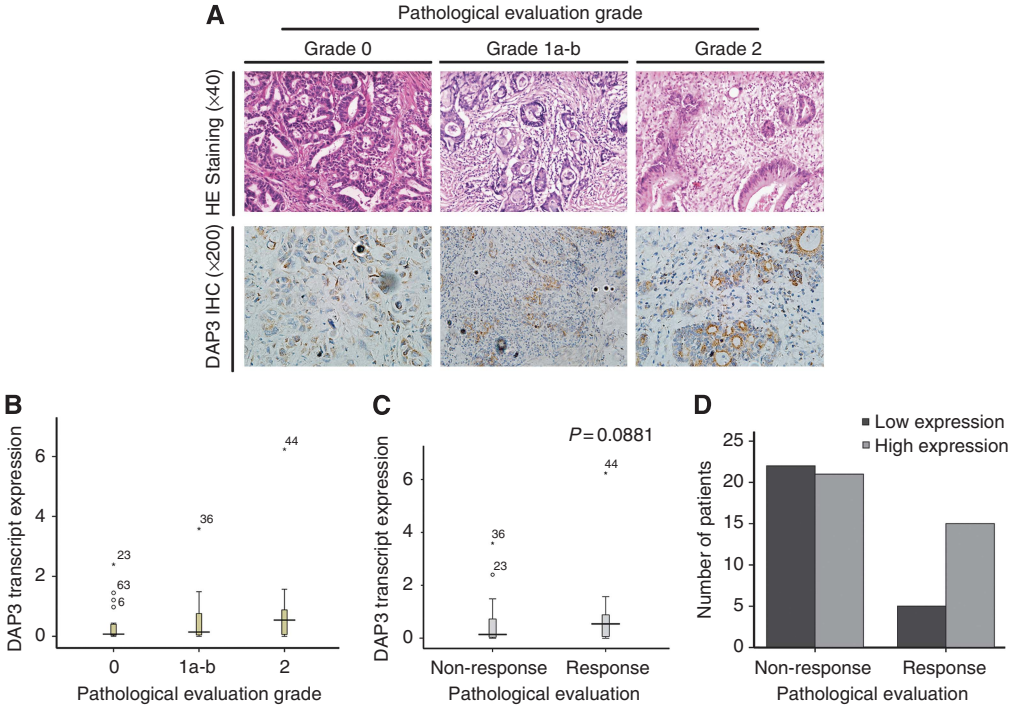

Figure 2. Correlation of DAP3 expression with pathological evaluation of chemotherapy effectiveness. (**A**) Immunohistochemical staining of DAP3 in different pathological evaluation grades and corresponding hematoxylin–eosin (HE) staining; pathological response was evaluated according to HE staining; higher DAP3 IHC staining was related to higher pathological response evaluation grade; (**B**) DAP3 transcript expression increased from lower to higher pathological evaluation grades; (**C**) DAP3 transcript expression was higher in patients exhibiting pathological response than in patients with no response; and (**D**) immunohistochemical staining of DAP3 in patients exhibiting pathological response and non-response to chemotherapy (Chi-square test, $P = 0.051$).

Effects of DAP3 knockdown on *in vitro* cell growth under concentration gradient of 5-FU and oxaliplatin. The knockdown of DAP3 expression in HGC 27 and AGS cell lines was confirmed using RT-PCR and western blotting assay (Figure 3A). Knockdown of DAP3 resulted in slightly decreased growth in both AGS and HGC27 cell lines (Figure 3B). However, under treatment with chemotherapeutic agents, the DAP3 knockdown cell line exhibited a faster rate of cell growth and higher tolerance compared with control cells (Figure 3D).

Influence of DAP3 knockdown on the adhesion, migration and invasion of gastric cancer cells. An *in vitro* adhesion assay was adopted to investigate the effect of DAP3 knockdown on the adhesive ability of gastric cancer cells. DAP3 knockdown AGS cells exhibited decreased adhesion, whereas such an effect was not seen in the DAP3 knockdown HGC27 cells (Supplementary Figure S1A).

The effect of DAP3 knockdown on migration was investigated using an *in vitro* wounding assay. Decreased DAP3 expression was significantly correlated with increased migration in both AGS and HGC 27 cell lines ($P < 0.05$; Figure 3C). Although DAP3 knockdown cells correlated with an increase in invasion of tumour cells in comparison with the control cells in both AGS and HGC27 cell lines, neither showed a significant difference (Supplementary Figure S1B).

Effects of DAP3 knockdown on apoptosis in response to chemotherapeutic agents. Treatment with 5-FU and oxaliplatin induced apoptosis in both AGS and HGC27 cells in a concentration-dependent pattern. The apoptosis index (AI) rose as the concentration increased. This induced apoptosis was suppressed by the DAP3 knockdown. DAP3 knockdown cells correlated with a significantly lower AI in both AGS and HGC27 cell lines under treatment with different concentrations of 5-FU and oxaliplatin (Figure 4A, B, C and D). In addition, the 17-KD subunit of caspase3 was decreased in the DAP3 knockdown cells following treatment with 5-FU and oxaliplatin (Figure 4E).

DISCUSSION

In the present study, immunohistochemical analysis and qRT-PCR analyses were used to investigate the protein and transcript expression of DAP3. Mann–Whitney test revealed that DAP3 transcript expression was significantly higher in patients with strong IHC staining than in patients with weak IHC staining, which suggests that expression of DAP3 was consistent at both mRNA and protein levels. Both assays showed that DAP3 expression in tumour cells was significantly lower than expression in the normal background tissue.

This is consistent with a similar study (Kissil *et al*, 1997): DAP3 was reported to be below the limit of detection in several other human tumours and human tumour cell lines, for example, 80% of B-cell leukemia cell lines had no DAP3 expression. The frequency of loss of DAP3 expression in breast, bladder and renal carcinoma cells is also well repored, ranging from 30 to 40%. Promoter hypermethylation of DAP3 was reported in various human tumours, including B-cell lymphoma, non-small cell lung cancer, head and neck cancer and colon cancer (Esteller *et al*, 1999; Katzenellenbogen *et al*, 1999; Sanchez-Cespedes *et al*, 2000).

In our study, the significant correlation of higher DAP3 expression with better prognosis could be observed at both mRNA and protein level, which suggested a negative role played by DAP3 during the development and progression of these malignancies.

The *in vitro* migration assay indicated that DAP3 knockdown may endow gastric cancer cell lines with more aggressive behaviour

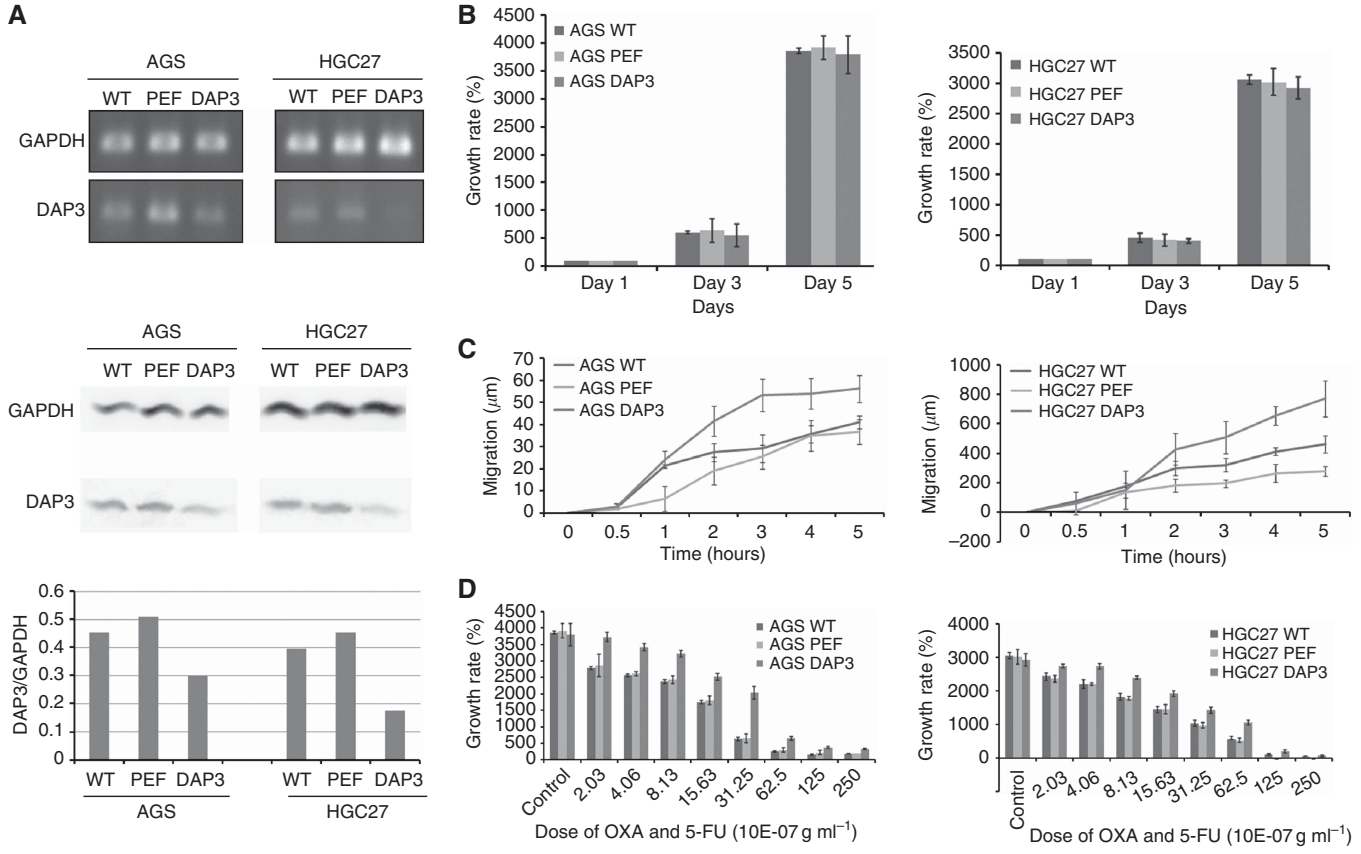

Figure 3. Confirmation of DAP3 expression knock down in AGS and HGC27 cell lines and *in vitro* cell function test. (**A**) DAP3 expression knock down at mRNA and protein level in AGS and HGC27 cell lines; WT: wild type cell line with no plasmid transfection, PEF: cell transfected with empty plasmid vector, DAP3: cell transfected with ribozyme transgenes; (**B**): *In vitro* growth test in AGS and HGC27 cell lines; AGS and HGC27 DAP3 knock down cell (AGS DAP3, HGC27 DAP3) grow slower than corresponding control cell lines (AGS PEF, HGC27 PEF), but no significant difference was observed ($P>0.05$); (**C**) *In vitro* wounding assay in AGS and HGC27 cell lines; in both cell lines, the lines from upper to lower, respectively, represent DAP3, WT and PEF cell lines; AGS and HGC27 DAP3 knock down cell (AGS DAP3, HGC27 DAP3) exhibit faster migration than corresponding control cell lines (AGS PEF, HGC27 PEF) ($P=0.004$, $P=0.022$); and (**D**) *In vitro* drug sensitivity test in HGC27 and AGS cell lines. AGS and HGC27 DAP3 knock down cell lines(AGS DAP3, HGC27 DAP3) were less sensitive to chemotherapy drugs than corresponding control cell lines (AGS PEF, HGC27 PEF) ($P=0.001$, $P=0.002$).

compared with the corresponding control cell line. This is consistent with the result of DAP3 expression analysis in the clinical gastric cancer cohort, which showed that patients developing local recurrence and/or distant metastases had lower expression of DAP3 in their tumours.

As a member of the DAP family, DAP3 has been characterised with a proapoptotic function according to the featured common domains of the DAP family. Knockout of DAP3 was reported to enable cells to survive treatment with many apoptosis-inducing compounds, such as IFN-γ and TRAIL (Kissil *et al*, 1995; Berger and Kretzler, 2002). Decreased DAP3 expression may allow cancer cells to escape from the immune system by increased resistance to apoptotic stimuli, which could partially explain the correlation between better prognosis and a higher level of DAP3 transcript expression. In addition to its proapoptotic role through the exogenous apoptotic pathway, DAP3 has also been found to be a mitochondrial protein with a highly conserved mitochondria-targeting sequence. It has a specific role in the regulation of mitochondria morphology during the apoptotic process. Together with the critical role of mitochondria in genomic damage-induced endogenous apoptosis, DAP3 protein may have a significant role in chemotherapeutic agent-induced apoptosis. In the present study, we focused on the proapoptotic function of DAP3 and its implication for the effectiveness of neoadjuvant chemotherapy.

All patients enrolled in our cohort received preoperative chemotherapy, followed by operation and postoperative chemotherapy. Pathological evaluation was used to evaluate the effectiveness of neoadjuvant chemotherapy. Preoperative chemotherapy aims to downsize and downstage locally advanced gastric cancer, which could enhance the rate of R0 resection and hence improve overall survival. Another potential advantage of neoadjuvant chemotherapy may be that it could predict the effectiveness of postoperative chemotherapy. If it was possible to predict, or make an early judgement on the effectiveness of neoadjuvant chemotherapy, it could allow a strategic decision about further combined therapy to be made. Currently, pathological evaluation is the most reliable criterion with which to judge the effectiveness of chemotherapy in gastric cancer. In this study, the responders (pathological evaluation 2, 3 grade) had significantly longer survival than non-responders (pathological evaluation 1b, 1a, 0 grades). Both IHC and qRT-PCR revealed that DAP3 expression increased as the effectiveness of neoadjuvant chemotherapy enhanced. The DAP3 transcript expression of responders was higher than that of non-responders, and the percentage of strong DAP3 IHC staining was higher in responders. Although the difference was not statistically significant, a correlation between DAP3 expression and the effectiveness of neoadjuvant chemotherapy could be observed.

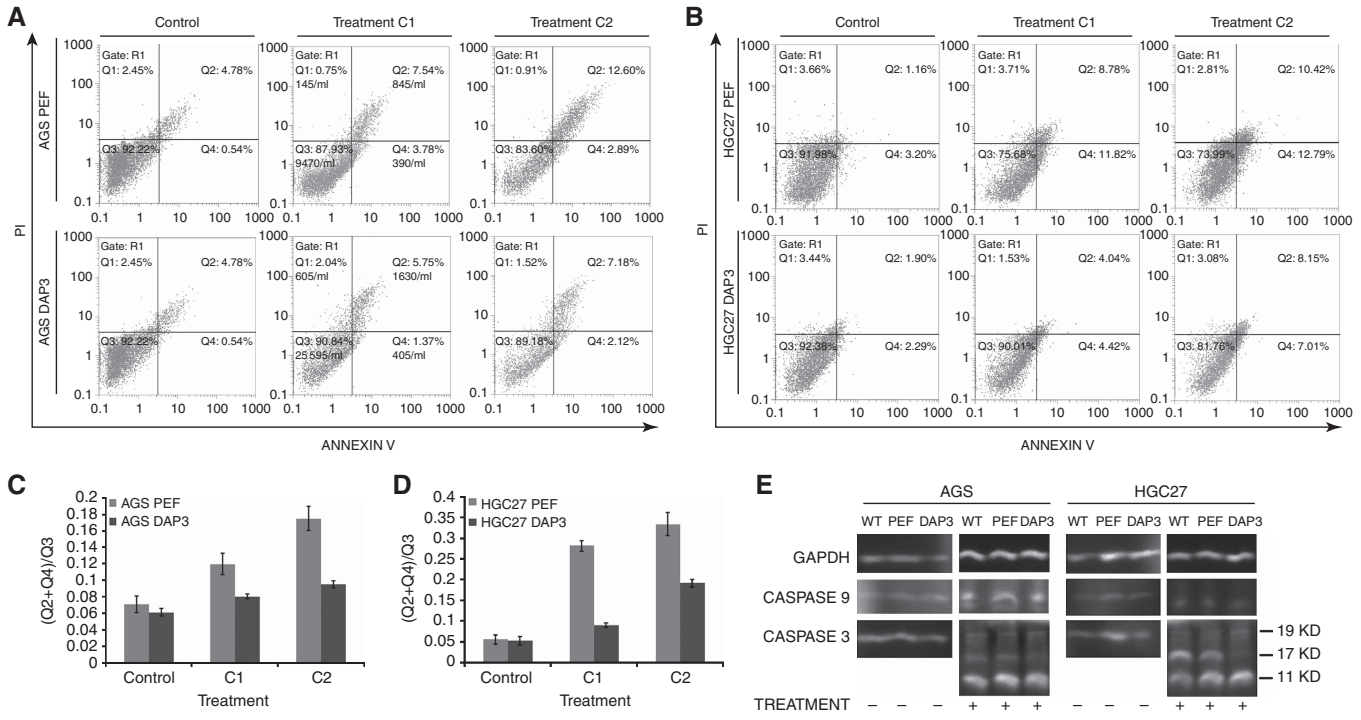

Figure 4. Apoptotic level and caspase protein expression following treatment with 5-FU and oxaliplatin differed in DAP3 knock down cell lines (DAP3) and the corresponding control cell lines (PEF). In both AGS and HGC27 cell lines, PEF and DAP3 knockdown cell lines were treated with a combination of 5-FU and oxaliplatin; then after 6 h treatment, the apoptotic population of the cells was determined using flow cytometry. The downstream effectors caspase 3 and 9 were also determined using western blotting. (**A**) Flow cytometric analysis of apoptosis of AGS PEF and DAP3 cell lines under normal conditions (C) and under treatment with 5-FU and oxaliplatin at lower (C1) and higher (C2) concentration; (**B**) flow cytometric analysis of apoptosis of HGC27 PEF and DAP3 cell lines under normal conditions (C1) and under treatment with 5-FU and oxaliplatin at lower (C1) and higher (C2) concentration. (**C**) In AGS cell line, apoptosis index (AI) was enhanced under treatment with 5-FU and oxaliplatin in contrast to that under normal conditions, and AI increased with increasing treatment concentrations (C1) to (C2). The AI of AGS DAP3 cell line was significantly lower than that of AGS PEF cell line following treatment ($P<0.05$); (**D**) In HGC27 cell lines, a similar trend could be observed. AI was enhanced under treatment with 5-FU and oxaliplatin and increased with increasing treatment concentrations (C1) to (C2). The AI of HGC27 DAP3 cell line was significantly lower than that of HGC27 PEF cell line following treatment ($P<0.05$); and (**E**) Expression of caspase 3 and 9 under normal conditions and under treatment with a combination of 5-FU and oxaliplatin in AGS and HGC27 cell lines. The 17-KD subunit of caspase 3 decreased in DAP3 knockdown cell compared with PEF cell line after treatment.

In order to investigate the underlying mechanism of DAP3-related chemotherapy insensitivity, the growth of different cell lines exposed to 5-FU and oxaliplatin was determined. Although DAP3 knockdown cells exhibited slightly slower growth rate in comparison with control cells in normal medium, these cells appeared to be less sensitive to 5-FU and oxaliplatin compared with control cells and exhibited a faster growth rate. This result indicated that down-regulation of DAP3 expression could endow tumour cells with a higher tolerance to treatment with geno-toxic agents.

In order to verify the impact of DAP3 knockdown on apoptosis, we measured the AI of different cell lines following 6 h of treatment with 5-FU and oxaliplatin. The AI was significantly lower in DAP3 knockdown cell lines treated with 5-FU and oxaliplatin, but no significant difference was found in untreated cells. Further investigation of caspase 3 and 9 showed similar results, which confirms the caspase cascade as a downstream event.

Thus we can conclude that downregulation of DAP3 expression level could prevent gastric cancer cell lines from undergoing apoptosis induced by chemotherapeutic agents and may contribute to their resistance to chemotherapy. This may be part of the reason for the correlation between high levels of DAP3 expression and longer survival of gastric cancer patients after combined therapy.

Although pathological evaluation is widely accepted as a measure of effectiveness of neoadjuvant chemotherapy, our previous study indicated that AI could be more sensitive and could be adopted to identify more potential responders. However, both these evaluation methods can only be performed after preoperative chemotherapy, and any adjustment or change to the postoperative chemotherapy regimen could then be made. Therefore many studies have sought a predictive biomarker for the effectiveness of chemotherapy such as thymidylate synthase or excision repair cross complimenting (ERCC1) (Lenz *et al*, 1996; Metzger *et al*, 1998), but none of them has been accepted as a standard predictive marker. This study has focused on DAP3 due to its role in apoptosis. However, our study has its limitations: DAP3 expression was determined from tumour tissue obtained after neoadjuvant chemotherapy and operation, but it would be preferable to investigate DAP3 expression in biopsy tissues before chemotherapy, in order to confirm the role of DAP3 in predicting the effectiveness of neoadjuvant chemotherapy.

In conclusion, in gastric cancer, DAP3 expression is significantly lower in tumour tissue than that in the normal background tissue, downregulation of DAP3 leads to increased cell migration and lower DAP3 expression is correlated with distant metastasis; DAP3 expression knockdown may also lead to a higher tolerance to chemotherapy-induced apoptosis, and a trend for DAP3 low expression patients to be less sensitive to 5-FU and oxaliplatin, and there is a significant correlation between DAP3 expression and overall survival in the gastric cancer cohort. Finally, DAP3 is a potential molecular marker to predict the effectiveness of preoperative chemotherapy and prognosis in patients undergoing combined therapy for gastric cancer.

ACKNOWLEDGEMENTS

We thank Cancer Research Wales and Albert Hung Foundation for supporting this study. YJ is a recipient of the Cardiff University's China Medical Scholarship.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Berger T, Brigl M, Herrmann JM, Vielhauer V, Luckow B, Schlondorff D, Kretzler M (2000) The apoptosis mediator mDAP-3 is a novel member of a conserved family of mitochondrial proteins. *J Cell Sci* **113**, Pt 20 3603–3612.

Berger T, Kretzler M (2002) TRAIL-induced apoptosis is independent of the mitochondrial apoptosis mediator DAP3. *Biochem Biophys Res Commun* **297**: 880–884.

Cavdar Koc E, Ranasinghe A, Burkhart W, Blackburn K, Koc H, Moseley A, Spremulli LL (2001) A new face on apoptosis: death-associated protein 3 and PDCD9 are mitochondrial ribosomal proteins. *FEBS Lett* **492**: 166–170.

Cohen O, Inbal B, Kissil JL, Raveh T, Berissi H, Spivak-Kroizaman T, Feinstein E, Kimchi A (1999) DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. *J Cell Biol* **146**: 141–148.

Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman J. G (1999) Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* **59**: 67–70.

Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* **281**: 1309–1312.

Inbal B, Cohen O, Polak-Charcon S, Kopolovic J, Vadai E, Eisenbach L, Kimchi A (1997) DAP kinase links the control of apoptosis to metastasis. *Nature* **390**: 180–184.

Japanese Gastric Cancer Association (2011) Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* **14**: 101–112.

Jia Y, Dong B, Tang L, Liu Y, Du H, Yuan P, Wu A, Ji J (2012) Apoptosis index correlates with chemotherapy efficacy and predicts the survival of patients with gastric cancer. *Tumour Biol* **33**: 1151–1158.

Jiang WG, Grimshaw D, Lane J, Martin TA, Abounader R, Laterra J, Mansel RE (2001) A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-MET and reduces migration and invasiveness of breast cancer cells. *Clin Cancer Res* **7**: 2555–2562.

Jiang WG, Hiscox S, Hallett MB, Scott C, Horrobin DF, Puntis MC (1995) Inhibition of hepatocyte growth factor-induced motility and *in vitro* invasion of human colon cancer cells by gamma-linolenic acid. *Br J Cancer* **71**: 744–752.

Jiang WG, Hiscox SE, Parr C, Martin TA, Matsumoto K, Nakamura T, Mansel RE (1999) Antagonistic effect of NK4, a novel hepatocyte growth factor variant, on *in vitro* angiogenesis of human vascular endothelial cells. *Clin Cancer Res* **5**: 3695–3703.

Katzenellenbogen RA, Baylin SB, Herman JG (1999) Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* **93**: 4347–4353.

Kissil JL, Deiss LP, Bayewitch M, Raveh T, Khaspekov G, Kimchi A (1995) Isolation of DAP3, a novel mediator of interferon-gamma-induced cell death. *J Biol Chem* **270**: 27932–27936.

Kissil JL, Feinstein E, Cohen O, Jones PA, Tsai YC, Knowles MA, Eydmann M. E, Kimchi A (1997) DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. *Oncogene* **15**: 403–407.

Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ* **7**: 1166–1173.

Kroemer G, Reed J. C (2000) Mitochondrial control of cell death. *Nat Med* **6**: 513–519.

Lenz HJ, Leichman CG, Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L (1996) Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J Clin Oncol* **14**: 176–182.

Lowy AM, Mansfield PF, Leach SD, Pazdur R, Dumas P, Ajani JA (1999) Response to neoadjuvant chemotherapy best predicts survival after curative resection of gastric cancer. *Ann Surg* **229**: 303–308.

Martinou JC, Green DR (2001) Breaking the mitochondrial barrier. *Nat Rev Mol Cell Biol* **2**: 63–67.

Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, Groshen S, Salonga D, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Konda B, Leichman L (1998) ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* **16**: 309–316.

Morgan CJ, Jacques C, Savagner F, Tourmen Y, Mirebeau DP, Malthiery Y, Reynier P (2001) A conserved N-terminal sequence targets human DAP3 to mitochondria. *Biochem Biophys Res Commun* **280**: 177–181.

Mukamel Z, Kimchi A (2004) Death-associated protein 3 localizes to the mitochondria and is involved in the process of mitochondrial fragmentation during cell death. *J Biol Chem* **279**: 36732–36738.

Parr C, Jiang WG (2009) Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer. *Eur J Cancer* **45**: 1673–1683.

Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D (2000) Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* **60**: 892–895.

Saveanu C, Fromont-Racine M, Harington A, Ricard F, Namane A, Jacquier A (2001) Identification of 12 new yeast mitochondrial ribosomal proteins including 6 that have no prokaryotic homologues. *J Biol Chem* **276**: 15861–15867.

Suzuki T, Terasaki M, Takemoto-Hori C, Hanada T, Ueda T, Wada A, Watanabe K (2001) Proteomic analysis of the mammalian mitochondrial ribosome. Identification of protein components in the 28S small subunit. *J Biol Chem* **276**: 33181–33195.

Ye L, Kynaston H, Jiang WG (2008) Bone morphogenetic protein-9 induces apoptosis in prostate cancer cells, the role of prostate apoptosis response-4. *Mol Cancer Res* **6**: 1594–1606.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)