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Expression of death-associated protein kinase during tumour progression of human renal cell carcinomas: Hypermethylation-independent mechanisms of inactivation

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ABSTRACT

Death-associated protein kinase (DAPK) is a pro-apoptotic Ca^{2+}/calmodulin-dependent serine/threonine kinase that is widely expressed in tissues but kept silent in growing cells. Downregulation of DAPK transcription by CpG methylation has been demonstrated in a variety of tumours, providing a selective growth advantage during tumour progression. As the in vivo expression of DAPK in human renal cell carcinomas (RCCs) has not previously been analysed, 72 RCCs were investigated using semi-quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). We found that almost 92% (66/72) of all primary RCCs express DAPK mRNA and results obtained from methylation-specific PCR analyses suggest that aberrant CpG methylation of the DAPK promoter is absent even in DAPK non-expressing tumours. Comparison of early/intermediate with advanced tumour stages of clear cell RCCs showed that no significant changes in the expression levels of DAPK were evident. Chromophilic/papillary RCCs display no significantly different expression patterns of DAPK compared with stage-adjusted clear cell RCCs. Furthermore, on analysing the DAPK enzyme activity in RCC cell lines with DAPK mRNA and protein expression, only 1 out of 11 cell lines showed basal DAPK activity in kinase activity assays, suggesting that DAPK, although expressed in RCC, remains largely inactive. Our study demonstrates the in vivo expression of DAPK in RCCs and reveals that, in contrast to other tumour types, RCCs may not downregulate DAPK mRNA expression during tumour progression. Despite persistent DAPK transcription and translation, however, the markedly reduced DAPK enzyme activity in our RCC cell lines suggested a post-translational inactivation of DAPK in RCCs.

1. Introduction

Apoptosis is a genetically controlled and evolutionary conserved mechanism of programmed cell death that is involved in a wide range of physiological processes, such as embryonic development, tissue homeostasis of multicellular organisms and immune regulation [1,2]. Apoptosis is hence considered to be a key biological regulation process. Deregulation of

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apoptosis may therefore result in irregular cell survival and has been implicated in various diseases including neurodegenerative disorders and cancer [3]. An abnormal pattern of cell death is frequently caused by an imbalance between pro-apoptotic and anti-apoptotic factors. Increased resistance to apoptosis due to such an imbalance plays a crucial role in tumour progression, resulting in prolonged cell survival, thereby facilitating the accumulation of transforming mutations and promoting resistance to immunosurveillance [3]. Although many anti-apoptotic genes and gene products, including Bcl-2 and members of the inhibitor of apoptosis protein (IAP) family such as XIAP and survivin, have been characterised with respect to contribution to the multi-step process of tumourigenicity [4–6], only a few positive mediators of cell death have been shown to link apoptosis to the development of cancer. Relevant observations for this include the tumour suppressor p53, whose pro-apoptotic function is lost in a wide range of human tumours [7] and death-associated protein kinase (DAPK), a death-inducing factor with putative tumour and metastasis suppressor activity [8]. The *DAPK* gene was initially isolated by a functional genetic screening approach and encodes for a 160 kDa calcium/calmodulin-dependent serine/threonine protein kinase [9]. Although its apoptosis-promoting mechanism is poorly understood, DAPK seems to be involved in p53-dependent suppression of tumour development [10] and it participates in a number of death signalling pathways, i.e. apoptosis triggered by interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), CD95 (Fas/Apo1-L) and transforming growth factor-β (TGF-β) [11–13]. Moreover, DAPK is involved in the formation of autophagic vesicles and takes part in mediation of membrane blebbing, presumably through phosphorylation of its direct substrate myosin-II regulatory light chain (RLC) [14,15]. The protein is composed of several domains, including a kinase domain and a conserved death domain, which are essential for cell death induction [11,16]. Recently, it was shown that, via its death domain, DAPK interacts with extracellular signal-regulated kinase (ERK) and that both proteins form a crucial regulatory circuit promoting the pro-apoptotic effect of DAPK [17]. DAPK mRNA is widely expressed in tissues, but the protein remains largely inactive because DAPK is negatively controlled by an autophosphorylation-based mechanism and is thereby kept silent in growing cells [18]. In contrast, loss of DAPK mRNA transcription by promoter CpG methylation has been demonstrated in a variety of tumours and tumour derived cell lines including B-cell malignancies, non-small cell lung cancer, head and neck cancer, thyroid lymphoma, advanced stage gastric cancer and other adenocarcinomas of the upper gastrointestinal tract [19–24]. In non-small cell lung cancer cells DAPK downregulation was also associated with a decreased sensitivity to treatment with death-inducing ligand TRAIL [25]. DAPK downregulation therefore seems to provide a selective advantage during tumour progression that could also affect the response to anticancer drugs. In the present study we analysed the in vivo expression of DAPK in human renal cell carcinomas (RCCs), screening 72 RCCs of different histological types and grades by semi-quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

In contrast to most other tested tumour types, RCCs may not downregulate DAPK mRNA expression by CpG hypermethylation during tumour progression, indicating that loss of DAPK mRNA is not a prerequisite for tumour progression in general. Despite persistent DAPK transcription and translation, however, the markedly reduced DAPK enzyme activity in our RCC cell lines suggested that constitutive DAPK expression might be tolerated by RCC tumours through a post-translational mechanism of DAPK inactivation.

2. Materials and methods

2.1. *Tumour material*

A total of 72 tumour tissue samples from RCCs of different histological types, stages and grades (Table 1) were obtained from patients who had undergone a nephrectomy. The tumour material was immediately flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction. To confirm that the proportion of tumour tissue was more than 80%, a consecutive tissue section was stained with haematoxylin

Table 1 – Data on the typing, grading and staging of 72 renal cell carcinomas (RCCs) with regard to the amount of samples with no DAPK mRNA expression[a]

RCC type	Grading	Samples with no DAPK expression	Staging	Samples with no DAPK expressions
Clear cell ($n = 58$)[b]	G1: $n = 0$ G2: $n = 50$ G3: $n = 7$	5	pT1: $n = 23$ pT2: $n = 21$ pT3: $n = 14$	 4 1
Chromophilic/papillary ($n = 12$)	G1: $n = 0$ G2: $n = 10$ G3: $n = 2$	1	pT1: $n = 10$ pT2: $n = 2$ pT3: $n = 0$	 1
Chromophobe ($n = 2$)	G1: $n = 0$ G2: $n = 2$ G3: $n = 0$		pT1: $n = 1$ pT2: $n = 1$ pT3: $n = 0$	
$\sum$	$n = 72$	6	$n = 72$	6

a For details see text.
b In one case the grading of the corresponding sample could not be determined, therefore the sum of all clear cell grading samples is 57.

and eosin (H&E) and examined under the microscope. Tumour typing, grading and staging were performed according to the principles outlined by the World Health Organisation (WHO) [26,27] and the International Union Against Cancer (UICC) [28]. The contingent of the different RCC types in the present study reflects the frequency of each tumour type in a large RCC series [27].

2.2. Cell lines and cell culture

All RCC cell lines used in this study were derived from typical representatives of the clear cell and chromophilic/papillary types of RCC, as established in our laboratory and previously described [29–31]. The RCC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Eggenstein, Germany) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma–Aldrich, Deisenhofen, Germany), 200 mg/l arginine, 72 mg/l asparagine (Serva Electrophoresis, Heidelberg, Germany), 10 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). HeLa cells were maintained in DMEM medium containing 10% (v/v) FBS, 2 mM glutamine and penicillin/streptomycin. Raji cells were grown in RPMI medium (Gibco) containing 10% (v/v) FBS and also supplemented with 2 mM glutamine and penicillin/streptomycin. All cells were incubated at 37 °C in an atmosphere containing 5% CO_2.

2.3. Laser capture microdissection (LCM) and RNA extraction

Paraffin-embedded RCC tumour tissue specimens were cut in 5 μm thick sections and slightly stained with haemalaun. Using the PixCell II LCM System (Arcturus Engineering, Mountain View, CA, United States of America (USA)) with a target voltage of 200 mV, a 7.5-μm laser spot diameter and a pulse power of 70 mW with a duration time of 3 ms, approximately 2000 tumour cells were collected in CapSure HS caps (Arcturus) by an experienced pathologist and immediately resolved in RNA lysis buffer (Qiagen, Hilden, Germany). Then, total RNA was isolated using the RNeasy FFPE Test Kit (Qiagen) according to the manufacturer's instructions including DNAse I treatment. Due to the low RNA amounts, during isolation 20 ng of a 1.2 kb Kanamycin control RNA (Promega, Heidelberg, Germany) was used as carrier RNA.

2.4. RNA extraction from frozen tumour material

Total RNA was isolated using the guanidinium thiocyanate procedure as described previously [32]. An additional purification step was performed using the RNeasy Mini Kit including DNAse I treatment according to the manufacturer's instructions (Qiagen). For all the tested samples, the RNA integrity was checked from the intact 18 S/28 S rRNA bands in agarose gel electrophoresis.

2.5. Reverse transcription

First strand cDNA synthesis was carried out using 1 μg DNAse I treated total RNA, 25 nmol dNTPs, 0.5 U of recombinant RNasin® ribonuclease inhibitor (Promega), 0.5 μg random primers (Promega), 5 mM $MgCl_2$ and 15 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) with the corresponding RT-buffer in a final volume of 20 μl. The reactions were incubated at 55 °C for 1 h and afterwards terminated by heat inactivation at 94 °C for 5 min.

2.6. Semi-quantitative real-time PCR

Amplification and quantification of PCR products were performed with the LightCycler (Roche Diagnostics, Mannheim, Germany) using the Fast Start DNA Master SYBR Green I Kit (Roche). To amplify and detect DAPK transcripts, 4 μl cDNA synthesis reaction mixture were transferred to a PCR assay composed of 2 μl 10 × Fast Start Reaction Mix (Roche; including the Taq DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix and SYBR Green I dye), 3 mM $MgCl_2$ and 0.5 μM forward primer (5′-TGA CCA CGG ACG GAA AGA C-3′) and reverse primer (5′-TGC GGC TCC TCA CAC TCA-3′; TIB MOLBIOL, Berlin, Germany), respectively, in a total volume of 20 μl. In addition, 1 unit of uracil-DNA-glycosylase (Roche) was added to avoid contamination by PCR products. During amplification, a PCR product of 368 bp was obtained corresponding to nt 2255 to nt 2623 of DAPK mRNA (NCBI accession number X76104). In addition, amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out (forward primer: 5′-ACC AGC GAC ACC CAC TCC TC-3′; reverse primer: 5′-GGA GGG GAG ATT CAG TGT GGT-3′; TIB MOLBIOL), which yielded a 257 bp fragment. Amplification included initial denaturation at 95 °C for 10 min followed by 50 cycles of 1 s denaturation at 95 °C, 10 s annealing at 66 °C and extension at 72 °C for 20 s performed at a temperature transition rate of 20 °C/s. As SYBR Green I dye is a non-specific intercalating dye in every PCR, the hot start was used in order to prevent the formation of primer dimers and non-specific products during the reaction set-up and the first denaturation step. In addition, PCR products were checked by agarose gel electrophoresis and confirmed by DNA sequencing using the ABI-Prism BigDye Terminator Cycle Sequencing Kit (ABI, Weiterstadt, Germany) with the respective forward and reverse primers according to the manufacturer's protocol. Sequence analysis was performed in an ABI PRISM® 310 genetic analyser.

2.7. Quantification of DAPK transcripts

The concentration of DAPK transcripts was calculated semi-quantitatively in relation to the amount of GAPDH transcripts. (Due to the lack of DAPK expression plasmids, an absolute quantification of total DAPK copy numbers was not possible.) For this relative quantification, GAPDH PCR on cDNA standards comprising a fivefold dilution series of HeLa cDNA was performed and used to generate a GAPDH standard curve (external standard) by plotting the crossing point (CP) values against the dilution factor, with every dilution being run at least in duplicate. DAPK and GAPDH expression values were normalised to the standard curve by GAPDH expression levels obtained from cDNA standards which were amplified along the samples in every run. The concentrations of transcripts were calculated by the LightCycler Software Version 3.5.3 according to the second derivative maximum method.

2.8. Statistical analysis

GAPDH expression values for each sample were used to normalise the respective amplification values of DAPK transcripts, calculating ratios of relative mRNA levels. Statistical analysis was carried out using the Mann–Whitney and Wilcoxon tests with the statistical software package SPSS 9.0.1 (SPSS, Chicago, IL, USA). Using this test, a *P*-value of less than 0.05 was considered to indicate statistical significance.

2.9. RT-PCR of laser microdissected RCC samples

Total RNA isolated from the respective laser microdissected tumour sample was used to perform cDNA synthesis as described above. Subsequently, 10 μl thereof were used in a 50 μl PCR mixture containing 10 × PCR buffer, 1.25 units Taq-polymerase (Qiagen), 200 μM of each dNTP (Promega) and 15 pmol of the respective DAPK primers (5′-TGC GGC CAA CAA CGG AAT C-3′ (+) and 5′-GGG TCG GGG CCA CAA ACA C-3′ (–)). The PCR conditions were as follows: hot start for 5 min at 95 °C and 50 cycles of 30 s at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C. During PCR a 496 bp fragment was generated corresponding to nt 2187 to nt 2683 of DAPK mRNA (NCBI accession number X76104) which was separated and visualised using a 1.5% ethidium bromide stained agarose gel.

2.10. DNA extraction and sodium bisulphite modification

Genomic DNA from freshly frozen primary tissue was extracted using the QiaAMP DNA Mini Kit (Qiagen) according to the manufacturer's instructions and subsequently modified by sodium bisulphite treatment as described previously [33]. In summary, 2 μg genomic DNA in a total volume of 50 μl was denatured with 0.2 M NaOH for 10 min at 37 °C. About 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulphite (pH 5.0) were added, mixed thoroughly and incubated at 50 °C for 16 h with mineral oil overlay. Modified DNA was cleaned using the QIAquick PCR purification kit (Qiagen) according to the manual's instructions and eluted with 50 μl H_2O. Thereafter, 5 μl of 3 M NaOH was added and the mixture was incubated for 5 min at RT. At last the modified DNA was precipitated by ammonium acetate precipitation, resuspended in 20 μl H_2O and immediately used as a template in methylation-specific PCR (MSP).

2.11. Methylation-specific polymerase chain reaction

Methylation-specific PCR (MSP) was carried out using two primer pairs specific for the methylated or sodium bisulphite converted unmethylated *DAPK* promoter DNA, respectively [34]. For each MSP, 3 μl modified DNA was used in a 50 μl reaction mixture containing 10 × PCR buffer, 1.25 units Taq-polymerase (Qiagen), 200 μM of each dNTP (Promega) and 15 pmol of the respective primers (5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′ (+) and 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (–) for detecting unmethylated DAPK promoter DNA and 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ (+) and 5′-CCC TCC CAA ACG CCG A-3′ (–) for methylated alleles). The PCR conditions were as follows: hot start for 5 min at 95 °C and 39 cycles of 60 s at 95 °C, 60 s annealing at 60 °C and 60 s extension at 72 °C. PCR products related to unmethylated DAPK promoter DNA displayed a 106 bp fragment and for methylated alleles a 98 bp fragment was generated; these were separated and visualised using 3% (v/v) ethidium bromide stained agarose gels. In all the experiments, HeLa DNA served as the positive control for unmethylated DAPK alleles and Raji DNA served as the positive control for methylation-specific DAPK amplification [20].

2.12. Preparation of protein extracts and Western blot analysis

Cells were washed twice with ice-cold PBS and afterwards incubated in ice-cold lysis buffer containing 20 mM HEPES, 10 mM EGTA, 40 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT) and 1% (v/v) NP-40 supplemented with a mixture of various protease inhibitors (Roche). Thereafter the cell lysate was harvested and centrifuged to isolate the protein fraction from intact cells and cell debris. Primary tissue samples were lysed in a buffer containing 100 mM NaCl, 10 mM Tris–HCl (pH 7.6), 1 mM EDTA and 1% (v/v) NP-40 supplemented with several protease inhibitors (Roche). After centrifugation, the supernatant was considered the protein fraction. Equal amounts of protein were separated by SDS–PAGE (8%) and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After membrane saturation in blocking buffer (3% (w/v) non-fat dried milk and 1% (w/v) BSA in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.2% (v/v) Tween 20), DAPK immunodetection was performed overnight at 4 °C using anti-DAPK monoclonal antibodies (Pharmingen, Heidelberg, Germany) in a 1:250 dilution. Visualisation of the DAPK protein was carried out by ECL detection reagents (Pharmacia Biotech, Freiburg, Germany), employing a horseradish peroxidase coupled mouse-anti-human secondary antibody (Sigma–Aldrich; 1:5000 dilution, incubation 1 h at room temperature).

2.13. Immunoprecipitation and kinase activity assays

For immunoprecipitation, supernatants containing 100 μg protein were incubated with 2 μg DAPK polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 4 °C and then coupled to Protein A agarose conjugate (Sigma–Aldrich) while rotating at 4 °C overnight. The immunoprecipitates were collected by centrifugation and washed twice in 100 μl lysis buffer and twice in the same volume of kinase assay buffer (12.5 mM MOPS; pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, and 0.5 mM NaF, 0.5 mM vanadate). They were subsequently incubated for 30 min at 37 °C in 15 μl kinase assay buffer supplemented with 2 μg DAPK substrate (Tocris Biotrend, Köln, Germany) and 10 μCi [γ-^{32}P]ATP. The reaction was stopped by adding SDS–PAGE loading buffer, and after denaturation for 3 min at 95 °C the samples were subjected to SDS–PAGE. Finally, the kinase activity was visualised by autoradiography of the [^{32}P]-labelled substrates.

2.14. *Immunohistochemistry*

Immunohistochemical detection of DAPK protein expression was performed on 4 μm thick paraffin sections from one paraffin block representative for the entire tumour. Sections were mounted on poly-L-lysine coated glass slides, dried at 58 °C for 12 h followed by deparaffinisation and rehydration. Subsequently, slides were immersed in 10 mM sodium citrate buffer (pH 6.0) and then heated in a pressure cooker overnight. After blocking of endogenous peroxidase activity by treatment with 0.5% hydrogen peroxide for 30 min, tissue sections were incubated with 5% normal horse serum (Sigma–Aldrich) to prevent non-specific antibody binding. Endogenous biotin was blocked using the SP-2001 avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions. Then, monoclonal IgG1 DAPK antibody (Pharmingen) was applied to sections at a dilution of 1:400 and incubated in a moist chamber at 37 °C for 60 min. Negative control was performed, replacing the primary antibody with an irrelevant monoclonal mouse antibody. Bound antibody was detected using the avidin–biotin complex peroxidase (ABC Elite Kit, Vector Laboratories). Staining reaction was performed with 3,3′-diaminobenzidine and H_2O. For mild counterstaining, Mayer's haematoxylin solution was used.

3. Results

3.1. *Detection and quantification of DAPK transcripts*

Semi-quantitative RT-PCR analysis of DAPK transcripts in 72 primary RCCs of different histological types, stages and grades (Table 1) using LightCycler technology revealed that 91.7% (66/72) of all the tested RCCs express DAPK mRNA, with GAPDH expression serving as an external standard. For relative quantification a standard curve was generated by performing GAPDH PCR on a fivefold dilution series of HeLa cDNA (Fig. 1). Comparing the DAPK/GAPDH ratios calculated by the LightCycler software according to the second derivative maximum method with the melting curve and agarose gel data, a relative mRNA level ratio of less than 5×10^{-4} indicated the absence of DAPK amplification products (data not shown). On that basis, we considered DAPK/GAPDH ratios of less than 5×10^{-4} as DAPK downregulation. This accounts for 8.3% of all the tested tumour samples. The total of 72 tumour samples comprised 58 samples of the clear cell type and 12 tumour samples of the chromophilic/papillary type, whereas 94.8% (55/58) of the clear cell RCCs and 91.7% (11/12) of the chromophilic/papillary type exhibit DAPK mRNA expression. Tumours of the rare chromophobic type are represented by 2 samples that also both display DAPK mRNA expression. In tumour samples with detectable DAPK transcripts (Fig. 2), samples referring to tumour stage pT1 displayed the average amplification at cycle 25, pT2 samples at cycle 32 and samples belonging to pT3 stages at cycle 29. As SYBR Green I is a sequence-unspecific intercalating dye, the integrity and length of all amplification products were checked by agarose gel electrophoresis in order to exclude false quantification due to additionally generated fragments.

3.2. *Stage dependent expression of DAPK mRNA in clear cell RCCs*

Comparing early and intermediate stages (pT1 + pT2) with late tumour stages (pT3) for clear cell RCCs (Fig. 3), no significant changes in the expression of DAPK became evident when levels were normalised to GAPDH mRNA expression levels using as an external standard. However, on analysing the relative (GAPDH-normalised) DAPK transcript levels of all tumour stages compared with one another, there is a marked decrease in DAPK expression levels from low (pT1) to intermediate (pT2) stages and a noteworthy rise in expression again from intermediate to

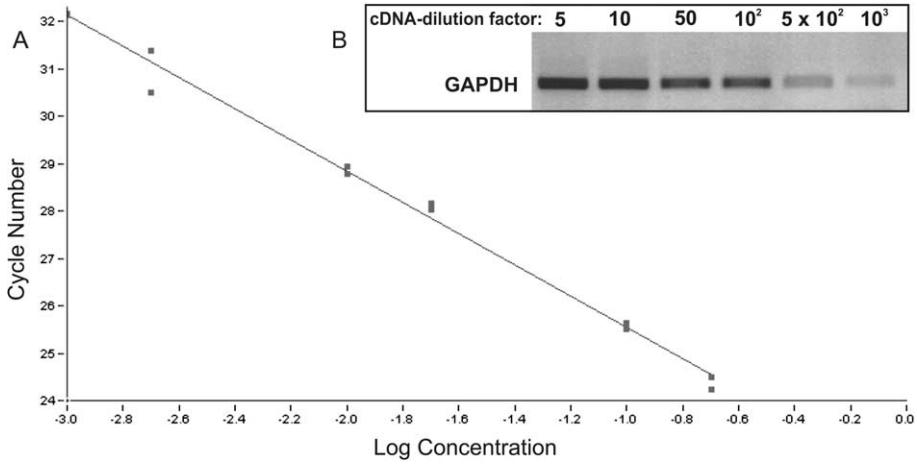

Fig. 1 – (A) LightCycler-based standard curve report for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification from diluted HeLa-cDNA samples (ranging from 5-fold to 1000-fold dilution, duplicate samples). The cycle number is plotted against the log of the concentration calculated by the second derivative maximum method. A correlation coefficient of $r = -1.00$ indicates a precise log linear relationship. (B) GAPDH calibration visualised on agarose gel (single dilution samples).

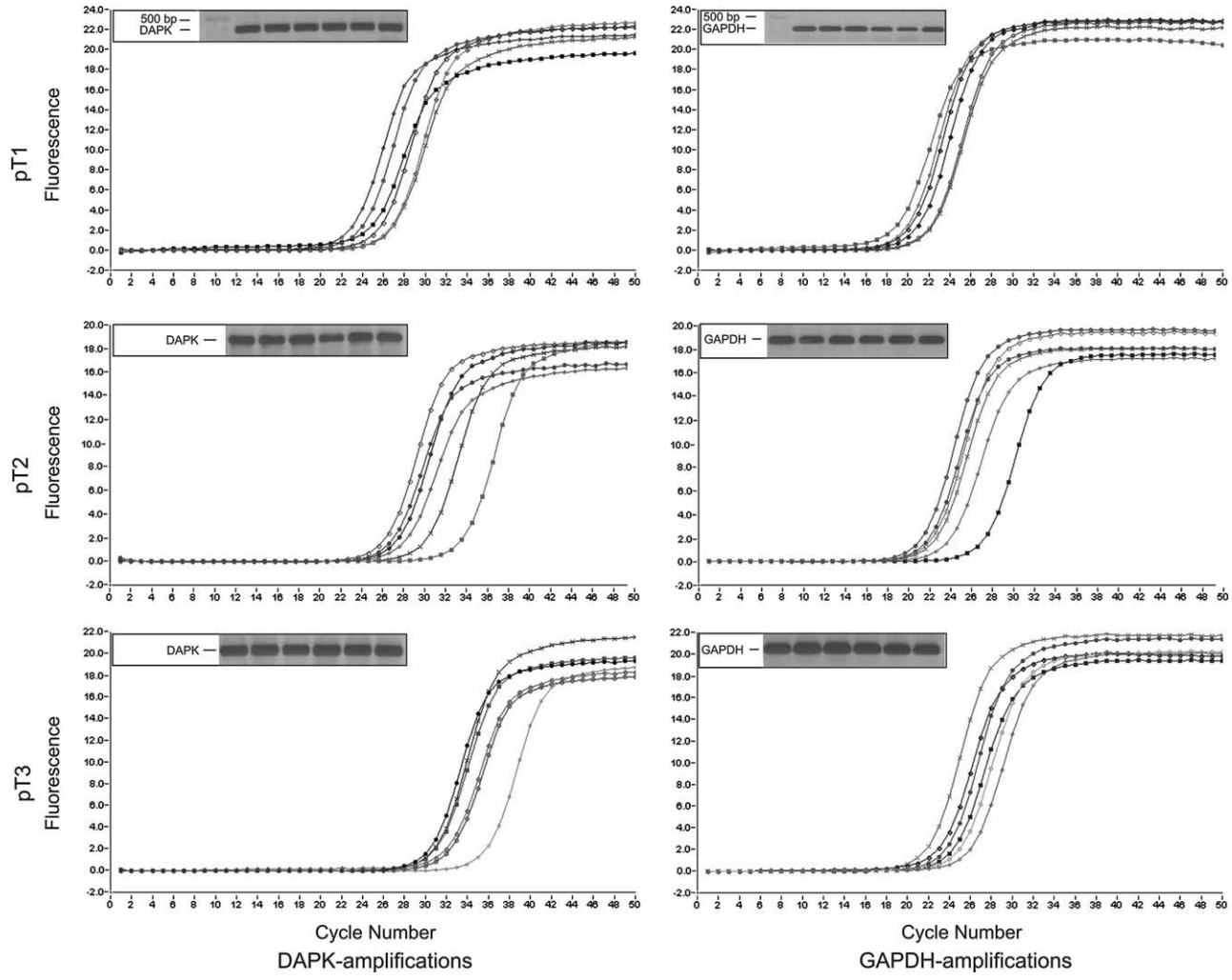

Fig. 2 – Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of death-associated protein kinase (DAPK) transcripts in renal cell carcinomas (RCCs) by LightCycler technology. SYBR Green I mediated fluorescence (*y*-axis) was measured once per cycle (*x*-axis). Representative DAPK and GAPDH amplification curves ($n = 6$) are shown for tumour stage pT1 (upper panel), pT2 (centre panel) and pT3 (lower panel). Same symbol indicates corresponding DAPK and GAPDH amplification curves. The specificity of amplification products by agarose gel electrophoresis is shown in the upper framed box.

advanced stages (pT3) (data not shown). This stage-dependent decrease in pT2 was also found for the expression of survivin [35], but not for smac/DIABLO (unpublished results), which indicates a gene-specific underlying effect.

3.3. *Expression levels of DAPK mRNA in clear cell and chromophilic/papillary RCCs*

Chromophilic/papillary RCCs are distinct tumour entities that differ from clear cell RCCs in aspects such as their histomorphology, cytogenetic aberrations and response to growth factors. They are known to be less aggressive than clear cell RCCs [31,36]. As shown in Fig. 4, chromophilic/papillary RCCs display no significantly different expression patterns for GAPDH-normalised DAPK mRNA levels compared with stage-adjusted clear cell RCCs. The similar number of DAPK expressing samples in each tumour entity (94.8% for clear cell RCCs and 91.7% for chromophilic/papillary RCCs) further indicates comparable DAPK expression in RCC irrespective of the histological type.

3.4. *Transcriptional repression of DAPK by promoter CpG methylation is absent in tested primary RCCs*

In many human tumours and tumour derived cell lines, DAPK mRNA transcription is silenced by CpG methylation [19–24]. In contrast, DAPK mRNA expression is maintained in all tumour stages (Fig. 3) to a greater extent in RCCs. We therefore examined the methylation profile of the DAPK promoter in 10 representative tumour samples and the corresponding non-neoplastic tissues, with special attention being put on samples showing no detectable DAPK mRNA expression levels. As shown in Fig. 5, aberrant promoter hypermethylation could not be detected in any of the tested tumour and non-neoplastic tissue samples as only PCR

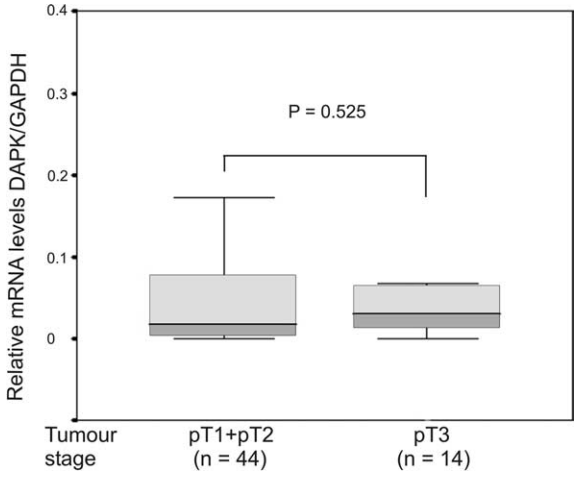

Fig. 3 – Comparison of relative death-associated protein kinase (DAPK) mRNA levels (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalised) between early and intermediate (pT1 + pT2) tumour stages and advanced tumour stages (pT3) of clear cell renal cell carcinomas (RCCs) indicates no significant change in the level of expression.

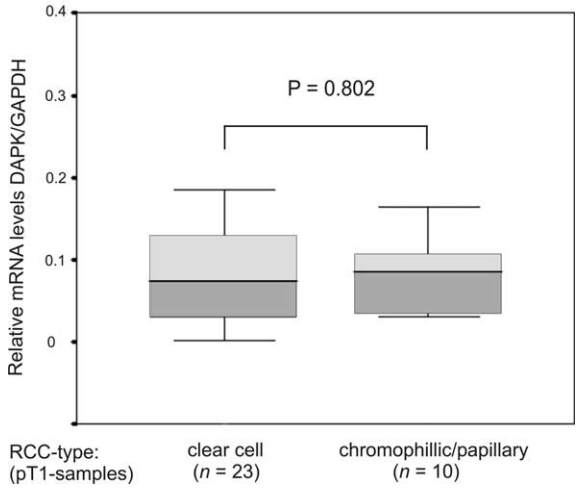

Fig. 4 – Comparison of relative (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalised) death-associated protein kinase (DAPK) mRNA levels between pT1 samples of clear cell renal cell carcinomas (RCCs) and pT1 samples of chromophilic/papillary RCCs reveal a similar DAPK expression pattern in both tumour entities.

products specific to methylated DAPK alleles were amplified in Raji cells, serving as positive control for DAPK hypermethylation [20]. These data suggest that DAPK promoter-methylation is not present in human RCCs and not responsible for the suppressed DAPK expression observed in 8.3% of the tested RCC samples.

3.5. *DAPK protein expression in primary RCCs*

In order to determine whether maintained DAPK mRNA expression during tumour progression could be further confirmed at protein level, we analysed the basal protein expression of DAPK in six randomly selected RCC samples of pT1 and pT3 stages (Fig. 6) by Western blot. We found that DAPK

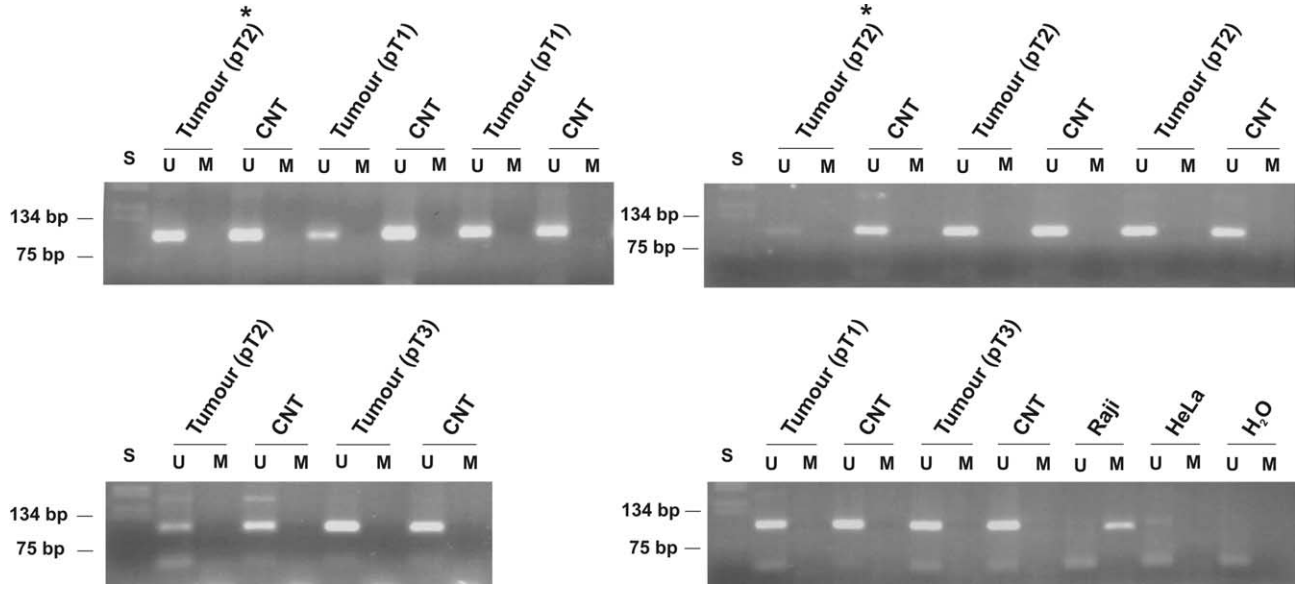

Fig. 5 – Methylation-specific PCR (MSP) of the death-associated protein kinase (DAPK)-promoter on genomic DNA derived from 10 representative primary renal cell carcinoma (RCC) tumour samples and the corresponding non-neoplastic tissues (CNT). For all tumour samples the respective stage is in parentheses. Probes with an asterisk indicate tumour samples with no detectable DAPK-mRNA transcripts using reverse transcriptase polymerase chain reaction (RT-PCR). DNA derived from Raji cells served as methylation-positive control [20] and HeLa-DNA as methylation-negative control. U, PCR with primers specific to unmethylated DAPK-sequences; M, PCR with primers specific to methylated DAPK-sequences [34]; S, DNA standard.

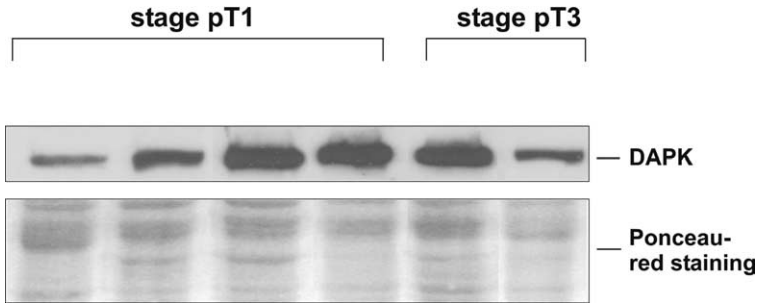

Fig. 6 – Death-associated protein kinase (DAPK) detection in randomly selected primary tumour samples of human renal cell carcinoma (RCC). One hundred μg of each tumour sample was subjected to Western blot and comparable amounts of protein were confirmed by Ponceau-red staining.

protein expression was present in all the tested samples, thereby corroborating the results obtained by semi-quantitative RT-PCR analysis. Ponceau-red staining was used to demonstrate that comparable amounts of protein were present.

To provide evidence that the observed DAPK expression of the tumour samples analysed in our study originated from tumour cells, a subset of tumour samples was further investigated by RT-PCR analysis after laser capture microdissection (LCM) of exclusive RCCs cells. In those nearly homogenous populations of tumour cells a DAPK mRNA expression could be also detected as exemplarily shown in Fig. 7. Additionally, immunohistochemistry was used to analyse DAPK protein expression in randomly selected RCC tissue sections. We found cytoplasmic as well as nuclear DAPK protein expression in the majority of clear cell RCC tumour cells. According to the ubiquitous expression pattern of DAPK, in adjacent non-neoplastic renal tissue, DAPK protein expression also became evident in tubular epithelial cells and in the glomerula (Fig. 8A and B). Together, this strongly suggests that the DAPK mRNA and protein expression observed in our study is originally mediated by tumour cells and not due to sample contamination by non-neoplastic cells.

3.6. *RCC cell lines reveal rare basal DAPK activity in vitro*

In addition to the described regulation of transcript levels by promoter hypermethylation and subsequent inhibition of mRNA expression, DAPK can be also regulated at protein-level.

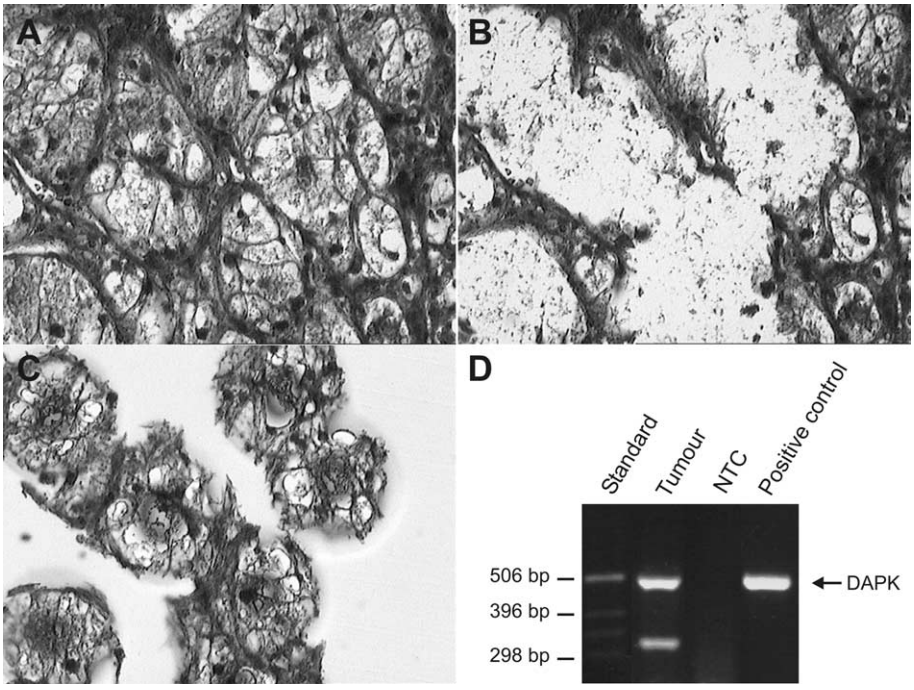

Fig. 7 – Death-associated protein kinase (DAPK) reverse transcriptase polymerase chain reaction (RT-PCR) analysis of renal cell carcinoma (RCC) cells after laser capture microdissection (LCM). Exemplary images of: (A) pre-captured and (B) post-captured tissue sections and (C) the captured RCC cells. (D) DAPK RT-PCR analysis of captured RCC cells reveals a specific 496 bp PCR fragment corresponding to nt 2187 to nt 2683 of DAPK mRNA (NCBI accession number X76104). A cDNA derived from RCC cell line clearCa-5, which clearly displays DAPK expression (Fig. 9) served as positive control and H_2O was used as negative control (NTC). Because of the primer binding sites, during PCR analysis a genomic DNA-based amplification product could be excluded. In this case product size would be greater than 20 kbp (NCBI accession number NC_000009).

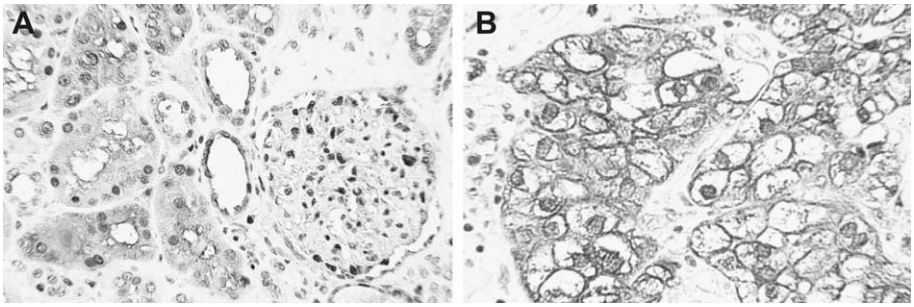

Fig. 8 – Immunohistochemical detection of death-associated protein kinase (DAPK) protein expression in non-neoplastic renal cortex and clear cell renal cell carcinoma (RCC). (A) Cytoplasmic and nuclear staining of DAPK protein in the majority of tubular epithelial cells and in cells of the glomerulum. (B) Clear cell RCC showing nuclear and cytoplasmic DAPK protein expression in the majority of tumour cells.

The next step was hence to investigate whether DAPK is silenced or activated in RCCs. Analysis of DAPK activity in various untreated RCC cell lines ($n = 11$) using in vitro kinase assays suggests that in RCC DAPK is largely inactive at basal level (Fig. 9A) although Western blot (Fig. 9B) and RT-PCR analysis (Fig. 9C) showed the expression of DAPK mRNA and protein in all tested RCC cell lines. Only in one cell line (clearCa 1) a marked activation of DAPK was noted (Fig. 9A). These observations, along with the results mentioned above, indicate that loss of DAPK expression in human RCCs does not appear to be a prerequisite during tumour progression. Furthermore, our observations suggest that post-translational inactivation of DAPK or compensatory events during tumour progression are responsible for silencing its cell death promoting activity.

4. Discussion

Death-associated protein kinase (DAPK) is a pro-apoptotic Ca^{2+}/calmodulin-dependent serine/threonine kinase that is widely expressed in normal tissues but epigenetically silenced by promoter hypermethylation in various types of cancer such as B-cell lymphomas, non-small cell lung cancer, head and neck cancer, thyroid lymphoma, advanced stage gastric cancer and adenocarcinomas of the upper gastrointestinal tract [19–24]. In the present study we analysed the in vivo expression of DAPK in 72 primary human RCCs of all major histological types using semi-quantitative real-time PCR, thereby extending previous reports on DAPK promoter methylation analyses in RCCs to real expression data [37]. We found that 91.7% of all tested RCCs irrespective of their histological type displayed DAPK mRNA expression and that selective tumours of the clear cell type and those of the chromophilic/papillary type also showed this expression pattern. Comparing early and intermediate stages with late tumour stages of clear cell RCCs, no significant changes in expression level of DAPK mRNA were obvious. Although the downregulation of DAPK seems to provide a selective growth advantage in tumours, our data revealed no correlation between DAPK expression and tumour progression in RCCs. In addition, for an example subset of tumour samples the maintained DAPK expression was also confirmed at protein level. Further investigation of 10 representative tumour samples and the corresponding non-neoplastic tissue samples by methylation-specific PCR (MSP) revealed that even in tumours with low

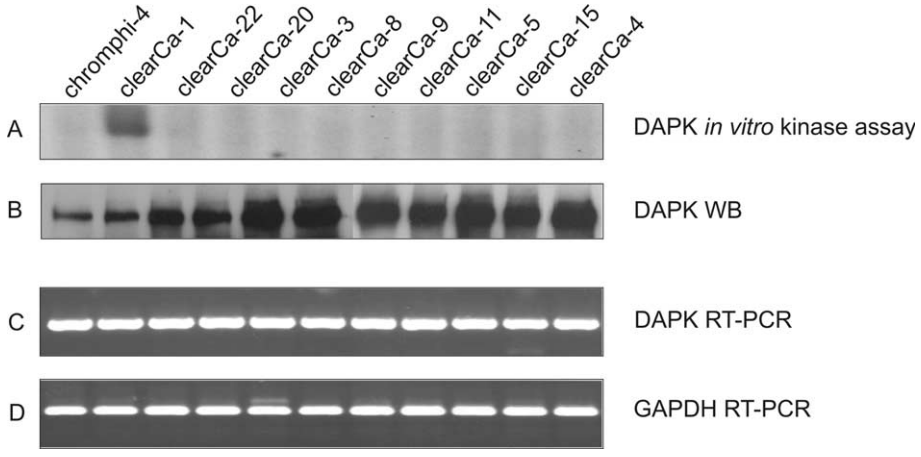

Fig. 9 – Detection of basal death-associated protein kinase (DAPK) activity in untreated renal cell carcinoma (RCC) cell lines ($n = 11$). Only one cell line (A) (clearCa-1) displayed in vitro DAPK activity at basal state, although (B) DAPK protein and (C) mRNA transcript are present in all cell lines. (D) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-RT-PCR was carried out as control. WB, Western blot; RT-PCR, reverse transcript polymerase chain reaction.

or absent DAPK mRNA expression no aberrant CpG-methylation was present. Although the amount of these samples is rather low and maybe too limited to entirely exclude methylation events occurring in the DAPK promoter of human RCCs, the sustained expression of DAPK in the vast majority of the analysed RCCs is concomitant with the observed absence of promoter hypermethylation in the respective samples. This indicates that RCCs may not downregulate DAPK expression by CpG-island-hypermethylation, supporting a general suggestion that kidney cancers display a more restricted hypermethylation pattern than other tumours, i.e. those originating from the gastrointestinal tract (oesophagus, stomach and colon) or lymphomas [38,39]. For the remaining 8.3% (6/72) of RCC tumour samples without DAPK expression the absence of hypermethylation, at least in the exemplarily confirmed samples (2/6), points to the existence of alternative mechanisms for DAPK downregulation, as has in part also been reported in lung and gastric cancer as well as in soft tissue leiomyosarcoma [40–42]. However, we cannot exclude the possibility that the hypermethylation of CpG islands not explored in our experimental setting is responsible for the silencing of expression in these samples; nevertheless our results suggest that, in general, loss of DAPK expression seems to be of minor importance in human RCCs. Moreover, data obtained by immunohistochemistry and RT-PCR analysis after LCM additionally confirm that the observed expression of DAPK is originally mediated by tumour cells, thereby excluding sample contamination by non-neoplastic cells as being responsible for the observed maintained expression of DAPK in the analysed RCCs.

At first sight, our results seem to be at variance with previous observations made by Morris and colleagues, who found that 24% of the primary RCCs they tested displayed aberrant DAPK promoter methylation, whereas tumours of the clear cell type were found to be less frequently methylated (19%) than those of the chromophilic/papillary type (43%). In addition, for Wilms' tumour, the most common childhood kidney tumour, they also detected a DAPK promoter methylation percentage of 11%. Nevertheless, they could not find any correlation between DAPK promoter methylation and clinicopathological parameters such as tumour stage, grade or age [37]. Possible heterogeneity of methylation due to different sample collectives or the relatively limited amount of samples tested by methylation-specific PCR in our study may be responsible for these differing results. In contrast, Harada and colleagues, who also examined the methylation status in Wilms' tumour, could not detect any promoter methylation, thus underpinning the results described here [43]. More importantly, recent studies suggest that methylation data obtained by methylation-specific PCR with the respective primers used by Morris and colleagues and in this study cannot strictly be linked to loss of expression [41,44,45]. A couple of reports have also suggested that complete gene silencing is dependent on the density and extent of methylation, which may vary according to the developmental stage of the tumour [46,47]. Therefore, for methylation to be biologically relevant, it should be confirmed by effective silencing of transcription.

The cell death promoting properties of DAPK are generally not mediated by the mere expression of the protein alone, but are also dependent on enzyme activity, which is negatively controlled by an autophosphorylation-based mechanism [18]. Therefore, we tested the basal DAPK activity in RCC derived cell lines by in vitro kinase assay. In general, DAPK was found to be inactive in the majority of RCC cell lines. Nevertheless, 1 out of 11 cell lines exhibited basal in vitro DAPK activity, indicating that even persistent DAPK activity may be tolerated in human RCCs, as the result of yet unknown compensatory events occurring during tumour progression. This points to a similar situation for DAPK in human RCC compared with that in other tumours, but instead of an epigenetically based silencing of DAPK, in human RCCs an activation-controlled mechanism seems to be sufficient for silencing the cell death promoting properties of DAPK.

In summary, this study has presented in vivo expression data of DAPK in a substantial number of primary RCCs. We have shown that RCCs, in contrast to other tumour types, may not downregulate DAPK expression by aberrant promoter hypermethylation during tumour progression and, in general, loss of DAPK expression seems to be a rare event in RCCs. Furthermore, for RCC cell lines it was shown that DAPK, although expressed, largely remains inactive at basal state in almost all RCCs. We therefore conclude that maintained DAPK expression in RCC is tolerated by post-translational inactivation or compensatory events during tumour progression, together indicating that loss of DAPK expression is not a universal prerequisite for tumour progression.

Conflict of interest statement

None declared.

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REFERENCES

1. Aravind L, Dixit VM, Koonin EV. The domains of death: evolution of the apoptosis machinery. *Trends Biochem Sci* 1999;**24**:47–53.
2. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell* 1997;**88**:347–54.
3. Rudin CM, Thompson CB. Apoptosis and disease: regulation and clinical relevance of programmed cell death. *Annu Rev Med* 1997;**48**:267–81.
4. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. *Oncogene* 2003;**22**:8581–9.
5. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003;**22**: 8590–607.
6. Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* 2003;**22**:8568–80.
7. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991;**351**:453–6.

8. Inbal B, Cohen O, Polak-Charcon S, et al. DAP kinase links the control of apoptosis to metastasis. *Nature* 1997;**390**:180–4.
9. Kimchi A. DAP genes: novel apoptotic genes isolated by a functional approach to gene cloning. *Biochim Biophys Acta* 1998;**1377**:F13–33.
10. Raveh T, Droguett G, Horwitz MS, et al. DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat Cell Biol* 2001;**3**:1–7.
11. Cohen O, Inbal B, Kissil JL, et al. DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. *J Cell Biol* 1999;**146**:141–8.
12. Deiss LP, Feinstein E, Berissi H, et al. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev* 1995;**9**:15–30.
13. Jang CW, Chen CH, Chen CC, et al. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 2002;**4**:51–8.
14. Inbal B, Bialik S, Sabanay I, et al. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 2002;**157**:455–68.
15. Bialik S, Bresnick AR, Kimchi A. DAP-kinase-mediated morphological changes are localization dependent and involve myosin-II phosphorylation. *Cell Death Differ* 2004;**11**:631–44.
16. Cohen O, Feinstein E, Kimchi A. DAP-kinase is a Ca2+/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J* 1997;**16**:998–1008.
17. Chen CH, Wang WJ, Kuo JC, et al. Bidirectional signals transduced by DAPK–ERK interactions promote the apoptotic effect of DAPK. *EMBO J* 2005;**24**:294–304.
18. Shohat G, Spivak-Kroizman T, Cohen O, et al. The pro-apoptotic function of death-associated protein kinase is controlled by a unique inhibitory autophosphorylation-based mechanism. *J Biol Chem* 2001;**276**:47460–7.
19. Kim DH, Nelson HH, Wiencke JK, et al. Promoter methylation of DAP-kinase: association with advanced stage in non-small cell lung cancer. *Oncogene* 2001;**20**:1765–70.
20. Nakatsuka S, Takakuwa T, Tomita Y, et al. Role of hypermethylation of DAP-kinase CpG island in the development of thyroid lymphoma. *Lab Invest* 2000;**80**:1651–5.
21. Sanchez-Cespedes M, Esteller M, Wu L, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 2000;**60**:892–5.
22. Kissil JL, Feinstein E, Cohen O, et al. DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. *Oncogene* 1997;**15**:403–7.
23. Chan AW, Chan MW, Lee TL, et al. Promoter hypermethylation of death-associated protein-kinase gene associated with advance stage gastric cancer. *Oncol Rep* 2005;**13**:937–41.
24. Schildhaus HU, Krockel I, Lippert H, et al. Promoter hypermethylation of p16INK4a, E-cadherin, O6-MGMT, DAPK and FHIT in adenocarcinomas of the esophagus, esophagogastric junction an proximal stomach. *Int J Oncol* 2005;**26**:1493–500.
25. Tang X, Wu W, Sun SY, et al. Hypermethylation of the death-associated protein kinase promoter attenuates the sensitivity to TRAIL-induced apoptosis in human non-small cell lung cancer cells. *Mol Cancer Res* 2004;**12**:685–91.
26. Thoenes W, Storkel S, Rumpelt HJ. Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. *Pathol Res Pract* 1986;**181**:125–43.
27. Mostofi FK, Davis CJ. *Histological typing of kidney tumors. WHO international classification of tumors*. 2nd ed. New York: Springer-Verlag; 1998.
28. Sobin LH, Wittekind CH. *TNM classification of malignant tumors*. 5th ed. New York: Wiley Liss; 1997.
29. Gerharz CD, Ramp U, Olert J, et al. Cytomorphological, cytogenetic, and molecular biological characterization of four new human renal carcinoma cell lines of the clear cell type. *Virchows Arch* 1994;**424**:403–9.
30. Gerharz CD, Moll R, Storkel S, et al. Establishment and characterization of two divergent cell lines derived from a human chromophobe renal cell carcinoma. *Am J Pathol* 1995;**146**:953–62.
31. Gerharz CD, Hildebrandt B, Moll R, et al. Chromophilic renal cell carcinoma: cytomorphological and cytogenetic characterisation of four permanent cell lines. *Brit J Cancer* 1996;**74**:1605–14.
32. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156–9.
33. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;**93**:9821–6.
34. Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* 1999;**93**:4347–53.
35. Mahotka C, Krieg T, Krieg A, et al. Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. *Int J Cancer* 2002;**100**:30–6.
36. Ramp U, Dejosez M, Mahotka C, et al. Deficient activation of CD95 (APO-1/Fas)-mediated apoptosis: a potential factor of multidrug resistance in human renal cell carcinoma. *Brit J Cancer* 2000;**82**:1851–9.
37. Morris MR, Hesson LB, Wagner KJ, et al. Multigene methylation analysis of Wilms' tumour and adult renal cell carcinoma. *Oncogene* 2003;**22**:6794–801.
38. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002;**21**:5427–40.
39. Meyer AJ, Hernandez A, Florl AR, et al. Novel mutations of the von hippel-lindau tumor-suppressor gene and rare DNA hypermethylation in renal-cell carcinoma cell lines of the clear-cell type. *Int J Cancer* 2000;**87**:650–3.
40. Satoh A, Toyota M, Itoh F, et al. DNA methylation and histone deacetylation associated with silencing DAP kinase gene expression in colorectal and gastric cancers. *Brit J Cancer* 2002;**86**:1817–23.
41. Toyooka S, Toyooka KO, Miyajima K, et al. Epigenetic downregulation of death-associated protein kinase in lung cancers. *Clin Cancer Res* 2003;**9**:3034–41.
42. Tamiya S, Iwamoto Y, Tsuneyoshi M. Death-associated protein kinase (DAP kinase) alteration in soft tissue leiomyosarcoma: promoter methylation or homozygous deletion is associated with loss of DAP kinase expression. *Hum Pathol* 2004;**35**:1266–71.
43. Harada K, Toyooka S, Maitra A, et al. Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. *Oncogene* 2002;**21**:4345–9.
44. Simpson DJ, Clayton RN, Farrell WE. Preferential loss of death associated protein kinase expression in invasive pituitary tumours is associated with either CpG island methylation or homozygous deletion. *Oncogene* 2002;**21**:1217–24.
45. Brankensiek K, Langer F, Kreipe H, et al. Low level of DAP-kinase DNA methylation in myelodysplastic syndrome. *Blood* 2004;**104**:1586–7.
46. Baylin SB, Herman JG, Graff JR, et al. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998;**72**:141–96.
47. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* 1997;**18**:869–82.