Oncogene (1997) 15, 403–407
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DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene

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DAP-kinase is a novel calmodulin dependent serine/threonine kinase that carries ankyrin repeats and the death domain. It was recently isolated, by a functional selection approach of gene cloning, as a positive mediator of programmed cell death. In this study the expression of DAP-kinase was examined in the cell lines derived from various human neoplasms. DAP-kinase mRNA and protein expression were below the limit of detection in eight out of ten neoplastic derived B-cell lines. In six out of 14 examined bladder carcinoma, in three out of five renal cell carcinoma, and in four out of ten tested breast carcinoma cell lines, the DAP-kinase protein levels were below detection limits or lower than 1% compared to the positive cell lines. Interestingly, DAP-kinase expression could be restored in some of the negative bladder carcinoma and B-cell lines by treatment of cells with 5′-azadeoxycytidine that causes DNA demethylation. The high frequency of loss of DAP-kinase expression in human tumor cell lines, and the occasional involvement of methylation in this process raise the possibility that this novel mediator of cell death may function as a tumor suppressor gene.

Keywords: serine/threonine kinase; death gene; tumor suppressor; methylation; 5′-azadeoxycytidine

Introduction

The active process of cell death is fundamental to the normal development, and maintenance of the multicellular organism. An increasing number of cell-death genes are implicated in various diseases. An abnormal pattern of cell death, caused by gain or loss of function mutations of death genes, is involved in different pathological conditions such as neurodegenerative disorders, lymphoproliferative diseases, and cancer.

Several lines of evidence indicate that the suppression of cell death is one of the critical steps in the multi-stage process of tumorigenesis (reviewed by Martin *et al*., 1994). The two most profound examples of death controlling genes involved in cancer are Bcl-2 and p53. The Bcl-2 gene, initially isolated from the breakpoint at the 14:18 chromosomal translocation in human follicular lymphomas (Tsujimoto *et al*., 1985), promotes cell survival. The overexpression of this gene was found to suppress cell death in response to a wide variety of cytotoxic insults (reviewed by Reed, 1994). In contrast to Bcl-2, the p53 protein is a positive mediator of apoptosis, and therefore its loss or inactivation often leads to the suppression of cell death. p53 was found to mediate cell death that is triggered by genotoxic damage (Lowe, 1993; Clarke *et al*., 1993). To date, p53 is the most commonly mutated tumor suppressor gene to be identified in various human tumors (reviewed by Haffner and Oren, 1995). Loss of function point mutations or deletions of the p53 gene, represent mechanisms by which tumors loose the functional death-inducing p53.

Recently we have reported that by using a functional approach of gene cloning which was developed in our laboratory (Deiss and Kimchi, 1991), we were able to isolate a number of novel genes that function as positive mediators of apoptosis (Deiss *et al*., 1995; Kissil *et al*., 1995). The genes were rescued by virtue of the fact that their undisrupted expression was necessary for the transduction of the apoptotic signals, and therefore their specific inactivation protected cells from programmed cell death. They were named DAP (Death Associated Protein) genes. Since positive mediators of cell death may be candidate tumor suppressor genes, as previously discussed for p53, we were interested in studying whether the DAP genes are prone to deletions, rearrangements, loss of function mutations or epigenetic changes that may abrogate their expression in tumors. To assess this possibility we set out to define, in the first stage, whether the expression of these genes was altered in any specific set of tumors. One of the genes we concentrated our efforts on was the DAP-kinase (DAPk) gene. The protein product of this gene is a 160 kDa calcium/calmodulin dependent serine/threonine kinase with a unique domain composition: an N-terminal typical serine/threonine kinase domain followed by a calmodulin binding domain, eight ankyrin repeats, two P-loop elements, several potential phosphorylation sites, a cytoskeleton binding region and a death domain (Deiss *et al*., 1995; Feinstein *et al*., 1995b; Cohen *et al*., 1997). DAP-kinase gene was mapped to chromosome 9q34.1 (Feinstein *et al*., 1995a), a locus that is frequently rearranged in human malignancies. Examples of 9q34.1 aberrations are found in various leukemias (Ellisen *et al*., 1991; de Klein *et al*., 1982; von Lindern *et al*., 1992) and loss of heterozygosity (LOH) at 9q34 is observed in the majority of bladder carcinomas (Knowles and Currie, 1993; Knowles *et al*., 1994; Tsai *et al*., 1990). In light of these data it became of interest to follow the pattern of DAP-kinase expression in cell lines established from various types of

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Received 30 September 1996; revised 24 March 1997; accepted 25 March 1997

carcinomas and leukemias. We report here that a large fraction of cell lines established from B cell lymphomas and leukemias, bladder carcinomas, renal cell carcinomas, and breast carcinomas lack the DAPk expression both at the mRNA and protein levels. In two bladder carcinoma cell lines and in one out of five tested B-cell neoplastic-derived cell lines, the expression of DAPk could be restored by treatment with 5′-azadeoxycytidine, suggesting that in these cases the loss of expression was due to epigenetic regulation, namely methylation, as previously reported for other tumor suppressor genes (Herman *et al*., 1994).

Results

When analysing the expression of the DAPk mRNA transcript in various cell lines it was found that it was not expressed in a substantial proportion of cell lines derived from human B-cell neoplasms. Ten different cell lines, representing different stages of B-cell maturation, were examined. Eight of them-SKW, 697, Daudi, RS4:11, MV4:11, SK-DHL, Raji and B380-failed to express detectable levels of the DAPk mRNA. In two other B-cell lines: pre-B ALL-1 cells (Erikson *et al*., 1986) and early progenitor B-1 cells (Erikson *et al*., 1986; Cohen *et al*., 1991), as well as in the EBV-immortalized B-cell lines established from normal peripheral B cells, GM1500 and GM607, the DAPk mRNA was present (Figure 1a and Table 1). DAPk remained below detection limits also when higher amounts of poly $(A)^+$ RNA (15 μg) from DAPk-negative cell lines were analysed (not shown).

Interestingly, the c-Abl mRNA transcripts were expressed in all the examined cell lines. The c-Abl gene is the closest known marker to DAPk (Feinstein *et al*., 1995a). The expression of c-Abl appeared to be normal and the two expected mRNA transcripts were observed in all the cell lines irrespective of whether they expressed DAPk mRNA (Figure 1b). The undisturbed pattern of c-Abl expression minimized the possibility that lack of DAPk expression is a consequence of gross rearrangements or deletions at 9q34.

We next analysed the expression of DAPk at the protein level. Cell lysates prepared from the various cell lines were examined for the presence of the DAPk protein by immunoblotting with anti-DAPk antibodies. It was found that in all the cell lines that did express DAPk mRNA transcript, the protein product with the expected size of 160 kDa, was detectable. In contrast, in cell lines in which DAPk transcript was not detected, there was no DAPk protein either (Figure

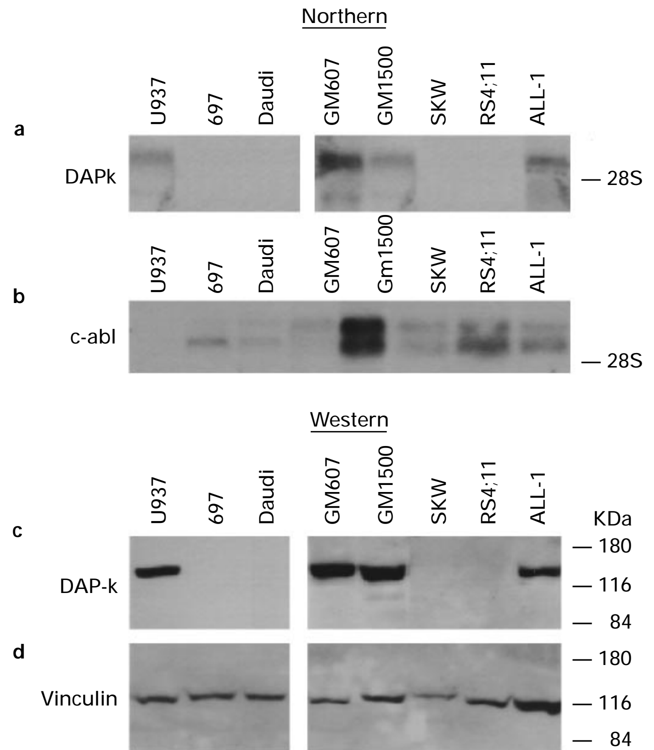

Figure 1 Analysis of DAP-kinase expression in various haematopoietic cell lines: (**a**) and (**b**) Northern blot analysis of poly$(A)^+$ RNA from various cell lines using probes for DAP-kinase and c-Abl, respectively. (**c** and **d**) Western blot analysis of DAP-kinase protein and vinculin (as an unrelated protein reference), respectively

Table 1 Expression of DAPk protein in various cell lines

Cell line origin	*DAP-K expression Positive*	*DAP-K expression Negative*	*DAP-K expression* $<1\%^a$
Normal B-cells (2)	(2) GM607, GM1500	(–)	(–)
B-cell neoplasms (10)	(2) ALL-1, B1	(8) Daudi, 697, RS4:11, B380, MV4:11, Raji SKW, SK-DHL	(–)
Bladder carcinomas (14)	(8) RT4, RT112, JO'N, 5637, HT1197, 253J, J82, SW1710	(4) SCa-BER, T24 609CR, HT1376	(2) UM-UC-3, VM-CUB-2
Breast carcinomas (10)	(6) MDA-MB-361, MDA-MB-175, SK-BR3, BT-549, ZR-75-30, MDA-MB-468	(3) T47D, MCF-7, MDA-MB-453	(1) ZR-75-1
Renal cell carcinomas (5)	(2) KTCM2, KTCM84	(2) KTCM54, KTCM60	(1) KTCM30

The data is grouped according to the cell line origin, and the expression pattern of DAP-kinase. [a] Determined by comparison of signal intensity on Western blot to signal levels of the DAP-kinase in the corresponding positive carcinoma cell lines

1c). The same immunoblots were also reacted with anti-vinculin antibodies as an internal control (Figure 1d). Together, the RNA and protein data indicate that the absence of DAPk expression is a genuine phenomenon and not an artifact of the assays.

The expression of DAPk protein was examined next in various cell lines of bladder, renal and breast carcinoma origin. This was done by Western blot analysis of protein extracts prepared from several different cell lines. Of 14 bladder carcinoma cell lines examined, eight cell lines expressed DAPk, four showed no detectable DAPk protein expression, and two expressed it at levels lower than 1% in comparison to the DAPk positive lines. Out of five tested renal cell carcinoma cell lines, DAPk was not expressed in two cell lines and in one cell line it was expressed at levels lower than 1% compared to DAPk positive cell lines. Three out of ten tested breast carcinoma cell lines were negative for DAPk protein expression and one cell line expressed levels lower than 1% (Table 1). These results indicate that complete loss, or a very significant reduction in the expression levels of DAPk also occur at a statistically significant frequency in bladder, renal and breast carcinoma cell lines.

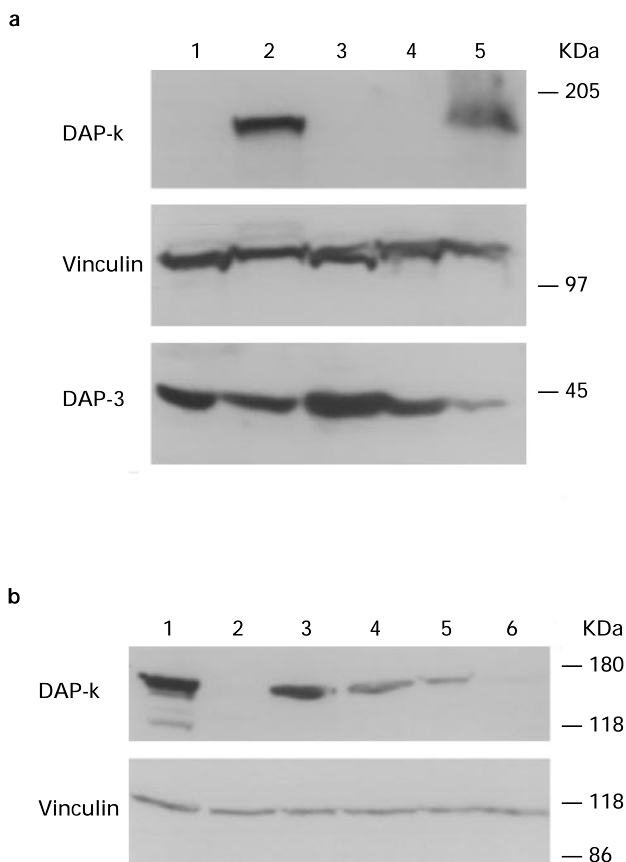

Figure 2 Western blot analysis of DAP-kinase expression in bladder carcinoma cell lines T24 and HT1376 and B-cell line SKW treated with 5′-azadeoxycytidine. (**a**) Protein extracts were loaded as follows: Lane 1 and 4- non treated T24 and HT1376 bladder carcinoma cells, respectively; lanes 2 and 5- T24 and HT1376 bladder carcinoma cells, treated with 5′-azadeoxycytidine and collected two passages after removal of the drug, respectively. Lane 3- T24 bladder carcinoma cells collected six passages after drug removal. The same blots were reacted with anti-vinculin and anti-DAP3 antibodies. (**b**) Lane 1- GM1500 cells used as a positive control, lane 2- untreated SKW cells, lanes 3 through 6- SKW cells treated with 0.5 μM 5′-azadeoxycytidine and collected after 1, 2, 4 and 6 passages, respectively. The same blot was reacted with anti-vinculin antibody as internal control

Two DAPk negative bladder carcinoma cell lines (T24 and HT1376), and five DAPk negative B-cell lines (SKW, MV4;11, RS4;11, 697 and Daudi), were treated with 5′-azadeoxycytidine in order to test whether the absence of DAPk expression was due to possible changes in the pattern of DNA methylation. Treatment of cells with 5′-azadeoxycytidine causes the removal of methyl groups from CpG dinucleotides and thus may reverse the hypermethylation dependent promoter shut-off/silencing, and restore expression of the corresponding gene(s) (Jones, 1985). Cells were treated with the drug for 24 h, washed, and protein extracts were prepared at early and late passages thereafter. The expression of DAPk was analysed by reacting the immunoblots with anti-DAPk antibodies. In the case of the two bladder carcinoma cell lines, it was found that while the DAPk was undetectable before treatment (Figure 2a, lanes 1 and 4), the addition of 5′-azadeoxycytidine to the growth medium restored DAPk expression and strong signals at the expected protein size were evident on western blots early after the drug treatment (Figure 2a, lanes 2 and 5). Among the five B-cell lines, in one- SKW, we found that drug treatment restored the expression of DAPk (Figure 2b, compare lane 2 to lane 3). The restored levels of DAPk expression in the drug treated SKW cells were similar to the average expression levels of DAPk in the DAPk positive cell lines (Figure 2b, compare lane 3 to 1). The other four B-cell lines tested remained null for DAPk after treatment with 5′-azadeoxycytidine at a wide range of concentrations (not shown). The expression of two other proteins, which unlike DAPk were initially present in these cell lines: vinculin, and DAP-3, was not influenced at all by the 5′-azadeoxycytidine treatment (Figure 2a, and b and data not shown). The effect of 5′-azadeoxycytidine on DAPk expression was transient, as can be seen by the fact that late after drug the treatment (after six passages), the expression of DAPk in T24 cells was again completely abolished, probably due to *de novo* methylation (Figure 2a, lane 3). The same was true in the case of the SKW cells, which displayed a gradual decline of the DAPk protein levels as a function of time from drug withdrawal (Figure 2b, lanes 3 through 6).

Discussion

In this work we have demonstrated that DAPk expression levels were below detection limits in eight out of ten leukemic cell lines of B-cell origin (80%), in three out of ten breast carcinoma cell lines (30%), in four out of 14 bladder carcinoma cell lines (29%), and in two out of five renal carcinoma cell lines (40%). In addition, among these groups extremely low levels (<1%) of DAPk protein were detected in two bladder carcinoma cell lines, one breast carcinoma cell line and one renal carcinoma cell line.

Frequent loss of gene expression in tumor derived cell lines has been shown to be a good predictive criterion for a tumor suppressive activity in the case of another candidate, the p16 inhibitor of cyclin-dependent kinases (Tulchinsky *et al*., 1995; Skolnick

et al., 1994). Moreover, as DAPk is a positive mediator of cell death (Deiss *et al*., 1995), its inactivation by loss of expression, would be beneficial for the neoplastic cell in a restrictive environment, as discussed before for p53. It should be emphasized that our screen was so far limited to cell lines, and thus there exists the possibility that the inactivation of DAPk does not represent a step in the multistage process of tumorigenesis, but is rather beneficial for the establishment of the immortalized cell line *in vitro*. Yet, DAPk was expressed in several other immortalized cell lines which originated from normal human cells including two B-cell lines that were established from normal peripheral B-cells (cell lines GM1500 and GM607 in Figure 1), and immortalized cell lines established from normal fibroblasts or epithelial cells such as SV80, 293 and HaCaT (not shown). Moreover, we have recently found that whereas the restoration of DAPk expression in a DAPk negative mouse Lewis lung carcinoma cell line increased the sensitivity of these cells to apoptotic stimuli, and as a consequence, greatly reduced their tumorigenicity *in vivo*, it had no effect on the continuous cell growth in culture (Inbal *et al*., submitted). This provides a direct link between the loss of DAPk, suppression of apoptosis, and the tumorigenic process on one hand, and minimizes the possibility that loss of DAPk expression was selected during the establishment of cell lines- on the other.

A second important finding that emerges from this work relates to the restoration of DAPk expression by demethylation in one out of five tested B-cell lines and in the two tested negative bladder carcinoma cell lines. The fact that loss of expression could be reversed by treatment with 5′-azadeoxycytidine indicates that DAPk gene was structurally intact, and that reversible epigenetic changes were involved in the selective shut-off of expression. This is consistent with our previous studies which showed that one of the bladder carcinoma cell lines used here, the T24 (EJ) cell line, has normal 9q status (Williamson *et al*., 1995). The treatment with 5′-azadeoxycytidine causes the removal of methyl groups from cytosine, thus suggesting that the lack of DAPk expression may involve methylation of CpG regions, probably in crucial DNA elements that influence the expression of the DAPk gene. Without direct assessment of the methylation pattern of the gene, one cannot distinguish at this stage between direct effects on cis-elements of the actual DAPk promoter region or other regulatory sequences, and indirect effects on the promoter(s) of some other gene(s) whose product(s) may effect DAPk expression in trans. The mechanism of transcription silencing by hypermethylation is well documented in the process of tumorigenesis and was shown for other tumor suppressor genes including p16, VHL and RB (Herman *et al*., 1994; Sheaff and Roberts, 1995; Sakai *et al*., 1991; Gonzalez *et al*., 1995). Thus, the discovery of DAPk hypermethylation-dependent shut-off further supports the possibility that this gene may be a novel tumor suppressor. Moreover, this work also demonstrates that methylation is not an exclusive mechanism responsible for loss of DAP-kinase expression, since DAPk protein levels were not restored in four 5′-azadeoxycytidine-treated B-cell lines. As in the cases of p16 and RB, other mechanisms of DAPk inactivation may also exist in tumors, such as mutations or deletions within the gene. These alternative mechanisms of DAPk inactivation are probably not limited specifically to the B-cell lines, and additional studies are required to determine the mode of DAPk shut-off in other types of cell lines.

Altogether, the loss of DAPk expression in the four studied sets of tumor derived cell lines that were analysed in this work, and the examples where expression can be restored by demethylation, open the possibility that this gene may function as a tumor suppressor whose loss or inactivation is a step in the multistage process of tumorigenesis.

Materials and methods

Cell lines and growth conditions

All haematopoietic cell lines (see ATCC for description of various lines) and renal carcinoma cell lines (see Simcha *et al*., 1996 for details) were grown in RPMI 1640 supplemented with 10% complement-inactivated FCS (Gibco-BRL), 100 IU/ml penicillin and streptomycin, and 2 mM L-glutamine, at 37°C and 5% CO_2. For the bladder and breast carcinoma derived cell lines (see ATCC for description of various lines), DMEM was used with 10% and 15% FCS, respectively.

Treatment with 5′-azadeoxycytidine

The bladder carcinoma cell lines were plated at $1E^5$ cells/100 mm dish, and treated 24 h later with 5′-azadeoxycytidine (Sigma Chemical Co., St. Louis, MO.) at final concentration of 1 μM. The haematopoietic cells were seeded at $1E^5$ cells/ml. The optimal concentration of 5′-azadeoxycytidine to be added was determined empirically for each cell line (ranging from $1E^{-7}$ M to 1 μM). The drug was removed by medium exchange 24 h after its addition and the medium was changed every 3 days thereafter. Cells were split 1:10 at stationary phase.

Northern blot analysis

Total RNA was extracted from the various cell lines using Trireagent (MRC). Samples of 3 μg poly(A)$^+$ RNA, prepared with oligo-dT Dynabeads (Dynal) as described by manufacturer, were separated on 1% formaldehyde-agarose gels, and blotted to Hybond-N nylon membranes (Amersham), as described (Sambrook, 1989). DNA probes were prepared using [α-^{32}P]dCTP with commercially available random priming kits (Boehringer Mannheim). Prehybridization, hybridization and washing of filters were performed as described (Sambrook, 1989).

Immunoblot analysis

Cells were harvested and protein extracts were prepared as previously described (Deiss *et al*., 1995). The protein extracts (400 μg/lane) were fractionated on 7.5% SDS–PAGE. The proteins were transferred to nitrocellulose filters (Schleicher and Schuell) with a semi-dry semi-phor blotter (Hoefer Scientific Instruments). The mouse anti-human DAP-kinase monoclonal antibodies were from (Sigma, Israel). Anti-human vinculin antibodies were from Sigma. Anti-human DAP3 were prepared as previously described (Kissil *et al*., 1995). Immunodetection was carried out using the ECL detection system (Amersham).

Acknowledgements
We thank Dr E Canaani for providing the B cell lines, Dr A Ben-Zeev for renal carcinoma cell lines and Z Dor for technical assistance. This work was supported by The Israel Science Foundation administered by The Israel Academy of Sciences and Humanities, the Cooperation Program in Cancer Research of the Deutsches Krebsforschungszentrum (DKFZ) and Israel's Ministry of Science (MOS), and by grant CA49758 from the National Cancer Institute. AK is an Incumbent of Helena Rubinstein Chair of Cancer Research. EF is a special fellow of Leukemia Society of America, and a fellow of the Israel Cancer Research Fund.

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