DAPK1 promoter hypermethylaiton in brain metastases and peripheral blood

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The *DAPK1* gene works as a regulator of apoptosis and is frequently inactivated in cancer by aberrant promoter hypermethylation. Loss of *DAPK1* expression is associated with a selective advantage for tumor cells to resist apoptotic stimuli, allowing them to separate from the original tumor; from this point of view, *DAPK1* could be considered a tumor metastases inhibitor gene. To verify the participation of *DAPK1* silencing in cerebral invasion, we analyzed its promoter methylation status in a series of 28 samples from cerebral metastases using MSP and sequencing of the MSP-product. We have found hypermethylation in 53.6% (15/28) metastatic tumor samples as well as in 27.8% (5/18) of its peripheral blood samples. Our data suggest an important role of *DAPK1* for silencing through promoter CpG island hypermethylation in the development of brain metastases from solid tumors. The detection of aberrant hypermethylation on *DAPK1* promoter from peripheral blood samples has potential clinical implications as a tumor prognosis marker.

Key words: DAPK1, Brain Metastases, Promoter Hypermethylation.

Metastatic brain tumors play a significant role in clinical practice. Most are carcinomas, counting lung (50%), breast (15–20%) and melanoma (10%) as the major primary lesions. Less common primary tumors include colorectal cancer, lymphoma and renal clear cell carcinoma [1]. The biological basis of the preferential spread of certain neoplasms to the nervous system remains to be elucidated. Probably, the metastatic development involves both epigenetic and classic genetic mechanisms. Frequent genetic alterations found consist of gains in chromosomic regions 1q23, 7p12, 8q24, 17q24-q25 and 20q13, and losses in regions 4q, 5q, 9p21, 17p12, 10q23-q24 and 18q21-q22 [2]. At the molecular level, expansions are regulated by developmentally nonessential genes physiologically involved in stress responses, inflammation, wound healing, and neovascularization [3]. Overexpression of *CD44R1*, *S100A4* (*p9ka*) and *c-erbB2* genes and loss of expression of *BAI1*, *DCC* and *MYO18B* has been related to tumor progression and increased metastatic potential [3].

In addition to the genes mentioned above, loss of expression of Death-associated protein kinase (*DAPK1*) gene has been directly related to suppression of apoptosis and metastases development. *DAPK1* codifies a calcium/calmodulin (Ca2+/CaM) serine/threonine kinase which positively mediates programmed cell death through the induction by interferon γ in a variety of cell systems. It has been suggested that loss of *DAPK1* expression could confer tumor cells a selective advantage to resist apoptotic stimuli and favours migration from the primary tumor. Thus, *DAPK1* can be considered as a metastatic suppressor gene [7–10].

Aberrant methylation of promoter CpG islands is known to be a major inactivation mechanism of tumor-related genes during tumorigenesis [11,12]. Numerous reports have shown that promoter CpG island hypermethylation contributes to *DAPK1* silencing in several human cancers [13–16]. A report described the relationship between *DAPK1* promoter hypermethylation and aggressiveness in stage I non-small-cell lung cancer [13].

We have previously found high rates of promoter hypermethylation for 10 tumor-related genes including *DAPK1*

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in a series of metastatic tumors [17]. To determine the potential participation of *DAPK1* in silencing brain invasion, we analyzed promoter methylation status in a larger series derived from brain metastases of solid tumors, as well as in samples of non-neoplastic brain tissue by methylation-specific PCR (MSP).

Materials and Methods

Fresh tumor tissue samples were obtained from 28 patients with metastatic brain tumors. The tumor cellular content represented about 85–95% of each sample. Pathological diagnosis was performed according to the WHO classification [18] and included metastatic tissue from 7 malignant melanomas, 8 lung carcinomas, 3 breast carcinomas, 4 miosarcomas, 2 ovarian carcinomas, one case each from colon, kidney and bladder carcinomas and 1 undifferentiated tumor. Peripheral blood was available from 18 patients. In addition, two samples from non-neoplastic brain obtained by autopsy and peripheral blood of healthy volunteers were studied as control. DNA was obtained from frozen tissues and blood using standard methods [19].

Bisulfite modification of genomic DNA was performed as reported [20]. Methylation Specific PCR was carried out for the methylated and unmethylated alleles in standard conditions with variable (55–66 °C) annealing temperatures. We used the primer sets described previously [21]. 10 µl of each PCR reaction was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV illumination. To verify the identity of PCR products, they were purified and sequenced bidirectionally (after PCR re-amplification with the same primer set) using the ABI P Big-Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on the ABI 3100 Avant, Applied Biosystems DNA sequencer.

Results

We analysed *DAPK1* promoter CpG island methylation status in 28 metastatic tumor samples and correspondent peripheral blood DNA samples available, as well as two samples from non-neoplastic brain and peripheral blood of healthy volunteers by Methylation Specific PCR and sequencing. None of the control brain and blood samples showed MSP product using *DAPK1* primers for methylated sequence. Hypermethylation of *DAPK1* promoter region was found in 15 (53.6%) metastases samples of which 5/8 were from primary lung carcinoma, 3/7 malignant melanoma, 2/4 miosarcoma, 2/3 breast carcinoma, 1/2 ovarian carcinoma, 1/1 colon carcinoma and 1/1 undifferentiated tumour (Table 1). All tumor samples showed MSP product using primers for unmethylated sequence. Positive samples displayed methylation of all CpG sites analysed by sequencing. Peripheral blood DNA samples available from 18 cases showed promoter hypermethylation in 5 (27.8%) cases: 1/6 lung carcinoma, 2/3 malignant melanoma, 1/3 breast carcinoma and 1/1 undifferentiated tumour. In the group of metastasis samples for which we also had peripheral blood DNA, 13/18 (72.2%) showed promoter hypermethylation and of those 5/13 (38.5%) also showed promoter hypermethylation in their peripheral blood DNA. None of the peripheral bloods corresponding to *DAPK1* promoter unmethylated metastases showed promoter hypermethylation for this gene. The hypermethylated promoter status for each MSP positive sample was confirmed by sequencing of a nested MSP amplification as shown in figure 1.

Table 1. Summary of methylation of DAP-kinase 1 in 28 brain metastases and their correspondent peripheral bloods.

Primary Tumour	Tumour	Peripheral Blood
	M / n	M / n
Lung carcinoma	5 / 8	1 / 6
Malignant melanoma	3 / 7	2 / 3
Miosarcoma	2 / 4	– / –
Breast carcinoma	2 / 3	1 / 3
Ovarian carcinoma	1 / 2	– / 2
Colon carcinoma	1 / 1	– / 1
Kidney carcinoma	– / 1	– / 1
Bladder carcinoma	– / 1	– / 1
Undifferentiated tumour	1 / 1	1 / 1
Total	15/28 (53,6%)	5/18 (27,8%)

M: Number of methylated samples; n: Samples analyzed.

Discussion

The described association between *DAPK1* loss of expression and metastases spreading together with the promoter hypermethylation found in our data suggest a potential role of *DAPK1* silencing through promoter CpG island hypermethylation in the development of brain metastases from solid tumors. This discovery is of special interest considering the relation between *DAPK1* silencing by promoter hypermethylation and aggressiveness in lung carcinoma [13], and the fact that 50% of metastatic brain tumors originate from primary lung lesions.

As previously documented for *DAPK1* in other neoplasms, the unmethylated sequence for this gene was detected in all the tumor samples, which could suggest the existence of unmethylated alleles or the presence of small amounts of non-tumor DNA (5–15% of the sample) [13,15]. In one of those tumor series, however, loss or reduced *DAPK1* gene expression was determined [13].

The detection of aberrant promoter hypermethylation for this gene in peripheral blood DNA samples from patients and not from healthy control volunteers might have potential clinical application as a prognostic tumor marker for cancer cells, since this could denote that there are tumour cells of these patients in their circulatory system. Tumor DNA in peripheral blood has been reported in several neoplasms as an important stage for metastases. However, current data suggest that *DAPK1* may be slightly methylated (ranging from

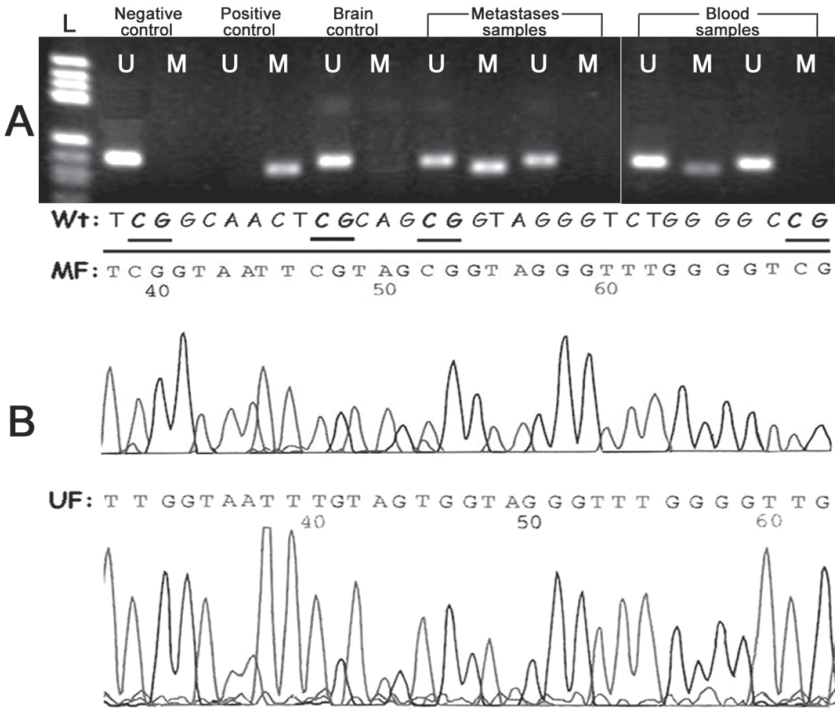

Figure 1. (A) MSP Methylation analysis of *DAPK1* gene. U: amplified product (106bp) with primers recognising unmethylated sequence; M: amplified product (98bp) with primers recognising methylated sequence. L: ladder. Positive control: In vitro methylated DNA from blood lymphocytes. Negative control: Untreated DNA. Brain control: DNA from non-malignant brain tissue. (B) Forward sequence of the methylated (MF) and unmethylated (UF) MSP products of *DAP-kinase* aligned against the wild-type (Wt) sequence, showing the change of C to T under bisulphite treatment. C in CpG islands that were methylated was unaffected.

0.003 to 1.181% by Q-MSP) in approximately 52% of normal or activated subpopulation of healthy B cells, which would act as a confounding factor in tumor detection techniques that use *DAPK1* hypermethylation as a tumor marker [22]. Measurement of aberrant promoter hypermethylation in serum or plasma, as it has been made already for *DAPK1* in head and neck cancer patients [23], appears more effective than in peripheral blood DNA. However, the data contributed here show an important role of *DAPK1* promoter hypermethylation in the development of cerebral metastasis. Further analyses are necessary to verify the effectiveness of this methodology for *DAPK1* in clinical practice.

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