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Loss of *caspase-8* mRNA expression is common in childhood primitive neuroectodermal brain tumour/medulloblastoma

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Abstract

Upon binding of tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), the agonistic TRAIL receptors DR4 and DR5 activate caspase-8 leading to apoptosis. In primitive neuroectodermal brain tumour (PNET) cell lines, TRAIL-induced apoptosis was recently shown to correlate with *caspase-8* mRNA expression (Grotzer MA, Eggert A, Zuzak TJ, et al. *Oncogene* 2000, **19**, 4604–4610). In this study, we analysed the expression of the TRAIL death pathway in 27 primary PNET/medulloblastoma. As shown by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR), all PNET/medulloblastoma evaluated expressed *DR5*, the adapter protein *FADD* and *caspase-3*, but only 48% expressed *caspase-8*. The mRNA expression of *caspase-8* was significantly lower in primary PNET/medulloblastoma compared with normal brain samples. PCR revealed >75% methylation of the *caspase-8* promoter region in three of seven PNET cell lines and in 55% of the primary PNET/medulloblastoma evaluated. In the PNET cell lines, the methylation status correlated with the *caspase-8* mRNA expression. We conclude that loss of *caspase-8* gene expression is common in PNET/medulloblastoma suggesting that suppression of death receptor induced apoptosis may play an important role in the pathogenesis of this common childhood brain tumour. © 2002 Published by Elsevier Science Ltd.

Keywords: Brain tumour; Primitive neuroectodermal tumour; Medulloblastoma; Pathogenesis; Apoptosis; TRAIL (Apo2L); Caspase-8

1. Introduction

Primitive neuroectodermal tumours (PNET) of the cerebellum, also termed medulloblastomas, and supratentorial PNET constitute more than 20% of all paediatric brain tumours, and are the most common malignant brain tumours in children [1]. PNET are characterised by their aggressive clinical behaviour and high risk of leptomeningeal dissemination. A majority of metastatic and most recurrent childhood PNET are resistant to current therapeutic approaches, including high-dose chemotherapy with autologous haematopoietic stem cell rescue [2–4]. Accordingly, the identification of new therapies remains a major goal.

Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a member of the TNF family. To date, TRAIL has been shown to interact with five receptors, only two of which (DR4 and DR5) have intracellular death domains essential for the induction of apoptosis following receptor ligation [5–7]. The TRAIL-receptors DcR1 (TRAIL-R3, TRID) and DcR2 (TRAIL-R4, TRUNDD) are believed to act as dominant-negative inhibitors of TRAIL-mediated apoptosis when overexpressed on TRAIL-sensitive cells [8–10]. A fifth TRAIL receptor, namely osteoprotegerin (OPG), inhibits osteoclastogenesis and regulates bone resorption and remodelling [11]. Binding of TRAIL to DR4 and DR5 enables the adapter protein Fas-associated death domain (FADD) and the caspase-8 (FLICE, MACH) to bind to the receptor. Recruitment of caspase-8 leads to its proteolytic activation, which initiates a cascade of caspases including the downstream executioner caspase-3, leading to apoptosis [12].

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The cellular FLICE inhibitory protein (cFLIP) is a protein homologue to caspase-8 that lacks catalytic activity [13]. It interacts with both FADD and caspase-8 to inhibit the apoptotic signal of TRAIL [14].

Recombinant soluble human TRAIL induces apoptosis in a broad spectrum of human cancer cell lines, but in few normal cell types, and exhibits potent antitumour activity without systemic toxicity in various cancer xenograft models using mice and non-human primates [15–17]. This general view has been challenged by recent reports that showed induction of apoptosis in cultured normal human hepatocytes and in human brain tissue upon treatment with polyhistidine-tagged recombinant human TRAIL [18,19]. However, different recombinant versions of TRAIL vary widely in biochemical properties and potential for cellular and whole animal toxicity. Lawrence and colleagues [17] demonstrated that human hepatocytes treated with a version of human recombinant TRAIL that lacks exogenous sequence tags showed significantly less toxicity compared with human hepatocytes treated with a polyhistidine-tagged recombinant version of human TRAIL. Therefore, the native anticancer-molecule TRAIL is still in serious consideration for use in the clinic.

Using a panel of human PNET cell lines, we have recently shown that three of eight cell lines tested were sensitive to TRAIL-induced apoptosis [20]. We have also shown that TRAIL-induced apoptosis correlates with *caspase-8* mRNA expression in PNET cell lines. To further characterise the TRAIL death pathway in PNET, we analysed in the present study the methylation status of the *caspase-8* gene and the expression of *TRAIL*, *TRAIL*-receptors, *FADD*, *caspase-8*, *cFLIP* and *caspase-3* in a representative panel of primary PNET/medulloblastoma samples.

2. Patients and methods

2.1. Tumour and tissue samples and tumour cell lines

78 patients were diagnosed with a PNET/medulloblastoma at the Children's Hospital of Philadelphia between January 1988 and December 1998. All diagnoses were confirmed by a histological assessment of a tumour specimen obtained at surgery by one neuropathologist. Frozen tumour tissue adequate to perform reverse transcriptase-polymerase chain reaction (RT-PCR) was available from 27 PNET/medulloblastoma patients. Tumour samples were snap-frozen in liquid nitrogen in the operating room and then stored at −80 °C until further analysis. The demographic and treatment characteristics of the 27 study patients are summarised in Table 1. They are comparable to those of

Table 1
PNET/medulloblastoma patient characteristics, treatment and survival outcomes

Patient no.	Sex	Age (years)	M-stage	Tumour resection	Chemotherapy	XRT (Gy) local/cs	Follow-up (months)	Outcome
1	m	4.4	M3	⩾90%	Yes	54/36	74	Alive, no PD
2	f	3.9	M0	⩾90%	Yes	55/36	37	Dead of PD
3	f	14.8	M0	⩾90%	Yes	54/36	19	Dead of PD
4	m	1.8	M0	⩾90%	Yes	50/18	6	Dead of PD
5	f	0.6	M0	⩾90%	Yes	0	15	Alive, no PD
6	m	9.7	M0	50–90%	No	54/35	21	Dead of PD
7	f	6.7	M0	⩾90%	Yes	56/40	122	Dead of PD
8	f	10.9	M3	⩾90%	Yes	55/23	47	Alive, no PD
9	m	12.1	M0	⩾90%	Yes	56/36	91	Alive, no PD
10	f	6.4	M0	⩾90%	Yes	54/36	90	Alive, no PD
11	m	7.6	M0	⩾90%	Yes	60/40	75	Dead of PD
12	m	8.6	M0	⩾90%	Yes	56/36	65	Dead of PD
13	m	1.4	M0	⩾90%	Yes	0	62	Alive, no PD
14	f	6.8	M0	⩾90%	Yes	56/36	70	Alive, no PD
15	m	6.4	M0	⩾90%	Yes	60/36	105	Alive, no PD
16	f	0.3	M1	50–90%	Yes	0	55	Alive, no PD
17	m	7.6	M0	⩾90%	Yes	56/23	38	Alive, no PD
18	m	3.5	M0	⩾90%	Yes	52/23	46	Alive, no PD
19	m	1.8	M3	⩾90%	Yes	52/36	15	Dead of PD
20	m	8.5	M0	⩾90%	Yes	56/36	90	Alive, no PD
21	m	10.7	M3	50–90%	Yes	54/36	70	Alive, no PD
22	m	7.2	M3	50–90%	No	56/36	17	Dead of PD
23	f	2.7	M0	50–90%	Yes	54/36	38	Alive, PD
24	m	1.5	M3	⩾90%	Yes	56/36	90	Alive, no PD
25	m	12.9	M0	50–90%	Yes	54/36	34	Alive, no PD
26	m	12.4	M3	⩾90%	Yes	56/36	52	Alive, no PD
27	m	1.3	M0	⩾90%	Yes	0	11	Dead of PD

XRT, radiation therapy; PD, progressive disease; m, male; f, female; cs, craniospinal.

the 51 PNET/medulloblastoma patients not included in the present study due to the lack of frozen tumour tissue (data not shown). Therefore, the subset of patients included in the present study can be considered as representative. The median age at diagnosis for these PNET/medulloblastoma patients was 6.7 years (range 0.3–14.8 years). 18 (67%) patients were male and 9 (33%) were female. Evidence of leptomeningeal metastasis (M1-3) was present in 8 (30%) patients, and 19 (70%) patients were M0. Tumour location was cerebellar in all patients.

Normal and near normal brain samples included fetal cerebellum, adult whole brain (Clontech, Palo Alto, CA, USA), normal cerebellum of a 4-year-old glioma patient, temporal cortex from a 4-year-old epilepsy surgery patient, temporal cortex from a 14-year-old epilepsy surgery patient, and occipital cortex from a 19-year-old epilepsy surgery patient. Normal non-commercial brain samples were snap-frozen in liquid nitrogen in the operating room and then stored at −80 °C until further analysis.

DAOY and PFSK human PNET cells were purchased from American Type Culture Collection (Rockville, MD, USA). D341, D425, D458 human PNET cells were a kind gift from Dr Henry Friedman, Duke University, Durham, NC, USA. UW228-2 human PNET cells were a kind gift from Dr John R. Silber, University of Washington, WA, USA. CHOP707 human PNET cells were a kind gift from Dr David Pleasure, The Children's Hospital of Philadelphia, PA, USA.

2.2. Isolation of total RNA, semiquantitative RT-PCR

RNA isolation, first strand cDNA synthesis, and semiquantitative RT-PCR were performed according to methods previously described in Ref. [21]. PCR primers for *TRAIL*, *TRAIL*-receptors, *cFLIP* and *caspase-8* have been previously described and their specificity has been confirmed [20]. Primers specific for *FADD* were 5′-GGT GGA GAA CTG GGA TTT GA-3′ (sense) and 5′-CAA CCA TCA CTG CCC CTA CT-3′ (antisense). Primers specific for *caspase-3* were 5′-ATG GAA GCG AAT CAA TGG AC-3′ (sense) and 5′-AAC ATC ACG CAT CAA TTC CA-3′ (antisense). The primers were designed to bracket cDNA sequences that cross an intron-exon boundary in genomic DNA and were biotinylated at their 5′ ends. To correct variations in the RT-PCR and chemiluminescence detection steps, the expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPD*) was used as an internal control. Expression levels of the target transcripts were expressed as target/*GAPD* signal ratios after densitometric analysis of transcript signals using the NIH Image program (US NIH; http://rsb.info.nih.gov/nih-image). A subset of samples was analysed three times and the assay-to-assay variability was less than 15%.

2.3. Isolation of genomic DNA, methylation-specific PCR

Adequate frozen tumour tissue to isolate genomic DNA and perform PCR was available from 11 PNET/medulloblastoma patients. Genomic DNA was isolated using the QIAmp DNA Mini kit (Qiagen Inc., Valencia, CA, USA) according to procedures recommended by the manufacturer. In brief, tumour tissue was disrupted with a sterile disposable tissue grinder (Sage Products Inc., Crystal Lake, IL, USA), lysed with Proteinase K, and incubated with RNase A. Genomic DNA was then purified using a silica-gel-based method. The yield of DNA from the various samples was calculated by spectrophotometry.

Methylation-specific PCR was performed as previously described in Ref. [22]. In brief, 1 μg of genomic DNA was modified by sodium bisulphite treatment that converts unmethylated, but not methylated, cytosine to uracil. The modified DNA was purified using the Wizard DNA purification Kit (Promega, Madison, WI, USA) according to procedures recommended by the manufacturer. Sodium bisulphite modified DNA was used as template for PCR. Two PCR primer sets were designed to the CpG-rich region of the *caspase-8* 5′ flanking region; one specific for modified methylated DNA (M) and one specific for modified unmethylated DNA (U). Primer sequences specific for modified methylated DNA were 5′-TAG GGG ATT CGG AGA TTG CGA-3′ and 5′-CGT ATA TCT ACA TTC GAA ACG A-3′. Primer sequences specific for modified unmethylated DNA were 5′-TAG GGG ATT TGG AGA TTG TGA-3′ and 5′-CCA TAT ATC TAC ATT CAA AAC AA-3′. The percentage methylation status was estimated by calculating M/U signal ratios after densitometric analysis of transcript signals.

3. Results

3.1. Expression of TRAIL and TRAIL-receptors in primary PNET/medulloblastoma

We used semiquantitative RT-PCR analysis to examine the mRNA expression of *TRAIL* and *TRAIL*-receptors in 27 primary PNET/medulloblastoma (Fig. 1). *TRAIL* mRNA was detected in 14 (52%) of the 27 primary PNET/medulloblastoma. In normal and near normal brain samples, TRAIL mRNA was detected in 100% of the samples. This is in accordance with a report by Frank and colleagues [23], who found *TRAIL* expression in normal brain tissue adherent to removed debris obtained from patients with penetrating brain injuries.

DR5 mRNA was detected in all primary PNET/medulloblastoma samples evaluated. In contrast, *DR4*

mRNA was detected only in 8 (30%) of the 27 primary PNET/medulloblastoma samples and mRNA expression levels of *DR4* were significantly lower than those of *DR5* (mean 0.01 versus 0.73; $P<0.0001$). This indicates that DR5 is the main agonistic TRAIL receptor in PNET/medulloblastoma. Northern blotting experiments to confirm these results have not been conducted due to a lack of sufficient tumour material. Therefore, differences in the relative efficiency of RT reactions can not be excluded. In normal and near normal brain samples, *DR5* mRNA expression was detected in 100% and *DR4* in 67%.

DcR1 mRNA was detected in 22 (81%) and *DcR2* mRNA in 25 (93%) of 27 primary PNET/medulloblastoma, respectively. *DcR1* mRNA expression showed a wide variance in expression levels and higher expression levels than *DcR2* in most of the tumours (mean 0.55 versus 0.07; $P<0.0001$). The vast majority of the primary PNET/medulloblastoma co-expressed the antagonistic TRAIL-receptors DcR1 and DcR2. In normal and near normal brain samples, *DcR1* mRNA expression was detected in 33% and DcR2 in 83%.

3.2. Expression of FADD, cFLIP, caspase-8 and caspase-3 in primary PNET/medulloblastoma

Fig. 2 shows the mRNA expression of *FADD*, *cFLIP*, *caspase-8* and *caspase-3* in primary PNET/medulloblastoma and normal/near normal brain samples as determined by semiquantitative RT-PCR. *FADD* mRNA was detected in all primary PNET/medulloblastoma samples evaluated, in seven of seven PNET cell lines (data not shown) and in all normal and near normal brain samples evaluated. *cFLIP* mRNA was

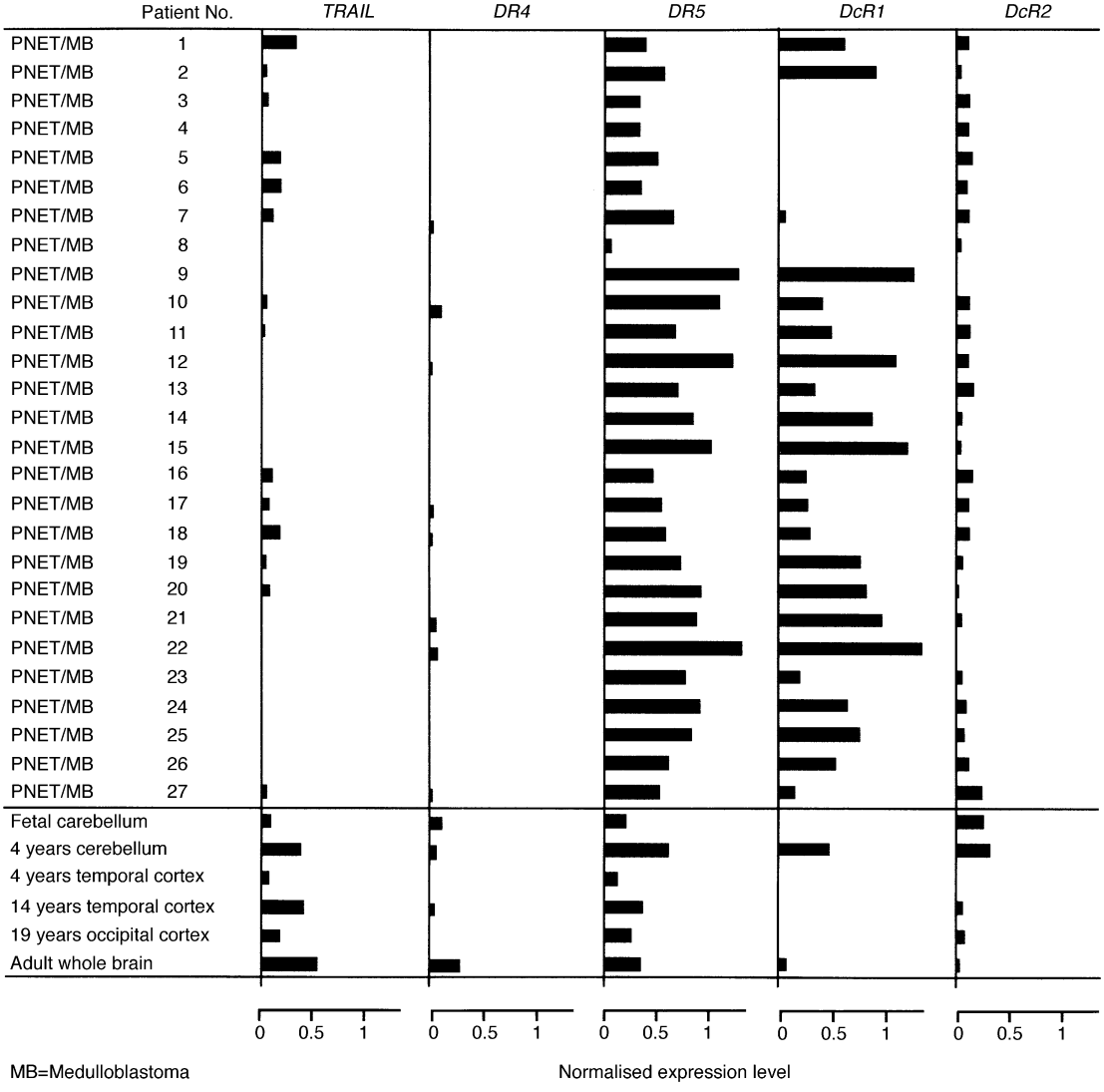

Fig. 1. mRNA expression of *TRAIL*, its agonistic (*DR4*, *DR5*) and antagonistic receptors (*DcR1*, *DcR2*) in primitive neuroectodermal tumours (PNET/medulloblastoma) and normal brain samples as determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Expression levels of the target transcripts were normalised by use of an internal glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) control.

detected at relatively high levels in all primary PNET/medulloblastoma samples. This is in accordance with our previous results showing *cFLIP* expression in seven of eight PNET cell lines [20]. In normal and near normal brain samples, *cFLIP* was detected in 100%. *Caspase-8* mRNA was detected in all normal and near normal brain samples, but only in 13 (48%) of the 27 primary PNET/medulloblastoma samples. Moreover, the *caspase-8* mRNA expression levels of primary PNET/medulloblastoma were significantly lower than those of normal and near normal brain samples (mean 0.023 versus 0.167; $P < 0.0001$). *Caspase-3* mRNA was detected at relatively high levels in all primary PNET/medulloblastoma evaluated, in seven of seven PNET cell lines (data not shown) and in all normal and near normal brain samples.

3.3. *Methylation status of* caspase-8 *in PNET cell lines and primary PNET/medulloblastoma*

Since aberrant methylation of CpG islands is a fundamental aspect of neoplasia associated with inhibition of transcription initiation [24,25], we analysed the methylation status of *caspase-8* in PNET cell lines and primary PNET/medulloblastoma by a methylation-specific PCR method. We used a CpG-rich region of the caspase-8 5′ flanking region in designing oligonucleotide primers for methylation-specific PCR analysis. One primer set contained adenine substituted for each guanine in the antisense strand, such that these primers only bound to a bisulphite-modified DNA sequence corresponding to unmethylated DNA (caspase-8 primers 'U'). The other primer set retained guanine at positions

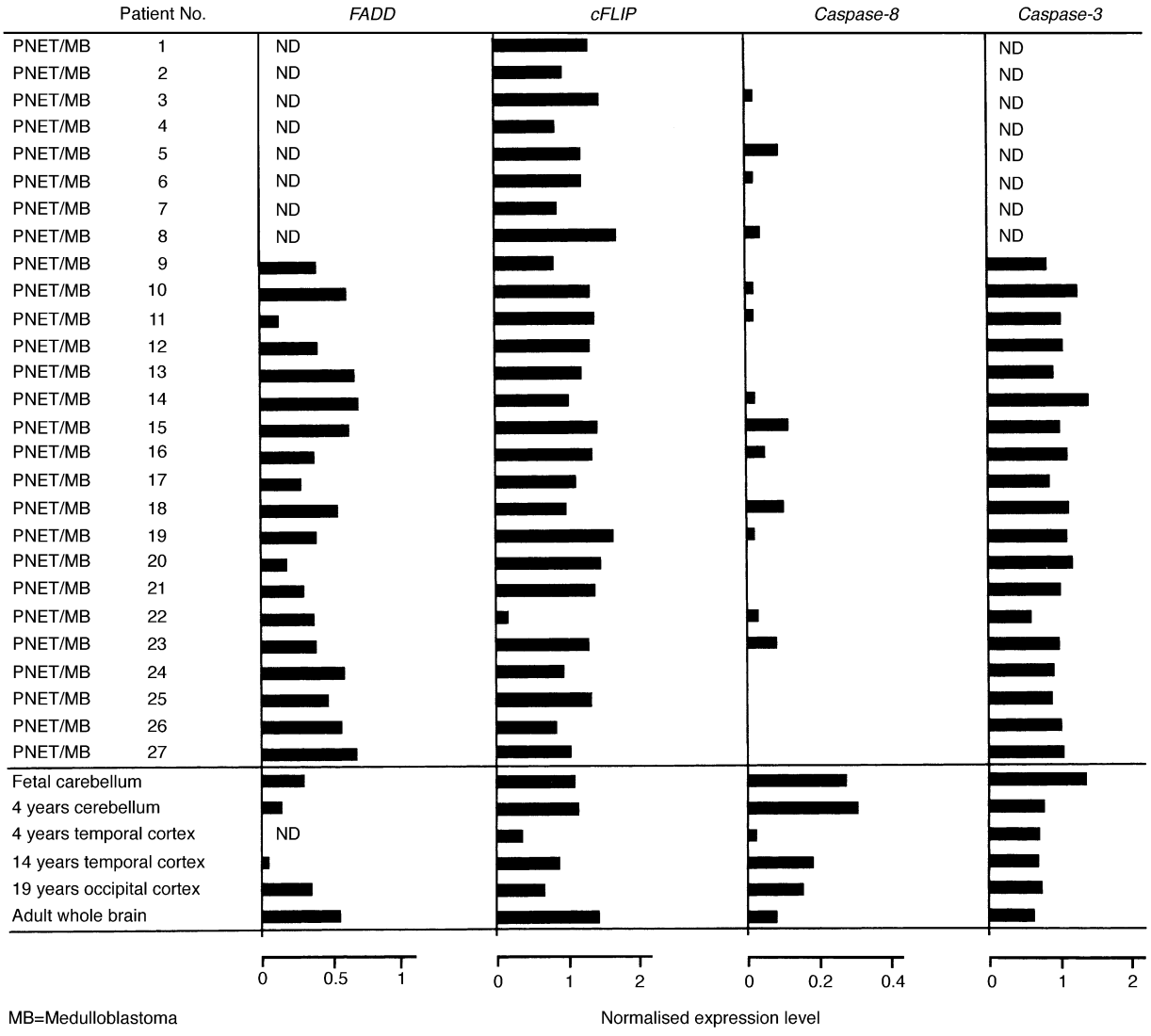

Fig. 2. mRNA expression levels of *FADD*, *cFLIP*, *caspase-8* and *caspase-3* in PNET/medulloblastoma and normal brain samples as determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Expression levels of the target transcript were normalised by use of an internal glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) control. ND, not done.

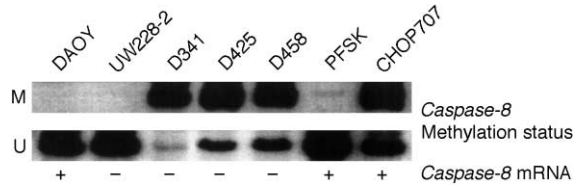

Fig. 3. Methylation status of *caspase-8* and its effect on the mRNA expression of *caspase-8* in PNET cell lines. DNA extracted from primitive neuroectodermal tumour (PNET) cells were amplified with primers specific to the unmethylated (U) or the methylated (M) CpG islands of *caspase-8* after modification with sodium bisulphite.

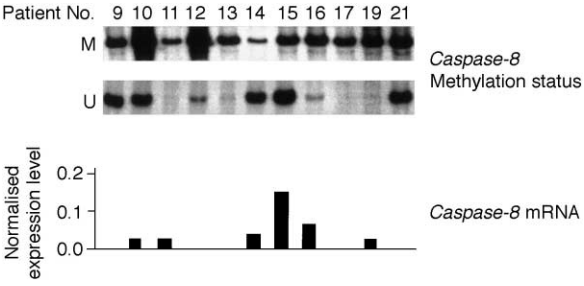

Fig. 4. Methylation status of *caspase-8* and mRNA expression of *caspase-8* in primary primitive neuroectodermal tumours (PNET)/medulloblastoma. DNA extracted from PNET/medulloblastoma were amplified with primers specific to the unmethylated (U) or the methylated (M) CpG islands of *caspase-8* after modification with sodium bisulphite.

corresponding to cytosine in CpG oligonucleotide sequences specific for methylated genomic DNA (caspase-8 primers 'M'), as these residues are protected by methylation from bisulphite modification.

Using caspase-8 primers 'U' for a PCR with bisulphite-treated genomic DNA from PNET cell lines as template, the 322-base-pair *caspase-8* product was detectable at high levels of expression in DAOY, UW228-2 and PFSK, and in lower levels of expression in D425, D458 and CHOP707 (Fig. 3). Use of the same bisulphite-treated template DNA with the *caspase-8* primers 'M' resulted in a 321-base-pair PCR product in the PNET cell lines D341, D425, D458 and CHOP707 indicating methylation of CpG sites in the *caspase-8* promoter region of these cell lines. Whereas D341 PNET cells displayed complete methylation of *caspase-8*, the PNET cell lines D425, D458 and CHOP707 contained PCR products in reactions amplified with both primers sets, indicating incomplete methylation of *caspase-8*. Interestingly, the *caspase-8* mRNA expression reported previously in Ref. [20] correlated with the methylation status of the *caspase-8* gene in the PNET cell lines. All PNET cell lines with no detectable *caspase-8* mRNA expression (D341, D425, D458) displayed $>75\%$ methylation of the *caspase-8* promoter region, whereas the 4 PNET cell lines with detectable *caspase-8* mRNA expression (DAOY, UW228-2, PFSK, CHOP707) displayed an unmethylated *caspase-8* promoter region in 3 cases and $<75\%$ methylation in 1 case indicating that aberrant methylation is a common reason for loss of *caspase-8* gene expression in PNET cells.

In primary PNET/medulloblastoma, use of caspase-8 primers 'M' for a PCR with bisulphite-treated genomic DNA resulted in a 321-base-pair PCR product in all PNET/medulloblastoma samples evaluated indicating that 5′CpG island methylation of the *caspase-8* gene is common in primary PNET/medulloblastoma. Use of the same bisulphite-treated template DNA with the *caspase-8* primers 'U' resulted in a 322-base-pair PCR product in 7 of 11 primary PNET/medulloblastoma samples (Fig. 4). Whereas 6 (55%) primary PNET/medulloblastoma samples displayed $>75\%$ methylation of *caspase-8*, 5 PNET/medulloblastoma samples contained PCR products in reactions amplified with both primers sets indicating incomplete and/or inhomogeneous methylation of *caspase-8* in these tumours. In contrast to the PNET cell lines, there was no correlation of the *caspase-8* methylation status and *caspase-8* mRNA expression in the primary PNET/medulloblastoma.

4. Discussion

Targeting TRAIL-death receptors to induce apoptosis in tumour cells is an intriguing therapeutic strategy because it has been shown that TRAIL induces apoptosis in a broad spectrum of human cancer cell lines and exhibits potent anti-tumour activity without systemic toxicity in various cancer xenograft models [15–17,26,27]. In our previous work [20], we have found that three of eight PNET cell lines evaluated were sensitive to TRAIL-induced apoptosis and that TRAIL-resistance correlated with a loss of *caspase-8* mRNA and protein expression. This is similar to recent findings in neuroblastoma, another embryonal tumour with important biological similarities to central nervous system (CNS) PNET [28–30]. In addition, other mechanisms of resistance to TRAIL-induced apoptosis in tumour cell lines have been demonstrated, including lack of agonistic TRAIL-receptors, enhanced expression of antagonistic TRAIL-receptors, or high expression levels of cFLIP, which inhibits the activation of caspase-8 [14,31,32].

In this study, we analysed, in a representative panel of primary PNET/medulloblastoma, important elements of the TRAIL death pathway to evaluate whether treatment with recombinant TRAIL deserves further investigation in PNET. It was found that all tumours expressed at least one agonistic TRAIL receptor (*DR5* in 100% and *DR4* in 30%), the adapter protein *FADD* and *caspase-3*. However, all primary PNET/medulloblastoma expressed also at least one antagonistic TRAIL-receptor (*DcR1* in 81% and *DcR2* in 93%) and

high mRNA expression levels of *cFLIP*. In addition, loss of *caspase-8* mRNA expression was detected in 52% and *caspase-8* mRNA expression levels were significantly lower in PNET/medulloblastoma compared with normal and near normal brain samples. This indicates the presence of different inhibitory mechanisms of TRAIL-induced apoptosis in PNET/medulloblastoma. Of these, loss of *caspase-8* seems to be of particular relevance since upregulation of *caspase-8* mRNA expression by treatment with 5-aza-2′-deoxycytidine restored TRAIL-sensitivity in D458 and CHOP707 PNET cells as reported before in Ref. [20].

Caspases are a family of cysteine aspartyl proteases that are activated in proteolytic cascades during apoptosis [33]. Caspase-3 and other effector caspases, which cleave essential intracellular substrates are activated by apical or initiator caspases that autoactivate when induced to oligomerise in response to a variety of pro-apoptotic signals, including cytotoxic drugs. For example, caspase-9 is activated when cytochrome c is released from mitochondria and binds to its adapter molecule, Apaf-1. In contrast, caspase-8 is activated after ligation of specific death receptors including TRAIL-receptors DR4/5 and CD95/Fas/Apo1 [34,35]. Mutant cell lines that lack *caspase-8* are resistant to TRAIL-induced apoptosis demonstrating that caspase-8 is essential for the transmission of DR4/5 mediated signals leading to apoptosis [12].

Common pathways leading to a loss of gene function in human cancers are alterations in the genetic code, deletions of genetic material and aberrant gene methylation. The human *caspase-8* gene is located on chromosome 2q22-34 [36]. Losses of chromosome 2 are rare in PNET as shown by comparative genomic hybridisation studies [37] and functional mutations of *caspase-8* have not been described in primitive neuroectodermal tumours of the peripheral nervous system (neuroblastoma) [29]. Therefore, we hypothesised that loss of *caspase-8* mRNA expression in PNET cell lines and primary PNET/medulloblastoma might be the result of aberrant *caspase-8* gene methylation. Using a methylation-specific PCR method, we found in the present study $>75\%$ methylation of the *caspase-8* promoter region in three of seven PNET cell lines and in 55% of the primary PNET/medulloblastoma, indicating that 5′CpG island methylation of the *caspase-8* gene is common in childhood PNET/medulloblastoma. The methylation status of *caspase-8* correlated with the expression of *caspase-8* mRNA in the PNET cell lines, indicating that promoter hypermethylation silences *caspase-8* in the PNET cells. In primary PNET/medulloblastoma, there was no correlation of the *caspase-8* methylation status and *caspase-8* mRNA expression. This might be explained by the relatively small sample size and the heterogeneity of the studied frozen tumour tissue. Indeed, preliminary studies indicate intratumoral variability of caspase-8 immunoreactivity in a subset of primary PNET/medulloblastoma (data not shown). In one single tumour, both areas with methylated and unmethylated *caspase-8* might exist. Additionally, mechanisms other than aberrant methylation might downregulate *caspase-8* in a subset of PNET/medulloblastoma, explaining the lack of *caspase-8* mRNA expression in tumours 9 and 21. Clearly, to address these points a larger study is needed in which different tumour areas are analysed separately, potentially using microdissection techniques.

While *de novo* methylation of CpG islands is rare in normal tissues, tissue culture cells and cancer cells both may have a genome-wide increase in CpG island methylation [24,38,39]. Methylation of CpG islands in the promoter regions of genes is a powerful mechanism of gene activity suppression. Indeed, the reciprocal relationship between the density of methylated cytosine residues and the transcriptional activity of a gene has been widely documented [24]. Aberrant methylation of individual CpG islands has been explained as the result of selective pressure [40]. However, abnormal methylation of individual CpG islands may also reflect a widespread loss of the protection of CpG islands against DNA methylation [24]. Costello and colleagues [41] performed a global analysis of the methylation status of 1,184 unselected CpG islands in each of 98 primary human tumours using restriction landmark genomic scanning [42]. Breast, head and neck and testicular tumours displayed relatively low levels of methylation, with many such tumour profiles showing no methylation. Whereas colon tumours, gliomas, acute myeloid leukaemias and PNET displayed a much higher frequency of methylation. Assuming 45 000 CpG islands per genome, they predicted that aberrant methylation was initiated in PNET at an average of 420 CpG islands (range 114–760).

Functional changes of critical genes that regulate cell proliferation and survival can cause cancer [43]. It is therefore of great clinical importance to determine how apoptosis becomes suppressed in cancer, as repair of a cancer cell's defective apoptotic machinery offers the promise of effective and specific anti-cancer therapy [44]. In the present study, we demonstrate that the TRAIL death pathway is interrupted in PNET cells by loss of *caspase-8* mRNA expression due to aberrant gene methylation. It is possible to reverse epigenetic changes by treatment with DNA methylation inhibitors to restore gene function to a cell. In our previous work, we demonstrated restoration of *caspase-8* mRNA expression and TRAIL-sensitivity in D458 and CHOP707 human PNET cells by demethylation with 5-aza-2′-deoxycytidine (Decitabine), an analogue of 2′-deoxycytidine with a nitrogen atom substituting for the carbon in the 5 position of the heterocyclic ring. Decitabine has entered clinical trials in humans displaying

activity in acute myelogenous leukaemia and myelodysplastic syndromes [45,46]. However, there are significant problems with the use of analogues, which are incorporated into patients DNA, as they have been shown to be mutagenic [47]. Therefore, there is clearly a need for the discovery of new inhibitors of DNA methylation. Ramachandani and colleagues [48] for example have obtained evidence that antisense oligodeoxynucleotides may inhibit methyltransferase and decrease tumorigenesis.

In conclusion, the present study characterises the expression profile of the TRAIL death pathway in primary PNET/medulloblastoma, characterises DR5 as the major agonistic TRAIL receptor and demonstrates that loss of *caspase-8* gene expression is common in PNET/medulloblastoma suggesting that suppression of death receptor induced apoptosis may play a role in PNET/medulloblastoma pathogenesis. Moreover, we demonstrate that aberrant methylation silences the *caspase-8* gene in PNET cells, thus indicating a novel molecular target in this common childhood brain tumour. It remains to be tested whether restoration of *caspase-8* by the use of demethylating agents, methyltransferase inhibitors or gene transfer increases the rate of spontaneous apoptosis and decreases tumour growth in PNET models. It also remains to be tested, whether restoration of *caspase-8* alters the chemosensitivity of PNET cells since *caspase-8* functions in the mitochondrial pathway as an amplifying executioner caspase [49]. Upon restoration of *caspase-8*, treatment with recombinant TRAIL remains an attractive novel therapeutic approach for childhood PNET that deserves further investigation.

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References

1. Gurney JG, Smith MA, Bunin GR. CNS and miscellaneous intracranial and intraspinal neoplasms. In *SEER Pediatric Monograph* (http://seer.cancer.gov/publications/). National Cancer Institute, 2000, 51–63.
2. Zeltzer PM, Boyett JM, Finlay JL, *et al*. Metastasis stage, adjuvant treatment, and residual tumor are prognostic factors for medulloblastoma in children: conclusions from the Children's Cancer Group 921 randomized phase III study. *J Clin Oncol* 1999, **17**, 832–845.
3. Graham ML, Herndon II JE, Casey JR, *et al*. High-dose chemotherapy with autologous stem-cell rescue in patients with recurrent and high-risk pediatric brain tumors. *J Clin Oncol* 1997, **15**, 1814–1823.
4. Dunkel IJ, Boyett JM, Yates A, *et al*. High-dose carboplatin, thiotepa, and etoposide with autologous stem-cell rescue for patients with recurrent medulloblastoma. Children's Cancer Group. *J Clin Oncol* 1998, **16**, 222–228.
5. Wu GS, Burns TF, McDonald 3rd ER, *et al*. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 1997, **17**, 141–143.
6. Walczak H, Degli-Esposti MA, Johnson RS, *et al*. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* 1997, **16**, 5386–5397.
7. Pan G, O'Rourke K, Chinnaiyen AM, *et al*. The receptor for the cytotoxic ligand TRAIL. *Science* 1997, **276**, 111–113.
8. Degli-Esposti MA, Smolak PJ, Walczak H, *et al*. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* 1997, **186**, 1165–1170.
9. Pan G, Ni J, Wei YF, Yu Gl, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997, **277**, 815–818.
10. Pan G, Ni J, Yu Gl, Wei YF, Dixit VM. TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* 1998, **424**, 41–45.
11. Emery JG, McDonnell P, Burke MB, *et al*. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* 1998, **273**, 14363–14367.
12. Bodmer JL, Holler N, Reynard S, *et al*. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* 2000, **2**, 241–243.
13. Irmler M, Thome M, Hahne M, *et al*. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997, **388**, 190–195.
14. Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* 1998, **161**, 2833–2840.
15. Bonavida B, Ng C-P, Jazirehi A, Schiller G, Mizutani Y. Selectivity of TRAIL-mediated apoptosis of cancer cells and synergy with drugs: the trail to non-toxic cancer therapeutics (review). *Int J Oncol* 1999, **15**, 793–802.
16. Ashkenazi A, Pai RC, Fong S, *et al*. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999, **104**, 155–162.
17. Lawrence D, Shahrokh Z, Marsters S, *et al*. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001, **7**, 383–385.
18. Jo M, Kim TH, Seol DW, *et al*. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000, **6**, 564–567.
19. Nitsch R, Bechmann I, Deisz RA, *et al*. Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet* 2000, **356**, 827–828.
20. Grotzer MA, Eggert A, Zuzak TJ, *et al*. Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* 2000, **19**, 4604–4610.
21. Grotzer MA, Hogarty MD, Janss AJ, *et al*. MYC messenger RNA expression predicts survival outcome in childhood primitive neuroectodermal tumor/medulloblastoma. *Clin Cancer Res* 2001, **7**, 2425–2433.
22. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG island. *Proc Natl Acad Sci USA* 1996, **93**, 9821–9826.
23. Frank S, Köhler U, Schackert G, Schackert HK. Expression of TRAIL and its receptors in human brain tumors. *Biochem Biophys Res Co* 1999, **257**, 454–459.
24. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999, **21**, 163–167.
25. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998, **72**, 141–196.

26. Petak I, Douglas L, Tillmman DM, Vernes R, Houghton JA. Pediatric rhabdomyosarcoma cell lines are resistant to Fas-induced apoptosis and highly sensitive to TRAIL-induced apoptosis. *Clin Cancer Res* 2000, **6**, 4119–4127.
27. Thomas WD, Hersey P. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J Immunol* 1998, **161**, 2195–2200.
28. Hopkins-Donaldson S, Bodmer JL, Bourlond KB, Brognara CB, Tschopp J, Gross N. Loss of caspase-8 expression in highly malignant human neuroblastoma cells correlates with resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res* 2000, **60**, 4315–4319.
29. Teitz T, Wei T, Valentine MB, *et al*. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med* 2000, **6**, 529–535.
30. Eggert A, Grotzer MA, Zuzak TJ, *et al*. Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res* 2001, **61**, 1314–1319.
31. Kim K, Fisher MJ, Xu S-Q, El-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* 2000, **6**, 335–346.
32. Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999, **59**, 2747–2753.
33. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998, **281**, 1312–1316.
34. Nagata S. Apoptosis by death factor. *Cell* 1997, **88**, 355–365.
35. Peter ME. The TRAIL DISCussion: it is FADD and caspase-8. *Cell Death Differ* 2000, **7**, 759–760.
36. Grenet J, Teitz T, Wei T, Valentine V, Kidd VJ. Structure and chromosome localization of the human CASP8 gene. *Gene* 1999, **226**, 225–232.
37. Russo C, Pellarin M, Tingby O, *et al*. Comparative genomic hybridization in patients with supratentorial and infratentorial primitive neuroectodermal tumors. *Cancer* 1999, **86**, 331–339.
38. Jones PA, Wolkowicz MJ, Rideout WM, *et al*. De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc Natl Acad Sci USA* 1990, **87**, 6117–6121.
39. Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* 1990, **62**, 503–514.
40. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* 1994, **7**, 536–540.
41. Costello JF, Frühwald MC, Smiraglia DJ, *et al*. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000, **24**, 132–138.
42. Hatada I, Hayashizaki Y, Hirotsune S, Komatsubara H, Mukai T. A genomic scanning method for higher organisms using restriction sites as landmarks. *Proc Natl Acad Sci USA* 1991, **88**, 9523–9527.
43. Evan G, Littlewood T. A matter of life and cell death. *Science* 1998, **281**, 1317–1322.
44. Juin P, Evan G. Caspase 8: the killer you can't live without. *Nat Med* 2000, **6**, 498–500.
45. Pinto A, Zagonel V. 5-Aza-2′-deoxycytidine (Decitabine) and 5-azacytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present and future trends. *Leukemia* 1993, **7**, 51–60.
46. Lübbert M, Haak HL, Kunzmann R, *et al*. Cytogenic response to low-dose 5′-aza-2′-deoxyctidine (DAC) in poor-risk myelodysplastic syndromes (MDS). *Blood* 1997, **90**(Suppl. 1), 582a.
47. Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R. Mutagenicity of 5-aza-2′deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc Natl Acad Sci USA* 1997, **94**, 4681–4685.
48. Ramchandani S, MacLeod AR, Pinard M, Von Hofe E, Szyf M. Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1997, **94**, 684–689.
49. Engels IH, Stpczynska A, Stroh C, *et al*. Caspase-8/FLICE functions as an executioner caspase in anticancer drug-induced apoptosis. *Oncogene* 2000, **19**, 4563–4573.