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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

KIT expression and methylation in medulloblastoma and PNET cell lines and tumors

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Abstract The stem cell factor/kit tyrosine kinase receptor pathway is related to tumor growth and progression in several cancers including Ewing sarcoma, a peripheral PNET (pPNET). Identifying additional groups of tumors that may use the pathway is important as they might be responsive to imatinib mesylate treatment. MB and central PNET (cPNET) are embryonal tumors of the CNS that share similar undifferentiated morphology with Ewing sarcomas and display aggressive clinical behavior. cPNET outcome is significantly lower than MB outcome, even for localized tumors treated with high-risk MB therapy. The elucidation of signaling pathways involved in MB and cPNET pathogenesis, and the discovery of new therapeutic targets is necessary to improve the treatment of these neoplasms. We analyzed KIT expression in 2 MB, one pPNET, one cPNET and 2 rhabdomyosarcoma (RMS) cell lines. Also, in 13 tumor samples (12 MB and one cPNET), we found KIT overexpression in the most aggressive cell lines (metastatic MB and pPNET). Hypermethylation of KIT was clear in the RMS non-expressing cell lines. Among MB tumors, we could see variable levels of KIT expression; a subset of them (25%) might be related in its growth pattern to KIT up-regulation. No methylated KIT was detected in the tumors expressing the lowest levels of KIT. Our results point to methylation as an epigenetic regulatory mechanism for KIT inhibition only in the KIT non-expressing RMS cell lines, and neither in the rest of the cell lines nor in the tumor samples.

Keywords KIT · Stem cell factor · Medulloblastoma · PNET · Methylation

Abbreviations

CNS	Central nervous system
EWS	Ewing sarcoma
MB	Medulloblastoma
MCA-Meth	Melting curve analysis-methylation
MSP	Methylation specific PCR
PNET	Primitive neuroectodermal tumor
SCF	Stem cell factor
RMS	Rhabdomyosarcoma

Introduction

KIT proto-oncogene is a member of the tyrosine receptor kinase subclass III family and is recognized by the stem

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cell factor (SCF) protein. Aberrant expression of KIT has been shown to be involved in the pathogenesis of several cancers. The stem cell factor/kit tyrosine kinase receptor pathway is important for tumor growth and progression in mast cell diseases, gastrointestinal stromal tumors, acute myeloid leukemias, small cell lung carcinomas, some gliomas and Ewing sarcomas [1].

Histologic and clinicopathologic studies indicate that Ewing sarcoma and PNET have overlapping features. In fact, the identification of a common translocation t(11;22)(q24;q12) that results in the formation of the EWS–ETS fusion gene in cases of Ewing sarcomas and peripheral PNET (pPNET) strongly supported the hypothesis that these tumors are related [2].

Regarding the 2007 WHO classification of CNS tumors [3], which differs significantly from the previous version [4], medulloblastoma (MB) and central PNET (cPNET) are two different entities of CNS embryonal tumors that share similar undifferentiated morphology. They have some overlapping features with pPNET and might be regulated by similar signaling pathways.

MB is the most common malignant brain neoplasm of childhood, representing 20% of all pediatric brain tumors [5]. They are extremely malignant and invasive tumors originated from primitive or poorly developed cells and localized in the cerebellum. PNET commonly arise in the cerebrum, and are seen less frequently in deep paraventricular or midline locations such as the diencephalon and basal ganglia [6]. The treatment of MB depends mainly on the age of the patient and includes surgical resection (particularly in children with localized disease), craniospinal radiation (not given to children younger than 3 years old due to the severe neurological morbidity) and chemotherapy (cisplatin, vincristine, lomustine, cyclophosphamide and oral etoposide are the most frequently used, either alone or in combination) [7]. However, only 60% of affected children are cured and most of them suffer from long-term side effects as a result of aggressive treatments [8]. Based on more aggressive clinical features, cPNET have traditionally received therapies designed for metastatic, high-risk MB, which involves more intensive chemotherapy and higher doses of radiation to the head and spine. cPNET outcome is significantly low even for localized tumors treated with high-risk MB therapy [9].

These observations stress the need for more thorough knowledge regarding cPNET tumorigenesis in order to be able to design more successful treatments and to amplify the range of drugs that can be used as treatment for MB and cPNET [10–12]. KIT expression has not been studied in detail in these tumors, and the mechanisms by which its expression is activated or inhibited is not so far well determined. With this purpose, we decided to analyze KIT expression in MB, cPNET, pPNET and rhabdomyosarcoma (RMS) cell lines, but also in MB tumor samples. Moreover, we evaluated whether KIT methylation was an epigenetic regulatory mechanism in those tumors and cell lines.

Materials and methods

Cell lines and culture conditions

PFSK-1 (derived from a cPNET), SK-PN-DW (a pPNET), DAOY (derived from a desmoplastic MB) and D283Med (derived from a metastatic MB) cell lines were obtained from the American Type Culture Collection (Manassas, USA). TE671 and TE671Sub2, both rhabdomyosarcoma (RMS) cell lines, were obtained from the Deutsches Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cell lines were cultured in RPMI + L-Glutamax medium containing 10% FBS, 4% Non-Essential Aminoacids, 1% Penicillin/Streptomycin and 0.1% Anphotericine B at 37°C in an atmosphere with 5% CO_2 to expand them.

Tumor specimens

Twelve MB (73, 112, 71509, 231, 386, 348, 374, 376, 429, 304, 319 and 397) and one cPNET (349) derived from human patients were collected from the University of Michigan Department of Neurosurgery. As a control, 2 samples of normal cerebellum were examined in the current study to compare the basal expression of the KIT gene. These studies were approved by the Internal Review Board of the University of Michigan Medical School.

KIT expression analysis in cell lines by RT-PCR

RNA from cell lines was purified using the QuickPrep Total RNA Extraction Kit (Amersham Biosciences), following manufacturer's instructions. RNA quality was checked by electrophoresis in a 1% agarose gel. Purity and quantity of total RNA was determined by spectrophotometer (SmartSpec™ Plus; BioRad, Hercules, CA, USA) as a 260/280 ratio with expected values between 1.8 and 2. One microgram of RNA was then retrotranscribed using the SuperScript™ II RNase H Reverse Transcriptase (Invitrogen).

The PCRs were carried out with 100 ng of cDNA in a total volume of 25 µl, which contained 2.5 µl 10× reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol forward and reverse primers, 5% DMSO and one unit of AmpliTaq Gold™ polymerase (Roche), in a T3 thermocycler of Biometra®. The TFR (Transferrine Receptor) gene was used as internal control of expression. cDNA obtained from normal brain RNA was used as positive control of expression.

The primers for the TFR gene (forward: 5′-GTCAATG TCCCAAACGTCACCAGA-3′, reverse: 5′-ATTTCGGGA ATGCTGAGAAAACAGACAGA-3′) and for the KIT gene (forward: 5′-GCAGAAGCCACCAACACC-3′, reverse primer: 5′-GAGAGGACAGCGGACCAG-3′) were designed by the Primer 3 software. The reactions were denatured at 94°C in a first PCR step for 10 min, then 30–40 cycles of 1 min at 94°C, 45–60 s at 60°C for TFR or 62°C for KIT, and 45 s–2 min at 72°C, followed by a final extension step at 72°C for 10 min. PCR products were visualized in an agarose 2% gel with ethidium bromide at a final concentration of 0.1 μg/ml.

KIT protein expression analysis in cell lines by Western blotting

Equal amounts of protein (50 μg) were electrophoresed on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated overnight with an anti-human c-kit antibody (cat. A4502; Dako) diluted 1/5,000 in TBST-5% non-fat milk. The secondary antibody, an anti-rabbit IgG (cat. ab6721; Abcam) diluted 1/5,000 was incubated 1 h at RT in TBST-5% non-fat milk. GAPDH protein was detected in the same membrane using the Reblot Plus MIld solution (cat. 2502; Chemicon). The primary antibody, mouse monoclonal GAPDH (cat. sc-32233; Santa Cruz Biotechnology) diluted 1/2,000, and the secondary antibody, anti-mouse IgG (cat. sc-2005; Santa Cruz Biotechnology) diluted 1/5,000, were incubated 1 h at RT in TBST-5% non-fat milk. ECL Reagent (cat. RPN2109) was used for revealing.

DNA extraction and bisulfite treatment

DNA from cell lines was purified by using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's instruction. Genomic DNA was then bisulfite modified by using CpGenome™ DNA Modification Kit (Chemicon® International), following manufacturer's protocol. Genomic blood DNA and in vitro methylated DNA (IMD) were used as negative and positive controls, respectively, for the methylation status of DNA.

MSP

The methylation status of the CpG island C (located closer to the exon 1) of the KIT gene was analyzed by methylation specific PCR due to its high CpG dinucleotide content. PCRs were carried out in a T3 thermocycler of Biometra® with 60 ng of bisulfite modified DNA in a total volume of 25 μl, which contained 2.5 μl 10× reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol forward and reverse primers, 5% DMSO and one unit of AmpliTaq Gold™ polymerase (Roche). The primers for unmethylated promoter (forward: 5′-GTAGTATTTTTTTGGTTT GGGAATGG-3′, reverse: 5′-CATCTAACCCCTACTCTT TCAACATA-3′) and for methylated promoter (forward: 5′-GGTAGTATTTTTTTGGTTCGGGAAC-3′, reverse: 5′-G TCTAACCCCTACTCTTTCGACGTA-3′) were designed using the MethPrimer software [13]. The reactions were heated at 94°C for 10 min in order to initiate the reaction, followed by 35–40 cycles of amplification (30 s at 94°C, 30 s at 60°C and 30 s at 72°C). A final extension at 72°C for 10 min completed the reaction. PCR products were visualized on 2% agarose gels.

MCA-Meth

The methylation status of CpG islands A and B (located farther from the exon 1) of the KIT gene promoter was assayed by MCA-Meth [14]. The Melting Curve Analysis-Methylation (MCA-Meth) assays were carried out with 10 ng of bisulfite modified DNA in a total volume of 25 μl, containing 12.5 μl of 2× IQ™ SYBR Green Supermix (Bio-Rad) and 2.5 pmol of primers. Bisulfite primers (with no CpG on its sequence) for KIT island A (forward: 5′-T TTAGGGGTGGAAAGGTGGAGAGA-3′, reverse: 5′-AA CAACAACCCCTCCTCCCAAC-3′) and KIT island B (forward: 5′-GGTGTTAGGAGTTTTTAATAGGTTTG-3′, reverse: 5′-CACCTTTCCACCCCTAAAATATAA-3′) were designed using the MethPrimer software [13]. The reactions were heated at 94°C in a first PCR step for 10 min and then 40–45 cycles of 30 s at 94°C, 30 s at the corresponding annealing temperature and 30 s at 72°C. After the amplification step, the melting curve analysis was carried out as follows: from 70 to 90°C, 30 s at every 0.5°C. Both the amplification step and the melting curve were carried out in an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad).

5-Aza-2′-deoxycytidine treatment and re-expression analysis

Cell lines were treated with 5-aza-2′-deoxycytidine at a final concentration of 2 μM for 72 h. The medium was changed daily. Untreated cells were used as a control. RNA was extracted, retrotranscribed to cDNA, and qRT-PCR was performed to check for re-expression of the KIT gene. The reactions were carried out in an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using IQ™ SYBR Green Supermix. The PCR conditions were as described above. The hypoxanthine phosphoribosyltransferase 1 (HPRT1) housekeeping gene was used as an internal control of expression.

KIT expression analysis in tumor specimens

The cDNA was obtained using the same protocol as before. The amplification reactions were carried out in an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each sample was analyzed in triplicate and contained 75 ng of template DNA, 12.5 µl SYBR Green I Master Mix buffer and 0.5 µl of each primer (2.5 µmol/l) in a 25 µl of total volume. Human HPRT was used to normalize the amount of cDNA. The relative quantification of target gene expression was performed by the comparative Ct method (ddCt).

KIT methylation analysis in tumor specimens

CpG island C was assayed for methylation in tumor samples displaying low expression of KIT. MSP was performed after bisulfite treatment following the same methodology as above.

Results

KIT expression in cell lines

The KIT transcript was greatly detected in SK-PN-DW (pPNET) and D283Med (metastatic MB) cell lines whereas the expression in PFSK-1 (cPNET) was much lower, and Daoy (desmoplastic MB) did not express KIT. TE671 and TE671Sub2 RMS cell lines were negative for KIT expression. Similar results were obtained when protein was detected by western blot. KIT protein in PFSK-1 cell line was undetectable probably in relation with the low amount of transcript detected by PCR (Fig. 1).

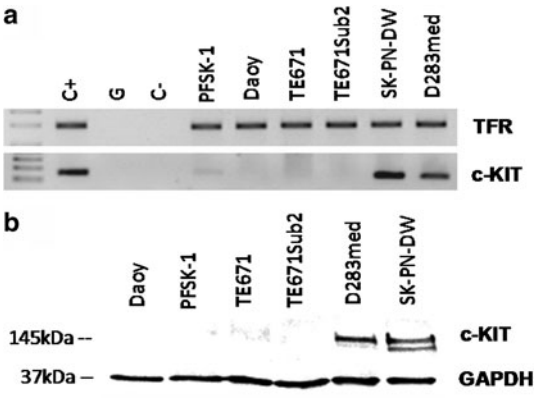

Fig. 1 **a** Expression analysis of the KIT gene by RT-PCR. *C+* Total brain cDNA, *G* genomic DNA. **b** Protein expression by western blot

KIT methylation in cell lines

There are three CpG islands on the first 1,000 bp of the KIT promoter (Fig. 2): island A (238 bp), island B (185 bp) and island C (352 bp). The last one is located closer to exon 1 of the KIT promoter and its sequence consisted of a high content of CpG dinucleotides; therefore, it was not possible to design MCA-Meth primers for this region, and MSP had to be carried out for CpG island C.

Regarding MSP, TE671 and TE671Sub2 cell lines (RMS) appeared to be hypermethylated at CpG island C, whereas Daoy and D283Med (both MB) harbored an unmethylated status. PFSK-1 (cPNET) and SK-PN-DW (pPNET) displayed a partially methylated promoter region. However, the percentage of methylation in these PNET cell lines is opposite: the CpG island of the PFSK-1 cell line is almost totally methylated and the SK-PN-DW one is practically unmethylated (Fig. 2).

Regarding MCA-Meth, similar methylation results were found in CpG islands A and B (Table 1; Fig. 3). Such results were in agreement with those obtained in the CpG island C analysis except for D283Med, which displayed an unmethylated CpG island C and partially methylated CpG islands A and B. Methylation of the CpG island C was greatly correlated to KIT expression levels displayed by the cell lines (Table 1).

KIT re-expression after demethylation treatment

The TE671 and TE671Sub2 cell lines highly expressed KIT after the 5-aza-2′-deoxycytidine treatment (Fig. 4), thereby demonstrating that methylation is responsible for lack of expression in these two cell lines. The expression of the KIT gene after the demethylation treatment was increased fourfold in the PFSK-1 cell line that displayed a partially methylated promoter in the three CpG island analyzed. The expression of KIT in SK-PN-DW and D283Med was similar before and after the demethylation treatment. KIT transcript remained undetectable even after treatment demonstrating that the lack of expression in this cell line was not due to methylation.

KIT expression and methylation in tumor samples

We found that all tumors expressed KIT (Fig. 5). However, we can divide the tumors in three groups according to the results obtained when expression levels where compared between tumors and controls: group 1, consisting of 7 MB (319, 112, 71509, 73, 231, 386 and 348) expressed lower levels of KIT than the two controls (normal cerebellum); group 2, of 2 MB (374, 397) and the cPNET (349), expressed levels similarly to the controls (higher expression levels than control 1 and lower levels than control 2);

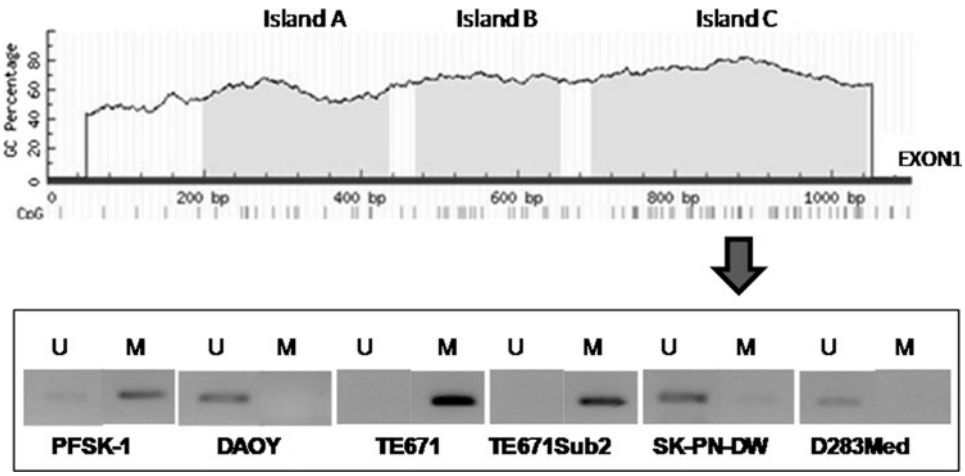

Fig. 2 **a** CpG islands on the first 1,000 bp of the KIT promoter. **b** Methylation status of the KIT promoter on CpG island C by MSP. *U* Unmethylated, *M* methylated

Table 1 Expression and promoter methylation results in KIT promoter in different cell lines analyzed

	PFSK-1	Daoy	TE671	TE671Sub2	SK-PN-DW	D283Med
Island A	PM	U	M	M	PM	PM
Island B	PM	U	M	M	PM	PM
Island C	PM	U	M	M	PM	U
KIT expression	±	−	−	−	+	+

M Methylated, *U* unmethylated, *PM* partially methylated

− No expression, + expression, ± low expression

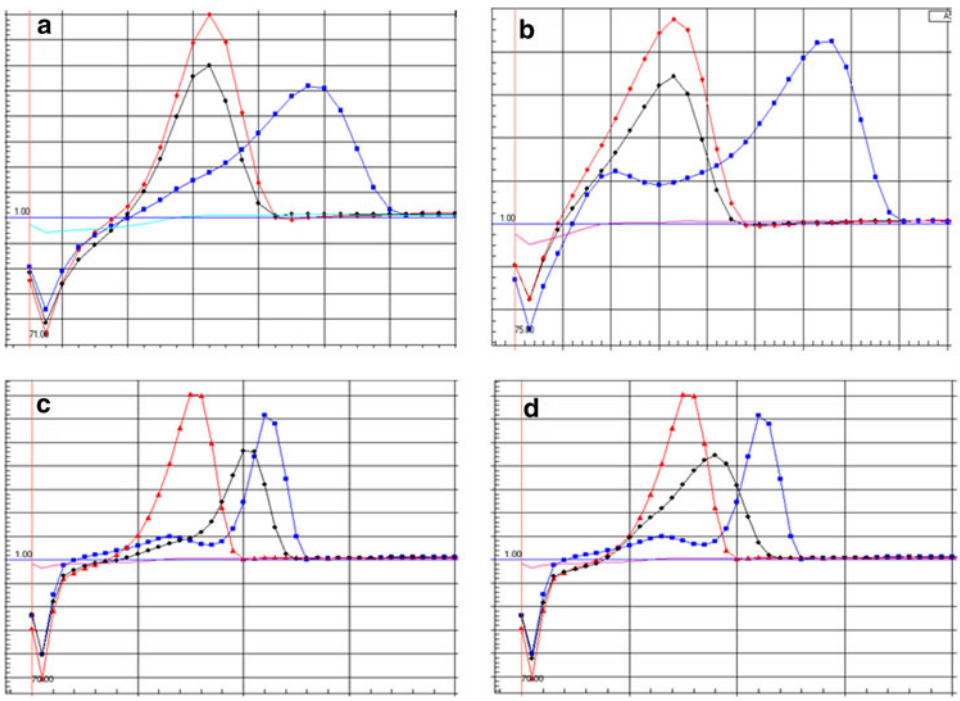

Fig. 3 Methylation analysis of inhibitors of CpG island B (**a,b**) and island A (**c,d**) of the KIT promoter. The *red line* represents the unmethylated control, the *blue line* represents the completely methylated DNA control and the *black line* is the corresponding sample. **a** TE671, **b** TE671Sub.2, **c** PFSK-1, **d** D283Med

and group 3, with 3 MB (376, 429, 304) expressed higher levels of KIT than both controls.

To test whether the down-regulated expression was due to the presence of a methylated KIT promoter, we analyzed CpG island C by MSP in the three tumor samples that displayed the lowest levels of expression of KIT (319, 112, 71509). As shown in Fig. 5, none of the tumor samples analyzed was methylated.

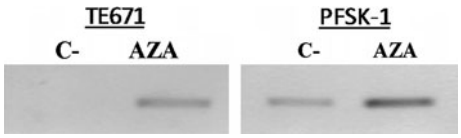

Fig. 4 KIT expression after demethylation treatment in the TE671 and PFSK-1 cell lines. *C-* represents the untreated samples and *AZA* represents the 5-aza-2′-deoxycytidine treated samples

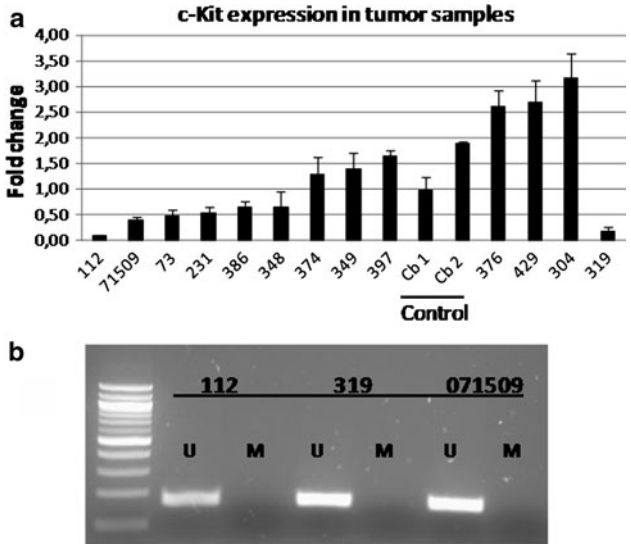

Fig. 5 Results obtained from the tumor samples. **a** KIT expression (*Cb* cerebellum controls). **b** MSP from KIT low-expressing tumors (*U* unmethylated, *M* methylated)

Discussion

KIT is a recognized receptor tyrosine kinase for initiating essential signal transduction pathways involved in the pathogenesis of many cancers. KIT implication in EWS (considered as a pPNET) led us to study its role in other embryonal tumor types with common features, such as cPNETs and MB. Despite their high similarity (undistinguishable in some cases), it is known that cPNET are more aggressive than MB [9]. In fact, some cPNET have received therapies designed for the treatment of high-malignant MB. We determined KIT expression in cell lines derived from both types of tumors, cPNET and MB. We also included two RMS cell lines: TE671 and TE671Sub2. We found that three of the cell lines (PFSK-1, SK-PN-DW and D283Med) expressed the KIT oncogene. Curiously, KIT was expressed in both PNET cell lines (PFSK-1 and SK-PN-DW) and also in the D283Med cell line which is the unique metastatic MB cell line used in this work. Thus, KIT might be associated to a higher aggressiveness of the tumor, being expressed mostly in PNET than in MB. To test this hypothesis, we analyzed the expression of KIT in a small collection of MB and cPNET (12 MB and 1 cPNET) and found that expression is broadly presented in MB, as we could establish three groups of tumors (lower, normal and higher expressing tumors) when comparing KIT expression between tumors and normal cerebellum controls. The only cPNET we could include in our series (349) expressed KIT at a level in between the levels of the two controls. Among the 12 MB, expression was very variable, and only in 3 of them (25%) was KIT overexpression manifested.

Chilton-Macneill et al. [15] examined KIT expression in 10 MB and looked for mutations in exons 9, 11 and 13. They observed that 9 of 10 MB tumors expressed KIT; however, no mutations were found. Amplification of the KIT oncogene has been found in some MB and cPNET in relation to a poor prognosis [16]. KIT expression could be a therapeutic advantage, maybe making possible the treatment of these tumors with tyrosine kinase inhibitors, such as imatinib mesylate [17, 18].

With regard to the regulation of this oncogene, little is known about the control of KIT expression. Yamamoto et al. [19] investigated the human KIT promoter, concluding that only the proximal 200 bp of the promoter was needed for cell-specific expression. Subsequently, Vandenbark et al. [20] discovered that there were several activator and repressor elements that regulated KIT expression by binding to a much bigger promoter sequence than 200 bp, more specifically to 5,500 bp. More than regulation by protein interactions, CpG island hypermethylation seems to be an alternative mechanism for gene inactivation. There are three CpG islands on the first 5,500 bp of the KIT promoter; all of them are located in the 800 bp closer to the exon 1 of the KIT gene. We investigated the possible role of the three CpG islands on KIT methylation and inactivation. A consistent correlation was found between KIT methylation status of the CpG island located closer to the exon 1 of the KIT gene and the expression pattern in all the cell lines except Daoy which displayed an unmethylated promoter and, even so, was not expressed. A repressing mechanism, different from promoter methylation, might be acting in the Daoy cell line.

Our results suggest that the lack of KIT expression by TE671 and TE671Sub2 cell lines and the low expression detected in PSFK-1 may be due to methylation, because the demethylation treatment produced the re-expression in TE671 and TE671Sub2 RMS cell lines and a fourfold increase of expression in PFSK-1 cPNET cell line. KIT promoter hypermethylation might also be an important epigenetic regulatory mechanism in tumors. In order to elucidate this hypothesis, we tested the promoter methylation status in three tumors expressing the lowest amount of KIT, finding that none of them were methylated. We also performed the MSP analysis on 10 additional MB

tumors (from which RNA was not available) obtaining the same answer. These results do not support an implication of KIT promoter methylation in MB tumorigenesis; however, additional studies, including more PNET and RMS are needed to confirm or refute any implication of KIT hypermethylation in these tumors.

KIT hypermethylation seems to be a widespread event in cell lines since we have found it in some RMS and PNET, and in glioblastoma and neuroblastoma cell lines (data not shown). Whether this is a tissue culture artifact or a true condition of some tumors waits for further confirmatory studies.

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