

Protein Expression of the Matrix Metalloproteinase (MMP-1, -2, -3, -9 and -14) in Ewing Family Tumors and Medulloblastoma of Pediatric Patients

Elvis Cueva Mateo^{1*}, Fábio J. Nascimento Motta², Rosane G.P. Queiroz¹, Carlos A. Scirileli¹ and Luiz G. Tone¹

¹Division of Pediatric Oncology, Department of Pediatrics, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil

²Department of Genetics, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil

Abstract

The matrix metalloproteinases (MMP) are endopeptidases performing proteolytic functions in the extracellular matrix and their overexpression has been suggested to be a characteristic of malignant tumors. Molecular changes such as the presence of chimeric protein EWS-FLI1 in the Ewing family of tumors (EFT) and the oncogenes *C-ERBB-2*, *N-MYC*, *C-MYC* in medulloblastomas (MB) promote the overexpression of MMP. In the present study, protein expression of MMP -1, -2, -3, -9 and -14 was qualitatively evaluated in 17 EFT and MB samples of children and adolescent by Western Blotting and optical densitometry, and the level of gene expression of some MMPs was determined by RQ-PCR. Five MB samples (45.4%) presented expression of the 5 MMPs and 6 samples (54.6%) presented expression of at least one of them. Four EFT samples (66.6%) presented expression of MMP-2, -9 and -14, and two samples (33.4%) presented expression of at least one of these MMPs, whereas the presence of MMP-1 and -3 was not observed. Gene analysis showed that *MMP-2* had a high expression in MB, while the expression of *MMP-9* and *MMP-14* was higher in EFT. It has been established that the expression of the MMPs might be related to a complex pathway of gene regulation.

Keywords: Matrix metalloproteinase; Ewing family of tumors; Medulloblastomas; Protein expression

Introduction

The matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent endopeptidases capable of degrading all extracellular matrix (ECM) components, a complex net of proteins and carbohydrate polymers that are secreted by connective tissue cells [1], and are expressed by fibroblasts, endothelial and tumor cells, and a variety of immune system cells [2]. The MMP family is divided into 5 subfamilies, i.e., the collagenases, gelatinases, stromelysins, matrilysins, and membrane type metalloproteinases (MT-MMP), for a total of approximately 25 human MMPs already described and new members that have not yet been investigated [3]. The levels of MMP expression are relatively low in normal conditions, whereas their expression is increased by determined physiologic conditions such as ECM remodeling during embryogenesis, tissue repair, and bone remodeling. The increased MMP expression is equally observed in several diseases, particularly in arthritis and neoplasms. Conversely, individual members of the MMP family are strictly regulated and their expression is specific for the type of tissue, though the regulation of MMPs can occur in three different ways: 1) regulation of MMP gene transcription, 2) proenzyme activation, and 3) inhibition by the action of tissue inhibitor of metalloproteinases (TIMPs) [4].

The Ewing family of tumors (EFT) represents a wide group of primitive neuroectodermal neoplasms. According to the degree of neural differentiation, they are named Ewing sarcomas (ES) when the tumor is undifferentiated or primitive neuroectodermal tumors (PNET) when they present characteristics of neural differentiation. The EFT is common in children and adolescents and primarily involves the bones, but may also affect the adjacent soft tissue [5]. The majority of EFT are characterized by a specific chromosomal translocation, t(11;22)(q24;q12). The fusion of the *EWS* gene, located at 22q12, with the *FLI1* gene, locus 11q24, results in the formation of the chimeric gene *EWS-FLI1* [6]. The *FLI1* gene belongs to a family of transcription factors ETS (Erythroblastic Transforming Sequence) implicated in cell proliferation, tumor development and gene control. Other members of the *ETS* gene family, such as *ERG*, *ETV1* and *E1AF* may also be combined with the

EWS gene in t(21;22), t(7;22) and t(17;22) translocations, respectively [7].

The two possible mechanisms by which the chimeric transcript may contribute to the biology of the tumor are: 1) primarily, the *EWS-FLI1* transcript can alter the transcription of some genes which are normally regulated by *FLI1* or 2) it can affect genes other than those regulated by *FLI1* [8]. It is estimated that the chimeric transcripts *EWS-FLI1* may activate the transcription of the MMP genes. Stromelysin 1 (MMP-3) has been proposed as a target of *EWS-FLI1* in studies of representational difference analysis [9]. The promoters of human collagenase 1 (MMP-1) and stromelysin 1 (MMP-3) have been reported to be activated by *ETS* family genes [10]. Additionally, *E1AF* can transactivate *MMP-1*, *MMP-3* and gelatinase B (*MMP-9*) [11]. Unexpectedly, the Ewing Sarcoma cells do not express *MMP-1* or *MMP-3*. This absence of expression seems to be a result of the loss of accessibility to the *ETS* recognition sites in the promoting region of these MMPs. Nonetheless, these cells express high levels of gelatinase B (*MMP-2*), *MMP-9* and type 1 membrane MMP (*MMP-14*) [12].

The medulloblastomas (MBs) are highly malignant neoplasms composed of undifferentiated neuroepithelial cells in the cerebellum. Of all central nervous system (CNS) neoplasms, they are the tumors with the highest probability of extraneural dissemination, especially to the bone, which represents over 80% of the metastases

***Corresponding author:** Elvis Cueva Mateo, Division of Pediatric Oncology, Department of Pediatrics, School of Medicine of Ribeirão Preto, University of São Paulo. Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, SP, Brazil, Tel: 55 16 36022671; Fax: 55 16 36022297; E-mail: ecuevamateo@yahoo.es

Received September 23, 2011; **Accepted** November 12, 2011; **Published** November 13, 2011

Citation: Mateo EC, Nascimento Motta FJ, Queiroz RGP, Scirileli CA, Tone LG (2011) Protein Expression of the Matrix Metalloproteinase (MMP-1, -2, -3, -9 and -14) in Ewing Family Tumors and Medulloblastoma of Pediatric Patients. *Pediatr Therapeut* S1:001. doi:10.4172/2161-0665.S1-001

Copyright: © 2011 Mateo EC, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

[13]. The main cytogenetic change found in MBs is the presence of isochromosome17q[i(17q)], which has been observed in 30 to 50% of cases [14]. The loss of heterozygosity of 17p has also been reported, especially involving the 17p13 region that holds the gene *TP53* locus [15]. Some oncogenes such as *C-ERBB-2*, *N-MYC*, *C-MYC* may possibly contribute to tumorigenesis by regulating MMP expression [16]. Other studies about the pattern of MMP expression in MBs have revealed an increased expression of MMP-2, -9, -3, -10, -14 and -15 in these tumors [17,18]. Due to the relevance of the participation of these enzymes in neoplastic processes, the objectives of the present study were: 1) to qualitatively evaluate the protein expression of MMPs: collagenase 1 (MMP-1), gelatinase A (MMP-2), stromelysin 1(MMP-3), gelatinase B (MMP-9), and type 1 membrane MMP (MMP-14) in EFT and MB samples from children and adolescents by Western Blotting and to observe the level of gene expression of some MMPs by RQ-PCR, 2) to quantify the protein expression of MMPs by optic densitometry.

Materials and Methods

Tumor samples

Seventeen MB and EFT tumor samples of children and adolescents were obtained from the tumor bank laboratory of Pediatric Oncology, University Hospital, School of Medicine of Ribeirão Preto, University of São Paulo. The 17 tumor samples studied presented the following distribution: 11 medulloblastomas (MB), 5 of which from girls and 6 from boys aged 2 to 14 years, and 6 Ewing family tumors (EFT) from 4 girls and 2 boys aged 5 to 17 years. The samples were collected at diagnosis, during the period from May 1996 to March 2005. The study was analyzed and approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP), protocol number 6055/2004.

Western blotting

The proteins of each sample were extracted with Trizol[®] reagent (Invitrogen, Carlsbad, CA, USA), and quantified by the method of Bradford, as described in the guide for protein quantification of Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Twenty µg of each sample was denatured and then submitted to electrophoresis to separate the proteins according to molecular weight. The proteins were then transferred to a nitrocellulose membrane (Amersham Biosciences) and immunodetection was performed using mouse monoclonal anti-MMP-1, anti-MMP-3, and anti-MMP-14 antibodies (Onocogene Research Products) and mouse monoclonal anti-MMP-2 and anti-MMP-9 antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The secondary antibody in the anti-mouse monoclonal form was then added (Amersham Biosciences). The membranes were submitted to radiographic labeling in a dark chamber with the ECL kit (Amersham Biosciences) and left in contact with the solution and with the X-rays for 3 minutes.

Optical densitometry

Optical densitometry was performed with a computerized densitometer (GS 800 Calibrated Densitometer, BioRad, Hercules, CA, USA). The images were digitized and analyzed with reading software (Quantity One Quantitation Software, BioRad) and the number of pixels/mm² was determined. This number is directly related to the concentration of labeled proteins.

Quantitative polymerase chain reaction(RQ-PCR)

Total RNA was extracted with Trizol[®] reagent (Invitrogen, Carlsbad,

CA, USA), and cDNA was then synthesized using a High Capacity Kit (Applied Biosystems, Foster City, CA, USA). All procedures were performed according to the manufacturer's specifications. The mRNA expression of *MMP-2*, *-9*, *-14* and the endogenous control gene β -glucuronidase (*GUS β*) was quantified by real-time PCR with the Gene Amp[®] 7500 sequence detection system (Applied Biosystems). The reactions were carried out using the TaqMan Universal PCR Master Mix Kit optimized for reaction with the TaqMan probe containing AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Blank and standard controls were run in parallel to verify the amplification within each experiment. The calibration curves were elaborated in quadruplicate from serial dilutions (1:1, 1:10, 1:100, 1:1000) of cDNA derived from the lineages. Dilutions of cDNA of the glioblastoma cell line U343 were used to construct the calibration curves of the *MMP-14* gene and dilutions of the cell line GLC4/Adr were used for the *MMP-2* gene. A normal medulla sample (MO 020) was used for the *MMP-9* gene. The *GUS β* gene was used as housekeeping or endogenous reference. Since it is similarly expressed in all cells, the *GUS β* gene is used as a control for material validation and normalization of cDNA concentration in different samples [19].

Statistical analysis

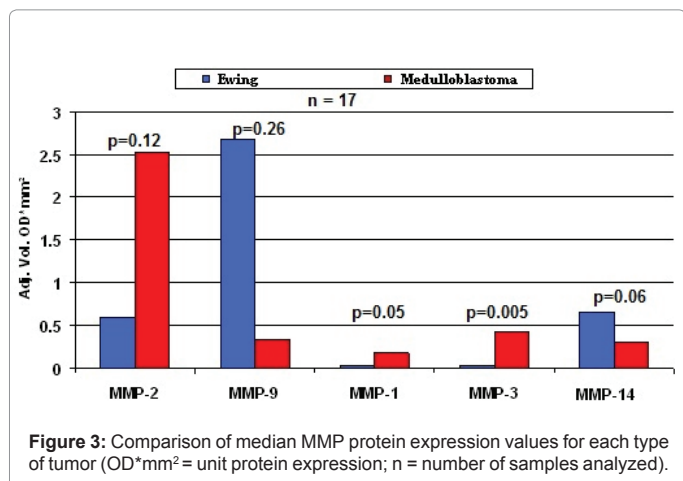
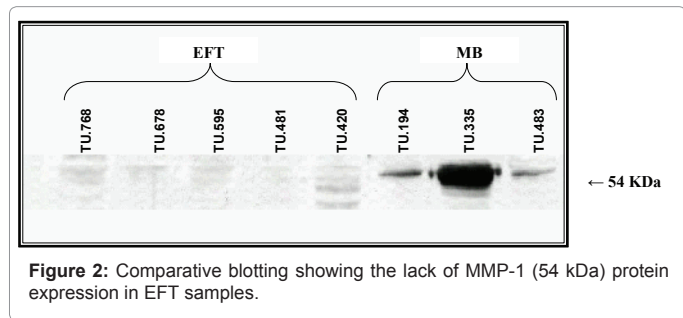
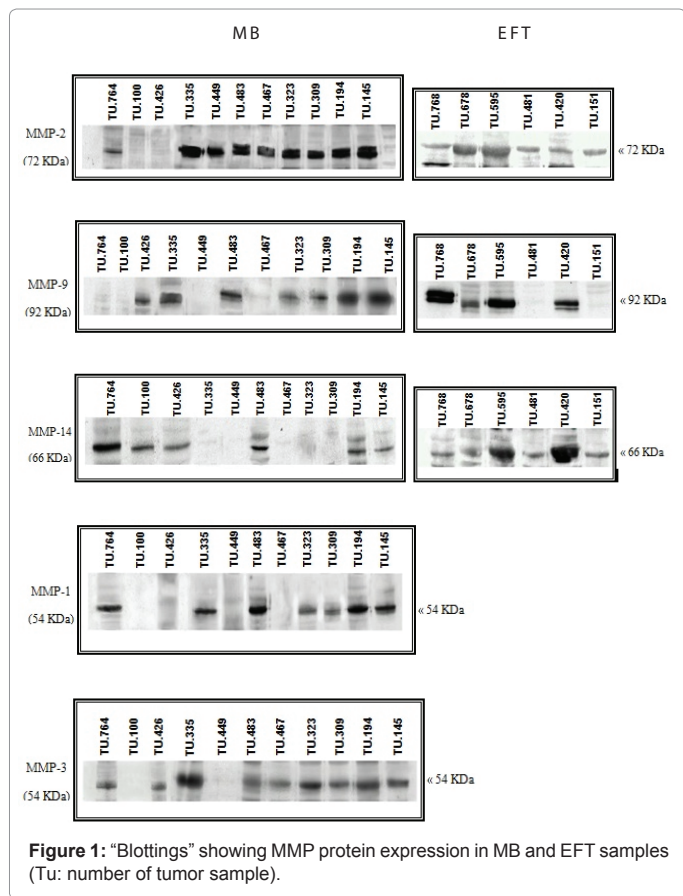
The nonparametric Mann-Whitney test was used to compare the MMP expression values for each type of tumor. The statistical tests were carried out using the GraphPad Prism Software, version 4.0 (GraphPad Prism Software, San Diego, CA, US). The level of significance was considered to be $P < 0.05$.

Results

Western blotting analysis revealed that the 5 analyzed MMPs presented a differential pattern of protein expression depending on the type of tumor. In MB, 5 samples (45.4%) expressed the 5 MMPs and 6 samples (54.6%) expressed at least one of them. In EFT, expression of MMP-2, -9, and -14 was observed in 4 samples (66.6%), and the expression of at least one of these MMPs was detected in the other 2 (33.4%) (Figure 1). MMP-1 and -3 expression was not observed in any EFT sample (Figure 2). The comparative expression obtained by the densitometry analysis for the 2 types of tumors revealed that MMP-2 presented a higher level of expression in the MBs; although the difference was not significant ($p=0.12$). MMP-9 and -14 presented the highest levels of expression in EFT ($p=0.26$ and $p=0.06$ respectively). MMP-1 and MMP-3 were identified only in the MBs; and this difference was significant ($p=0.05$ and $p=0.005$, respectively) (Figure 3) (Table 1). RQ-PCR revealed that the expression profile of the *MMP-2* gene was higher in MBs than in EFT; although the difference was not significant ($p=0.36$). The *MMP-9* and *MMP-14* genes presented a significantly higher level of expression in EFT ($p=0.04$ and $p=0.01$, respectively) (Figure 4) (Table 2).

Discussion

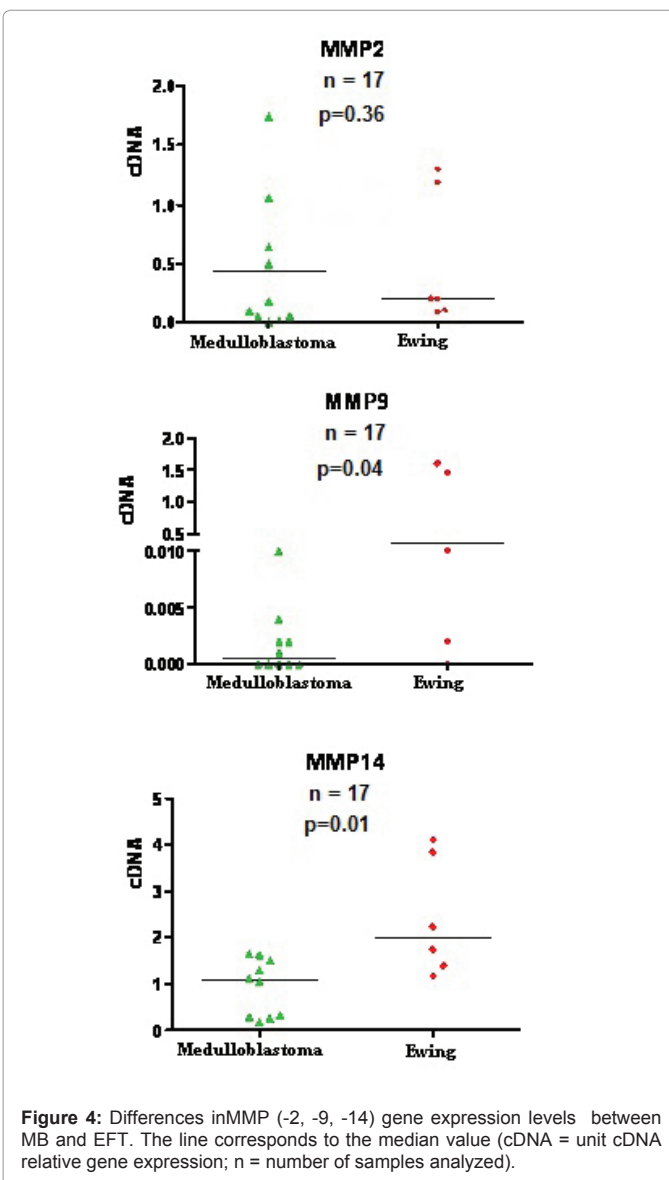
Degradation of the extracellular matrix creates space for the growth of the tumor mass, facilitates intra-tumor angiogenesis and reduces the adherence of the cells and the matrix components, thus facilitating the invasion of subjacent tissues and the process of tumor metastasis [20-22]. Numerous studies have demonstrated high levels of MMP expression in malignant tumors compared to normal tissues and have proposed proposing its participations in the process of tumor invasion [20,21]. Previous studies using the northern blot technique, immunohistochemistry and densitometry found higher levels of MMP-2, -9 -1 -3, -7, -14, and -15 in MB samples [18]. In our study, 5



	MB patients		EFT patients		P
	mean	median	mean	median	
MMP-1	1,38	0.18	0.04	0.04	0.05*
MMP-2	2,41	2,52	0.70	0.60	0.12
MMP-3	0.88	0.43	0.04	0.04	0.005*
MMP-9	1,33	0.33	5,04	2,68	0.26
MMP-14	0.38	0.30	0.94	0.66	0.06

*: significant P

Table 1: Median and mean values of MMP (-2, -9, -14) protein expression (OD*mm²) in MB and EFT.



MB samples showed expression of all MMPs and 6 showed expression of at least one enzyme, with a high expression of MMP-2 and -9. It is known that MMP-2 and -9 differ from the other MMPs for their ability to interact in the form of proenzymes with the other MMPs for their activation [23]. Moreover, other studies on MBs have established the participation of the oncogenes *C-ERBB-2*, *N-MYC* and *C-MYC* in the

	MB patients			EFT patients			P
	mean	median	P25-P75	mean	median	P25-P75	
MMP-2	0.432	0.133	0.340-0.849	0.512	0.204	0.098-1,235	0.3676
MMP-9	0.001	0.0005	0.000-0.003	0.5420	0.094	0.001-1,531	0.0420*
MMP-14	0.925	1,087	0.270-1,563	2,415	1,990	1,282-3,975	0.0110*

P25-P75: 25th to 75th percentile; *: significant P

Table 2: Median and mean values of MMP (-2, -9, -14) gene expression (cDNA relative gene expression) in MB and EFT.

regulation of MMP expression [24]. These data may explain the high levels of MMP-2 and -9 compared to the other MMPs in our study.

Hiroki [12] using the northern blot technique, showed that Ewing sarcomas express MMP-2 and MMP-9, but surprisingly do not express MMP-1 or MMP-3. In our study we found that 4 EFT samples expressed MMP-2, MMP-9 and MMP-14 and 2 samples expressed at least one of these MMPs. None of the samples showed the expression of MMP-1 or MMP-3. Besides being of pathologic significance, the presence of MMP-2, MMP-9 and MMP-14 in the EFT samples is related to the possibility that the genes from the MMPs are activated by the product of the chimeric gene *EWS-FLI1*. The regulation of MMP gene expression has been studied using mRNA and the participation of members of the family of transcription factors (ETS), for example FLI1, in MMP activation has been demonstrated. The FL1 protein activates the MMPs through its affinity for regulatory elements such as, AP-2, AP-1, PEA3, found in the promoter region [6]. Our results were consistent with these studies and could be interpreted on the basis of these mechanisms of differential MMP activation. The loss of expression of MMP-1 and MMP-3 in Ewing sarcoma is an unexpected result because these genes also present regulatory elements and would be expected to be activated by the chimeric protein EWS-FLI1. To prove this lack of expression of MMP-1 and MMP-3 in the EFT samples, we performed a comparative “blotting”, in which we used MB samples known to present high levels of expression and EFT samples. The result confirmed the lack of expression of MMP-1 and -3. Hiroki [12] explained that there is a loss of accessibility of the chimeric protein to the regulatory elements of these MMP genes. This loss could be explained by two mechanisms: 1) high chromosomal condensation of the promoter region, which would exclude the access of the chimeric protein, and 2) aberrant methylation of the promoter region, which would also exclude the transcription of these two MMPs.

The histological analysis of the MBs and EFT suggests that they have a common origin, i.e. the PNET [25]. Based on this characteristic, it is possible that these tumors present similar patterns of MMP expression. In the present study, we analyzed the levels of protein expression of 5 MMPs by comparing the median values of MMP expression between the two types of tumors. MMP-2 presented a higher level of expression in MBs, in contrast to what was observed in the EFT. MMP-9 and MMP-14 presented higher expression levels in the EFT. Alternatively, MMP-1 and MMP-3 were detected only in the MBs. These data show that the MMP expression profile has a different behavior according to the type of tissue, indicating that the expression of MMPs may be related to a complex gene regulation pathway in both tumors. The results of the gene expression profile analysis of some MMPs were consistent with the protein expression findings; although in this analysis the *MMP-2* gene was more expressed in MBs than in the EFT, while *MMP-9* and *MMP-14* were more expressed in the EFT. Elevation in the *MMP-2* and *MMP-9* expression rates and its clinical-biologic implications in other tumors have been reported in several studies [26-29]. It is important to mention that, despite our expressive results, the number of samples

analyzed was limited (particularly the EFT samples) and the level of statistical significance was not achieved.

References

- Giancotti FG, Ruoslahti E (1999) Integrin signaling. *Science* 285: 1028-1032.
- Curran S, Murray GI (2000) Matrix metalloproteinases: molecular aspects of their roles in tumor invasion and metastasis. *Eur J Cancer* 36: 1621-1630.
- Pei D, Kang T, Qi H (2000) Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. *J Biol Chem* 275: 33988-33997.
- Yong VW, Krekoski CA, Forsyth PA, Bell R, Edwards DR (1998) Matrix metalloproteinases and diseases of the CNS. *Trends Neuroscience* 21: 75-80.
- Zamora P, García de Paredes ML, González Barón M, Díaz MA, Escobar Y, et al. (1986) Ewing's tumor in brothers. An unusual observation. *Am J Clin Oncol* 9: 358-360.
- May WA, Lessnick SL, Braun BS, Klemsz M, Lewis BC, et al. (1993) The Ewing's sarcoma *EWS/FLI-1* fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than *FLI-1*. *Mol Cell Biol* 13: 7393-7398.
- Urano F, Umezawa A, Hong W, Kikuchi H, Hata J (1996) Novel chimera gene between *EWS* and *E1A-F*, encoding the adenovirus *E1A* enhancer-binding protein, in extraosseous Ewing sarcoma. *Biochem Biophys Res Commun* 219: 608-612.
- de Alava E, Panizo A, Antonescu CR, Huvos AG, Pardo-Mindán FJ, et al. (2000) Association of *EWS-FLI1* type 1 fusion with coger proliferative rate in Ewing's sarcoma. *Am J Pathol* 156: 849-855.
- Braun BS, Frieden R, Lessnick SL, May WL, Denny CT (1995) Identification of target genes for the Ewing sarcoma *EWS/FLI1* fusion protein by representational difference analysis. *Mol Cell Biol* 15: 4623-4630.
- Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, et al. (1998) The *Ets-1* and *Ets-2* transcription factors activate the promoters for invasion associated urokinase and collagenase genes in response to epidermal growth factor. *Int J Cancer* 77: 128-137.
- Higashino F, Yoshida K, Noumi T, Seiki M, Figinaga K (1995) *Ets*-related protein *E1A-F* can activate three different matrix metalloproteinases gene promoters. *Oncogene* 10: 1461-1463.
- Yabe H, Fukuma M, Urano F, Yoshida K, Kato S, et al. (2002) Lack of matrix metalloproteinase (MMP)-1 and -3 expression in Ewing sarcoma may be due to loss of accessibility of the MMP regulatory element to the specific fusion protein in vivo. *Biochem Biophys Res Commun* 293: 61-71
- Potter R, Czech TH, Dieckmann K, Slavc I, Winberger-Prayer D, et al. (1998) Tumor of the central nervous system. In: Voute, PA, Kalifa C, Barrett A. *Cancer in Children: clinical management*. 4thed. New York: Oxford University Press 12: 170-184.
- Goussia AC, Bruner JM, Kyritsis AP, Agnantis NJ, Fuller GN (2000) Cytogenetic and molecular genetic abnormalities in primitive neuroectodermal tumors of the central nervous system. *Anticancer Res* 20: 65-73.
- Biegel JA, Rorke LB, Janss AJ, Sutton LN, Parmiter AH (1995) Isochromosome 17q demonstrated by interphase fluorescence in situ hybridization in primitive neuroectodermal tumors of the central nervous system. *Genes Chromosome Cancer* 14: 85-96.
- Giunciuglio D, Culty M, Fassina G, Masiello L, Melchiori A, et al. (1995) Invasive

- phenotipo of MCF10A cells overexpressing c-Ha-ras and c-erbB-2 oncogenes. *Int J Cancer* 63: 815-822.
17. Ozlem O, Bjarne K, Bernhard H, Arnulf P, Hans K, et al. (2004) Expression of matrix metalloproteinases and their inhibitors in medulloblastomas and their prognostic relevance. *Clin Cancer Res* 10: 4746-4753.
 18. Vince GH, Herbold C, Klein R, Kuh J, Pretsch T, et al. (2001) Medulloblastoma displays distinct regional matrix metalloproteinase expression. *J Neurooncol* 53: 99-106.
 19. Scrideli CA, Cazzaniga G, Fazio G, Pirola L, Callegaro A, et al. (2003) Gene expression profile unravels significant differences between childhood and adult Ph+ acute lymphoblastic leukemia. *Leukemia* 17: 2234-2237.
 20. Vihinen P, Kahary VM (2002) Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *Int J Cancer* 99: 157-166.
 21. Poincloux R, Lizarraga F, Chavrier P (2009) Matrix invasion by tumor cells: a focus on MT1-MMP trafficking to invadopodia. *J Cell Sci* 122: 3015-3024.
 22. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, et al. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2: 737-744.
 23. Okada A, Belloq JP, Rouyer N, Chenard MP, Rio MC, et al. (1995) Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast and head and neck carcinomas. *Proc Natl Acad Sci USA* 92: 2730-2734.
 24. Batra SK, McLendon RE, Koo JS, Castelino-Prabhu S, Fuchs HE, et al. (1995) Prognostic implications of chromosome 17p deletions in human medulloblastomas. *J Neurooncol* 24: 39-45.
 25. Bayani J, Zielenska M, Marrano P, Kwan K, Taylor MD, et al. (2000) Molecular cytogenetic analysis of medulloblastoma and supratentorial primitive neuroectodermal tumors by using conventional banding, comparative genomic hybridization, and spectral karyotyping. *J Neurosurg* 93: 437-448.
 26. Kunishio K, Okada M, Matsumoto Y, Nagao S (2003) Matrix metalloproteinase-2 and -9 expression in astrocytic tumors. *Brain Tumor Pathol* 20: 39-45.
 27. Roebuck MM, Helliwell TR, Chaudhry IH, Kalogrianitis S, Carter S, et al. (2005) Matrix metalloproteinase expression is related to angiogenesis and histologic grade in spindle cell soft tissue neoplasm of the extremities. *Am J Clin Pathol* 123: 405-414.
 28. Cho YB, Lee WY, Song SY, Shin HJ, Yun SH, et al. (2007) Matrix metalloproteinase-9 activity is associated with poor prognosis in T3-T4 node-negative colorectal cancer. *Hum Pathol* 38: 1603-1610.
 29. Motta FJN, Valera ET, Lucio-Eterovic AKB, Queiroz RGP, Neder L, et al. (2008) Differential expression of E-cadherin gene in human neuroepithelial tumors. *Genetic and Molecular Research* 7: 295-304.

This article was originally published in a special issue, **Pediatric Oncology: Diagnosis and Therapy** handled by Editor(s). Dr. Anna Petryk, University of Minnesota, USA