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

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Abstract

The matrix metalloproteinases (MMPs) 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 RT-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, although 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 primary 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 factorsETS (ErythroblasticTransformic 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 promoter region of these MMPs. Nonetheless, these cells express high levels of gelatine 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...
concentration of labeled proteins. The number of pixels/mm² was determined. This number is directly related to the (Quantity One Quantitation Software, BioRad) and the number of densitometer (GS 800 Calibrated Densitometer, BioRad, Hercules, CA, USA). Optical densitometry was performed with a computerized densitometer (GS 800 Calibrated Densimeter, BioRad, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane (Amersham Biosciences) and left in contact with the membranes were submitted to radiographic labeling in a dark chamber. The secondary antibody in the anti-mouse monoclonal anti-MMP-1, anti-MMP-3, and anti-MMP-14 antibodies (Santa Cruz Biotechnology, CA, USA). The proteins were then transferred to a nitrocellulose membrane (Amersham Biosciences) and left in contact with the membrane to observe the level of gene expression of some MMPs by RQ-PCR. 2) to quantify the protein expression of MMPs by optical 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 (Onogene 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 Densimeter, BioRad, Hercules, CA, USA). The images were digitized and analyzed with reading software (Quantity One Quatification 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) 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 gene was used as housekeeping or endogenous reference. Since it is similarly expressed in all cells, the GUS gene is used as a control for material validation and normalization of cDNA concentration in different samples.

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 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
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. FLI 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


