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TRANSCRIPTION ANALYSIS OF *TIMP-1* AND *NM23-H1* GENES IN GLIOMA CELL INVASION

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ABSTRACT - *Purpose:* To evaluate using transcription analysis the presence and importance of two genes: *NM23-H1* and *TIMP-1* on control of tumor cell invasion in diffuse astrocytomas (WHO II) and *glioblastoma multiforme* (WHO IV). *Method:* Northern blot analysis of *NM23-H1* and *TIMP-1* was performed. Eight diffuse astrocytomas and 19 glioblastomas (WHO IV) were analyzed to determine if *TIMP-1* and *NM23-H1* were candidates to inhibition of tumor cell invasion quantitated RNA levels. The samples were collected directly from operating room. Total cellular RNA was extracted from frozen tissue samples using guanidinium-isothiocyanate and cesium chloride gradients. Total RNA (10 μg per sample) from tumor tissue were size fractionated through 1% agarose-formaldehyde gel and transferred to nylon filters and then hybridized to ^{32}P-labeled DNA probes and placed for autoradiography. Levels of specific RNAs were determined by computer-assisted laser densitometry. Blot filters were sequentially hybridized to nm23 and *TIMP-1* probes in addition to GAPDH, as a control. Statistical analyses were carried out according to t-test for equality of means. *Results: NM23-H1* were detected in each sample, however it did not correlate with malignancy and invasiveness. On the other side *TIMP-1* gene expression showed a clear correlation between low expression and invasiveness. *Conclusion:* The data suggest that *TIMP-1* is an inhibitor of high grade gliomas invasion. *NM23-H1* was present in the entire gliomas sample, but it did not vary in diffuse astrocytomas and glioblastomas.

KEY WORDS: tumor cell invasion, *TIMP-1, NM23-H1,* RNA levels, gliomas.

Análise transcricional dos genes *TIMP-1* e *NM23-H1* na invasão celular em astrocitoma difuso e glioblastoma multiforme

RESUMO - *Objetivo:* Comparar através da análise da expressão dos níveis de RNA, a presença e a relevância dos genes *NM23-H1* e *TIMP-1* no controle da invasão celular tumoral dentro do tecido cerebral normal em: astrocitoma difuso (OMS II) e glioblastoma multiforme (OMS:IV). *Método:* Análise em "Northern blot" dos genes *NM23-H1* e *TIMP-1.* Oito astrocitomas fibrilares difusos (OMS II) e 19 glioblastomas multiformes foram analisados para determinar se *TIMP-1* e *NM23-H1* estavam relacinados à inibição da invasão tumoral nas neoplasias do sistema nervoso central, quantificando os níveis de RNA dos respectivos genes extraídos diretamente dos tumores. 10 μg por amostra de RNA total foram fracionados de gel de formaldeído e transferidos para os filmes de hibridação. Níveis específicos de RNAs foram determinados na espectrofotometria. Valores das razões entre *NM23-H1*/*GAPDH* e *TIMP-1*/*GAPDH* foram submetidos à análise de variabilidade das médias. *Resultados:* A análise da expressão do gene *TIMP-1* mostrou supressão em tumores gliais malignos. *Conclusão:* Os resultados indicam que existe relação direta entre níveis baixos de *TIMP-1* e malignidade dos gliomas. O gene *NM23-H1* foi detectado em todas as amostras, mas não foi possível relacionar sua subexpressão ou superexpressão com algum fenótipo de invasividade.

PALAVRAS-CHAVE: invasão celular tumoral, *TIMP-1, NM23-H1,* níveis de RNA, gliomas.

Tumor cell invasion into normal brain is a major component of brain tumor malignancy. In contrast to other tumors, brain tumors are often invasive, but rarely metastasize to distant sites. An understanding of the genes which control tumor cell invasion will allow the development of therapeutic regimens designed to counteract this complex property. Although there are reports of genes that may modulate the ability of a brain tumor cell to invade adjacent normal brain, the molecular control of brain tumor inva-

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siveness is still not well-defined. Specific secreted proteases including heparinases plus serine-,thiol- and metal-dependent proteinases have been implicated in tumor cell invasion[1].

The most well-studied of these enzymes are the metalloproteinases. Metalloproteinases are a family of protein-degrading enzymes that digest components of the extracellular matrix (ECM), the supporting material that functions to cement cells in place within tissues. These proteins have been implicated in wound healing, uterine, involution, embryogenesis, tissue remodeling, angiogenesis, parasite and bacterial invasion, rheumatoid arthritis and tumor cell invasion. The metalloproteinases are sub grouped into three categories: interstitial collagenases, type IV collagenases and stromelysins (transin). These enzymes are secreted as inactive proenzyme forms and must be activated by removal of an amino terminal fragment. Left unchecked, the activated metalloproteinases would destroy the structural integrity of tissues. Cells have involved mechanisms to protect themselves and their surroundings by excreting proteins that inactivate the metalloproteinases. Important representatives of this group of proteins are the tissue inhibitors of metalloproteinases *(TIMP). TIMP-1, TIMP-2, TIMP-3* and *TIMP-4* genes have been isolated and their role in tumor and normal cell invasion is being investigated[2-4]. The production of *TIMP* in a small sample of cultured brain tumor explants and cell lines has been examined in tumor tissues, normal brain, low passage number tumor-derived cell lines and cultured normal astrocytes[5]. Another gene that could function to inhibit tumor cell invasion is the *NM23-H1.* This gene is expressed at higher levels in tumor tissues derived from primary site melanomas and breast carcinomas compared to distant metastases[6-8]. Although a direct functional role for this gene in the establishment of metastases has not yet been experimentally determined, it appears that reduced levels of gene product could aid metastatic spread. It is unclear if the gene is involved in tumor cell invasion or in seeding of invaded tumor at distant sites. The cloned human *NM23-H1* gene encodes nucleoside diphospate (NDP) kinase A[9] and has a nucleic acid sequence homology to NDP-kinase from *Drosophila melanogaster.* NDP kinase functions in the synthesis of nucleotide triphosphates others than ATP.

Since *NM23-H1* could be involved in tumor cell invasion inhibition, we have examined the levels of *NM23-H1* in diffuse astrocytomas and glioblastoma multiforme (GMB).

METHOD

Tumor samples – 27 tumor tissues were removed from patients undergoing neurosurgical procedures at the Neurological Institute of Columbia University. A portion of the tumor was sent for histopathological diagnosis by a neuropathologist. Detailed reports of the tumor specimen were the basis for tumor classification. Another portion was snap-frozen in liquid nitrogen and stored at –70°C for subsequent molecular characterization. A subset of tumors was divided into a third portion and these tissues were established as tumor-derived cell lines. Cells for culture were dispersed in trypsin: EDTA and grown in plastic tissue culture dishes in Dulbecco's minimal essential medium containing 20% fetal bovine serum.

Extraction of RNA – Total celular RNA and DNA were simultaneously extracted from frozen tissue samples using guanidinium-isothiocyanate and cesium chloride gradients

Northern blot – Total RNA (10 μg per sample) from tumor tissues were size-fractionated through 1% agarose-formaldehyde gels and transferred to Amersham nylon filters. RNA immobilized on the filters was hybridized to 32P-labeled DNA probes and placed next to X-ray film for autoradiography. Levels of specific RNAs were determined by computer-assisted laser densitometry (Molecular Dynamics, Inc.) of the autoradiographs. Blot filters were sequentially hybridized to radiolabeled *NM23-H1* and *TIMP-1* probes in addition to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* probe to determine normal continually expressed gene RNA levels. The *GADPDH* control RNA levels were important to accurately compare the various samples to one another since they can reflect differences in RNA loading onto gels and spectrophotometric quantitation errors. The *GAPDH* levels were compared to ethidium bromide-stained total RNAs to insure the accuracy of using GAPDH RNA level standardization among samples.

Statistical analysis was carried out according to t-test for equality of means.

RESULTS

The bands from the autoradiographs are depicted in Figures 1A-4A and graphs comparing relative gene expression, based on computer-assisted laser densitometry of the autoradiographic signals, are presented in Figures 1B-4B. The relative levels of RNAs were different depending on the probe used in the blot hybridization. *TIMP-1* RNA levels were more variable from tumor to tumor compared to levels of *NM23-H1* RNA. GAPDH RNA levels were used to accurately determine the physical amount of sample loaded into each gel. Total RNAs were visualized after gel electrophoresis and documented by photography. The 28S and 18S ribosomal RNAs served as size markers to determine the size of the mRNAs that were detected as bands after Northern blot analysis.

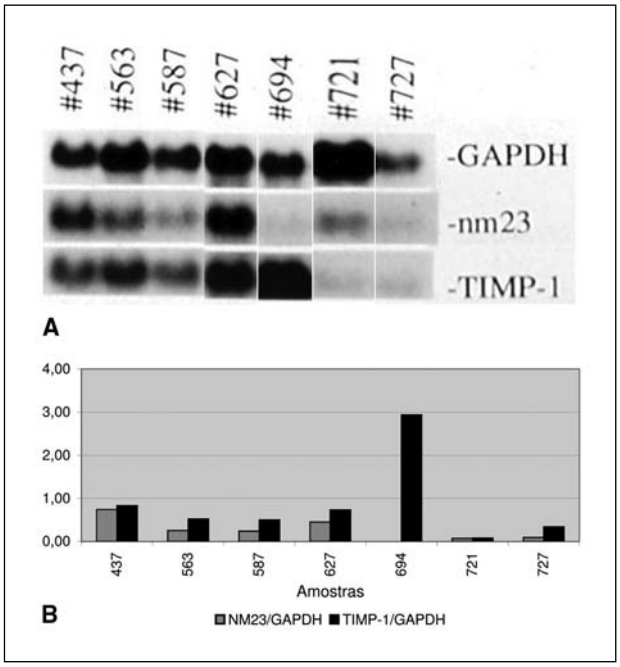

Fig 1. Analysis of TIMP-1, NM23-H1 GAPDH RNA levels in GBM tissue samples. Tumors were obtained at time of surgery. A) Northern blot analysis: 10 μg of each RNA sample was size-fractionated and analyzed. Ribosomal RNAs were visualized by ethidium bromide staining to determine RNA quality and relative differences in the physical amount of RNA loaded per lane. B) Quantitation of band signals: The amount of band signal on each autoradiograph was quantitated by computer-assisted laser densitometry as described in Figure 1B.

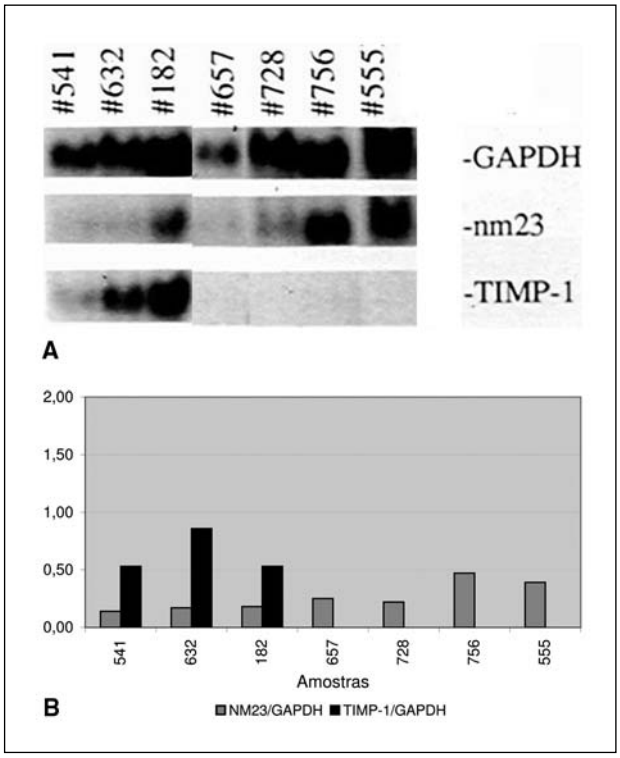

Fig 2. Analysis of TIMP-1, NM23-H1 and GAPDH RNA levels in diffuse astrocytoma and GBM tissue samples. Tumors were obtained at time of surgery. A) Northern blot analysis. B) Quantitation of band signals: diffuse astrocytoma (541,632,182) and GBMs (555,657, 728,756).

The ribosomal RNAs also served to document differences in the physical amounts of RNAs loaded per sample. There were variations in the amounts of RNA loaded per lane and these variations were in 100% correspondence with the variations detected after laser densitometry of bands generated using a *GAPDH* probe. For each RNA sample, absorbance values of *TIMP-1*-specific bands or *NM23-H1*-specific bands were divided by the absorbencies of GAPDH bands to generate relative gene expression values.

Figures 1A and B, 4A and B depict the bands detected after Northern blot analyses of RNA extracted from 27 primary tumor tissues removed at time of surgery. Total cellular RNA was used in these analyses.

Figures 1A and B depicts RNAs from seven GBM samples.

Regarding to *TIMP-1* gene expression: RNA sample (694) revealed a significant *TIMP-1* increase relative to *GAPDH.* Lower levels were obtained in four samples according to laser densitometry and RNA sample (721) was significantly decreased relative to *GAPDH.*

Regarding to *NM23-H1* gene expression: Higher RNA level was detected in one sample (437) while three samples (694, 721 and 727) were less than half of the mean densitometry ratios.

Figures 2A and B depicts RNAs from three diffuse astrocytomas (541,632,182) and four GBMs (555,657, 728,756).

TIMP-1 RNA levels in diffuse astrocytomas were extremely high compared to levels in all other samples analyzed in this blot. The GBMs samples either severely down-regulated expression of *TIMP-1* or they shut off its expression completely.

In contrast to *TIMP-1*, *NM23-H1* RNA levels were easily detected and there were no major differences in the levels of expression in tumors from the astrocytic lineage.

Figures 3A and B contains eight GBM RNA samples.

Two tumors contained high levels of *TIMP-1* RNA (735,782). While six other tumors had low levels, less than half of the mean densitometry ratios.

NM23-H1 RNA levels in these samples were not as variable. Only one sample (788) contained high level of *NM23-H1* compared to the mean. The other

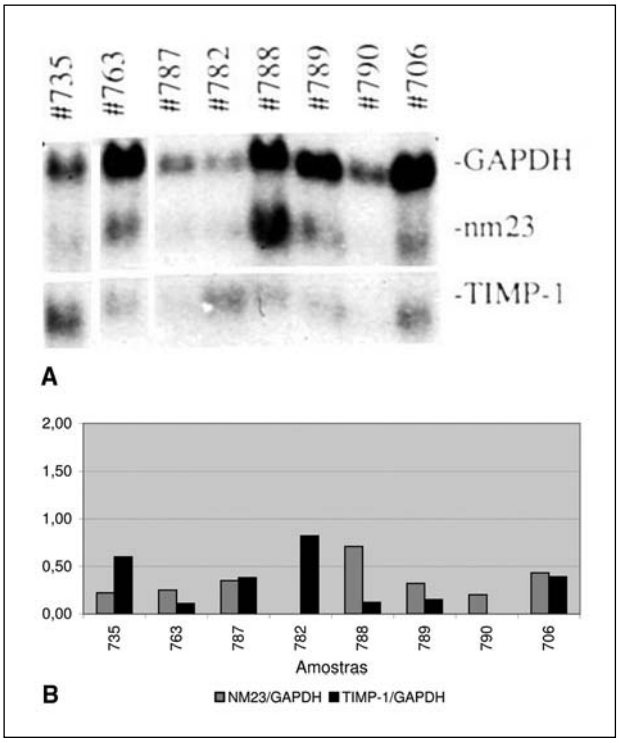

Fig 3. Analysis of TIMP-1 NM23-H1 and GAPDH RNA levels in eight GBM tissue samples. Tumors were obtained at time of surgery. A) Northern blot analysis. B) Quantitation of band signals.

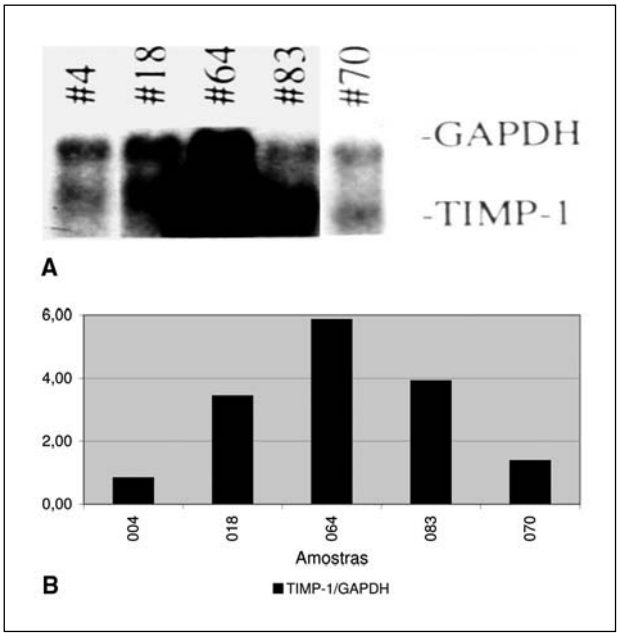

Fig 4. Analysis of TIMP-1 and GAPDH RNA levels in five diffuse astrocytoma tissue samples. Tumors were obtained at time of surgery. A) Northern blot analysis: B) Quantitation of band signals.

tumors did not appear too significantly up-regulate or down-regulate *NM23-H1* gene expression at RNA levels.

Figures 4A and B depicts RNAs from five diffuse astrocytomas.

There was variable *TIMP-1* gene expression in this set of tumors. Sample (64) contained significantly higher amounts of *TIMP-1* RNA compared to the other tumors in the series. Tumor sample (4,70) contained an intermediate amount of this RNA.

Statistical analyses were carried out according to t-test for equality of means (Tables 1, 2, 3).

TIMP-1 and diffuse astrocytoma showed that the mean ratio *TIMP-1/GAPDH* was in 2.17 and standard deviation (SD) 1.99. The confidence interval with level of significance of 5% presented with values from 0.5 and 3.83. *TIMP-1* and GBM the mean ratio *TIMP1/GAPDH* was 0.45 and SD 0.67. The confidence interval with level of significance of 5% presented limits of 0.13 and 0.77. Analysis of equality of variances (0.05 significance level was considered for all analysis) found to *TIMP-1* gene the rejection of hypothesis of equality of variances, then it was used t test to

Table 1. Descriptive statistics (Laser densitometry ratios NM23-H1 and TIMP-1 RNA levels comparing with control gene RNA levels in gliomas).

		Number observations	Mean	SD	Std. Error	95% confidence interval of mean		Lower	Upper
						Lower limit	Higher limit		
NM23-H1	ASTRO	3	0.16	0.02	0.01	0.11	0.22	0.14	0.18
	GBM	19	0.30	0.20	0.05	0.20	0.40	0.00	0.74
	Total	22	0.28	0.19	0.04	0.19	0.37	0.00	0.74
TIMP-1	ASTRO	8	2.17	1.99	0.70	0.50	3.83	0.53	5.86
	GBM	19	0.45	0.67	0.15	0.13	0.77	0.00	2.94
	Total	27	0.96	1.42	0.27	0.40	1.52	0.00	5.86

Table 2. Levene 's test for equality of variances.

	Levene statistic	Degrees of freedom 1	Degrees of freedom 2	Significance
NM23	3.52	1	20	0.075
TIMP-1	22.16	1	25	0.000

Table 3. Statistical analysis t-test for equality of means NM23-H1 and TIMP-1 in gliomas.

		Levene's test for equality of variances		t-test for equality of means						
		F	Significance	t	Degrees of freedom	Significance (2-tailedl)	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	Upper
NM23	Equal variances assumed	3.52	0.08	–1.11	20.00	0.279	–0.13	0.12	–0.38	0.12
	Equal variances not assumed			–2.77	19.68	0.012	–0.13	0.05	–0.23	–0.03
TIMP-1	Equal variances assumed	22.16	0.00	3.41	25.00	0.002	1.72	0.50	0.68	2.76
	Equal variances not assumed			2.39	7.68	0.045	1.72	0.72	0.04	3.39

equality of means taking in account unequal variances. The results showed that the means of these two groups were different ($p<0.05$).

Statistical analysis of *NM23-H1* and diffuse astrocytoma showed that the mean ratio *NM23-H1/GAPDH* was 0.16 and SD 0.02. The confidence interval with level of significance of 5% presented limits of 0.11 and 0.22. *NM23-H1* and GBM the mean ratio *NM23-H1/GAPDH* was 0.30 and SD 0.20. The confidence interval with level of significance of 5% presented limits of 0.20 and 0.40. Analysis of homogeneity of variances (level of confidence: 5%) found to *NM23-H1* was not rejected the hypothesis of equality of variances, then it was used t test to equality of means taking in account same variances. The results showed that the means of two groups were the same ($p> 0.05$).

DISCUSSION

Invasion of normal brain tissue by a glial tumor cell has been a goal of many studies[10]. *TIMP-1* interacts with metalloproteinases to inhibit its collagenase activity. The balance between collagenase and collagenase inhibitor appears to determine whether a tumor cell can invade adjacent tissue. Most tumors and normal cells produce a number of collagenases[11]. Our aim was to determine it there was a differential invasiveness into adjacent normal brain as part of their malignant phenotype. Our initial approach to the study of *TIMP-1* correlated with protein levels and anticollagenase activity in other tumor systems. There was a clear difference in the amounts of *TIMP-1* RNA in histopathologically defined well-differentiated astrocytomas compared to malignant astrocytomas, we have quantitated the levels in 27 different samples from different patients. In the malignant astrocytomas, there was a significant trend toward the down-regulation of *TIMP-1* gene expression as evidenced by the lower amounts of accumulated *TIMP-1* RNA. This expression trend correlated with the invasive capacity of the tumor. Apodaca examined collagenases and TIMP protein excretes into culture medium of five malignant glioma cell lines and from cultured fetal astrocytes[1]. In agreement with

our RNA data, they detected collagenase and *TIMP-1* activity in conditioned medium from all of these cells.

Halaka et al. examined conditioned medium from eight glioma explants cultured up to seven days[5]. They reported varying amounts of *TIMP* production and a tendency for invasive tumors to release less *TIMP-1* into culture medium. Nakagawa et al. confirmed the *TIMP-1* involvement with gliomas and meningiomas, they did protein experiments and their results are similar as ours[12]. Nakano and his group published a very elegant study showing their conclusions about the relationship of metalloproteinases, *TIMP-1* and *TIMP-2,* and also the interaction with PMA, *TNF*α*, EGF, IL-1*α*, IL-6* and *TGF* β*1.* They did Northern blot analysis in glioma cells and chemoinvasion assay[13].

Invasion is a complex interaction between metalloproteinases and inhibitors, in addition with the modulation of some growth factors and cytokines[14]. *TIMP-1* RNA and *NM23-H1* RNA in diffuse astrocytomas and GBM samples extracted from tissue culture are mostly the same, because it is not a good model for something which is very active. Other mechanisms are clearly involved and there is likely to be a continual balance between the metalloproteinases and their inhibitors[15].

There are individual tumors that contained significantly higher amounts of *TIMP-1* RNA. It is possible believe the existence of chemical mediators of *TIMP-1* overexpression. It is supported by the fact of high levels of *TIMP-1* RNA are influenced by external stimuli, internal cell regulatory control or a combination of both gene expression control mechanisms.

Since *TIMP-1* levels are lower in specific malignant astrocytomas, this gene may be involved in regulating tumor cell invasion. It is possible that therapeutic regimens could be devised to deliver *TIMP-1* protein or increase *TIMP-1* gene expression to destroy the activity of the *TIMP-1* target, collagenase, and ameliorate tumor cell invasion. Increased expression of *TIMP-1* mediated by the addition of exogenous DNA can decrease the metastatic potential of the genetically engineered cell[16,17]. The efficacy of *TIMP-1* gene replacement therapy will be dependent on useful gene expression vectors that can be easily delivered to patients[18].

The role of *NM23-H1* in tumor cell invasion was determined by Liotta[19]. This gene is overexpressed in primary melanomas compared to their respective metastases and decreased in human primary infiltrating ductal breast carcinomas that exhibit lymph node involvement. RNA expression of this gene in glial tumors has not been evaluated. The levels of *NM23-H1* in glial tumors samples were not as varied as those of *TIMP-1* RNA in our data. We did not detect a significant trend toward low expression of *NM23-H1* in the invasive astrocytomas. However, *NM23-H1* was present in all glial tumor sample. Our results did not show any variation in *NM23-H1* gene expression from diffuse astrocytomas and glioblastoma cells. Researchers have suggested that the *NM23-H1* is responsible by the internal control of the invasion in malignant tumors[19-23].

The data presented in this paper suggest that *TIMP-1* is a candidate inhibitor of brain tumor invasion. We presented samples from GBM in which low or undetectable amounts of *TIMP-1* RNA suggest that one of the known mechanisms to inhibit tumor cell invasion is impaired in these tumors. Replacement of this gene product may be of therapeutic value to patients with brain tumors, slowing or statically inhibiting their disease[24,25].

NM23-H1 was present in the entire glioma samples, but it did not vary in diffuse astrocytomas and glioblastomas.

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