

Cathepsin L in human meningiomas

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Background. Although meningiomas are considered as benign tumours, about 10% comprise a subgroup of atypical meningiomas, classified as WHO grade II, with greater likelihood of recurrences and/or aggressive behaviour, including the possibility of brain tissue invasion. The lysosomal cysteine endopeptidase cathepsin L plays a role in tumour cell invasion and malignant progression of cancer, and has been suggested as a prognostic marker for certain types of tumours.

Results. In our study, we compared the expression of cathepsin L in 30 meningiomas with their clinical invasiveness. Cathepsin L was determined by immunohistochemical analysis, quantitative real-time RT-PCR and Northern blot. We showed that expression of cathepsin L protein was significantly higher ($p=0.019$) in 9 atypical than in 21 benign meningiomas. Within the group of benign meningiomas, expression of cathepsin L was significantly lower in the transitional histological subtype. We measured the levels of cathepsin L A type of RNA splicing variants: LA, LAI and LAII, but not LAIII and not the LB variant, the latter being several times lower than the L A type. In contrast to protein levels, the levels of cathepsin L A, AI, AII RNA variants did not differ between histological subtypes or between benign and atypical meningiomas. The expression of total measured cathepsin L A, AI, AII RNA variants in the samples, taken from the centre and the periphery of the tumours, also showed no statistically significant differences.

Conclusions. These results indicate that cathepsin L protein over-expression may contribute to the development of the aggressive and possibly invasive character of atypical meningiomas and that it may be up regulated at the translational level.

Key words: meningioma - pathology; cathepsin L; reverse transcriptase, polymerase chain reaction; neoplasm invasiveness

Introduction

Meningiomas are tumours of the central nervous system that derive from the coverings of the brain. Meningiomas are generally slow growing, benign tumours attached to the dura matter and composed of neoplastic meningotheial (arachnoidal) cells.¹ Meningioma have a wide range of histopathological

Received 25 April 2003

Accepted 20 May 2003

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appearances. However, of the 11 subtypes, the three most common are meningothelial, fibrous and transitional meningioma.^{1,2}

Most meningiomas are benign and can be graded into WHO grade I. Certain histological subtypes are associated with a less favourable clinical outcome and correspond to WHO grades II and III classification of histological diagnosis of brain tumours. Atypical meningiomas corresponding to grade II have moderately high labelling indices of nuclear proliferation index (PI) and have likely the following features: frequent mitosis, increased cellularity, small cells with high nuclear-cytoplasmic ratio and/or prominent nucleoli, uninterrupted pattern-less or sheet-like growth and foci of 'spontaneous' or geographic necrosis.¹ These criteria have been shown to correlate with higher recurrence rates. Anaplastic malignant meningiomas (grade III) exhibit malignant histological features in excess of the abnormalities noted in atypical meningiomas. The latter constitute about 6%, whereas anaplastic malignant meningiomas account for between 1.0 and 2.8% of all meningiomas.²

Cathepsin L (EC 3.4.22.15) is a cysteine endopeptidase belonging to the evolutionarily well conserved clan CA/family CI proteases (<http://www.merops.co.uk>). It is localised mainly to lysosomes, mediating intracellular protein turnover, but under certain physiological conditions it also regulates the function of some cytosolic and extracellular proteins. Cathepsin L gene is activated by a variety of growth factors and some oncogenes. In NIH3T3 cells (mouse embryonal fibroblasts), cathepsin L gene is activated by platelet derived growth factor (PDGF), epidermal growth factor (EGF), tumour promoter phorbol 12-myristate 13-acetate (PMA), cAMP and oncogenes, including *v-ras*, *v-src* and *v-mos*.⁴ Enzyme activity is regulated specifically by endogenous inhibitors of the cystatin family, thyropins and p41 fragment of invariant chain (Ii).⁵

Gottesman³ first reported an increased synthesis and secretion of the so called »major excreted protein, MEP« in NIH3T3 fibroblasts on viral transformation, the protein later being identified as cathepsin L.⁶ Many subsequent studies have supported the hypothesis that increased levels and eventual secretion of cathepsin L are related to malignant transformation. Increased expression of RNA, protein and activity of cathepsin L was found in gastric⁷, colorectal, ovarian, breast, head and neck thyroid, lung endometrial carcinomas and gliomas.⁸ It was shown that cathepsin L is progressively expressed with increasing tumour malignancy in breast⁹ and brain tumours^{10,11} and has prognostic relevance for some cancers.^{8,12} The role of cathepsin L in the invasion process was confirmed in *in vitro* invasion assays by the use of selective inhibitor Click-148, which also reduced *in vitro* invasion of tumour cells^{13,14} and reduced bone metastasis in an *in vivo* mouse model.¹⁵ Collectively, this data suggests that cathepsin L plays an important role in tumour invasion.

The present study is based on the hypothesis that, due to the observed association of cathepsin L with tumour progression and invasiveness, this lysosomal endopeptidase may be a biological marker for differentiation between benign and the more aggressive atypical meningioma. This suggested the further question as to whether cathepsin L expression would be increased at the invasive edge of meningiomas.

Materials and methods

Patients

Thirty patients (19 female and 11 male) with diagnosed meningioma were selected randomly for the study. They ranged in age from 26-80 years. Immediately after surgical removal, samples from the centre and periphery of the tumours were removed and snap frozen in liquid nitrogen. The rest of the tu-

mour tissue was fixed in formaldehyde and embedded in paraffin. The histological slides were analyzed and categorized according to the WHO classification of brain tumours. The study comprised 22 benign and 8 atypical meningiomas (Table 1). The study was approved by the Ethics Committee at the Ministry of Health of the Republic of Slovenia.

Immunohistochemistry

Immunohistochemical labelling was performed using standard techniques.¹⁶ Paraffin-embedded tissue sections were deparaffinised and rehydrated. The sections were boiled in 100mM EDTA buffer (pH 8.0) for 5 minutes for antigen retrieval. The slides were first blocked with an inhibitor of endogenous peroxidase and then incubated with primary anti-cathepsin L mouse monoclonal antibodies (CatLHLM1, clone N135) purchased by Krka d.d., Novo mesto, Slovenia, at 1:10 dilution of the stock concentration of 100µg/ml for 26min at 40 °C. Subsequent IHC reaction was performed with biotinylated goat anti-mouse secondary antibodies, avidin with bound horseradish peroxidase and diaminobenzidine as chromogen. The immunohistochemistry and counterstaining with haematoxylin, was performed in an NexES IHC Staining Module (Ventana Medical Systems, USA). The intensity and frequency of immunostaining was considered independently by two pathologists for grading (0; 0.5; 1; 1.5; 2; 2.5; 3).

Proliferation index: immunolabelling with MIB-1 antibodies

Proliferation index (PI) in meningiomas was determined by the IHC procedure, described above, using MIB 1 antibodies (mouse anti-human Ki-67, clone MIB-1, No 7240, Dako Corporation). The MIB-1 solution (200µg/ml) was diluted 1:20 prior to use. Positive IHC staining indicated proliferating cells and was counted under a microscope using the computer program Lexica Q Product (Leica, Germany). PI was calculated as the percentage of proliferating vs. all cells in the preparation.

Quantitative real time RT-PCR

A fluorescence-based real-time quantitative RT-PCR method, developed by Perkin Elmer ABI (TaqMan), was used to measure cathepsin L RNA levels in meningioma tissue. For each sample 1 µg of total RNA was reverse transcribed in a 50µl reaction using a High-Capacity cDNA Archive Kit (Applied Biosystem, USA). After an initial step at room temperature for 10 min, reverse transcription was performed at 37 °C for 2h. Quantitative PCR reaction was carried out with a cDNA equivalent of 2ng total RNA per reaction, using the TaqMan Universal PCR Master Mix (Applied Biosystems, USA): 1x TaqMan Universal PCR Master Mix, 200nM of each primer and 100nM TaqMan (final concentration) fluorescent probe in a 10 µl final reaction volume. PCR reactions were carried out in an ABI Prism 7900 PCR machine (Applied

Table 1. Histological diagnosis of meningiomas

Histological diagnosis	WHO grade	No of tumours analysed by IHC	No of tumours analysed by RT-PCR
Benign meningioma	I		
Fibroblastic	I	6	6
Meningothelial	I	6	6
Transitional	I	8	6
Psammomatus	I	1	1
Secretory	I	1	1
Atypical meningioma	II	8	7

Biosystems, USA), with a step at 50°C for 2min for AmpErase UNG activity, hot start at 95°C for 10min, followed by 40 cycles with denaturation at 95°C for 15s and annealing/elongation at 60°C for 1min. To normalize the signal for cathepsin L A type variants, amplification of 18S ribosomal RNA was performed as an internal control in a duplex reaction together with amplification of cathepsin L cDNA. Primers and probes for 18S rRNA were purchased from Applied Biosystem in Pre-Developed TaqMan Assay Reagent Control Kit.

We designed probe and primers to measure simultaneously cathepsin LA, cathepsin LAI and cathepsin LAII RNA splicing variants, but not LAIII RNA variant.¹⁷ The following primers were synthesized (Laboratories Eurobio, France): forward primer for cathepsin L A variants: AGC GTC TAC CCC GAA CTC TG, located at bp 158-178 in exon1 and reverse primer for cathepsin L A variants: TTG TGC ATC GCC TTC CAC T, located at bp 372-387 in exon 2. The nucleotide sequence for cathepsin L A variants probe, labelled with FAM reporter molecule, ACT CAT CCT TGC TGC CTT TTG CCT GG, is located in exon 2 at bp 372-387. Cathepsin L B variant does not have exon 1, therefore the forward primer was located in intron 1, bp 161-186, extending 4 nucleotides into exon 1, whereas the reverse primers and the probe were the same as for the cathepsin L A variants.

Northern blot of selected tumour samples

Total RNA was isolated with TRIzol (Gibco BRL, USA) according to the supplier's instructions. The RNA concentration was estimated by A_{260nm}. 20µg of total RNA per lane was mixed with ethidium bromide and electrophoresed on 1% formaldehyde-agarose gel for 3h at 70V. Northern transfer was performed overnight in 20x SSC (Standard Saline Citrate, 3M sodium chloride, 0.3M sodium citrate, pH 7.0) on a rapid downward transfer system using the turboblotter appa-

ratus and Nytran membrane (Schleicher and Schuell, Germany). RNA was cross-linked to the membrane by UV irradiation. The membrane was photographed under UV light and the photographs used for signal normalization. The blots were pre-hybridized for 3h at 50°C in »high SDS« buffer (5x SSC, 2% blocking solution, 50mM sodium phosphate, 0.1% (w/v) lauroylsarcosine, 7% (w/v) SDS, 50% formamide) as suggested by the supplier of the blocking solution (Roche, Switzerland). Hybridization was performed overnight in the same buffer containing 30ng/ml DIG-labelled full length cDNA probe. Blots were washed twice with 2x washing buffer (2xSSC, 0.1%, w/v, SDS) and twice with 0.5x washing buffer (0.5xSSC, 0.1% (w/v) SDS). All washing steps were performed for 10min at room T. The signals were detected by chemiluminescence using the CDP Star System (Roche, Switzerland) according to the supplier's instructions. The signals of cathepsin L RNA on Northern blots were analyzed densitometrically with program Lucia G 4.21 (Laboratory Imaging Ltd, GB) and normalized to 18S rRNA signal.

Statistics

For statistical calculation we used SPSS 10.1 for Windows (SPSS Inc, USA) program. The differences in cathepsin L expression were analyzed by the Mann-Whitney and Kruskal-Wallis non-parametric test. The significance of differences between histological subtypes of meningiomas was given as p values. P<0.05 was considered to be significant.

Results

Immunohistochemistry

Cathepsin L immunostaining was evaluated in 30 meningiomas, characterised histologically (Table 1). Figures 1A and 1B show weak (score 1.0) and strong (score 3.0) IHC staining of cathepsin L in tumour cells of benign tran-

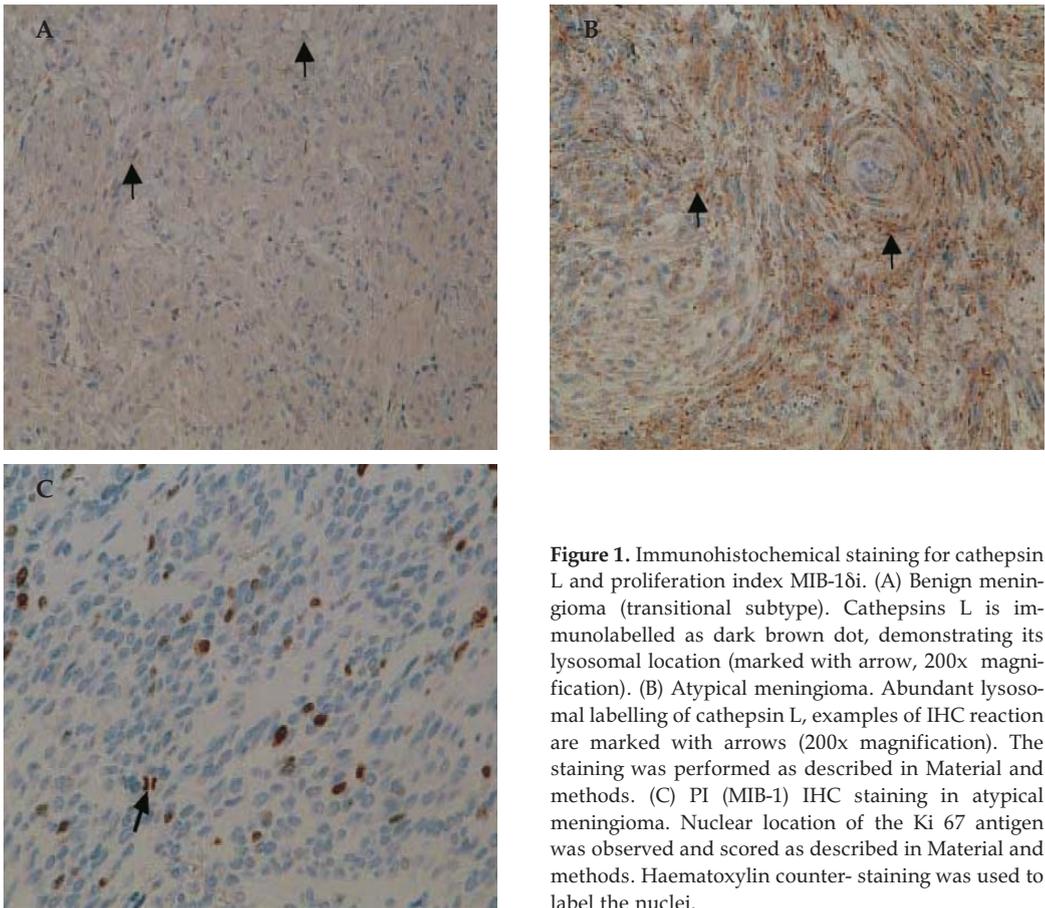


Figure 1. Immunohistochemical staining for cathepsin L and proliferation index MIB-1*δ*i. (A) Benign meningioma (transitional subtype). Cathepsins L is immunolabelled as dark brown dot, demonstrating its lysosomal location (marked with arrow, 200x magnification). (B) Atypical meningioma. Abundant lysosomal labelling of cathepsin L, examples of IHC reaction are marked with arrows (200x magnification). The staining was performed as described in Material and methods. (C) PI (MIB-1) IHC staining in atypical meningioma. Nuclear location of the Ki 67 antigen was observed and scored as described in Material and methods. Haematoxylin counter-staining was used to label the nuclei.

sitional and an atypical meningioma, respectively. No statistically significant differences in cathepsin L levels were observed between meningeothelial and fibrous meningioma, but significantly lower levels were observed in transitional meningiomas (Figure 2A). When all benign subtypes were grouped and compared with all atypical meningiomas, we found significantly higher ($p=0.009$) scores of IHC staining in the more invasive atypical meningiomas (Figure 2B).

Quantitative real time RT-PCR

Cathepsin L is encoded by four major RNA species: cathepsin LA, cathepsin LAI, cathepsin LAII RNAs, the newly discovered cathepsin LAIII RNA, and cathepsin LB RNA.¹⁹

Cathepsin L RNA levels were analysed by quantitative RT-PCR using specifically designed probes, as described in Material and methods. We found that the levels of cathepsin LB RNA in meningiomas were between 3 and 5 cycles less (average about 8 times lower) than with cathepsin LA RNA variants (not shown), therefore the LB variant was not determined in this study. The primers and the probe which detects all previously mentioned cathepsin L A type RNA variants, but not the recently discovered cathepsin LAIII splice variant, were designed. No statistically significant differences were found in the amount of cathepsin L A, AI, AII type splice variants between different histological subtypes of meningiomas (Figure 3). Also, no statistically

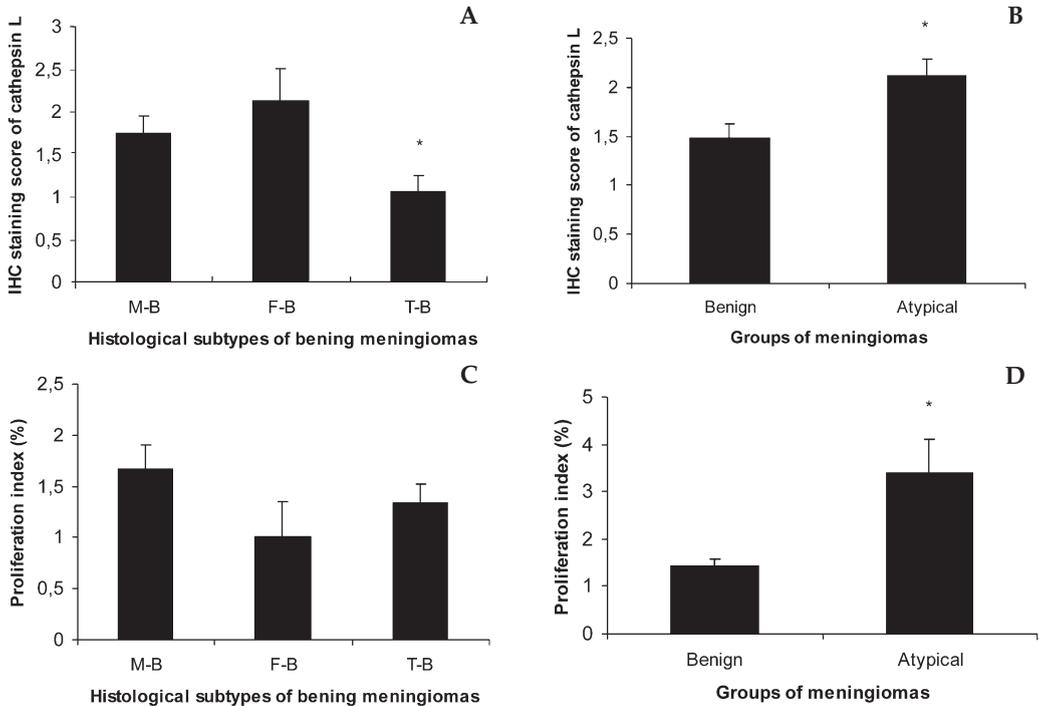


Figure 2. Immunohistochemical scoring of cathepsin L and PI in different histological subtypes of meningiomas. (A) Comparison of the scores of cathepsin L within benign tumours: M-B meningothelial benign, F-B fibroblastic benign and T-B transitional benign. (B) Comparison of mean IHC scores in all benign and all atypical meningiomas. IHC scoring was performed according to the frequency and intensity of cathepsin L labeling on a scale from 0 - 3, as described in Methods. (C) Immunohistochemical scoring of PI within benign tumors: M-B meningothelial benign, F-B fibroblastic benign and T-B transitional benign meningioma. (D) Immunohistochemical scoring of PI in all benign and all atypical meningiomas, presented as mean scores. Results are presented as mean \pm standard error. The stars indicate statistically significant differences ($p < 0.05$) between the groups.

significant differences were found between the expression of cathepsin LA RNA variants in atypical and benign meningiomas. The comparison between the samples taken from the periphery and the centre of the tumours revealed no difference in the amount of cathepsin L A type variants (not shown).

Northern blot of selected tumour samples

To determine whether total cathepsin L mRNA expression, using the cDNA probe of cathepsin L, differs between histological subtypes types and between the centre and the periphery of the tumours, Northern blot analysis was performed in two meningothelial (samples 2,6), one fibroblast (sample 1),

three transitional (samples 3,7,8) and two atypical meningiomas (samples 4,5). Samples from the centre and periphery of the tumours were loaded separately (Figure 4A). Expression in cathepsin L RNA samples varies, even within the same subtype of tumour, for example samples 2 and 6, which are both classified as meningothelial subtype. Densitometric analysis (Figure 4B) showed slightly lower expression in fibroblastic and transitional benign subtypes, but from this we cannot deduce any significant differences between these meningiomas. As observed with cathepsin LA RNA variants, the expression is very similar in the centre and in the periphery of the tumours.

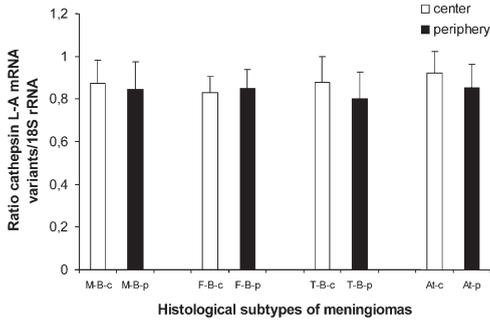


Figure 3. Cathepsin LA RNA variants determined by real time RT PCR. The expression of cathepsin LA variants was quantitated and related (normalized) to the levels of 18S rRNA in different histological subtypes of meningioma in the center (c) and at the periphery (p) of the meningiomas: M-B meningothelial benign, F-B fibroblastic benign, T-B transitional benign, At-atypical. Results are shown as mean \pm standard error.

Correlation of cathepsin L measurements

Cathepsin L protein expression (IHC score) and the expression measured by cathepsin L A variants RNA (mRNA/18srRNA) levels correlated statistically significantly ($p < 0.02$) regardless of the location of the sample measurements, i.e. tumour centre ($r = 0.45$), tumour periphery ($r = 0.43$) or total tumour ($r = 0.48$).

Proliferation index (PI)

The proliferation index indicates the relative (%) expression of antibodies labelling a nuclear antigen, which is greatly increased in all phases of the cell cycle, except in Go. It is used as a histopathological marker indicating the degree of cell proliferation (Figure 1C). Our data confirm previous reports¹⁸ that PI discriminates between benign and atypical meningiomas, but not between different histologies of benign meningioma (Figure 2 C, D). It has been also reported to be good prognostic marker for relapse of meningiomas. In our study, PI correlated moderately ($r = 0.30$), although statistically significantly ($p = 0.003$), with the expression of cathepsin L protein.

Discussion

Meningiomas are among the most common tumours encountered in neurosurgical practice^{1,2,19} and the incidence is estimated to constitute between 13% and 26% of primary intracranial tumours, with an annual incidence of approximately 6 per 100 000 population.^{1,2} Meningiomas are in 90% of cases benign (WHO grade I), whereas 6-8 % are atypical, 1-2 % malignant anaplastic meningiomas, the rest comprising other types of grade II and III meningiomas.¹ In spite of the existence of certain morphological, histopathological and a few biological parameters, discrimination between clear benign, border benign and atypical meningioma remains the key problem.^{19,21} Therefore, new markers of malignant progression of atypical meningioma, which could lead clinicians towards a more informed treatment of patients, are needed.

Proliferation index is such a parameter which has been clearly shown to distinguish between benign and invasive atypical and anaplastic meningioma¹⁸, and this has been confirmed in this study. It has been found to correlate with cathepsin L antigen in atypical *vs* benign, but not within the benign meningioma group, suggesting that cathepsin L may not be related to increased proliferation rate.

Proteolytic enzymes have long been proposed as prognostic factors for disease-free and overall survival of cancer patients (recently reviewed in 25), including those with brain tumours^{26,27,28}, due to their role in invasion processes and metastases. The metalloprotease stromelysin was also suggested as a marker for invasiveness of meningioma²⁹ and the same was confirmed for lysosomal cysteine cathepsins in our previous study.²¹ We analyzed the expression of cathepsin B and cathepsin L in 67 benign and 21 atypical meningiomas and found that the protein levels of both cathepsins were significantly ele-

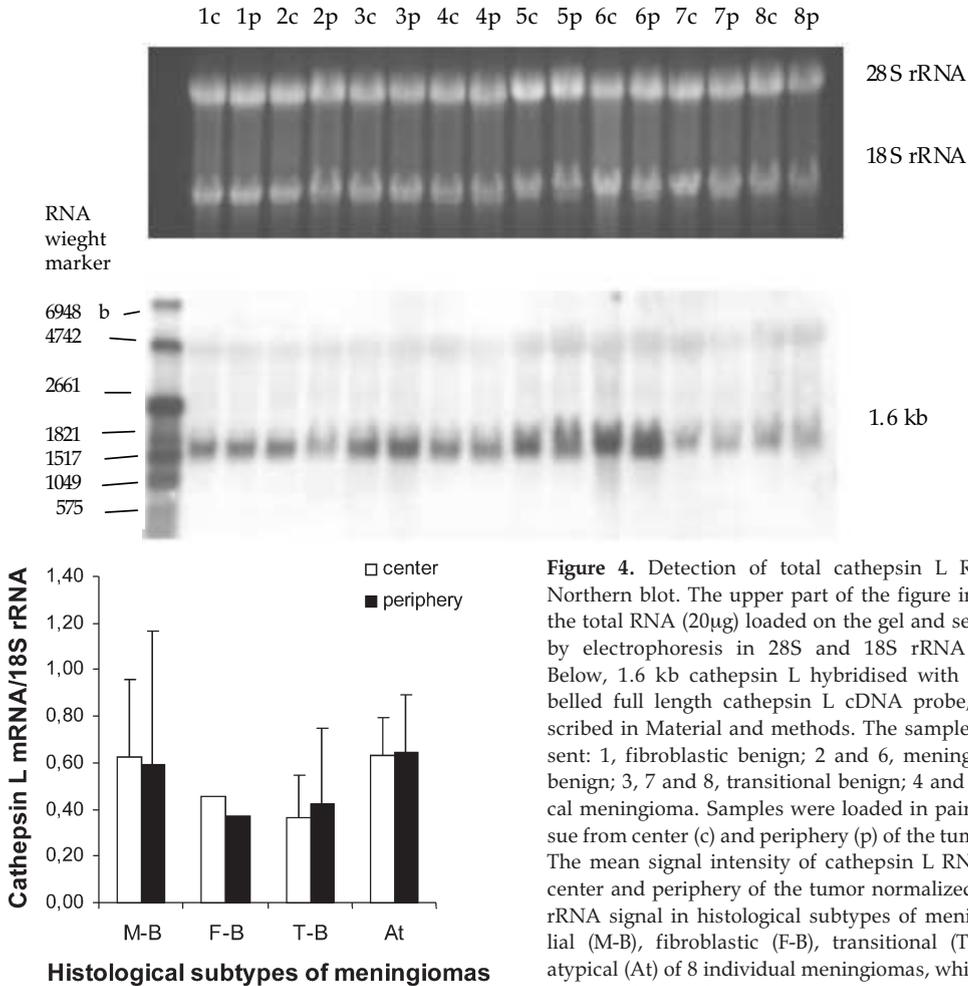


Figure 4. Detection of total cathepsin L RNA by Northern blot. The upper part of the figure indicates the total RNA (20µg) loaded on the gel and separated by electrophoresis in 28S and 18S rRNA bands. Below, 1.6 kb cathepsin L hybridised with DIG labelled full length cathepsin L cDNA probe, as described in Material and methods. The samples represent: 1, fibroblastic benign; 2 and 6, meningothelial benign; 3, 7 and 8, transitional benign; 4 and 5 atypical meningioma. Samples were loaded in pairs of tissue from center (c) and periphery (p) of the tumors. (B) The mean signal intensity of cathepsin L RNA from center and periphery of the tumor normalized to 18S rRNA signal in histological subtypes of meningothelial (M-B), fibroblastic (F-B), transitional (T-B) and atypical (At) of 8 individual meningiomas, which were analyzed by Northern blot experiment above (A).

vated in atypical tumours. Moreover, among 67 benign tumours, nine had certain features of malignancy, classified as borderline benign meningioma, and significantly higher expression of cathepsin B was also found in the borderline benign tumours compared with clear benign tumours. However, cathepsin L could only discriminate between atypical and clear benign tumours. This suggested that cathepsin L was a less selective diagnostic marker for distinguishing between histomorphologically benign but invasive, and histomorphologically clear benign tumours. However, several recent *in vitro* studies have confirmed a

close link between the invasive potential of tumour cells and increased cathepsin L expression. In brain tumours, first reports that cathepsin L may be related to brain tumour invasion came from Sivaparvathi *et al.*²², who found that cathepsin L expression paralleled increased malignancy of astrocytoma and glioblastoma cell lines. Moreover, the authors prevented glioblastoma cells from invading Matrigel by using specific cathepsin L antibodies. We have confirmed these results in an astrocytoma cell line model, using different extracellular matrices and a variety of synthetic cathepsin B and L inhibitors.¹¹

Recently, we have also demonstrated stable transfection of an IPTG glioblastoma cell line with whole length cDNA of cathepsin L, which resulted in significant, 80%, inhibition of cell invasion in Matrigel compared with control cells transfected with the vector-GFP.²³ This was confirmed in other cell lines by others.²⁴

In this study, protein expression of cathepsin L was significantly lower in transitional benign meningiomas than in other benign types of meningiomas. At present we cannot explain this, since the transitional subtype has features transitional between those of meningothelial and fibrous meningiomas. Strojnik *et al.*²¹ also found no variations in the subtypes of benign meningioma. However, the levels of cathepsins were more related to their intracranial localisation and were the highest in parasagittal and convexity meningioma, which were also clinically more aggressive.

In our previous study of glioblastoma invasion, we detected high concentrations of cathepsin B at the invasive edges of invading tumours²⁷, confirming earlier reports that intra-tumour and intracellular localisation of cathepsins at the plasma membrane may be important in facilitating lytic interactions between the tumours and surrounding stroma.²⁵ In this study we failed to observe different expression of cathepsin L between the centre and the periphery of the tumour, either by immunohistochemistry, Northern blot or RT-PCR methods. This suggests that cathepsin L may not be directly involved in degradation of extracellular matrix proteins at the cell (tumour surface), but may play a different role in the proteolytic events leading either to tumour cell invasion and/or other features of malignancy.

In the present study we found significantly higher levels of cathepsin L protein in atypical (grade II) compared with benign meningioma (grade I), indicating possible correlation of cathepsin L with invasiveness and/or

clinical aggressiveness of grade II meningiomas. However, we have not succeeded in demonstrating increased cathepsin L RNA in atypical meningioma. Although total cathepsin L RNA was only assayed in a few meningiomas, the cathepsin L A splicing variants (LA, LAI and LAII) measured in all 30 patients, also did not differ significantly between benign and atypical meningiomas. Chauhan *et al.*³⁰ first reported two splicing variants of the single cathepsin L gene, LA and LB variants, expressed concurrently at a similar ratio in several different cell lines, LB variant being higher than LA. This is in contrast to our results in meningioma tissue, where we found about 8 times lower LB variant and therefore did not quantify this variant. The discrepancy between messenger RNA (LA variants) and protein levels of cathepsin L can be explained either by an increased rate of translation of cathepsin L protein in atypical meningioma or by the fact, that we have not determined the LAIII splicing variant. Abula *et al.*³¹ has recently demonstrated that LAIII is predominant in all tissues, including malignant tumours, and showed the highest expression and translational rate *in vitro* and *in vivo*.^{17,31} It is possible, that this variant is also the most active in atypical and possibly anaplastic malignant meningioma, resulting in higher protein levels of cathepsin L, as observed in this study and previously.²¹ This needs to be further investigated.

Brain invasion may occur in histologically benign, atypical or anaplastic malignant meningiomas.¹ The presence of brain invasion connotes a greater likelihood of recurrence with brain-invasive histologically benign meningioma having clinical courses similar to atypical meningioma.¹ This clearly indicates that new markers of invasiveness, such as cathepsin L, are needed to predict malignant clinical behaviour of histologically classified benign tumours.

In conclusion, we have demonstrated sig-

nificantly higher levels of cathepsin L protein in atypical meningiomas. As cathepsin L is a marker of invasion but not of cell proliferation of malignant tumours, we may hypothesize that atypical meningioma acquire invasive behaviour. In contrast to protein, the levels of cathepsin L (LA, LAI and LAII) splicing RNA variants were not higher in atypical meningioma, strongly suggesting that the rate of RNA translation increased in atypical compared to benign meningioma. Particularly, the level and translational rate of LAII splicing variant, may be responsible for increased protein concentration of cathepsin L in atypical meningioma, what needs to be further investigated. We confirmed our hypothesis that cathepsin L is a biological marker for differentiation between benign and the more aggressive atypical meningioma and may be used to predict clinically observed aggressive behaviour of meningiomas.

Acknowledgements

This study was supported by the Project J3-2409 (granted to V.V.Dolenc,P.I.) and Programme 105/509 (granted to T.T.Lah) from the Ministry of Education, Science and Sports of Republic of Slovenia. We thank Prof. Dr. Roger Pain for fruitful discussion and careful reading the paper.

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