

## Expression of cathepsin B is related to tumorigenicity of breast cancer cell lines

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**Background.** The lysosomal cysteine proteases cathepsins B (CatB) and L (CatL) and their endogenous inhibitors, stefins A (StA) and B (StB), are widely thought to be involved in the progression of human breast carcinoma. Previously we showed that, in model breast carcinoma cell lines, the reported tumorigenicity was not directly related to their *in vitro* invasive potential.<sup>1</sup> However, CatL expression was positively related to the invasiveness of the cells and inversely related to the levels of StA. Here we challenge the hypothesis that imbalance between CatB and the two stefins is associated either with the invasiveness or the reported tumorigenicity of the panel of selected breast carcinoma cells.

**Results.** We investigated levels of mRNA, protein and activity for CatB in the panel of human breast carcinoma cell lines whose tumorigenicity *in vivo* increased in the order MCF-7 < MDA-MB468 < MDA-MB231 < MDA-MB435, the most invasive being MDA-231. Levels of expression of mRNA, protein and activity for CatB were highly correlated and increased progressively with cell tumorigenicity. The ratio of CatB to stefins was shifted in favour of CatB in the more tumorigenic cell lines.

**Conclusions.** Since CatL has been shown previously to be associated with invasive potential and, in this study, CatB expression was found positively associated with the tumorigenicity of the same breast carcinoma cell lines, the two cathepsins in these cells do not appear to be regulated in a coordinated manner. CatB expression and the ratio between CatB and stefins increased progressively with tumorigenicity of the cells and suggests a similar situation in human tumours *in vivo*.

*Key words:* breast neoplasms; cathepsin B; neoplasm invasiveness; tumor cells, cultured

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### Introduction

Lysosomal cathepsins comprise a variety of peptidases of different clans, among which ClanA (CA) includes the cysteine peptidases cathepsins (Cats) B and L, which belong to so-called papain family.<sup>2</sup> The association of the two cathepsins with tumour progression is

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well established in the literature.<sup>3,4</sup> In clinical studies of breast carcinoma patients, elevated levels of CatB are associated with bad prognosis of patient survival.<sup>5-9</sup>

The activities of cysteine cathepsins are regulated by their endogenous inhibitors, a large superfamily of cystatins.<sup>10</sup> The stefin (St) family comprises intracellular inhibitors, of which levels of StA and StB were found to be altered in tumour cells<sup>11</sup> and in clinical samples from cancer patients.<sup>3,7,9,12-13</sup> Therefore, the molecular mechanisms responsible for the biological roles of CatB and CatL in tumour cells, together with their inhibitors, StA and StB, need to be elucidated at the molecular level.

Tumour metastasis is a multi-step process, starting by detachment of tumour cells from the primary tumours, invasion through the extracellular matrix and/or basement membranes of vasculature, to reach the blood flow, which carries the tumour cells to distant organs where organ selective invasion and growth of tumour cells into the secondary site takes place. Invasion is therefore the common denominator of many metastatic steps, a process which is associated with tissue remodelling. Presumably, this is induced by tumour cells which are triggered to express, secrete and/or activate a battery of proteolytic enzymes at their cell surface. Extracellularly and at the plasma membrane, metalloproteinases and the plasminogen activator/plasmin system may initiate the extracellular matrix degradation. However, it has been proposed that Cats B and L initiate the proteolytic cascade by specific activation of pro-urokinase and/or metalloproteinases.<sup>14</sup> Furthermore, intracellular degradation of extracellular matrix components occurs during the invasion process<sup>15-16</sup> which involves activation of lysosomal cathepsins, including CatB.<sup>17</sup> Moreover, recent reports show that the invasion of tumour cells is significantly impaired when the intracellular activity of cysteine cathepsins is blocked.<sup>16-18</sup>

Tumorigenicity is a key characteristic of the malignant cancer cell, although the potential to form tumours at the secondary site may not directly reflect its invasive potential. In the two models of breast carcinoma cell lines, we have demonstrated, that the tumorigenicity of the cell lines was not strictly related to their *in vitro* invasiveness in Matrigel.<sup>1,19</sup> Here, we used a model of four selected human breast cancer lines: MCF7, MDA-MB468, MDA-MB231 and MDA-MB435, which have been reported to differ in tumorigenicity and metastasis *in vivo*.<sup>20,21</sup> Their phenotype varies from epithelial (MCF7) to mesenchymal (MDA-MB435), with highly increased expression of vimentin and downregulated expression of estrogen receptors.<sup>22</sup> However, not much is known about the expression of lysosomal proteinases, except our recent report on the positive association of CatL mRNA and protein expression, and an inverse correlation of StA expression, with the invasiveness of the cells in this model.<sup>1</sup> In the present study we used the same cell model with the aim (a) to determine mRNA, protein and activity levels for CatB, (b) to relate protein expression of CatB to those of StA and StB and (c) to relate these to the invasiveness and progressive tumorigenicity of the human breast carcinoma cell lines.

## Materials and methods

### *Cells and their characteristics: tumorigenic and invasive potentials*

Human breast carcinoma cell lines were obtained from the ATCC cell bank and cultured under conditions recommended by the supplier. The cells range from poorly to highly tumorigenic and metastatic in the order, MCF7 < MDA-MB468 < MDA-MB231 < MDA-MB435.<sup>20</sup> The MCF7 cell line is poorly tumorigenic and non-metastatic, MDA-MB468 cells exhibit low tumorigenic and low metastatic activities, while MDA-MB231 and

MDA-MB435 cell lines are both highly tumorigenic and metastatic, the latter producing the highest number of distant metastases.<sup>21</sup> The invasiveness of this panel of cell lines was determined in vitro, using the Matrigel assay.<sup>1</sup> Invasiveness ranged from MCF7, MDA-MB468, MDA-MB435 to MDA-MB231 which was the most invasive cell. Thus invasiveness does not parallel tumorigenicity in the two most tumorigenic cell lines.

#### *RNA analysis - Northern analysis and real time PCR*

Total RNA was extracted from cells by TRIzol Reagent, according to the instructions of the supplier (Gibco, UK). CatB mRNA was determined by Northern analysis and by quantitative RT-PCR. For Northern analysis, 15µg of RNA was electrophoresed through agarose/formaldehyde gel and hybridized with full length CatB cDNA probe that was non-radioactively labelled with digoxigenin, according to the instructions of the supplier. The cDNA probe was kindly provided by Dr. Boris Turk, Josef Stefan Institute, Ljubljana, Slovenia. The signals were detected by chemiluminescence, using CDP Star<sup>TM</sup> System (Boehringer, Germany).

A fluorescence-based real-time, quantitative RT-PCR method developed by Perkin Elmer ABI (TaqMan), was used to measure CatB RNA levels in cell extracts. 1µg of total RNA was reverse transcribed using High-Capacity cDNA Archive Kit and PCR amplified with TaqMan Universal PCR Master Mix according to the instructions of the supplier (both Applied Biosystems, USA). The sequence of the CatB forward primer was 5'-CTCTATGAATCCCATGTAGGGTGC-3', 5'-CCTGTTTGTAGGTCCGGGCTG-3' for the reverse primer and 5'-CCCTGTGAGCACACGTCAACGG-3' for the TaqMan probe. Amplification of 18S ribosomal RNA was performed as an internal control.

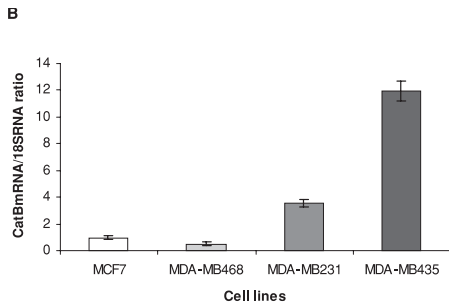
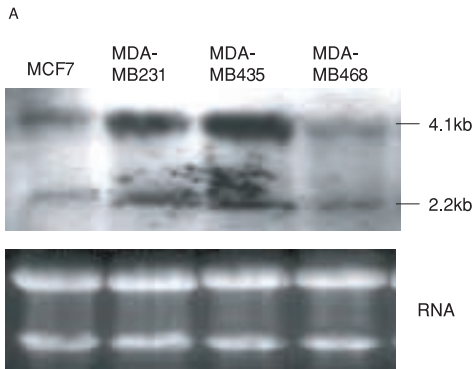
#### *Protein concentration*

Cells were homogenised in Tris buffer (50mM Tris, pH 6.9, 0.05% Brij 35, 0.5mM DTT, 5mM EDTA, and 10µM pepstatin A) at 4°C using Tissue Tearor<sup>TM</sup> (Biospec Products Inc., USA). The homogenates were centrifuged at 12,000 rpm for 15 min and supernatants (cell lysates) were stored at -20°C. Total protein concentration was determined by Bio-Rad protein assay (Bio-Rad, USA) using bovine serum albumin as standard. Protein concentration of CatB was measured in cell lysates using a specific ELISA kit (Krka d.d., Slovenia). The ELISAs recognised total CatB protein, i.e. the precursor and the active forms as well as CatB complexed with the inhibitors.

#### *Cathepsin B activity*

The activity of CatB was determined using the fluorogenic substrate Z-Phe-Arg-AMC (modified from *Lah*<sup>12</sup>), which is not selective for CatB, but also measures other cathepsins, such as CatL. First, the total cysteine peptidase activity was determined as the difference between the total activity and the background activity of the non-cysteine peptidases; this was determined using the general cysteine peptidase inhibitor, E64c, at a final concentration of 16µM. CatB activity was measured by adding its selective inhibitor, 10µM CA-074, and determining the residual activity. CatB activity was obtained as the difference between total cysteine protease activity and residual activity. One enzyme unit (EU) is defined as the quantity releasing 1µmol of AMC per min. Specific activity is expressed as mEU/mg of total protein in the cell lysates.

Statistical significance between measurements was determined by the two tailed t-test and  $p < 0.05$  was considered as significant.

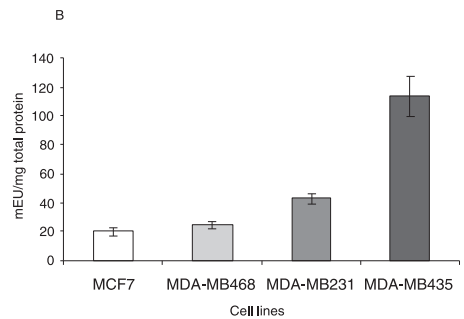
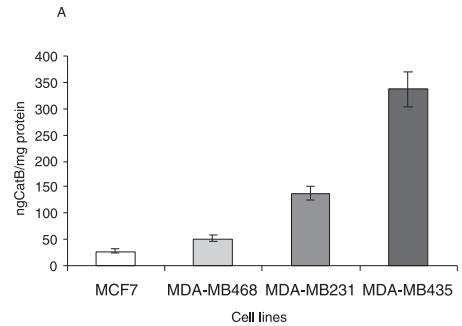


**Figure 1.** CatB mRNA expression in MCF7 and MDA-MB cell lines determined by Northern analysis (A) and quantitative RT-PCR (B). (A) For Northern analysis the total RNA was electrophoresed and hybridised with full length CatB DNA probe, as described in Material and methods. Total RNA stained with EtBr served as loading and transfer control (shown underneath). CatB expression is shown typically as two bands, one at 4.1kb and the other by 2.2.bp. (B) For quantitative analysis the total RNA was reverse transcribed and PCR amplified using CatB specific primers, as described in Material and methods. The results were normalised to 18S RNA. Error bars depict standard error of the mean. CatB mRNA levels were similar in MCF7 and MDA-MB468, but significantly higher in MDA-MB231 and MDA-MB435 cells.

## Results

### *Expression of cathepsin B in four human breast carcinoma cell lines*

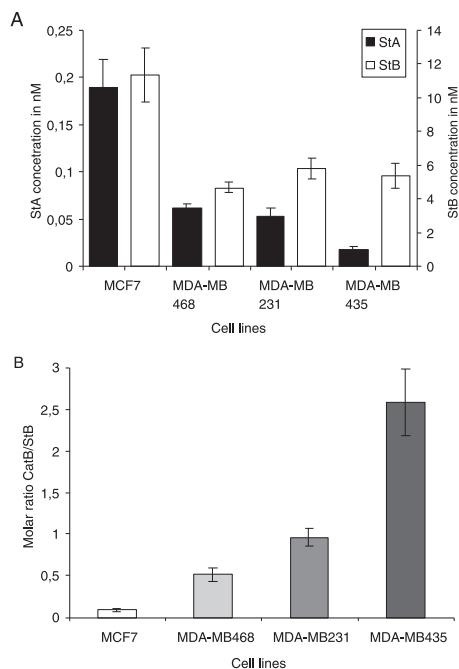
Expression of CatB was determined in the four breast cancer cell lines that differ in their tumorigenicity and invasive potential. Levels of CatB mRNA, protein and enzyme activity correlated highly ( $r > 0.99$ ). All three levels in-



**Figure 2.** Protein concentration (A) and enzymatic activity (B) of CatB in MCF7 and MDA-MB cell lines. Cell lysates were prepared as described in Material and methods. (A) CatB protein was measured by ELISA, and expressed as ng/mg total protein. The differences in protein concentration were statistically significant. (B) CatB activity was determined as described in Materials and methods. CatB enzymatic activities were significantly different, except between MCF7 and MDA-MB468 cells. Error bars depict standard error of the mean.

creased with the degree of tumorigenicity of these breast carcinoma cells (Figures 1 and 2).

Figure 1 shows CatB mRNA expression in MCF7 and the three MDA-MB cell lines determined by Northern analysis (A) and quantitative RT-PCR (B). The highest CatB mRNA level was detected in the most tumorigenic MDA-MB435 cells, slightly less in the most invasive MDA-MB231 cells, whereas both poorly tumorigenic cell lines MCF7 and MDA-MB468 expressed significantly lower levels of CatB. However, the difference between the latter two cell lines was not statistically significant.



**Figure 3.** The molar concentrations of StA and StB (A) and the molar ratio between CatB and StB (B) in MCF7 and MDA-MB cell lines. Protein concentrations of StA, StB and CatB were measured in cell lysates with ELISA (see above) and their molar concentrations were calculated. (A) Both stefins were the highest in MCF7 cells and significantly lower in metastatic MDA-MB cell lines. Note the difference in scale. (B) The molar ratio between CatB and StB increased progressively with tumorigenicity of the cells. All the differences between the cell lines were statistically significant. Error bars depict standard error of the mean.

Concentrations of CatB protein in MCF7 and the three MDA-MB cell lines are presented in Figure 2A. The lowest concentration was observed in the non-metastatic and poorly tumorigenic MCF7 cells and increased progressively from MDA-MB468, MDA-MB231 to MDA-MB435, the most tumorigenic cell line. All the differences were statistically significant. The protein concentration of CatB in cell lysates increased more than 14 fold, from 25 ng/mg (cca 1 nM) in MCF7 cells to 350 ng/mg protein (14 nM) in MDA-MB435 cells.

Figure 2B shows CatB proteolytic activities

in MCF7 and the three MDA-MB cell lines. As for protein concentration, specific activity of CatB was the lowest in MCF7 cells and increased progressively with tumorigenicity of the cells. The difference is statistically significant between the high and low tumorigenic lines, but not between the poorly tumorigenic cell lines MCF7 and MDA-MB468.

#### *Correlation between cathepsin B and stefins in four breast carcinoma cell lines*

Protein concentrations of StA and StB in the same breast cancer cell lines have been shown to be lower in cells with higher tumorigenicity.<sup>1</sup> As shown in Figure 3A, StB in the cell lysates was 2.4 fold lower in MDA-MB435, the most tumorigenic cell line, (59 ng/mg; 5.4 nM) than in MCF7 (about 126 ng/mg; 11 nM), whereas the levels of StA were about 10 fold lower, at 2 ng/mg protein (0.19 nM) and 0.2 ng/mg protein (0.018 nM) respectively. The molar ratio of the protein concentration of CatB to that of StB in the lysates of MCF7 and the three MDA-MB cell lines was determined (Figure 3B). This ratio was lowest in the MCF7 cell line and increased with tumorigenicity of the cells, as was observed with CatB levels alone.

## Discussion

Cathepsin B and CatL were initially considered to be products of single copy house-keeping genes, their expression being necessary for normal protein turnover in the cells. Surprisingly, homozygous CatB-deficient mice have an apparently normal phenotype,<sup>23</sup> suggesting redundancy of the genes, whereas CatL-deficient mice have periodic shading of fur and abnormal skin, but are otherwise viable,<sup>24</sup> suggesting cell-specific functions of this enzyme. Their expression is regulated at the gene level,<sup>25-26</sup> by mRNA splicing<sup>27-28</sup> and by posttranslational modification (reviewed by Frosch<sup>29</sup>). In the present

study, we have determined the expression of cathepsin B at the mRNA, protein and activity levels in MCF7, MDA-MB468, MDA-MB231 and MDA-MB435 breast cancer cell lines of increasing tumorigenicity *in vivo*.<sup>20</sup> CatB expression at all three levels is highly correlated. This suggests that the initial regulation of CatB occurs at the genetic level, the regulation of CatB transcription during development of the tumorigenic phenotype being most probably modulated through multiple GC boxes.<sup>25</sup> This needs to be elucidated further. However, in a model comprising cells derived from MCF-10A manipulation to result in distinct invasive and tumorigenic phenotypes, we observed that CatB was significantly related to invasiveness but not tumorigenicity,<sup>19</sup> although again all three levels of CatB expression correlated well. Presumably, expression of other gene profiles in the two panels of breast carcinoma cell lines is responsible for up regulation of CatB in relation to the invasive and/or tumorigenic potential.

There are also differences in the regulation of CatB and CatL. We found previously that, in contrast to CatB, CatL was highly increased at mRNA and protein levels, but was lowered at the activity level in the most invasive of the four cell lines, MDA-MB231.<sup>1</sup> This may be due to selective inhibition of CatL activity by endogenous inhibitors, or by another defect in its intralysosomal processing. Similar differences in expression of CatL between different levels were observed in the MCF10A model, suggesting that, in contrast to CatB, CatL regulation of expression also occurs posttranslationally. This supports our previous observation on differential regulation of cathepsins in breast cancer cell lines<sup>30</sup> and in clinical samples of breast tumours.<sup>7,8</sup>

The ultimate regulation of CatB and CatL activities in the cells results from binding by the endogenous inhibitors, the stefins.<sup>31</sup> Alteration of their levels in tumours, presumably downregulation, was reported.<sup>3,11</sup> In the cell line model used here, we have shown pre-

viously that StA levels decrease significantly with increasing tumorigenicity, in line with the hypothesis that imbalance between cysteine proteinases and their inhibitors facilitates tumour progression. StB levels were higher in the MCF-7 cells than in the other three invasive and tumorigenic cell lines.<sup>1</sup> It is noteworthy that the molar concentration of stefins was higher in low tumorigenic cells lines, but was lower than that of CatB in the most invasive and tumorigenic cells. This would suggest insufficient inhibition of CatB activity, since the complexes between cysteine cathepsins and cystatins are equimolar, as shown by crystallography<sup>31</sup> and kinetic measurements.<sup>32</sup>

Although measurements in cell lysates may not completely parallel the situation in living cells, the result is a good indication that the balance between cathepsins and stefins is drastically altered in this panel of cell lines. We conclude that, in this model of breast carcinoma cells, upregulation of CatB is a characteristic of the highly tumorigenic cell phenotype. Together with our previous studies on this cell model, the results presented here confirm that cathepsins B and L are important in the processes of tumorigenicity and invasiveness of the cells, respectively, but are not regulated in a coordinated manner. Furthermore, the imbalance between proteolytic enzymes and their inhibitors may facilitate the development of a malignant phenotype in breast cancer. If confirmed by further studies, both cathepsins could constitute potential targets for anti-invasive therapy.

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