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High expression of the cysteine proteinase legumain in colorectal cancer – Implications for therapeutic targeting

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Abstract ***Background:*** The cysteine proteinase legumain is highly expressed in cancer. Legumain is a potential biomarker and has been suggested to be utilised for prodrug activation in cancer therapy. However, to define the suitability of legumain for such purposes, detailed knowledge of cell type-specific and subcellular expression together with proteolytic activity patterns in tumour tissue is necessary.
Methods: Expression of legumain was examined in a panel of 277 primary tumours from colorectal cancer (CRC) patients using immunohistochemistry. Tumour (cytoplasmic diffuse, cytoplasmic granulated, and nuclear) and stromal cell expression of legumain was quantified, and associations with clinicopathological parameters and outcome were analysed. Additionally, normal colon tissue and spontaneous mouse tumours were stained for legumain.
Results: Legumain was highly expressed in tumour and stromal cells. Nuclear legumain was detected in 30% of the tumours. In colon cancer patients, high legumain expression was associated with overall and metastasis-free survival (OS; MFS) in uni- and multivariate analysis. Nuclear legumain was associated with poor OS, but not MFS in the colon cancer subgroup. Cytoplasmic granulated or diffuse expression was not associated with OS or MFS. Normal

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epithelial cells exhibited granulated legumain mainly at the apical pole, and legumain was highly expressed in CD68 positive macrophages.

Conclusions: Legumain is a highly expressed proteinase in CRC and associated with poor outcome in colon cancer. Diversified localisation of legumain expression in tumour and stromal cells suggests multiple functions in CRC, representing both a challenge and an opportunity for use in therapeutic targeting.

1. Introduction

The cysteine endo-proteinase legumain (also known as asparaginyl endopeptidase AEP) is unique in cleaving substrates N-terminally of asparagine [1]. Recent observations also describe exopeptidase activity of legumain [2]. The proteinase is translated as a pro-enzyme which undergoes a complex series of maturation steps to reach full proteolytic activity, and the mature form is regulated by endogenously expressed inhibitors, the cystatins, which compete with substrates for the active site [3]. Legumain is over-expressed in a number of cancers, and has been shown to be associated with tumour invasion and metastasis development [4–11]. Furthermore, blocking of legumain by means of inhibitors or antibodies has been shown to constrain tumour progression and metastasis [11]. Traditionally, legumain was thought to be expressed mainly in the endo-lysosomes, but it has become clear that legumain also is present extracellularly, and we recently described its prominent nuclear localisation and activity in colorectal cancer (CRC) cells [12]. The general up-regulation of legumain in cancer combined with its unique substrate recognition makes legumain an attractive candidate for tumour-specific targeting and has motivated the use of legumain-specific molecules in various therapeutic approaches. The enzymatic activity of legumain has been exploited by synthesising inert cytotoxic prodrugs and monoclonal antibodies that are activated locally in the tumour upon legumain cleavage [4,13–16]. This approach requires the presence of legumain as well as favourable conditions for proteolytic activity in the targeted cells, extracellularly or in relevant subcellular compartments. Thus, utilisation of legumain in prodrug therapeutics demands detailed knowledge about cellular expression patterns and subcellular localisation in stromal or tumour cells.

In the present work, using CRC as a model disease in which legumain expression is prevalent, we investigated its presence in tumour samples from 277 CRC patients, thereby focusing on the subcellular distribution patterns of legumain. Associations between legumain expression and clinical parameters, patient outcome, and also with expression of the metastasis-related protein S100A4 were analysed.

2. Materials and methods

2.1. Patient cohort and immunohistochemistry

Between September 1998 and July 2000 tumour tissue was prospectively collected from patients undergoing primary surgery for assumed or verified CRC ($n = 277$). Follow-up data were collected from consecutive reports from the participating hospitals. Survival data were obtained from the National Registry of Norway and updated by October 1st 2008, and median follow-up of patients still alive was 9.1 years (range 8.2–10.0) [17]. The study was approved by the Regional Ethics Committee (#S-98080) and written informed consent was obtained from the patients. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections from the primary tumours, using a previously validated polyclonal goat anti-human legumain antibody (R&D Systems; AF2199) in a 1:300 dilution with the biotin–streptavidin–peroxidase method as described previously [12,18]. Negative controls were performed by replacement of the primary antibodies with normal goat IgG antibody (Supplementary Fig. S2). Blinded primary tumour sections were evaluated by the study pathologist (JMN) and cytoplasmic granular, cytoplasmic diffuse, nuclear and stromal staining were reported as separate variables and scored (0–3) according to the numbers of legumain-positive carcinoma cells, i.e. 0 (0%), 1 (<10%), 2 (10–50%) and 3 (>50%). In subsequent statistical analyses all variables with a score $\geqslant 2$ were grouped as positive, except for cytoplasmic diffuse legumain staining where only score 3 was grouped as positive, due to a small number of cases with low score. For survival analyses, a binary variable 'total legumain expression' in tumour cells was generated by grouping the cases as positive or negative according to the cut off-values described above, in which positivity indicates simultaneous positivity for all possible locations of legumain presence, which is nuclear, cytoplasmic diffuse and granular.

2.2. Statistical analysis

Associations between legumain staining and clinicopathological variables and S100A4 were tested using