

Cathepsins H and L in colorectal cancer.

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ABSTRACT: Cysteine cathepsins are important regulators and signaling molecules of an unimaginable number of biological processes while they also play an essential role in cancer progression, invasion and metastasis. The purpose of our study was: first to compare the expression levels of cathepsins H and L in the supernatants of colon cancer tissues from 74 patients versus the same enzymic expressions of the supernatants of the adjacent normal colorectal tissues and second to correlate our findings to the grade of the malignancy by using an enzyme-linked immunosorbent assay (ELISA). The results indicated that the cathepsins H and L of all malignant tissues presented significant higher expression's values than the corresponding control. Specifically the concentration of cathepsin H that has been found increased significantly as malignancy proceeded, was higher than the corresponding control as following: 155% in B1 stage and 204,44% in D stage. Between the two investigated proteases cathepsin L has showed the greatest increase, which in D stage was 261,03% higher than the corresponding control. According to these results, the expression of cysteine proteases H and L could be of critical value in the diagnosis and progression of colon cancer.

Key Words: Colorectal cancer, Cathepsin H, Cathepsin L.

INTRODUCTION

Cathepsins have a vital role in mammalian cellular turnover. In living organisms their activity depends on a delicate balance of expression, targeting, zymogen activation, inhibition by protein inhibitors and degradation. They constitute a large family which is involved in various cellular processes such as MHC antigen presentation, protein degradation, hormone regulation and TNF induced apoptosis¹. Cathepsins' family is composed of at least fifteen discrete members, which characterized by their structure and substrate specificity^{1,2}. Based on these properties, cathepsins are divided into three different groups: the serine proteases (cathepsins A and G), the aspartic proteases (cathepsins D and E) and the cysteine proteases (cathepsins B, C, F, H, K, L, O, S, U, W and X). These proteases are found inside the cellular organelles mainly in lysosomes and peroxisomes, as inactive proenzymes³. However, recent studies have shown that active cathepsins are located in other cellular

compartments, such as nucleus, cytoplasm and plasma membrane, where they contribute in protein turnover and degradation of polypeptides. It was shown that the catalytically active variants of cathepsin L localized in the nucleus play a role in the regulation of cell-cycle progression and the proteolytic processing of the N-terminus of the histone H3 tail.

Following a cell death signal, cathepsins are released outside the cell and trigger the degradation of the extracellular matrix and subsequent cell apoptosis^{4,5,6,7}.

On the other hand, cathepsins participate in pathological and inflammatory processes including Alzheimer's disease and tumor development and invasion. Increased cathepsins' expression in cancer cells causes tumor cell growth, invasion, and metastasis⁵ while the precise role of each cathepsin in carcinogenesis remains unclear. Promising targets for anticancer therapy seem to be cathepsins B, C, H, L, S and X/Z. The levels of these cathepsins are elevated in cancer

cells and cells involved in angiogenesis. Cathepsins H and L are overexpressed in cancer cells only, in contrast to cathepsins B, C, S and X/Z, which are also found to be overexpressed in non-malignant cells in tumors. As Cathepsin L has been studied most thoroughly but yet their functions are still not well-defined and as there is growing evidence that the expression of cathepsin H is increased in malignant diseases including breast, colorectal and prostate carcinoma⁶, we decided to estimate the expression levels of the two cysteine cathepsins H and L in human colon cancer tissue and to correlate the findings to the grade of this malignancy.

MATERIALS AND METHODS

Tissue sample collection

The study comprised 74 patients with colorectal cancer (CRC), who underwent colorectal resection in Theagenio Cancer Hospital of Thessaloniki, 35 males, about 69 years old and 39 females, about 68,9 years old. Patients were not undergoing chemotherapy, radiotherapy, or any other adjuvant therapy for CRC, before the colorectal resection. Clinical data of the patients and histology of tumors were registered accurately. In addition, all patients were monitored after surgery.

The resected tumors have been sub grouped according to their location (colon cancer, n = 48; rectal cancer, n = 26), but mainly they have been histologically classified according to Astler-Coller staging system: in stage A the tumor has been confined to mucosa (n = 3); in stage B1 the tumor has extended into muscularis propria but it hasn't penetrated it and nodes haven't been involved (n = 13); in stage B2 the tumor has penetrated muscularis propria, but nodes haven't been involved (n = 18); in stage C1 the tumor has extended into muscularis propria, it hasn't penetrated through, but nodes have been involved (n = 11); in stage C2 the tumor has penetrated muscularis propria, and nodes have been involved (n = 15); finally, in stage D the tumor has been associated with distant metastases (n = 14) (Table 1).

The samples have been taken from the resected tissues. The malignant samples have been collected from different areas of the lesion that was macroscopically evident, as well as from the adjacent normal tissue

Table 1. Clinical characteristics of the colorectal cancer patients included in the study.

<i>Patients Characteristics</i>	<i>Data</i>
Number of patients	74
Mean Age	± 69 years
Male	35
Female	39
Period	2009-2010
Tumour Site	Colon and rectum
Colon	48
Rectum	26

(controls). As normal tissue has been characterized the tissue where there haven't been macroscopically dead or hemorrhagic foci. Presence or absence of lesion was confirmed histopathologically in all cases according to current guidelines. After the collection, all tissue samples have been frozen in liquid nitrogen and stored at -80°C until used.

ELISA assay for quantitative detection of cathepsins H and L

Prior to assay, the frozen samples have been brought to room temperature, washed in isotonic solution of sodium chloride (CAS No: 8028-77-1), cut into pieces, weighed and homogenized in 4°C with lysis buffer [50 mM HEPES (CAS No: 7365-45-9) pH 7.4, 5 mM CHAPS (CAS No: 75621-03-3), 5 mM 1 DTT (CAS No: 7634-42-6)], in proportion: 1 gr tissue/10 ml lysis buffer. The homogenized tissues have remained at -20°C for 24 hours and then they have been centrifuged at 20000 g for 15 min at 4°C. Supernatants (10 µl per well) have been used for quantitative detection of cathepsins H and L by enzyme-linked immunosorbent assay.

Anti-human cathepsin-H and cathepsin-L coating antibodies have been adsorbed onto microwells. Aliquots of supernatants (10µl per well) have been bound to corresponding adsorbed antibodies. Biotin-conjugated anti-human cathepsin-H and cathepsin-L antibodies have been added and bound to corresponding cathepsin captured by the first antibodies. After

incubation unbound biotin-conjugated antibodies have been removed during a wash step and streptavidin-HRP has been added and bound to the biotin-conjugated anti-human antibodies. Following incubation unbound streptavidin-HRP has been removed by washing and substrate solutions reactivated with HRP has been added to the wells. Colored products have been formed in proportion to the amount of cathepsins present in the samples. The reactions have been terminated by addition of 100µl 1M phosphoric acid to each well. A spectrofluorometer microplate reader (Perking Elmer LS 50 B, using illumination at 440 nm-measuring emission at 370 nm) has been used to measure the absorbance of the ELISA assay at 450 nm. Tree standard curves have been prepared from standard dilutions of the two human cathepsins (H and L) and the concentrations of corresponding cathepsins of the samples have been determined in ng/mg homogenated tissue.

RESULTS

The Graphs 1 and 2 show respectively the expression (ng/mg homogenated tissue) of cathepsins H and L in human colon cancer tissues. Each graph presents the expression of corresponding cathepsin in the tissues of the cancerous stages B1, B2, C1, C2 and D and of their adjacent normal tissues (controls). The bars of B1 stage illustrate the mean value of the expression of the corresponding cathepsin from 13 different patients, the B2 bars have been created from the mean value of the expression of the corresponding cathepsin from 18 different patients, the C1 bars is the mean value of the expression of the corresponding cathepsin from 11 different patients, the C2 bars become from the mean value of the expression of the corresponding cathepsin from 15 different patients and the D bars show the mean value of the expression of the corresponding cathepsin from 14 different patients. The expressions of these two cathepsins in all cancerous tissues have been found significantly higher than those of the controls.. Cathepsin H has been estimated statistically significant increased in all the malignant stages compared to that of the adjacent normal tissues and as the malignancy has proceeded, the elevation has being higher as following: 155%, 160,71%, 193,19%, 208,20% and 204,44% in stages B1, B2, C1, C2 and D respectively than the adjacent

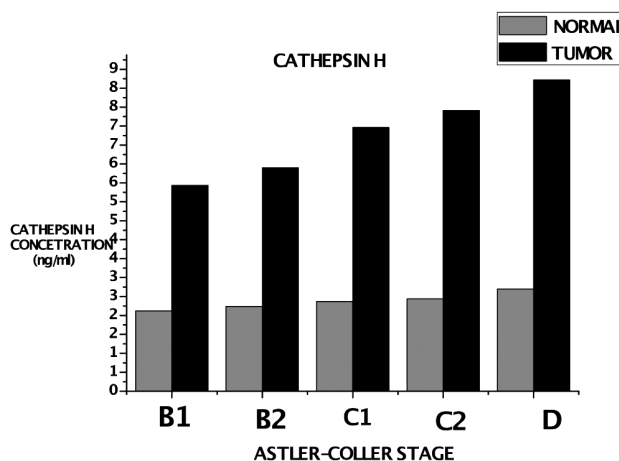


Figure 1. Comparison of the expression (ng/ mg) of cathepsin H in colon cancer patients vs controls L in different stages (B1, B2, C1, C2, D) of colon cancer. The data are shown as mean ± SD of the samples from each group.

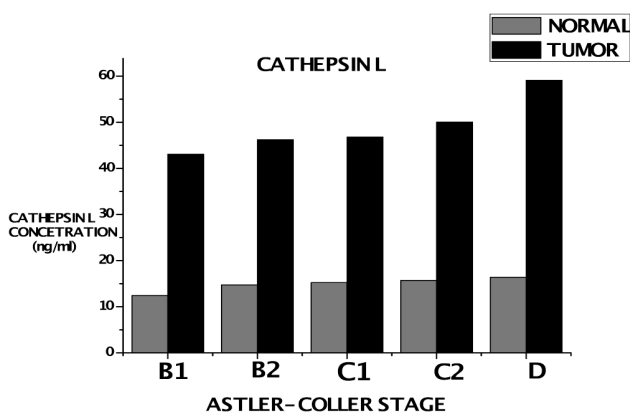


Figure 2. Comparison of the expression (ng/ mg) of cathepsins L in colon cancer patients vs control L in different stages (B1, B2, C1, C2, D) of colon cancer. The data are shown as mean ± SD of the samples from each group.

normal tissues. Between the two investigated proteases cathepsin L has showed the highest increase in all stages of malignancy, which was 246,42% in stage B1, 213,99% in stage B2, 206,95% in stage C1, 218,52% in stage C2 and 261,03% in stage D, compared to the normal adjacent tissues.

Statistical Analysis

Student’s t-test and one-way ANOVA test were performed for the statistical evaluation of cathepsins

H and L expression, in order to determine whether any values deviated significantly from the controls ($p < 0,01$). Results were given as mean value of expression (ng/ml homogenated tissue) \pm standard deviation (SD). All statistical analyses were conducted using the GraphPad 5,1 statistical software package (GraphPad, Europe).

DISCUSSION

As cancer continues to be one of the most serious health issue and colorectal cancer (CRC) the most common gastrointestinal cancer in the Western world causes of cancer-related death, there is a great need for searching of sensitive and specific markers of the disease. Proteases have been suggested by several researchers as tumor markers in CRC^{8,9}.

Clear evidence that cysteine cathepsins aren't only lysosomal proteases and that they have also non-lysosomal/endosomal roles implicates them in an unimaginable number of biological processes involving the invasion and the migration of cancerous cells, but also proliferation, apoptosis and angiogenesis that characterize the malignant tumours¹⁰. Loss of cell-cell and cell-matrix adhesion and degradation of extracellular matrix (ECM) components are involved in invasion and migration¹¹. Cysteine cathepsins can be expressed at the cell surface and secreted into the extracellular space, where they can degrade components of the ECM and remodel the microenvironment of tumour processes. In addition, they may play roles in the regulation of the action of certain growth factors, growth factor-binding proteins and growth factor receptors, vital participants in human colorectal cancer growth.^{12,13,14,15} On the other hand cysteine cathepsins could contribute in tumour progression, affecting intracellular tumorigenic processes, as programmed cell death¹⁶.

Cathepsin B is the first cathepsin that has been demonstrated to be linked to cancer 30 years ago⁵. Since then it has been found to be expressed in the vast majority of colon cancers and adenoma. Other investigators have revealed that cathepsins B, C, S and X/Z overexpress also in non-malignant cells in tumours, while overexpression of cathepsins H and L has been identified in cancer cells only^{14,17,18}.

As it is obvious, the conclusions of the investigation about the precise biological role of cysteine ca-

thepsins in CRC do not coincide. On the other hand numerous clinical reports and results from experimental works have supported that cysteine cathepsins H and L, play a crucial role in both tumour progression and invasion^{18,19,20}, but none of them has investigated these two proteases concomitantly in the same malignant tissue, using the same assay method and comparing with the same adjacent normal tissue. Therefore we were determined to carry out the present study.

Cathepsin H influences several important tumorigenic processes including degradation of the extracellular matrix, proteolytic processing of chemokines and activation of other enzymes. Numerous clinical studies have reported correlations between elevated cathepsin H levels and malignant progression however its specific functions in tumour development and progression is not fully understood.^{21,22,23} Gocheva et al have demonstrated for the first time the important tumour-promoting role for cathepsin H in vivo using a mouse model of human cancer²¹. They have found that deletion of cathepsin H action in crossed cathepsin H-deficient mice with the RIP1-Tag2 model of pancreatic islet carcinogenesis has significantly impaired angiogenic switching of the pre-malignant hyperplastic islets and resulted in a reduction in the subsequent number of tumours that formed.^{24,25} When Schweiger et al. have measured cathepsin H in preoperative sera from 324 patients with colorectal cancer by ELISA they have found that its level was significantly increased and that there was a weak association of cathepsin H levels with patient age but not with stage (Astler-Coller), sex or the level of carcinoembryonic antigen (CEA)²⁶. According to these findings and to the results of the survival analysis Schweiger et al have concluded that the prognostic information and the role during the malignant progression of cathepsin H differed from those of the related cathepsin L²⁶. In the present work the expression of CRC cathepsin H has been estimated statistically significant increased ($P < 0,01$) in all the malignant stages compared to that of adjacent normal tissues and the elevation has been increased as the malignancy proceeded (155%, 160,71%, 193,19%, 208,20% and 204,44% in stages B1, B2, C1, C2 and D respectively). This last finding testifies the participation of cathepsin H in tumour development and progression and its different behaviour as cancer proceeds, in comparison to cathepsin L.

The expression of cathepsin L has presented the highest increase in all malignant stages. Specifically the expression of this cathepsin has been estimated 246,42% in B1 stage, slightly decreased in B2 (213,99%), C1 (206,95%) and C2 (218,52%) stages, but slightly elevated (261,03%) in stage D, compared to the adjacent physiological tissues. As the differences among the cathepsin L expression of stages B2, C1 and C2 have been negligible, we would agree with Herszényi L. et al.²⁷ According to them cathepsin L may be involved in the progression from premalignant colorectal adenoma into CRC. Nevertheless our results about cathepsin L are in agreement with the findings that have been referred in the review of Jacqueline Lankelma et al.¹¹ They have evaluated the state of affairs concerning cathepsin L as a possible target in cancer treatment, as its activity has exclu-

sively been increased in malignant cells, in contrast to that of other cathepsins.

Our parallel investigation about the expression levels of cysteine cathepsins H and L in the supernatant of colon cancer tissue from 74 patients indicated that these two cathepsins of all malignant tissues presented significant higher expressions' values than those of the corresponding controls. Between the two investigated proteases, cathepsin L showed the highest increase in all the malignant stages, although the expression of cathepsin H increased significantly as malignancy progressed. So these data which are in agreement with the results of significant, corresponding researches, could suggest that the expression of cysteine proteases H and L could be of critical value in the diagnosis and progression of colon cancer.

Καθεψίνες H και L στον καρκίνο του παχέος εντέρου.

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ΠΕΡΙΛΗΨΗ: Οι καθεψίνες, ως πρωτεάσες, ρυθμίζουν σημαντικό αριθμό φυσιολογικών βιολογικών διεργασιών, ενώ διαδραματίζουν επίσης πρωταγωνιστικό ρόλο τόσο στην ανάπτυξη του καρκίνου, όσο και στη μετάσταση του. Σκοπός της μελέτης μας ήταν κατ' αρχή να συγκριθούν τα επίπεδα έκφρασης των καθεψινών H και L στο υπερκείμενο ομογενοποιημένου καρκινικού ιστού παχέος εντέρου από 74 ασθενείς, με την έκφραση των ίδιων ενζύμων από το υπερκείμενο αντίστοιχων φυσιολογικών ιστών, γειτνιαζόντων με τους καρκινικούς, και δεύτερον να συσχετιστούν τα αποτελέσματά αυτά με το βαθμό κακοήθειας της νόσου. Οι δραστηριότητες των καθεψινών H και L, οι οποίες προσδιορίστηκαν με τη μέθοδο ELISA, όλων των καρκινικών ιστών παρουσίασαν τιμές σημαντικά υψηλότερες συγκρινόμενες με τις τιμές των αντίστοιχων φυσιολογικών ιστών. Συγκεκριμένα, η έκφραση της καθεψίνης H παρουσίαζε σημαντική αύξηση, καθώς αυξάνονταν το στάδιο της κακοήθειας. Ενδεικτικά αναφέρεται ότι η συγκέντρωση της καθεψίνης H ήταν υψηλότερη από την αντίστοιχη του μάρτυρα κατά 155% στο στάδιο B1 και κατά 204,44% στο στάδιο D. Μεταξύ των δύο εξεταζόμενων πρωτεασών η καθεψίνη L έδειξε τη μεγαλύτερη αύξηση, η οποία στο στάδιο D ήταν 261,03% υψηλότερη από την αντίστοιχη του μάρτυρα-φυσιολογικού ιστού. Σύμφωνα με τα αποτελέσματα αυτά, οι μεταβολές της έκφρασης των κυστείνοπρωτεασών H και L των καρκινικών ιστών παχέος εντέρου θα μπορούσαν να διαδραματίσουν σημαντικό ρόλο στην διάγνωση και την εξέλιξη του καρκίνου του παχέος εντέρου.

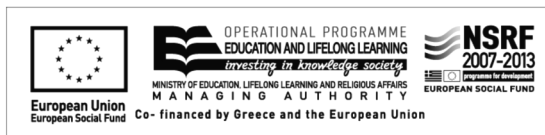
Λέξεις κλειδιά: Καρκίνος παχέος εντέρου, Καθεψίνη H, Καθεψίνη L.

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