Introduction

The first known cancer family would be described today as involving hereditary nonpolyposis colorectal cancer, in which, as in familial adenomatous polyposis, the emergence of a malignancy requires a seemingly unlikely sequence of somatic mutations, superimposed on a risk-conveying inheritance. Events identified in the inherited syndromes are proving crucial in learning precisely how nonfamilial tumors develop. In this study, effect of the expression of cysteine proteases will be discussed on the development of colorectal cancer, and the interaction between chicken cstatin and cathepsins in the tumor tissues may be used in a future as pro-drug against colorectal cancer.

1. Expression of cathepsin B and L in colorectal cancer patients associated with disease progression

Among cancer diseases associated with the digestive system, the most thoroughly studied, with respect to cysteine cathepsins (CCs) involvement, is colorectal carcinoma (CRC). Similarly to other cancers, the best investigated is cathepsin B, whose increased activity or immunostaining has been found in cancerous tissues (compared with normal tissues) from CRC patients [1–5]. Interestingly, Kim et al. [6] have observed that cathepsin...
B and L up-regulation might result from ras oncogene alteration; colorectal carcinomas demonstrating changed N-ras protein forms displayed significantly higher cathepsin B and L activities compared to carcinomas with normal N-ras protein. Diffuse distribution of cathepsin B (both protein and activity) in the cytoplasm has been observed in CRC tissues by Campo et al. [2] and Hazen et al. [7], especially in poorly differentiated carcinomas. Moreover, similar localization as well as high activity of the enzyme has been detected particularly in the cells at the invasive fronts of the cancer [1, 7], which would support the concept about cathepsin B participation in invasive processes of CRC. However, experiments examining the relation of the protease with tumor progression have revealed ambiguous results. In some studies, no correlation between cathepsin B (nor cathepsin L) activity and tumor stage has been found [3], in other higher activities of both cathepsins B and L have been observed in the initial stages of cancer development (Dukes’ A and B) [4, 8]. Higher levels of cathepsin B and L have been detected in metastatic versus non-metastatic cancer (performed with ELISA assay in tissue homogenates). On the other hand, in the latter study, increased level of cathepsin L has been shown to correlate with shorter survival of patients. Another CC studied in CRC is cathepsin H whose higher activity in cancers in comparison to control mucosa has been shown by del Re et al. [8]. Besides, unlike cathepsins B and L displaying elevated activities in Dukes’ A and B stages, cathepsin H activity was increased in later stages of cancer development (Dukes’ B and C) [9]. In gastric cancer (GC), both cathepsin B and L expression has been demonstrated in invasive and non-invasive human cell lines [10]. Cathepsins B and L levels have been shown to be elevated in cancerous tissues from GC patients as compared to paired normal mucosa [11, 12]. Moreover, cathepsin B expression was significantly associated with TNM stage, nodal status and histological grade. However, no prognostic significance has been observed for cathepsin B and L, due to the lack of correlation with patients survival rates [11]. Contrary results have been obtained by Herszenyi et al. [13], who found significantly higher cathepsin B concentration in cancer tissue from the patients with shorter survival rates. Moreover, cathepsin B level correlated better with survival than with TNM stage and tumor grading. Khan et al. [14] have found a significant association between increased cathepsin B staining and higher tumor stage in gastric carcinomas. In human tissues of pancreatic ductal adenocarcinoma, cathepsin B immunohistochemical staining has been found in 70% of invasive cancers, whereas noninvasive cancers displayed no enzymatic expression [15]. Moreover, diffused expression of cathepsin B within the cytoplasm of cancerous cells has been observed [15]. Also, cathepsin H expression has been shown to be upregulated in pancreas cancer cultures, in comparison to normal pancreas tissue or cultured cells [16]. In human hepatocellular carcinoma (HCC) and cholangiocarcinoma diffuse pattern of cathepsin B cytoplasmic distribution has been observed [17]. However, no differences between cathepsin B activity in HCC and normal tissue have been determined [18]. Also, serum cathepsin B activity remained at the same level in-patients after surgery, in comparison to serum activity before surgery [18]. Herszenyi et al. [13], Farinati et al. [19] and Shuja et al. [4] have proposed that cathepsins B and L could be markers reflecting progression from precancerous to cancerous lesions in gastrointestinal cancer. Shuja et al. [4] have observed normal cathepsin B activity in adenomas from colorectal cancer patients, whereas in cancers the protease activity was enhanced. Farinati et al. [19] have demonstrated significantly higher cathepsin B and L concentrations in chronic atrophic gastritis than in controls. Additionally, cathepsin B level was significantly higher in chronic atrophic gastritis with dysplasia, as compared to gastritis without dysplasia. Herszenyi et al. [13] have examined the usefulness of cathepsin B serum level in identification of patients with gastrointestinal tract cancers (GC, CRC, HCC, pancreatic cancer). The best sensitivity values were obtained for pancreatic cancer (100%) and gastric cancer (81%).

2. Expression of cystatin C in colorectal cancer tissues

Cystatins are reversible, tightly-binding competitive inhibitors of cysteine peptidases, e.g. catepsins B, H, L, which can promote cancer cell invasion and metastasis [20]. The cystatin superfamily of cysteine peptidase inhibitors is comprised of three major families: Type 1 cystatins, which are cytosolic and include stefins A and B; Type 2 cystatins, which are present in most bodily fluids and include cystatins C, D, E, F, and S; and Type 3 cystatins in plasma which include the kininogens and fetuin [21]. In physiological conditions, cysteine peptidases within the cells are regulated by: stefin A and B and cystatin C [20-22]. In the present study we concentrated on cystatin C because it possesses high affinity for cathepsin B. In inflammatory conditions or conditions with tissue breakdown, cystatin C free in the blood or other body fluid inhibits cysteine peptidases and thereby suppresses tissue damage. Cystatin activity or concentration has been shown to be higher, similar, or lower in different cancer tissue homogenates compared to normal [23]. Cystatin C inhibits motility and in vitro invasiveness of cancer cells, supporting the hypothesis that cystatins play a role in the maintenance of cell differentiation [24]. It has been reported that in colon and breast cancers (colon, breast, ovary) overexpressing cathepsin B, the surrounding tissue demonstrates increase in cystatin C level [25-27]. Potential use of cystatin C in tumour therapy was also suggested [28-31].

3. Expression of cathepsin D in colorectal cancer tissues (CDC)

Cathepsin D expression is a predictor of lymph node metastasis in sub-mucosal colorectal cancer. Cathepsin D expression was examined immunohistochemically in
cancer and stromal cells located at the deepest portion of 254 invasive tumours that had been resected from patients with submucosal CRC. In cancer cells, the expression was classified according to differences in intracellular localisation: polarity positive, apical type (PA); polarity positive, basal type (PB); polarity negative (PN); or no expression (NE). Lesions with PN or NE expression showed a significantly higher incidence of lymph node metastasis than those with PA or PB expression. Alternatively, lesions with positive expression in stromal cells showed a significantly higher incidence of lymph node metastasis than that of those with negative expression. None of the lesions with PA or PB expression and negative expression in stromal cells had metastasised to the lymph node. In conclusion, analysis combining cathepsin D expression in cancer and stromal cells may be a quite useful predictor of lymph node metastasis and may broaden the indications for curative endoscopic treatment of submucosal CRC [32]. Endoscopic mucosal resection (EMR; the so-called strip biopsy) allows for the complete resection of not only polyoid lesions but also flat or depressed lesions, and relatively large elevated lesions in the colorectum [33-34]. Previous reports indicate that, among cases of early colorectal cancer (CRC), intramucosal lesions show no lymph node metastasis [35]. Submucosally invasive lesions, however, show lymph node metastasis in 3.6-16.2% of patients with this condition [36-41]. The success of curative EMR of early CRC depends on presence of no or very limited lymph node metastases. We previously reported that histological grade at the deepest invasive portion was the most important risk factor for lymph node metastasis in submucosal CRC [39-41]. This is because the deepest invasive portion is the invasive tumour region with the highest malignant potential, being the part that ultimately will invade, spread locally, and metastasise. Our previous results indicate that scanty submucosal invasion (sm-s) and a histological grade of well or moderately to well differentiated adenocarcinoma at the deepest invasive portion are the appropriate indicators for curative EMR of submucosal CRC because of the very low incidence of lymph node metastasis [39-42]. At present, however, CRCs with massive submucosal invasion (sm-m) or histological grade of moderately to poorly differentiated, poorly differentiated or mucinous adenocarcinomas usually have undergone additional surgical resection, even after complete EMR, because there are few of negative lymph node metastases in these lesions [40, 42]. If more useful and strict predictors of lymph node metastases are established, we can broaden the curative conditions after EMR and provide a better quality of life for patients with submucosal CRC. Cathepsin D expression has been reported as a useful prognostic factors in patients with cancer of various organs [43-46]. As for CRC, several studies have demonstrated the significance of cathepsin D expression [47-48].

4. Mechanism of interaction between cystatin and cysteine proteinase

Cystatins were observed with tight interactions with certain target enzymes, for which reversibility have been difficult to verify and dissociation equilibrium constants are difficult to determine accurately by equilibrium methods [49, 50]. Separate measurements of association and dissociation rate constants have however demonstrated the reversibility also of these very tight interactions [51], and have enabled determination of Ki values as low as~10 fM. Recombinant human cystatin C and two of its mutants were expressed in Escherichia coli. The recombinant inhibitor was found to be identical to authentic cystatin C, as judged by isoelectric focusing (pI 9.2). Kinetics of inhibition of papain and human cathepsins B, H, and L: N-terminal truncation of 8 residues resulted in a decrease of isoelectric point (pI 7.8), but the inhibitory properties were similar to those of recombinant cystatin C, suggesting that Leu9 is a critical residue for the inhibition. The mutation of Trp106 to Ser, however, resulted in a decreased affinity of the inhibitor for the enzymes tested, with the largest effect on cathepsin B inhibition (approximately 100-fold increase in Ki) [52]. A review of kinetic and structural data has enabled the authors to reconsider the definition of substrate binding sites in papain-like cysteine proteases. The location and definition of substrate binding sites beyond S3 and S2 are even more questionable [53]. These results clearly indicate differences in the specificity of interaction between the N-terminal region of cystatin C and cathepsins B, H, L, and S, and that, although cystatin C has evolved to be a good inhibitor of all of the mammalian cysteine proteinases, more specific inhibitors of the individual enzymes can be engineered [54]. Stopped-flow kinetics showed that the inhibition of the lysosomal cystein proteinase, cathepsin B, by its endogenous inhibitor, cystatin C, occurs by a two-step mechanism, in which an initial, weak interaction is followed by a conformational change. The initial interaction most likely involves binding of the N-terminal region of the inhibitor to the proteinase. The presence of this loop, which allows the enzyme to function as an exopeptidase, thus complicates the inhibition mechanism, rendering cathepsin B much less susceptible than other cysteine proteinases to inhibition by cystatins [55]. The N-terminal region of human cystatin C has been shown to be of crucial importance for the interaction of the inhibitor with cysteine proteinases. These results show that bovine cystatin C has 118 residues, in contrast to 110-112 residues reported previously, and has an N-terminal region analogous to that of human cystatin C. This region presumably is of similar importance for tight binding of target proteinases as human proteases [56]. Inhibition of calpain by human kininogen domain 2 requires the correct conformation and combination of several contact sites, and suggests that the N-terminus and the first hairpin loop play a major role in this ensemble. Remarkably, hybrid sc2-KD2 exhibited 5 or 150 times stronger inhibi-
tion of actinidin compared to native chicken cystatin or to proteolytically isolated human kininogen domain 2, respectively. This indicates an important role of the first hairpin loop of cystatins in the interaction with actinidin, impaired along inhibition of cathepsin L, papain, actinidin, cathepsin B and calpain by the hybrids sc1/3-KD2, sc2/3-KD2 and sc1/2/3-KD2. These results support the hypothesis that all three predicted contact regions of kininogen domain 2 contribute to binding in the active site clefts of papain-like enzymes in a finely balanced manner [57]. The binding of proteinsases to kininogen has been the subject of some dispute. Although it has been shown that two of three isolated kininogen domains have inhibitory activity when studied separately [58], and that the kininogens thus have the potential for binding two molecules of cysteine proteinsases, the evidence regarding the binding stoichiometry of the intact molecules was conflicting [59]. Recently, both intact LMK and HMK have been shown by several methods to bind two molecules of papain, cruzipain and cathepsins, H, L and S, thus clarifying the dilemma. Moreover, the two binding sites on HMK and LMK bind proteinsases with different binding rates [60]. The three kininogen separates (HMK, LMK and TK) have closely similar inhibitory properties [58], indicating very small differences between their enzyme binding regions. The inhibitions of endopeptidases papain, cathepsins S and L and cruzipain by animal cystatins are extremely tight and ripped whereas the inhibition of exopeptidases cathepsins B and H [61], is considerably weaker. The active site cleft of known endopeptidases is free to accommodate the inhibitors, whereas in the case of exopeptidases the active site cleft contains additional enzyme residues. In carboxydipeptidase cathepsin B its occluding loop partly occupies the active site cleft and needs to be displaced in order to accept a cystatin molecule [62]. It was suggested that the mini-chain of aminopeptidase cathepsin H, which is attached to the enzyme via a disulphide bridge in the vicinity of the active site, partly fills the active site cleft, thereby offering sterical hindrance to the binding of inhibitors [63].

Cystatins are the natural inhibitors of cysteine proteinases [51]. Conventionally, they are divided in three major families according to their amino acid sequence [64] as shown in Table 1. However, this classification is based only on animal cystatins, because many studies have been conducted to clarify the physiological and clinical significance of cystatins in animal tissues and products. Cystatins have received much attention in the last two decades due to their potential in regulating the function of cysteine proteinsases [51]. The major characteristics of mammalian cystatins are summarized in Table 1. During the last decade, a fourth group belonging to the cystatin superfamily has emerged, that is, the plant cystatins. The first plant cystatin was found by [65] in rice seeds and is called oryzacystatin. Several other plant cystatins were discovered in soy, corn, wheat, potato, ragweed, cowpea, avocado, and papaya. Homology searches show that plant cystatins, except for potato cystatin, resemble family 2 cystatins of animal origin, but lack disulphide bonds like family 1 cystatins [66]. Despite the high protein sequence homology, the gene organization of oryzacystatins is markedly different to that seen in animal cystatins. Therefore, it has been suggested that a new cystatin family exist, namely the phytocystatins [67].

Most cystatins are reversible, tight binding competitive inhibitors of cysteine proteinsases, which form equimolar complexes with their target enzyme [68]. Their general mechanism of action is based on three domains that show highly conserved amino acids se-

Table 1. Major characteristics of mammalian cystatins

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Family 1</th>
<th>Family 2</th>
<th>Family 3</th>
</tr>
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<tbody>
<tr>
<td>Amino acid residues</td>
<td>About 100</td>
<td>115–120</td>
<td>3 cystatin like domains</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>About 11</td>
<td>13–14</td>
<td>High: 88–114</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low: 50–68</td>
</tr>
<tr>
<td>Disulfide bonds</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Glycosylated</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Location</td>
<td>Mainly intracellularly</td>
<td>Mainly extracellularly</td>
<td>Intravascularly</td>
</tr>
<tr>
<td>Cystatins</td>
<td>Human: A (stefin A), B (stefin B)</td>
<td>Rat: cystatin α and β</td>
<td>Human, rat, bovine: L-kininogen, H-kininogen</td>
</tr>
<tr>
<td></td>
<td>Human: C, D, S, S1, S2, SN, SA, D</td>
<td>Mouse: C</td>
<td>Rat: T-kininogen</td>
</tr>
<tr>
<td></td>
<td>Rat: C, S</td>
<td>Chicken egg white, bovine colostrums, ox, Drosophila</td>
<td>Ox: kininogen</td>
</tr>
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quences. These are important for the inhibitory activity. These domains consist of 10 amino acid residues in the amino terminus, a β-hairpin loop containing the conserved –QVVAG– residues, and a second β-hairpin loop containing the conserved residues Leu102, His 104 in family 1 and Trp104 in family 2 cystatins [69]. This wedge penetrates and covers the active site in such a fashion to block the papain or other cysteine proteinase’s active site cysteine residue [68, 69]. The interactive elements of this complex are represented having chicken egg-white cystatin as model in Figure 1 [70].

![Figure 1](image)

**Fig. 1.** Scheme of the proposed “trunk model” for the interaction of chicken egg-white cystatin and papain

Ryc. 1. Proponowany model interakcji cystatyny białka jaj i papainy (Turk and Bode, 1991) [71].

5. Regulation of cysteine peptidases by chicken cystatin

Kos et al. [71] reveal a correlation between high levels of extracellular cysteine protease inhibitors and short survival in patients with colorectal cancer, and the data thus support previous studies suggesting a contributing role of protease inhibitors in the progression of cancer, strongly implicate cystatin C in the invasiveness of human glioblastoma cells and suggest that sense transcripts of cystatin C may prove useful in cancer therapy [72]. Cystatin C displays the strongest inhibitory activity among cystatins toward lysosomal cysteine peptidases in general and has a widespread distribution in human tissues and body fluids [73, 74]. Serum cystatin C concentration has been proposed as a marker for glomerular filtration rate and kidney function [75], while altered serum cystatin C or cathepsin B: cystatin C complex concentrations were suggested as diagnostic and prognostic indicators for cancers including colorectal cancer [76, 77, 78]. In tumour cells cysteine peptidase, mainly cathepsins B and L, are overexpressed and translocated to the plasma membrane or secreted from the cells, and take part in the degradation of components of the extracellular matrix and basement membrane which is deemed to be a crucial step in the metastatic process. Angiogenesis can be considered as a controlled invasive process in which endothelial cells produce a number of proteolytic enzymes, such as cathepsin B.

Cystatin C expression was significantly evident in cancers of stomach (75%; 6/8 cases), uterus (71%; 5/7 cases), prostate (67%; 2/3 cases), colon (55%; 6/11 cases), and kidney (47%; 7/15 cases) [79]. Imbalance between CB and CPI may contribute to cancer invasion and metastasis and was observed for another cancer [80]. Additionally, knowledge about the balance between endogenous CPI and different papain-type cysteine peptidases is also needed for better understanding of the regulation of cysteine-dependent proteolysis in tumour progression. Prognostic significance of cathepsin B levels in sera of patients with colorectal cancer was not found despite the link between cathepsin B protein expression and metastasis [81]. However, the complex signalling between cathepsin B and antigen involving translocation of lysosomal vesicles from the perinuclear region to the plasma membrane, possible fusion and subsequent release of active cathepsin at the cell surface is an alternative explanation demonstrated on the outer surface of cancer cells [82, 83]. In conclusion, we have observed a wide range of cathepsin B and D expression patterns in colorectal tumor that may represent different subsets of colorectal tumor with developmental stages of the disease. Also from the results above we have provided convincing evidence that cystatin might contribute to the mechanisms of invasion of colorectal cancer.

**References**


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