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Assessment of the Roles of Cathepsins B, H and L in the Progression of Colorectal Cancer*

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ABSTRACT

Cysteine cathepsins are important regulators and signaling molecules of an unimaginable number of biological processes, while they concurrently play an essential role in cancer progression, invasion and metastasis. The purposes of our study were to: a) compare the expression levels of cathepsins B, H and L in the supernatants of colon cancer tissues from 74 patients versus the corresponding enzymic expressions of supernatants in the adjacent normal colorectal tissues; and b) correlate our results to the grade of the malignancy by using an enzyme-linked immunosorbent assay (ELISA). The findings indicated that cathepsins B, H and L of all malignant tissues exhibited significantly higher expression levels than their corresponding controls. Furthermore, cathepsin B expression levels doubled in all tumor samples and this increase remained quite steady with tumor stage advancement, in contrast to cathepsin H expression which rose significantly as malignancy progressed. Specifically, cathepsin H concentration was higher than the corresponding control: 155% in B1 stage and 204.44% in D stage. Among the three investigated proteases, cathepsin L has shown the highest increase, which in D stage stood 261.03% higher than the corresponding control. The results at hand suggested that cysteine protease H and L expression levels could be of critical value in the diagnosis and progression of colon cancer.

Keywords: Colorectal Cancer; Cysteine Cathepsins; Cathepsin B; Cathepsin H; Cathepsin L

1. Introduction

Cathepsins have a vital role in mammalian cellular turnover. In living organisms, their activities depend on a delicate balance of expression, targeting, zymogen activation, inhibition by protein inhibitors and degradation. They constitute a large family of molecules involved in various cellular processes, such as MHC antigen presentation, protein degradation, hormone regulation and TNF induced apoptosis [1]. The cathepsin family includes at least fifteen discrete members distinct in structure and substrate specificity [2,3]. Based on these properties, cathepsins are divided into three different groups: serine proteases (cathepsins A and G), aspartic proteases (cathepsins D and E), and cysteine proteases (cathepsins B, C, F, H, K, L, O, S, U, W and X). These proteases are found inside cellular organelles, mainly in lysosomes and peroxisomes, as inactive proenzymes [3]. Recent studies, however, have shown that active cathepsins are also localized in other cellular compartments, such as the nucleus, the cytoplasm and plasma membrane, where they contribute to protein turnover and polypeptide degradation. To this end, it has been shown that catalytically active variants of cathepsin L localized in the nucleus play a role in the regulation of cell-cycle progression and proteolytic processing of the N-terminus of the histone H3 tail. In response to cell death signals, cathepsins are released outside the cell and trigger degradation of the extracellular matrix and cell apoptosis [4].

Furthermore, cathepsins participate in pathological and inflammatory processes, such as those in Alzheimer's disease, tumor formation and invasion. Increased production of cathepsins in cancer cells causes tumor cell growth, invasion, and metastasis [5], while the precise role of each cathepsin in carcinogenesis remains unclear. On the other hand, cathepsins B, C, H, L, S and X/Z emerge as promising targets in anticancer therapy. The levels of these cathepsins are elevated in cancer cells and

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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. In addition, cathepsin B has been shown to be involved in tumor initiation, hyperproliferation and de-differentiation, being up-regulated in early human colon adenomas, carcinomas and metastatic lesions. In view of the facts that a) even though cathepsins B and L have been studied most thoroughly their functions are still not well-defined; and b) there is growing evidence that cathepsin H expression rises in malignant diseases, including breast, colorectal and prostate carcinoma [6], we decided to determine the expression levels of the three cysteine cathepsins B, H and L in human colon cancer tissues and correlate the findings to the grade of this malignancy.

2. Materials and Methods

2.1. Tissue Sample Collection

The study involved 74 patients with colorectal cancer (CRC), who underwent colorectal resection in Theagenio Cancer Hospital of Thessaloniki: 35 males of ~69 years of age and 39 females of ~68.9 years of age. Patients did not undergo chemotherapy, radiotherapy or any other adjuvant therapy for CRC prior to colorectal resection. Clinical data for the patients and the histology of tumors were recorded accurately. All patients were monitored after surgery.

The resected tumors were subdivided in groups according to their biological site (colon cancer, n = 48; rectal cancer, n = 26), but in particular they were histologically classified according to the Astler-Coller staging system [7]: in stage A, the tumor was confined to the mucosa (n = 3); in stage B1 the tumor extended into the muscularis propria, but it had not penetrated it and nodes were not involved (n = 13); in stage B2, the tumor penetrated the muscularis propria, but nodes were not involved (n = 18); in stage C1, the tumor extended into the muscularis propria, it had not penetrated through, but nodes were involved (n = 11); in stage C2, the tumor penetrated the muscularis propria, and nodes were involved (n = 15); finally, in stage D, the tumor was associated with distant metastases (n = 14) (**Table 1**).

Samples were recovered from resected tissues. Malignant samples were collected from a) different areas of the lesion that was macroscopically evident, and b) the adjacent normal tissue (controls). Tissue bearing no macroscopically dead or hemorrhagic foci was characterized as normal tissue. In all cases, the presence or absence of lesion(s) was confirmed histopathologically according to current guidelines. Following collection, all tissue samples were frozen in liquid nitrogen and stored at −80˚C until further use.

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

Patients Charasteristics	*Data*	*P value*
Number of patients	74	<0.001
Mean Age	± 69 years	0.001
Male	35	
Female	39	
Period	2009-2010	
Tumor Site	Colon and rectum	
Colon	48	
Rectum	26	
Astler-Coller Stage	B1, B2, C1, C2, D	

2.2. ELISA Assay for Quantitative Detection of Cathepsins B, H and L

Prior to assaying, frozen samples were brought to room temperature. Subsequently, they were washed in an isotonic solution of sodium chloride (CAS No: 8028-77-1), cut into pieces, weighed and homogenized at 4˚C in lysis buffer [50 mM HEPES (CAS No: 7365-45-9) pH 7.4, 5 mM CHAPS (CAS No: 75621-03-3), 5 mM DTT (CAS No: 7634-42-6)]. Sample processing took place in a mass scale of 1 g tissue/10 ml lysis buffer. The homogenized tissues remained at −20˚C for 24 h and then centrifuged at 20,000 g for 15 min at 4˚C. Supernatants (10 μl per well) were used for quantitative detection of cathepsins B, H and L through enzyme-linked immunosorbent assays.

Anti-human cathepsin-B, cathepsin-H and cathepsin-L coating antibodies were adsorbed onto microwells. Aliquots of supernatants (10 μl per well) were bound to corresponding adsorbed antibodies. Biotin-conjugated anti-human cathepsin-B, cathepsin-H and cathepsin-L antibodies were added and bound to corresponding cathepsins captured by the first antibodies. Following incubation, unbound biotin-conjugated antibodies were removed during a wash step and streptavidin-HRP was added and bound to the biotin-conjugated anti-human antibodies. Following further incubation, unbound streptavidin-HRP was removed by washing and substrate solutions reactive toward HRP were added to the wells. Colored products formed commensurate to the amount of cathepsins present in the samples. The reactions were terminated by adding 100 μl 1 M phosphoric acid to each well. A spectrofluorimeter microplate reader (Perking Elmer LS 50 B, using illumination at 440 nm-measuring emission at 370 nm) was used to measure absorbance in the ELISA assay at 450 nm. Three standard curves were prepared from standard dilutions of the three human cathepsins (-B, -H and -L) and concentrations of the cor-

responding cathepsins in the samples were determined in ng/mg of homogenized tissue.

2.3. Statistical Analysis

Results from the obtained clinical data were expressed as mean ± SD. Student's t test and one-way ANOVA were used for statistical analyses of the data. All statistical analyses were run on GraphPad 5.1 statistical software package (GraphPad, Europe).

All data reported were verified through at least three independent experiments and expressed as mean ± standard deviation (SD). Cases with P values of <0.05 or <0.01 were considered statistically significant. Student's t-test was performed for the statistical evaluation of cathepsin B, H and L activities, in order to determine whether any values deviated significantly from the controls ($p < 0.01$). Results are presented as mean±standard deviation (SD).

3. Results

Figures 1-3 show the B, H and L cathepsin expression level (ng/mg homogenized tissue), respectively, in human colon cancer tissues. Each graph presents the expression level of the corresponding cathepsin in tissues of the cancerous stages B1, B2, C1, C2 and D, and that of their adjacent normal tissues (controls). The bars in the B1 stage illustrate the mean value of the expression of the corresponding cathepsin from 13 different patients, the B2 bars were generated 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 emerged from the mean value of the expression of the corresponding cathepsin from 15 different patients, and the D bars reflect the mean value of the expression of the corresponding cathepsin from 14 different patients. **Figures 4-8** show the expression levels of these three cathepsins in all cancerous tissues were significantly higher than those of their corresponding controls. Cathepsin B expression was found to be approximately double in all stages of the CRC tissues. In the B1, B2, C1, C2 and D stages, the rise was 96.77%, 99.54%, 101%, 102.34% and 103.59%, respectively, compared to that of the adjacent normal tissues. It appears that the high level of cathepsin B expression remains practically invariable in all stages, in contrast to the expression levels of H and L cathepsins, which project a different behavior with malignancy progression. Cathepsin H expression showed a statistically significant increase in all malignant stages compared to that of the adjacent normal tissues and as malignancy progressed the rise followed suit in the following percentile fashion: 155%, 160.71%, 193.19%, 208.20% and 204.44% in

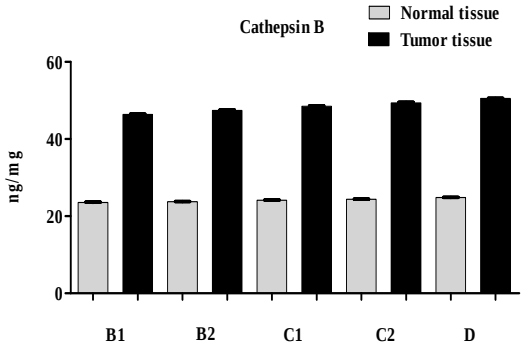

Figure 1. Comparison of cathepsin B concentrations (ng/mg) in the sera of colon cancer patients vs control patients at different stages (B1, B2, C1, C2, D) of colon cancer carcinogenesis. The data are presented as mean ± 0.2487 (SD) of samples for each group involved.

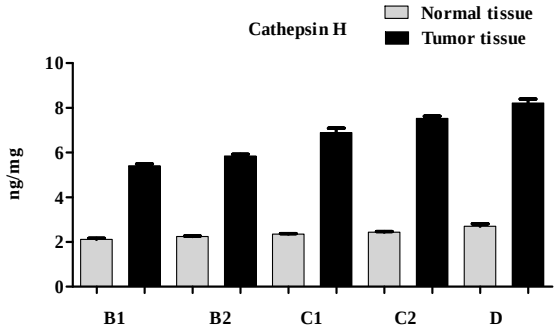

Figure 2. Comparison of cathepsin H concentrations (ng/mg) in the sera of colon cancer patients vs control patients at different stages (B1, B2, C1, C2, D) of colon cancer carcinogenesis. The data are presented as mean ± 0.0875 (SD) of samples for each group involved.

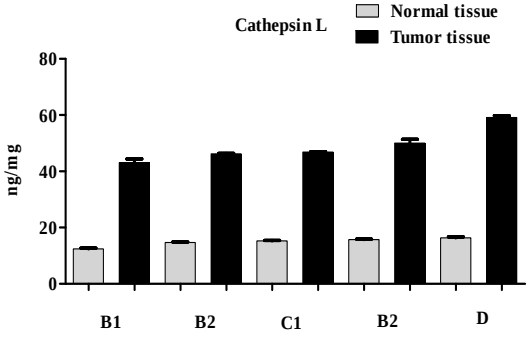

Figure 3. Comparison of cathepsin L concentrations (ng/mg) in the sera of colon cancer patients vs control patients at different stages (B1, B2, C1, C2, D) of colon cancer carcinogenesis. The data are presented as mean ± 0.4527 (SD) of samples for each group involved.

stages B1, B2, C1, C2 and D, respectively, compared to the adjacent normal tissues. Among the three investigated proteases, cathepsin L 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%

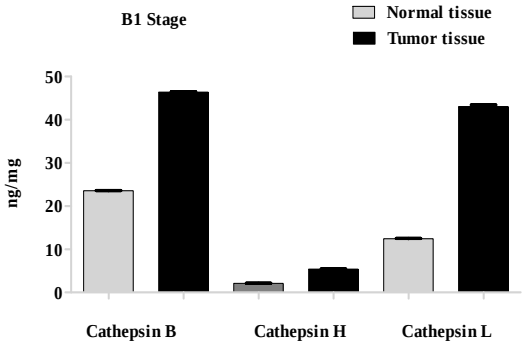

Figure 4. Evaluation of cathepsin B, H and L concentrations (ng/mg) in the sera of colon cancer patients vs control patients in the B1 stage of colon cancer progression. The data are presented as mean ± 0.3502 (SD) of samples for each group involved.

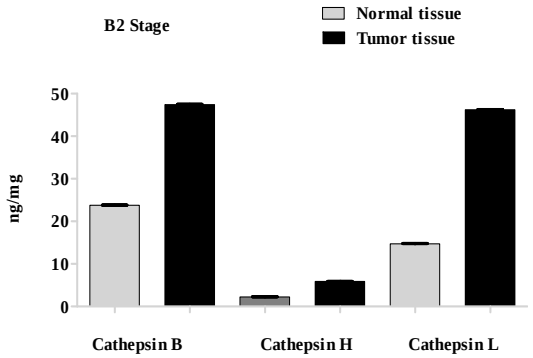

Figure 5. Evaluation of cathepsin B, H and L concentrations (ng/mg) in the sera of colon cancer patients vs control patients in the B2 stage of colon cancer progression. The data are presented as mean ± 0.1361 (SD) of samples for each group involved.

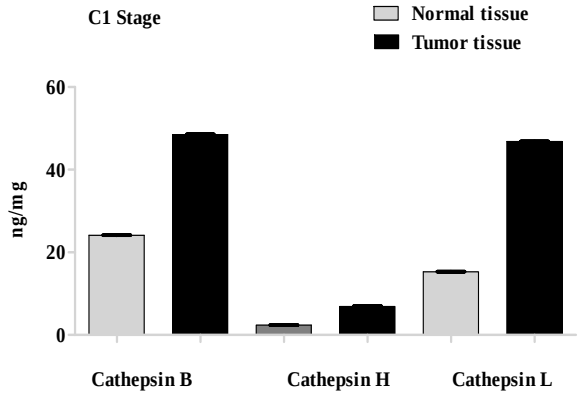

Figure 6. Evaluation of cathepsin B, H and L concentrations (ng/mg) in the sera of colon cancer patients vs control patients in the C1 stage of colon cancer progression. The data are presented as mean ± 0.1529 (SD) of samples for each group involved.

in stage C2, and 261.03% in stage D, compared to the normal adjacent tissues.

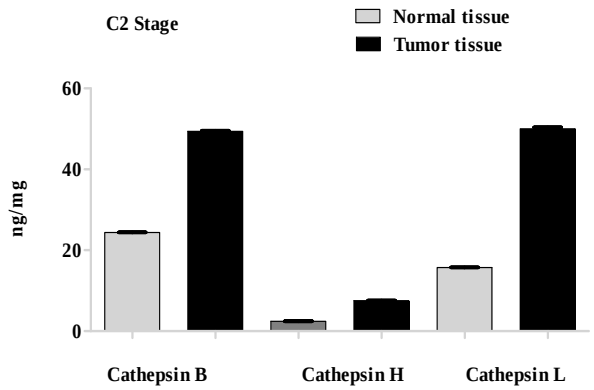

Figure 7. Evaluation of cathepsin B, H and L concentrations (ng/mg) in the sera of colon cancer patients vs control patients in the C2 stage of colon cancer progression. The data are presented as mean ± 0.3983 (SD) of samples for each group involved.

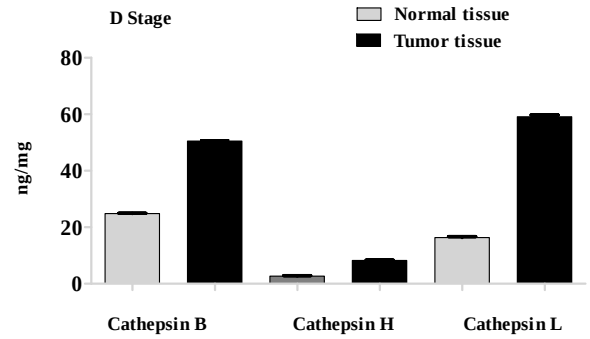

Figure 8. Evaluation of cathepsin B, H and L concentrations (ng/mg) in the sera of colon cancer patients vs control patients in the D stage of colon cancer progression. The data are presented as mean ± 0.2778 (SD) of samples for each group involved.

4. Discussion

As cancer continues to be one of the most serious health issues, colorectal cancer (CRC) is the second leading cause of cancer related deaths worldwide and the third most common cancer in men and in women in Europe. There is a great need for sensitive and specific biomarkers of the disease. Proteases have been suggested by several researchers as tumor markers in CRC [8,9].

A clear evidence that cysteine cathepsins are not only lysosomal proteases, but also possess non-lysosomal/endosomal roles implicates them in a plethora of biological processes involving invasion and migration of cancerous cells, as well as proliferation, apoptosis and angiogenesis that characterize malignant tumors [10]. Loss of cell-cell and cell-matrix adhesion and degradation of extracellular matrix (ECM) components are involved in invasion and migration [11]. Cysteine cathepsins can be expressed on the cell surface and secreted into the extracellular space, where they can degrade components of the ECM [12-15]

and remodel the microenvironment of tumor 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, all vital participants in human colorectal cancer growth. On the other hand, cysteine cathepsins could contribute to tumor progression, affecting intracellular tumorigenic processes, such as programmed cell death [16].

Cathepsin B is the first cathepsin demonstrated to be linked to cancer as far back as 30 years ago. Since then, it has been found to be expressed in the vast majority of colon cancers and adenoma, although other investigators have shown that cathepsins B, C, S and X/Z were also overexpressed in non-malignant cells in tumors, while overexpression of cathepsins H and L had been indentified in cancer cells only [14,17,18].

The results of ongoing investigations suggest no coincidence of the precise biological role among individual cysteine cathepsins in CRC. On the other hand, numerous clinical reports and literature experimental work support the notion that cysteine cathepsins B, H and L play a crucial role in both tumor progression and invasion. None of them, however, has thus far investigated these three proteases: a) concurrently in the same malignant tissue; b) using the same assay to peruse their activity; and c) comparing their expression levels with the corresponding levels in the adjacent normal tissue [18-20]. To this end, a compelling need arose to carry out the present study and establish cathepsin activity relationships with CRC stage nature and progression.

Duplication of cathepsin B expression in all malignant stages found in the present research (96.77%, 99.54%, 101%, 102.34% and 103.59% in B1, B2, C1, C2 and D stages, respectively, compared to that of the adjacent normal tissues) is in agreement with recent findings by Chan *et al*. in a cohort study of 558 men and women with colon cancer and involving tumors amenable to immunohistochemical assessment [21]. They demonstrated that cathepsin B is expressed in the vast majority of colon cancers, independent of stage, supporting a possible role of this protease in early alterations leading to tumor formation. Nevertheless, in previous studies, cathepsin B antigen levels in CRC tissue, found to be significantly higher than those in the corresponding normal mucosa, had also been positively correlated with differentiation grade and Astler-Coller stage but not nodal status [1,4, 12]. Later, Herszényi *et al*. demonstrated that antigen levels of cathepsins B and L were significantly higher in blood samples of patients with colorectal adenomas compared to controls [17,22-24]. When they correlated the rise of cathepsin antigens with Astler-Coller stage, they ascertained that cathepsin B had exhibited a significant increase in patients with advanced stage, while cathepsin L had not shown any correlation with stage. Based on these results, they concluded that cathepsins B and L may be involved in the progression from premalignant colorectal adenoma into CRC [24-26]. Moreover, other studies suggest that cathepsin B expression or activity may actually peak in an early stage of the cancer process and decline with disease advancement. The importance of cathepsin B expression and activity across stages of the neoplasia process have also been validated by previous data showing uniform cathepsin B expression and high-enzyme activity in intestinal adenomas generated in mouse models. In any case, the statistically significant ($P < 0.001$) increase of cathepsin B expression compared to the corresponding value for B in normal tissues, in all stages of CRC found in the present work, the correlation between mutations in KRAS and BRAF genes and the upregulation in the expression of cathepsin and its role in activation of the pro-urokinase-type plasminogen activator (pro-uPA) suggest that cathepsin B may play a crucial role not only in the invasive process of cancer, but also in the progression of colorectal precancerous lesions into cancer [27].

Cathepsin H, like cathepsin B, 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 tumor development and progresssion are not fully understood [28-30]. Gocheva *et al.* have demonstrated for the first time the important tumor-promoting role for cathepsin H *in vivo* using a mouse model of human cancer [23]. They 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 tumors that formed. When Schweiger *et al*. measured cathepsin H in preoperative sera from 324 patients with colorectal cancer through ELISA, they found that its level had significantly increased and that there was a weak association between cathepsin H levels and patient age but not Astler-Coller stage, sex or level of carcinoembryonic antigen (CEA) [31]. In line with these findings and the results of the survival analysis, Schweiger *et al.* concluded that the prognostic information and role during malignant progression for cathepsin H differed from those of the related cathepsins B and L. In the present work, the increase in the expression of CRC cathepsin H a) was shown to be statistically significant ($P < 0.001$) in all malignant stages compared to that of adjacent normal tissues; and b) followed the progression of the malignancy (155%, 160.71%, 193.19%, 208.20% and 204.44% in stages B1, B2, C1, C2 and D, respectively). This last

finding attests to the participation of cathepsin H in tumor development and progression and its varying behavior as cancer proceeds, in comparison to cathepsins B and L. Cathepsin B expression levels doubled in all tumor samples, with the increase remaining stable with tumor stage advancement, while cathepsin L expression showed the highest increase in all malignant stages compared to the other two cathepsins. Specifically, cathepsin L expression was 246.42% in the B1 stage, slightly degreased in B2 (213.99%), C1 (206.95%) and C2 (218.52%) stages, and slightly elevated (261.03%) in stage D, compared to the adjacent physiological tissues. As differences in cathepsin L expression among stages B2, C1 and C2 were negligible, the results tend to agree with those by Herszényi *et al*. [22]. According to those results, cathepsins B and L may be involved in the progression from premalignant colorectal adenoma into CRC. Nevertheless, our results on cathepsin L are in agreement with the findings reported by Lankelma *et al*. [11]. They have evaluated the state of affairs on cathepsin L as a possible target in cancer treatment, as its activity had been exclusively elevated in malignant cells, in contrast to that of other cathepsins.

The present investigation on the expression levels of cysteine cathepsins B, H and L in the supernatants of colon cancer tissues from 74 patients indicates that the three cathepsins of all malignant tissues exhibit significantly higher expression levels than the corresponding controls ($P < 0.001$). Among the three investigated proteases: a) cathepsin L showed the highest increase in all malignant stages; b) cathepsin B expression levels doubled in all tumor samples, but the observed increase remained about stable with tumor stage advancement; and c) cathepsin H expression increased significantly as malignancy progressed. Collectively, the data at hand are in agreement with previously reported literature findings, suggesting that: a) the expression of cysteine proteases H and L could be of critical value in the diagnosis and progression of colon cancer; while b) cathepsin B has a major prognostic impact on patients with colorectal cancer.

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