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Expression of Cathepsin B and L antigen and activity is associated with early colorectal cancer progression

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Abstract

Cathepsin B and Cathepsin L are cysteine proteases important in the process of invasion and metastasis. The aim of our study was to assay antigen and activity levels of these enzymes and to correlate these with established clinical and pathological prognostic parameters including patient survival. 99 patients undergoing operations for colorectal cancer were included in this study. We quantitated cathepsin B and L levels in matched normal mucosa and cancer samples using an enzyme-linked immunosorbent assay (ELISA) and specific activity assays and expressed the results as tumour/normal ratios. Significant correlations were found between tumour/normal cathepsin B and L antigen and activity ratios. Cathepsin B and L tumour/normal activity ratios were greater than 1 in early stage disease and there were gradual reductions in cathepsin B ($P = 0.02$) and L ($P = 0.03$) activity ratios with advancing tumour stage. Survival of patients with potentially curative disease was inversely related to both cathepsin B ($P = 0.007$) and L ($P = 0.001$) activity ratio, in addition to cathepsin L antigen ratio ($P = 0.008$). Our findings suggest that cysteine proteases play an important role in colorectal cancer progression.

Keywords: Cathepsin; protease; colorectal cancer; prognosis; tumour stage

1. Introduction

The malignancy of solid tumours is largely related to their capacity for invasion and metastasis, and proteases may play a major role in this process. The lysosomal cysteine protease, cathepsin B, found in both tumour epithelium and stroma [1], is known to degrade components of the extracellular matrix and basement membrane [2] and has been measured in both primary and metastatic colorectal cancers [3]. It converts inactive pro-urokinase-type plasminogen activator (uPA) to active uPA [4], and uPA and its inhibitor plasminogen activator inhibitor 1 (PAI-1) have been shown to be closely associated with outcome in many tumour types including colorectal, breast, gastric and lung cancers [5]. Levels of both the active and precursor forms of cathepsin B are elevated compared with normal tissue in many cancer types, as well as in transformed cells in culture [6–9] and the specific activity of cathepsin B has been found to correlate with tumorigenicity in a number of cell lines [10]. Cathepsin L levels have also been shown to be elevated both in cancer cell lines and in colorectal tumours [8,9,11–13].

Although cathepsin B and L have previously been correlated with clinico-pathological features in a number of malignant tumours [5], information on their prognostic value is limited in relation to colorectal cancer. In this study, we aimed to correlate cathepsin B and L activity and antigen levels with clinical and pathological features and with cancer-specific survival in a large cohort of colorectal cancer patients.

2. Materials and methods

2.1. Patients and tissue collection

Ninety nine patients (median age 71 years, range 31–84 years; 59 male) undergoing colorectal cancer surgery

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in St Vincent's University Hospital, Dublin between September 1991 and December 1994 were included in this prospective study which was approved by the hospital's ethics committee. For the purposes of this study, colorectal carcinoma tissue and normal colorectal mucosa (macroscopically normal at least 5cm away from the tumour margin) from the same patient was resected immediately following surgery, snap-frozen and stored at −70 °C. In addition, all histopathological slides were reviewed to confirm tumour stage [14]. Patients were followed-up at regular intervals and the median follow-up in patients alive at the end of the study period was 6.3 years (range for follow-up: 0.2–9.1 years).

2.2. Tissue extraction

Between 100 and 300 mg of frozen tissue was finely chopped and homogenised (3×20 s at maximum speed) using a Silverson homogeniser in Tris–HCl with 1.0% Triton-X-100 at pH 7.4.

Homogenates were centrifuged at 10000*g* for 20 min at 4 °C. Supernatant was removed and stored at −70 °C for analysis. Protein concentrations were determined using the Biorad Dye binding assay according to the manufacturer's instructions.

2.3. Activity assay

Cathepsin B activity was measured fluorometrically using the fluorescent substrate Z–Arg–Arg–AMC (Sigma Chemicals, Perth, Australia) at pH 6.8. Assays were carried out using a final concentration of 10 μM substrate in 0.1 M phosphate buffer containing 1 mM dithiothreitol (DTT) and 2 mM ethylene diamine tetra acetic acid (EDTA) in a final volume of 1 ml. Mixtures were incubated at 37 °C for 1 h and the reaction stopped by adding 200 μl 1.8 M acetic acid. Released 7-amino-4-methylcoumarin (NHMEC) was measured using a Perkin–Elmer fluorescent spectrophotometer (Perkin–Elmer, CA, USA) with excitation set at 370 nm and emission at 440 nm. Specific activity was expressed as μMol AMC/min/mg protein at 37 °C. Cathepsin L activity was measured using the fluorescent substrate *Z*-phenylalanine–Arginine–AMC at pH 4.0. In order to demonstrate that the measured activities were due to cysteine protease, the active-site titrant E-64 was used as a control [15]. Cathepsin B activity was measured after the addition of E-64 to a final concentration of 0.1 M.

2.4. Antigen assay

Cathepsin B and L antigen was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) assay from KRKA (Novo Mesto, Slovenia). This sandwich ELISA assay utilised a rabbit anti-human Cathepsin B polyclonal capture antibody followed by a sheep anti-human Cathepsin B detection antibody. The detection antibody was conjugated with horseradish peroxidase. On addition of TMB substrate, the coloured product was measured at 450 nm. These ELISAs detected inactive pro-cathepsins, active single- and double-chain cathepsins and cathepsins complexed to endogenous inhibitors.

2.5. Data presentation and statistical analysis

Cathepsin B and L antigen levels were expressed as ng antigen per mg protein. Proteolytic activity was expressed as units of enzyme activity per mg of protein. Cancer to normal colorectal mucosal ratios were calculated for each case so that normal colorectal tissue acted as an internal control in each case. As an example, patient number 1 had a tumour cathepsin B antigen level of 10.5 ng/mg protein and a normal mucosal cathepsin B antigen level of 1.8 ng/mg protein, and thus a tumour/normal cathepsin B antigen ratio of 5.8. Additionally, this patient had tumour and normal mucosal cathepsin B activity levels of 0.17 and 0.36 U/mg protein, respectively, and thus a tumour/normal cathepsin B activity ratio of 0.47.

Continuous data are presented as medians and interquartile ranges and were analysed using the Spearman rank correlation coefficient, Kruskal–Wallis one-way analysis of variance or Cuzick's test for trend [16], as appropriate. Kaplan–Meier survival curves were constructed with cancer related death as the endpoint. The maximal logrank test was used to dichotomise continuous data relating to cathepsin antigen and activity ratios for the purposes of constructing survival curves. The logrank test was used to assess the effect of categorical variables, such as tumour stage, on survival. Cox's proportional hazards model was used to correct for the effect of tumour stage on tumour/normal cathepsin ratios and survival within the multivariate survival analyses. Analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL). All *P* values are two-tailed.

3. Results

3.1. Cathepsin antigen and activity expression in normal and cancer tissues

Cathepsin B antigen ranged from 0.09 to 60.0 ng/mg protein in normal mucosa and from 0.02 to 104.0 ng/mg protein in colorectal cancer. Cathepsin B activity varied from 0.013 to 3.4 U/mg protein in normal tissue and from 0.007 to 20.0 U/mg protein in cancer tissue. Cathepsin L antigen expression in normal mucosa and cancer tissues ranged from 0.20 to 17.0 and from 0.02 to 60.7 ng/mg protein, respectively. Cathepsin L activity

ranged from 0.02 to 4.8 U/mg protein in normal tissue and from 0.01 to 3.8 U/mg protein in cancer tissue. Cathepsin B antigen levels, cathepsin B activity levels and cathepsin L activity levels appears to be higher in tumour tissues taken from those with localised (stage I/II) disease compared with normal tissues or tumour tissues from those with nodal or distant metastases (stage III/IV) (Table 1). Cathepsin L antigen levels were similar in normal, early cancer and advanced cancer tissues.

3.2. Antigen and activity ratio correlations

There was a positive association between cathepsin antigen and activity ratio measurements in all cases (Table 2). Correlation coefficients varied from 0.38 for the correlation between cathepsin B antigen ratio and cathepsin L activity ratio ($P < 0.001$) to 0.53 for the correlation between cathepsin B antigen ratio and cathepsin L antigen ratio ($P < 0.0001$).

3.3. Cathepsin ratios, tumour site and stage

There was no association between cathepsin antigen or activity tumour/normal ratios and tumour site (Table 3). Fig. 1 shows the relationship between cathepsin tumour/normal ratios and tumour stage. Cathepsin B and L antigen ratios remained relatively constant as the pathological stage progressed. In contrast, there was a gradual and significant trend towards decreased cathepsin B activity ($P = 0.02$) and L activity ($P = 0.03$) tumour/normal ratios with advancing stage.

3.4. Tumour/normal cathepsin ratios and clinical disease progression

Long-term survival was closely associated with tumour stage (logrank test, $P < 0.0001$) (Fig. 2). Fig. 3 shows long-term survival in patients with locoregional and distant disease stratified by tumour/normal cathepsin antigen ratios. Survival was unrelated to cathepsin B antigen ratios in those patients with early and intermediate cancer (stage I–III) (Logrank test $P = 0.17$; stage-corrected, $P = 0.93$) and in those with advanced (stage IV) cancer ($P = 0.72$). In contrast, survival was inversely related to tumour/normal cathepsin L antigen ratios in those with stage I–III tumours ($P = 0.008$; stage-corrected, $P = 0.008$), but not for those with distant metastases ($P = 0.96$).

Fig. 4 shows that cathepsin B activity ratio was especially associated with outcome in patients with stage I–III cancers ($P = 0.05$; stage-corrected, $P = 0.007$), but was unrelated to outcome in those with stage IV disease ($P = 0.33$). Similarly, cathepsin L activity ratio was closely related to outcome in stage I–III tumours ($P = 0.05$, stage-corrected, $P = 0.001$), but was also unrelated to outcome in patients with advanced disease ($P = 0.84$).

Secondary analyses were performed following the calculation of activity/antigen ratios for each case. No significant correlations or additional results of interest were found (data not shown).

4. Discussion

The breakdown of natural barriers to tumour spread is an essential component of invasion and metastasis. A number of proteolytic enzymes have been implicated in this process including serine, aspartyl and matrix metalloproteases. Cysteine proteases, such as cathepsin B and L, are also believed to play a central role by catalysing the degradation of several components of the extracellular matrix. Cathepsin B and L have previously been found to correlate with metastatic potential in a number of model tumour systems, both *in vivo* and *in vitro* [13,17]. They have also been associated with outcome in a number of human cancers [5], although information on the association between cysteine protease antigen and activity and survival following colorectal cancer is limited.

A number of previous studies have examined the relationship between cysteine proteases and colorectal cancer evolution [8,12,18–23]. However, advances in assay technology allied to other methodological changes over the past 10 years make it difficult to compare contemporary and previous results. Our study used a highly sensitive and specific ELISA assay that has been well characterised [24]. We also used specific assays to assess proteolytic activity in each sample. In addition,

Table 1
Cathepsin B and L antigen and activity (median [interquartile range]) in normal colonic mucosa and tumour tissues

	Normal tissue	Tumour tissue		P value[a]
		Stage I–II	Stage III–IV	
Cathepsin B antigen	4.4 (2.1–9.7)	7.8 (4.5–14.7)	6.2 (3.7–10.1)	0.005
Cathepsin B activity	0.2 (0.1–0.4)	0.3 (0.2–0.8)	0.2 (0.1–0.6)	0.06
Cathepsin L antigen	3.8 (2.1–6.7)	4.0 (2.0–6.6)	3.6 (2.0–8.9)	0.91
Cathepsin L activity	0.3 (0.2–0.5)	0.5 (0.2–0.7)	0.3 (0.2–0.6)	0.05

[a] Kruskal–Wallis test.

Table 2
Correlation (*r* values [95% Confidence Intervals (C.I.)]) between tumour/normal antigen and activity ratios

	Cathepsin B (*r* value[a] [95% C.I.])		Cathepsin L (*r* value[a] [95% C.I.])	
	Antigen	Activity	Antigen	Activity
Cathepsin B antigen	–	0.45 (0.26–0.60)	0.53 (0.37–0.66)	0.38 (0.19–0.55)
Cathepsin B activity	0.45 (0.26–0.60)	–	0.45 (0.27–0.60)	0.47 (0.29–0.61)
Cathepsin L antigen	0.53 (0.37–0.66)	0.45 (0.27–0.60)	–	0.49 (0.32–0.64)
Cathepsin L activity	0.38 (0.19–0.55)	0.47 (0.29–0.61)	0.49 (0.32–0.64)	–

All correlations were significant with a P value < 0.001.
[a] Spearman rank correlation coefficient.

Table 3
Cathepsin B and L antigen and antibody tumour/normal ratios (median [interquartile range]) stratified by tumour site

	n	Rectum	*n*	Left colon	*n*	Right colon	*P* value[a]
Cathepsin B antigen ratio	45	1.6 (0.6–3.7)	21	1.8 (0.7–2.5)	32	1.6 (0.9–5.3)	0.61
Cathepsin B activity ratio	43	1.5 (0.7–2.3)	21	1.1 (0.5–4.2)	27	1.5 (0.8–3.1)	0.70
Cathepsin L antigen ratio	43	1.1 (0.8–2.0)	21	1.1 (0.7–2.6)	32	1.1 (0.6–1.8)	0.45
Cathepsin L activity ratio	43	1.5 (0.6–3.3)	21	1.2 (0.6–1.7)	27	1.1 (0.5–1.5)	0.15

Some data are missing for some patients.
[a] Cuzick's test for trend.

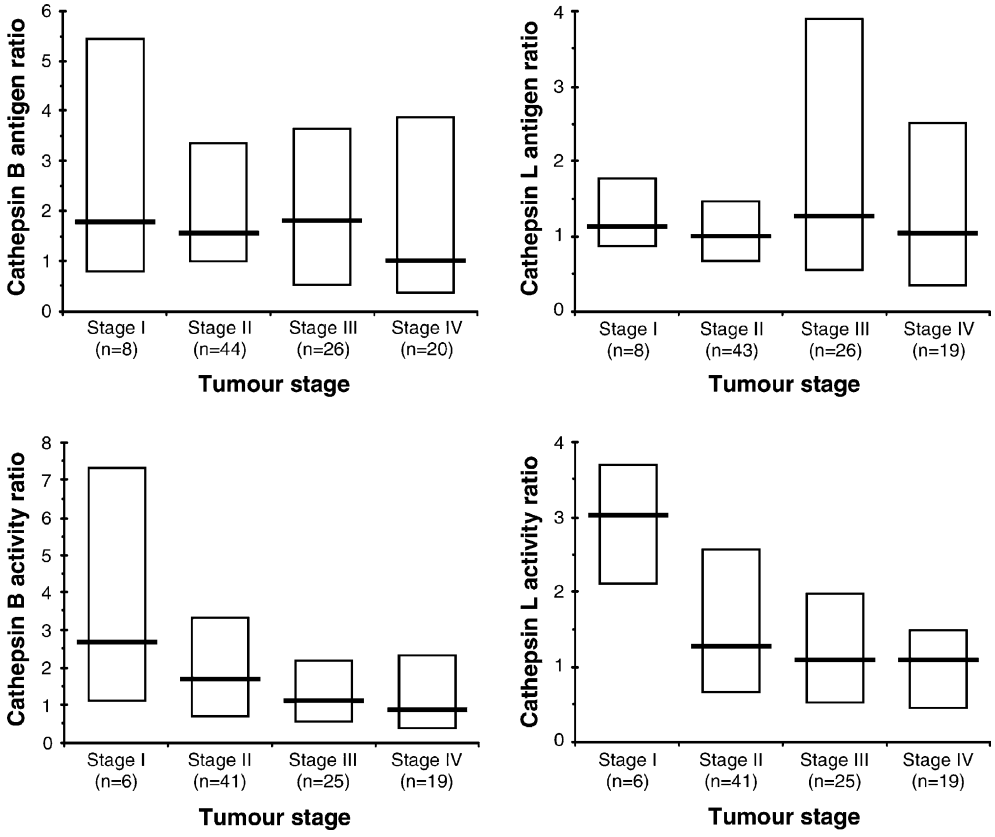

Fig. 1. Cathepsin antigen and activity tumour/normal ratios stratified by tumour stage. Top left, Cathepsin B antigen ratio (Cuzick's test for trend, $P = 0.13$). Top right, Cathepsin L antigen ratio ($P = 0.82$). Bottom left, Cathepsin B activity ratio ($P = 0.02$). Bottom right, Cathepsin L activity ratio ($P = 0.03$). Some data are missing for some patients.

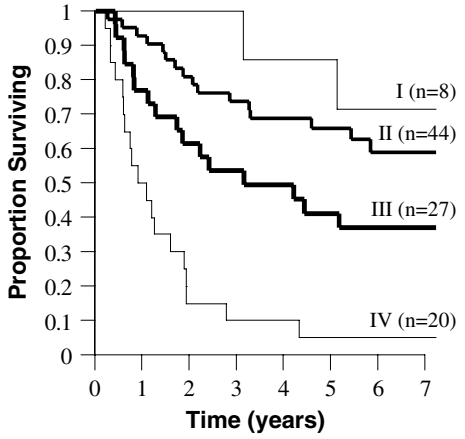

Fig. 2. Survival stratified by tumour stage.

we measured both antigen and activity levels in normal as well as tumour tissues so that each normal colorectal mucosa acted as an internal control for individual patients. Finally, rigorous pathological review and comprehensive long-term follow-up allowed us to draw conclusions with regard to the value of these assays in assessing clinical and anatomical progression.

We found that cathepsin B and L antigen ratios did not change significantly with advancing stage and that cathepsin L, but not cathepsin B, antigen ratio was closely related to clinical progression and long-term outcome. Murnane and colleagues [20,25] using Western blotting, previously reported high levels of the 28 and 27 kDa cathepsin B protein in Duke's stage A and B cancers when compared with advanced disease and, like Murnane and colleagues, we used tumour/normal ratios so that each patient served as their own internal control for protease expression. In contrast, the only previous study to measure antigen levels of cathepsin B and L contained 60 patients and found higher absolute levels of both B and L antigen in metastatic compared with localised tumours [11]. In this study, cathepsin L, but not cathepsin B, antigen was closely related to patient outcome. Overall, the above results relating to clinical outcome are similar to ours. However, it will be difficult to compare results between different institutions in any more than a superficial manner until interested researchers agree upon strict methodological guidelines and analytical criteria.

The main findings of this study were that cathepsin B and L activity tumour/normal ratios were relatively high in early disease and declined steadily with advancing tumour stage. Since stage is closely related to outcome, one might expect that declining activity ratios would be similarly associated with poor survival. However, the opposite was the case, since those patients with low activity ratios undergoing potentially curative surgery tended to have a significantly better outcome than those with higher ratios. What is the pathophysiological explanation for these apparently dichotomous results? The inverse relationship between cathepsin activity and stage confirms previous assertions that such activity is important in the early breakdown of barriers to tumour spread from localised to regional and metastatic disease [8,20,26]. This has previously been referred to as "phenotypic drift", whereby clonally derived tumours, with continued somatic mutation, generate an admixture of distinct phenotypes with selection for those cells which provide advantageous characteristics at each stage [8]. However, our finding that cysteine protease activity is inversely related to long-term survival also appears to indicate that, although levels decrease with anatomical progression, high levels are associated with particularly aggressive disease independent of anatomical spread or other conventional pathological parameters. It is also clear from a review of Figs. 3 and 4 that antigen and activity ratios were unrelated to outcome in the first two years after surgery and that their effects on survival only became noticeable in the medium to long term. This is interesting, not only from a pathophysiological perspective, but also from a clinical and methodological viewpoint as it seems that extended follow-up periods will be necessary when assessing the prognostic impact of specific proteases and that some studies may be falsely negative simply as a result of inadequate follow-up. Our results agree with two recent studies [1,3] which suggested that measurement of activity rather than

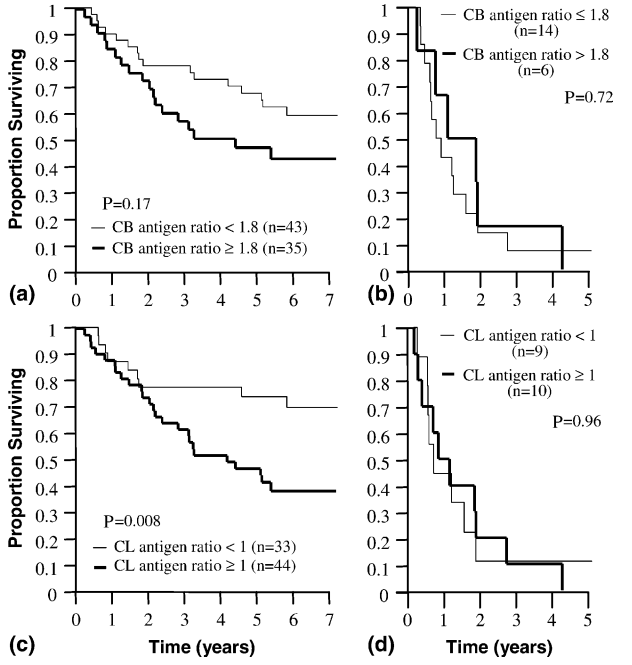

Fig. 3. Survival stratified by cathepsin B and L antigen tumour/normal ratios. Top left (a), cathepsin B antigen in stage I–III disease. Top right (b), cathepsin B antigen in stage IV disease. Bottom left (c), cathepsin L antigen in stage I–III disease. Bottom right (d), cathepsin L antigen in stage IV disease. CB, cathepsin B. CL, cathepsin L. Some data are missing for some patients.

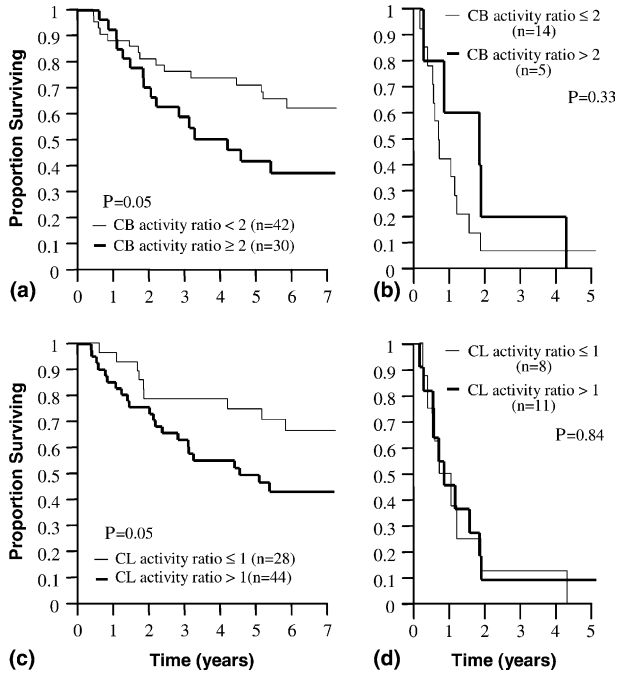

Fig. 4. Survival stratified by cathepsin B and L activity tumour/normal ratios. Top left (a), cathepsin B activity in stage I–III disease. Top right (b), cathepsin B activity in stage IV disease. Bottom left (c), cathepsin L activity in stage I–III disease. Bottom right (d), cathepsin L activity in stage IV disease. CB, cathepsin B. CL, cathepsin L. Some data are missing for some patients.

protein might be more important in the assay of proteases. From these studies, it would also appear that localisation of activity may also be important. However, these are technically demanding using conventional techniques and require fresh tissue [1]. Antibodies recognising active and inactive enzyme forms might further clarify their role in malignant progression.

The concept of a biochemical staging system for cancer is attractive, since its focus would be on the function of tumour cells rather than simply their anatomical spread at the time of resection. The protease cascade, hypothesised on the basis of work done on experimental cell systems [27], suggests that the process of tumour progression requires a multiplicity of proteolytic enzymes, each with a different function at various times during the evolution of cancer. Our results, demonstrating elevated cathepsin B and L activity at specific disease stages provide direct evidence from human primary tumours to support this theory. Recent work on sera from colorectal cancer patients may also support this hypothesis [28]. Our results implicate cathepsin B and L in the early stages of disease progression and support *in vitro* findings that cathepsin B and L may be involved in activation of other proteases involved in later stage disease. More specifically, as stage I and II tumours are in the process of invading surrounding tissue, our results suggest that cathepsin B and L activity might facilitate local invasion. Further work on a variety of protease classes might help us develop a more complete insight into the precise mechanism of tumour initiation, spread and metastasis and allow us to begin investigating biochemical staging systems for colorectal cancer.

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