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Effect of preoperative radiotherapy on *matrilysin* gene expression in rectal cancer

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Abstract

Matrilysin, a member of matrix metalloproteinase family, is believed to play a significant role in the growth and proliferation of colon cancer cells. Overexpression of the *matrilysin* gene has been shown to correlate with Dukes' stage and increased metastatic potential in colorectal cancer. The aim of this study was to evaluate the effect of preoperative high-dose radiotherapy (25 Gy in five fractions over 5 days) on matrilysin (*MMP-7*) gene expression, in patients with resectable rectal cancer, by a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Biopsy samples of tumour ($n=30$) and distant normal mucosa ($n=12$) from 15 patients were obtained pre- and post-radiotherapy. Messenger (m)RNA was extracted from all of the tissue samples and reverse transcribed to double-stranded cDNA. Quantitative RT-PCR was performed to study the effect of preoperative radiotherapy on *matrilysin* gene expression in both the tumour and normal mucosal specimens. *Matrilysin* mRNA values were expressed relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for each sample. In 14 out of 15 cases, *matrilysin* mRNA was detected in the cancerous tissue. Although all six normal mucosal specimens expressed *matrilysin* mRNA, the levels were approximately 10-fold lower compared with those seen in the paired tumour samples. Preoperative radiotherapy led to a significant 6- to 7-fold increase ($P=0.001$) in the expression of *matrilysin* mRNA in rectal cancer tissue. In contrast, there was no significant change in the *matrilysin* mRNA expression of normal mucosal specimens post-radiotherapy. Preoperative high-dose radiotherapy upregulates *matrilysin* gene expression in rectal cancer. Matrilysin inhibition may be a useful preventive or therapeutic adjunct to radiotherapy in rectal cancer.

Keywords: Rectal cancer; Radiotherapy; Matrix metalloproteinase; Matrilysin

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes, which degrade all components of both the basement membrane and extracellular matrix. They are believed to play a causal role in cancer invasion and spread [1–3]. This role is supported by several *in vitro* and *in vivo* experiments demonstrating inhibition of invasion and metastasis by specific inhibitors of the MMPs [4–6]. There is growing evidence suggesting a crucial role for MMPs in the process of tumour angiogenesis, indicating a complementary route by which they influence the growth and behaviour of cancer cells [7,8]. On the basis of substrate specificity, MMPs have been grouped into interstitial collagenases, gelatinases, stromelysin and membrane type MMPs (MT-MMP).

Matrilysin (MMP-7), a member of the stromelysin subgroup, degrades proteoglycans, gelatin, elastin, and glycoproteins such as fibronectin, laminin and entactin, including type IV collagen [9,10]. MMP-7 is a unique member of the MMP family in that it is expressed exclusively in malignant epithelial cells [11], in contrast to most other MMPs which are expressed not only in tumour cells, but also in the stromal cells surrounding the tumour [12]. The matrilysin protein has also been purified from a human rectal carcinoma cell line [13]. Overexpression of the *matrilysin* gene has been shown to correlate with Dukes' stage and increased metastatic potential in colorectal cancer [14,15]. There is considerable evidence to support a role for matrilysin, in the development and growth of very early stages of colorectal cancer [16,17]. Transfection of colon cancer cells with cDNA for *MMP-7* led to increased tumorigenicity and progression of recipient cells, but had no or little effect on invasion or metastatic potential [16]. Furthermore, in mice with a germline deficiency in the *APC* gene, the absence of matrilysin led to a reduction in the size and

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number of intestinal adenomas [17]. A number of studies [14,15,18] have localised *matrilysin* mRNA in the epithelial component of benign stages of colorectal cancer. These observations argue for the use of MMP inhibitors in the prevention of early-stage colorectal cancer.

Preoperative high dose short-term radiotherapy is increasingly used as an adjuvant treatment for patients with resectable rectal cancer in Europe. The aim of preoperative high dose radiotherapy is to sterilise the resection margins and to destroy microscopic collections of cancer cells situated outside the mesorectum, in presacral lymph nodes and at lateral pelvic walls, a potential focus of local recurrence and, a source of distant metastasis. Most of the studies [19–21] using a higher dose of radiation equivalent to 25 Gy or more, with variable fraction schedules have demonstrated a significant reduction in local recurrence compared with surgery alone. A significant number of patients still suffer from local recurrence (11% in Swedish Rectal Cancer Trial, 1997) [21] and distant metastasis even after adjuvant radiotherapy. This may be due to the inherent capacity of the cancer cell to repair radiation-induced damage, re-oxygenation, and the rate of re-population and re-distribution of the cells in the sensitive phases of the cell cycle. In addition, induction of many other genes may modify behaviour of the remaining viable cancer cells after radiotherapy.

Radiotherapy has been shown to induce MMPs in different types of cell lines [22,23]. Previously, we have demonstrated via gelatin zymography that preoperative high-dose radiotherapy (5×5 Gy), a commonly used adjuvant treatment in rectal cancer, led to a significant increase in the levels of gelatinase protein, along with the activation of MMP-2 in malignant rectal mucosa with no change observed in the normal mucosa [24]. We proposed that overexpression of these enzymes, in response to the treatment, may be responsible for re-establishing a blood supply and resurrecting the invasive edge of remaining viable cancer cells in the pelvis, modulating the behaviour of cancer cells. This study focused on *matrilysin* gene expression in rectal cancer based on its tumour specificity and its role in stimulating tumour cell growth in the early stages of colorectal cancer. The aim of this study was to investigate the effect of preoperative high-dose short-term radiotherapy (25 Gy in five fractions in 5 days), a commonly used adjuvant treatment, on *matrilysin* gene expression in rectal cancer.

2. Patients and methods

15 patients with resectable adenocarcinoma of the rectum, located within 15 cm of the anal verge, were included in the study. All patients underwent standard preoperative radiotherapy, which was delivered to the pelvis with a three-beam technique. The dose delivered was 25 Gy in five fractions over 5 days. Surgery (either anterior resection or abdomino-perineal resection) was performed within a week following completion of the radiotherapy. This study was carried out with Hospital Ethical Committee approval and all patients provided written informed consent to participate in the study.

2.1. Tissue samples

Biopsy samples of tumour ($n=30$) and distant normal mucosa ($n=12$) were obtained pre- and post-radiotherapy. Specimens were obtained from the tumour edge at different sites by biopsy through a rigid sigmoidoscope, avoiding the necrotic centre of the tumour. Multiple specimens from the same tumour were pooled to give a uniform representation of the tumour. The post-radiotherapy biopsies were performed 48 h after completion of radiotherapy and just before surgery. The second biopsy was taken prior to surgery to eliminate the changes in matrilysin expression induced by ischaemia. The post-irradiated biopsies were taken from multiple sites close to those used for the pre-radiotherapy biopsies and in case of normal mucosa, the second biopsy was performed from within the irradiated field. All tissue samples were cryopreserved in liquid nitrogen, following their removal from the patients, and stored at -80 °C. Ten-micron cryostat sections were cut from each tissue sample and Haematoxylin and Eosin staining performed to validate the presence of tumour epithelium and stroma in both tumour samples and normal epithelium. Computer generated image analysis of malignant or normal epithelium/stroma was performed and the ratio was found to be comparable in pre- and post-radiotherapy sections.

2.2. RNA extraction and cDNA synthesis

The frozen tissue was first weighed (0.1 mg of tissue per 0.6 ml of extraction buffer) and total RNA extracted with guanidium thiocyanate and phenol-chloroform procedure, using QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). The method used for cDNA manufacture was the Riboclone cDNA Synthesis System M-MLV (H-) (Promega). The first strand synthesis is driven by a reverse transcriptase and an Oligo-(dt)-containing primer (this is a string of T-deoxyribonucleotides) which anneals to the poly (A) tail of the mRNA. This therefore selectively primes cDNA synthesis from mRNA only. Second strand synthesis is achieved by 'replacement synthesis' where the hybridised RNA is cut with RNase H and the ends of the RNA fragments are extended as DNA using DNA polymerase. After second-strand synthesis, the cDNA was extracted using phenol and the pellet of cDNA

precipitated and left to air dry before suspension in 30 μl sterile water.

2.3. Polymerase chain reaction

Complimentary DNA was screened by performing a polymerase chain reaction [25]. The standard cDNA (details as described by Wells and colleagues [25] and primer pairs for *MMP-7* and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were kindly donated by British Biotech Ltd. (Oxford, UK). The following primer sequences were used: *GAPDH*. 5′-TGC CGT CTA GAA AAA CCT GC-3′ and 5′-ACC CTG TTG CTG TAG CCA AA-3′: *MMP-7*. 5′-TTT GAT GGG CCA GGA AAC AC-3′ and 5′- GGG GAT CTC CAT TTC CAT AG-3′. The 50-μl reaction mix contained 3.5 mM $MgCl_2$, 25 μl Taq buffer (50 mM Tris–HCl, pH 9.1, 16 mM ammonium sulphate and 150 μg/ml bovine serum albumin, BSA), 200 μM each deoxynucleotide triphosphates (dNTPs), 50 pmols each primer, 1 μl of cDNA for each primer and Taq DNA polymerase (Helena Biosciences) 1 unit per 100 μl of reaction mix [26].

To check for possible artifacts based on the contamination of RNA by genomic DNA during RT-PCR, PCRs were performed under the same conditions, but with no reverse transcription step.

2.4. Competitive reverse transcription-polymerase chain reaction (RT-PCR)

Competitive polymerase chain reaction, as optimised by Wells and colleagues [25], was performed on samples, which had expressed MMPs in the non-competitive screen. In this technique, a synthetic standard DNA template acts as a competitor with the cDNA to be amplified. The standard DNA was produced at British Biotech Ltd (Oxford, UK), as 28 overlapping oligonucleotides (from R&D System) and would result in a band at the 300 bp mark. To avoid variations in the efficiency of the amplification procedure due to a known quantity of standard in one reaction and an unknown level of target DNA in the other [27], the target and the standard cDNA were co-amplified in the same reaction. In such a technique the standard DNA template acts as a competitor with the cDNA to be amplified, but yields products of differing sizes [28–30].

Three-fold serial dilutions of the standard multicompetitor cDNA starting at 200 pg were combined with a constant amount of cellular cDNA made from the rectal cancer and mucosal samples (equivalent to 2 ng of starting mRNA) in 50 μl reactions which also contained 1 μ Ci [^{32}P] α-deoxycytidine triphosphate (dCTP) (Amersham). Cycling conditions were identical for both primer pairs, 35 cycles of 95 °C, 30 s/57 °C, 30 s/72 °C, 120 s hold at 4 °C.

Gel electrophoresis was performed by loading 10 μl of the reaction mix post-PCR onto a precast 6% acrylamide trisborate EDTA (TBE) gel (Novex) and the bands were visualised by staining with ethidium bromide (0.5 μg/ml) (Fig. 1). The bands were excised and 400 μl of Microscint 40 (Packard) scintillation fluid was added to each band in a well. The counts per minute (cpm) (due to [^{32}P] α-dCTP in the reaction mix) obtained from the band representing standard multicompetitor cDNA were divided by that from the corresponding sample cDNA band to obtain a ratio. The resulting ratios of the standard cDNA to cellular cDNA were plotted against the known dilution of standard multicompetitor cDNA on a log–log scale (Fig. 2). The value of the dilution, when the ratio of standard to cellular cDNA was equal

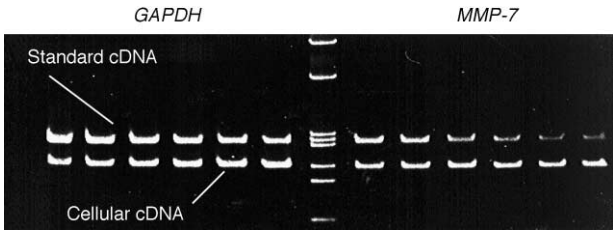

Fig. 1. Gel showing competition between standard cDNA (top row) and cellular cDNA (bottom row) with primer pairs for *GAPDH* (lanes 1–6) and *MMP-7* (lanes 8–13) in a representative rectal cancer specimen. Lane 7 shows DNA molecular weight marker. Three-fold serial dilutions of standard cDNA were used.

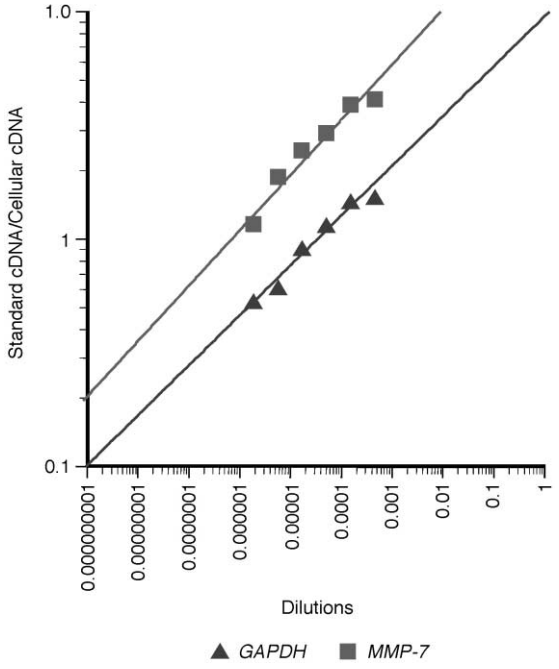

Fig. 2. Plot showing log–log scale with dilutions of the standard (*x*-axis) against the ratio of the standard cDNA to cellular cDNA (*y*-axis). The value of the dilution (both for *GAPDH* and *MMP-7*) when the ratio of standard to cellular cDNA was equal to 1, was calculated using a curve fitting program on Delta Graph Professional. *MMP-7* mRNA value was normalised to *GAPDH*.

to 1, was calculated using a curve fitting program (software graphics package, Delta Graph Professional). Using this method it was possible to obtain values for *MMP-7* mRNA present in a sample relative to *GAPDH* for that sample.

2.5. Statistical analysis

All data were found to be non-parametrically distributed and therefore the *P* values for comparison of mRNA values were calculated using the Wilcoxon signed ranks test. Median values of the mRNA were used in these analyses.

3. Results

15 fresh frozen rectal cancers and six samples of normal rectal mucosa from the same patients, pre- and post-radiotherapy, were evaluated by competitive RT-PCR assay. Haematoxylin and Eosin staining confirmed both malignant epithelium and stroma in all the cancer specimens and normal epithelium and stroma in all the normal mucosal specimens.

mRNA was successfully extracted from all 30 tumour samples and 12 normal mucosal tissue samples. Fourteen of 15 rectal cancer specimens studied showed expression of *matrilysin* mRNA before radiotherapy, whereas the remaining tumour sample did not express *matrilysin* mRNA. All six normal mucosal samples studied expressed *matrilysin* mRNA, but the levels were approximately 10-fold lower compared with those seen in tumour samples. Following radiotherapy, there was 6- to 7-fold increase in the levels of *matrilysin* mRNA and this difference was statistically significant ($P = 0.001$). Fig. 3 shows the median value of *matrilysin* mRNA relative to *GAPDH*. Table 1 shows the values of *matrilysin* and *GAPDH* mRNA in all 15 rectal cancer specimens. In contrast, radiotherapy led to no significant changes in the levels of *matrilysin* mRNA of the normal mucosal specimens.

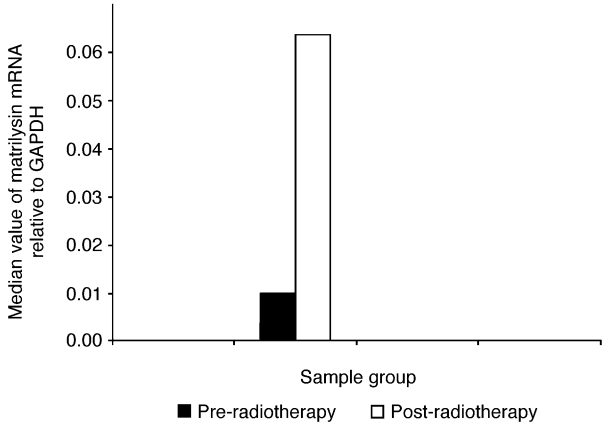

Fig. 3. Bar chart comparing median values (relative to *GAPDH*) of *matrilysin* mRNA ($P = 0.001$) in pre- and post-radiotherapy rectal cancer specimens.

4. Discussion

Preoperative high-dose radiotherapy led to a significant 6- to 7-fold increase in the expression of *matrilysin* mRNA in rectal cancer tissues. In contrast, there was no significant change in *matrilysin* mRNA expression of normal mucosal specimens. The results of this study support the previously published data [11] that most colorectal cancers express *matrilysin* mRNA. In the present study, 14 of 15 tumour samples showed expression of *matrilysin* mRNA. Although *matrilysin* expression was seen in six normal mucosal specimens studied, the levels were approximately 10-fold lower compared with those seen in the paired tumour samples. This finding is consistent with the previous report demonstrating a greater *matrilysin* mRNA signal in colorectal carcinoma than in paired adjacent mucosal samples [15].

There is growing evidence to suggest that matrilysin is involved in the regulation of growth of tumours, both at the primary site and metastases [31]. It has been suggested that matrilysin may stimulate cancer cell growth and proliferation through different mechanisms. High levels of *matrilysin* mRNA have been observed in proliferating glands of normal human and monkey endometrium [32], suggesting a direct role for matrilysin in cellular proliferation in an autocrine fashion. In primate endometrium, matrilysin expression co-localised with the expression of the antigen Ki-67, a marker of cellular proliferation. *Matrilysin* mRNA levels are increased

Table 1
Values of *GAPDH* and *MMP-7* mRNA in rectal cancer specimens

Patient no.	Pre-radiotherapy (mRNA expression)		Post-radiotherapy (mRNA expression)	
	GAPDH	*MMP-7*	*GAPDH*	*MMP-7*
1	1	9.2×10^{-2}	1	6.4×10^{-3}
2	1	1.9×10^{-5}	1	6.9×10^{-3}
3	1	2.3×10^{-2}	1	2.7×10^{-2}
4	1	4.8×10^{-3}	1	4.0×10^{-3}
5	1	1.3×10^{-1}	1	9.0×10^{-1}
6	1	1.8×10^{-1}	1	2.8×10^{-1}
7	1	1.0×10^{-2}	1	2.0×10^{-2}
8	1	4.0×10^{-6}	1	2.0×10^{-3}
9	1	1.0×10^{-1}	1	1.6
10	1	1.7×10^{-4}	1	2.5×10^{-3}
11	1	1.9×10^{-1}	1	23.3
12	1	1.0×10^{-2}	1	1.0×10^{-1}
13	1	5.0	1	45
14	1	ND	1	ND
15	1	1.0×10^{-2}	1	1.0×10^{-1}

ND, not detected.

when SW620 and WiDR human colon cell lines are treated with the mitogen epidermal growth factor [33]. Furthermore, degradation of the basement membrane and extracellular matrix by matrilysin may release basement membrane and matrix bound growth factors and cytokines, resulting in an indirect effect on cell proliferation. Matrilysin may also mediate alterations in cell-matrix interactions, which have been shown to affect the proliferative state of endothelial cells [34]. Alternatively, matrilysin may affect tumour cell growth and proliferation as a result of an increase in angiogenesis. All of this information put together suggests that matrilysin may alter the growth rate of the tumour cells *in vivo*.

It has been demonstrated that the introduction of matrilysin into colon cancer cells markedly upregulated their *in vivo* invasive and metastatic potential, suggesting that matrilysin may play a crucial role in colorectal cancer progression [35]. This may be due to its direct proteolytic effect, as this proteinase exhibits a wide spectrum of substrate specificity and effectively degrades several components of the basement membrane and extracellular matrix [10,36]. Alternatively, it may occur through an indirect effect of matrilysin on other MMPs, such as activation of MMP-2 [37]. Previously, we have demonstrated that preoperative radiotherapy led to a significant upregulation of *MMP-2* and *MMP-9* gene and protein expression, along with the activation of MMP-2 [24,38]. Metastasis is a complex process and requires the changes in expression of more than one gene and therefore overexpression of the *matrilysin* gene alone may not be sufficient to acquire the full ability to metastasise [16]. However, matrilysin may modify the metastatic potential of the cancer cells by activating MMP-2 and through its interaction with other genes such as *MMP-2* and *MMP-9*.

The mechanism by which preoperative radiotherapy upregulates matrilysin gene expression is speculative at this point. There is some evidence to suggest that interleukin-1 (IL-1) and epidermal growth factor (EGF) levels are increased after radiotherapy for brain tumours [39]. MMP genes have been shown to be induced by many kinds of growth factors and cytokines such as IL-1, tumour necrosis factor α (TNFα), transforming growth factor (TGF) α and β, EGF [40,41]. Furthermore, stromelysin-1, a related metalloproteinase, expression is responsive to EGF stimulation [42]. Upregulation of *matrilysin* gene expression in rectal cancer tissue, seen after radiotherapy, may be due to the presence of tumour-derived cytokines such as IL-1 and EGF in response to the treatment. Further work is underway to evaluate this hypothesis.

This report provides the first evidence that preoperative high-dose radiotherapy upregulates *matrilysin* gene expression in rectal cancer. This may be responsible for growth and proliferation and promotion of angiogenesis of the remaining viable cancer cells in the pelvis, modulating the behaviour of the cancer cells. Further studies are needed to validate these findings at the protein level. In future, matrilysin inhibition may be a useful preventive or therapeutic adjunct to radiotherapy in rectal cancer.

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