Colocalisation of matrix metalloproteinase-9-mRNA and protein in human colorectal cancer stromal cells

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Summary The matrix metalloproteinases (MMPs) are perceived as essential for tumour invasion and metastases. The purpose of this study was to determine the expression and localisation of the 92 kDa type IV collagenase (MMP-9) protein and mRNA in human colorectal cancer (CRC). In CRC and matched normal mucosa specimens from 26 CRC patients, Northern blot hybridisation and Western blot analyses provide convincing evidence that MMP-9 is expressed in greater quantities in CRC than in normal tissue. The MMP-9 tumour to normal mucosa fold-increase (T/N) was 9.7±7.1 (mean ± s.d.) (P<0.001) for RNA and 7.1±3.9 (P<0.001) for protein. The sites of MMP-9 mRNA and protein synthesis were colocalised in tumour stroma by in situ hybridisation and immunohistochemistry in 26 CRC samples. Both MMP-9 mRNA and protein signals were strongest in the population of stromal cells concentrated at the tumour–stroma interface of an invading tumour. Furthermore, MMP-9-positive cells were identified as macrophages using an anti-macrophage antibody (KP1) in serial sections from ten CRC samples. Given the persistent localisation of MMP-9-producing macrophages to the interphase between CRC and surrounding stroma, our observations suggest that MMP-9 production is controlled, in part, by tumour–stroma cell interactions. Further studies are needed to determine the in vivo regulation of MMP-9 production from infiltrating peritumour macrophages.

Keywords: 92 type IV collagenase; matrix metalloproteinase; colorectal cancer; macrophage

Crucial steps in tumour invasion and metastases are the breaching of the basement membrane (BM) and degradation of the extracellular matrix (ECM) (Liotta et al., 1991; Matrisian, 1992). These processes are likely to involve numerous proteolytic enzymes, including matrix metalloproteinases (MMPs), a family of extracellular matrix-degrading enzymes (Liotta et al., 1991). To date, at least 14 members of the MMP family have been described by substrate specificity (Birkedal-Hansen, 1995). Based on substrate preference, MMPs can be subclassified into interstitial collagenases (MMP-1, MMP-8 and MMP-13), type IV collagenases/gelatinase (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), membrane-type MMPs [MT-MMP1 (Sato et al., 1994), MT-MMP2 (Will and Hinzmann, 1995), MT-MMP3 (Takino et al., 1995) and MT-MMP4 (Puente et al., 1996)]. However, several MMPs including, stromelysin 3 (MMP-11) (Noel et al., 1995) and macrophage metalloelastase (MMP-12) (Woesnser, 1994; Birkedal-Hansen, 1995) do not appear to belong to any one group. An important role for MMPs in invasion and metastases is supported by studies demonstrating a correlation between elevated MMP levels and the metastatic phenotype in cell cultures (Ballin et al., 1988; Moll et al., 1990; Turpeenniemi-Hujanen et al., 1985; Yamagata et al., 1988), animal models (Nakajima et al., 1990) and several human tumours (Muller et al., 1991; McDonnell et al., 1991; Hamdy et al., 1994; Levy et al., 1991; Stearns and Wang, 1993; Rao et al., 1993; Yoshimoto et al., 1993; Kossakowska et al., 1993; Brown et al., 1993; Naylors, 1994).

Since type IV collagen is a major component of BM, the 72 kDa (MMP-2) and 92 kDa (MMP-9) type IV collagenase are of particular interest. In order to understand further in vivo type IV collagenase regulation, knowledge of the in vivo sources of MMP-2 and MMP-9 production is essential. However, owing to conflicting reports localising MMP-2 mRNA to peritumour stromal fibroblasts (Poulosom et al., 1992; Pyke et al., 1993) and MMP-2 protein to CRC cells themselves (Levy et al., 1991), the cellular origin of MMP-2 within CRC specimens remains unclear. Similarly, although microdissection studies on human CRC specimens localise increased pro-MMP-9 enzyme to the invasive CRC edge (Emmert-Buck et al., 1994), the cellular source of MMP-9 remains uncertain, since MMP-9 mRNA signals have been localised to the peritumour stromal compartment of breast (Tryggvason et al., 1993; Davies et al., 1993a), skin (Pyke et al., 1992), bladder (Davies et al., 1993b) and colorectal cancers (Pyke et al., 1993; Newell et al., 1994; Zeng and Guillem, 1995), while MMP-9 immunostaining has been localised within CRC cells as well as peritumour stromal neutrophils and macrophages (Jeziorska et al., 1994). Furthermore, in human osteoclastomas, both MMP-9 RNA and protein have been localised to multinucleated giant cells (Wucherpfennig et al., 1994). In order to clarify the discrepancy in the cellular origin of CRC MMP-9, we examined the simultaneous mRNA and protein pattern of MMP-9 expression in human CRC specimens.

Materials and methods

Tissue preparation

Twenty-six colorectal cancers and paired normal tissue used in this study were obtained from the operating room immediately after resection with the approval of the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center. They were quick frozen in liquid nitrogen and stored at −80°C until processed. Samples were handled and stored under strict RNAase-free conditions. Frozen-section tissue was embedded in OCT (Miles, Elkhart, IN, USA) and frozen in 2-methylbutane cooled with liquid nitrogen. For in situ hybridisation, specimens were fixed in RNAase-free 4% paraformaldehyde overnight at 4°C, sequentially dehydrated with 50%, 70%, 85%, 95% and 100% ethanol and embedded in paraffin.

Northern blot hybridisation

RNA was extracted by the guanidium isocyanide–caesium chloride methods, as previously described (Guillem et al., 1990). Total RNA (10 μg) were electrophoresed on 1.0%
agaroase–formaldehyde gel and blotted onto a Duralon-UV membrane (Stratagene). MMP-9 DNA probes were radio-labelled with $[^{35}]$PdCTP by the random primer technique. A 28S oligo probe was used as an internal control for equal RNA loading and ethidium bromide staining of gels to confirm equal RNA transfer.

In situ hybridisation

Our technique for MMP-9 in situ hybridisation has been previously described in detail (Zeng and Guillem, 1995). Briefly, the sense and antisense transcripts were prepared from a human MMP-9 cDNA insert (1059 bp extending from nucleotides 7 to 1066) subcloned in Bluescript KS (Stratagene). The MMP-9 sense probe was generated by T3 polymerase following digestion with XbaI, while the antisense probe was generated by T3 polymerase after EcoRI template digestion respectively. Transcribed RNA was labelled with $[^{35}]$SUTP (1200 Ci mmol$^{-1}$, DuPont NEN). DNA template was removed by incubating with RNAsase-free DNAase. tRNA (10 μg) was added and samples were extracted with phenol–chloroform. RNA probes were hydrolysed with sodium carbonate buffer, pH 10.2, for 60 min at 60°C, neutralised and ethanol precipitated.

Paraffin sections (5–10 μm thick) were dried, deparaffinised and rehydrated. Slides were incubated with proteinase K (100 μg ml$^{-1}$), washed in 2 mg ml$^{-1}$ glycerol (w/v), and finally, washed in triethanolamine buffer containing 0.25% acetic anhydride. Sections were covered with $[^{35}]$S-labelled RNA probe and sealed with a coverslip and incubated at 57°C overnight. After hybridisation, slides were washed in 4 × saline sodium citrate (SSC) for 1 h and then in 2× SSC, 0.1% formamide, 10 mM DTT solution for 40 min at 68°C. Slides were then treated with 20 μg ml$^{-1}$ RNAase-A in 20 mM Tris, pH 7.5, 0.5 mM sodium chloride, 1 mM EDTA at 37°C for 30 min, followed by washing in the same buffer without RNAase-A for 30 min. The final washes were in 2× SSC, 50% formamide, 1 mM DTT for 40 min at 68°C, 2× SSC for 5 min at room temperature and 0.1× SSC for 15 min at 50°C. Slides were dehydrated through graded ethanol and air dried.

Autoradiographic detection of the hybrids was carried out by dipping in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA) at 42°C under safelight and dried. Slides were developed and fixed after 10–14 days exposure. Tissue sections were counterstained with haematoxylin and eosin (H&E). The sections were examined by dark-field microscopy.

Monoclonal antibody

The monoclonal antibody (Mab) MMP-9 (Ab-2) (clone 7–11C) was obtained from Oncogene Science, Inc. (Manhasset, NY, USA). This Mab is generated by immunising mice with MMP-9 protein partially purified from the conditioned media of PMA-stimulated HT1080 human fibrosarcoma cells (Ramos-DeSimone et al., 1993). MMP-9 (Ab-2) recognises the latent (92 kDa) form of human MMP-9. The active (82 kDa) form of human MMP-9 cannot be recognised by this antibody (Ramos-DeSimone et al., 1993). Mab MMP-9 (Ab-2) was used for both Western blot and immunohistochemistry. The anti-human macrophage monoclonal antibody, CD-68 (KP-1), was obtained from Dako Corporation (Glostrup, Denmark). This antibody is known to react with macrophages in a wide variety of human tissues, including Kupffer’s cells and macrophages in the red pulp of the spleen, in the lamina propria of the gut, in lung alveoli and in bone marrow (Pulford et al., 1989). However, not all haematopoietic cells react with KP1 antibody (Thalmeyer et al., 1994).

Western blot analysis

The tissue was homogenised in Tris buffer (50 mM Tris-HCl, pH 7.5, containing 75 mM sodium chloride) and centrifuged at 5000 × g for 20 min as previously described (Zeng et al., 1994a). The supernatant of tumour and paired normal mucosa (25 μg) were electrophoresed on a 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel using a MINIGEL apparatus (Bio-Rad, Richmond, CA, USA). Separated proteins were transferred to nitrocellulose membranes (Amersham, Bucks, UK) in Tris/glycine buffer (2.5 mM Tris, 192 mM glycine and 20% methanol) at 4°C and 100 V using a MINI system. Non-specific binding sites were blocked for 1 h at room temperature in 10 mM Tris buffer containing 150 mM sodium chloride and 0.5% Tween 20 (TBS-T) with 4% bovine serum albumin (BSA). The blots were incubated overnight at 4°C in a 1:500 dilution of MMP-9 MAb Ab-2. The blot was washed several times with TBS-T, followed by an incubation step with horseradish peroxidase-labeled anti-rabbit antibody (1:5000 in TBS-T for 30 min at room temperature). After washing with TBS-T, an enhanced chemiluminescence detection system (ECL, Amersham) was used. For molecular weight determination, ECL protein molecular weight and rainbow coloured protein molecular weight markers (Amersham) were used.

Immunohistochemistry

For immunohistochemical staining, the slides were processed by use of the Vectastain ABC Elite kits (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer’s protocol as previously described (Zeng et al., 1994b). Immediately before staining, frozen sections were fixed in 4°C acetone then rinsed with phosphate-buffered saline (PBS).

For the reduction of non-specific background staining, slides were incubated with diluted normal blocking serum for 20 min at room temperature. The serum was drained off and sections were incubated with the MAb MMP-9 (Ab-2) at a concentration of 200 ng ml$^{-1}$ at 4°C overnight. After washing, the slides were incubated with diluted biotinylated secondary antibody solution for 30 min, rinsed with PBS and then incubated with Vectastain Elite ABC reagent for 30 min. Following this, tissue sections were rinsed in PBS and immunostaining was developed by immersion in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution dissolved in 0.5% Triton-X/PBS for 4 min. Sections were counterstained with modified Harris-Haematoxylin (Fisher) and 0.3% ammonia water and passed through graded alcohols and xylene to dehydrate. Slides were then observed by conventional light microscopy.

Tissue sections adjacent to those used for MMP-9 staining were stained by a MAb KP-1 (CD-68).

Densitometric quantitation

MMP-9 RNA and protein levels were quantitated by measuring the intensities of the appropriate autoradiographic bands using LKB XL laser densitometry (Phar- macia LKB Biotechnology, Uppsala, Sweden).

The RNA results were expressed as the fold-increase of a 2.8 kilobase MMP-9 transcript in tumours to that in the paired normal tissues. The 28S transcript was used as an internal control:

\[
\frac{N_{28S}}{N_{28S}_{\text{Normal}}} = \frac{T_{\text{MMP-9}_{28S}}}{T_{\text{MMP-9}_{28S}}_{\text{Normal}}}
\]

The MMP-9 protein levels are expressed as the fold-increase in expression of the 92 kDa bands in tumour relative to that measured in the corresponding adjacent normal mucosa.

Differences of both RNA and protein levels between tumour and paired normal tissue were analysed by Student’s t-test.

Results

Determination of tumour–normal MMP-9 RNA level

Total cellular RNA of human CRC and paired adjacent normal mucosa from 26 CRC patients was examined for expression of MMP-9 RNA by Northern blot hybridisation.
Figure 1 is a representative Northern blot hybridisation of five CRC patients. The 2.8 kb MMP-9 transcript was strongly expressed in tumour compared with the extremely low levels noted in normal mucosa. Densitometric analyses of blots indicated that MMP-9 was overexpressed in all primary CRCs when compared with corresponding normal mucosa. The T/N fold-increase of MMP-9 RNA ranged from 1.3 to 25.7 with a mean ± standard deviation (s.d.) of 9.7 ± 7.1. MMP-9 RNA expression was significantly increased in primary CRC relative to adjacent normal mucosa (P<0.001).

**Determination of tumour–normal MMP-9 protein level**

The expression of MMP-9 protein in the CRC was detected by Western blot analysis. A 92 kDa band representative of MMP-9 protein was increased in the tumour compared with normal mucosa (Figure 2). Based upon MMP-9 protein densitometric quantitation, the mean MMP-9 protein tumour to normal mucosa fold-increase was 7.1 ± 3.9 (mean ± s.d.) (P<0.001).

**Cellular localisation of MMP-9 mRNA by in situ hybridisation**

The cellular localisation of MMP-9 RNA within CRC tissues was examined by in situ hybridisation using an antisense MMP-9 RNA probe. Eighteen out of 26 (69.2%) CRCs had detectable signals for MMP-9 mRNA. The predominance of MMP-9-positive cells was limited to the interface between tumour and surrounding normal mucosa and shows a punctate pattern of localisation (Figure 3a and b). MMP-9-positive signals were located in stromal cells encircling the tumour epithelial cells but not in tumour cells themselves (Figure 3c and d). No

**Figure 1** A representative Northern blot hybridisation of five colorectal cancer patients. MMP-9 expression is higher in primary tumour (T) than in adjacent normal mucosa (N). Total RNA was first hybridised with an MMP-9 cDNA probe (top). The blot was subsequently stripped and rehybridised to a 28S probe as an internal control (bottom).

**Figure 2** Western blot analysis of MMP-9 protein in colorectal cancer. Tumour (T) and normal mucosa (N) extracts from each patient were separated on an 8% SDS–PAGE gel and transferred to a nitrocellulose membrane, which was incubated with an anti-MMP-9 monoclonal antibody and visualised as described in Materials and methods. The position of MMP-9 is noted by arrows.

**Figure 3** In situ hybridisation of MMP-9 mRNA in colorectal cancer. Sections were hybridised with a 35S-labelled anti-sense RNA probe specific for MMP-9. Microphotographs demonstrate MMP-9 mRNA are located in malignant tumour stroma, not in cancer cells themselves. Paired dark-field (a) and bright-field (b) display that MMP-9 mRNA are most abundant in the interface between tumour (T) and normal mucosa (M) and show a punctate pattern of localisation. Paired bright (c) and dark-field (d) photomicrographs reveal that MMP-9 RNA signals within the stroma are circular in shape. Scale bars = 100 μm.
Colocalisation of MMP-9 mRNA and protein in colon cancer stroma

ZS Zeng and JG Guillem

Colocalisation of MMP-9 mRNA and protein in colon cancer stroma

ZS Zeng and JG Guillem

1164

Figure 4 Immunohistochemical localisation of MMP-9 protein in colorectal cancer and normal mucosa. a, Normal colon mucosa without any MMP-9 staining, scale bar = 50 μm. b, Higher magnification of a, scale bar = 25 μm. c, MMP-9 protein concentrated at the invading front between tumour and normal mucosa, scale bar = 100 μm. d, Higher magnification of area bordered by arrows in c, scale bar = 50 μm. e, MMP-9 protein is located in the tumour stroma and not in cancer cells, scale bar = 50 μm. f, Higher magnification of area bordered by arrows in e; MMP-9 protein-positive cells located within peritumour stroma have a macrophage-like morphology, scale bar = 25 μm.

overexpression was detected in adjacent normal mucosa. In situ hybridisation using an MMP-9 sense probe revealed only a background level of signals (figures not shown).

Cellular localisation of MMP-9 protein by immunohistochemistry

Immunohistochemical staining with an anti-MMP-9 monoclonal antibody revealed positive brown signals in most CRC tissue sections. Figure 4 demonstrates typical results with anti-MMP-9 MAb in normal colonic mucosa and CRC tissue sections. Figure 4a and b (higher magnification of Figure 4a) demonstrate absence of staining in normal mucosa. Control sections processed with preimmune serum also did not stain (data not shown).

The pattern of MMP-9 protein cellular localisation in CRC was similar to the MMP-9 mRNA. Figure 4c, d, e and f demonstrate the typical pattern of tumour stroma MMP-9 staining without tumour cell staining. A colon cancer with surrounding normal mucosa (Figure 4c and d higher magnification, bordered by arrows in Figure 4c) demonstrates MMP-9 antigen concentration at the invading front between tumour and normal mucosa. Figure 4e and f (higher magnification, bordered by arrows in Figure 4e) further emphasises that MMP-9 protein-positive cells are located principally in the peritumour stroma. Higher magnification (Figure 4d and f) reveals that MMP-9-positive cells have a macrophage-like morphology.

To identify the cells expressing MMP-9 protein, serial sections from ten CRC samples were examined immunohistochemically with both MMP-9 and macrophage-specific antibodies (CD-68) (Pulford et al., 1989). Figure 5a and c reveals anti-MMP-9 MAb and anti-macrophage MAb staining respectively. As noted, MMP-9-positive cells and macrophages have a similar distribution. At higher magnification (Figure 5b and d), MMP-9-positive cells with a macrophage-like morphology correspond directly with the cells staining positive for macrophages.
Discussion

Based upon both Northern and Western blot data, as well as in situ hybridisation and immunohistochemical staining, our results provide convincing evidence that MMP-9 is significantly overexpressed in CRC and that the sites of production are the peritumour stromal cells rather than the cancer cells themselves. These results, along with previous zymographic studies demonstrating increased levels of active MMP-9 (82 kDa) in CRC specimens from patients with metastases (Zeng et al., 1995), suggest an important role for MMP-9 production in CRC invasion and metastases.

In addition to studies demonstrating a correlation between MMP-9 expression and invasion and metastases (Bullin et al., 1988; Moll et al., 1990; Turpeenniemi-Hujanen et al., 1985; Yamagata et al., 1988), a recent transfection study directly links MMP-9 expression to the metastatic phenotype (Bernhard et al., 1994). Although in vitro, TGF-α, EGF and TGF-β up-regulate MMP-9 expression (Birkedal-Hansen et al., 1993), and natural tissue inhibitor proteins, such as tissue inhibitor of metalloproteinase (TIMP) (Liootta et al., 1991; Matsriasan, 1992) inhibit MMP activity, the in vivo regulation of MMP-9 remains undefined. This is, in part, owing to the uncertainty of the cellular origin of MMP-9 and its site of activation.

In vitro, a variety of cell types including fibroblasts, endothelial cells, keratinocytes, macrophages and eosinophils produce MMP-9 (Werb and Alexander, 1993; Saarialho-Kere et al., 1993). In human squamous cell carcinomas, Pyke et al. (1992) have demonstrated that MMP-9 mRNA is expressed by stromal macrophages, whereas Stahle-Backdahl and Parks (1993) noted that tissue eosinophils produce MMP-9. In human CRC, data from Pyke et al. (1993) and our own (Zeng and Guillem, 1995) demonstrate that MMP-9 mRNA may be produced by peritumour macrophages. In the present study, we demonstrate that MMP-9 protein, detected with an MMP-9 monoclonal antibody, also localises to the peritumour macrophages. The lack of a complete concordance between the immunohistochemical staining pattern of MMP-9-positive cells and macrophages suggests that either serial adjacent sections are not 100% morphologically identical or other stromal cells, such as neutrophils (Jeziorska et al., 1994; Nielsen et al., 1996), eosinophils (Stahle-Backdahl and Parks, 1993), fibroblasts or endothelial cells may also be producing MMP-9.

Although the in vivo regulation of MMPs remains undefined, the localisation of several MMPs to tumour stromal cells rather than tumour cells themselves (Pyke et al., 1992; 1993; Cottam and Rees, 1993; Davies et al. 1993a,b; Canete-Soler et al., 1994; Zeng and Guillem, 1995) suggests that stromal cells may be involved in this process. Basset et al. (1990) and Wolf et al. (1993) first demonstrated both mRNA and protein expression of MMP-11 (stromelysin) in the tumour stroma of human breast carcinoma. However, current evidence suggests that the mRNA and protein expression of other MMPs may be in either tumour or stromal cells. In CRC, matrilysin (MMP-2) mRNA is produced by benign and malignant cells (Cottam and Rees, 1993). In contrast, mRNA of other MMPs, such as MMP-2, 3, 7, 9, 11 appear to be restricted to tumour stromal cells (Pyke et al., 1993; Cottam and Rees, 1993; Zeng and Guillem, 1995). Although MMP-2 mRNA has been found exclusively in fibroblasts, MMP-2 protein has been localised to the surface of cancer cells (Pyke et al., 1992, 1993). Discrepancies between MMP-2 mRNA and protein cellular expression suggest distinct sites of MMP-2 RNA production.
and MMP-2 protein utilisation (Levy et al., 1991; Mackay et al., 1992). This concept is supported by the detection of an MMP-2 cell surface binding protein on cancer cell lines (Emonard et al., 1992), as well as a transmembrane MMP (MT-MMP) capable of activating MMP-2 (Sato et al., 1994).

Although evidence supports a complex membrane-associated regulation of MMP-2, involving TIMPs, the recently described membrane activators (MT-MMP) and a cell surface MMP-2 binding protein (Birkedal-Hansen, 1995; Young et al., 1995; Yu et al., 1995), less is known about MMP-9 activation. In vitro, stromelysin-1 (MMP-3), TIMP-2, plasmin, kallikrein (Birkedal-Hansen, 1995), and most recently, MMP-2 (Fridman et al., 1995), have been shown to activate MMP-9. However, the localisation of MMP-9 mRNA to the stroma of breast (Davies et al., 1993a), bladder (Davies et al., 1993b) and skin cancer (Pyke et al., 1992) and CRC (Pyke et al., 1995; Zeng and Guillem, 1995) suggests a stromal source of MMP-9 in several human cancers. Our present results demonstrating colocalisation of both MMP-9 mRNA and protein to peritumour macrophages suggest that peritumour macrophages are both a source of MMP-9 production and a site of localisation and, therefore, a possible site of utilisation.

The localisation of MMP-9 around tumour blood vessels (Davies et al., 1993b) also suggests that MMPs may facilitate angiogenesis via enhanced ECM turnover. Furthermore, the localisation of MMP-9 signals to the interface between cancer and stromal cells suggests probable cancer–stromal cell interactions in the regulation of macrophage MMP-9 production. Although certain factors produced from cancer cells, such as tumour collagenase-stimulating factor (TCSF), can stimulate fibroblast MMP-2 production (Kataoka et al., 1993), it does not appear to stimulate MMP-9 production. However, the observation that mitogens, such as lipopolysaccharide (LPS), can stimulate the production of both MMP-2 and MMP-9 from murine peritoneal macrophages (Sledge et al., 1995), as well as our recent coculture experiments demonstrating induction of monocyte-MMP release by metastatic CRC cells (Swallow et al., 1996), support the notion that in vivo CRC cells may activate macrophage MMP production in a paracrine-like manner. Alternative explanations include CRC cell-mediated chemotraction of activated macrophages, which express MMP-9, or induction of MMP-9 gene expression in resident macrophages (Mantovani and Semeraro, 1995).

In summary, we have demonstrated that MMP-9 RNA and protein expression are significantly elevated in CRC when compared with corresponding normal mucosa. The distribution of MMP-9 mRNA and protein in CRC tissue is similar and appears to localise to macrophages at the interface between cancer cells and surrounding normal tissue. Further studies are needed to determine the in vivo regulation of MMP-9 production from infiltrating peritumour macrophages as well as to delineate cancer–stromal cellular interactions.

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