Membrane Type MMPs Show Differential Expression in Non-Small Cell Lung Cancer (NSCLC) Compared to Normal Lung; Correlation of MMP-14 mRNA Expression and Proteolytic Activity

JM Atkinson[1], CJ Pennington[2], SW Martin[1], VA Anikin[3], AJ Mearns[3], PM Loadman [1], DR Edwards[2], JH Gill[1*].

[1]Institute of Cancer Therapeutics, University of Bradford; UK [2]School of Biological Sciences, University of East Anglia, UK; [3]Department of Cardiothoracic Surgery, Bradford Royal Infirmary; UK.

*Corresponding Author: Dr JH Gill, Institute for Cancer Therapeutics, University of Bradford, Bradford, UK. E-mail: j.gill1@bradford.ac.uk

KEYWORDS:
Matrix Metalloproteinases; Membrane-Type Metalloproteinases; Lung cancer; Real-Time PCR; Carcinoma, Non-Small Cell Lung, Cancer Therapeutics

ABSTRACT:

Improved understanding of the involvement of matrix-metalloproteinases (MMPs), including membrane-type MMPs (MT-MMPs), in human tumours has potential diagnostic, prognostic and therapeutic implications. We assessed the relationship between MT-MMP expression and clinicopathological parameters in human non-small cell lung cancer (NSCLC) and histologically normal lung tissue by quantitative Real Time PCR (qRT-PCR). All MT-MMPs (MMPs 14-17, 24 and 25) were detected by qRT-PCR with significantly higher MMP-14, -15 and -17 expression observed in tumour relative to normal lung specimens. MMP-16 was undetectable in normal lung but expressed in 8% tumours. MMP-15 demonstrated significant overexpression in adenocarinomas relative to squamous cell carcinomas and normal lung tissue. MMP-14 mRNA expression strongly correlated to MMP-14 proteolytic activity in preclinical tumour models, indicating that qRT-PCR may predict MMP-14 activity levels in NSCLC. These data suggest that MMP-14, -15 and -17 may be good markers of disease, or therapeutic targets for treatment of human NSCLC.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the major cancer type in developed countries. Early stage NSCLC, without distant metastases or heavy regional nodal involvement, is often amenable to surgical resection and adjuvant chemotherapy. However, response to chemotherapy in NSCLC and prediction of tumour pathogenesis is often poor. As a consequence, better understanding of the molecular pathology of NSCLC, determination of novel tumour markers and identification of targets for therapeutic exploitation are central to improving diagnosis and treatment.

Matrix metalloproteinases (MMPs) are a family of at least 24 zinc-dependent endopeptidases, with the collective ability to degrade the basement membrane and all protein components of the extracellular matrix (ECM) (1-3). However, MMP activity is not restricted to ECM degradation and is essential for several processes involved in tumorigenesis including neovascularisation, induction of the epithelial-mesenchymal transition and regulation of growth factor and chemokine activity to name but a few (1-8). The human MMP family is divided into eight distinct structural groups: five of which are secreted and three which are membrane-type MMPs (MT-MMPs) (2). MT-MMPs are membrane-tethered proteolytic enzymes and constitute the largest family of MMPs identified so far. The type 1 transmembrane structural class of MT-MMPs contains MMP-14 (*MT1-MMP*), MMP-15 (*MT2-MMP*), MMP-16 (*MT3-MMP*) and MMP-24 (*MT5-MMP*). The glycosyl phosphatidylinositol (GPI)-anchored structural group encompasses MMP-17 (*MT4-MMP*) and MMP-25 (*MT6-MMP*). Very little is known regarding the third MT-MMP group, the type II transmembrane MMPs, which contains only MMP-23 (*cysteine-array MMP*), a member structurally different from all other MMPs (9). Overexpression of MT-MMPs has been observed in many forms of cancer (10). The functions of MT-MMPs are known to include roles in activation

of other MMP family members, pericellular proteolysis, modulation of cellular signalling, cellular migration, regulation of cellular proliferation and apoptosis, the angiogenic response and regulation of tumour invasion metastasis (4, 10-13). Given the vast range of cellular processes influenced by the MT-MMPs, evaluation of their expression and activity in human tumours may be important in terms of diagnosis, prognosis and treatment.

A number of recent studies have analysed genetic expression of MMP-14 in several human tumours types, including tumours of the breast, colon, head and neck, oral cavity, prostate and importantly for this study, pulmonary tumours including bronchopulmonary and NSCLC (14-20). The majority of these studies were semiquantitative and did not evaluate expression of the other MT-MMP members.

Data regarding the expression of the MT-MMP subfamily in NSCLC is limited, therefore, the aim of the present study was to (a) assess the mRNA expression of the MT-MMPs by qRT-PCR in human preclinical tumour models and paired clinical human NSCLC tumour and histologically normal lung tissue; (b) to evaluate the correlation between MMP-14 mRNA expression and proteolytic activity.

MATERIALS AND METHODS

Human Tissue Samples

A total of 39 freshly resected and snap frozen specimens of NSCLC were used for this study. In addition, the study also included paired histologically normal human lung tissue specimens for 15 of the tumours, excised distant to the tumor mass. Patient ages ranged from 47 to 85 years (median 68 years). Informed consent was obtained from all patients prior to specimen collection and all patient details were anonymised to ensure confidentiality. All experiments were performed after first obtaining consent from the local research and ethics committee according to Medical Research Council Regulations.

The presence of tumour (or lack in terms of normal tissue specimens) and histopathological classification was confirmed by pathologists at Bradford Royal Infirmary, UK. The panel of tumours comprised 28 adenocarcinomas and 11 squamous cell carcinomas (SCC) and a range of tumour stages (4 pT1; 30 pT2; 4 pT3 and one pTx).

Cell culture

Cell lines were grown in monolayer culture in complete RPMI 1640 containing 10% v/v FBS, 1% v/v L-glutamine and 1% v/v sodium pyruvate (Sigma, Dorset, UK). All cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were maintained in continuous exponential growth by passage every 5-7 days. Cells were either harvested when subconfluent by trypsinisation (Trypsin-EDTA; Sigma) and stored as pellets at -20°C. Cell lines used in these studies include; HT1080 (fibrosarcoma); MCF-7 and MDA-MB-231 (breast carcinoma); HT29, BE, HCT116, COLO205, DLD1 (colorectal carcinoma); A549, H661, H460 (NSCLC); PC3 and

LNCAP (prostate carcinoma). All cell lines were obtained from ECACC (Sailsbury, Wiltshire, UK) or ATCC (Manassas, Virginia, USA).

Human tumour xenografts

Human tumour cell lines were xenografted in mice under a project license issued by the UK Home Office, following UKCCCR guidelines. Female mice (nu/nu from an inbred colony, B and K Universal, Hull, UK) 6-8 weeks old were implanted subcutaneously with 2-3 mm^3 fragments of H460, A549 (NSCLC); MCF7 (breast carcinoma); BE, COLO 205, DLD1, HCT116, HT29 (colorectal carcinoma); PC3 (prostate adenocarcinoma) or HT1080 (fibrosarcoma) tumors. Resultant tumors were removed, snap-frozen in liquid nitrogen and stored at -70°C.

Determination of MMP-14 mRNA expression by semi-quantitative RT-PCR analysis

TRI REAGENT™ (Sigma) was used to isolate RNA from cell pellets and tissue samples. Reverse transcription was performed using Advantage™ RT-for-PCR kit (BD Bioscience, Oxford, UK) according to the manufacturer's instructions. PCR was performed using the Titanium™ Taq PCR kit (BD Bioscience) according to the manufacturer's instructions and the primers described below (Invitrogen, Paisley, UK). Primers were based on previous studies (21, 22), and were: MMP-14 (sense) 5′-CGC TAC GCC ATC CAG GGT CTC AAA-3′; MMP-14 (antisense) 5′-CGG TCA TCA TCG GGC AGC ACA AAA-3′; GAPDH (sense) 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; GAPDH (antisense) 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′. PCR amplification of MMP-14 involved; initial denaturation of 94°C for 4 min followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec

PCR for GAPDH involved denaturation at 94°C for 4 min followed by 25 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec. All cycles were terminated with a 10 min incubation at 72°C to ensure complete elongation prior to incubation and storage at 4°C. RT-PCR products were separated through a 1% w/v agarose gel containing 0.01% v/v ethidium bromide and visualised under ultraviolet (UV) illumination.

Quantification of MT-MMP expression by Real-Time RT-PCR (qRT-PCR)

For qRT-PCR, RNA extraction was carried out as for RT-PCR. For cDNA synthesis, a total of 1µg of total RNA was reverse transcribed using random hexamers (Amersham, Buckinghamshire, UK) and Superscript II reverse transcriptase (Invitrogen), according to supplier's instructions. For PCR reactions, specific primers and fluorogenic probes for all MT-MMPs were designed using Primer Express 1.0 software (Applied Biosystems, Warrington, UK) and synthesised by Applied Biosystems. Where possible, to prevent the amplification of genomic DNA and to ensure the PCR signal was generated from cDNA, primers were generated with sequences within different exons, close to intron-exon boundaries. BLASTN searches (23) were conducted on all primer/probe nucleotide sequences to ensure gene specificity. The 18S ribosomal RNA gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample, using previously validated procedures (24); 18S rRNA primers and probes were purchased from Applied Biosystems. PCR reactions were performed as described previously (24). To determine relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using cDNA from one sample and making two-fold serial

dilutions covering the range equivalent to 20-0.625 ng RNA (for 18S analyses, the range was 4-0.125 ng).

Determination of MMP-14 protein levels by ELISA assay

MMP-14 protein activity levels were measured using the Biotrak Activity Assay System (Amersham Biosciences), carried out according to manufacturers instructions. Briefly, xenograft tissues were weighed, incubated for 15 min at 4ºC in membrane extraction buffer (50mM Tris-HCl pH 7.6, 1.5mM NaCl, 0.5mM CaCl, 1µM ZnCl, 0.01% v/v Brij-35, 0.25% v/v Triton X-100), homogenised on ice and incubated for a further 15 min at 4°C. The resultant supernatants and MMP-14 standards (ranging from, 0.125ng/ml - 32ng/ml) were incubated overnight at 4ºC on an anti-MMP-14 antibody coated microplate. Following incubation, all wells were washed (phosphate buffered saline containing 0.05% v/v Tween-20), the detection reagent added and the plate agitated and incubated at 37ºC for 6 hours. The absorbance of each sample was read at 405nm and the concentration of MMP-14 in the samples calculated from the standard curve.

Data Analysis

For comparison of MT-MMP expression between NSCLC specimens and histologically normal tissue, the data did not satisfy normality or equal variance, so nonparametric statistical tests were used. The Mann-Whitney U test was carried out to compare malignant and non-malignant samples. For all tests, a P-value of less than 0.01 was considered statistically significant.

RESULTS

Expression of MT-MMPs in human tumour cell lines and xenografts

The expression of the MT-MMPs was assessed in a panel of human cell lines and human tumour xenografts representing several different human tumour types by qRT-PCR. All MT-MMPs were detected successfully (Figure 1) and were in agreement with expression observed by semi-quantitative RT-PCR (data not shown). MMP-14 and MMP-15 were expressed at high levels in all the tumour types represented in the screen (Figure 1). MMP-16, -17, -24 and -25 demonstrated differential expression throughout the preclinical tumour panel suggesting potential associations between these MMPs and tumour origin or tumour type (Figure 1). In contrast to the other MT-MMPs, the type II transmembrane MMP, MMP-23, was expressed at relatively low levels in both lung cell lines and xenografts (Figure 1) and was not therefore analysed in subsequent studies.

MT-MMP expression in NSCLC specimens and histologically normal lung tissue

The high level and range of MT-MMP expression observed in preclinical models of lung cancer (Figure 1) led us to evaluate the expression of MMP-14, -15, -16, -17, -24 and -25 (MT[1-6]-MMP) in clinical specimens of NSCLC and associated histologically normal lung tissue by qRT-PCR. MMP-14 was expressed in 100% NSCLC tumour specimens analysed in our study at levels significantly higher than that observed in histologically normal lung tissue (Figure 2; $P< 0.01$). Of the other type I transmembrane MMPs, MMP-15 demonstrated significantly higher expression in NSCLC compared to histologically normal lung tissue (Figure 2; $P< 0.01$), MMP-16 was absent or expressed at extremely low levels in all clinical specimens evaluated

(Figure 2) and MMP-24 demonstrated no significant difference in expression between NSCLC and histologically normal lung tissue (Figure 2; P = 0.5).

The GPI-anchored MT-MMPs, MMP-17 and -25 were also expressed in both NSCLC and the associated histologically normal lung tissue (Figure 2), although a significant difference between tumour and normal was detected for MMP-17 but not MMP-25 (P< 0.01 and P= 0.9, respectively)

Relationship between MT-MMP expression and tumour pathology

In contrast to the other MT-MMPs evaluated, expression of MMP-15 demonstrated differential expression between NSCLC tumour subtypes (Figure 3). In addition to being significantly higher than that observed in histologically normal lung tissue (p< 0.01), levels of MMP-15 in adenocarcinoma (n=28) were also significantly higher than that detected in squamous cell carcinoma (SCC) (n=11; p< 0.01) (Figure 3). In contrast, although MMP-14 also demonstrated significantly higher expression in NSCLC compared to histologically normal lung, no significant difference was observed between tumour types (Figure 3).

Although expression of MMP-14, -16, -17, -24 and -25 were detected in human NSCLC, no significant correlation was observed between the expression of the individual MMPs and either tumour stage (pT status), tumour grade or presence of lymph node invasion (pN status) (Data not shown).

Correlation between MMP-14 activity and MMP-14 qRT-PCR expression

Levels of proteolytically active MMP-14 were determined in human tumour cell lines and xenografts using the fluorogenic MT1-MMP (MMP-14) Biotrak assay. MMP-14 activity levels detected using this assay ranged from 1.1–0.02 ng MMP-14 per mg

tissue, with the highest levels detected in HT1080 (1.12±0.10 ng MMP-14 per mg tissue) and lowest in H460 (0.02±0.01 ng MMP-14 per mg tissue) (Figure 4). Comparison of MMP-14 activity levels with MMP-14 mRNA expression, as measured by qRT-PCR, demonstrated an excellent correlation between the levels of active MMP-14 and MMP-14 mRNA expression in both cell lines (data not shown) and xenografts (Figure 4; $R^2 = 0.9$).

DISCUSSION

MMPs are central regulators in the tumorigenic process and the major proteases involved in ECM breakdown and remodelling (1). The original hypothesis that MMPs were involved purely with ECM degradation and consequently tumour invasion and metastasis was demonstrated to be too simplistic and the MMPs are now known to be a major force in the phenotypic evolution of cancer (25). Growth-factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands, and angiogenic factors are just some examples of the diversity of substrates targeted by the MMPs (3).

Identification of proteolytic enzyme expression and localisation in human cancers is critical in understanding the characteristics of individual cancer types, referred to as the cancer degradome (26). Identification of specific MMPs important in the various stages of cancer progression, will lead to improved understanding of different cancers and may represent new markers of disease or may lead to the identification and validation of new therapeutic targets.

The purpose of this study was to comprehensively analyse expression of the membrane-type MMP (MT-MMP) subfamily in human NSCLC and to evaluate the relationship between genetic expression of MMP-14 and the presence of active MMP-14. We have shown differential expression of these MMPs in NSCLC and

demonstrated significantly higher levels of several family members in tumours compared to histologically normal lung tissue (Figure 2).

MMP-14 was expressed in 100% NSCLC tumour specimens analysed in our study (Figure 2). The high levels of MMP-14 expression quantified in NSCLC in this study reinforces previous studies implicating increased MMP-14 expression in lung cancer by semi-quantitative *in-situ* hybridisation and cDNA array analysis (15, 27). Although in our study MMP-14 was detected at low levels in histologically normal lung tissue, expression was significantly higher in NSCLC specimens ($p < 0.01$; Figure 2). The elevated levels of MMP-14 we observed in NSCLC demonstrated no relationship to tumour stage, tumour grade, presence of tumour lymph node invasion or differential between histological tumour type (data not shown).

Data focussing on the expression of MMP-15 (MT2-MMP) in NSCLC is sparse. As for MMP-14, MMP-15 displayed a statistically significant difference ($P<0.01$) in expression between tumour tissue and histologically normal lung (Figure 2). This is supported by a previous study in which MMP-15 levels were suggested to be higher in adenocarcinoma cells than either type II alveolar cells or bronchiolar epithelial cells (28). Further to demonstrating increased MMP-15 expression in NSCLC relative to histologically normal lung tissue, we have also shown significantly higher expression in lung adenocarcinoma relative to lung SCC ($P<0.01$; Figure 3), a previously unreported observation. No such differential was observed for MMP-14 (Figure 3). This strongly suggests that MMP-15 may be a viable marker for molecular diagnostics of NSCLC and as such warrants further investigation. Similarly, the suggestion that MMP-15 has an anti-apoptotic mechanism and may connect tumour metastasis and apoptotic resistance (11) is also of relevance to our study and worthy

of further investigation due to the high levels of MMP-15 we observed in NSCLC specimens.

There is very little data concerning the expression of MMP-16, MMP-17, MMP-24 or MMP-25 (MT(3-6)-MMP) in human tumours and no specific evidence for their involvement in NSCLC. MMP-16 was absent or expressed at extremely low levels in all clinical samples evaluated (Figure 2). It is interesting to note the expression profile of MMP-16 in preclinical human tumour models, the differential in expression observed between tumour types and between cells grown *in vitro* or as *in vivo* tumour xenografts (Figure 1). These observations could be the consequence of the degree of xenograft vascularisation or invasiveness, as supported by the recent associations between MMP-16 and both tissue vascularisation (29) and β-catenin mediated tumour invasion (30). Identification of any such relationship and their clinical significance have yet to be undertaken. In the case of the lung cell lines, MMP-16 was detected in the H460 cells grown *in vitro* but not *in vivo* (Figure 1), which is interesting in light of the fact that MMP-16 was absent in the majority of NSCLC samples.

Increased expression of MMP-17 was observed in NSCLC and did demonstrate a statistically significant difference from histologically normal lung (Figure 2). Due to the low levels of MMP-17 detected in the majority of samples, caution should be adopted in the significance of this difference and the relationship to protein levels. MMP-24 and MMP-25 display differential expression between tumour and normal tissue although these failed to reach statistical significance ($P = 0.5$ and $P = 0.9$, respectively). Whilst we would predict that the expression of the MT-MMPs might be related to the stage and development of cancers due to their known involvement in metastasis, cell migration and activation of other MMP family

members, no correlation was observed between tumour stage, grade or lymph node invasion and the expression of individual MT-MMPs (data not shown). These findings are perhaps surprising given the well documented role of the MMPs, including MT-MMPs, in various stages of cancer development and tumour spread (13) .

The excellent correlation (R^2= 0.9) noted in this study between qRT-PCR and activity data for MMP-14 indicates that qRT-PCR is a proportional measurement of the level of MMP-14 protein present in a sample (Figure 4). This correlation thereby adds value to the qRT-PCR clinical sample data for the MT-MMPs. Due to the nature of this assay, it does not take into account any potential intrinsic regulation of MT-MMP activity, such as by TIMPs, and may therefore signify the levels of MMP-14 protein and total potential MMP-14 activity rather than actual activity. Since both the qRT-PCR and activity assay capture a single snapshot in the tumour, it would be interesting to assess the potential changes in expression and activity during tumourigenesis and tumour progression. In support of this, it would be desirable to evaluate the expression and sub-tumoral distribution of specific MT-MMPs using *in situ* hybridisation or immunohistochemistry, should specific and appropriate antibodies become available. In addition, it would also be interesting to differentiate the levels of actual MMP-14 activity from total potential MMP-14 activity in this context, to further assess MMP-14 as a disease marker or target for therapeutic exploitation in NSCLC.

Currently there are no assays available to study the activity of MT(2-6)MMP in samples and we have not found any commercially available antibodies suitable for the development of such assays (data not shown). It will be important to investigate

the relationship between qRT-PCR data and protein expression for all MT-MMPs to ascertain if a similar relationship is observed as is seen for MMP-14.

This study is the first analysis of all 6 MT-MMPs in NSCLC clinical specimens. Our data identifies a number of the MT-MMPs as being differentially expressed in NSCLC relative to histologically normal lung tissue, suggesting roles in tumourigenesis and/or as markers of disease. Using an MMP-14 activity assay, we have demonstrated the value of qRT-PCR as a predicator of protein expression. In summary, this study indicates the potential use of MMP-14 and MMP-15 as disease markers and therapeutic targets.

ACKNOWLEDGEMENTS

This work was supported by Cancer Research UK (Programme Grant A5953), The Big C Appeal and the Cancer Degradome Integrated Project Grant LSHC-CT-2003-503297 from the European Union Framework Programme 6.

CONFLICT OF INTEREST STATEMENT

None of the authors disclose any financial or personal relationships with other people or organisations that could inappropriately influence the present work.

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Figure Legends:

Figure 1. MMP expression in human tumour cell lines and xenografts as measured by quantitative RT- PCR (qRT-PCR). Values of gene output are probe, and therefore gene specific, thus precluding comparison of expression between genes.

Figure 2. Differential expression of the MT-MMP subfamily in human NSCLC and histologically normal lung tissue by qRT-PCR. Values are after normalisation to 18S-rRNA and are therefore gene specific. Expression data is plotted as mean expression ± Standard Error (S.E.).

Figure 3. Elevated expression of MMP-15 by qRT-PCR in lung adenocarcinomas compared to SCC ($P<0.01$) and histologically normal lung ($P<0.01$).

Figure 4. MMP-14 activity levels determined by ELISA (A) correlate strongly to MMP-14 expression by qRT-PCR (B), as measured in human tumour xenografts. A close correlation ($R^2=0.9$) is observed between activity and expression (C).

Atkinson et al. Figure 1

Origin	Cell line	MMP 14	15	16	17	23	24	25
Fibrosarcoma	HT1080							
Breast	MCF7							
	MDA-MB-231							
Colon	HT29							
	BE							
	HCT116							
	COLO205							
	DLD1							
Lung	A549							
	H661							
	H460							
Prostate	PC3							
	LNCAP							

Origin	Xenograft	14	15	16	17	23	24	25
Fibrosarcoma	HT1080							
Breast	MCF7							
Colon	BE							
	HCT116							
	COLO205							
	DLD1							
	HT29							
Lung	A549							
	H460							
Prostate	PC3							

Expression key:

Negative | Low | Moderate | High | Very high

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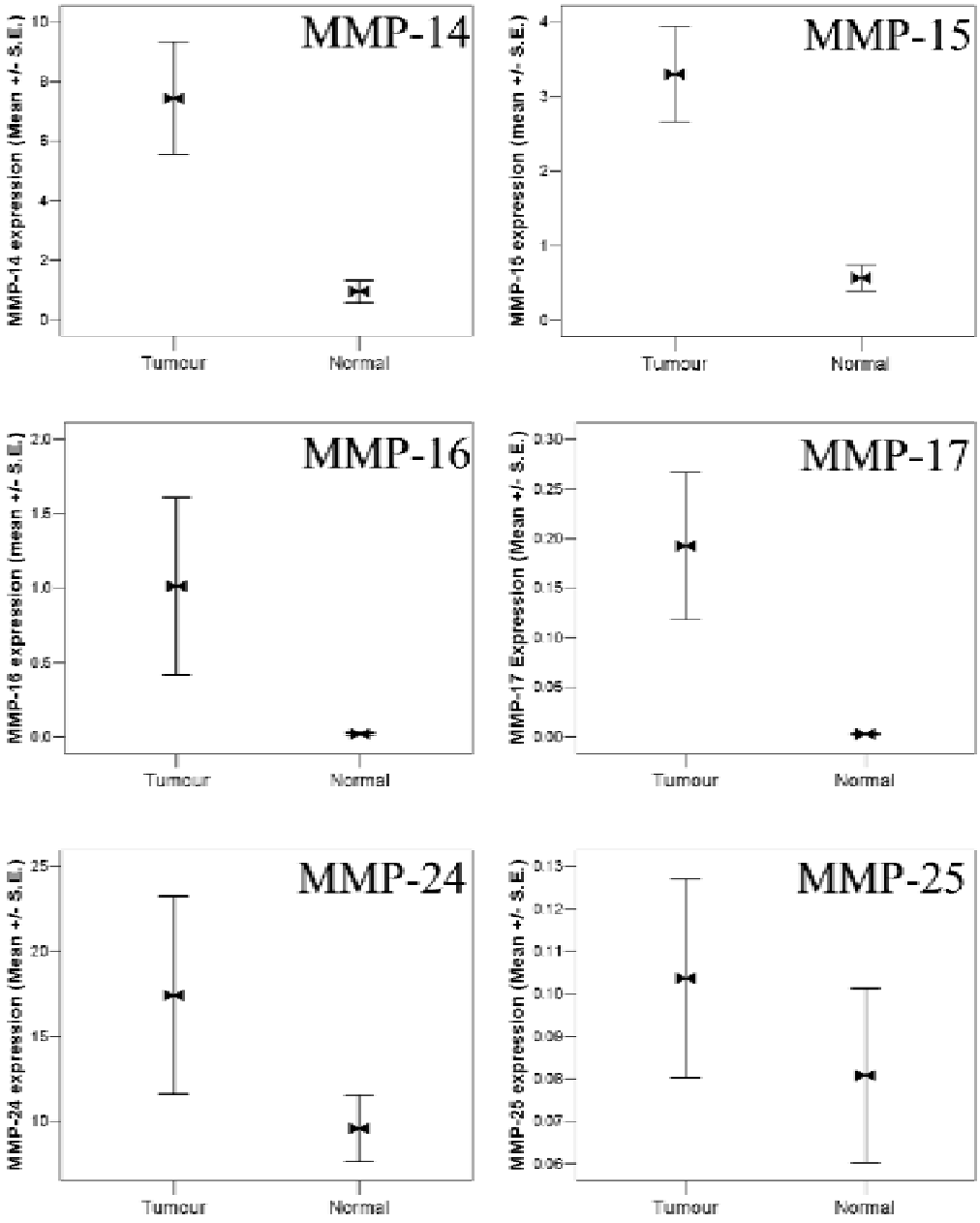
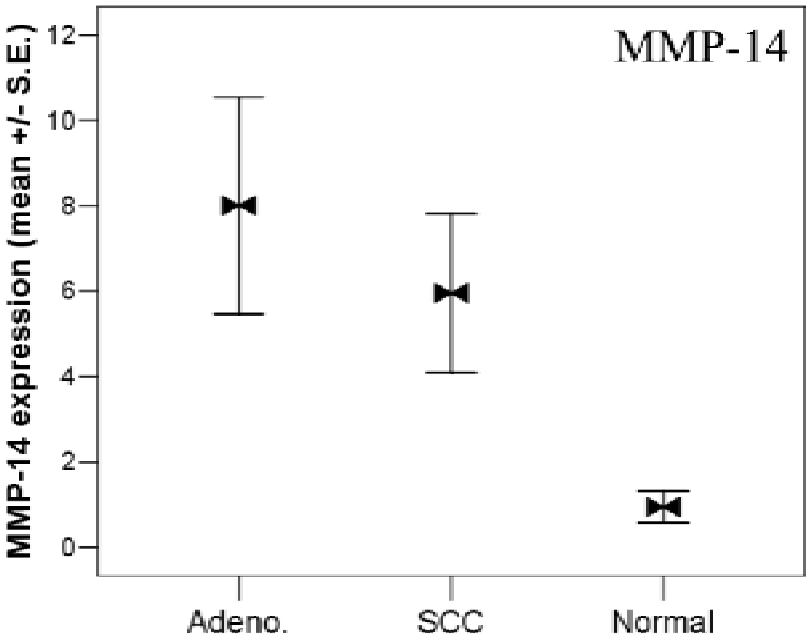
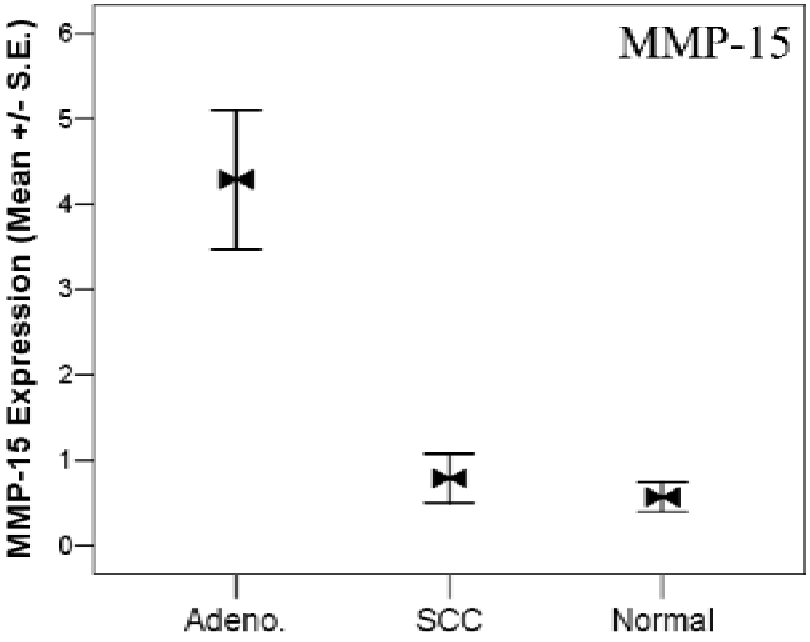
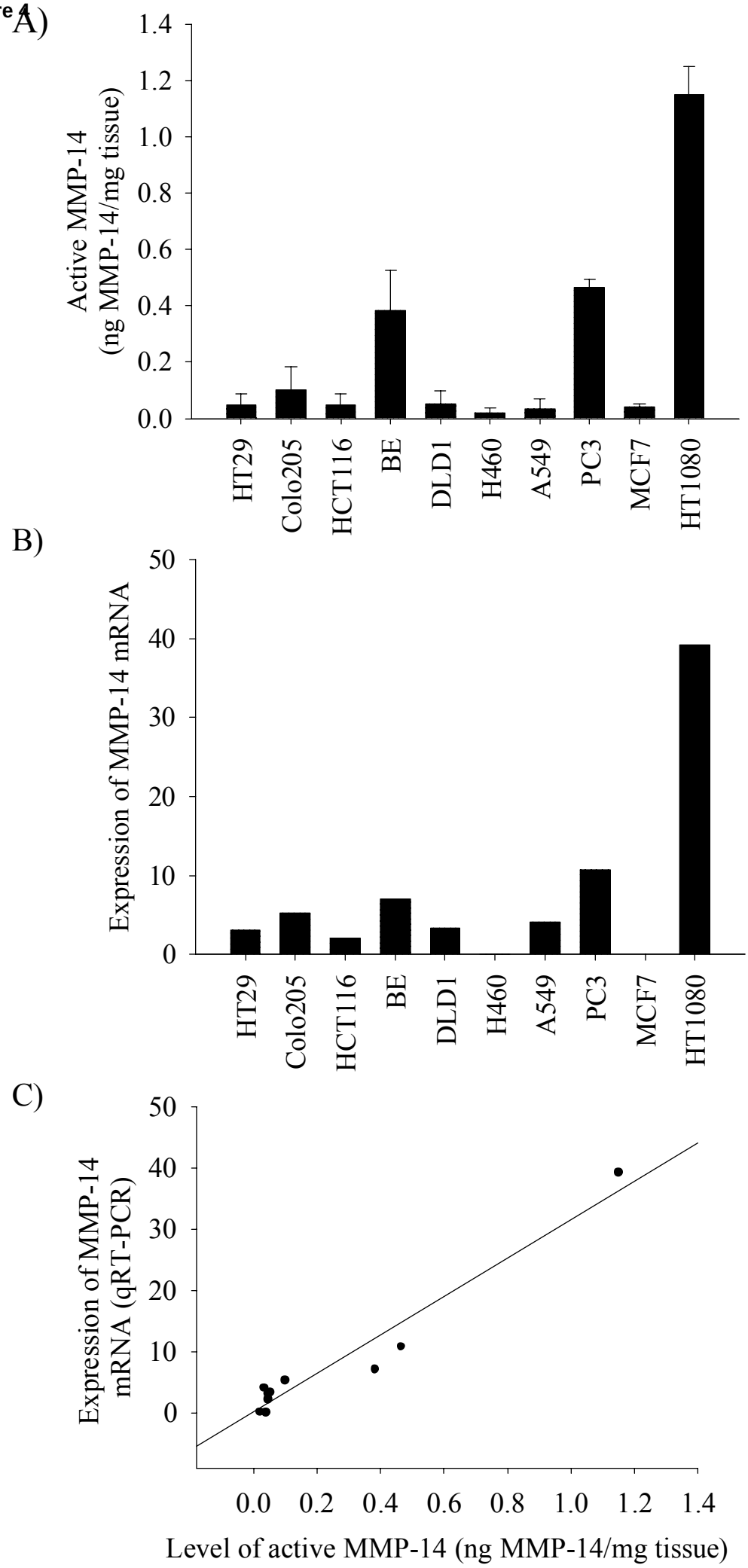


Atkinson et al. Figure 3






A)
Active MMP-14 (ng MMP-14/mg tissue)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HT29
Colo205
HCT116
BE
DLD1
H460
A549
PC3
MCF7
HT1080
B)
Expression of MMP-14 mRNA
50
40
30
20
10
0
HT29
Colo205
HCT116
BE
DLD1
H460
A549
PC3
MCF7
HT1080
C)
Expression of MMP-14 mRNA (qRT-PCR)
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Level of active MMP-14 (ng MMP-14/mg tissue)

CONFLICT OF INTEREST STATEMENT

None of the authors disclose any financial or personal relationships with other people or organisations that could inappropriately influence the present work.

ACKNOWLEDGEMENTS

This work was supported by Cancer Research UK (Programme Grant A5953), The Big C Appeal and the Cancer Degradome Integrated Project Grant LSHC-CT-2003-503297 from the European Union Framework Programme 6.