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***TIMP-1* expression in human colorectal cancer is associated with *TGF-B1*, *LOXL2*, *INHBA1*, *TNF-AIP6* and *TIMP-2* transcript profiles**

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ABSTRACT

The balance of activity between the endogenous enzyme inhibitors known as tissue inhibitors of metalloproteinases and their targets, the matrix metalloproteinases, in the extracellular matrix is thought to play an important role in tumour cell invasion. Supporting this notion, we have shown that colorectal cancer patients have increased plasma levels of the tissue inhibitor of metalloproteinases-1 (*TIMP-1*), and that high plasma *TIMP-1* levels are associated with short colorectal cancer patient survival. However, although *TIMP-1* has been extensively studied in cancer, very little is known about how it is regulated. To further elucidate potential mechanisms of regulation of this protein, we did a number of experiments to look at associations between the transcript profile of *TIMP-1* with known matrix metalloproteinases (*MMPs*) as well as with expression profiles of other genes differentially regulated in human colorectal cancer (*CRC*) and the other *TIMPs* 2–4, which have also been associated with the progression of colorectal cancer. Genome-wide expression profiling of 172 *CRC* and normal mucosa samples was used to identify transcript changes for the genes under investigation. We found that *TIMP-1* was up-regulated in *CRC* samples compared with normal tissue, while *TIMP-2* was down-regulated. Eight *MMPs* were up-regulated in *CRC* compared with normal tissue. Correlating up-regulated genes with the *TIMP-1* transcript, we identified 13 that were also up-regulated in cancerous tissue. Among these were genes associated with the synthesis of extracellular matrix, genes involved in the *TGF-beta* signalling pathway, and genes that are likely transcribed by the tumour cells. These insights add to the complex picture emerging about the regulation of *TIMPs* in colorectal cancer.

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1. Introduction

A better biological understanding of colorectal cancer (*CRC*) biology, the third most frequent cancer type in the Western

world, is imperative for improving patient treatment and thereby patient prognosis. We and others have focused our work on the biological role of the naturally occurring endogenous Tissue Inhibitors of Metalloproteinases (*TIMPs*) in

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cancer. The TIMPs, now encompassing TIMP-1 to -4, can complex with most of the metalloproteinases (MMPs) and thereby inhibit proteolysis. This inhibition should, in theory, inhibit cancer progression. Moreover, it has been suggested that the TIMPs are co-regulated with the MMPs, i.e., that high MMP activity would induce a comparable high TIMP activity. However, the TIMPs have been shown to have functions being distinct from their anti-proteinase functions. For TIMP-1, these functions include stimulation of cell proliferation (Westbrook et al., 1984), inhibition of apoptosis (Guedez et al., 1998; Liu et al., 2003) and regulation of angiogenesis (Akahane et al., 2004), all functions which could facilitate cancer progression. In relation to apoptosis recent studies have suggested that TIMP-1 could be associated with resistance to chemotherapy-induced apoptosis in cancer cells (Davidsen et al., 2006; Schrohl et al., 2006; Sorensen et al., 2007) and one recent study has shown that in MCF10A cells TIMP-1 exerts its anti-apoptotic mechanism by interaction with CD63 and integrin beta 1 (ITGB1) on the plasma membrane (Jung et al., 2006). That TIMP-1 in particular is involved in tumour progression is further supported by many clinical studies demonstrating a highly significant association between high tumour tissue or blood TIMP-1 concentration and poor patient prognosis (Holten-Andersen et al., 2000, 2002; Schrohl et al., 2004). However, much is still to be learned on the biological role of TIMPs in cancer progression.

The aim of this study was to investigate the regulation of TIMP-1 in CRC as well as investigate the relationship and interactions between transcripts of MMPs and TIMPs in CRC. To gain further insight into the mechanisms that may influence TIMP-1 expression we also included the expression data of genes that were found correlated with the expression of TIMP-1.

2. Experimental procedures

2.1. Tissue samples and patient information

A total number of 172 CRC patient tissue samples obtained from hospitals in Denmark, Finland and The Netherlands, comprising 10 normal colon mucosae from the resection edge, 149 stage II (Dukes B) and 13 stage III (Dukes C) adenocarcinomas from patients with sporadic CRC was previously analysed on microarrays. This dataset was previously used in two publications, and clinical information such as age, sex, microsatellite stability/instability (MSS/MSI) status can be found in the supplementary (Birkenkamp-Demtroder et al., 2007; Mansilla et al., 2007). Studies were approved by the local ethical committees of all the involved clinics, and patients gave informed consent prior to surgery.

An independent set of samples comprising 25 normal mucosae and 22 adenocarcinomas from the proximal and distal colon were also analysed. Ten of the 25 normal samples have been previously used for quantitative PCR analysis (Mansilla et al., 2007). The 22 adenocarcinomas comprised Dukes A (2), B (6), C (9), and D (5). Nine of the 22 adenocarcinomas were accompanied by their paired normal mucosa. The 22 adenocarcinomas and 15 of the normal samples had

previously been analysed on HU6800 microarrays comparing gene expression in distal versus proximal colon (Birkenkamp-Demtroder et al., 2005).

2.2. Genome-wide microarray expression profiling

Genome-wide microarray expression profiling was performed using Affymetrix U133.plus.2.0 GeneChips and data were normalized as previously described (Kruhoffer et al., 2005). Expression values are given in “log₂” where indicated. All sequences of the TIMP-1 to -4 Affymetrix probe sets were BLASTed and the probes located at the 3' end of the coding region or the 3' UTR region of the human TIMP sequences.

Identification of genes correlating with the TIMP-1 transcript profile was selected based on the following criteria: Pearson correlation coefficient >0.6; significantly differentially expressed between normal mucosa and tumour tissue ($p < 0.05$, two-tailed Student's *t*-test), log₂ ratio normalized to zero >1.1 or <-1.1 and expression level of log₂ >3.

2.3. Ingenuity pathway analysis (IPA)

Transcript expression values (log₂) from 10 normal colon mucosae, 149 stage II and 13 stage III colon adenocarcinomas were normalized around zero. Probe sets with an intensity value of log₂ < 2 were excluded from analysis. Normalized ratios given as ([-INF, -1] and [+1, +INF]) were submitted to IPA (2000–2008 Ingenuity Systems).

2.4. Quantitative PCR

Differences in gene expression between tumour tissue and normal tissue were verified by quantitative PCR (Q-PCR) for selected genes: TIMP-1, TGF-B1, TNF-AIP6, LOXL-2, Activin A (INHBA) and MMP28. For these analyses RNA was extracted from 22 CRC tumours and 25 normal mucosa samples using Trizol (Gibco Life Technologies, Invitrogen Corporation, Carlsbad, CA) and 12 µg RNA was reverse transcribed to cDNA using the SuperScript Choice System (Invitrogen Corporation, Carlsbad, CA) as previously described (Birkenkamp-Demtroder et al., 2005). All primers were intron spanning to avoid detection of contaminating DNA and beta-actin was used as reference gene. The primer sequences were: TIMP-1 forward: 5'-CTT CTG GCA TCC TGT TGT TG-3', TIMP-1 reverse: 5'-GGT ATA AGG TGG TCT GGT TG-3'; TGF-B1 forward: 5'-GAG CCT GAG GCC GAC TAC TA-3', TGF-B1 reverse: 5'-GGG TTC AGG TAC CGC TTC TC-3'; TNF-AIP6 forward: 5'-ACC ACA GAG AAG CAC GGT CT-3', TNF-AIP6 reverse: 5'-CAA CTC TGC CCT TAG CCA TC-3'; LOXL-2 forward: 5'-CCG GGT GGA GGT GTA CTA TG-3', LOXL2 reverse: 5'-TCG TTG CCA GTA CAG TGG AG-3'; INHBA forward: 5'-CCT CGG AGA TCA TCA CGT TT-3', INHBA reverse: 5'-CCC TTT AAG CCC ACT TCC TC-3'; MMP28 forward: 5'-CAA TGA ACA GGT CCC CAA AG-3', MMP28 reverse: 5'-GGC CGC ATA ACT GTT GGT AT-3' and beta actin forward: 5'-CGT GGG CCG CCC TAG GCA CCA-3', beta actin reverse: 5'-TTG GCC TTA GGG TTC AGG GGG G-3'. All samples were measured in duplicate. Q-PCR was carried out using SYBR Green I detection and the LightCycler 480 (Roche Diagnostics, Hvidovre, Denmark). Reactions were carried out in 96 well plates with 2 µl cDNA in 20 µl reaction volumes

consisting of 1× FastStart Master SYBR Green Mix and 0.5 μM of the gene-specific primers. The amplification programme was as follows: Preincubation for Fast Start Polymerase activation at 95 °C for 5 min, followed by 45 amplification cycles (95 °C for 10 s (20 °C/s), 60 °C for 10 s (20 °C/s), and 72 °C for 10–12 s (20 °C/s). SYBR Green fluorescence was acquired at 72 °C in each amplification cycle. After the end of the last cycle the melting curve was generated by starting the fluorescence acquisition at 65 °C and taking measurements every 0.1 s until 95 °C was reached. Relative quantification was done using the Relative Quantification software (LightCycler, Roche, Denmark).

2.5. Statistical analysis

Statistical Analysis was performed using STATA9.2 (Statacorp, Texas, USA). A two-tailed Student's t-test was applied and p-values <0.05 were considered as statistically significant.

3. Results

The *TIMP-1* to -4 transcript expression levels were determined by genome wide microarray transcript profiling of 10 normal colon mucosae, 149 stage II, and 13 stage III colon adenocarcinomas as shown in Figure 1. The *TIMP-1* transcript was significantly up-regulated >5-fold in stage II ($p = 4.9E-23$) and stage III ($p = 2.2E-06$) colon adenocarcinomas, as compared with normal mucosa, while the *TIMP-2* transcript was down-regulated 2-fold in stage II ($p = 1.9E-02$) and stage III ($p = 2.2E-02$) cancers. *TIMP-3* was not differentially expressed between cancer and normal tissue and the *TIMP-4* transcript was below detection level in all samples. Detailed expression values of *TIMP-1* to -4 are given in Table 1.

The encoded proteins of the *CD63* and *ITGB1* genes are known to interact with *TIMP-1* (Jung et al., 2006). Figure 2 shows that the *CD63* transcript as determined by the genome wide microarray expression profiling is significantly down-regulated in stage II (median, $p = 3.2E-17$) and to a lesser

extent in stage III (median, $p = 2.1E-04$) in adenocarcinomas as compared with normal mucosa. *ITGB1* was not found to be differentially expressed.

Spearman correlation analysis was done for *TIMP-1* to -4 as shown in Table 2. The strongest correlation was seen between *TIMP-2* and *TIMP-3* ($r = 0.77$). A strong correlation was also found between *TIMP-1* and *TIMP-2* ($r = 0.62$) and to a lesser extent between *TIMP-1* and *TIMP-3* ($r = 0.49$).

A Pearson correlation analysis was then performed for *TIMP-1* to -4 and the 23 MMPs represented on the GeneChips as shown in Table 3. MMPs marked with an asterisk were significantly different between tumour and normal mucosa and showed a correlation coefficient >0.5 with at least one of *TIMP-1* to -3. The transcripts of *TIMP-1* to -3 showed a relatively good correlation with the transcripts of MMPs 2, 9, 11, 14 and 19. *TIMP-1* correlated very well with the transcripts of MMPs 1, 2, 9, 11 and 14. In particular, MMP2 was highly correlating with *TIMP-1* to -3, the best correlation was seen for *TIMP-2* ($r = 0.88$). None of the MMPs correlated with *TIMP-4*.

Of the 23 different MMPs, the transcript levels of 8 MMPs were identified to be significantly up-regulated from normal to tumour, whereas only one, MMP28, was significantly down-regulated as shown in Table 3. The most remarkable differences were detected for MMPs 1, 3, 7, and 28.

In addition to the correlation of *TIMP-1* with other *TIMPs* and MMPs, we identified a group of 13 genes correlating with the *TIMP-1* transcript profile as shown in Table 4.

Ingenuity pathway analysis was performed using median log2 transcript expression data from the 172 colon tissue samples. Data were applied to the 36 genes listed in Tables 3 and 4; 18 of these were correlating with *TIMP-1* expression. Hereof, about 50% are known to be associated with cell migration, invasion and/or adhesion and for the majority of these genes a direct or indirect interaction with *TIMP-1* was identified as depicted in Figure 3A. Most of the genes affecting *TIMP-1* (Figure 3B) or genes affected by *TIMP-1* (Figure 3C) were identified to be differentially expressed in colorectal cancer when compared to normal mucosa.

Using quantitative PCR on the independent sample set, we verified the up-regulation of *TIMP-1* in tumour samples as compared with normal tissue (ratio 4.5, $p = 0.0001$). Furthermore, we verified the up-regulation of *TGF-B1* (ratio 3.7, $p = 0.004$), *TNF-AIP6* (ratio 9.3, $p = 0.02$), *LOXL-2* (ratio 8.1, $p = 0.0001$) and *Activin A* (*INHBA*) (ratio 69, $p = 0.0001$). The four genes were selected based on either a high correlation with *TIMP-1* or a very low p-value in the array analysis. We also verified the correlation of the selected genes with *TIMP-1* in the tumour samples. For *TGF-B1* ($r = 0.64$) and *LOXL2* ($r = 0.67$) we found correlation values comparable to the ones obtained in the array analysis. For *TNF-AIP6* ($r = 0.54$) and *INHBA* ($r = 0.55$) the correlation values were lower compared with what was found in the array analysis.

As MMP28 was the only down-regulated gene we also included this in the quantitative PCR analysis. A tendency towards down-regulation in tumour tissue of MMP28 (ratio 0.34, $p = 0.09$) was found. No correlation between MMP28 and *TIMP-1* ($r = 0.17$) could be demonstrated which is in agreement with the array-analysis.

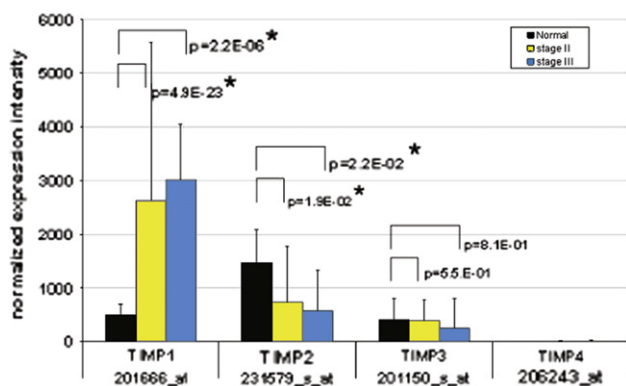


Figure 1 – U133plus.2.0 microarray transcript profiling of *TIMP-1* to -4. *TIMP-1* was significantly up-regulated in stage II and stage III colon adenocarcinomas as compared with normal colon mucosa, while *TIMP-2* was significantly down-regulated in the tumour tissue.

Table 1 – Detailed expression values of *TIMP-1* to *-4*

	Mean	Std. dev.	Median	Std. err.	[95% conf. interval]	
Normal (n = 10)						
TIMP-1	521.87	200.39	498.81	63.37	378.52	665.23
TIMP-2	1628.59	610.48	1472.14	193.05	1191.88	2065.30
TIMP-3	536.53	396.67	408.32	125.44	252.77	820.30
TIMP-4	7.06	0.94	7.04	0.29	6.39	7.74
Stage II (n = 149)						
TIMP-1	3426.92	2940.06	2633.45	240.85	2950.95	3902.88
TIMP-2	1061.69	1046.37	736.02	85.72	892.29	1231.08
TIMP-3	456.55	368.2655	398.19	30.16	396.93	516.17
TIMP-4	12.23	13.88	7.03	1.13	9.98	14.47
Stage III (n = 13)						
TIMP-1	2849.46	1030.91	3014.31	285.92	2226.49	3472.43
TIMP-2	926.57	746.19	736.02	206.95	475.65	1377.49
TIMP-3	488.99	551.74	259.57	153.02	155.58	822.41
TIMP-4	19.11	23.22	13.58	6.44	5.07	33.14

4. Discussion

TIMP-1 has recently gained increasing interest as a protein biomarker in cancer, either analysed in tumour tissue or in corresponding plasma samples (Holten-Andersen et al., 2000, 2002; Schrohler et al., 2004, 2006; Sorensen et al., 2007). Furthermore, recent studies have implicated *TIMP-1* as an important new target for anti-cancer therapy (Davidsen et al., 2006). However, little is at present known about the regulation of *TIMP-1* in cancer. The aim of this study was therefore to gain further insight into regulatory mechanisms

being associated with transcript expression of *TIMP-1* in cancer patients. This was accomplished by 1) analysing genome wide expression profiles of 172 tissue samples from colorectal adenocarcinomas and normal colon mucosae from the resection edge and then correlating the expression of *TIMP-1* with *TIMP-2* to *-4* and MMPs and 2) identifying other gene transcripts which were correlated with the expression profile of *TIMP-1*.

The transcript profiling dataset used in this study has previously been used for expression profiling studies of *DHHC9* and *KRT23*, both genes strongly up-regulated in colon adenocarcinomas showing expression differences between MSS and MSI tumours. In these two publications we did not analyse MMPs or TIMPs (Birkenkamp-Demtroder et al., 2007; Mansilla et al., 2007).

The relationship between expression of MMPs and TIMPs in CRC has been the subject of intensive studies (reviewed by Zucker and Vacirca, 2004). However, no study has to our knowledge analysed changes in gene expression of all MMPs and TIMPs simultaneously.

The main findings of the present study were that *TIMP-1* was found to be the only of the TIMPs being significantly up-regulated in CRC. Furthermore, among the MMPs, eight were found up-regulated and one down-regulated in CRC. When looking for interactions, a significant association was found between *TIMP-1* and *TIMP-2* and *-3*. For the MMPs the

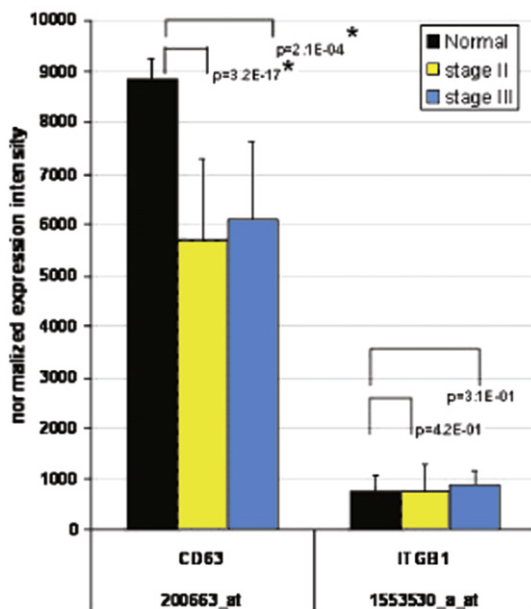


Figure 2 – Microarray transcript profiling of *CD63* and *ITGB1*. *CD63* was significantly down-regulated in stage II and stage III colon adenocarcinomas as compared with normal colon mucosa (marked with asterisk), while *ITGB1* showed the same expression level in all three groups.

Table 2 – Spearman correlation analyses performed on transcript expression values of 162 colon adenocarcinomas (149 stage II, 13 stage III)

	r	95% CI	p-Value
TIMP-1/TIMP-2	0.62	0.51–0.71	<0.0001
TIMP-1/TIMP-3	0.49	0.36–0.60	<0.0001
TIMP-1/TIMP-4	0.05	–0.10–0.20	0.509
TIMP-2/TIMP-3	0.77	0.69–0.82	<0.0001
TIMP-2/TIMP-4	0.08	–0.07–0.24	0.286
TIMP-3/TIMP-4	0.03	–0.12–0.18	0.696

Table 3 – Pearson correlation analysis of 23 different MMPs with the four TIMPs

Gene	Probe set	Median log2		p-value	log2 ratio	Pearson correlation coefficient							
		Normal	Tumour			TIMP-1		TIMP-2		TIMP-3		TIMP-4	
						201666_at	231579_s_at	201150_s_at	206243_at				
MMP1	204475_at	5.16	10.43	8.36E-09	5.26	↑ *	0.52	0.40	0.38	0.00			
MMP2	201069_at	7.96	8.62	1.97E-03	0.66	↑ *	0.67	0.88	0.70	0.05			
MMP3	205828_at	3.38	9.08	1.05E-32	5.71	↑	0.44	0.28	0.29	-0.05			
MMP7	204259_at	2.69	6.22	1.04E-12	3.53	↑	0.22	0.17	0.17	0.01			
MMP8	207329_at	2.07	2.05	2.70E-01	-0.01		0.19	0.15	0.12	0.02			
MMP9	203936_s_at	5.02	7.19	2.06E-04	2.17	↑ *	0.58	0.58	0.42	-0.03			
MMP10	205680_at	2.49	3.87	2.38E-26	1.38	↑	0.30	0.08	0.03	0.02			
MMP11	203878_s_at	4.27	5.77	2.34E-13	1.50	↑ *	0.32	0.50	0.54	-0.14			
MMP12	204580_at	9.43	10.34	1.50E-01	0.91		0.36	0.33	0.22	-0.01			
MMP13	205959_at	2.41	2.41	4.23E-04	0.00		0.31	0.28	0.27	0.05			
MMP14	202827_s_at	2.88	5.38	1.09E-40	2.50	↑ *	0.51	0.56	0.44	0.05			
MMP15	203365_s_at	7.18	6.60	8.22E-02	-0.58		-0.31	-0.27	-0.26	0.06			
MMP16	207012_at	2.36	2.33	1.98E-01	-0.03		-0.06	-0.08	-0.09	0.04			
MMP17	206234_s_at	2.34	2.34	2.04E-01	0.00		0.03	0.10	-0.02	-0.03			
MMP19	204575_s_at	3.15	3.17	6.37E-02	0.02		0.55	0.60	0.51	0.01			
MMP20	207599_at	2.50	2.47	6.83E-01	-0.03		0.02	0.12	0.07	-0.03			
MMP21	1552592_at	2.62	2.58	1.68E-01	-0.04		-0.21	-0.29	-0.17	0.05			
MMP23A/MMP23B	207118_s_at	2.01	2.01	3.35E-01	-0.01		0.12	0.22	0.17	0.00			
MMP24	213171_s_at	2.92	2.91	8.90E-01	-0.01		0.02	-0.02	0.02	0.00			
MMP25	207890_s_at	2.97	2.96	9.23E-01	-0.01		0.37	0.21	0.14	0.08			
MMP26	220541_at	2.06	2.05	2.50E-01	-0.01		-0.02	-0.01	-0.08	-0.06			
MMP27	220783_at	2.17	2.15	2.08E-02	-0.02		-0.13	-0.13	-0.07	0.06			
MMP28	239272_at	8.11	3.80	2.38E-10	-4.31	↓	-0.05	0.17	-0.02	0.12			

Five of the nine matrix metalloproteinases, marked with an asterisk, are differently regulated from normal to tumour tissue and correlate significantly with at least one of the TIMPs. Median log2 expression values were normalized around zero. Genes significantly regulated are marked with an arrow.

most significant correlation was found between MMP2 and TIMP-1, -2 and -3. Thus, our data speaks against a general co-regulation of MMPs and TIMPs as an explanation to the findings of high TIMP-1 levels in CRC. Other genes associated with the expression of TIMP-1 were genes involved in the regulation of the extracellular matrix and genes involved in activation of MMPs.

In line with other published papers we found that TIMP-1 is up-regulated (Guillem et al., 1990) while TIMP-2 is down-regulated (Baker et al., 2000) in the tumour tissue. We did not find any significant regulation of TIMP-3 and the expression of TIMP-4 was almost at the detection limit of the analysis. Other studies have shown that TIMP-3 expression is absent in poorly differentiated carcinomas as compared with normal tissue (Powe et al., 1997). This difference to our study may be explained by the fact that the majority of samples analysed in the present study were obtained from well-differentiated stage II tumours.

The MMPs are subdivided into six subgroups based on their structure and substrate specificity: collagenases (MMP1, 8 and 13), gelatinases (MMP2 and 9), stromelysin and stromelysin-like MMPs (MMP3, 7, 10, 11 and 12), Membrane-type MMPs (MMP14, 15, 16, 17, 24 and 25), and other MMPs (MMP19, 20, 21, 23 and 28) (Nagase and Woessner, 1999; Bister et al., 2004). We identified nine MMPs that were differently regulated in tumour samples as compared with normal colon mucosa. While MMP28 was down-regulated in tumour tissue, the remaining MMPs belonging to the group of collagenases (MMP1), stromelysins (MMPs 3, 7, 10 and 11) or gelatinases

(MMPs 2 and 9) were significantly up-regulated. These findings are in agreement with other studies (for review see Zucker and Vacirca, 2004). It is thus clear that a broad range of MMPs involved directly in the breakdown of the extracellular matrix are up-regulated in the CRC tumour tissue. In contrast, MMP14 was the only one of the membrane type MMPs being up-regulated, suggesting that this group of enzymes may not play an important role in the progression of CRC.

When looking for a correlation between MMPs and TIMPs we observed the strongest correlation between MMP2 and TIMP-1 to -3, even though MMP2 only showed a minimal but significant increase in expression level.

It has been shown that TIMP-1 has other effects besides inhibiting the action of the MMPs, including inhibition of apoptosis (Liu et al., 2005; Davidsen et al., 2006). One study by Jung et al. (2006) showed that the TIMP-1 protein may exert its anti-apoptotic function by interaction with the CD63 cell surface antigen and Integrin Beta 1 (ITGB1). In our study a Pearson correlation analyses did not show any correlation between the transcripts of TIMP-1, CD63 and ITGB1. However, the CD63 transcript was significantly down-regulated in CRC as compared with normal mucosa.

We also identified 13 genes the expression of which correlated with the TIMP-1 transcript profile (Table 4). Among these were a number of genes associated with the synthesis of extracellular matrix (collagen, biglycan and spondin 2). It has been shown that TIMP-1 mRNA and protein is localized to the fibroblasts located at the invasive front of CRC tissue (Holten-Andersen et al., 2005). Since these fibroblasts are the

Table 4 – Pearson correlation analysis of 13 genes correlating with the *TIMP-1* transcript

Gene	Gene name	Median log2		p-value	log2 ratio	Pearson correlation coefficient	
		Normal	Tumour			TIMP-1 201666_at	
BGN	biglycan biglycan/serologically defined colon cancer	4.9	6.7	1.34E-07	1.8	↑	0.648
BGN/SDCCAG33	antigen 33	4.2	6.8	8.80E-07	2.6	↑	0.660
COL1A1	collagen, type I, alpha 1	8.1	11.2	5.30E-10	3.1	↑	0.653
COL6A2	collagen, type VI, alpha 2	7.0	8.8	3.21E-03	1.8	↑	0.652
SPON2	spondin 2	5.8	7.8	4.12E-07	2.0	↑	0.609
LOXL2	lysyl oxidase-like 2	5.5	7.5	1.88E-11	2.1	↑	0.653
TGFB1	transforming growth factor, beta 1	2.9	5.2	4.81E-24	2.3	↑	0.631
INHBA	inhibin, beta A (activin A)	3.2	6.1	2.09E-13	2.9	↑	0.711
PLAU	urokinase plasminogen activator	4.6	7.3	1.08E-05	2.7	↑	0.613
TNFAIP6	tumour necrosis factor, alpha-induced protein 6	3.2	4.4	1.47E-04	1.2	↑	0.687
NNMT	nicotinamide N-methyltransferase	4.9	6.8	4.23E-03	2.0	↑	0.646
ICAM1	intercellular adhesion molecule 1 (CD54)	3.8	5.7	9.75E-05	1.9	↑	0.637
THY1	Thy-1 cell surface antigen	4.8	6.8	2.82E-06	2.0	↑	0.627

Median log2 expression values were normalized around zero. All are significantly up-regulated (arrow) in tumour tissue compared to normal mucosa.

likely source of extracellular matrix as well, it is not surprising that the expression of these three genes correlates with the *TIMP-1* gene expression.

Two of the other *TIMP-1* correlating genes (*TGF-B1* and *INHBA1*, the latter encoding inhibin A (activin A)) belong to the family of TGF beta genes and are thereby involved in the TGF beta signalling pathways. Both of these genes have previously been reported to be over-expressed in CRC tumour cells (Wildi et al., 2001; Daniel et al., 2007). *TGF-B1* is known to regulate extracellular matrix production by stimulation of fibroblasts (Matsushita et al., 1999) and to induce the expression of urokinase plasminogen activator (uPA) and MMP2 in epithelial cancers (Sieuwerts et al., 1998; Ellenrieder et al., 2001; Tanaka et al., 2004). The uPA protein, encoded by urokinase plasminogen activator gene

(*PLAU*) was also found to be up-regulated and to correlate with *TIMP-1*. *INHBA* is involved in the TGF beta signalling pathway as it binds to the transforming growth factor beta receptor and has been reported to be over-expressed in colorectal cancer (Wildi et al., 2001).

Lysyl oxidase 2 (*LOXL2*) has been shown to be produced by cancer cells (Fong et al., 2007). It has several functions, among which one is biogenesis of connective tissue, another is down-regulation of E-cadherin, and *LOXL2* thus has the potential to promote tumour progression (Peinado et al., 2005). The nicotinamide N-methyl transferase (*NNMT*) transcript was also correlated to *TIMP-1* expression. *NNMT* has previously been shown to be up-regulated in colon adenocarcinomas as compared with normal mucosa (Birkenkamp-Demtroder et al., 2002).

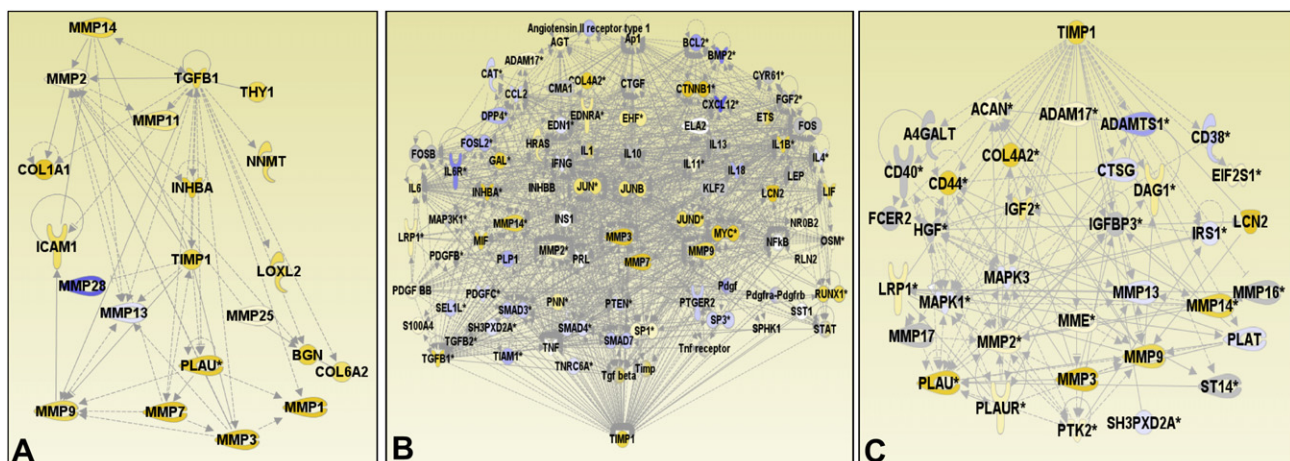


Figure 3 – Ingenuity pathway analysis. Transcripts up-regulated in colon adenocarcinomas ($n = 162$) compared to normal mucosa ($n = 10$) are shown in yellow, those down-regulated in the tumours are shown in blue. (A) A network comprising 20 genes directly or indirectly interacting with *TIMP-1* and investigated in this study. (B,C) The majority of genes located upstream (B) or downstream (C) from *TIMP-1*, directly or indirectly being affected by *TIMP-1* or affecting the *TIMP-1* expression or activity, respectively, were identified to be differentially expressed in colorectal cancer.

Thus these genes are all known to be up-regulated in cancer. If the correlation to *TIMP-1* is due to specific mechanisms regulating transcription or a result of malignancy independent of *TIMP-1* transcription remains unknown.

Quantitative PCR validated the up-regulation of *TIMP-1*, *TGF-B1*, *LOXL2*, *TNF-AIP6* and *INHBA* in tumour samples as compared with normal mucosa and corroborated the correlation of these genes with the *TIMP-1* transcript as found by the genome wide expression profiling. Quantitative PCR yielded a higher ratio between tumour and normal samples compared to the ratio obtained from Genechip. However, the two methods differ in sensitivity and sequence and location of probes and primers within the transcript and the variation is likely due to these differences.

In conclusion, *TIMP-1* (and -2 and -3) correlates with the expression of a limited number of MMPs, in particular *MMP2*. Other genes that correlated with *TIMP-1* in CRC are either genes expressed in fibroblasts (the cells that express *TIMP-1* and which produce the main components of the extracellular matrix) or genes found in the tumour cells.

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