Differences in the Gene Expression Profile of Matrix Metalloproteinases (MMPs) and their Inhibitors (TIMPs) in Primary Colorectal Tumors and their Synchronous Liver Metastases

BERNHARD GENTNER[1*], AXEL WEIN[1], ROLAND S. CRONER[2], ISABEL ZEITTRAEGER[1], RALPH M. WIRTZ[3], FRANZ ROEDEL[4], ARNO DIMMLER[5], LAURE DORLAQUE[1], WERNER HOHENBERGER[2], ECKHART G. HAHN[1] and WOLFGANG M. BRUECKL[1§]

Departments of [1]Medicine 1 and [2]Surgery, Friedrich Alexander University Erlangen-Nuremberg, Erlangen; [3]Siemens Medical Solutions GmbH, Leverkusen; [4]Department of Radiation Therapy, Goethe University Frankfurt; [5]Department of Pathology, University Hospital Karlsruhe, Germany

Abstract. *Background: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been strongly implicated in the pathogenesis of many types of human cancer. We wanted to specifically define their role in established colorectal cancer liver metastases. Patients and Methods: The MMP/TIMP expression profiles of N=9 colorectal primary tumour liver metastasis tissue pairs were determined using oligonucleotide-based arrays. Expression levels for the most relevant MMPs were confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR). Additionally, unsupervised clustering using the MMP/TIMP profile of N=25 colorectal cancer liver metastases was performed and the response to palliative 5-fluorouracil (5-FU)-based chemotherapy was assessed using radiological response criteria. Results: When comparing the primary tumors to their synchronous liver metastases, a statistically significant ($p<0.05$) down-regulation of MMP1, -2, -3 and -12 was found in the metastases. Unsupervised clustering using the MMP/TIMP profiles of 25 liver metastases revealed two distinct subgroups with different responses to palliative, 5-FU-based chemotherapy (response rates: 22% vs. 56%, respectively). In particular, higher MMP7, TIMP1 and TIMP2 levels were found in the unfavourable group, while higher expression of MMP2, -9, -11 and -14 was associated with a more favourable response to chemotherapy. Conclusion: Colorectal cancer liver metastases show a distinctive MMP/TIMP profile with predictive implications.*

Colorectal cancer (CRC) represents the second leading cause of cancer related deaths in the European Union. One million people worldwide are diagnosed with this cancer annually and about half of them will succumb, mostly to metastatic disease (1). Though much is known about the genetic pathways leading to colorectal neoplasia, the exact molecular mechanisms underlying tumour growth, local invasion, angiogenesis, intravasation and finally metastasis remain poorly understood.

In this context, the family of matrix metalloproteinases (MMPs) as the main extracellular matrix remodelling enzymes has been studied extensively. There are at least 24 members of the MMP family that can degrade all constituents of connective tissue and thus facilitate invasion. MMPs can be grouped into collagenases (*e.g.* MMP1, -8, -13), gelatinases (*e.g.* MMP2, -9), stromelysins (*e.g.* MMP3, -10, -11) and matrilysins (*e.g.* MMP7) according to their substrate specificity. Newer classification systems discriminate 8 classes of MMP on the basis of common structural motifs (2).

MMP activity *in vivo* is tightly controlled by transcriptional activation, by a complex proteolytic activation cascade and by

*Current address: San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy.

§Current address: State Hospital Mittersill, Teaching Hospital of the Paracelsus Medical University Salzburg, Mittersill, Austria.

Correspondence to: Wolfgang M. Brueckl, MD, Assistant Professor of Medicine, Department of Medicine, State Hospital Mittersill, Felberstr. 1, A-5730 Mittersill, Austria. Tel: +43 656245360, Fax: +43 65624536601, e-mail: w-brueckl@krankenhaus-mittersill.at

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an endogenous system of tissue inhibitors of metalloproteinases (TIMPs). Numerous studies have established that MMP expression is higher in colorectal cancer tissue compared to normal mucosa, and some have shown direct correlations of MMP levels with tumour stage, grade, invasion, metastasis and prognosis, suggesting a pivotal role of these enzymes in the development of a malignant phenotype (3-5). Furthermore, observational and experimental studies in mice strongly implicate these MMPs in tumour progression as well as metastasis (6-9), and preclinical studies using synthetic MMP inhibitors have revealed marked antitumour activity (10-12). However, this sharply contrasts with the lack of efficacy of MMP inhibitors in clinical phase III trials where patients with advanced disease were treated (13).

It has become increasingly clear that the biological role of MMPs is not confined to their ability to degrade the extracellular matrix. They also participate in the regulation of cellular processes such as differentiation, proliferation, angiogenesis, migration, invasion and apoptosis by interacting with growth factors, cytokines, integrins and cell surface receptors (14), suggesting a complex *in vivo* function that remains poorly understood. Most of the studies correlating MMP expression with cancer outcome analyzed tissue specimens from the primary tumour (15, 16). There is, however, a lack of data regarding MMP expression in metastatic tissue.

The scope of this paper was to characterize the expression pattern of multiple members of the MMP family in advanced colorectal cancer (CRC) and analyse how expression levels differ between the primary tumours and their corresponding liver metastases. Additionally, as most of the patients received a palliative, first-line 5-flourouracil (5-FU)-based chemotherapy after surgery, we aimed to associate clinical response with MMP/TIMP gene expression levels.

Patients and Methods

Patient and tumour characteristics. The study was approved by the Ethics Committee of the University of Erlangen-Nuremberg. Written consent was obtained from eligible patients and the research was conducted in accordance with the principles of the Declaration of Helsinki.

Biopsy samples from synchronous liver metastases were collected intraoperatively from 25 patients with UICC stage IV colorectal carcinoma at the time of resection of the primary tumour. Additionally, 9 of these patients contributed fresh biopsies from the primary CRC. Fresh frozen material from the primary tumours was not available from the other 16 patients, so they were excluded from the comparative analyses between primary and metastatic tumour. Rectal cancer was defined in both study cohorts according to the definition of the International Documentation System (IDS) as elaborated in the Working Party Report to the World Congresses of Gastroenterology (17).

Chemotherapy. Patients received 500 mg/m^2 folic acid (FA) together with 2,600 mg/m^2 5-FU administered *i.v.* as a 24-h infusion once weekly [according to the Arbeitsgemeinschaft Internistische Onkologie (AIO) regimen] *via* a miniature pump system, with or without biweekly application of 85 mg/m^2 oxaliplatin *i.v.* in the context of a phase II study (18). One cycle comprised six weekly infusions followed by two weeks of rest. In the cases of therapy control and good tolerability, chemotherapy was applied until disease progression or the time of secondary metastatic resection. After every cycle, a follow-up examination comprising a blood count, serum test for carcino-embryogenic antigen (CEA) and cancer antigen 19-9 (CA 19-9), an abdominal computed tomography (CT) scan and a chest X-ray were performed. Response to chemotherapy was evaluated in all cases after each cycle of chemotherapy by CT scans, in accordance with WHO criteria (19). Partial remission (PR) was defined as a >50% size reduction of a reference metastasis during chemotherapy, while complete remission (CR) required the absence of radiographic evidence for metastasis. Stable disease (SD) was defined as a reduction of <50% or an increase of <25% in maximum diameter of the reference metastasis, while a >25% increase was scored as progressive disease (PD).

Response data were available for 22 patients. Two patients did not receive palliative chemotherapy because they refused consent (1 patient) or died before application of chemotherapy (1 patient). One patient died before the first follow-up examination. A summary of the clinical data is provided in Table I.

Sample preparation. Intraoperatively obtained biopsies from colorectal primary tumours and liver metastases were shock-frozen with liquid nitrogen (within one minute after removal) and then stored at –80˚C. The frozen tissues were cut into 8 μm sections using a cryostat and then stained with haematoxylin and eosin for histological examination. Laser capture microdissection (LCM) was performed immediately after staining and dehydration. Tumour areas of interest were selected with the help of an experienced pathologist (A.D.) and excised using a 0.6 mm laser beam (32 mW, 30 Hz, 0.8 s pulse). Each sample yielded approximately 10,000 cells. Captured cells were dissolved in RLT buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany) and RNA was extracted as described below. Tumour material from two primary colon tumours (patients no. 6 and no. 9 (for clinical details see Table I)) and one liver metastasis (patient no. 9) were differentially microdissected into stromal cells and tumour cells.

RNA extraction. Total RNA was isolated with the use of commercial kits (RNeasy-Mini Kit; Qiagen) according to the manufacturer's instructions As part of this procedure, DNAse digestion (Qiagen) was included before elution from the columns. The quantity and quality of the purified total RNA was measured with the use of the RNA Nano 6000 Assay Chip (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA, USA).

RNA amplification. Each biopsy yielded up to 800 ng of total RNA. After several rounds of T7 promotor-based RNA amplification, each sample typically provided a final yield of 50-100 μg of amplified RNA (aRNA). Reverse transcription was performed with the MessageAmp aRNA Kit (Ambion, Huntingdon, UK) followed by *in vitro* transcription (20). During this latter step, a biotin label was added. The overall quality of the aRNA was assessed using the RNA Nano 6000 Assay Chip.

Table I. *Clinical data of the 25 patients for whom gene expression analysis from liver metastases was performed. Internal numbers correspond to the numbers in the figures.*

No.	Internal no.	Gender	Age at surgery (years)	Location of CRC	TNM	CTx	Response	P*	M*
1	01	M	66	Caecum	pT3pN2M1	AIO	PR	x	x
2	02	M	58	Asc. c.	pT3pN2M1	AIO	PD	x	x
3	04	M	58	Desc. c.	pT2pN2M1	AIO	SD	x	x
4	05	M	57	Sigmoid c.	pT3pN0M1	AIO+Ox	PR		x
5	07	F	74	Caecum	pT3pN2M1	AIO	PD		x
6	09	M	72	Rectum	pT3pN2M1	AIO+Ox	PD	x	x
7	10	M	72	Caecum	pT3pN2M1	n.d.	n.d.	x	x
8	12	M	74	Sigmoid c.	pT2pN2M1	n.d.	n.d.	x	x
9	15	M	70	Sigmoid c.	pT3pN0M1	AIO+Ox	PR	x	x
10	16	F	71	Rectum	pT3pN2M1	AIO	SD	x	x
11	19	F	55	Asc. c.	pT3pN2M1	AIO	PD		x
12	20	M	77	Rectum	pT3pN0M1	AIO	PD	x	x
13	23	M	56	Rectum	pT4pN0M1	AIO+Ox	SD		x
14	25	M	85	Sigmoid c.	pT4pN0M1	AIO+Ox	PR		x
15	26	F	43	Rectum	pT3pN1M1	AIO	PR		x
16	28	M	53	Rectum	pT3pN0M1	AIO+Ox	PR		x
17	31	M	82	Transv. c.	pT3pN2M1	n.d.	n.d.		x
18	33	M	58	Rectum	pT4pN2M1	AIO+Ox	PD		x
19	35	M	58	Rectum	pT3pN0M1	AIO	PR		x
20	37	M	46	Sigmoid c.	pT4pN2M1	AIO+Ox	PR		x
21	40	M	57	Rectum	pT3pN0M1	AIO	SD		x
22	41	M	62	Sigmoid c.	pT4pN2M1	AIO+Ox	PR		x
23	43	M	55	Sigmoid c.	pT3pN1M1	AIO+Ox	PR		x
24	45	M	52	Sigmoid c.	pT3pN1M1	AIO	PR		x
25	47	M	60	Desc. c.	pT3pN1M1	AIO	SD		x

P*, Microarray analysis of primary CRC available (x); M*, microarray analysis of liver metastasis available (x); CTx, chemotherapy; AIO, chemotherapy regimen of 5-flourourcil (5-FU) and folinic acid as a 24-h infusion d 1, 8, 15, 22, 29, 36 *qd* 57 according to the Arbeitsgemeinschaft Internistische Onkologie (AIO); AIO+Ox, 5-FU/FS as 24-h infusion d 1, 8, 15, 22, 29, 36, *qd* 57 and oxaliplatin every d 1, 15, 29 *qd* 57 (details in Materials and Methods); PR, partial remission; SD, stable disease; PD progressive disease; n.d., not determined.

GeneChip hybridisation. Samples were hybridised to Affymetrix HG U133-A high-density oligonucleotide-based arrays (Affymetrix, Santa Clara/CA, USA) targeting 22,230 probe sets representing about 16,000 genes. From each biopsy, 15 μg of either cRNA or aRNA were loaded onto an array following the recommended procedures for prehybridization, hybridization, washing and staining with streptavidin-phycoerythrin. The arrays were scanned on an Affymetrix GeneChip Scanner (Agilent). The fluorescence intensity was measured for each microarray and normalised to the average fluorescence intensity of the entire microarray.

Statistical analysis. The raw, unnormalized data-sets were analyzed by MicroArray Suite (Affymetrix) for normalization and estimation of expression values. Signal intensities and detection calls for statistical analysis and hierarchical clustering were determined using the GeneChip 5.0 software (Affymetrix) and Expressionist™ software (Genedata). Unsupervised two-dimensional hierarchical cluster analysis of the MMP and TIMP data were performed using the between-groups linkage method with the χ^2 measure for ordinal data to identify individual groups of tumours with specific MMP/TIMP profiles. Significance levels of microarray results were calculated using the Mann-Whitney *U*-test. A *p*-value of <0.05 was regarded as significant.

For box plots, the median expression values as well as the first and third quartiles were calculated. Variances between box blots were calculated using the F-test. A *p*-value of <0.05 was regarded as significant.

RT-PCR. The expression levels of *MMP1*, *MMP2* and *MMP3* mRNA were confirmed by RT-PCR. Total cellular RNA was extracted as described above and 2.5 μg of total RNA was reversely transcribed. RT-PCR was performed with 0.2 units of Taq DNA polymerase according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany) in a final volume of 25 μl. Cycling was as follows: For MMPs: 40 cycles with 30 s of denaturation at 94˚C, 1 min of primer annealing at 59˚C and 45 s of extension at 72˚C. For GAPDH: 40 cycles with 2 min of denaturation at 94˚C, 30 s of primer annealing at 59˚C and 30 s of extension at 72˚C. The primers for MMP1, MMP2, MMP3 and GAPDH were as follows: MMP1 sense (5'-ATG CAC AGC TTT CCT CCA CTG-3') and antisense (5'-CAG CCC AAA GAA TTC CTG CAT T-3'); MMP2 sense (5'-GCT ACG ATG GAG GCC CTA ATG-3') and antisense (5'-TCT CCT TGG GGC AGC CAT-3'); MMP3 sense (5'-GAG TCT TCC AAT CCT ACT GTT GCT G-3') and antisense (5'-AGG AAC TTC TGC ATT TCT CGG AT-3'); GAPDH sense (5'-ATG GGG AAG GTG AAG GTC GG-3') and antisense (5'- TGA TTT TGG AGG GAT CTC GCT C-3').

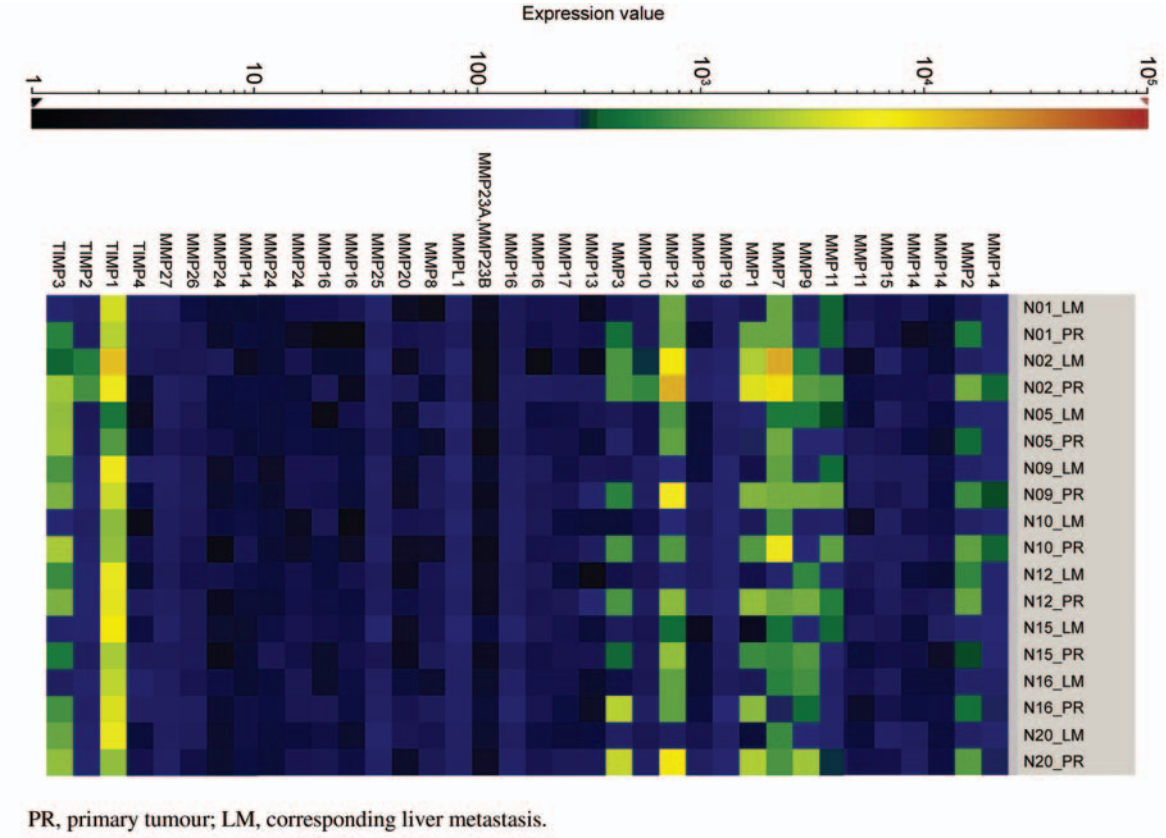

Figure 1. *Heat map, showing expression values of all available MMP/TIMP probes contained on the array. Each column corresponds to an MMP or TIMP probe, lines represent the liver metastasis (LM) or primary tumour (PR) sample of a patient. Individual patients were coded as N01, N02 etc. Expression values were colour coded. Note that most MMPs and TIMPs are down-regulated in the synchronous liver metastases compared to the corresponding primary CRC.*

Results

Global MMP expression profile. Microarray expression data were generated from 9 pairs of primary tumours and their corresponding liver metastases. All samples were enriched for tumour cells by microdissection, thus reducing possible biases that could arise from differences in stroma content and necrotic areas within individual tumours. The MMP/TIMP expression profile readily distinguished between primary tumour and liver metastasis (Figure 1).

Notably, the average and median expression values were lower for all MMPs in the liver metastases compared to their corresponding primary tumours (Figure 1). In particular, *MMP1*, *-2*, *-3* and *-12* showed a statistically significant ($p<0.05$) down-regulation in the liver metastasis specimens. TIMPs showed a mixed picture. *TIMP1* and *-2* were equally expressed or up-regulated in liver metastases, while *TIMP3* was down-regulated. TIMP4 was not expressed.

To confirm the array results, semiquantitative RT-PCR was performed on the same cDNA material using primers detecting *MMP1*, *-2* and *-3*. In line with the microarray data, primary tumours showed a more intense signal than their corresponding liver metastases, while expression levels of the housekeeping gene *GAPDH* were comparable. Representative RT-PCR results for MMP1 are displayed (Figure 2).

To estimate the impact of the microdissection procedure on MMP expression levels, we microdissected tumour material from two colon primary tumours and one liver metastasis differentially into stromal and tumour cells, the latter fraction being comparable with the material used for microarray analysis. Qualitative RT-PCR for *MMP1*, *-2*, *-7* and *-9* showed that these MMPs were produced in both tumour and stroma fractions of the primary tumour and the liver metastasis (data not shown). All three patient samples yielded a higher stroma signal for *MMP2* and *MMP9* compared to *MMP1* and *MMP7*, suggesting that the former two MMPs were mainly stroma derived.

Predictive power of the MMP/TIMP profile in liver metastases. To test for a possible biological significance of a specific MMP/TIMP profile in individual liver metastases,

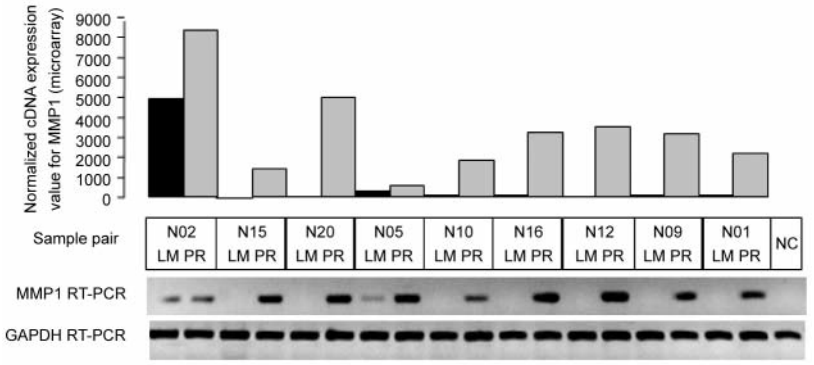

Figure 2. *Comparison of MMP1 expression values derived from microarray data (upper half, columns) and semiquantitative RT-PCR (lower half, ethidium bromide-stained agarose gel) for 9 colorectal cancer primary tumour (PR)–liver metastasis (LM) pairs.*

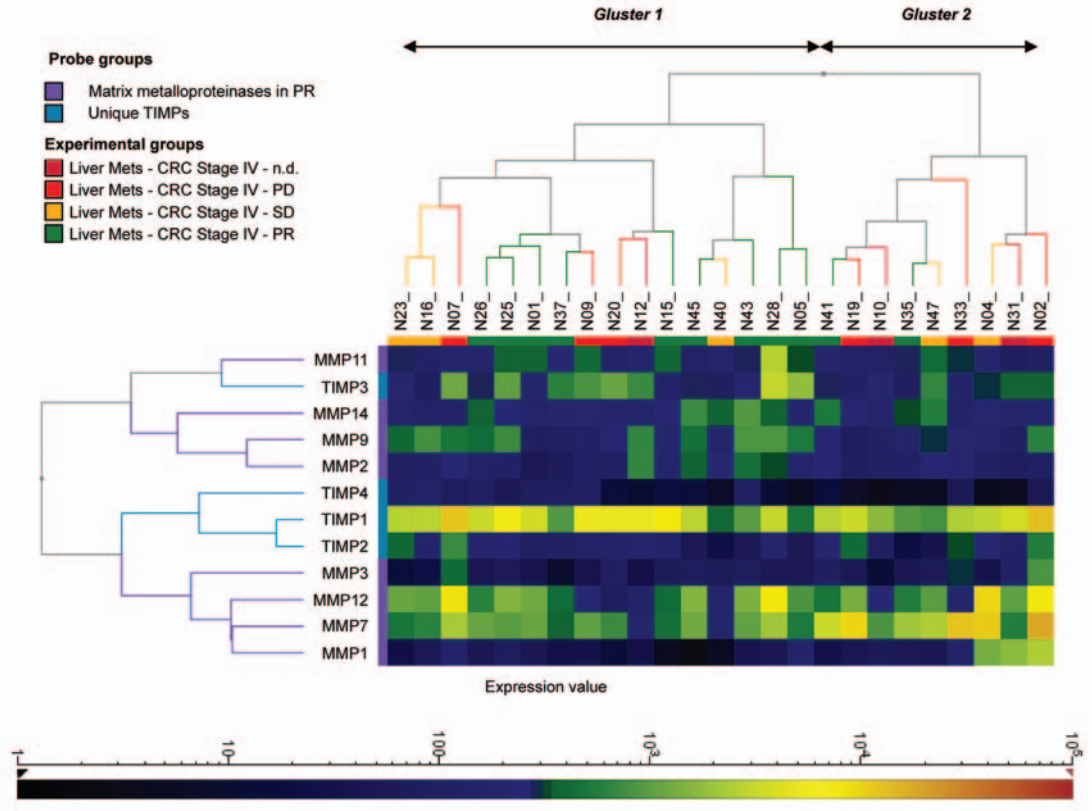

Figure 3. *Two-dimensional unsupervised hierarchical cluster analysis of MMP/TIMP profile in liver metastases of CRC. On the top and on the left the dendrograms produced by the hierarchical cluster analysis. Each column corresponds to an individual patient liver metastasis sample. Rows show the colour-coded expression values of the indicated probe for the different patient samples. Probes were chosen based on expression of MMP/TIMPs in at least one colorectal/liver metastasis pair from Figure 1. Above the columns, chemotherapy response is indicated by green (partial response, PR), yellow (stable disease, SD) and red (progressive disease, PD) bars. Note that patient samples divide into two first order clusters with differences in chemotherapy response.*

we correlated the MMP/TIMP expression profiles of 25 colorectal cancer liver metastases with the response to a first line, palliative 5-FU-based chemotherapy. To this aim, we performed an unsupervised, hierarchical cluster analysis using a panel of 11 relevant MMPs/TIMPs (MMPs 1, 2, 3, 7, 9, 11, 12, 14 and TIMPs 1, 2, 3, 4) that were expressed in at

least one of the liver metastases (expression level >500) shown in Figure 1. Two main clusters were formed, the first containing 9 liver metastases and the second one containing the remaining 16 tumours (Figure 3). In the first cluster, there were only 2 patients (28%) with (partial) response (PR) under chemotherapy, while the remaining 5 patients were non-responders; 2 patients were not available for response assessment. In the other cluster, there were 9 patients with PR (60%), and 6 non-responders; 1 patient was not available for response assessment. Comparing the median MMP/TIMP expression values in the 2 subgroups, we found higher *MMP7*, *TIMP1* and *TIMP2* levels in the unfavourable group, while higher expression of *MMP2*, *-9*, *-11* and *-14* was associated with a more favourable response to chemotherapy. Expression values of *MMP11*, *MMP14* and *TIMP2* differed significantly ($p<0.05$) between responders and non-responders.

Discussion

There is a huge line of evidence that MMPs are involved in the tumorigenesis of CRC. We studied MMP expression in a patient cohort with metastastic CRC, comparing the primary colorectal tumour to its liver metastasis.

To our knowledge, the expression of multiple MMPs in colorectal primary tumour-metastasis pairs has not been compared so far. We show that multiple MMPs are down-regulated at the mRNA expression level in CRC liver metastases. For some MMPs, there is existing evidence in the literature for such a down-regulation in the liver metastasis.

Grau *et al*. determined MMP7 expression levels in paired CRC orthotopic xenograft and metastasis samples. Five out of seven metastases showed reduced MMP7 expression compared to the primary tumours (21). Chan *et al*. analyzed MMP2 expression levels in 65 advanced colorectal cancers using ELISA, Western blot and *in situ* hybridization, and found an increase of MMP2 in the primary tumour but a decrease in the liver metastasis (22). A recent study found high MMP9 expression at the invasive front in all analysed CRC primary tumours, but a marked drop in number and cluster size of MMP9-positive cells in CRC liver metastases (23).Our study lends further support to the supposition that MMPs are generally down-regulated in CRC liver metastases.

MMP activity is the result of interactions between the tumour cells and the microenvironment, *i.e*. the stroma component. This idea is supported by cell culture experiments showing different MMP inducibility in fibroblasts from different organs (24). Thus, CRC cells colonizing the liver are biologically diverse from the cells present in the primary tumour, and the former ones might adopt different proteolytic mechanisms, not relying on MMPs. Other groups have found down-regulation of various MMPs in metastatic prostate cancer (25, 26). This may explain the observation that synthetic MMP inhibitors are only effective when given early in the phase of tumour establishment but not once metastatic disease is present (4).

It is important to note that our microarray-based study has, however, some limitations. First, our mRNA expression levels cannot differentiate between active and latent forms of MMPs. Some groups have shown selective localization of active MMPs to tumour areas, while normal tissue mainly contained inactive forms of the MMP (27). Second, we did not perform an *in situ* expression analysis in order to quantify the cellular source of MMP/TIMP expression. It has been hypothesized that differences in stroma content could account for the variation of MMP levels in different prognostic groups (28). We tried to reduce this bias by microdissecting both primary and metastatic tumours, thus eliminating necrotic/scar tissue.

By analysing median expression values, one does not consider individual tumours that do not fit into the scheme of MMP down-regulation in metastasis. As an example, two liver metastases (N02, N16) in our study showed markedly increased *MMP7* mRNA levels compared to the primary tumour. We asked the question whether there was a significance to such outliers and thus compared the MMP/TIMP cluster analysis to clinical outcome parameters. Our preliminary data indicate that the MMP/TIMP profile in individual liver metastases seems to distinguish different predictive groups concerning response to a palliative first-line 5-FU-based chemotherapy. These data might be clinically relevant, as in contrast to the primary CRC (which will be resected in nearly all cases), the liver metastases remain in the patient's body and are the target of palliative chemotherapy. To our knowledge this is the first report of an association of MMP/TIMP expression in liver metastases with response to chemotherapy. Therefore, we acknowledge that larger studies are necessary to corroborate this correlation and establish the biological basis behind this observation.

However, our gene expression data are in concordance with serum data of MMP2/TIMP1 during palliative chemotherapy of metastastic colorectal carcinoma (29). In this recently published study, we showed that high levels of MMP2 and low levels of TIMP1 were significantly associated with episodes of tumour control, while samples with episodes of tumour progression displayed the opposite pattern. Additionally, serum analyses of MMP2 and MMP9 from Rauvala *et al*. support our findings (30). Patients with ovarian carcinoma responding to chemotherapy had increased *MMP2* and -9 mean serum values. Sutnar *et al*. measured mRNA expression of *MMP7* and *-9* and *TIMP1* and *-2* in biopsies of CRC liver metastases which were radically resected (31). The increased expression of *TIMP1* was associated with a short disease-free survival and a high tendency to early recurrence. Additionally, Ogawa *et al*. performed immunohistochemical analyses of MMP7 in colorectal carcinoma and tried to correlate them with

occurrence of liver metastases (32). They showed that patients with synchronous liver metastases had a positive staining in 72%, whereas cases with metachronous liver metastases or those being free of metastases after 5 years of observation expressed MMP7 to a lower extent (47% and 51%, respectively).

To further validate the molecular signature of metastases as far as drug sensitivity or resistance is concerned, our results should be verified in a study with a large independent patient cohort. If our response signature is confirmed, we envision immediate clinical application. The MMP/TIMP signature will allow "non-response" to 5-FU-based chemotherapy to be predicted and more fitting anticancer drugs to be actively selected, leading to an individualized, tailored tumour therapy, thus maximizing the chance of response (33). This will come along with an increasing number of patients becoming eligible for curative metastatic surgery. It is also feasible that metastasis-specific gene expression profiles will identify relevant targets for novel anticancer drugs.

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