Maspin is a marker for early recurrence in primary stage III and IV colorectal cancer

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Background: Little is known about the factors that drive metastasis formation in colorectal cancer (CRC). Here, we set out to identify genes and proteins in patients with colorectal liver metastases that correlate with early disease recurrence. Such factors may predict a propensity for metastasis in earlier stages of CRC.

Methods: Gene expression profiling and proteomics were used to identify differentially expressed genes/proteins in resected liver metastases that recurred within 6 months following liver surgery vs those that did not recur for >24 months. Expression of the identified genes/proteins in stage II (n = 243) and III (n = 176) tumours was analysed by immunohistochemistry on tissue microarrays. Correlation of protein levels with stage-specific outcome was assessed by uni- and multivariable analyses.

Results: Both gene expression profiling and proteomics identified Maspin to be differentially expressed in colorectal liver metastases with early (<6 months) and prolonged (>24 months) time to recurrence. Immunohistochemical analysis of Maspin expression on tumour sections revealed that it was an independent predictor of time to recurrence (log-rank P = 0.004) and CRC-specific survival (P = 0.000) in stage III CRC. High Maspin expression was also correlated with mucinous differentiation. In stage II CRC patients, high Maspin expression did not correlate with survival but was correlated with a right-sided tumour location.

Conclusion: High Maspin expression correlates with poor outcome in CRC after spread to the local lymph nodes. Therefore, Maspin may have a stage-specific function possibly related to tumour cell dissemination and/or metastatic outgrowth.

Five-year survival rates in colorectal cancer (CRC) vary dramatically from ~93% in patients with localised stage I tumours to ~6% in patients with metastasised inoperable disease (Siegel et al., 2012). The decision to administer adjuvant chemotherapy in CRC is predominantly stage-dependent. All stage III and high-risk stage II patients are recommended to receive adjuvant treatment. In contrast, patients with stage I and low-risk II disease generally do not receive adjuvant chemotherapy after resection of the primary tumour, as 5-year survival rates are high and there is only minimal benefit in unselected patients (O’Connell et al., 2004; Edge and Compton, 2010). Recently, gene expression profiling has been used to identify high-risk stage II patients who can possibly benefit from adjuvant chemotherapy. Two published gene signatures have proven to be able to select stage II cancer patients for adjuvant chemotherapy.
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Patients and samples

Microarray and mass-spectrometry analysis. Biopsies of 30 liver metastases were used to identify genes and proteins correlating with early recurrence in stage IV CRC patients. Patient, tumour and surgical characteristics were derived from our prospectively collected database. Thirty frozen tumour biopsies were collected between July 2003 and August 2008 at the University Medical Centre in Utrecht, the Netherlands (Data set 1) (Figure 1). Samples were included if patients were aged >18 years and received curative resection for histologically confirmed liver metastases from CRC. Patients with a history of non-colorectal malignancies, extra hepatic disease or microscopic residual disease (R1) after surgery and patients who received local ablation therapy or chemoembolization alone or in combination with resection were excluded. Only those specimens were included that were snap-frozen in liquid nitrogen within 30 min after resection and stored at −80 °C. The study protocol was approved by The Medical Ethical Committee (MEC) of the University Medical Center Utrecht as recognised by article 16 of the WMO (Dutch Law on Medical Research with human subjects). Written informed consent was obtained from all patients in Data set 1.

Tissue microarray study. Between 1996 and 2005, 419 patients underwent surgical colon cancer resection at the Kennemer Gasthuis Hospital in Haarlem, the Netherlands, that were classified as stage II (T1–4, N0, M0) or stage III (T1–4, N1–3, M0) according to the fourth edition of the TNM classification system (Data set 2) (Figure 1). These tumour samples were used to examine any stage-specific role of the biomarkers retrieved from Data set 1. Patient, tumour and surgical characteristics were retrospectively drafted from clinical and pathology reports. Collection, storage and use of tissue and patient data were performed in agreement with the Code for Proper Secondary Use of Human Tissue in The Netherlands (available at http://www.federa.org/codes-conduct) (Belt et al, 2011).

Gene expression profiling. RNA isolation, labelling and hybridisation to whole-genome oligonucleotide high-density microarrays were performed as previously described (Snoeren et al, 2012). In short, two expression profiles in dye-swap experiments were generated for each sample. The samples were compared against a commercial reference (Universal Human Reference RNA catalogue #740 000, Stratagene, La Jolla, CA, USA). The Human Array-Ready
Oligo set (version 2.0) was purchased from Qiagen (Venlo, Netherlands) and spotted on Codelink slides (GE Healthcare, Little Chalfont, UK) in a dust-filtered and humidity controlled clean room. The microarrays contained 70-mer oligonucleotides representing 21,329 human genes and expressed sequence tags (ESTs), as well as 3,871 additional spots for control purposes. We applied total RNA and cRNA quality control criteria in accordance with the Tumour Analysis Best Practices Working Group (2004). Gene expression in metastases showing recurrence within 6 months after resection was compared with metastases that did not show recurrence for >2 years by using ANOVA (Wu et al., 2003).

In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2 test in which residuals were shuffled 5,000 times globally. Genes with $P < 0.05$ after family-wise error correction were considered significant.

### Mass-spectrometry analysis

**Tissue homogenisation.** Frozen tumour tissue sections of five stage IV patients with short time to recurrence (<6 months) and five patients with prolonged time to recurrence (>24 months) were used for mass-spectrometry analysis. All patients were selected from Data set 1. The frozen tumour tissue sections were cut into pieces of ~20 mg, after which they were solubilized in 800 μl SDS sample buffer (containing 62.5 mm Tris-HCl, 2% w/v SDS, 10% v/v glycerol, 0.0025% bromphenol blue, 100 mm DTT, 1% w/v BSA and 1 mm DTT).

### Table 2. Clinical and pathological characteristics of patients included in microarray analysis

| Variable                   | Characteristics | DFS < 6 months (n = 17) | DFS > 24 months (n = 13) | P-value |
|----------------------------|-----------------|-------------------------|--------------------------|---------|
| Sex                        |                 |                         |                          |         |
| Male                       | 10 (58.8%)      | 7 (53.8%)               | 0.785                    |         |
| Female                     | 7 (41.2%)       | 6 (46.2%)               |                         |         |
| Age (years)                | (Mean; median; s.d.) | 62.24; 65.00; 14.70    | 63.84; 64.00; 8.09      | 0.715   |
| Tumour location primary    |                 |                         |                          |         |
| Right-sided                | 5 (29.4%)       | 6 (46.2%)               | 0.636                    |         |
| Left-sided                 | 6 (35.5%)       | 3 (23.1%)               |                         |         |
| Rectum                     | 6 (35.5%)       | 4 (30.8%)               |                         |         |
| Histological grade        |                 |                         |                          |         |
| Well                       | 3 (17.6%)       |                         | —                        |         |
| Moderate                   | 12 (70.6%)      | 12 (92.3%)              | 0.714                    |         |
| Poor                       | 2 (11.8%)       | 1 (7.7%)                | —                        |         |
| Unknown                    | —               | —                       | —                        |         |
| Nodal stage                |                 |                         |                          |         |
| N0                         | 8 (47.1%)       | 7 (53.8%)               | 0.713                    |         |
| N1                         | 9 (52.9%)       | 6 (46.2%)               |                         |         |
| Unknown                    | —               | —                       | —                        |         |
| Interval between primary and LM |              |                         |                          |         |
| Synchronous                | 8 (47.1%)       | 3 (23.1%)               | 0.184                    |         |
| Metachronous               | 9 (52.9%)       | 10 (76.9%)              |                         |         |
| Neoadjuvant chemotherapy   |                 |                         |                          |         |
| Yes                        | 5 (29.4%)       | 2 (15.4%)               | 0.375                    |         |
| No                         | 12 (70.6%)      | 11 (84.6%)              |                         |         |
| Adjuvant chemotherapy      |                 |                         |                          |         |
| Yes                        | 16 (94.1%)      | 6 (53.8%)               | 0.025                    |         |
| No                         | 1 (5.9%)        | 7 (46.2%)               | —                        |         |
| No. of metastases         | (Mean; median; s.d.) | 2.41; 2.00; 1.77      | 1.54; 1.00; 0.88        | 0.131   |
| Size liver metastases     | (Mean; median; s.d.) | 5.18; 4.21; 3.37      | 4.25; 3.70; 2.52        | 0.408   |
| Preoperative CEA          | (Mean; median; s.d.) | 111.46; 54.28; 134.77 | 37.26; 33.30; 30.81     | 0.125   |

Bold value denotes significant P-value ($P < 0.05$).
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Table 3. Differentially expressed genes found by microarray analysis in early (<6 months) vs late (>24 months) recurrence groups

| Upregulated genes in early recurrence group | Fold change | P-value |
|--------------------------------------------|-------------|---------|
| SERPINB5 Serpin B5                          | 2.1         | 0.0118  |
| COLEC11 Collectin-T1 Precursor             | 1.4         | 0.0018  |
| LAPT4M4A Lysosomal-associated transmembrane protein 4A | 1.3         | 0.0000  |
| C13orf3 Spindle and kinetochore-associated protein RAMA1 | 1.3         | 0.0016  |
| LYPLAL1 Lyosphospholipase-like protein 1    | 1.3         | 0.0000  |
| GTF3C3 General transcription factor 3C polypeptide 3 | 1.3         | 0.0204  |
| AMPD1 AMP deaminase 1                       | 1.3         | 0.0000  |
| ARL6IP5 PRAT1 family protein 3              | 1.3         | 0.0000  |
| SMYD2 SET1 and MYND domain-containing protein 2 | 1.2         | 0.0118  |
| ASAP2 Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2 | 1.2         | 0.0356  |
| FYTTD1 Forty-two-three domain-containing protein 1 | 1.2         | 0.0026  |

| Downregulated genes in early recurrence group | Fold change | P-value |
|-----------------------------------------------|-------------|---------|
| THEM2 Thioesterase superfamily member 2       | 0.8         | 0.0000  |
| BNIP3 BCL2/adenovirus E1B 19kDa protein-interacting protein 3 | 0.7         | 0.0096  |
| GDF15 Growth/differentiation factor 15 Precursor | 0.6         | 0.0000  |

Figure 2. Microarray analysis of patients with short and prolonged time to recurrence identifies Maspin to be differentially expressed in stage IV CRC. (A) Median expression levels of Maspin in the early (<6 months, n = 17) and prolonged (>24 months, n = 13) time-to-recurrence groups (P = 0.012). (B) Kaplan–Meier curves of 46 patients in Data set 1, illustrating time to recurrence in stage IV CRC liver metastasis patients with high Maspin expression (> median) and low Maspin expression (< median) (P = 0.005, log-rank test). Median Maspin expression was used as a cutoff (168.6). (C) Kaplan–Meier curves illustrating time to recurrence in primary stage IV CRC patients with high Maspin expression (> median, n = 27) and low Maspin expression (< median, n = 26) using a previously published data set (Smith et al, 2010) (P = 0.005). Median Maspin expression was used as the cutoff value (259.6). (D) Kaplan–Meier curves illustrating overall survival in 53 primary stage IV CRC patients with high Maspin expression (> median) and low Maspin expression (< median) using the study by Smith et al (2010) (P = 0.029). Median Maspin expression was used as the cutoff value (259.6).
Fractionation using gel electrophoresis. Equal amounts of protein (50 μg) were separated on NuPAGE Bis-Tris Mini Gels (Invitrogen, Bleiswijk, Netherlands). Gels were stained with Coomassie brilliant blue G-250 (Pierce, Rockford, IL, USA, Bleiswijk, Netherlands), washed and each lane was sliced into 10 bands using a band pattern to guide the slicing. The gel slicing and in-gel digestion was performed in a laminar flow under keratin-free conditions.

In-gel digestion, NanoLC-MS/MS analysis. In-gel digestion and NanoLC-MS/MS analysis was performed as described by van Houdt et al. (2011).

Protein identification. MS/MS spectra were searched against IPI human 3.62 (83947 entries) using Sequest version 27, rev 12 (Thermo, San Jose, CA, USA). Cysteine carbamidomethylation and methionine oxidation were treated as variable modifications. Peptides precursor ions were searched with a maximum mass deviation of 10 p.p.m. and fragment ions with a maximum mass deviation of 1 Da. Sequest output files were imported in Scaffold 2.06.1 (Proteome software, Portland, OR, USA) and search results of the 10 gel bands per biological sample were combined. A protein was considered identified when at least two unique peptides were identified in one of the samples. Peptides were identified with a PeptideProphet (Keller et al, 2002) probability score of >95% and a ProteinProphet (Nesvizhskii et al, 2003) probability score of >99%. Proteins were (label-free) quantified by spectral counting (Liu et al, 2004; Pham et al, 2010), that is, the sum of all MS/MS spectra for each identified protein. For each biological sample the spectral counts for each protein were normalised on the sum of the counts for that sample and multiplied by the average of the sums across samples. Subsequently, the beta-binominal test was applied to detect significantly different proteins between early and late recurrence samples.

Tissue microarray construction. Tissue microarrays (TMAs) were constructed from stage II and III colon cancer samples as described previously (Simon et al, 2004; Belt et al, 2011). In summary, formalin-fixed, paraffin-embedded specimens of resected colon cancer tumours were used as donor blocks. Three 0.6-mm cores were taken from the centre of the tumour and three cores from the periphery of the tumour and transferred into recipient TMA paraffin blocks, resulting in six cores per tumour. The maximum number of samples that was transferred to a single TMA was 264.

Immunohistochemistry. Formalin-fixed paraffin-embedded cores were deparaffinized with xylene and rehydrated in decreasing ethanol dilutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was achieved by boiling slides in citrate buffer (pH 6) for 20 min. Slides were then incubated (overnight 4 C) with the monoclonal anti-Maspin antibody (clone G167-70; Pharmingen, San Jose, CA, USA) at a 1:1000 dilution. For detection, goat anti mouse poly-HRP (Powervision, Immunologic, Immunovation Technologies, Brisbane, CA, USA) was used. All slides were developed with diaminobenzidine (DAB). Slides were counterstained with filtered hematoxylin, dehydrated through a graded series of ethanol, immersed in xylene and mounted.

Immunoreactivity for Maspin was assessed and scored by two independent investigators who were blinded to clinical, pathological or survival data. The presence of nuclear and cytoplasmic staining in the tumour cells was assessed separately in each sample and divided into high and low scores. The immunohistochemistry scoring system used is based on scoring systems used in previous studies (Bettstetter et al, 2005; Dietmaier et al, 2006). A combination of intensity (negative, 1+, 2+ or 3+) and percentage of stained cells was used to score each core biopsy. Intensity score of 2+ in >50% of cells or 3+ in >10% of cells was considered high (Figure 4). In case of discrepancies between the scoring by the two investigators, the slides were reviewed and a consensus was reached. For analytical purposes protein expression was divided into high and low scores combining cytoplasmic and nuclear scores (see Table 1).

Western blotting. The homogenised tumour tissue from all five patients with short time to recurrence (<6 months), which was used for mass-spectrometry analysis, was used for western blot validation. Equal amounts of protein was run out on NuPAGE Novex Tris-Acetate Mini Gel (Invitrogen) and was analysed by western blotting using primary antibodies directed against Maspin (clone G167-70; Pharmingen) and β-Actin (AC-15, Novus Biologicals, Littleton, CO, USA) in combination with secondary antibody peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). For detection of antibodies Amersham ECL Western Blotting Detection Reagent was used (GE Healthcare Life Sciences).

Follow-up and survival. All patients were subjected to routine follow-up. The follow-up data were updated by letters and telephone calls to referring physicians and general practitioners. The duration of the follow-up and the time between surgery and...
Table 4. Differentially expressed proteins (P<0.05) found by mass-spectrometry analysis in early (<6 months) vs late recurrence (>24 months) groups

| Protein       | Description                                                        | Fold change | P-value |
|---------------|-------------------------------------------------------------------|-------------|---------|
| Upregulated proteins in early recurrence group |
| SERPINB5      | Serpin peptidase inhibitor, clade B (ovalbumin), member 5         |             | 0.01    |
| RNASET2       | Ribonuclease T2                                                    |             | 0.01    |
| ICAM1         | Intercellular adhesion molecule 1                                 |             | 0.02    |
| EIF2B1        | Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26 kDa |             | 0.02    |
| TPSAB1        | Tryptase alpha/beta 1                                             |             | 0.02    |
| SF3A3         | Splicing factor 3a, subunit 3, 60 kDa                             |             | 0.05    |
| OLFM4         | Olfactomedin 4                                                    | 31.9        | 0.05    |
| IGFALS        | Insulin-like growth factor binding protein, acid labile subunit   | 8.8         | 0.02    |
| PTK7          | PTK7 protein tyrosine kinase 7                                    | 8.3         | 0.02    |
| LANCL1        | LanC lantibiotic synthetase component C-like 1 (bacterial)        | 7.8         | 0.01    |
| API5          | Apoptosis inhibitor 5                                             | 5.8         | 0.05    |
| XPO7          | Exportin, tRNA (nuclear export receptor for tRNAs)                | 5.8         | 0.05    |
| LSP7          | Ubiquitin specific peptidase 7 (herpes virus-associated)          | 5.3         | 0.04    |
| ACSL3         | Acyl-CoA synthetase long-chain family member 3                    | 4.4         | 0.01    |
| NUP155        | Nucleoporin 155 kDa                                               | 4           | 0.01    |
| CLIC4         | Chloride intracellular channel 4                                   | 3.9         | 0.01    |
| KRT6B         | Keratin 6B                                                        | 3.6         | 0.04    |
| MYL9          | Myosin, light chain 9, regulatory                                  | 3.3         | 0.03    |
| VARS          | Valyl-tRNA synthetase                                             | 3.2         | 0.0      |
| CYFIP1        | Cytoplasmic FMR1 interacting protein                              | 3.1         | 0.03    |
| SYNE2         | Spectrin repeat containing, nuclear envelope 2                    | 2.8         | 0.05    |
| NUDT21        | Nudix (nucleoside diphosphate linked moiety X)-type motif 21      | 2.6         | 0.05    |
| CKBPA         | Cytoskeleton-associated protein 4                                  | 2.3         | 0.02    |
| VIL1          | Villin 1                                                          | 2.2         | 0.04    |
| HSD17B12      | Hydroxysteroid (17-beta) dehydrogenase 12                         | 2           | 0.05    |
| YWHAQ         | Tyrosine 3-monoxygenase                                           | 2           | 0.04    |
| Downregulated proteins in early recurrence group |
| PTTG1IP       | Pituitary tumour-transforming 1 interacting protein               |             | 0.011   |
| DUT           | Deoxyuridine triphosphatase                                       |             | 0.013   |
| CASP1         | Caspase 1, apoptosis-related cysteine peptidase                    |             | 0.018   |
| HSD17B4       | Hydroxysteroid (17-beta) dehydrogenase 4                          |             | 0.024   |
| VWF           | von Willebrand factor                                             |             | 0.024   |
| RBM14         | RNA binding motif protein 14                                       |             | 0.026   |
| LACTB2        | Lactamase, beta 2                                                 |             | 0.028   |
| AMBP          | Alpha-1-microglobulin/bikunin precursor                           |             | 0.042   |
| ANKRD22       | Ankyrin repeat domain 22                                           |             | 0.046   |
| ADCK3         | AarF domain containing kinase 3                                   |             | 0.001   |
| GYR1          | Glycogenin 1                                                      | 9.0         | 0.003   |
| AKR1C3        | Aldo-keto reductase family 1, member C3                            | 6.2         | 0.053   |
| PTGES2        | Prostaglandin E synthase 2                                        | 5.4         | 0.053   |
| SCCP1D        | Saccharopine dehydrogenase (putative)                             | 2.8         | 0.007   |
| MRPL14        | Mitochondrial ribosomal protein L14                               | 2.7         | 0.011   |
| CUL4A         | Cullin 4A                                                         | 2.7         | 0.051   |
| SDCBP         | Syndecan binding protein (syntenin)                               | 2.6         | 0.029   |
| CAST          | Calpastatin                                                      | 2.1         | 0.019   |
| MCC2          | Methylcrotonoyl-CoA carboxylase 2 (beta)                           | 1.9         | 0.004   |
| HMCN1         | Hemicentin 1                                                      | 1.7         | 0.027   |

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the detection of recurrence were obtained, in addition to CRC-specific survival.

Statistical analysis. Time-to-recurrence and CRC-specific survival data were calculated from the day of surgery to the day of the first recurrence or the day of death caused by CRC. Median time to recurrence and CRC-specific survival were estimated by the Kaplan–Meier method. To determine the influence of possible risk factors on time to recurrence and CRC-specific survival, a univariable Cox regression analysis was performed. A multivariable Cox proportional hazards model, containing the factors that displayed P-values <0.2 in univariable analysis, was used to determine the independent prognostic impact of all variables on time to recurrence and CRC-specific survival. Statistical significance was assumed for P-values <0.05. Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA).

RESULTS

Gene expression profiling identifies Maspin as a marker for recurrence in stage IV CRC. Forty-six patients from Data set 1 fulfilled the inclusion- and quality control criteria. To identify genes that are differentially expressed, patients with metastases showing early (<6 months, n = 17) and prolonged (>24 months, n = 13) time to recurrence were selected (Table 2). Gene expression profiling of these 30 tumours revealed that 14 genes were significantly differentially expressed between the two groups: SERPINB5, THEM2, GDF15, LYLPA1, AMPD1, ARL6IP5, LAPTMM4A, C13orf3, COLEC11, FYTTD1, Bnip3, SMYD2, GTF3c3 and ASAP2. Of these genes, 11 were upregulated in the group with time to recurrence of <6 months (Table 3). SERPINB5, also known as Maspin, was the gene most upregulated in patients with a short time to recurrence. Maspin expression was ~2.1 times higher in tumours of patients with a short time to recurrence vs tumours in patients with a prolonged time to recurrence (Figure 2A; P = 0.01).

The Kaplan–Meier survival curves of all 46 patients from Data set 1 (including the 30 patients used for gene expression profiling) (Figure 2B) show that the 2-year recurrence-free survival probability of patients with Maspin-high tumours (>median 168.6) is an estimated 0.10 (95% CI = 0.00–0.20) compared with 0.39 (95% CI = 0.18–0.60) in patients with Maspin-low tumours (<median 168.6) (Cox regression analysis P = 0.007, HR = 2.65, 95% CI = 1.307–5.380). Multivariable analysis shows that Maspin is an independent predictor of early recurrence (P = 0.02, HR = 2.971, 95% CI = 1.168–7558) in our training set. Adjuvant treatment was the only other independent predictor of early recurrence in multivariable analysis (P = 0.05, HR = 0.329, 95% CI = 0.109–0.994) (Supplementary Table 1).

This result was validated using an independent microarray data set from a cohort of 53 patients with stage IV primary CRC (Smith et al, 2010). Two Kaplan–Meier graphs, generated by the R2 microarray analysis and visualisation platform (http://r2.amc.nl) show that high levels (>median 259.6) of Maspin are significantly correlated with disease-specific (Figure 2C; P = 0.005) and overall survival (Figure 2D; P = 0.029) in primary stage IV CRC patients.

Mass-spectrometry analysis confirms Maspin as marker for early recurrence in stage IV CRC. In parallel to gene expression profiling, we also analysed differentially expressed proteins in patients, from Data set 1, showing early (<6 months) and prolonged (>24 months) time to recurrence. To this end, mass-spectrometry analysis was performed on five tumours from each group (Supplementary Table 2). Protein-containing lysates of these tumour tissue samples were fractionated on an SDS–PAGE gel, followed by in-gel tryptic digestion (Figure 3A). Analysis of the extracted peptides was performed by Nano-LC-MS/MS, followed by database searching. In total, 2097 unique proteins were identified in all patient samples. Forty-six proteins were present in significantly different amounts in the two groups, of which 26 were overrepresented and 20 were underrepresented in the tumours of patients with early recurrence (Table 4). The sets of 14 genes and 46 proteins that showed a significant association with short or prolonged recurrence times contained only 1 overlapping factor: Maspin (Figure 3B). Validation of the mass-spectrometry results by western blotting showed that the number of spectral counts detected by mass-spectrometry analysis correlates very well with Maspin protein levels (Figure 3C). These results demonstrate that Maspin is differentially expressed on both mRNA and protein level in stage IV CRC patients with early and late recurrence.

Tissue microarray analysis reveals that Maspin staining in the tumour centre is a stage-specific marker in CRC. The above results indicate that Maspin could be a prognostic marker for early recurrence in stage IV CRC patients. This prompted us to assess whether this marker had a similar prognostic power in stage II and III CRC patients. To this end, a total of 243 stage II and 166 stage III tumours on TMA cores were stained with specific monoclonal Maspin antibody (Bettstetter et al, 2005; Dietmaier et al, 2006; Fung et al, 2010). The presence
of nuclear and cytoplasmic staining in both central tumour cores and peripheral tumour cores was assessed in each sample and divided in high and low scores. A high score was defined as an intensity score of 2+ in >50% of cells or 3+ in >10% of cells (Table 1, Figure 4).

In stage II CRC tumours (n = 243), Maspin staining in the central tumour cores showed no significant differences in time-to-recurrence and CRC-specific survival times (Table 5). Kaplan–Meier curves of Maspin are depicted in Figure 5A and B. High Maspin expression was found to be correlated with a right-sided tumour location (P = 0.001) (Supplementary Table 3).

In stage III CRC patients (n = 166), Maspin staining in the central tumour cores was an independent predictor of time-to-recurrence and CRC-specific survival (Table 6, Figure 5C and D). Patients with combined low cytoplasmic and nuclear staining score had estimated 2-year recurrence-free and CRC-specific survival probabilities of 0.74 (95% CI = 0.64–0.84) and 0.83 (95% CI = 0.74–0.92; log-rank P = 0.011), respectively. Patients with a combined high cytoplasmic and nuclear score did markedly worse with estimated 2-year recurrence-free and CRC-specific survival probabilities of 0.42 (95% CI = 0.25–0.59) and 0.55 (0.38–0.72; log-rank P = 0.000), respectively. The low-cytoplasmic and

Table 5. Uni- and multivariate analysis of factors influencing disease-free survival in stage II CRC patients

| Variable                                      | Hazard ratio | 95%CI      | P-value | Hazard ratio | 95%CI      | P-value |
|-----------------------------------------------|--------------|------------|---------|--------------|------------|---------|
| Sex                                           | 0.854        | 0.498–1.464| 0.566   | 0.803        | 0.434–1.484| 0.483   |
| Age (years)                                   | 1.008        | 0.984–1.032| 0.535   | 1.021        | 0.991–1.051| 0.169   |
| Tumour size (mm)                              | 0.990        | 0.975–1.006| 0.215   | 0.996        | 0.979–1.013| 0.605   |
| Tumour location (right-sided/left sided/rectum)| 1.456        | 0.830–2.553| 0.190   | 1.271        | 0.678–2.380| 0.455   |
| Nodal stage (N0/N1/N2)                        | n.a.         | n.a.       |         | n.a.         | n.a.       |         |
| No. of lymph nodes retrieved                  | 0.960        | 0.907–1.015| 0.152   | 0.970        | 0.911–1.033| 0.343   |
| Histological grade (well/moderate/poor)       | 1.925        | 1.056–3.511| 0.033   | 1.820        | 0.923–3.591| 0.084   |
| Mucinous differentiation (present/absent)     | 1.152        | 0.616–2.155| 0.657   | 1.243        | 0.623–2.481| 0.537   |
| Ulceration (present/absent)                   | 1.003        | 0.536–1.876| 0.992   | 0.998        | 0.489–2.036| 0.995   |
| Angioinvasion (present/absent)                | 2.301        | 1.156–4.581| 0.018   | 2.322        | 1.072–5.028| 0.033   |
| Adjuvant chemotherapy (yes/no)                | 1.289        | 0.943–1.762| 0.112   | 1.259        | 0.869–1.768| 0.184   |
| Microsatellite stability status (MSS/MSI)     | 0.685        | 0.458–1.025| 0.066   | 0.791        | 0.514–1.215| 0.284   |

Central tumour cores

Maspin staining

| Low nuclear, low cytoplasmic | 1.015 | 0.733–1.405 | 0.930 | 0.937 | 0.639–1.375 | 0.740 |
| Low nuclear, high cytoplasmic | 0.792 | 0.282–2.224 | 0.658 | 0.999 | 0.307–3.255 | 0.999 |
| High nuclear, high cytoplasmic | n.a. | n.a.         | n.a.  | n.a.  | n.a.        | n.a.  |

Peripheral tumour cores

Maspin staining

| Low nuclear, low cytoplasmic | 0.987 | 0.694–1.404 | 0.943 | 0.717 | 0.440–1.168 | 0.201 |
| Low nuclear, high cytoplasmic | 1.291 | 0.359–4.643 | 0.696 | 2.074 | 0.396–10.859| 0.388 |
| High nuclear, high cytoplasmic | n.a. | n.a.         | n.a.  | n.a.  | n.a.        | n.a.  |

Multivariable analysis

| Variable                                      | Hazard ratio | 95%CI      | P-value | Hazard ratio | 95%CI      | P-value |
|-----------------------------------------------|--------------|------------|---------|--------------|------------|---------|
| Histological grade                           | 2.028        | 1.097–3.749| 0.024   | 2.149        | 0.998–5.043| 0.060   |
| Angioinvasion                                 | 2.338        | 1.153–4.743| 0.019   | 2.497        | 1.150–5.421| 0.021   |
| MMS–MSI                                       | 0.806        | 0.533–1.220| 0.308   | —            | —          | —       |
| Tumour location                              | 1.146        | 0.636–2.064| 0.650   | —            | —          | —       |
| No. of lymph nodes retrieved                 | 0.966        | 0.913–1.022| 0.228   | —            | —          | —       |
| Adjuvant chemotherapy                        | 1.229        | 0.901–1.676| 0.193   | 1.265        | 0.878–1.824| 0.208   |
| Age                                           | —            | —          | —       | 1.021        | 0.989–1.053| 0.201   |

Bold value denotes significant P-value (P < 0.05).
Maspin is a prognostic marker in colorectal cancer

**DISCUSSION**

In this report we identified Maspin as a marker for early recurrence after surgery for colorectal liver metastases. Maspin is a prognostic factor for early recurrence in stage III and IV CRC patients but not in stage II patients. Maspin is a member of the serine protease inhibitor (serpin) family. In breast cancer, Maspin acts as a tumour suppressor by inhibiting tumour cell motility, invasion and tumour growth (Zou et al., 1994). In breast, ovarian and lung cancer Maspin expression is correlated with a relatively good prognosis (Marioni et al., 2005; Zheng et al., 2008). By contrast, studies in lung, breast, gastric and pancreatic cancer show that Maspin expression is associated with more aggressive disease (Umekita et al., 2002; Cao et al., 2007; Woenckhaus et al., 2007; Yu et al., 2007). The reasons for these discrepancies are presently unknown.

Mixed-stage studies in CRC have failed to reach consensus whether Maspin is associated with good or poor prognosis (Song et al., 2002; Bettstetter et al., 2005; Markl et al., 2010). A study in patients with only stage III disease has shown that high nuclear Maspin is significantly correlated with poor overall survival (Dietmaier et al., 2006), which is in line with the results presented in this report. We used a combined nuclear and cytoplasmic expression score. The group with high-cytoplasmic and high-nuclear staining is associated with early recurrence. By contrast, the groups with low-nuclear and either high- or low-cytoplasmic staining are associated with late recurrence. This suggests that high-nuclear Maspin staining is the major variable determining the association with early recurrence. This is in line with earlier reports (Dietmaier et al., 2006; Markl et al., 2010). In contrast to the results presented here, a previous study failed to detect a significant association of nuclear or cytoplasmic Maspin staining with outcome in a large cohort of stage III CRC patients (Fung et al., 2010). As all three studies made use of the same antibody, the different outcome of the study of Fung et al. (2010) may be related to the use of a different scoring system. The cutoffs used in this study described above were selected based on the results of the study presented here.
study resulted in a very unevenly distributed number of samples per group, in which the vast majority (75%) of samples were allocated to one group. Moreover, this study did not take the intensity of the staining into account, which has previously been associated with the amount of Maspin expression and clinical outcome (Dietmaier et al., 2006). However, when we apply the Fung scoring system to our own data set, we still find that nuclear Maspin expression is associated with disease-free and CRC-specific survival in stage III CRC patients ($P = 0.031$ and $P = 0.001$; data not shown). The same significant association between high levels of Maspin and early-recurrence and colorectal-specific survival was seen when applying the Dietmaier et al. (2006) scoring system ($P = 0.040$ and $P = 0.019$; data not shown).

Tumours with high Maspin expression respond significantly better to adjuvant 5-FU chemotherapy in stage III CRC (Dietmaier et al., 2006). This trend was also observed in our study, although patient numbers were too low for this association to reach significance (Data not shown). Maspin may therefore not only have value as a prognostic marker but possibly also as a marker for predicting response to adjuvant treatment in advanced stage CRC.

**Table 6. Uni- and multivariate analysis of factors influencing disease-free survival in stage III CRC patients**

| Variable                          | Disease-free survival | Colorectal-specific survival |
|-----------------------------------|-----------------------|-----------------------------|
|                                   | Hazard ratio | 95%CI            | P-value | Hazard ratio | 95%CI            | P-value |
| Sex                               | 1.054        | 0.665–1.671      | 0.821   | 1.418        | 0.854–2.354      | 0.177   |
| Age (years)                       | 0.999        | 0.979–1.018      | 0.890   | 1.011        | 0.988–1.035      | 0.366   |
| Tumour size (mm)                  | 1.015        | 1.002–1.029      | 0.021   | 1.015        | 1.000–1.031      | 0.048   |
| Tumour location (right sided/lefthanded/rectum) | 0.808 | 0.512–1.275 | 0.360 | 0.691 | 0.416–1.148 | 0.153 |
| Nodal stage (N0/N1/N2)            | 2.110        | 1.328–3.353      | 0.002   | 2.262        | 1.355–3.777      | 0.002   |
| No. of lymph nodes retrieved      | 0.989        | 0.942–1.038      | 0.651   | 0.992        | 0.940–1.047      | 0.765   |
| Histological grade (well/moderate/poor) | 0.673 | 0.412–1.099 | 0.113 | 0.643 | 0.367–1.127 | 0.123 |
| Mucinous differentiation (present/absent) | 1.093 | 0.628–1.902 | 0.752 | 1.131 | 0.612–2.092 | 0.694 |
| Ulceration (present/absent)       | 0.807        | 0.463–1.404      | 0.447   | 0.560        | 0.320–1.007      | 0.053   |
| Angioinvasion (present/absent)    | 3.226        | 2.029–5.130      | 0.000   | 3.107        | 1.860–5.189      | 0.000   |
| Adjuvant chemotherapy (yes/no)    | 1.127        | 0.914–1.390      | 0.264   | 0.950        | 0.739–1.221      | 0.689   |
| Microsatellite stability status (MSS/MSI) | 1.025 | 0.713–1.472 | 0.895 | 0.365 | 0.523–1.269 | 0.365 |

**Central tumour cores**

**Maspin staining**

| Low nuclear, low cytoplasmic | 1.088 | 0.814–1.454 | 0.568 |
| Low nuclear, high cytoplasmic | 2.150 | 1.159–3.987 | 0.009 |
| High nuclear, high cytoplasmic | — | — | — |

**Peripheral tumour cores**

**Maspin staining**

| Low nuclear, low cytoplasmic | 0.869 | 0.653–1.156 | 0.334 |
| Low nuclear, high cytoplasmic | 1.754 | 0.822–3.742 | 0.146 |
| High nuclear, high cytoplasmic | — | — | — |

**Multivariable analysis**

| Variable                          | Disease-free survival | Colorectal-specific survival |
|-----------------------------------|-----------------------|-----------------------------|
|                                   | Hazard ratio | 95%CI            | P-value | Hazard ratio | 95%CI            | P-value |
| Diameter                          | 1.014        | 1.001–1.027      | 0.038   | —            | —                | —       |
| Angioinvasion                     | 2.973        | 1.792–4.932      | 0.000   | 2.733        | 1.548–4.826      | 0.001   |
| Combined Maspin staining (central tumour cores) | 1.415 | 1.044–1.919 | 0.025 | 1.534 | 1.176–2.350 | 0.005 |
| Histological grade               | 0.963        | 0.545–1.701      | 0.897   | 0.971        | 0.541–1.742      | 0.920   |
| Tumor location                   | —            | —                | —       | 0.676        | 0.388–1.178      | 0.167   |
| Nodal stage                      | —            | —                | —       | 1.525        | 0.843–2.758      | 0.163   |
| Sex                              | —            | —                | —       | 1.165        | 0.662–2.051      | 0.597   |
| Ulceration                       | —            | —                | —       | 0.571        | 0.305–1.068      | 0.079   |
| Adjuvant                         | —            | —                | —       | 1.066        | 0.801–1.419      | 0.661   |

Bold value denotes significant P-value ($P < 0.05$).
Whether Maspin itself could be a drug target depends on whether it is causally involved in facilitating the metastatic process.

In this study, Maspin was highly upregulated using two different high-throughput screens suggestive of a key role in predicting time to recurrence after surgery of colorectal liver metastasis. To our knowledge, this is the first study reporting a possible predictive role for Maspin in stage IV CRC. However, it must be noted that identification of Maspin and validation was performed in a relatively small cohort.

Experimental studies show that high Maspin expression is correlated with increased apoptosis resistance in CRC cells (Payne et al, 2011). Furthermore, it has been shown that circulating tumour cells in peripheral blood of CRC patients express high levels of Maspin (Findeisen et al, 2008). We did not find a difference in Maspin expression at the periphery of the tumour compared with that at the centre; however, Bettstetter et al (2005) did find that Maspin expression levels were higher at the invasive tumour font when compared with that at the more central parts of CRC tumours. This discrepancy can probably be explained by the fact that we scored peripheral cores containing mainly tumour bulk, but not necessarily cells invading the adjacent stroma, whereas Bettstetter et al (2005) only scored Maspin expression in cell clusters detaching from the peripheral tumour bulk.

These results combined with the results of our study, demonstrating that high Maspin expression is associated with poor prognosis in CRC that has metastasised to local lymph nodes or beyond, point to a potential role for Maspin in facilitating the metastatic process. Future work should therefore address whether (and how) high Maspin levels promote metastasis in advanced-stage CRC.

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