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mRNA expression profiles of colorectal liver metastases as a novel biomarker for early recurrence after partial hepatectomy

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ABSTRACT

Background: Identification of specific risk groups for recurrence after surgery for isolated colorectal liver metastases (CRLM) remains challenging due to the heterogeneity of the disease. Classical clinicopathologic parameters have limited prognostic value. The aim of this study was to identify a gene expression signature measured in CRLM discriminating early from late recurrence after partial hepatectomy.

Methods: CRLM from two patient groups were collected: I) with recurrent disease ≤12 months after surgery (N = 33), and II) without recurrences and disease free for ≥36 months (N = 30). The patients were clinically homogeneous; all had a low clinical risk score (0–2) and did not receive (neo-) adjuvant chemotherapy. Total RNA was hybridised to Illumina arrays, and processed for analysis. A leave-one-out cross validation (LOOCV) analysis was performed to identify a prognostic gene expression signature.

Results: LOOCV yielded an 11-gene profile with prognostic value in relation to recurrent disease ≤12 months after partial hepatectomy. This signature had a sensitivity of 81.8%, with a specificity of 66.7% for predicting recurrences (≤12 months) versus no recurrences for at least 36 months after surgery (X^2 $P < 0.0001$).

Conclusion: The current study yielded an 11-gene signature at mRNA level in CRLM discriminating early from late or no relapse after partial hepatectomy.

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1. Introduction

Colorectal cancer is one of the most commonly diagnosed cancers worldwide (Torre et al., 2015). Approximately 15–25% of patients with colorectal cancer (CRC) present with synchronous liver metastases and another 20% have a metachronous disease development (van der Pool et al., 2012). For patients presenting with isolated liver metastases, partial hepatectomy is the only potentially curative treatment option. Reported 5-year survival rates are 40–60% (Dols et al., 2009; Rees et al., 2008; Primrose, 2010). A substantial number of patients develop recurrent disease after liver surgery, underlining the need for prognostic biomarkers (D'Angelica et al., 2011; Butte et al., 2015; de Jong et al., 2009). Such prognostic biomarkers may allow a more personalised treatment strategy. In recent years, several clinicopathological prognostic variables in patients with isolated colorectal liver metastases (CRLM) have been identified predicting the risk of relapse after a metastasectomy (Matias et al., 2015). These variables have been integrated in various clinical risk scores (CRS) (Matias et al., 2015; Fong et al., 1999; Konopke et al., 2009; Nagashima et al., 2004; Nordlinger et al., 1996). The CRS according to Fong et al. is the most widely used and validated score, able to distinguish between high risk and low risk patients in terms of survival outcomes (Fong et al., 1999). This score is composed of 5 prognostic variables: positive lymph node status of the primary tumour, diagnosis of liver metastases within 12 months after resection of primary tumour, serum CEA $\geq$200 ng/ml, $>$1 liver metastases, a metastasis of $>$5 cm diameter. Each variable accounts for 1 point. Patients with 0–2 points are categorised as low risk, patients with 3–5 points as high risk. Still, outcomes after surgery remain heterogeneous: low risk patients may develop early recurrences – approximately 50% of patients with a low CRS develop metastases within 12 months after surgery – while high risk patients may remain disease free (Poston, 2008; Poston et al., 2008). Unravelling the biological properties characterising tumours may be pivotal to designing individualised therapies, based on biological predictors of outcome rather than or in addition to clinical predictors. Various groups have established molecular subtypes in primary cancers with distinct biology, predictive and prognostic value (Guinney et al., 2015;Paik et al., 2004; Hoshida et al., 2008; Albain et al., 2010; Budinska et al., 2013; Sadanandam et al., 2013). Biological markers may improve patient selection for (neo-) adjuvant therapies in addition to surgical management or intensive surveillance schemes.

The ability to analyse tumours at DNA-, RNA-, and protein-level promises to revolutionize our understanding of the malignant disease process, and hopefully this will herald new (superior) biomarkers. The aim of the current study was to identify a prognostic gene signature at mRNA level in patients with a low CRS, effective in identifying patients at high risk of early recurrence after surgery for CRLM.

2. Methods

2.1. Patient and treatment

Erasmus MC Cancer Institute is a tertiary referral centre for liver surgery. In the current retrospective study, patient characteristics were collected from a prospectively maintained database. All patients undergoing resection for CRLM are prospectively entered into an institutional database. This database includes standard clinicopathological variables. Patients selected for the current study had a low risk profile (Fong's clinical risk score 0–2 (Fong et al., 1999)) and did not receive treatment with (neo-) adjuvant chemotherapy for the resectable CRLM in line with the Dutch guidelines that do not support routine administration of chemotherapy/biologicals in the case of primary resectable colorectal liver-only metastases. Patients were further selected according to the following criteria: I) patients with recurrent disease within 12 months after hepatectomy, and II) patients without recurrent disease and a disease free survival of at least 36 months after hepatectomy. Thus, "two extremes" were selected in terms of recurrent disease. All resections were performed between 2000 and 2009. Hepatic parenchymal resection was performed with an ultrasonic surgical aspirator and a monopolar coagulator. R0-resections were defined by the absence of microscopic tumour invasion of the resection margins, and R1-resections were defined by the presence or microscopic tumour invasion of the resection margins (Ayez et al., 2012).

During follow-up, patients visited the outpatient clinic every 4 months in the first 2 years after CRLM resection for clinical examination and CEA-determination. Thereafter, patients visited the outpatient clinic every 6 months and were discharged from follow up after 5 years. Abdominal imaging (CT of thorax and abdomen) was performed twice a year during the first 3 years and thereafter annually. If disease recurred, a decision on whether to initiate chemotherapy treatment or to perform local therapy was made by a multidisciplinary team. Disease free survival (DFS) was defined as the interval in months between resection of CRLM and recurrence.

2.2. Tissue collection and assessment

After resection of CRLM, tumour tissue is standardly fixed on formalin and embedded in paraffin in the department of pathology according to standard protocols, and stored. For the current study, tumour samples (N = 80) of CRLM were retrieved from the selected patient groups. In the case a patient had more than one metastasis, there were no additional selection criteria in terms of which tumour to analyse. The formalin fixed, paraffin embedded (FFPE) samples were evaluated by a pathologist for colon tumour cell content: only specimens with at least 30% tumour cells in the tissue block were included (N = 63). The final study population consisted of 33 samples for group I with disease recurrence within 12 months and 30 samples for group II without disease recurrence and a DFS of 36 months.

The established tumour growth patterns are assessed by a dedicated pathologist and at least one additional observer in all resected CRLM in Erasmus MC Cancer Institute (Vermeulen et al., 2001; Van den Eynden et al., 2013). Three tumour growth patterns have been reported in literature, with a distinct growing pattern (Vermeulen et al., 2001; Van den Eynden et al., 2013). These patterns consist of a pushing type, a replacing type and a desmoplastic type. Briefly, in the pushing type the metastasis has a displacing interaction with the normal liver parenchyma, and is separated from normal cells by a thin layer of reticulin fibres. The replacing type infiltrates the normal liver parenchyma. The desmoplastic type has a band of desmoplastic tissue that separates tumour cells from the liver parenchyma.

On a patient level, the growth patterns were classified by two methods for analysis in relation to outcomes. First, when a pattern was expressed in >75% of the CRLM the patient was classified as such. If no pattern was expressed in >75%, the growth pattern was classified as a "mixed type". Second, based on prognostic evidence reported in the literature, if any percentage of the pattern was a replacement type, the patient was classified as such (Van den Eynden et al., 2012; Okano et al., 2000; Lunevicius et al., 2001; Eefsen et al., 2015). Tumour differentiation and inflammation at the leading edge of the tumour were also objectified, for the current study specifically.

2.3. RNA extraction and purification

Depending on the size of the FFPE samples, total RNA was extracted from 3 to 6 × 20 μm sections. Following paraffin removal with xylene the high-pure RNA paraffin kit was used according the supplier's instructions (Roche, Mannheim, Germany). Following isolation, RNA was stored in RNase/DNase-free water at −80 °C. Quality control was performed as previously described (Mustafa et al., 2015).

2.4. Gene expression profiles

Illumina Whole Genome-cDNA-mediated Annealing, Selection, Extension and Ligation (WG-DASL) V4 assay is an array-based method for expression profiling of partially degraded RNA molecules such as those isolated from Formalin-Fixed Paraffin-Embedded samples. In the HumanHT-12 v4 BeadChip assay 29,285 annotated transcripts corresponding to 27,253 coding transcripts with well-established annotations are measured. The WG-DASL assay was performed according to the manufacturer's instructions. In summary, 1000 ng total RNA was used from the 63 FFPE samples. 500 ng of total RNA from a pool of fresh frozen tumour RNA samples (I-scan control) was included in each individual hybridisation experiment of 11–23 samples to evaluate possible inter-assay differences (Supplementary 1). Total RNA was converted to cDNA using biotinylated oligo-dT_{18} and random nonamer primers. The biotinylated cDNA was annealed to the DASL Assay Pool (DAP) probe groups, which contain oligonucleotides specifically designed to interrogate each target sequence of the transcript. The DAP was annealed to targeted cDNA during a 16 h temperature gradient (70°–37 °C) incubation. Hybridisation of these oligonucleotides to the targeted cDNA site, followed by enzymatic extension and ligation was used to create a Polymerase Chain Reaction (PCR) template that was amplified with a set of universal PCR primers (Fan et al., 2004). Cy3-coupled primers were used to facilitate the precipitation of the single stranded labelled products, which were hybridised to the whole genome HumanHT-12 v4 BeadChips containing 12 identical microarrays each. The microarrays were scanned using a confocal type imaging system with Cy3 (532 nm) laser illumination Illumina I-scan reader (N0262). Fluorescent intensities were read and images were extracted using software version 1.8.13.5. Each sequence type is represented by an average of 30 beads on the array.

Eight hybridisations did not meet our criteria of an average intensity signal of at least 500 prior to background correction and normalisation and were re-measured at an input of 2000 ng total RNA.

2.5. Data analysis

Scanned data were uploaded into GenomeStudio software version 2011.1 via the Whole Genome DASL gene expression module for further analysis. The average signal, detection P-value, Bead standard error and average beads were used to quantile normalise the data in the statistical language R (www.r-project.org) using the "lumi" package (Du et al., 2008). The expression raw data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/entry nr.: GSE81423).

2.6. Statistics

A leave-one-out cross validation (LOOCV) was performed using Biometric Research Branch ArrayTools (BRB-ArrayTools, http://linus.nci.nih.gov/BRB-ArrayTools.html), starting with the top 25% most variable genes (N = 7101) in all samples as input. Samples were classified in two classes: recurrences ≤12 months (class 1) or no recurrences and a disease free survival ≥36 months (class 2). In each round of the LOOCV, genes with a univariate P-value <0.001 were selected to differentiate between class 1 and class 2 (patients with and without recurrent disease). The linear prediction rule was defined by the inner sum of the weights (Wi) and expression (Xi) of these significant genes. In the prediction model, a sample was classified to class 2 if the sum was greater than the established threshold ($\sum iWiXi >$ threshold). From the available prediction algorithms, the "Support Vector Machine" (SVM) proved the most accurate classifier (75% correct classification, Supplementary 2), resulting in an 11-gene signature (Table 3). Through this algorithm, each patient could be classified as "high risk" or "low risk" on basis of the identified expression profile (molecular risk).

Descriptive values are expressed as median (interquartile range (IQR)). Variables were compared by means of Chi-square analysis or Fischer's exact test (depending on the sample size) or with the independent Student's t test or Mann–Whitney U test when appropriate. The SPSS statistical package (version 21.0, Chicago, IL, USA) was used for statistical analysis; a two-sided P-value of ≤0.05 was considered statistically significant.

2.7. Ethical approval

Of all patients, an informed consent was available, to use residual tissue for research purposes. The data and tissue used in the current study was employed in an anonymous fashion. As prescribed by national regulations, the current study was not subject to the "Medical Research Involving Human Subjects Act".

3. Results

3.1. Patients

Clinicopathological features of both patient groups (with recurrences ≤12 months and without recurrences and a DFS ≥36 months) are outlined in Table 1. The groups were homogeneous in terms of clinicopathological characteristics, as expected since all patients were selected to have a low CRS according to Fong (Matias et al., 2015). There was no difference in tumour differentiation, histological growth pattern and inflammation (at the leading edge of the tumour). The respective molecular risk groups did not differ on basis of the assessed biological (pathological) characteristics.

3.2. Genes associated with early recurrence

Through a LOOCV analysis, an 11-gene profile was constructed capable of discriminating patients at high-from low risk of recurrence (Table 3 and Supplementary 3). Clinicopathological features of patients by the identified molecular risk groups (low- and high-risk) are depicted in Table 2. These groups differed on basis of location of primary tumour and, inherently, the administration of neo-adjuvant radiotherapy for primary CRC. Of the 37 patients with at high molecular risk, 27 developed recurrent disease within 12 months. This yielded a sensitivity of the signature of 81.8%, with a specificity of 66.7% (X^2 $P < 0.0001$, Table 4a). From the group of patients with recurrences within 12 months, the subgroup of patients with hepatic recurrences was identified ($N = 17$). All patients with hepatic recurrences were at high molecular risk based on the 11-gene signature, resulting in a 100% sensitivity and 56% specificity for hepatic recurrences specifically (X^2 $P < 0.0001$, Table 4b).

In the KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html) and Gene Ontology Consortium database (http://geneontology.org) the respective genes were searched and pathways in which they are known to be involved are depicted in Supplementary 4 (KEGG) and Supplementary 5 (Gene Ontology). Two genes, CLRN3 and KIAA0219, have not been described and not been registered in both databases.

4. Discussion

The clinical and biological diversity of CRLM urges the need for prognostic biomarkers and tailor-made treatment strategies (Poston et al., 2008). Despite improvement of therapies for liver-only stage IV CRC resulting in improved survival rates, knowledge on treatment response and risks of relapse or progression is still scarce. A substantial number of patients develop recurrent disease following resection of CRLM, underlining the need for prognostic factors (D'Angelica et al., 2011; Butte et al., 2015; de Jong et al., 2009). More insights into biological tumour behaviour may result in better understanding of treatment failure and may yield biomarkers for risks of relapse or prediction of response to therapy. This could improve identification of patients who will or will not benefit from tailored treatment strategies, e.g. more intensified (neo-) adjuvant treatments for those with a high risk for relapse and potentially less intensified approaches for those with a low risk profile. Currently, prognostication and prediction in resectable CRLM is solely based on clinical parameters, with sub-optimal performance. As an exception, KRAS/BRAF mutation status may impact response to treatment and outcome in CRLM as in primary colorectal cancer (Passiglia et al., 2016; Karagkounis et al., 2013; Lin et al., 2014; Loes et al., 2016; Margonis et al., 2015; Vauthey et al., 2013). Nevertheless, both clinical and the latter mutational status fails to impact clinical management of CRLM (Zakaria et al., 2007).

In the present study, mRNA expression profiles in CRLM were objectified in low risk patients who underwent hepatectomy with curative intent, without (neo-) adjuvant chemotherapy. All patients were homogeneous in terms of clinical risk, as defined by current standards (Matias et al., 2015). Within this homogeneous group with respect to clinical risk, we were still able to select two opposite ends of the clinical spectrum: patients with recurrences within 12 months after surgery and patients without recurrences for at least 36 months post-surgery. Analysis of differential gene expression of CRLM of these 2 adverse patient groups resulted in the identification of an 11-gene expression profile, able to discriminate between patients with early versus late or no recurrences after partial hepatectomy.

The fact that we were still able to identify two extremes (in terms of time to recurrence) in a clinically homogeneous group confirms the shortcomings of classic clinical risk scoring. The selection of these specific groups provided the opportunity to find molecular differences involved in outcome in a cohort where clinical parameters are incapable to do so. As all patients were chemo naive, true prognostic impact (tumour biology) could be researched. Chemo-naivety ruled out potential influences of the systemic regimens on the RNA expression in the tumour samples. Comparable studies lack true focus on prognostics, since the majority of these patients underwent pre- or postoperative systemic treatment (Ito et al., 2013; Snoeren et al., 2012; Balachandran et al., 2016). The current chemo naïve patient cohort is unique, and the molecular risk profile identified in the current study therefore promising.

There is a strong potential for gene expression based-biomarkers such as the one identified in the current study. The 11-gene signature may serve as a novel blueprint for individualised therapies; either in combination with or without the classic clinical risk scores. Identification of patients for neo-adjuvant (preoperative) therapy is certainly possible since prognostic gene expression profiles may be detected in liquid biopsies before surgery (Mostert et al., 2013, 2015). Currently the clinical risk scores do not impact clinical management, although some retrospective reports have suggested they

Table 1 – Clinicopathological characteristics of patients by recurrence.

		DFS ≤12 Months (N = 33)		DFS ≥36 + No recurrence Months (N = 30)			All patients (N = 63)	
		Value	%/IQR	Value	%/IQR	*P-value*	Value	%/IQR
Male		19	58%	18	60%	0.845[a]	37	59%
Age	Median	67	58–71	63.5	58-72	0.895[b]	65	58–72
Primary tumour								
Location (right sided)		6	18%	4	13%	0.599[a]	10	16%
Rectal cancer		17	52%	12	40%	0.360[a]	29	46%
T stage 3/4		25	76%	23	77%	0.933[a]	48	76%
Positive lymph node (pN+)		17	52%	14	47%	0.701[a]	31	49%
Adjuvant CTx		8	24%	6	20%	0.686[a]	14	22%
Neo-adjuvant RTx		10	32%	6	20%	0.277[a]	16	26%
Liver metastases								
CEA > 200		2	6%	0	0%	0.164[a]	2	3%
Synchronous	DFI < 12	11	33%	9	30%	0.777[a]	20	32%
Diameter > 5 (cm)		6	18%	3	10%	0.354[a]	9	14%
Number of mets > 1		7	21%	6	20%	0.905[a]	13	21%
Bilobar		6	18%	4	13%	0.599[a]	10	16%
R1 resection		5	15%	1	3%	0.110[a]	6	10%
Growth pattern 1	Replacement	23	70%	16	53%	0.284[a]	39	62%
	Desmoplastic	3	9%	7	23%		10	16%
	Pushing	1	3%	0	0%		1	2%
	Mixed	6	18%	7	23%		13	21%
Growth pattern 2	Replacement (any)	28	85%	22	73%	0.259[a]	50	79%
Differentiation	Good	4	13%	2	7%	0.657[a]	6	10%
	Moderate/good	3	10%	6	21%		9	15%
	Moderate	6	19%	5	18%		11	19%
	Poor/moderate	11	36%	11	39%		22	37%
	Poor	7	23%	4	14%		11	19%
Inflammation	Increased	5	16%	5	18%	0.321[a]	10	17%
	Moderate/increased	3	10%	8	29%		11	19%
	Moderate	10	32%	8	29%		18	31%
	Decreased/moderate	6	19%	2	7%		8	14%
	Decreased	7	23%	5	18%		12	20%

DFS = Disease Free Survival; pN+ = Pathological Node Positivity; CTx = Chemotherapy; RTx = Radiotherapy; CEA=Carcinoembryonic Antigen; R1 = Microscopic Irradical.
a Pearson X^2.
b Mann–Whitney U test.

may be effective (Ayez et al., 2015a; Rahbari et al., 2014) (this is prospectively investigated at present in the CHARISMA trial (Ayez et al., 2015b)). There may be a synergistic effect between the clinical risk score and the molecular score of the current study. As all patients developing liver recurrences in the current study were at high molecular risk, the 11-gene signature may also play a role in identifying patients that benefit from regional chemotherapy specifically (e.g. hepatic arterial infusion pump (Kemeny et al., 1999)). Therefore, after thorough validation, the current biomarker may be effective in selecting patient groups for various treatment strategies.

There was no clear link between the mRNA expression profiles and other previously identified pathological features in CRLM, such as the tumour growth patterns. As stated earlier, three types of CRLM growth patterns can be observed: a pushing type, a replacing type and a desmoplastic type (Vermeulen et al., 2001; Van den Eynden et al., 2013). The clinical impact of these growth patterns is still under investigation as their pathological presence is widely recognised. The molecular risk groups of the current study may be associated with a corresponding distinctive phenotype, possibly in the form of any of the established growth patterns. If such apparent tumour phenotypes exist, one could hypothesise that obvious differences may be recognisable at molecular level accordingly. In the current study, there was a trend towards an association between the high molecular risk group and the replacing growth pattern. A replacing growth pattern has repeatedly been associated with worse outcomes as compared to the desmoplastic growth pattern (Eefsen et al., 2015; Nielsen et al., 2014; Pinheiro et al., 2014). In the current study the association is argumentative. A possible explanation for the lack of significance may be that these growth patterns are a specific characteristic of the leading edge of tumours. The gene expression data from the tumour samples in the current study are not exclusively retrieved from tumour tissue present in the leading edge. Currently, gene expression profiles for each of the growth patterns are assessed in an on-going study through laser macro-dissection of representative parts of the tumour.

Some of the functional annotations of the 11 genes in the signature provided insight into underlying biological mechanisms involved in recurrence, yet no evident common pathways could be discerned (see Supplementaries 4 and 5). JARID1A, one of the 11 genes, is part of the "KDM5 family" of histone demethylases removing tri- and di-methylation

Table 2 – Clinicopathological characteristics of patients by molecular risk.

		High risk (N = 37)		Low risk (N = 26)			All patients (N = 63)	
		Value	%/IQR	Value	%/IQR	*P-value*	Value	%/IQR
Male		21	57%	16	62%	0.704[a]	37	59%
Age	Median	64	57–70	68	60–72	0.718[b]	65	58–72
Primary tumour								
Location (right sided)		4	11%	6	23%	0.190[a]	10	16%
Rectal cancer		21	57%	8	31%	0.042[a]	29	46%
T stage 3/4		30	81%	18	69%	0.277[a]	48	76%
Positive lymph node (pN+)		20	54%	11	42%	0.359[a]	31	49%
Adjuvant CTx		7	19%	7	27%	0.452[a]	14	22%
Neo-adjuvant RTx		13	37%	3	12%	0.025[a]	16	26%
Liver metastases								
CEA > 200		2	6%	0	0%	0.222[a]	2	3%
Synchronous	DFI < 12	12	32%	8	31%	0.889[a]	20	32%
Diameter > 5 (cm)		7	19%	2	8%	0.210[a]	9	14%
Number of mets > 1		7	19%	6	23%	0.688[a]	13	21%
Bilobar		5	14%	5	19%	0.541[a]	10	16%
R1 resection		5	14%	1	4%	0.198[a]	6	10%
Growth pattern 1	Replacement	27	73%	12	46%	0.106[a]	39	62%
	Desmoplastic	4	11%	6	23%		10	16%
	Pushing	1	3%	0	0%		1	2%
	Mixed	8	31%	5	14%		13	21%
Growth pattern 2	Replacement (any)	29	78%	21	81%	0.817[a]	50	79%
Differentiation	Good	4	11%	2	9%	0.975[a]	6	10%
	Moderate/good	5	14%	4	17%		9	15%
	Moderate	6	17%	5	22%		11	19%
	Poor/moderate	14	39%	8	35%		22	37%
	Poor	7	19%	4	17%		11	19%
Inflammation	Increased	5	14%	5	22%	0.513[a]	10	17%
	Moderate/increased	5	14%	6	26%		11	19%
	Moderate	11	31%	7	30%		18	31%
	Decreased/moderate	6	17%	2	9%		8	14%
	Decreased	9	25%	3	13%		12	20%

DFS = Disease Free Survival; pN+ = Pathological Node Positivity; CTx = Chemotherapy; RTx = Radiotherapy; CEA = Carcinoembryonic Antigen; R1 = Microscopic Irradical.
a Pearson X^2.
b Mann–Whitney U test.

marks of lysine 4 of histone H3 at transcription start site in actively transcribed genes. We find JARID1A upregulated in patients with early recurrences in the current study which is in line with growing evidence for a causal role of this marker in relation to cancer progression (Rasmussen and Staller, 2014). ERN1 (endoplasmic reticulum to nucleus signalling 1) is an important endoplasmic reticulum (ER) stress sensor. ERN1 signalling is a pro-angiogenic mechanism (Rahbari et al., 2014) and since we found ERN1 increased in patients with early recurrences, angiogenesis may be a contributing factor. Natural killer group 2, member D ligand ULBP2 and

Table 3 – The identified 11-gene signature.

Nr.	Parametric P-value	Fold-change	Unique ID	Name
1	5.53e-05	0.55	ILMN_1786920	JARID1A
2	0.0003634	0.51	ILMN_1698404	ERN1
3	0.0004586	0.47	ILMN_1683082	RPUSD1
4	0.0004769	0.59	ILMN_2067408	CLRN3
5	0.0009447	0.53	ILMN_1668374	ITGB5
6	0.0007405	2.07	ILMN_1678061	CASS4
7	0.0006431	1.79	ILMN_1684183	RAD9A
8	0.0004545	1.8	ILMN_3238676	ULBP2
9	0.0002883	1.98	ILMN_2381758	G3BP2
10	0.0002758	2.03	ILMN_1783636	COX6A1
11	0.0002593	1.87	ILMN_1656042	KIAA0319

P-value, Relative fold change (DFS ≤12 months vs. no recurrence and DFS ≥36 months), ID, Names (annotations) of genes.

Table 4a – Prognostic impact of the molecular risk profile for all site recurrences.

True recurrence	Molecular risk		
	Low	High	Total
No	20	10	30
Yes	6	27	33
Total	26	37	63
Sensitivity			81.8%
Specificity			66.7%
PPV			73%
NPV			76.9%

Pearson X^2: $P < 0.0001$.
PPV=Positive Predictive Value; NPV=Negative Predictive Value.

Table 4b – Prognostic impact of the molecular risk profile for hepatic recurrences.

True recurrence	Molecular risk		
	Low	High	Total
No	26	20	46
Yes	0	17	17
Total	26	37	63
Sensitivity			100%
Specificity			56%
PPV			46%
NPV			100%

Pearson X^2: $P < 0.0001$.
PPV=Positive Predictive Value; NPV=Negative Predictive Value.

Ras-GAP binding protein G3BP2 are two extrinsic stress induced proteins contributing to progression. ULBP2, whose expression is low in patients having an early recurrence and whose receptor is on the surface of natural killer (NK) cells and specific T-cells, implies immune modulation (Ayez et al., 2015b) in recurrence. G3BP2 is known to affect matrix stiffness as does RPUSD1 (RNA pseudouridylate synthase domain containing 1) by controlling lateral growth of collagen II fibrils. G3BP2 and RPUSD1, with decreased and increased expression in the current study respectively, suggest that extracellular remodelling may affect the occurrence of recurrences as well. Potentially connected to the latter we find integrin subunit beta 5 (ITGB5), which is overexpressed in higher stages of CRC (Kemeny et al., 1999) and which modulates adhesion phenomena, and CASS4 the less studied signalling scaffold of the CAS (Crk-associated substrate) family which affects motility. Expression of these genes was elevated (ITGB5) and decreased (CASS4) in patients with early recurrence in the current study implying a role for migration, invasion and possibly progenitor cell function (Nielsen et al., 2014) and inhibition of apoptosis in cancer recurrence as well. The barely studied KIAA0319L and transmembrane protein clarin 3 (CLRN3) as well as COX6A1, which is involved in oxidative phosphorylation, affect recurrence rate but for now we cannot connect these proteins mechanistically to disease progression. Finally, the RAD9A checkpoint protein is required for proper localization of topoisomerase II-binding protein 1 (TopBP1) regulating cell cycle checkpoints, DNA repair, telomere stability and apoptosis (Greer Card et al., 2010; Broustas and Lieberman, 2012; Lieberman et al., 2011) thereby preserving genomic integrity in all types of DNA aberrations (Greer Card et al., 2010; Broustas and Lieberman, 2012; Lieberman et al., 2011). In the current study, RAD9A was relatively downregulated in patients with early recurrences suggesting loss of genomic integrity is another contributing factor to recurrence (Broustas and Lieberman, 2012). Overall, we can conclude that recurrence of metastatic colorectal cancer in the liver is influenced by multiple complementary factors.

Limitations of the current study are its retrospective nature, the selection bias in terms of DFS and a relatively small sample size. Based on the current study, it is challenging to provide advice regarding treatment management for the patient group 36 > DFS > 12. The present molecular marker profile therefore needs extensive validation in a larger independent cohort. This cohort should consist of patients representing the complete (continuous) spectrum in relation to recurrent disease, and possibly (but not necessarily) with both high- and low-clinical risk scores. The current setting with two extremes in terms of recurrences was chosen as a first step in establishing a prognostic signature. If any relevant expression profiles exist in relation to recurrent disease, they are most likely to be identified within these extremes. KRAS and BRAF status would have been informative in terms of assessment of baseline risk for relapse. It is a timely topic of interest in CRLM. These molecular entities were not available in the current cohort. Ideally, in a validation study for the current molecular biomarker, all known prognostic molecular factors should be assessed (including other established signatures) such that all respective molecular markers can be put into context (Passiglia et al., 2016; Karagkounis et al., 2013; Lin et al., 2014; Loes et al., 2016; Margonis et al., 2015; Vauthey et al., 2013; Ito et al., 2013; Snoeren et al., 2012; Balachandran et al., 2016). A general point of discussion related to this type of translational research is the impact of inter- and intra-tumour heterogeneity on the reproducibility of results. Multiple studies show that even within single tumours heterogeneity exists (Marusyk et al., 2012; Tabassum and Polyak, 2015). Despite any consensus on what lesion to analyse (e.g.: the largest) or what area within a tumour (e.g.: leading edge or core), heterogeneity will affect the generated results. Interestingly, these features of heterogeneity are known to have prognostic associations by itself in resected colorectal liver metastases (Sveen et al., 2016). Future studies should possibly also address spatial and temporal tumour heterogeneity, in addition to identification of a new biomarker.

5. Conclusion

In summary, in the current study a prognostic signature was constructed with the mRNA expression profiles of tumour tissue from resected CRLM. The signature consists of 11 genes of which the expression-patterns were able to discriminate between patients with early recurrences (≤12 months) versus no recurrences (≥36 months) after partial hepatectomy. This biomarker requires validation in a larger cohort representative of the complete clinical spectrum in terms of relapse and treated without (neo-) adjuvant therapy, including any other established prognostic molecular markers.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.09.002.

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