

# Comprehensive genomic analysis contrasting primary colorectal cancer and matched liver metastases: a retrospective study

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## Research article

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# Abstract

## (Background)

Recent studies have revealed that colorectal cancer (CRC) displays intratumor genetic heterogeneity, and the cancer microenvironment play an important role in the proliferation, invasion, and metastasis of CRC.

## (Methods)

We performed genomic analysis on 22 paired patients with primary CRC and synchronous colorectal liver metastasis (CRLM) using whole-exome sequencing, cancer gene panels, and microarray gene expression profiling.

In addition, immunohistochemical analysis was used to confirm the protein expression of genes identified as highly expressed in CRLM by DNA microarray analysis.

## (Results)

A total of 22 paired patients were enrolled in this study, including 13 (59.1%) colon and 9 (40.9%) rectal cancer patients. All patients had synchronous liver metastasis and did not have any other extrahepatic metastases.

We identified 11 probes (10 genes) that were highly expressed in CRLM compared to CRC, from 36022 probes obtained from primary CRC, CRLM, and normal liver tissues, using gene expression analysis with DNA microarray.

Of the 11 probes (10 genes), 5 genes classified as encoding “matricellular proteins” (osteopontin, periostin, thrombospondin-2, MGP, and GPNMB) were selected for immunohistochemical analysis.

Osteopontin was strongly expressed in CRLM (6 of 22 cases: 27.3%) but not in CRC (0 of 22: 0%;  $p=0.02$ ). Periostin also showed strong immunoreactivity in CRLM (17 of 22: 68.2%) compared to 7 of 22 (31.8%) in CRC ( $p=0.006$ ). Thrombospondin-2 showed strong immunoreactivity both in CRC and CRLM (54.5% in CRC, 45.5% in CRLM;  $p=0.55$ ). GPNMB and MGP were rarely positive for both CRC and CRLM. A comparison of immunoreactive positive factors for these 5 genes revealed the complexities of gene expression in CRLM. Of the cases, 16 (72.7%) CRC revealed zero or only one positive immunoreactivity. On the other hand, CRLM revealed more frequent multiple immunoreactivity; 16 cases (72.7%) shared 2 or more factors, which was statistically more frequent in CRLM than in CRC ( $p=0.007$ ).

## (Conclusions)

This study revealed the genomic heterogeneity between paired primary CRC and CRLM in terms of cancer cell microenvironment. This finding will lead to new diagnostic and therapeutic targets in the era of genome-guided personalized cancer treatment.

(Trial registration)

This study was retrospectively registered.

## Background

Colorectal cancer (CRC) is a common malignancy; it has the third highest incidence rate among all cancers and is the second leading cause of cancer-related deaths worldwide [1]. Despite advances in the understanding of the clinicopathological features of CRC as well as improved diagnosis and treatment, mortality from CRC is expected to continue to increase, with approximately 700,000 deaths each year [2]. Unfortunately, at the time of diagnosis, 18.2% of patients with primary CRC had concurrent distant metastases, with the highest frequency in liver (10.7%), peritoneum (5.0%), and lung (1.6%) [3]. The prognosis of patients with liver metastasis from colorectal cancer (CRLM) has dramatically improved with the availability of new effective cytotoxic and targeted agents as well as aggressive surgical resection. Moreover, recent remarkable improvements in innovative agents have led to an increased response rate in unresectable CRLM. The surgical resection rate after downstaging in initially unresectable CRLM has been reported to be up to 40% (so called “conversion therapy”) [4]. In spite of these developments, the oncological outcomes for patients with CRLM remains unsatisfactory; CRLM recurrence after hepatectomy is common, with a 50% rate in remnant liver [5–7].

Advances in molecular biology over the past decade have enabled a greater understanding of the development of several kinds of cancers, and a more precise use of innovative targeted therapies. Recent studies have revealed that several cancers display intratumor genetic heterogeneity. Given the intratumor genetic heterogeneity described in several cancers, the metastatic process itself may result in clonal selection in the progression from primary to metastatic disease. The proliferation, invasion, and metastasis of cancer cells are not defined by the nature of the cancer cell itself, but rather by the adaptation of the microenvironment via interactions between the cancer cell and its surrounding tissues [8, 9].

Elucidation of intratumor genetic heterogeneity will lead to new diagnostic and therapeutic methods for CRC. Herein, we performed molecular analysis on paired patients with primary CRC and synchronous CRLM, resected at the same institute. In this study, we investigated the molecular characteristics using whole-exome sequencing, cancer gene panels, fusion gene panels and microarray gene expression profiling. A notable feature of this study is that it fully matches all clinicopathological data, as the cases are from the same medical institution. Most previously reported studies used metachronous or unpaired patient samples. Such analysis could be biased by intertumoral heterogeneity and the administration of chemotherapy between the resection of primary CRC and metastatic sites, which might alter the genetic clones. Moreover, the studies used a limited set of biomarkers.

To our knowledge, no other study has analyzed such a completely paired sample of primary CRC and synchronous CRLM using a major complement of exhaustive genetic analysis with next-generation sequencing.

## Methods

### Clinical data

Surgically resected tumor specimens and corresponding peripheral blood samples were obtained from 22 consecutive patients who underwent both colectomy and hepatectomy for CRC and synchronous CRLM between January 2014 and March 2015. All patients were enrolled in Project HOPE (High-tech Omics-based Patient Evaluation), a study launched at Shizuoka Cancer Center with the aim of evaluating the biological characteristics of cancer by multiomics-based analyses [10]. The clinicopathological data of patients were retrospectively reviewed. A prospective colorectal database, containing information regarding patient characteristics, preoperative assessment, operative characteristics, postoperative complications, pathological characteristics, and oncological outcomes, maintained at the hospital was used for this retrospective analysis.

### Project HOPE (High-tech Omics-based Patient Evaluation)

In the present study, we evaluated fresh frozen tumor tissues obtained from both primary CRC and CRLM using whole-exome sequencing (WES), cancer gene panel sequencing, fusion gene panel sequencing and microarray-based gene expression profiling (GEP). We also acquired peripheral blood samples from patients and paired them with the corresponding resected tissue samples from the HOPE study. DNA was extracted from blood and tissues by the same method using a QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany), except that tissues were treated with Proteinase K (QIAGEN) [11]. A board-certified pathologist determined that the tumor purity was > 50%. DNA was quantified using Nano-drop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

### Ethical considerations

The research plan of Project HOPE was designed according to the revised Ethical Guidelines for Human Genome/Gene Analysis Research in Japan ([http://www.lifescience.mext.go.jp/files/pdf/n1115\\_01.pdf](http://www.lifescience.mext.go.jp/files/pdf/n1115_01.pdf)), and the study protocol was approved by the Institutional Review Board of Shizuoka Cancer Center (approval number #25–33). Written informed consent was obtained from all participants. This retrospective study was also approved by the same board (approval number #30-5-30-1-2).

### Next-generation sequencing

The exome library used for WES was prepared using an Ion Torrent AmpliSeq RDY Exome Kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. A total of 100 ng of DNA was used for target amplification under the following conditions: 99 °C for 2 min, followed by 10 cycles at 95 °C for 15 s and 60 °C for 16 min, and a final hold at 10 °C. Incorporated primer sequences were partially digested using a proprietary method. Ion Torrent Proton adapters were ligated to the amplicons at 22 °C for 30 min, followed by 72 °C for 10 min, and the library was purified with Agencourt Ampure XT beads (Thermo Fisher Scientific). Libraries were quantified using a quantitative polymerase chain reaction (qPCR), and DNA (8 pM) was sequenced using the semiconductor DNA sequencer (Ion Torrent Proton

Sequencer, Thermo Fisher Scientific) according to the manufacturer's protocol. Matched tumor–normal pair somatic variants were identified using Ion Reporter ver. 4.4 software (Thermo Fisher Scientific) [12] after base calling, quality trimming, and mapping to the hg19/GRCh37 reference genome using Torrent Suite software ver. 4.4 (Thermo Fisher Scientific) [13]. In this step, sequence data derived from tumor and blood samples were analyzed separately, and the latter were used as matched controls. In this process, only somatic variants remain after the subtraction of variants from blood data from those acquired from tumor data. In this variant-call workflow, we identified somatic mutations that satisfied the thresholds quality score  $\geq 60$  or depth of coverage  $\geq 20$ . Somatic variants were manually inspected using the Integrative Genomics Viewer [14]. Annotation of detected single nucleotide variants (SNVs) was performed using databases that included germline and somatic variants as follows: COSMIC [15], ClinVar [16], dbSNP [17], UniProt [18] and DrugBank [19].

## Comprehensive gene expression analysis using DNA microarray

Fresh tumor and adjacent normal tissues were soaked in RNAlater reagent (Thermo Fisher Scientific), and total RNA was isolated and purified using a miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. RNA quality was evaluated by RNA integrity number, which was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples with an RNA integrity number  $\geq 6.0$  were used for gene expression analysis. Gene expression analysis was performed on a SurePrint G3 Human Gene Expression 8  $\times$  60K v2 Microarray (Agilent Technologies) using a One-color Low Input Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's instructions. Data processing to generate raw signal intensity data was performed with GeneSpring version 13.1.1 software (Agilent Technologies). The raw signals were log-transformed and normalized (GeneSpring software, Agilent Technologies), and the difference in the normalized microarray signal intensities (fold change) between the tumor and adjacent normal tissues was calculated.

## Immunohistochemical analysis

Routine pathological diagnosis was achieved using surgically resected tumors fixed in 10% formalin and embedded in paraffin. Paraffin sections (3  $\mu\text{m}$  thickness) containing representative histology of the tumor were used for immunohistochemical analysis. Immunohistochemical staining was performed using the Bond III automated stainer and BOND Polymer Refine Detection kit (Leica Biosystems, Bannockburn, IL, USA). The sections were pretreated with Bond Epitope Retrieval Solution for 20 min at 100 °C and then reacted with the primary antibodies (Bond Epitope Retrieval Solution 1 for osteopontin and MGP; Bond Epitope Retrieval Solution 2 for periostin, thrombospondin 2, and GPNMB). After reaction with diaminobenzidine chromogen, the sections were counterstained with hematoxylin. The stained sections were independently evaluated by two investigators (A.S. and T.S.) blinded to patient data. Primary antibodies for osteopontin, periostin, thrombospondin-2, and glycoprotein nonmetastatic melanoma protein B (GPNMB) were obtained from Abcam (Cambridge, UK). Primary antibodies for matrix gla protein (MGP) were obtained from Thermo Fisher Scientific.

# Statistical analysis

Parametric variables are reported as medians (range). For comparisons between two groups, the chi-squared and Wilcoxon signed rank test were used. A *p*-value < 0.05 was considered to be significant. All statistical analyses were performed using IBM SPSS Statistics software for Windows, version 24 (SPSS Inc., Chicago, IL, USA).

## Results

### Patients' characteristics

The data set consisted of 22 pairs of primary CRC and CRLM. The patients' characteristics are shown in Table 1. A total of 22 paired patients were enrolled in this study, including 13 (59.1%) colon and 9 (40.9%) rectal cancer patients. Right-sided colon was defined as caecum, ascending, and transverse colon. Left-sided colon was defined as descending and sigmoid colon. All patients had synchronous liver metastasis and did not have any other extrahepatic metastasis. Patients with multiple cancers such as synchronous or metachronous malignancy (within 5 years) other than carcinoma in situ, familial adenomatous polyposis, and appendiceal cancer were excluded. The median number of CRLM of patients was 2 (1–14). The median size of CRLM was 30 (17–110) mm. Of the cases, 21 of 22 (95.5%) received two-stage hepatectomy. The median interval between resection of primary CRC and CRLM was 6 (0–15) weeks. No patients received adjuvant/neoadjuvant chemo/chemoradiotherapy before and after the resection for primary CRC or hepatectomies for CRLM.

### Comparison of tumor mutation burden between primary CRC and CRLM

The tumor mutation burden (TMB), also referred to as “mutation load”, represents the number of single nucleotide variations (SNV) per mega base, and has received increasing attention owing to its potential responses to immune checkpoint inhibitors. TMB was determined for the 22 paired samples (Fig. 1). The median TMB in primary CRC was 2.8 (0.9–6.4), whereas it was 3.8 (2.0–6.6) in CRLM. The TMB counts in CRLM tended to be more frequent than those of primary CRC, although there were no statistically significant differences (*p* = 0.07).

### Gene mutation profiling

Genomic alterations contributing to tumorigenesis were analyzed using an analysis pipeline called “Shizuoka Multi-omics Analysis Protocol (SMAP) [10]” developed in-house in primary CRC and CRLM. Detected genomic alterations are listed in Table 2. In primary CRC, the alteration of TP53, APC, and KRAS was detected in 16 (72.7%), 14 (63.6%), and 9 (40.9%) cases, respectively. In CRLM, the alteration of TP53, APC, and KRAS was detected in 16 (72.7%), 14 (63.6%), 9 (40.9%) cases, respectively. Mutations in KDM6A, FAT3, FBXW7, and LAMA5 were specific to CRLM.

### Concordance of mutations in matched pairs of primary CRC and CRLM

The concordance of genomic alterations between paired primary CRC and CRLM varied from 15.8 to 80.6% in each paired case (median concordance, 49.2%; Fig. 2A). The mutational concordance of KRAS, which is known as an important factor related to tumor metastasis was 100%. On the other hand, the concordance of APC was only 59.1% (Fig. 2B).

## Gene expression analysis using DNA microarray

To select candidate genes that showed significantly higher expression in CRLM than primary CRC, gene expression was compared using the average expression value of 36022 probes in primary CRC, CRLM, and normal liver tissues (i.e., non-cancerous tissues of liver metastasis cases). First, we selected 1794 probes whose expression was up to 1.5 times higher in CRLM than primary CRC. Second, to remove contamination of normal liver tissues in liver metastasis samples, we compared expression levels in primary CRC with those in non-cancerous liver tissues and selected 198 probes whose expression was higher in primary CRC than normal liver. Third, we chose 20 probes (19 genes) as highly expressed probes in CRLM with a normalized ratio  $> 1.0$ . Finally, we examined the difference in expression level of these 20 probes in paired samples of CRC and CRLM, and selected 11 probes (10 genes) that showed significantly higher expression in CRLM than CRC (Table 3 and Fig. 3). Figure 4A shows the characteristic expression profiles of the 11 probes in the 22 paired cases. Figure 4B shows the comparison of gene expression between primary CRC and CRLM; all 11 probes showed significantly higher expression in CRLM (Wilcoxon signed rank test,  $p < 0.001$ ).

## Immunohistochemical analysis

Immunohistochemical analysis was performed to confirm the protein expression of genes identified by DNA microarray analysis. Among the 10 genes that were highly expressed in CRLM, 5 genes classified as encoding “matricellular proteins”, i.e., osteopontin, periostin, thrombospondin-2, MGP, and GPNMB, were selected for this analysis. GPNMB is a type I transmembrane protein with three unique domains: an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain is composed of two regions with distinct properties: the integrin-binding motif and the polycystic kidney disease domain. Thus, we included GPNMB as a matricellular protein in this study. Representative images of immunohistochemical staining are shown in Fig. 5. First, immunoreactive scores were defined independently according to the intensity of staining and the proportion of stained structures. Staining intensity was scored as: 0, no staining; 1, weak staining; and 2, strong staining. Proportions of stained tumor cells / extracellular matrix (ECM) were classified as: 0,  $\leq 5\%$  positive cells; 1, 6–25% positive cells; 2, 26–50% positive cells; and 3,  $\geq 51\%$  positive cells. Total scores for intensity and proportion were used to signify the levels of protein expression. A score of  $\leq 3$  was considered to represent negative expression, and a score of  $\geq 4$  was considered positive expression in this study. Table 4 shows the positive immunoreactivity scores for these 5 proteins.

Osteopontin showed strong immunoreactivity in tumor cells and ECM of CRLM (6 of 22 cases: 27.3%) compared to no immunoreactivity in primary CRC (0 of 22 cases: 0.0%). The immunoreactivity score was significantly higher in CRLM than primary CRC ( $p = 0.02$ ).

Periostin also showed strong immunoreactivity in the ECM of CRLM (17 of 22 cases: 68.2%) compared to that of primary CRC (7 of 22 cases: 31.8%). Periostin showed no immunoreactivity in the tumor cells of primary CRC and CRLM. Periostin was not expressed with high frequency in primary CRC. However, when expressed, it showed strong immunoreactivity in the peripheral invasive part of primary lesions.

Thrombospondin-2 showed strong immunoreactivity in tumor cells and ECM of both primary CRC and CRLM, and there were no statistically significant differences ( $p = 0.55$ ).

GPNMB showed weak immunoreactivity in tumor cells but strong immunoreactivity in ECM of both primary CRC and CRLM. These expressions were also observed in macrophages. However, there were no statistically significant differences.

MGP showed strong immunoreactivity in ECM of primary CRC (3 of 22 cases: 13.6%) and CRLM (4 of 22 cases: 18.2%). No statistically significant differences were observed.

#### Comparison of number of immunoreactive factors

To determine whether differences in the complexity of expression patterns between primary CRC and CRLM can be verified, and to determine genomic heterogeneity, we compared the number of positive immunoreactivities for 22 pairs of primary CRC and CRLM (Table 5). Sixteen cases (72.7%) of primary CRC showed zero or one positive immunoreactivity, and 6 case (27.3%) revealed positivity for 2 or more factors. On the other hand, CRLM revealed more frequent multiple immunoreactivities, 16 cases (72.7%) shared 2 or more factors, which was significantly more frequent in CRLM than in primary CRC ( $p = 0.007$ ).

## Discussion

In the present study, we clearly demonstrated genomic heterogeneity between paired primary CRC and CRLM by using a large complement of exhaustive genetic analyses with next-generation sequencing. No other study has analyzed such a completely paired sample of primary CRC and synchronous CRLM. Elucidation of the heterogeneity of microenvironment-related factors on the proliferation, invasion, and metastasis of cancer cells will lead to novel diagnostic and therapeutic targets for CRC in the era of genome-guided personalized cancer treatment.

Recent advances in next-generation sequencing technologies have made it possible to analyze large numbers of sequences, leading to international cancer genome analysis projects such as the Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). CRC can be classified into four gene expression-based subtypes with distinguishing features, the consensus molecular subtypes (CMSs): CMS1 (microsatellite instability immune, 14%), CMS2 (canonical, 37%), CMS3 (metabolic, 13%), and CMS4 (mesenchymal, 23%) [20]. This intertumoral heterogeneity has led to the finding that different subtypes of CRC are represented by different genetic makeup, clinical behavior, pathological features, and responses to treatment [21–23].

In addition to the intertumoral heterogeneity mentioned above, intratumoral heterogeneity relates to the genetic heterogeneity between cancer cells within a single tumor. During carcinogenesis, genetic abnormalities accumulate continually, allowing the cells an increased ability to expand and invade. As a result of this continuous process, cancers become genetically heterogeneous, with an indeterminate number of coexisting genomic clones. These clones have different functional characteristics such as the ability to form metastases or respond to chemotherapy.

Cancer cells survive and proliferate in a microenvironment created by the cells themselves, various stromal cells, and the stromal tissue. The stromal cells that form cancer tissues include fibroblasts, vascular and lymphangial endothelial cells, lymphocytes, and macrophages. Both cells interact with cancer cells, imparting their characteristic biological features on the cancer [24, 25]. Distant metastasis of cancer has also been implicated in interactions between cancer cells and stromal cells in the cancer microenvironment [8, 9].

In this study, we determined that many highly expressed genes by DNA microarray analysis were classified as encoding “matricellular proteins”, which interact with the ECM. Periostin is a secreted adhesion-related protein expressed in the periosteum and periodontal ligaments, which acts as a critical regulator of the formation and maintenance of bone and teeth, and also plays an important role in tumorigenesis [26]. Recent studies have shown that periostin is highly expressed in various human cancers and have suggested that periostin promotes tumor growth and metastasis [27–29]. Moreover, periostin is reported to enhance the metastatic growth of colon cancer by both preventing stress-induced apoptosis in cancer cells and augmenting endothelial cell survival to promote angiogenesis. The expression level of periostin is reported to be noticeably higher in metastatic tumors than that in the matched primary colon cancer [30].

Osteopontin is a multifunctional ECM phosphorylated glycoprotein (glycol-phosphoprotein) classified into the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family, and is reported to play an important role in the tumorigenesis, progression and prognosis of various cancers by regulating cell-matrix interactions and cell signaling through binding with integrins and CD44 receptors [31–36]. A pooled data analysis showed that high osteopontin expression was significantly associated with high tumor grade, invasion, lymph node metastasis, tumor distant metastasis and poor survival in CRC [37].

Thrombospondin-2 (THBS2) is a member of the ECM glycoproteins that mediate ECM assembly, cell-matrix interactions, degradation of matrix metalloproteinases (MMP)-2 and MMP-9, and interact with multiple cell receptors and growth factors. The implication of THBS2 expression in CRC has been controversial. Several studies reported an inverse correlation between THBS2 expression level and malignancy grade [38, 39]. In contrast, recent studies reported that THBS2 expression in CRC was positively correlated with TNM stage and is a strong prognostic indicator [40, 41].

GPNMB gene is reported to be overexpressed in numerous cancers and is often associated with the metastatic phenotype [42–46]. The extracellular domain of GPNMB interacts with integrins to facilitate the recruitment of immune-suppressive and proangiogenic cells to the tumor microenvironment, thereby enhancing tumor migration and invasion [47]. GPNMB expressed in immune cells such as macrophages and dendritic cells [14,29] may impair T-cell activation to down-modulate anti-tumor immune responses [48–50]. However, the role of GPNMB is complex; it appears to have an inhibitory role in some cancers but may promote metastasis in others.

MGP is an ECM protein containing post-translationally modified γ-carboxyglutamate residues due to vitamin K-dependent carboxylation. MGP was initially thought to be involved in the inhibition of

calcification of arteries and cartilage. Further investigation demonstrated that MGP had a wider range of activities, which were dependent of the phosphorylation-carboxylation status, protein expression and variants. Recent studies showed that MGP has a role in tumor angiogenesis by increasing vascular endothelial growth factor gene expression [51–53]. Recently, MGP was reported to be upregulated in a variety of tumors, including ovarian, breast, urogenital and skin cancer. However, in colon and lung cancers, an inverse correlation between MGP expression and survival was observed [54].

In the present study, exhaustive genetic analysis using next-generation sequencing and comparison of immunoreactive factors revealed the complexities of gene expression in CRLM. It is especially notable that CRLM has greater genomic heterogeneity associated with the ECM compared to primary CRC. This result suggests our hypotheses. To proliferate in the liver, which differs environmentally from the original colorectal tissue in which they naturally exist, cancer cells must modify their microenvironment to make it more amenable for survival. A suitable microenvironment cannot be regulated by a single factor; instead, complex factors are involved, especially in metastatic sites. The complexity of intratumor heterogeneity, which we revealed in this study, may be an underlying cause of the resistance to treatment for metastatic disease.

Differences in genomic mutational profiling between primary and metastatic sites have been investigated in several studies [55–57]. However, most studies have used metachronous or unpaired patient samples. Moreover, a limited set of biomarkers was employed to show that metastatic sites have more inherited mutations than primary CRC. These studies have potential biases related to both intra- and intertumoral heterogeneity.

It has been reported that the several kinds of systemic chemotherapy could alter the genomic landscape in several cancers [58, 59]. Therefore, adjuvant chemotherapy has the potential to alter the genomic mutational profiles of recurrent cancers, so that they would differ from those of primary tumors [60]. Previous analyses evaluating the heterogeneity of metachronous tumors could be biased by the administration of chemotherapy between the resection of primary CRC and metastatic sites, potentially producing alterations in genomic clones [55, 61]. Our study eliminates this bias since we selected paired samples of synchronous tumors.

This study has two important limitations. First, the study included a relatively small number of patients. We plan to continue our analysis using an increased number of cases in the future. Next, although the gene expression of thrombospondin-2, GPNMB, and MGP in CRLM was more frequent than in primary CRC according to the DNA microarray analysis, the immunohistochemical analysis revealed no differences in expression. We suggest that the discrepancy between the tissue regions analyzed by DNA microarray and those subjected to immunohistochemical analysis led to this result. The relatively low immunoreactivity scores of GPNMB and MGP may be attributed to the timing of degradation and wash-out of these proteins from the tissue [53].

There are three future perspectives from this study. First, we also obtained data of surgically resected tumor specimens and corresponding peripheral blood samples not only for CRLM, but also pulmonary

metastasis, peritoneal metastasis, ovarian metastasis and so on, through WES, cancer gene panel sequencing, fusion gene panel sequencing and microarray-based GEP under the framework of “project HOPE”. Thus, we will be able to perform further investigations using these samples. Second, studied of circulating tumor cells (liquid biopsy) may be useful in establishing early CRLM diagnosis. Additionally, it may lead to better predictive biomarkers to identify patients who might benefit from adjuvant chemotherapy. Third, the genes that were overexpressed in this study have the potential to be new therapeutic targets. For example, administration of anti-periostin antibody significantly inhibited the growth of primary tumors as well as metastatic tumors in a murine model of breast cancer [26]. Osteopontin-inhibition is also reported to be a favorable therapeutic approach to metastatic disease [62–65]. An antibody-drug conjugate targeting GPNMB, called glembatumumab vedotin (CDX-011), is currently in clinical studies for various cancers [66].

## Conclusions

In conclusion, we revealed the genomic heterogeneity between paired primary CRC and CRLM in terms of the microenvironment on the proliferation, invasion, and metastasis of cancer cells. This will lead to new diagnostic and therapeutic targets for CRC in the era of genome-guided personalized cancer treatment.

## Abbreviations

CRC  
colorectal cancer  
CRLM  
colorectal liver metastasis  
Project HOPE  
High-tech Omics-based Patient Evaluation  
WES  
whole-exome sequencing  
GEP  
gene expression profiling  
SNV  
single nucleotide variants  
GPNMB  
glycoprotein nonmetastatic melanoma protein B  
MGP  
matrix gla protein  
THBS2  
thrombospondin-2  
TMB  
tumor mutation burden  
SMAP

Shizuoka Multi-omics Analysis Protocol

ECM

extracellular matrix

CMS

consensus molecular subtypes

TCGA

the Cancer Genome Atlas

ICGC

the International Cancer Genome Consortium

SIBLING

small integrin-binding ligand N-linked glycoprotein

MMP

matrix metalloproteinases

## Declarations

### Ethics approval and consent to participate

The research plan of Project HOPE was designed according to the revised Ethical Guidelines for Human Genome/Gene Analysis Research in Japan ([http://www.lifescience.mext.go.jp/files/pdf/n1115\\_01.pdf](http://www.lifescience.mext.go.jp/files/pdf/n1115_01.pdf)), and the study protocol was approved by the Institutional Review Board of Shizuoka Cancer Center (approval number #25-33).

Written informed consent was obtained from all participants.

This retrospective study was also approved by the same board (approval number #30-5-30-1-2).

### Consent for publication

Consent for publication were obtained from all participants.

### Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interest.

### Funding

We obtained no funding for this study.

## **Authors' contributions**

Akio Shiomi, Masatoshi Kusuhara and Takashi Sugino designed the study.

Akio Shiomi and Teiichi Sugiura enrolled patients.

Akio Shiomi, Masatoshi Kusuhara, Takashi Sugino, and Takeshi Nagashima analysed and interpreted the data.

Akio Shiomi drafted the manuscript.

All authors reviewed and revised the paper and approved the submitted version.

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## Tables

Table 1. Patient characteristics (n=22)

Gender		
	Male	12
	Female	10
Age (year)		64.5 (27-86)
	<50	3
	≥50	19
Primary tumor site		
	Colon	13
	Right side	5
	Left side	8
	Rectum	9
CEA (ng/ml)		24.7 (1.8-1421)
		7
	>5.0	15
Histological type of primary site		
	Differentiated	21
	Undifferentiated	1
pT stage		
	pT1	0
	pT2	1
	pT3	9
	pT4	12
pN stage		
	pN0	3
	pN1	9
	pN2	10
Synchronous presentation of CRLM		
	Absent	22

	Present	0
Lymphatic invasion		
Absent		12
Present		10
Venous invasion		
Absent		4
Present		18
Median number of CRLM		2 (1-14)
Largest size of CRLM (cm)		30 (17-110)
Multiple CRLM		
Absent		9
Present		13
Extrahepatic disease		
Absent		22
Present		0
Neoadjuvant chemo(radio)therapy		
Absent		22
Present		0

Values for Age, CEA, and Largest size of CRLM are provided as the median (range).

CEA: carcinoembryonic antigen, CRLM: colorectal liver metastasis

Table 2. Gene mutation profiling of primary CRC and CRLM

CRC	n	CRLM	n
TP53	16	TP53	16
APC	14	APC	14
KRAS	9	KRAS	9
CSMD3	5	CSMD3	5
EPHA5	3	EPHA5	4
FAT4	3	FAT4	4
FBXW7	3	<b>KDM6A</b>	4
KMT2D	3	<b>FAT3</b>	3
PIK3CA	3	<b>FBXW7</b>	3
TERT	3	<b>LAMA5</b>	3
TSHZ2	3	TERT	3
		TSHZ2	3

Bold letters indicate gene mutations found only in liver metastases

CRC: colorectal cancer CRLM: colorectal liver metastasis

Table 3. Gene expression analysis using DNA microarray

Gene Symbol	Gene name	Location	Variance	Variance	Bonferroni
			F value	tvalue	<0.05/20=0.0025
SPP1	Osteopontin	ECM	55.8088	0.0001	0.0025
TIMP1	tissue inhibitor of metalloproteinase 1	secreted	48.9451	0.0001	0.0025
THBS2	Thrombospondin-2	ECM	25.8758	0.0001	0.0025
POSTN	periostin	ECM	24.8512	0.0001	0.0025
GPNMB	glycoprotein nonmetastatic melanoma protein B	membrane (with ECM domain)	24.5809	0.0001	0.0025
MGP_2	Matrix Gla protein	ECM	19.8335	0.0001	0.0025
MGP	Matrix Gla protein	ECM	19.1511	0.0001	0.0025
COL10A1	Collagen type X alpha 1 chain	ECM	13.0898	0.0008	0.0025
CCDC80	Coiled-coil domain containing 80	cytosol	12.5409	0.001	0.0025
CCDC146	Coiled-coil domain containing 146	nucleus	11.3416	0.0016	0.0025
CCL18	Chemokine (C-C motif) ligand 18	secreted	10.4601	0.0024	0.0025

Table 4A. Expression status of each factor by immunostaining

	Positive		Negative		<i>p</i> -value
	n	%	n	%	
<b>Osteopontin</b>					
primary CRC	0	0	22	100	0.02
CRLM	6	27.3	16	72.7	
<b>Periostin</b>					
primary CRC	7	31.8	15	68.2	0.006
CRLM	17	68.2	5	31.8	
<b>Thrombospondin-2</b>					
primary CRC	12	54.5	10	45.5	0.55
CRLM	10	45.5	12	54.5	
<b>GPNMB</b>					
primary CRC	4	18.2	18	81.8	1
CRLM	5	22.7	17	77.3	
<b>MGP</b>					
primary CRC	3	13.6	19	86.4	1
CRLM	4	18.2	18	81.8	

CRC: colorectal cancer CRLM: colorectal liver metastasis

Table 5. Comparison of number of positive immunoreactivities between primary CRC and CRLM

	Primary CRC (n=22)	CRLM (n=22)	<i>p</i> -value
0 or 1 factor	16 (72.7%)	6 (27.3%)	0.007
2 or more factors	6 (27.3%)	16 (72.7%)	

CRC: colorectal cancer CRLM: colorectal liver metastasis

## Figures

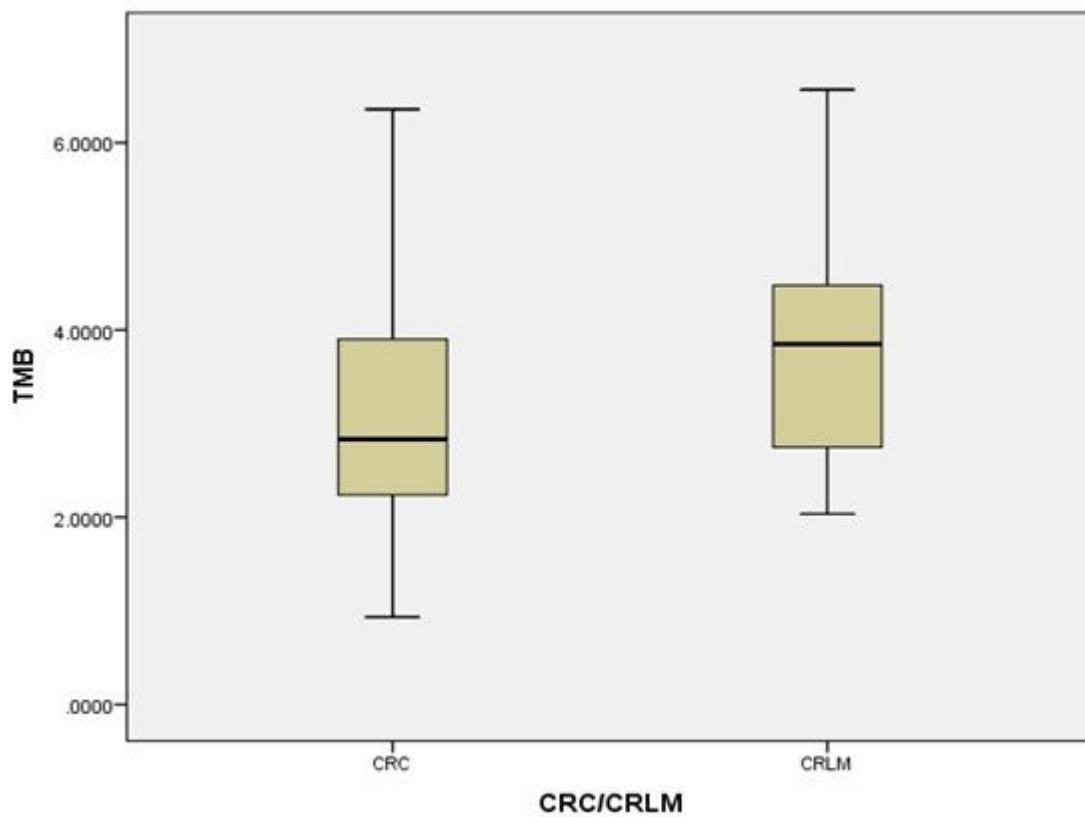
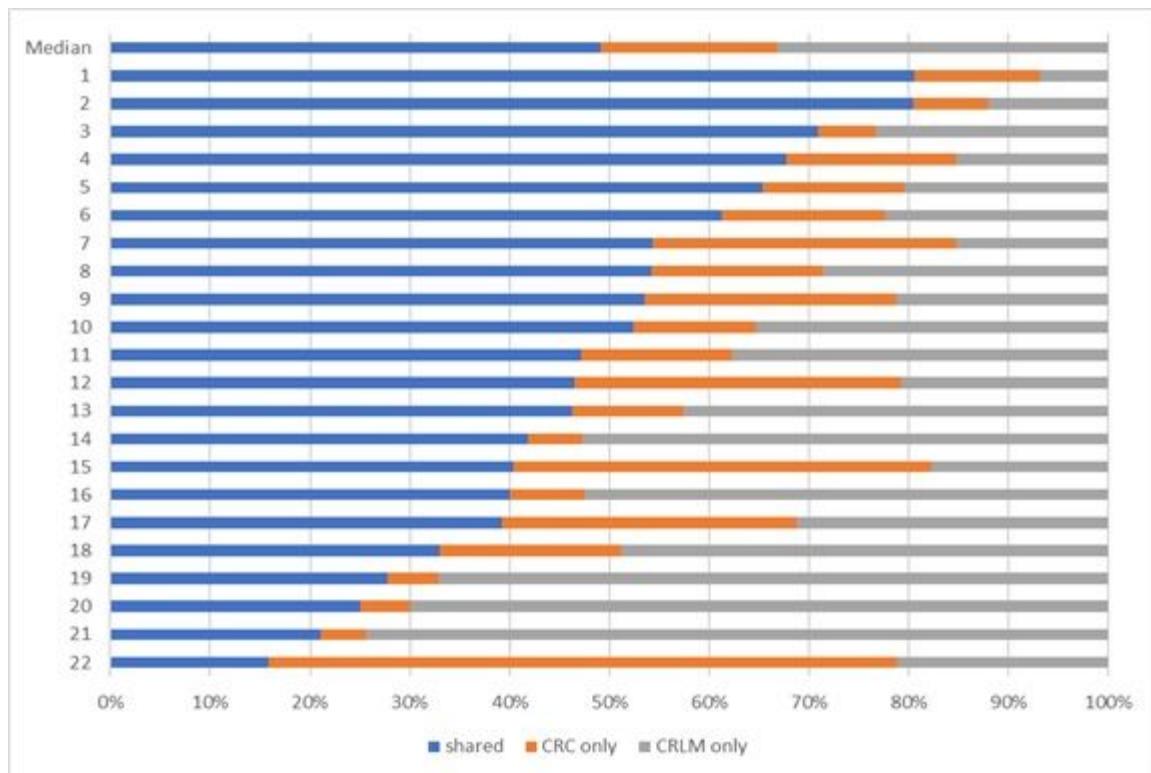


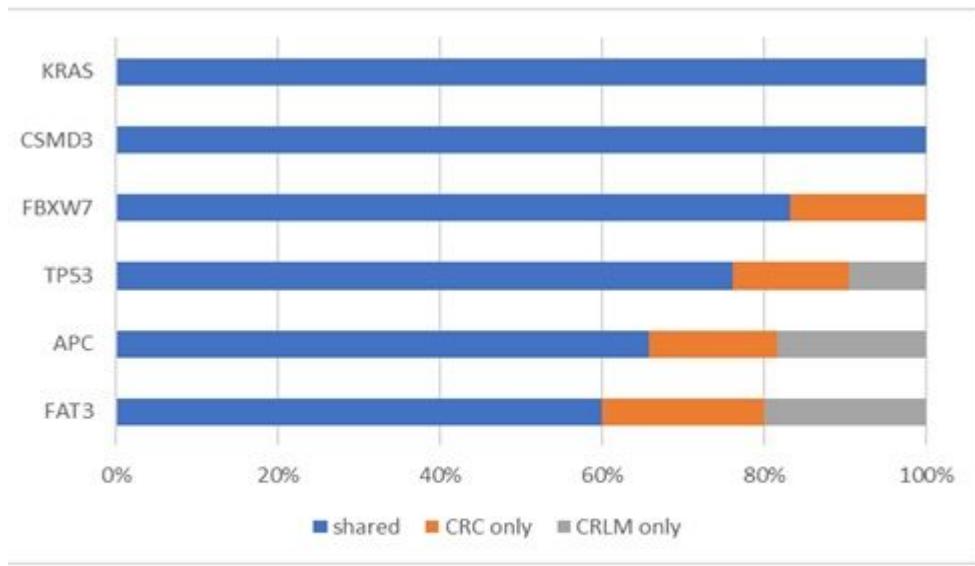
Figure 1

Comparison of tumor mutation burden between primary CRC and CRLM



**Figure 2**

Concordance of genomic mutations in matched pairs of primary CRC and CRLM



CRC: colorectal cancer CRLM: colorectal liver metastasis

**Figure 3**

Concordance of genomic mutations in matched pairs of primary CRC and CRLM

36022 probes

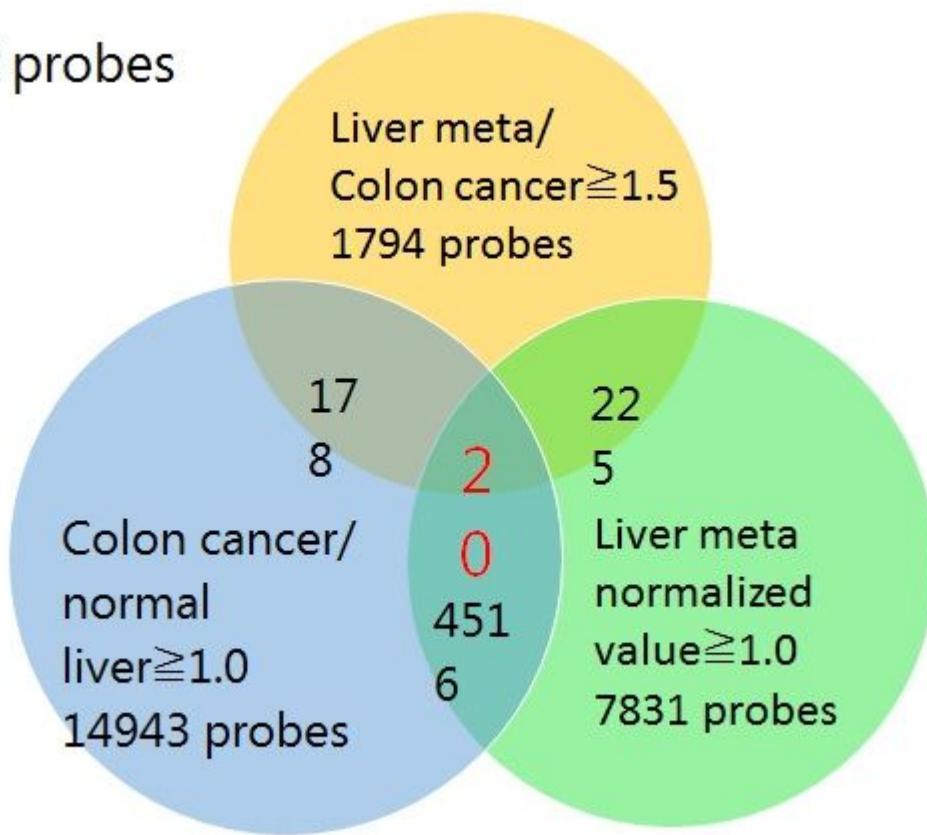


Figure 4

Selection of candidate genes showing significantly higher expression in CRLM than CRC

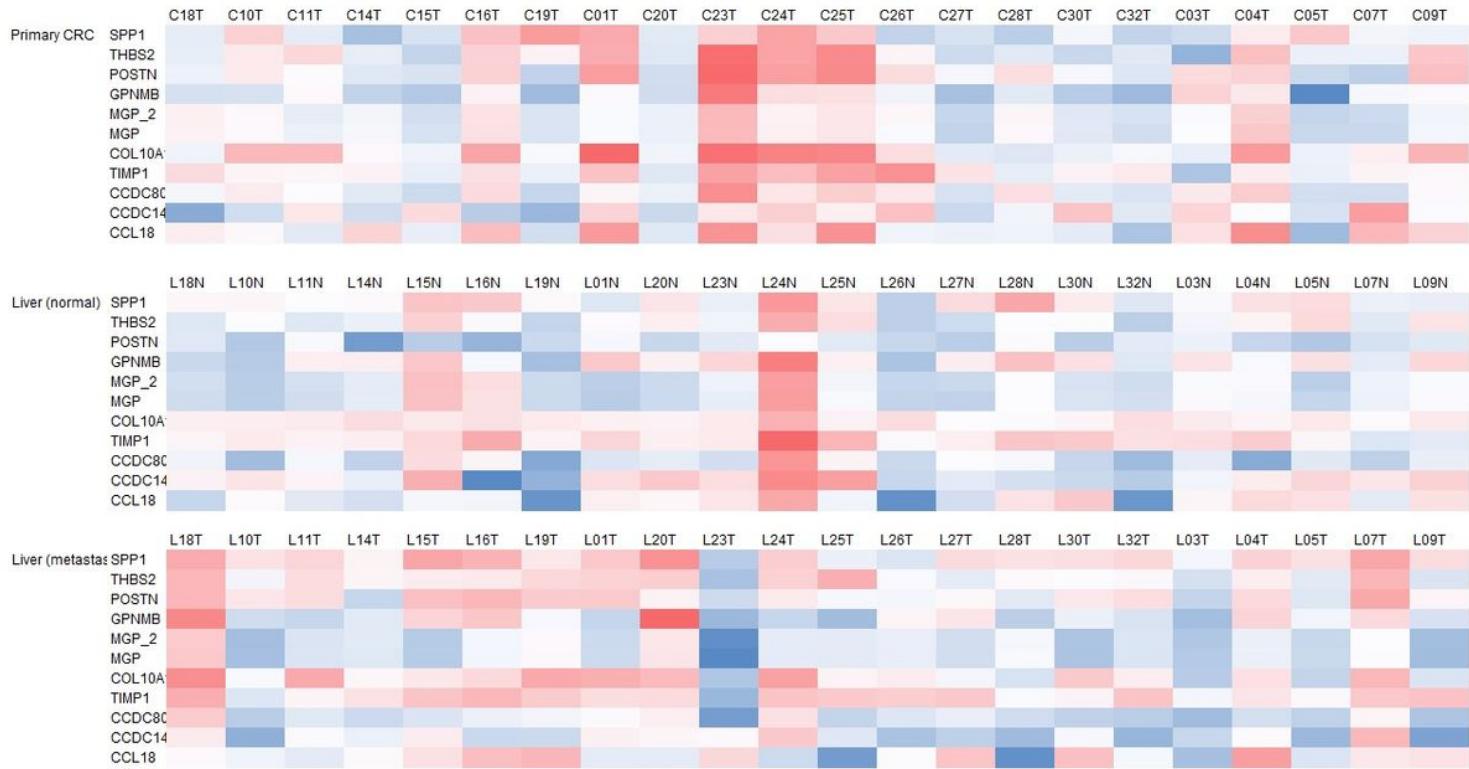
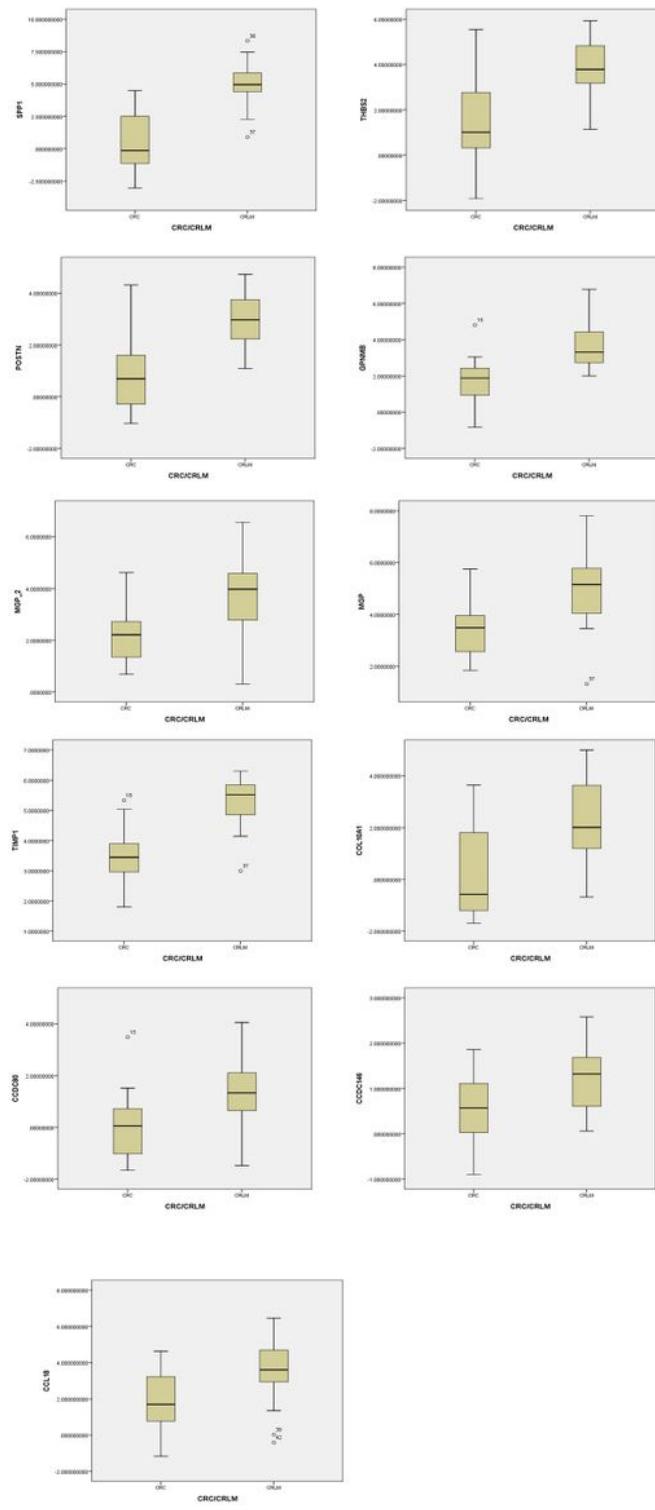


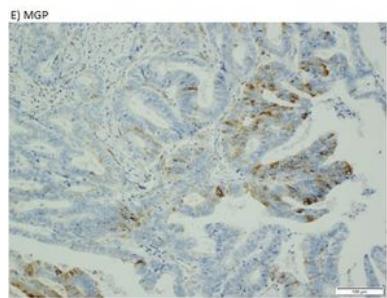
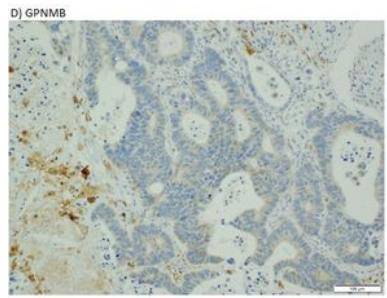
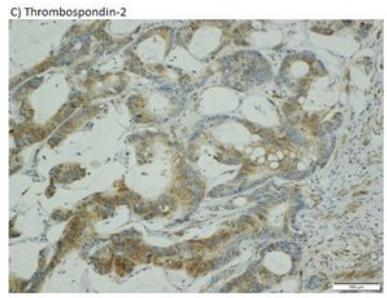
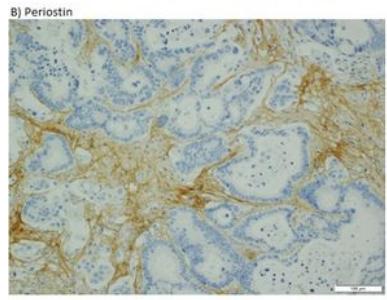
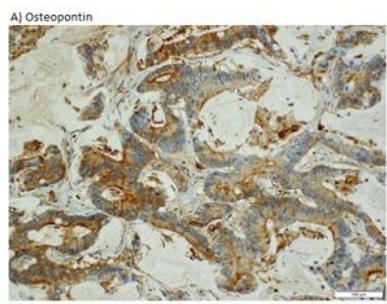
Figure 5

## Heat map of the expression profiles from the microarray analysis



**Figure 6**

Comparison of gene expression between primary CRC and CRLM



**Figure 7**

Representative images of immunohistochemical staining for osteopontin, periostin, thrombospondin-2, GPNMB, and MGP in CRLM