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Author manuscript

*Clin Exp Metastasis*. Author manuscript; available in PMC 2017 August 21.

Published in final edited form as:

*Clin Exp Metastasis*. 2013 March ; 30(3): 309–316. doi:10.1007/s10585-012-9538-5.**Protein pathway activation mapping of colorectal metastatic progression reveals metastasis-specific network alterations****Alessandra Silvestri,**

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Electronic supplementary material: The online version of this article (doi:10.1007/s10585-012-9538-5) contains supplementary material, which is available to authorized users.

**Conflict of interest:** The authors are inventors on US Government and University assigned patents and patent applications that cover aspects of the technologies discussed. As inventors, they are entitled to receive royalties as provided by US Law and George Mason University policy. VC, MP, LL, and EP are consultants to and shareholders of Theranostics Health, LLC.

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## Abstract

The mechanism by which tissue microecology influences invasion and metastasis is largely unknown. Recent studies have indicated differences in the molecular architecture of the metastatic lesion compared to the primary tumor, however, systemic analysis of the alterations within the activated protein signaling network has not been described. Using laser capture microdissection, protein microarray technology, and a unique specimen collection of 34 matched primary colorectal cancers (CRC) and synchronous hepatic metastasis, the quantitative measurement of the total and activated/phosphorylated levels of 86 key signaling proteins was performed. Activation of the EGFR–PDGFR–cKIT network, in addition to PI3K/AKT pathway, was found uniquely activated in the hepatic metastatic lesions compared to the matched primary tumors. If validated in larger study sets, these findings may have potential clinical relevance since many of these activated signaling proteins are current targets for molecularly targeted therapeutics. Thus, these findings could lead to liver metastasis specific molecular therapies for CRC.

## Keywords

Colorectal cancer; Proteomics; Metastasis; Protein pathway activation mapping; Tumor microenvironment; Reverse phase protein microarray

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## Introduction

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the Western countries [1]. Approximately 35 % of patients seeking medical attention for colorectal cancer present with stage IV disease, and 20–50 % of the patients with stage II or III CRC develop metastatic lesions within 5 years from the initial diagnosis [2]. In the past two decades, the introduction of new molecularly targeted therapies and the improvement of surgical techniques has doubled the 5-year overall survival time of patients with metastases from 9 to 20 % [3].

Since metastasis in general, and liver metastasis specifically in CRC, is the major cause of death, the identification of molecular targets that are metastasis-specific is an important strategy to address the urgent need to improve patient survival.

Metastatic progression is a multi-step phenomenon. It is estimated that less than 0.01 % of the cancer cells are able to leave the site of origin, survive in the systemic circulation, colonize secondary organs, and grow into a viable metastatic lesion [4]. According to Paget's "Seed and Soil" theory, organ selectivity is based on a successful interaction between the tumor cells (seed) and the microenvironment of the secondary location (soil) that supports extravasation, survival, and growth of the metastatic colony [5].

To date, the analysis of metastatic tumors has focused mostly on the identification of genomic-based prognostic signatures of primary tumors [6–11]. Comparatively little is known about the protein signal pathways that mediate the interaction between the metastatic colony and the organ tissue microenvironment. Since signal transduction is underpinned by kinase-driven post-translational modifications, a direct investigation of signaling within the metastatic microenvironment may best be studied using proteomic technologies that can measure the activation state (e.g. phosphorylation) of a given protein [12–14]. The analysis of post-translational modifications that drive metastatic progression represents a novel frontier for the characterization of the molecular mechanisms underlying tumor progression and for the identification of molecular targets responsible for the formation of the secondary lesions. Such analyses are complementary to genomic studies, since gene expression has shown little correlation with protein expression and activation [15, 16].

This study provided a unique opportunity for broad-scale characterization of the signaling architecture of primary CRC compared to synchronous matched hepatic metastasis. We investigated whether the proteomic profile of the primary tumor and the metastatic lesion are homogeneous and can be used interchangeably for the selection of appropriate targeted treatment. Moreover, this analysis sought to identify molecular mechanisms that drive the metastatic progression in a host organ. We first focused on EGF receptor analysis since it is considered an important drug target for CRC. Not only expression but also activation status of the receptor was measured using the tyrosine residue 1173 due to the central role of this site in receptor-mediated signaling [17].

We then performed a broadened pathway activation profile of the matched set to better characterize molecular alterations involved in metastatic progression.

The promise of personalized therapy is the molecular fingerprint of a patient tumor that becomes the basis for targeted therapy. In current clinical practice, the fingerprinting is done on the primary tumor, not on the metastasis, which is the lethal aspect of the disease. Thus, we set out to explore whether the organ tissue microenvironment influences the levels of phosphorylated and activated signaling networks within the metastatic microenvironment compared to the matched primary tumor.

## Materials and methods

### Clinical sample collection and handling

Primary colorectal cancer tissues and matched synchronous liver metastases from 34 untreated patients were collected at the time of surgery between 1992 and 2002. Tissue was obtained after Institutional Review Board approval under informed consent from the Clinica

Chirurgica II, Padua, Italy. Surgical specimens were coded, frozen in liquid nitrogen within 2 h of surgical removal and stored at  $-80^{\circ}\text{C}$  until processed for molecular analysis. A board certified pathologist confirmed the presence of tumor in each sample. Clinical information and pathological characterization of the subjects enrolled in the study were collected by the enrolling institution.

### Laser capture microdissection (LCM)

Highly enriched (greater than 90 %) CRC epithelial cell populations were obtained using LCM as described previously [18, 19]. Briefly, several  $8\ \mu\text{m}$  frozen tissue sections were prepared for each sample. Before LCM, sections were fixed in 70 % ethanol, stained with hematoxylin, and dehydrated in 70, 95, and 100 % ethanol followed by xylene. Complete mini protease inhibitor tablets (Roche Applied Science, Indianapolis, IN, USA) were added to 70 % ethanol, deionized water, and hematoxylin to avoid protein degradation. Using a Pixcell II Laser Capture Microdissection System (Arcturus, Mountain View, CA, USA), approximately 20,000 tumor epithelial cells were isolated from each sample. Isolated cells were lysed in a 1:1 solution of Tris–Glycine SDS sample buffer (Invitrogen, Life Technologies, Carlsbad, CA, USA) and Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) solution supplemented with 2.5 %  $\beta$ -mercaptoethanol. Cellular lysates were boiled for 8 min and stored at  $-80^{\circ}\text{C}$ .

### Reverse phase protein microarray analysis

Pathway activation mapping was performed by reverse phase protein microarray (RPMA). Each sample was printed onto nitrocellulose arrays in duplicate, in 5-point dilution curves to assure that for each endpoint a linear detection range was achieved [20–23]. All antibodies used in these studies were rigorously validated for single band specificity by western blotting prior to use [20–23]. A complete list of all endpoints measured is provided in Online Resource Table 1. Measured analytes were chosen based on their involvement in key aspects of cellular survival, mitogenesis, adhesion, motility, and inflammation. All analytes values were normalized to total protein concentration to ensure that changes in levels of protein expression/phosphorylation were not due to differences in overall protein concentration or spotting variances. Total protein concentration was assessed as previously described [24]. This method of normalization has been determined to be superior to normalization to any specific “housekeeping” protein [25]. Stained slides were scanned on a standard flatbed scanner as 16-bit images/600 dpi and spot intensities were calculated and normalized using Micro-Vigene| software (Vigenetech, Carlisle, MA).

### Western blotting

To validate RPMA data, five matched primary tumors and synchronous liver metastases were randomly selected and subjected to independent LCM procurement of a separate area of the tumor specimen. Laser capture microdissected cells were lysed directly in SDS sample buffer and subjected to western blotting [26]. Blotting was performed with anti-phospho AKT S473 (Cell Signaling, Danver, MA) and anti phospho c-KIT Y703 (Zymed Laboratories, Life Technologies, UK) antibodies at a 1:1,000 dilution. Intensity signals of  $\beta$ -actin expression were used to normalize the total protein content in each sample.

Immunobands were quantified using ImageQuant v5.2 software (Molecular Dynamics, Sunnyvale, CA).

### Statistical analysis

The correspondence between patients rank order of primary tumor and liver metastasis for EGFR expression and activation was evaluated using GraphPad Prism Version 5.00 (GraphPad Software, Inc., La Jolla, CA). Only primary tumors with the highest and the lowest values (above the 75th and below the 25th percentile) were considered and the rank order of the corresponding liver metastasis was analyzed. The data obtained by RPMA analysis of the metastatic lesion self-organized into two major groups consisting of high and low intensity EGFR total and phosphorylated values. Concordance between the patient-matched primary tumor and metastatic lesions was assessed using these data (Fig. 1).

Statistical analyses were performed on RPMA intensity values obtained using SAS version 9 software (SAS Institute, Cary, NC). Initially, the distribution of variables was checked. If the distribution of variables for the analyzed groups (e.g. primary vs. metastasis) was normal, a two-sample *t* test was performed. If the variances of two groups were equal, two-sample *t* test with a pooled variance procedure was used to compare the means of intensity between two groups. Otherwise, two-sample *t* test without a pooled variance procedure was adopted. For non-normally distributed variables, Wilcoxon rank sum test was used. All significance levels were set at  $p = 0.05$ .

### Results

A unique study set (Table 1) of 68 tissue samples (34 pairs) of patient-matched primary colorectal cancers and hepatic metastases taken at the same time of surgery was used for this study. To evaluate the overall concordance between the signaling networks within the primary tumor and its matched hepatic metastasis, the expression and activation levels of epithelial growth factor receptor (EGFR) were compared between matched lesions (Fig. 1). While only 4/18 of the patients switched from one group to another (concordance = 77.8 %) when measuring for total EGFR (Fig. 1, left), the concordance between the primary tumor and matched liver metastasis was much lower when activated/phosphorylated EGFR was evaluated (concordance = 55.6 %) (Fig. 1, right).

Reverse phase protein microarray based pathway mapping was then utilized to evaluate the activation status of 86 key signaling proteins (Online Resource Table 1) belonging to important cancer-related signaling networks that control motility, growth, survival, apoptosis, and differentiation. Our results, shown in Table 2, reveal that the level of expression/activation of 30 of the signaling proteins measured was significantly different between primary tumor and hepatic metastasis ( $p < 0.05$ ). Analysis of the significant alterations showed that most of the differences were based on metastasis-specific elevation of the phosphorylation of receptor tyrosine kinases and their downstream effectors, in particular the activation of the PI3K/AKT pathway. The phosphorylation levels of FAK, PRAS, FKHR/FKHRL, and PYK2, all members of the PI3K/AKT pathway, as well as proteins involved in MAPK signaling, were increased in the liver metastases compared to the primary tumors (Table 2; Fig. 2).

Reverse phase protein microarray findings for key signaling proteins were validated by western blotting. As shown by the bar graphs in Fig. 3, the phosphorylation level of AKT (S473) (Fig. 3, top) and c-KIT (Y703) (Fig. 3, middle) correlated well with the RPMA results.

## Discussion

Little is known about the molecular rearrangements that cancer cells acquire either within the “seed” microenvironment or within the “soil”. During metastatic colonization, adaption to a new organ provides tumor cells with a selective pressure to acquire survival mechanisms through the activation of specific cellular pathways [27, 28]. Our unique study set of patient-matched primary and synchronous metastatic lesions provided a novel opportunity to explore the signaling events that occur in an isogenic setting during the metastatic progression. The use of a powerful protein pathway mapping approach uncovered systemic activation of specific pathway networks elevated only in the metastatic setting in most patients. Understanding the molecular changes that are involved in the establishment of secondary lesions represents the most important step for the development of therapeutic strategies to specifically treat metastatic disease.

Targeting EGFR signaling using kinase inhibitors in CRC is an important standard of care therapy option. As part of our study, we evaluated EGFR expression and its phosphorylation/activation in liver metastases. Indeed, we found that the overall expression and activation (phosphorylation) of EGFR differed between primary tumor and metastatic lesion (concordance of 78 and 56 % respectively). These findings are in keeping with past results where investigators found significant changes in the total levels of the EGFR protein in the metastatic lesion compared to the primary tumor [29]. These results indicate that measurement of EGFR and its activation/phosphorylation from the primary tumor cannot be used to accurately determine the level of this kinase drug target in the metastatic lesion and suggest that a direct sampling of the metastasis is required for accurate patient therapy stratification.

Genomic analysis has been conducted in the past to compare mutation status of key components of the EGFR pathway, such as K-Ras and B-Raf. Some studies reported a high correlation between the primary tumor and the metastatic lesions (95 and 97 % concordance respectively) [30, 31]. In contrast, Vermaat and colleagues [32], reported substantial genetic variability between primary tumor and patient-matched liver metastasis when a more broad approach for genomic analysis was used. In keeping with our protein data, they found significant genetic alterations in the metastatic mass that were not present in the primary tumor.

Beyond metastasis-specific activation of EGFR, our results reveal overarching network activation from other receptor tyrosine kinase (RTK) mediated signaling events within the metastatic lesion. Statistically significant increased phosphorylation of c-KIT, c-ABL, and PDGFR was observed in the metastatic cells concomitant with downstream FAK, PYK2, SHC, SRC, and PI3K/AKT signaling increases. AKT signaling has been shown to be a central player in the CRC metastatic setting as previously reported [33, 34]. Increased AKT

phosphorylation (on both T308 and S473) as well as a large number of AKT downstream substrates such as the Forkhead family, PRAS, STAT kinases were all significantly elevated in the metastasis compared to the primary tumor.

Aside from RTK-driven PI3K/AKT activation, we observed a greatly reduced level of PTEN phosphorylation (S380), which is a well-known primary control mechanism for AKT activation. Thus, it appears that both RTK activation and loss of AKT inhibitory mechanisms are major signaling events in the metastatic process. Past work has revealed a similar lack of concordance between AKT phosphorylation in the primary CRC tumor and matched metastases [35].

Due to the retrospective nature of this analysis, the sample collection protocol was not established a priori. Therefore, collection and freezing time might not have been consistent within and between subjects, although all samples were collected within 2 h. For this reason, cellular signaling might have been impacted by pre-analytical variables that were hard to control when broad pathway analysis was performed [36]. However, since we found systemic differences in the signaling architecture between primary and metastatic tissues that transcended any collection differences between patients, it does not appear that the variations in tissue collection time could account for the consistent pathway activation we found in this set. Moreover, the collection of the intra-patient primary tumor and synchronous metastatic lesion was random so that any collection biases would be normalized over the population set.

Finally, these findings are based on a relatively small cohort of patients ( $n = 34$ ). A major factor affecting the sample size is the need to simultaneously collect patient-matched primary colorectal cancers and synchronous liver metastases. Collection of matched primary tumors and metastases is a rare event in the clinical setting, highlighting the unique nature of the study set that was used in this analysis. In addition, all samples were subjected to careful laser capture microdissection to isolate the tumor epithelium from the surrounding tissue prior to analysis. Tissue microarrays or similar study-sets are not commercially available and thus new samples will require specific prospective collection, which is ongoing.

The systemic activation of the RTK-PI3K/AKT axis in the metastatic lesion could be a cause or a consequence of the metastatic colonization process. The significance of the activation needs to be further evaluated through in vivo models. However, the systemic nature of the activation for so many members of a biochemically-interlinked network across a large number of patients highlights the probable importance of the activation of these molecular events.

Previous data published by our research group [26] showed that specific members of the signaling architecture within tumor epithelium differ between primary CRC presenting with synchronous liver metastasis and primary tumors that never developed secondary lesions. In particular, we identified the hyper-activation of the EGFR/COX2 signaling pathway in primary tumors from patients with advanced disease compared to the tumor epithelium from primary CRC from patients that remained metastasis-free. Although this previous work differs from our work presented herein that interrogates the signaling architecture of the

metastatic and primary lesions from patients with synchronous disease, the findings as a whole reinforce the concept that both “seed and soil” are involved in metastatic progression and that sequential changes at the molecular level are necessary for a tumor to disseminate to distant organs. According to our data, selective pathways that are activated in the primary tumor may promote migration and local invasion, but further molecular alterations within the tumor epithelium are necessary to adapt in the new “soil” in the secondary organ.

The underlying mechanisms regulating the network activation we have observed in the present study are unknown at this time. Recent genomic analysis of matched primary and metastatic CRC tumors [32] has shown that PTEN mutation changes do not correlate with the metastatic phenotype, thus the loss of PTEN protein expression/phosphorylation and associated AKT activation is not explained by mutational events alone. Given the large number of RTKs that were activated in the metastatic lesion (EGFR, PDGFR, c-KIT, etc.), much of the concomitant downstream signaling is likely to be driven by activation of these upstream receptors.

In conclusion, our results demonstrate that specific cell signaling pathway activations are associated with human CRC tumor cells that have colonized the liver. We can hypothesize that these pathways assist the tumor cell to survive in the new host microenvironment. If this hypothesis is true, then suppression of these pathways would be expected to reduce the growth, or kill the metastatic lesions.

Overall, these data support the need for new clinical trials in which molecularly targeted therapies are selected based on the metastasis-specific signaling pathways, a clinical approach that is supported by Vermaat and colleagues [32]. In addition, these data point to the importance of collecting metastatic tissue and matched primary tumors for further molecular profiling studies, to better understand mechanisms that drive the adaptation of cancer cells to a new host environment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by the Italian Istituto Superiore di Sanita' in the framework of the Italy/USA cooperation agreement between the US Department of Health and Human Services, George Mason University, and the Italian Ministry of Public Health, as well as the generous support of the College of Science, George Mason University.

## Abbreviations

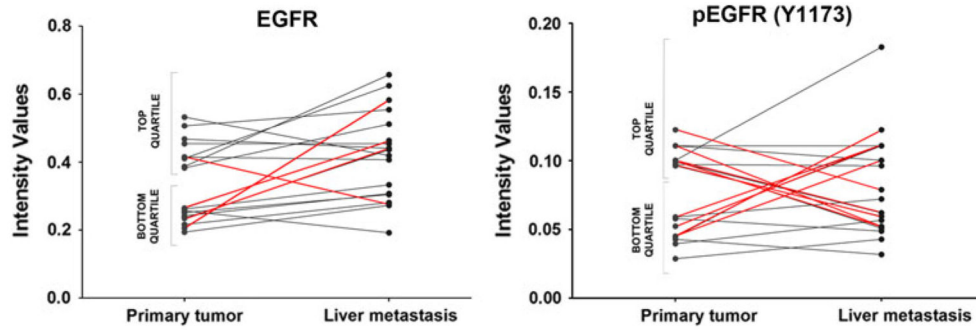
<b>CRC</b>	Colorectal cancer
<b>LCM</b>	Laser capture microdissection
<b>RPMA</b>	Reverse phase protein microarray
<b>EGFR</b>	Epithelial growth factor
<b>RTK</b>	Receptor tyrosine kinase



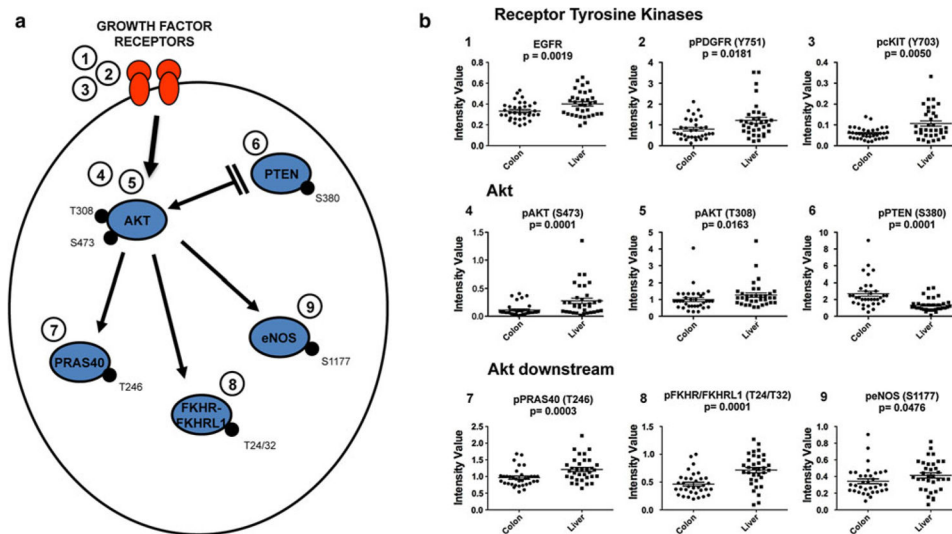
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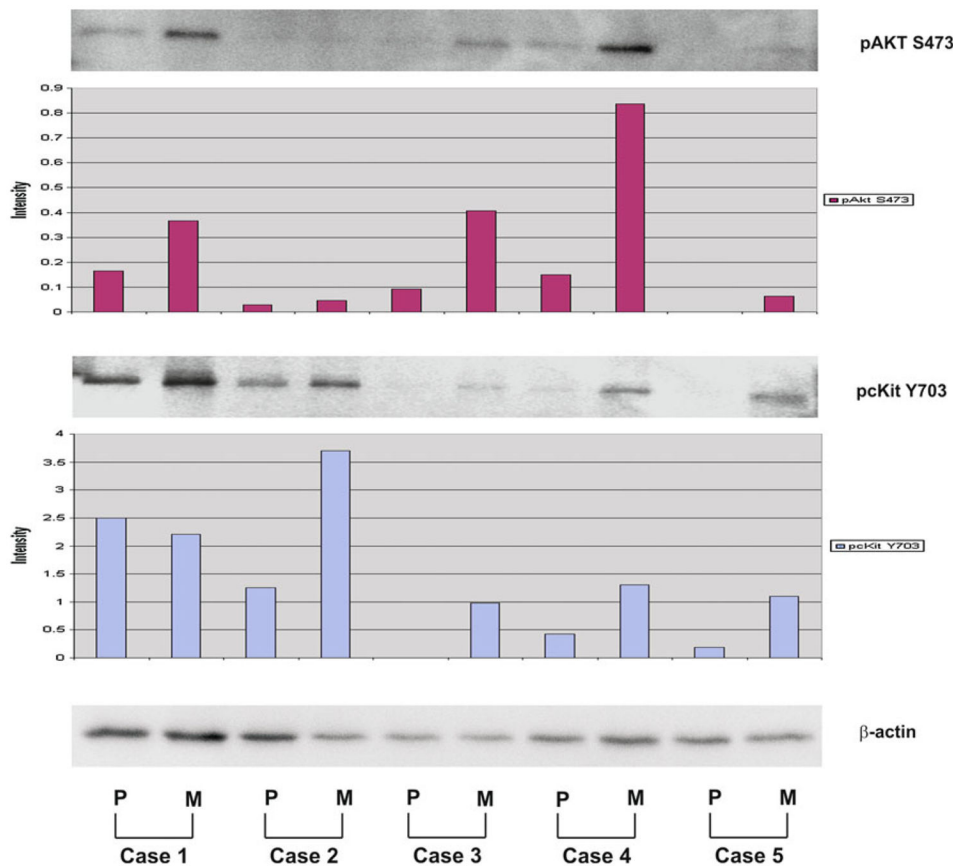
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**Fig. 1.** Scatter plots comparing EGFR expression (*left*) and EGFR Y1173 activation (*right*) in primary CRC tumors and matched liver metastasis. RPMA-generated relative intensity values were plotted for primary tumor and matched liver metastasis. Only patients in 25th and 75th percentile were included in the analysis. Changes in the rank order between the two lesions are shown by the connecting lines. *Red lines* show pairs for which protein expression/activation level switched between groups. (Color figure online)



**Fig. 2.** Endpoints involved in the RTKs-PI3K/AKT pathway that were statistically different between the primary tumor and the matched hepatic lesions. **a** The PI3K/AKT pro-survival pathway and downstream substrates were statistically significantly higher in the synchronous liver metastases compared to the primary colon cancer. **b** Scatter plot of RPMA-generated relative intensity values for primary tumor and patient matched liver metastasis showing that most of the effectors involved in the PI3K/AKT pathway are more activated in the liver metastasis compared to the primary tumor



**Fig. 3.** Western blot analysis RPMA data were confirmed by western blot analysis of five patient-matched primary CRC and hepatic metastases. All samples were subjected to independent LCM. Blots were stained for pAKT S473, pcKIT Y703 and  $\beta$ -actin. The *bar graphs* illustrate the intensity value of each sample after normalization for the total amount of  $\beta$ -actin. Western blot analysis confirmed increased activation of both endpoints in the liver metastasis compared to the primary tumor

**Table 1**

## Patient clinicopathological information

	<b>Total</b>
Number of patients	
Female	12
Male	22
Site of primary tumor	
Right colon	7
Transverse colon	1
Left colon	10
Rectum	16
T stage	
T2	5
T3	21
T4	8
N stage	
N0	4
N1	15
N2	14
N3	1
M stage	
M+	34
Tumor grade	
G1	2
G2	24
G3	8
Stage	
Stage IV	34

Clinical description of the 68 samples collected from 34 patients matched primary CRC and synchronous liver metastasis

**Table 2**

Statistically significant analytes

Variable	P value	Regulation in liver metastases
EGFR	0.0019	↑
p4EBP1 S65	0.0326	↑
pAbl Y245	0.0037	↑
pAKT S473	0.0001	↑
pAKT T308	0.0163	↑
pBAD S136	0.0087	↓
pcKit Y703	0.0050	↑
peIF4G S1108	0.0416	↑
peNOS S1177	0.0476	↑
pErk 1/2 T202/204	0.0021	↑
pFADD S194	0.0222	↑
pFAK Y576/577	0.0001	↑
pFKHR/FKHRL1 T24/T32	0.0001	↑
pIKBa S32/36	0.0212	↓
pp70S6 S371	0.0006	↓
pPDGFR $\beta$ Y751	0.0181	↑
pPDK1 S241	0.0051	↓
pPKC theta T538	0.0182	↑
pPKC zeta/lambda T410/403	0.0001	↓
pPKC $\alpha$ / $\beta$ II T638/641	0.0017	↓
pPRAS40 T246	0.0003	↑
pPTEN S380	0.0001	↓
pPyk2 Y402	0.0001	↑
pShc Y317	0.0001	↑
pSMAD2 S465/467	0.0043	↑
pSrc Y527	0.0001	↑
pSTAT3 Y705	0.0225	↑
pSTAT5 Y694	0.0159	↑
pVEGFR Y951	0.0001	↑
pVEGFR2 Y1175	0.0481	↑

List of analytes that showed a significant difference in expression/activation level between the primary CRC and synchronous liver metastases. Significance level was set at  $p = 0.05$ . The right column reports the class-specific trend in the hepatic lesions compared to the matched primary colorectal cancer. Phosphorylation site is shown when appropriate