Chapter 3
Gene Signatures in CRC and Liver Metastasis

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Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related death with a worldwide incidence of almost a million cases annually in both males and females [1].

The accelerated decrease in CRC incidence rates from 1998 to 2006 largely reflects the advances in diagnosis and treatment that have enabled to detect and remove precancerous polyps [2]. However, the screening technology has not resulted in major improvements in the prognosis of patients with advanced cancer, and liver metastasis remains the major cause of death in CRC [3]. Approximately 25% of patients have detectable liver metastasis at diagnosis, that are classified as "synchronous" lesions and approximately 70% of patients develop a liver recurrence during the course of their disease – identified as "metachronous" lesions [4]. Despite the development of different treatment modalities, the outcome for patients with unresectable metastatic lesions is still unfavorable and the metastatic spread to the liver is the major contributor to mortality in CRC [5]. Therefore, elucidation of the molecular mechanism involved in the development of metastases, by the identification of a specific gene signature for liver metastasis in CRC, could allow prediction of the onset of metastatic disease in patients with localized tumors and lead to designing new strategies for diagnosis and treatment of CRC.

Molecular Nature of Liver Metastasis in CRC

Most cancer, including CRC, might have a single clonal origin at the initial stage of the disease; however, a malignant tumor contains multiple cell populations with different properties and acquires the ability to invade and develop metastases. This heterogeneity determines a variation in clinical patterns and treatment efficacies because these cells have acquired the ability to regulate their adhesion or motility. Clones with metastatic potential show genetic properties that are different than clones without metastatic potential. Therefore, in recent years many researchers aimed to identify genetic markers of metastatic clones.

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Although little is known, two models have been proposed to explain the biological mechanisms of metastases. The genetic selection model proposes that only a subset of tumor cells acquire metastatic potential during the late stages of multistep tumorigenesis [6, 7]. Alternately, another model proposes that tumor cells acquire metastatic capacity during a relatively early stage of tumorigenesis depending on the genetic background. Recent gene expression analyses of clinical tumor samples support this hypothesis [8, 9]. Several biomarkers for CRC have been identified, including KRAS2, p53, p21/WAF/CEP1, cyclin D1, PCNA, COX-2, MMP-9, CD44, CK-19, VEGF-C, and E-cadherin [10–12].

Liver metastasis in CRC occurs in multiple steps, including the ability of cancer cells to release the primary site, to achieve a specific type of tissue through blood flow, and to establish a distant secondary tumor [13]. This event has been characterized as a complex process in which each step is characterized by several changes in gene expression profiling [14, 15] and in which multiple genes play a significant role. Carcinogenesis and progression of CRC involves multiple genetic and epigenetic changes in many genes, including common alterations in *TP53*, *K-RAS*, b-*catenin*, *APC*, and *AXIN2* [16, 17].

Microarray studies, through the analysis of gene expression profiling, have successfully shed light on various aspects of the molecular mechanisms involved in the development of different human tumors [18]. In CRC, the DNA array technology has allowed the identification of differences in gene expression profiles between normal mucosa, benign adenoma, and malignant carcinoma, and the creation of a molecular model of multistep carcinogenesis [19]. However, although much is known about genes in which mutation is responsible for the onset of CRC [16, 20], less is known about the specific molecular events that are crucial for the development of CRC liver metastasis. Nevertheless, although the mechanisms responsible for metastasis of CRC to the liver are yet to be elucidated, different recent microarray studies have identified several genes that are crucial for metastasis formation, leading to the identification of a possible gene expression signature that could differentiate between tumors that do or do not metastasize [21].

Gene Expression Profile Studies Related to CRC Liver Metastasis

The acquisition of a metastatic signature is often related to the loss of various activities, and genes associated with metastasis may already be expressed in early tumors. In order to identify specific metastatic gene signatures that can be used as a prognostic marker of metastatic spread using microarray technologies, several studies have been carried. The first studies were conducted comparing the gene expression profile of primary CRC from metastasis-free patients to those of patients affected by metastatic disease during a 5-year follow-up period [5].

Bertucci et al. have identified a total of 219 genes down- and 25 genes up-regulated, respectively, in metastatic samples as compared to nonmetastatic samples and additionally have identified a gene signature of 46 genes that discriminate CRC with and without lymph node metastases [22].

D'Arrigo et al. have found a different gene expression signature between ten primary CRCs from patients who did not develop metastases within a 5-year follow-up period and ten primary CRCs from patients with synchronous liver metastasis. This study identified 37 genes differentially expressed between the two groups of primary tumors, of which 29 genes distinguished nonmetastasizing tumors from metastases. In support of the existence of specific gene expression profiling distinguishing primary tumors from CRCs with a metastatic potential, the genes encoding for mannosyl (a-1,3-)-glycoprotein b-1, 4-*N*-acetyl-glucosamynyl-transferase were significantly up-regulated in metastatic tumors [23].

To identify specific metastatic gene signatures, another approach entails comparing the gene expression profile in primary tumors and in corresponding metastases.

These studies show the identification of a similar gene expression profile between primary tumors and their matched metastases, supporting the notion that the metastatic potential is genetically encoded in primary tumors [24].

To investigate whether the expression profile in primary CRC reflected the metastatic status, Yamasaki et al. have analyzed the chronological gene expression profiles of 104 colorectal samples corresponding to oncogenic development, including normal mucosa, localized and metastatic tumors, and liver metastasis, using microarray technologies. The hierarchical clustering obtained has shown that the metastatic potential of CRC is not acquired in correlation to cancer progression, but rather the gene expression profile in the primary tumors reflect the ability to trigger the metastatic process [21].

In their work, Lin et al. evaluated the gene expression changes in primary CRC and CRC after metastasis to the liver, in order to determine how the cancer cells adapt to the liver microenvironment [25]. Using genome-wide microarray analysis, the authors have compared the gene expression profile of 48 primary tumors and 28 liver metastases and have identified 778 genes differentially expressed. The gene ontology analysis has revealed that the genes involved in immune response (innate and adaptive) and tissue remodeling are statistically up-regulated in liver metastasis relative to primary tumors. The genes involved in immune response, including proteoglycan 3 (*PRG3*), natural killer cell receptor (*NCR3*), B-cell membrane protein CD22, chemokine receptor (*CXCR4*) and its ligand *CXCL12*, and the genes associated with tissue remodeling mainly encode extracellular matrix proteins such as fibronectin, osteopontin, and ADAM17. Additionally, this study has shown that the genes associated with proliferation are down-regulated in liver metastasis suggesting that the tumor cells are not proliferating in the liver metastasis as rapidly as in the primary tumors [25].

In order to identify genes that may be involved in tumor progression and liver metastasis of CRC patients, Ki et al. have analyzed gene expression profiles of 27 colorectal primary tumors, corresponding to 27 liver metastasis tumors, 25 normal colon mucosa, and 13 liver tissues from CRC patients with liver metastasis [26]. In this study, the investigators identified 46 liver metastasis-specific genes with an accuracy of 83.3% by comparing the gene expression levels of paired primary colorectal tumors and liver metastases using a cDNA microarray containing 17,104 known genes. These selected genes could help to more effectively pinpoint therapeutic targets for CRC. Several known oncogenes were included among the 46 selected genes. Of the 46 identified genes, 36 seem to be down-regulated and 8 up-regulated in liver metastasis tumors. *WNT5A*, a gene involved in carcinogenesis and several developmental processes, showed significantly lower expression in liver metastasis tumors and higher expression levels in primary tumors than in the normal colon.

Conversely, the most significantly up-regulated gene in liver metastasis was tissue inhibitor of metalloproteinase 1 (*TIMP1*), an inhibitor of *MMP-1* that plays an important role in cancer metastasis. Other identified cancer-related genes that showed variation in gene expression levels were *MMP-1*, *MMP-2*, *COX-2* and *HIF-1a, MMP-1* and *MMP-2*, known to be up-regulated in carcinogenesis, were up-regulated in primary tumors but not in normal colon tissue. However, they showed decreased expression levels in liver metastasis tumors. Furthermore, 21 of the 46 genes were differentially expressed in primary tumors with synchronous liver metastasis compared with primary tumors without liver metastasis. These 21 genes were mainly involved in the regulation of cellular process (47.1%), cell development (35.3%), and cellular morphogenesis (17.7%).

Moreover, 4,583 selected organ-specific genes, differentially expressed between 13 paired normal colon and normal liver tissue, were identified; 2,236 of these genes were up-regulated and 2,347 were down-regulated in normal liver compared with the normal colon tissues. The genes showing differences in gene expression were those related to metabolism, cellular physiological process, cell communication, response to stimulus, and organism physiological processes [26].

In their study, Pantaleo et al. have evaluated the gene expression profiling of ten synchronous and eight metachronous liver metastatic lesions, by using the Affymetrix platform [27]. The gene

expression analysis identified 49 up-regulated genes in metachronous and 55 up-regulated genes in synchronous metastases under which the main alterations were found in two pathways – EGFR signaling and eicosanoid metabolism. The key genes involved in this pathway are EGFR, COX-2, and COX-1; specifically, EGFR was overexpressed in metachronous lesions and the COX-2 gene was overexpressed in synchronous metastases. These results showed that the molecular background of liver metastases may be differentially related to differential expression of these genes and this observation may have clinical implications, allowing a more targeted treatment of patients with synchronous or metachronous lesions. Therefore, the identification of a specific metastatic gene signature could result in the choice of selective and differential chemotherapy treatment [27].

In a recent study, Koh et al. have analyzed 12 matched primary and metastatic colorectal carcinomas, and have identified 80 genes differentially expressed by means of a supervised hierarchical clustering [28]. The metastasis was distinguished from the corresponding primary tumor in half of the metastases analyzed using a specific set of genes identified. Among 80 genes, *MMP1*, *MMP-2*, *MMP3*, *MMP-13*, *COL1A2*, and *CXCL3* were up-regulated in primary colon carcinoma, and *SOX15*, *LIMS1, SERPINA3, CYP1B1, NAT5*, *SPP1*, and *SERPINA1* were up-regulated in metastatic colon carcinoma. SERPINA1 and SERPINA3 were associated with a poor prognosis in colon cancer and are overexpressed in metastatic breast carcinoma. Furthermore, the up-regulated genes in metastatic colon cancer include genes involved in embryonic development (*GA17*), cell adhesion (*ADRM1*), RNA binding (*SNRPB2*), transcriptional activity (*TWIST1* and *ETV4*), cell cycle and proliferation (*CKS2*), DNA repair (*RPA3*), signal transduction (*PRDX4*), and prefolin complex (*VBP1*). Down-regulated genes in metastatic colon cancer included genes involved in the cell–cell adhesion (*ICAM4*), extracellular region (*GUCA2A*) and carbonate dehydratase activity (*CA4*). Genes defined with high metastatic potential encode proteins influencing cell growth and proliferation (GAS1, ITLN1, IL1B, and IL24), extracellular proteases (MMP family), cell adhesion (COL12A1 and PAP), and cell motility (VIP and WNT5A). This molecular profiling may be useful for the differentiation of primary and metastatic carcinoma and the proteins encoded by these genes could be effective biomarkers for early metastasis detection.

Recently, Fritzmann et al. conducted a gene expression-profiling experiment to identify genetic markers of risk and to elucidate the molecular mechanisms of CRC metastasis. In particular, they have compared the gene expression patterns between metastatic and nonmetastatic matched CRC by microarray analysis. This study allowed the identification of 115 gene signatures that differentiated metastatic from nonmetastatic primary tumors. Among these, the transforming growth factor (TGF) b inhibitor BAMBI was noted to be highly expressed in half of the metastatic primary tumors and metastases but not in nonmetastatic tumors and it was observed an inverse correlation between the level of BAMBI expression and metastasis-free survival time of patients. BAMBI is a target of Wnt signaling and its inhibition of TGF-b pathway could directly drive the metastasis formation in CRC. Therefore, the metastatic gene signatures obtained in this work made it possible to identify a specific gene that could be used as a powerful prognostic indicator in CRC [29]. The most important genes involved in liver metastasis formation in CRC and reported in several studies are shown in Table 3.1.

Gene Signature: Potential and Limits

Studies of gene signatures have the potential to identify specific genes that may play a key role in the onset of liver metastases and, therefore, be used both to clarify the molecular mechanisms responsible for the development of metastases, and as prognostic indicators in CRC. However, the determination of metastatic expression signatures by microarray technologies must take into consideration different factors that can lead to differences among the results obtained by various groups.

Table 3.1 The most important genes involved in liver metastasis formation whose expression is altered in colorectal cancer

Gene	Gene name	Function
PRG3	Proteoglycan 3	Immune response
NCR3	Natural killer cell receptor	Immune response
CD22	T-cell surface antigen Leu-14	Immune response
CXCR4	Chemokine receptor 4	Response to stimulus
CXCL12	CXCR4 ligand	Response to stimulus
CXCL3	Chemokine (C–X–C motif) ligand 3	Response to stimulus
FN1	Fibronectin 1	Tissue remodeling
OPN	Osteopontin	Tissue remodeling
ADAM17	A disintegrin and metalloproteinase domain 17	Tissue remodeling
TIMP1	Tissue inhibitor of metalloproteinase 1	Tissue remodeling
MMP-1	Matrix metallopeptidase 1	Proteolysis
MMP-2	Matrix metallopeptidase 2	Proteolysis
MMP-3	Matrix metallopeptidase 3	Proteolysis
MMP-7	Matrix metallopeptidase 7	Proteolysis and invasion
MMP-13	Matrix metallopeptidase 13	Proteolysis
COX-1	Cyclooxygenase 1	Cellular metabolism
COX-2	Cyclooxygenase 2	Cellular metabolism
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Cellular metabolism
HIF-1	Hypoxia-inducible factor 1	Signal transduction
BAMBI	BMP and activin membrane-bound inhibitor homolog	Signal transduction
WNT5A	Wingless-type MMTV integration site family, member 5A	Signal transduction and cell motility
PRDX4	Peroxiredoxin 4	Signal transduction
GUCA2A	Guanylate cyclase activator 2A	Signal transduction
COL1A2	Collagen, type I, alpha 2	Cell adhesion
LIMS1	LIM and senescent cell antigen-like domains 1	Cell adhesion
ADRM1	Adhesion regulating molecule 1	Cell adhesion
ICAM4	Intercellular adhesion molecule 4 (Landsteiner–Wiener blood group)	Cell adhesion
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	Blood coagulation
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Blood coagulation
SNRPB2	Small nuclear ribonucleoprotein polypeptide B	RNA binding
TWIST1	Twist homolog 1 (Drosophila)	Transcriptional activity
ETV4	ETS variant 4	Transcriptional activity
RPA3	Replication protein A3	DNA repair
VBP1	Von Hippel-Lindau binding protein 1	Transport
CA4	Carbonic anhydrase IV	Carbonate dehydratase activity
VIP	Vasoactive intestinal peptide	Cell motility
CKS2	CDC28 protein kinase regulatory subunit 2	Cell cycle and proliferation
GAS1	Growth arrest-specific 1	Cell growth and proliferation
ITLN1	Intelectin 1 (galactofuranose binding)	Cell growth and proliferation
IL1B	Interleukin 1, beta	Cell growth and proliferation
IL24	Interleukin 24	Cell growth and proliferation
EGFR	Epidermal Growth Factor Receptor	Cell growth
VEGF	Vascular Endotelial Growth Factor	Angiogenesis
SOX15	Sex determining region Y (SRY)-box 15	Embryonic development

The main differences are due to the use of different array platforms (Affymetrix, cDNA nylon membranes) or experimental conditions. The ability to have tissue samples suitable for the analysis is a major problem. Availability of frozen tissues is not the norm in many institutions. Formalin-fixed or paraffin-embedded tissues usually yield low quality RNA and/or DNA. This stressed the need for creating frozen-tissue tumor banks. In addition, different methodologies for RNA isolation can lead to varying results, and the number of samples used varies enormously among the different studies. Selection of homogeneous samples among heterogeneous tumors can often also be a problem. Macrodissection techniques include tumor tissue with both tumor cells and tumor stroma, which may justify the variability of CRC gene expression profiles obtained. Microdissection techniques help to avoid this problem. Laser capture microdissection (LCM) allows isolation of only tumor cells and is considered the gold standard in microdissection procedures [30].

The analysis of data obtained by microarray is another point that may lead to impairment of the results of gene signature. Analysis of differentially expressed genes can be altered by the use of different normalization procedures, different baseline references for ratio calculations, and arbitrary criteria for cut-off values applied to fold-change and significance level. During this selection, information about the quantitative levels of gene expression can be lost. For this reason, different selection algorithms should be tested in order to improve the accuracy of the gene analyzed [19].

In conclusion, to obtain a genetic signature for liver metastases in CRC need to be implemented different measures in order to improve reproducibility, to increase consistence of data and to validate the results.

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