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Clinical applications of molecular profiling in colorectal cancer: Review of the literature

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ABSTRACT

Despite the developments in the diagnostic and management strategies, a considerable number of colorectal cancer (CRC) patients present with disease recurrence after curative surgery. Moreover; there are no reliable indicators to determine the prognosis and response of CRC patients to therapy. By harnessing recent technological advances in molecular profiling techniques, it is anticipated that greater insight to the various combinations of genetic events or alternative pathways underlying carcinogenesis will be gained. By carrying out literature search, we were able to identify a comprehensive list of genes with high differential expression patterns in colorectal cancer that could serve as molecular markers to complement existing histopathological factors in diagnosis, follow up and therapeutic strategies for individualized care of patients.

Keywords: Molecular Profiling in Colorectal Cancer; Gene Expression in Colorectal Cancer; Colorectal Cancer Genetics; Colorectal Cancer

1. INTRODUCTION

At the molecular level, activation of oncogenes and inactivation of tumour suppressor genes [1] are processes known to be involved in colorectal carcinogenesis. Additionally, abrogation of mismatch repair systems [2] contributes to some colorectal cancers. Nevertheless, exactly how those genetic alterations bring about the development and progression of colorectal carcinomas remains to be resolved. To complicate the picture, accumulations of mutant genes in neoplasms tend to be accompanied by other genetic and epigenetic changes including loss of heterozygosity, inactivation of important genes by methylation or loss of imprinting [3] or gene amplifications, all of which can alter gene expression profiles. Therefore, genome wide monitoring of gene expression is of great importance if we are to disclose the numerous and diverse events associated with carcinogenesis. Molecular profiling, a tool of genome monitoring, is an attempt to identify the different combinations of genetic events or alternative pathways that may be represented by cancers of a similar type. Examples of the type styles are provided throughout this document and are identified in italic type, within parentheses, following the example. Some components, such as multi-leveled equations, graphics, and tables are not prescribed, although the various table text styles are provided. The formatter will need to create these components, incorporating the applicable criteria that follow.

2. GENE EXPRESSION PROFILING IN CRC

Molecular biology represents one of the most interesting topics in medical oncology, because it provides a global and detailed view on the molecular changes involved in tumour progression, leading to a better understanding of the carcinogenesis process, to discovering new prognostic markers and novel therapeutic targets. Despite of clinical and pathological parameters are available for the classification and prognostic stratification of cancer, they may be inadequate in everyday practice due to the great biologic and genetic heterogeneity of this multiform disease.

CRC represents an interesting field of molecular profiling research for several reasons: CRC is considered a biological model of tumourigenesis, because clinical progression from adenoma to early stage carcinoma until advanced stage carcinoma seems to parallel distinctive molecular alterations [4]. In addition; traditional clinical and pathological parameters are not always sufficient to discriminate high risk from low risk CRC and validated

molecular markers with prognostic value are still not available. The studies on molecular profiling in CRC have mainly focused on carcinogenesis process, disease prognosis prediction and therapeutic targets and response prediction. (**Table 1**)

2.1. Diagnostic and Prognostic Biomarkers

The application of gene expression profiling on carcinogenesis studies purposes to identify specific alterations on gene expression according to tumour development and to diagnose and classify tumours on the basis of molecular features. Studies comparing gene expression between normal mucosa, adenoma and carcinoma or between primary tumour and metastases, as well as between left-side and right-side tumours are performed, in order to discover distinctive genetic signatures belonging to each. Furthermore; studies on prognosis prediction aim to identify specific alterations to the gene expression profile that may be useful to discriminate high risk from low risk CRC, to provide a molecular stratification according to the clinical outcome and to predict the metastatic potential of the primary tumour.

Table 1. Genes that are consistently represented in CRC literature. Of many published studies on colorectal gene expression profiling, little correlation exists between validated candidate genes associating with disease status. Some candidate genes are consistently represented in the literature however, examples are shown below.

Gene	Function	Expression Level
FABP1	Lipid transport & metabolism	D
CA2	Zinc metallo-enzyme	D
IL8	Neutrophils activation	U
GPX2	GIT protection	U
ADH1A	Alcohol metabolism	U
COL1A2	Cell growth & maintenance	D
ITGA5	Cell adhesion	D
HSP90B1	Protein folding & degradation	U
PLAU	Haemostasis & cell migration	U
MMP1	Collagenase	D
MMP2	Gelatinase	D
COL5A2	Cell growth & maintenance	D
COL4A2	Cell growth & maintenance	D
CDH17	Cell adhesion & transport	D
CXCL12	Immunity	U
CDK6	Cell proliferation	U
CDK8	Cell proliferation	U
MUC2	Immunity	U
EPOR	Cell proliferation	U
ATP6V0E	Cell transport	U
PDCD4	Tumour suppressor	U
AXIN2	Signal transduction	U
IGFBP7	IGF availability & function	D

Several studies were set to investigate the difference in gene expression levels between tumour and normal colorectal tissues. In 1999, Alon and colleagues reported a clustered data set of 2000 genes able to separate 22 normal and 40 tumour colon tissues with the highest minimal intensity across samples. Subsequently many studies reported other sets of genes that were differentially expressed between cancer and normal tissue and therefore potentially involved in the development of colorectal carcinogenesis [5-9]. In addition, some studies reported significant differences in gene expression profile between adenoma and normal mucosa, suggesting that different mechanisms of development of these precancerous lesions may exist [10,11]. Furthermore, and in order to clarify the molecular modifications underlying the development of metastases, some studies compared the gene expression profile of primary tumours with their corresponding metastases [12-16]. Agrawal *et al*. reported that among all genes associated with disease progress, osteopontin expression seemed to be the leading candidate [12]. Moreover; Yanagawa *et al*. [14] analysed genome-wide expression profiles of 10 primary CRCs and their corresponding liver metastasis and identified 40 genes whose expression was commonly up-regulated in metastatic lesions, and 7 that were commonly down-regulated. On the other hand; Watanabe *et al*. studied 89 CRC patients to identify a set of discriminating genes that can be used for characterization and prediction of lymph node metastasis and identified 73 genes in which expression was significantly different between patients with and without lymph node metastasis [17]. Using this gene set, they established a model to predict the presence of lymph node metastasis with an accuracy of 88.4%. In addotion, the controversial data on the benefit of adjuvant chemotherapy in stage II CRC led to the identification of molecular prognostic factors that may identify stage II CRC patients who develop disease recurrence and may benefit by adjuvant treatment. Wang *et al*. studied the gene expression profile in this set of patients and, using two supervised class prediction approaches of analysis, they identified a 23-genes signature that may predict recurrence in stage II CRC with 78% accuracy [18].

Some studies have also investigated differences in gene expression between CRC of the right side and left side, due to their epidemiological, morphological and pathogenetic diversity and found distinct profiles according to the anatomical stratification. Birkenkamp-Demtroder *et al*. [19] investigated the difference in gene expression between the caecum vs sigmoid and rectosigmoid and identified 58 genes to be differentially expressed between the normal mucosa of caecum and the sigmoid and rectosigmoid.

2.2. Therapeutic Targets and Treatment Response Prediction

While gene expression profiling has been widely applied to CRC for diagnosis, classification and prognosis prediction based on molecular patterns of expression, its application to response prediction to medical treatment is still lacking reliable results due to few currently available studies [20-24]. In a panel of 30 colon carcinoma cell lines Mariadason *et al*. identified 420 genes correlated with response to 5-fluorouracil (5-FU) and involved in two main biological processes, DNA replication and repair and protein processing/targeting [22]. The predictive value of 50 genes best correlated with 5-FU response was subsequently validated using a leave one out cross validation approach and it was higher than the traditional markers, such as thymidylate synthase, thymidine phosphorylase, mismatch repair and *p*53 status. Furthermore they also found that 149 genes best-correlated with CPT-11-induced apoptosis significantly predicted response of colon cancer cell lines to this agent. In addition; Del Rio *et al*. analyzed gene expression profile of 21 primary advanced CRC tissues, in order to identify an expression pattern that could predict response to leucovorin, fluorouracil and irinotecan as first-line treatment: 14 genes were found expressed differently between responders and non responders and were able to predict treatment response with 95% accuracy [23]. In the same year Khambata-Ford *et al*. investigated gene expression pattern of metastatic biopsies of 80 advanced CRC patients treated with cetuximab to identify genes whose expression correlates with best clinical response [24]. They found that, among 629 genes expressed differently between 25 patients with disease control and 55 non responders, the top candidate markers based on lowest p value were epiregulin and amphiregulin, both ligands for epidermal growth factor receptor (*EGFr*), suggesting that these markers could select patients for cetuximab therapy.

Some studies evaluated the ability of gene expression profiling for predicting response of advanced rectal cancer (RC) to preoperative chemoradiotherapy [20,25-27]. Ghadimi *et al*. analyzed gene expression signatures of biopsies from 30 locally advanced RC and found 54 genes differentially expressed between responders and non responders [20]. Kim *et al*. reported 261 genes differentiated between 20 partial response and 11 complete response patients affected by locally advanced RC treated with preoperative chemoradiotherapy. In their study 95 genes predicted complete and partial response with an 84% accuracy [25]. Similarly another study identified a gene expression signature of 42 genes that was able to distinguish responder from non responder locally advanced RC patients with a 71% accuracy [26]. Recently; Spitzner *et al*. were able to identify a gene expression signature for chemoradiosensitivity of colorectal cancer cells [27]. They exposed 12 colorectal cancer cell lines to of 5-fluorouracil and radiation therapy. The differences in treatment sensitivity were then correlated with the pretherapeutic gene expression profiles of these cell lines. Their data have suggested a potential relevance of the insulin and Wnt signalling pathways for treatment response, and they also identified *STAT*3, *RASSF*1, *DOK*3, and *ERBB*2 as potential therapeutic targets [27].

Although colorectal cancer (CRC) is still one of the leading causes of cancer related death, the introduction of new therapeutic options like oxaliplatin and irinotecan in addition to 5-fluorouracil, the standard therapeutic for CRC has increased the overall survival of affected patients from 10 to 18 - 24 months. Furthermore, the “biological” therapeutics cetuximab, an IgG1 chimeric monoclonal antibody against epidermal growth factor receptor (*EGFR*), and bevacizumab, a monoclonal antibody against vascular endothelial growth factor (*VEGF*), have augmented the course of the disease and brought in the new era of targeted therapy against cancer specific molecular pathways [28-31]. Although these biologicals have entered clinical routine due to their encouraging results, their effect has been shown to be limited due to adaptation or previously existing resistance of tumour cells. This has been clearly shown in the case of patients with mutations of K-ras, which lead to resistance against cetuximab. Therefore, several new pathways are currently investigated for therapeutic targeting in CRC. These include WNT-signaling, downstream mediators of *EGFR* as the mitogen-activated protein kinase (*MAPK*) or the phosphatidylinositol 3-kinase (PI3K)-pathway, the hypoxia response system involving hypoxia inducible factor-1 (*HIF*-1), mechanisms of tumour development following chronic inflammation, and many others [32].

3. MICRORNA EXPRESSION PROFILING IN CRC

MiRNAs have recently emerged as an exciting new class of disease biomarker with further potential as the next generation of cancer therapeutics. Although elucidating their mechanisms of action is still in its infancy, the discovery of miRNAs has uncovered an entirely new and

exciting repertoire of molecular factors upstream of gene expression, with great potential for new developments in current diagnostic and therapeutic strategies in the management of cancer patients. MiRNAs are small 19 to 22 nucleotide sequences of RNA found in both prokaryotes and eukaryotes that are intimately involved in cell differentiation, cell cycle progression, and apoptosis. Hence, miRNAs may be useful tools for characterizing specific cancers and for determining patient prognosis and response to therapy. The study of miRNA has been extended into many types of cancer, including leukemias, lung, breast, and colon cancer. The first description of miRNA appeared in 1993 by Lee *et al*., who proved that *lin*-4 is involved in controlling the temporal progression of cell differentiation in *C. elegans* [33]. Discoveries of other miRNAs that regulate apoptosis, proliferation, and differentiation in Drosophila, mice, and humans soon followed [34,35]. Calin *et al*. [36] published the first study to link miRNAs to cancer in 2002. These authors demonstrated that *miR*-15 and *miR*-16 are located on chromosome 13 in a position where deletion of a putative tumour suppressor, known to be associated with greater than half of chronic lymphocytic leukemia cases, was identified. Researchers have proposed that specific miRNA expression patterns could help identify human solid tumours, suggest patient prognosis, and even represent a novel molecular target for cancer treatment.

3.1. miRNA Expression and Function in CRC

In 2003 Michael *et al*. published the first report to profile miRNAs in CRC. Using cloning technology followed by Northern blotting, they reported consistently reduced accumulation of the specific mature *miR*-143 and *miR*-145 in the adenomatous and carcinoma stages of colorectal tumours [37]. Thereafter, several studies were set to investigate the role of miRNAs in colorectal cancer. MiRNAs with tumour suppressor properties which are under-expressed in CRC specimens, and thus potentially function as tumour suppressors, include *miR*-31, *miR*-34*a*, *miR*-96, *miR*-143, *miR*-145, and *let*-7*a* [38,39]. *MiR*-34*a* is a well described tumour suppressor miRNA which regulates the *p*53 pathway and when overexpressed induces apoptosis and acute senescence. Conversely reduction of *miR*-34 expression and function attenuates *p*53-mediated cell death and is therefore implicated in tumourigenesis, including initiation of CRC [40, 41]. It is postulated therefore that loss of *miR*-34*a* expression in colorectal biopsy specimens may be an early biomarker of CRC. Other miRNAs like m*iR*-31, *miR*-96, *miR*-135*b*, and *miR*-183 have been found to be upregulated in colorectal neoplasm. The transcription factor *CHES1* which is involved in repressing apoptosis *is a* potential target of *miR*-96. Schetter *et al*. identified miRNAs which can distinguish cancerous from normal colon tissue, with *miR*-21 over-expressed in 87% of colon cancers [38]. Subsequent mechanistic investigations provide evidence for the oncogenic role of *miR*-21 in CRC by demonstrating how it suppresses the cell cycle regulator *CDC*25*A* [42], and can also target and repress the tumour suppressor gene *PDCD*4 thus inducing invasion, intravasation and metastatic potential [43]. *MiR*-21 may also target *PTEN* and *TPM*1. In addition; m*iR*-135*a* and *miR*-135*b* are upregulated, and this upregulation correlates with reduced expression of the *APC* [44]. Moreover; *miR*-143 and *miR*-145 are both downregulated in colorectal cancer. The genes encoding these miRNAs are both located on 5q23, and these miRNAs possibly originate from the same primary miRNA [37, 39]. *MiR*-126 promotes cell proliferation through modulation of phosphatidylinositol 3-kinase signaling [45]. *MiR*-133*b* is also downregulated, and one of its putative targets is *KRAS* [46], which is a member of the Ras family of proteins, that regulates signaling pathways involved in cellular proliferation, differentiation, and survival. Moreover; over-expression of the oncogenic *miR*-17-92 cluster is also implicated in the etiology of CRC, specifically in adenoma to adenocarcinoma progression.

3.2. Clinical Value of Mirna Expression in CRC

Accumulating evidence shows that miRNA expression patterns are unique to certain cancers and may be used clinically as prognostic and diagnostic factors as well as therapeutic targets.

3.2.1. Diagnostic and Prognostic Value

To test the function of miRNAs in the pathogenesis of CRC, expression of 156 miRNAs was measured in both tumour and normal tissues from patients with CRC and cell lines [46]. Expression of 13 miRNAs was significantly altered, and the most significantly dysregulated miRNAs were *miR*-31, *miR*-96, *miR*-133*b*, *miR*-135*b*, *miR*-145 and *miR*-183. In addition, the expression level of *miR*-31 was significantly correlated with tumour stage. Xi *et al*. [47] analysed patients with adenocarcinoma of the colon and rectum and found that tumours expressing high levels of *miR*-200*c* are correlated with poorer prognosis, regardless of tumour stage: approximately 12 months decreased survival compared with patients whose tumour expresses low levels of *miR*-200*c*. Furthermore; Arndt *et al*. [48] identified 37 miRNAs that were differentially expressed between CRC and normal tissues. They also reported that loss of *miR*-133*a* and gain of *miR*-224 are associated with tumour progression. Overexpression of miR-21 was shown in many reports to be associated with worse prognosis, lymph node and distant metastasis and poor response to chemotherapy in CRC. Moreover, Asangani *et al*. [43] reported that overexpres-

sion of miR-21causes tumour cells to invade and metastasize more aggressively when implanted into mouse models. In addition; a study by Motoyama and colleagues [49] showed that expression of *miR*-31, *miR*-183, *miR*-17-5*p*, *miR*-18*a* and *miR*-92 were significantly higher in tumour tissues compared to normal, while expression of *miR*-143 and *miR*-145 in cancer were lower than in normal tissues. They also showed that *miR*-18*a* expression was associated with poor disease prognosis. Moreover, *miR*-31 expression was positively related to advanced TNM stage and tumour invasion suggesting its role in CRC initiation and progression [50]. Of further interest, Lanza *et al*. [51] identified a molecular signature consisting of 27 differentially expressed genes, inclusive of 8 miRNAs that can correctly distinguish high microsatellite instable (MSI-H) vs microsatellite stable (MSS) colon cancers.

3.2.2. Therapeutic Potential

The synthesis and functions of miRNAs can be manipulated with various oligonucleotides that encode the sequences complementary to mature miRNAs [52]. Overexpression of miRNAs can be induced either by using synthetic miRNA mimics or chemically modified oligonucleotides [53]. Conversely, miRNAs can be silenced by antisense oligonucleotides and synthetic analogues of miRNAs [54,55]. Cross-sensitivity with endogenous miRNAs and lack of specificity for target miRNAs can cause non-specific side-effects with miRNA modulation therapy. However, the use of an effective delivery system and less toxic synthetic anti-miRNA oligonucleotides may minimize such side-effects. The role of miRNAs in pathogenesis of cancer makes them important targets for therapeutic intervention. Gene therapies may be designed to treat colorectal cancers and to block the progression of precursor lesions by manipulating the tumour suppressor or promoter miRNAs [56]. Such manipulation may control the tumour growth rate and have potential as a new therapy for both early and advanced cancers.

Studies have revealed that inhibition of *miR*-21 and *miR*-17-92 activity is associated with reduced tumour growth, invasion, angiogenesis and metastasis [57,58]. Targeting such miRNAs may help to prevent the recurrence of disease in high-risk tumours and may control the growth of advanced metastatic tumours. Overexpression of *miR*-21 is associated with low sensitivity and poor response to chemotherapy [56]. Its inhibition may improve the response to chemotherapy. In addition; some drugs were found to alter the expression of miRNAs. Rossi *et al*. reported a suggestive pattern of miRNA rearrangement in HT-29 and HCT-116 human colon cancer cell lines after exposure to 5-FU [59]. It leads to down-regulation of *miR*-200, which is a microRNA known to inhibit a tumour-suppressor gene, protein tyrosine phosphatase, non-receptor type 12 (*PTPN*12) [60]. 5-FU treatment also induces up-regulation of *miR*-133*a*, which is thought to inhibit the proto-oncogene K-ras. Strangely, treatment with 5-FU also causes up-regulation of microRNA known to be mitogenic. To this, Rossi suggests that the cytotoxic effect of 5-FU induces cells to express anti-apoptotic factors, of which are these abnormally up-regulated, and tumourigenic, miRNAs. Besides these, 5-FU treatment leads to significant elevation in expression of many other miRNAs, and it remains to be seen what genes these miRNAs target. 5-FU induces *p*53 protein expression at a posttranscriptional level without correspondingly elevated mRNA level in a pattern that has become a hallmark for microRNA involvement. When wild-type HCT-116 cells are treated with 5-FU, they express high levels of certain miRNAs, and a great majority of these affected microRNA have a binding site for *p*53 in the gene. When HCT-116 cells knocked out for *p*53 are treated with 5-FU, these miRNAs are not up-regulated. These results suggest that 5-FU acts as a switch to turn on *p*53 and, through *p*53, a cascade of miRNAs that may act with or independently of *p*53.

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