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Review

Biomarkers in Colorectal Cancer

ANDREW J. YIU[1] and CHU Y. YIU[2]

[1]*Guy's, King's and St. Thomas' School of Medical Education, King's College London, London, U.K.;*
[2]*Department of Surgery, Queen Elizabeth Hospital, London, U.K.*

Abstract. *Colorectal cancer is the third most common cancer worldwide, with 1.36 million people diagnosed in 2012. The prognosis of colorectal cancer is better with an earlier diagnosis. The outcome of colorectal cancer may also be improved by targeting pathways involved in colorectal cancer formation, such as anti-epidermal growth factor receptor (EGFR) therapy. An understanding of colorectal carcinogenesis is essential for the design of molecular targeting. Recent advances in the molecular subtypes of colorectal cancer, methylation of DNA in colorectal cancer, and micro-RNA biogenesis, and their involvement in colorectal cancer have resulted in the identification of many new colorectal biomarkers. Such biomarkers may be used for earlier diagnosis of, selection of 'personalised' therapy for, and prognosis of colorectal cancer. Many of these biomarkers appear promising in small-scale studies. However, validation of their effectiveness with large-scale clinical trials is needed before routine clinical application. To this end, the recently established consensus molecular subtypes of colorectal cancer would enable like-for-like comparisons of the treatment outcomes of clinical trials.*

Colorectal cancer is the third most common cancer worldwide, with 1.36 million people diagnosed in 2012 (1). The prognosis of colorectal cancer is related to the stage at diagnosis, with a 5-year survival rate of 90% at early diagnosis and less than 10% when distant metastases develop (2). Advances in the understanding of colorectal carcinogenesis offer opportunities to identify biomarkers for earlier diagnosis, selection of 'personalised' treatment strategy and in providing prognostic markers for colorectal cancer, with a potential to improve the outcome of this disease.

Herein recent advances in the molecular classification of colorectal cancer based on gene expression, the use of aberrant DNA methylation markers and microRNA biogenesis and microRNA markers in the management of colorectal cancer are being reviewed.

Correspondence to: Mr C. Y. Yiu, Department of Surgery, Queen Elizabeth Hospital, Stadium Road, London SE18 4QH, U.K.

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Recent Advances in the Molecular Subtyping of Colorectal Cancer

Since Fearon and Vogelstein (3) formulated the multi-step events of the molecular pathway of colorectal cancer formation involving oncogenes and tumour suppressor genes, there have been considerable advances in the understanding of colorectal carcinogenesis. Four different genomic and epigenomic instabilities have been described for colorectal cancer. These are chromosome instability accounting for 85% of colorectal cancer, microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and DNA global hypomethylation (4). Advances in the understanding of colorectal carcinogenesis continue to progress through research on gene expression by many groups. Various colorectal subtypes based on gene expression have been reported. These subtypes appear to be dissimilar and could be a result of the different patient populations employed, methods used and choice of gene-expression platforms, as there is a lack of a standard protocol for colorectal cancer subtyping. To resolve these inconsistencies the CRC Subtyping Consortium (CRCSC) was formed (5). The CRCSC consists of six international expert teams each with its description of colorectal cancer subtypes.

By collaborating in large-scale data sharing and analytics, involving 18 colorectal cancer datasets from the public, The Cancer Genome Atlas (TCGA) and proprietary sources, the CRCSC found common features among six independent classification systems and was able to agree on four consensus molecular subtypes (CMSs) with distinguishing characteristics for colorectal cancer. These are designated as CMS1, CMS2, CMS3 and CMS4. However, no single

molecular event is confined to a subtype. There remained 'mixed or indeterminate' samples amounting to 13% which had heterogeneous patterns of CMS mixtures but did not constitute a fifth subtype.

CMS1. CMS1 (microsatellite instability or MSI, immune) was found in 14% of samples (5). It was characterized by microsatellite instability, CIMP-high, hypermutation, *BRAF* mutations, increased expression of genes related to diffuse immune infiltration consisting mainly of TH1 and cytotoxic T-cells, and activation of immune evasion pathway, low somatic copy number alterations (SCNAs) and with a worse prognosis after relapse. CMS1 included most MSI carcinomas with overexpression of DNA damage-repair proteins and impaired DNA mismatch repair ability. CMS1 was frequently found in right-sided tumours with high histological grade in females.

CMS2. CMS2 (canonical) comprised 37% of samples (5). It had a high frequency of SCNA alterations indicating higher chromosome instability. CMS2 had the greater number of copy number gains in oncogenes and copy number losses in tumour-suppressor genes compared with other subtypes. It was associated with a marked up-regulation of WNT and downstream targets associated with colorectal cancer. CMS2 carcinomas were found mainly on the left side. It was also related to better survival on tumour recurrence.

CMS3. CMS3 (metabolic) accounted for 13% of samples (5). It exhibited mixed MSI status, low SCNA and low CIMP. About 30% of samples were hypermutated and overlapped with MSI status. In TCGA samples, there were more frequent CIMP-low clusters with intermediate levels of gene hypermethylation. There was over-representation of *KRAS* mutations and metabolic deregulation.

CMS4. CMS4 (mesenchymal) was present in 23% of samples (5). It was associated with high SCNA confirming chromosome instability, stromal infiltration, TGFβ activation and angiogenesis, matrix remodelling pathways and complement-mediated inflammatory system, as shown by up-regulation of epithelial-to-mesenchymal transition. Patients with CMS4 carcinomas were usually diagnosed at more advance stages of III and IV and had worse overall survival and worse relapse-free survival, taking into consideration clinicopathological features, MSI status and *KRAS* and *BRAF* mutations.

There were correlations between gene-expression subtypes and protein levels, with CMS1 exhibiting up-regulation in levels of immune response proteins and CMS4 significant overexpression of proteins involved in stromal invasion, mesenchymal activation and complement pathways. Supervised microRNA analysis revealed significant subtype-specific miRNA regulations. Comparison between gene-expression patterns in colorectal cancer specimens and adjacent normal colon and left colon specimens from individuals without colorectal cancer showed a clear distinction. The CMS of colorectal cancer will help improve clinical translation and management of patients.

Biomarkers in the Treatment of Colorectal Cancer

Mismatch-repair deficiency. Mismatch-repair deficiency causing many somatic mutations may produce 'non-self' immunogenic antigens or neoantigens, as shown by the expression of immune checkpoint ligands PD1, PD-LI, CTLA-4, LAG-3 and IDO (6). It was postulated that mismatch repair-deficient tumours might respond to immune checkpoint blockage because of these neoantigens. Le *et al*. performed a phase II study using pembrolizumab, an anti-programmed death 1 immune checkpoint inhibitor, to test this hypothesis (7). Pembrolizumab was administered to 41 patients with advancing metastatic carcinoma. Some cases had mismatch repair deficiency while others did not. In patients with mismatch repair-deficient cancer, the immune-related objective response rate was 40% (four out of 10 patients) and progression-free survival rate was 78% (seven out of nine patients). In patients without mismatch-repair deficiency, the corresponding response rates were 0% (none of eight patients) and 11% (two out of 18 patients), respectively. This study showed that mismatch-repair status of a tumour could predict response to therapy with pembrolizumab.

Epidermal growth factor receptor (EGFR). In high-risk stage II and III colorectal cancer, a combination of therapies such as 5-fluorouracil (5-FU), leucovorin and oxaliplatin (FOLFOX) or capecitabine with oxaliplatin (XELOX) are used (8). For metastatic colorectal cancer FOLFOX or 5-FU/leucovorin/irinotecan (FOLFIRI) are standard treatment (9, 10). An increased understanding of colorectal carcinogenesis pathways has led to the addition of monoclonal antibodies to EGFR, cetuximab or panitumumab, to block EGFR, thereby preventing activation of signal transduction pathways involving RAS, PI3K-AKT and SRC kinase by ligands such as EGFR (13). The effectiveness of these monoclonal antibodies has been confirmed by phase II and III clinical trials. However, subset analyses of these trials show that patients with *KRAS* mutations in exon 2 codon 12 or 13 do not respond to this treatment. The *KRAS* mutation status should, therefore, be determined before treatment with anti-EGFR therapy (11-13). Further studies showed that *KRAS* mutations may also occur at codon 61 in exon 3 and 2% at codon 146 in exon 4 (14). *NRAS* mutations were found in 2.6% of samples, mainly in codon 61. These carcinomas had a significantly lower response rate to cetuximab and chemotherapy (15, 16).

BRAF. BRAF is an oncogene present in the RAS-RAF-MAPK pathway (17). In colorectal cancer, the most commonly reported mutation is V600E (18). *BRAF* mutation has an adverse prognosis irrespective of treatment (19). The value of *BRAF* mutation as a marker in the treatment of colorectal cancer remains unclear.

PIK3CA. *PIK3CA* is a proto-oncogene encoding phosphatidylinositol-3-kinases (PI3K) involved in the EGFR tyrosine-kinase domain, which may lead to phosphorylation of AKT and the activation of AKT-mTOR signaling pathway (20). Colorectal cancer studies have produced conflicting results on the use of *PIK3CA* as a predictive marker for therapy. Recent meta-analysis showed that mutations in exon 20 of *PIK3CA* may act as a marker for resistance to anti-EGFR therapy (21, 22).

PTEN. *PTEN* is a tumour-suppressor gene encoding a phosphatase protein that suppresses the PI3K-AKT signalling pathway. The role of PTEN as a predictive marker for anti-EGFR therapy lacks consensus (23).

The role of BRAF, PIK3A and PTEN are subjects of the Focus4 study, a molecularly stratified randomized controlled trial based in the UK, which started recruitment in 2014 (www.focus4trial.org).

DNA Methylation as a Cause of Colorectal Cancer

Gene expression may be influenced by changes in the DNA sequence that are not permanent changes, as in mutations. Such epigenetic changes include DNA methylation, histone modifications and post-transcriptional gene regulation through non-coding RNAs, and are part of normal cell function and activity. Disturbance of these epigenetic mechanisms may lead to cancer formation (24).

DNA methylation occurs when a methyl group is covalently added to the 5' position of the pyrimidine ring of cytosines within the CpG dinucleotides (25). Methylation of CpG islands in the promoter region of a gene may prevent access to the transcriptional process, and consequently affect gene-expression levels (2). DNA methylation generally acts to suppress gene transcription. In colorectal cancer aberrant DNA methylation is found to be involved in many genes, for example, DNA mismatch-repair genes, the WNT signalling pathway genes and cell cycles regulating genes (2, 26, 27). A subgroup of colorectal cancer with extensive methylated genes is known as CIMP+ (28).

DNA Methylation Biomarkers for the Diagnosis of Colorectal Cancer

Methylated DNA in stool. Imperiale *et al*. compared a non-invasive multitarget stool DNA test consisting of a quantitative molecular assay for *KRAS* mutations, aberrant NDGR4 and BMP3 methylation and β-actin with a faecal immunochemical test (FIT) (29).

The study involved 9,989 asymptomatic individuals with average risk for colorectal cancer, aged between 50 to 84 years, who were due to have screening colonoscopy. All participants provided a stool specimen before bowel preparation for DNA and FIT analysis. It was found that 65 (0.7%) had colorectal cancer and 757 (7.6%) had advanced precancerous lesions (advanced adenomas or sessile serrated polyps ≥1 cm) on colonoscopy. Stool DNA test performed significantly better than FIT in diagnosing both these types of neoplastic lesions: it detected 92.3% of colorectal cancer compared with 73.8% by FIT (p=0.002); stool DNA test detected 42.4% of advanced precancerous lesions, while FIT detected 23.8% (p<0.001). Similarly, for polyps with high-grade dysplasia, stool DNA test detected 69.2% and FIT 46.2% (p=0.004) of these lesions. For serrated sessile polyps ≥1 cm, 42.4% were detected by stool DNA test and 5.1% by FIT (p<0.001).

In participants with non-advanced lesions or negative findings, the specificity with DNA testing was 86.6% and with FIT 94.9% (p<0.001). In those with negative findings on colonoscopy, the specificity with DNA testing was 89.8% and with FIT 96.4% (p<0.001). In order to detect one colorectal cancer case, 154 individuals would need to be screened using colonoscopy, 166 with stool DNA test, and 208 with FIT. These findings suggest a role for the multitarget stool DNA test for screening of colorectal cancer. In August 2014, the US Food and Drug Administration approved the use of these stool DNA markers for the screening of colorectal cancer. By incorporating an immunochemical assay for human haemoglobin, these stool DNA markers are now commercially available for the screening of colorectal cancer.

Zhang *et al*. performed a meta-analysis of methylated genes in the detection of colorectal cancer in stool by analysing 37 articles with 4,484 patients (30). *SFRP2* methylation for detection of cancer had a sensitivity of 79% [95% confidence interval (CI)=75-82%], a specificity of 93% (95% CI=90-96%), the diagnostic odds ratio was 47.57 (95% CI=20.08-112.72), and the area under the receiver operating characteristic curve was 0.9565. For adenoma detection, *SFRP2* methylation had a sensitivity of 43% (95% CI=38-49%), a specificity of 94% (95% CI=91-97%), the diagnostic odds ratio was 11.06 (95% CI=5.77-21.18), and the area under the curve was 0.9563. *SFRP2* methylation, therefore, has the potential to be used as a non-invasive screening test for colorectal cancer.

Methylated DNA in the circulation. Church *et al*. prospectively assessed the value of circulating methylated *SEPT9* DNA for detecting colorectal cancer in a screening

population (31). A total of 7,941 asymptomatic men (45%) and women (55%) aged ≥50 years, with a mean age of 60 years, who were due for screening colonoscopy at 32 US and German clinics took part. Blood plasma was taken before bowel preparation. Methylated *SEPT9* DNA of all patients with colorectal cancer and stratified random samples of other individuals, not the whole study population, were analyzed blindly using commercially available assay in three different laboratories. A standardised sensitivity of 48.2% (95% CI=32.4-63.6%; crude rate=50.9%) was obtained from 53 patients with colorectal cancer and from 1,457 individuals without. In patients with colorectal cancer stage I, methylation of *SEPT9* DNA had a sensitivity of 35.0%, for stage II 63%, stage III 46.0% and stage IV 77.4%. The specificity was 91.5% (95% CI=89.7 to 93.1%; crude rate=91.4%). However, for advanced adenomas the sensitivity was only 11.2%.

Pedersen *et al*. compared two methylated DNA markers *BCAT1* and *IKZF1* from 74 patients with colorectal cancer and 144 healthy controls (32). Methylation-specific polymerase chain reaction assays were developed to measure the level of these markers extracted from plasma. Methylated *BCAT1* detected 48 (65%) out of 74 carcinomas and methylated *IKZF1*, 50 (68%) out of the 74. By combining both markers, 57 out of 74 cases of cancer (77%) were detected. In the 144 healthy controls, only five (4%) were positive with methylated *BCAT1* and seven (5%) with methylated *IKZF1*. When both markers were combined, only 11 out of 144 (7.6%) controls became positive. An increasing level of methylated DNA was found to correlate with advanced stage of colorectal cancer. A combination of these two methylated DNA biomarkers therefore improved the detection rate of colorectal cancer, with little change in specificity.

DNA Methylation Biomarkers for Therapy of Colorectal Cancer

Ouchi *et al*. studied genome-wide methylation status in two groups of patients with metastatic colorectal cancer which expressed wild-type *KRAS*, and their response to anti-EGFR therapy (33). Paraffin-embedded tumour specimens were used for this study. There were 45 patients in the first group and 52 in the second group. Each group was divided into highly-methylated colorectal cancer (HMCC) and low-methylated colorectal cancer (LMCC) by unsupervised clustering analyses. The clinical outcome in both groups was significantly better in the LMCC subgroup than the HMCC subgroup (response rate: 35.7% *vs*. 6.3%, $p=0.03$; disease control rate: 75% *vs*. 31.3%, $p=0.005$; hazard ratio for progression-free survival=0.27; 95% CI=0.13-0.57, $p<0.001$ and overall survival=0.19; 95% CI=0.06-0.54, $p<0.001$). Genome-wide methylation status was, therefore, a predictive marker for both progression-free and overall survival.

Other markers for resistance to treatment with 5-FU, irinotecan and oxaliplatin include hypermethylation of the gene for transcription factor AP-2 epsilon (34) and SPARC coding for the matricellular protein osteonectin (35).

DNA Methylation Biomarkers for Prognosis of Colorectal Cancer

Methylated DNA in tissues. There is conflicting evidence for the prognostic value of O(6)-methylguanine-DNA-methyltransferase (*MGMT*) methylation in colorectal cancer. Li *et al*. performed a meta-analysis to address this uncertainty. In this analysis, 14 studies were included after 120 articles were assessed (36). Pooled hazard ratios and odd ratios with 95% CIs were calculated using fixed- or random-effect models depending on the heterogeneity between studies. MGMT methylation was not significantly correlated with overall survival of patients with colorectal cancer. However, it was significantly increased in adenomas compared to normal tissues, confirming the adenoma–carcinoma sequence of colorectal carcinogenesis. *MGMT* methylation has no prognostic value in colorectal cancer.

Methylated DNA in stool. Kisiel *et al*. examined the presence of methylated *NDRG4* and *BMP3* in stool before and after colorectal resection in 22 patients and from 80 controls who had normal colonoscopy (37). The target genes were extracted from stool, treated with bisulphite and assayed by quantitative allele-specific real-time target and signal amplification. Results were dichotomised at 95% specificity cut-offs. They found that after colorectal cancer resection, levels of methylated *NDRG4* and *BMP3* fell significantly. In 14 out of 22 patients who had raised preoperative markers levels, 13 patients had normal range postoperatively. In one patient with a rapid rise in NDRG4 level following colon cancer resection, recurrent disease was diagnosed. These markers may be of value in postoperative surveillance.

There are other prognostic methylation biomarkers in colorectal cancer. Homeodomain-only protein X-β (*HOPXB*) gene-promoter methylation was found to be associated with poor prognosis in stage III colorectal cancer by analyzing cancer samples using quantitative methylation-specific PCR (38). The presence of methylation of microRNA *miR-128* in metastases to lymph nodes and peritoneal metastases was correlated with a poor prognosis (39).

Recent Advances in the Understanding of the Biogenesis of MicroRNAs

miRNAs are small, single-stranded, non-coding RNAs. miRNAs suppress gene expression through their interaction with mRNA by binding with complementary sequences in the 3' untranslated region (40). Each miRNA has the potential to

interact with many different mRNAs, which in turn may be suppressed by many miRNAs. miRNAs, therefore, influence cellular functions involved in malignant transformation, angiogenesis, cell growth or inflammatory response (41).

Recent advances show that the biogenesis of miRNAs involves the transcription of primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus. Pri-miRNAs are capped, spliced and polyadenylated (42). They are then cleaved by the microprocessor formed by the enzyme DROSHA and cofactor DGCR8, generating precursor miRNAs (pre-miRNAs) (43-46). Pre-miRNAs are translocated to the cytoplasm from the nucleus by exportin 5 (47), where they are cleaved to form miRNA duplexes consisting of a mature miRNA and a complementary passenger strand miRNA*, which is degraded (48) by the RNAse III enzyme DICER1 (44, 49). miRNA* may have an unknown function (50). The mature miRNA combines with a ribonucleoprotein effector, forming the RNA-induced silencing complex that silences genes (51) by binding with the target mRNA, causing the mRNA to degrade or to fail translation (52).

miRNA genes are located in chromosomal regions prone to damage through deletion, amplification or translocation (53, 54). Such damage may result in cancer formation. Dysregulated miRNAs may arise from a defective mechanism in miRNA biogenesis, leading to cancer development (41). miRNAs are involved in many types of cancer, including colorectal cancer, in which miRNAs may act as tumour suppressors or as oncogenes. miRNAs remain stable after prolonged storage, exposure to high or low pH levels or boiling, and are detectable in archival tissues and serum (55, 56). miRNAs can be extracted for analysis from blood, plasma, serum and various body fluids, in frozen or paraffin-embedded tissues (57). In colorectal cancer, miRNAs have been found to act as markers for diagnosis, response to therapy and prognosis.

miRNAs as Diagnostic Markers for Colorectal Cancer

miR-21 and miR-92a and have been investigated in serum in patients with colorectal cancer and adenoma (58). They were found at significantly higher levels in patients with colorectal cancer and advanced adenoma than those in healthy controls. They have lower sensitivities and specificities in stool than in serum for the detection of colorectal cancer, with a sensitivity of 71.6% and a specificity of 73.3% for *miR-92a* and sensitivity of 55% and a specificity of 73.3% for *miR-21* in stool (59).

Serum miR-92a had an independent prognostic significance in colorectal cancer. A high miR-92a expression was correlated with poor survival (p=0.03; hazard ratio=4.36; 95% CI=1.64-11.57) (58). miR-21 is not specific to colorectal cancer and has also been found in the plasma of patients with many other cancer types (60). In order to detect colorectal cancer with more specificity, Kanaan *et al*. used a panel of eight plasma miRNAs (*miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532*, and *miR-652*) (61). These were able to identify polyps from controls. Another panel of three plasma miRNAs (*miR-431, miR-15b*, and *miR-139-3p*) was able to distinguish stage IV colorectal cancer from controls.

An miRNA, *miR-135b*, was found at higher levels in colorectal cancer and adenomas when compared to normal adjacent colon. In a study on stool specimens, *miR-135b* detected colorectal cancer with a sensitivity of 78%, advanced adenomas with 73%, and 62% in adenomas, with a specificity of 68%. There was an increase of level of *miR-135b* from adenomas to colorectal cancers when compared with patients with inflammatory bowel disease or healthy controls. *miR-135b* levels in stool decreased significantly following surgery for these colorectal neoplastic lesions. *miR-135b* may act as a non-invasive biomarker for early-stage colorectal cancer (62).

In another study on stool specimens, the addition of *miR-106a* to a faecal occult blood test increased the sensitivity of colorectal cancer detection from 60.7% to 70.9% but specificity was slightly reduced from 98.1% to 96.3% compared to testing without *miR-106a*. *miR-106a* remained stable in stool after storage for 5 days at 4˚C (63).

Ahmed *et al*. performed global microarray expression studies on stool from 15 persons consisting of three controls and 12 with colon cancer, with three in each group of stage 0-I, stage II, stage III and stage IV (64). They found 141 miRNAs preferentially increased in expression and 61 reduced. Twenty miRNAs were selected for further study on stool specimens from 60 individuals, consisting of 20 controls, 20 with stage 0-I, 10 with stage II, five with stage III, and five with stage IV colon cancer. This showed that 12 miRNAs had raised expression in colorectal cancer (*miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p* and *miR-214*). The expression of these 12 miRNAs increased with the advancing TNM stages of colon cancer. Similarly, the eight miRNAs (*miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR146a, miR-222* and *miR-938*) with a reduced expression in colon cancer also had diminished expression with progression of TNM stage. These findings encourage the development of a chip for molecular screening of colon cancer (64).

miRNAs as Therapeutic Markers for Colorectal Cancer

Mlcochova *et al*. investigated miRNAs as markers for response to anti-EGFR treatment in patients with metastatic colorectal cancer with wild-type RAS, using cetuximab and panitumumab (65). Nine miRNAs with significantly different expression

between responders and non-responders to cetuximab therapy were identified ($p \leq 0.01$). Further studies showed that *miR-31-3p* ($p<0.001$) and *miR-31-5p* ($p<0.001$) were strongly associated with time to progression in patients treated with cetuximab but not those treated with panitumumab.

Simmer *et al*. analyzed the levels of 22 miRNAs and the DICER protein in primary tumours from patients with metastatic colorectal cancer treated with first-line capecitabine monotherapy in the CAIRO trial of the Dutch Colorectal Cancer Group, and progression-free survival (66). They found an increase in median progression-free survival in patients with primary tumours with a low-level expression of miR-143, compared to those with high expression. In addition, an ion transport regulator, *FXYD3*, and a putative target of *miR-143*, also showed an association with progression-free survival.

Perez-Carbonell *et al*. conducted a systematic discovery and validation of miRNA biomarkers in two clinical trial cohorts of patients with colorectal cancer (67). During the 'discovery' phase, Affymetrix miRNA expression arrays were used to investigate stage III colorectal cancer in patients with and without recurrent cancer at 3 years (n=50 per group). All patients were treated with FOLFOX (*i.e*. adjuvant 5-FU and oxaliplatin). During the 'validation' phase, miRNAs were analyzed by quantitative RT-PCR in another cohort of 237 patients with stage II-IV colorectal cancer treated with 5-FU-based chemotherapy, and in normal colonic mucosa from 20 healthy individuals. Cox proportional hazard models were used to analyze disease recurrence, disease-free survival and overall survival. miR-320e was found to be a prognostic biomarker indicating poor clinical outcome in patients with stage III colorectal cancer treated with 5-FU-based adjuvant chemotherapy.

miRNAs as Prognostic Markers for Colorectal Cancer

miRNAs are associated with epithelial-to-mesenchymal transition during carcinogenesis. Such miRNAs might act as prognostic markers for colorectal cancer. In a three phase-study, Toiyama *et al*. selected miRNAs associated with metastasis by analyzing four miR-200 family members (*miR-200b, miR-200c, miR-141* and *miR-429*) in serum from 12 patients with stage I and IV colorectal cancer (68). Candidate miRNAs were then validated in 182 patients with colorectal cancer and 24 controls. Finally, the expression of selected miRNAs was analyzed in 156 matched tumour tissues from 182 patients with colorectal cancer and in a different set of 20 colorectal cancers and their liver metastases in order to locate the source of these miRNAs. *miR-200c* was found at higher levels in liver metastases than in primary cancer. It was the best serum marker for metastasis, with expression levels significantly higher in stage IV compared to stage I-III colorectal cancer, in lymph nodes, in distant metastasis and prognosis. *miR-200c* is also an independent marker for lymph metastasis, cancer recurrence and an independent prognostic marker for colorectal cancer.

Ling *et al*. used miRNA microarrays to analyze primary colorectal cancer tissues from patients with and without metastasis (69). Selected miRNAs were tested in 85 colorectal specimens by quantitative real-time PCR. Metastatic activity of these miRNAs was further examined. By means of prediction algorithms, quantitative real-time PCR, western blot and luciferase assays, the targets for these miRNAs were revealed. *miR-224* expression was analyzed in 449 patients in six sets of colorectal cancer cases and the Cancer Genome Atlas Network (70). It was found that in colorectal cancer, *miR-224* expression rises persistently with cancer burden and microsatellite-stable status. SMAD4 was found to be a target of miR-224 and they are negatively correlated. It was concluded that *miR-224* facilitates colorectal cancer metastasis by involving SMAD4. Patients whose tumours expressed a high level of *miR-224* had poorer prognosis with a shorter overall survival.

Many other potential colorectal prognostic markers have been reported, for example, *miR-214* (71), *miR-182* (72), *miR-124* (73), *miR-30b* (74) and *miR-155* (75). Further studies are required to confirm their clinical values.

Future Role of miRNAs in Colorectal Cancer

New colorectal miRNA markers are being continuously identified (52, 57, 76, 77). These markers require further studies and validation before they can be routinely applied in the clinic. It is likely that a combination of markers rather than individual markers will be more effective when applied in the clinical setting. Standardisation of miRNA extractions from various sources and quality control will reduce variations in reported miRNA levels from different investigators, enabling more meaningful comparison of results (77). In this regard, *miR-1228* has been shown to act as a stable endogenous control for circulating miRNAs (78).

Discussion

The gene expression-based CMS of colorectal cancer formulated by an international consortium of six expert groups is a significant development in the classification of colorectal cancer (5). Through this collaborative effort, the pattern of colorectal carcinogenesis, and the pathways involved, has become clearer. The classification of the four CMS brings together seemingly disparate findings of molecular events in colorectal cancer formation by different research groups. By correlating with clinical outcomes, CMS classification of colorectal cancer has provided valuable information on the clinical behaviours, prognosis, and responses to treatment of the four colorectal cancer CMS (5). A better understanding of processes involved in the

carcinogenesis of these four subtypes also facilitates the identification of biomarkers and the design of targeted therapies for each subtype.

Colorectal biomarkers are being discovered at a rapid pace through research into the many processes involved in colorectal carcinogenesis by various groups. Many of these biomarkers, such as methylated DNAs and microRNAs, appear promising in small-scale studies. However, before their routine clinical application, validation with large-scale clinical trials is required. To this end, CMS classification of colorectal cancer would enable like-for-like comparisons of the treatment outcomes of different therapeutic regimes. Collaborative efforts, as illustrated by the formation of CRC Subtyping Consortium (5) in the subtyping of colorectal cancer serve as a model for collaboration between groups in colorectal cancer research. Such collaborations would accelerate advances in many areas to bring laboratory research into the clinical arena more readily and quickly.

Anti-EGFR therapies demonstrate the value of applying biomarkers in 'precision medicine' in the treatment of advanced colorectal cancer (17). Experience gained from many clinical trials continues to refine the optimal use of anti-EGFR treatment to obtain maximal therapeutic effects in patients with the best-fit molecular profile for such therapies. However, other promising markers such as *BRAF, PIK3CA, PTEN* and vascular endothelial growth factor (*VEGF*) require further elucidation. Some of these markers are currently being investigated in FOCUS4, a stratified randomised controlled trial based in the UK for advanced inoperable colorectal cancer, started in 2014. The cohorts included in this trial are: i: *BRAF*-mutant tumours; ii: *PIK3CA*-mutant tumours/*PTEN* loss; iii: *KRAS*- or *NRAS*-mutant tumours; iv: EGFR-dependent (*BRAF, PIK3CA, KRAS, NRAS* wild-type) tumours; and v: non-classified type. It aims to register 2,400 patients and randomise 1,536 patients over 4-5 years (www.focus4trial.org).

The use of aberrant methylated DNAs or miRNAs offers opportunities to screen, diagnose, follow-up after resection, or to act as prognostic markers by non-invasive means through testing blood, serum, plasma, or stool specimens for colorectal cancer. It is likely that a panel of markers is needed to improve detection rates. This is illustrated by the adoption of *KRAS* mutations, aberrant *NDGR4* and *BMP3* methylation and β-actin in the population screening of colorectal cancer (29). The stability and extractability of miRNAs under different conditions and temperature, in body fluid, blood, stool, fresh and frozen tissues, and in archival paraffin-embedded materials (55-57) makes them ideal candidates as biomarkers over less stable ones.

Novel biomarkers are also being investigated. Hamm *et al*. reported that circulating monocytes are plastic cells (79). They respond to soluble factors released by colorectal cancer. This monocyte signature, expressed early in carcinogenesis, is maintained as the cancer progresses. It was specific for the monocytic fraction of mononuclear cells. This process was reversible as the modified genes returned to normal expression following successful treatment of the cancer. This opens the possibility of using such tumour-educating circulating monocytes for diagnosis and follow-up of patients with colorectal cancer.

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