MicroRNAs are Novel Biomarkers for Detection of Colorectal Cancer

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1. Introduction

Incidence of Colorectal Cancer: Colorectal cancer (CRC) is the third most common neoplasm worldwide. According to the International Agency for Research on Cancer (IARC), approximately 1.24 million new cases of CRC were detected worldwide in 2008 (Ferlay, et al, 2008). It is the third most common cancer in men (10.0% of the total) and the second commonest in women (9.4% of the total) worldwide. IARC data have shown that more than half of all CRC cases occur in the developed regions of the world i.e. Europe, America and Japan (Ferlay, et al, 2008). In the European Union (EU27) alone 334,000 new cases of CRC were detected in 2008 and approximately 38,000 people were diagnosed with CRC in the UK alone (National UK Statistics). The incidence of CRC is on rise in Europe, particularly in southern and Eastern Europe, where rates were originally lower than in Western Europe (Coleman, et al, 1993 & Bray, et al, 2004). Contrary to the current trend in Europe, the incidence rate of CRC in the USA has fallen in the last two decades (NCI-SEER, 2006). Epidemiological studies have identified that a rapid trend of ‘Westernization’, with change in diet and life style has resulted in increased incidence rates of CRC in developing countries (Marchand, et al, 1999, Flood, et al, 2000, Boyle, et al, 2008, & Ferlay, et al, 2010). The occurrence of CRC is strongly related to age, with nearly 80% of cases arising in people who are 60 years or older, although there has been a recent increase in incidence in people younger than 60. The lifetime risk for developing CRC in men is 1 in 16 whereas in women it is 1 in 20 (National Statistics, UK).

2. The need for improved biomarkers

The survival and prognosis of patients suffering from CRC depends on the stage of the tumour at time of detection. "Five year survival" significantly reduces from 93% for localized early cancerous lesions (Dukes A) to < 15% for advanced metastatic cancers (Dukes D). Unfortunately, approximately one third of patients with CRC have regional or distant spread of their disease at time of diagnosis (Ferlay, et al, 2008). Currently, bowel
cancer screening programmes in Europe use either flexible sigmoidoscopy (FS) or guaiac-based faecal occult blood testing (FOBT) as the primary screening tool, with the current gold standard colonic imaging modality of colonoscopy being reserved for patients testing positive. Both primary screening tests have proven to be of benefit in reducing the death rate from CRC in randomised controlled trials but are generally considered to lack the desired convenience or accuracy for use as a general screening test (Hewitson, et al, 2007). A comparative study of diagnostic sensitivities of FOBT, faecal immunochemical stool testing (FIT), flexible sigmoidoscopy (FS), colonoscopy and CT colonography (CTC) has revealed 20%, 32%, 83.3% 100% and 96.7% sensitivity, respectively for the detection of CRC and advanced adenomas (Graser, et al, 2009). Endoscopic and radiological diagnostic modalities are expensive and are associated with risks such as bleeding, infection, bowel perforation and exposure to radiation. This explains why there is still a need for an improved, reliable, accurate and non-invasive biomarker for colorectal cancer detection.

3. Colorectal cancer development

The development of CRC follows the sequential progression from adenoma to the carcinoma (Vogelstein, et al, 1988). Carcinogenesis pathways for colorectal neoplasia have become much clearer and precise in the past two decades. The common pathway for CRC development is dependent on Adenomatous Polyposis Coli (APC) & Tumour Protein-53 (TP53) gene mutations and is initiated through WNT signalling (Segditsas, et al, 2006). In this pathway colonic carcinoma originates from the colonic epithelium as a consequence of accumulation of genetic alterations in the tumour suppressor gene TP53 and oncogenic APC genes. The initial genetic alterations result in adenoma formation in which cells exhibit autonomous growth. During the further course of carcinogenesis, intestinal epithelial cells acquire the characteristics of invasion and the potential for metastasis. Another carcinogenesis pathway has recently gained acceptance and is commonly named as the serrated-neoplasm pathway. This pathway is for the most part APC and TP53 independent and shows distinct molecular features of somatic mutations such as BRAF mutation and concordance with high CpG islands methylation phenotype (CIMP-H), microsatellite instability (MSI+) and MutT homologue 1 (MLH1) methylation (Casey, et al, 2005 & Spring, et al, 2006) . Sequential progression of colorectal neoplasia from adenoma to carcinoma highlights that opportunities exist to improve cancer specific survival by altering the natural course of disease development. Such interventions could potentially be chemo preventive for high risk individuals, the early detection of colorectal neoplasia, chemotherapy to down stage the cancer prior to surgical resection and therapy for palliation of symptoms in advanced stage cancer. Recent advances in proteomics and genomics provide a vast amount of information about the role of micro-molecules in several cancer related pathways. These advances have focused on the detection of micro molecules released from tumour cells and their utility as diagnostic biomarkers. The discovery of tumour specific microRNAs (miRNAs) has opened a new era of biomarker research that holds great potential for future cancer detection strategies.

4. What are MicroRNAs

MicroRNAs are single-stranded, evolutionarily conserved, small (17–25 ribonucleotides) noncoding (Lee, et al, 1993) RNA molecules. MiRNAs function as negative regulators of
target genes by directing specific messenger RNA cleavage or translational inhibition through the RNA induced silencing complex (RISC) (Bartel, et al, 2004 & 2009). So far around 1400 mature human miRNAs have been described in the Sanger miRBase version 17 (An international registry and database for miRNA nomenclature, targets, functions and their implications in different diseases). In the database, each mature miRNA in human and non-human species is assigned a unique identifier number for universal standardization. For example human microRNA 21 is designated as hsa-miR-21. Table 1 summarizes the different types of RNAs by size, mechanism of action and function in human cells.

<table>
<thead>
<tr>
<th>Types of Non Coding RNA</th>
<th>Size No of Nucleotides</th>
<th>Mechanism of Action</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA (miRNA)</td>
<td>17-23</td>
<td>RNA induced silencing complex (RISC)</td>
<td>Translational Inhibition</td>
</tr>
<tr>
<td>Messanger RNA (mRNA)</td>
<td>900-1500</td>
<td>Conveys genetic information from DNA to the ribosomes</td>
<td>Protein synthesis</td>
</tr>
<tr>
<td>Small interfering RNA (SiRNA)</td>
<td>20-25 Double stranded</td>
<td>RNA interference and RNA interference related pathways</td>
<td>Interference with gene expression</td>
</tr>
<tr>
<td>Piwi-interacting RNA (piRNA)</td>
<td>26-31</td>
<td>RNA-protein complex formation with piwi proteins</td>
<td>Transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells</td>
</tr>
<tr>
<td>Small Nucleolar RNA (SnoRNA)</td>
<td>70-200</td>
<td>Act as ribonucleoprotein (RNP) complexes to guide the enzymatic modification of target RNAs at sites determined by RNA:RNA antisense interactions</td>
<td>Chemical modifications of other RNAs e.g methylation, pseudouridylation</td>
</tr>
<tr>
<td>Transfer RNA (tRNAs)</td>
<td>73 to 93 Clover Leaf</td>
<td>Transfers a specific active amino acid to a growing polypeptide chain at the ribosomal site of Protein</td>
<td>Amino acid carriers and protein synthesis during translation.</td>
</tr>
<tr>
<td>Ribosomal RNA ( rRNA)</td>
<td>120-5050</td>
<td>Decode mRNA into amino acids</td>
<td>Protein synthesis in ribosomes</td>
</tr>
</tbody>
</table>

Table 1.

5. MicroRNA biogenesis in human cells

MiRNAs are mostly transcribed from intragenic or intergenic regions by RNA polymerase II into primary transcripts (pri-miRNAs) of variable length (1 kb- 3 kb). In the nucleus Pri-miRNA transcript is further processed by the nuclear ribo-nuclease enzyme ‘Drosha’ thereby resulting in a hairpin intermediate of about 70–100 nucleotides, called pre-miRNA. The pre-miRNA is then transported out of the nucleus by a transporting protein exportin-5.
In the cytoplasm, the pre-miRNA is once again processed by another ribonuclease enzyme ‘Dicer’ into a mature double-stranded miRNA. The two strands of double stranded miRNA (miRNA/miRNA* complex) are separated by Dicer processing. After strand separation, the mature miRNA strand (miRNA- also called the guide strand) is incorporated into an RNA-induced silencing complex (RISC), whereas the passenger strand, denoted with a star (miRNA*) is commonly degraded (Hammond, et al, 2000, Lee, et al, 2003, Bohnsack, et al, 2004 & Thimmaiah, et al, 2005). This miRNA/RISC complex is responsible for miRNA function. If on miRNA cloning or array the passenger strand is found at low frequency (less than 15% of the guide strand) it is named miR*. However, if both passenger and guide strand are equal in distribution, then these two strands are named 3p and 5p version of miRNA depending on their location to either 5' or 3' of the miRNA molecule. In this case both strands can potentially incorporate in RISC complex and have a biological role. Nevertheless, quite a few miRNA* strands are found to be conserved and play an important role in cell homeostasis. However, only recently studies have focussed on the functional role of the miRNA* strand. Well-conserved miRNA* strands may prove important links in cancer regulation networks (Stark, et al, 2007, Okamura, et al, 2008, Zhou, et al, 2010 & Guo, et al, 2010). Figure 1 illustrates the biogenesis of miRNAs in the cellular nucleous, its transport to cytoplasm, and processing by Drosha and Dicer Enzymes. Figure 1 also illustrates the RISC incorporation of miRNAs for functional activity in different pathways of translational inhibition or activation.

Fig. 1.

6. Mechanism of action & cellular function of MicroRNA

The specificity of miRNA targeting is defined by Watson–Crick complementarities between positions 2 to 8 from the 5 primed end of miRNA sequence with the 3' untranslated region
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(UTR) of their target mRNAs. When miRNA and its target mRNA sequence show perfect complementarities, the RISC induces mRNA degradation. Should an imperfect miRNA–mRNA target pairing occur, translation into a protein is blocked (Bartel, et al, 2004 & 2009). Regardless of which of these two events occur, the net result is a decrease in the amount of the proteins encoded by the mRNA targets. Each miRNA has the potential to target a large number of genes (on average about 500 for each miRNA family). Conversely, an estimated 60% of the mRNAs have one or more evolutionarily conserved sequences that are predicted to interact with miRNAs (Friedman, et al, 2009). MiRNAs have been shown to bind to the open reading frame or to the 5’ UTR of the target genes and, in some cases, they have been shown to activate rather than to inhibit gene expression (Ørom, et al, 2008). It has also been reported that miRNAs can bind to ribonucleoproteins in a seed sequence and a RISC-independent manner and then interfere with their RNA binding functions (decoy activity) (Eiring, et al, 2010). MiRNAs can also regulate gene expression at the transcriptional level by binding directly to the DNA (Khraiwesh, et al, 2010) as illustrated in Figure 1.

7. Methods of MicroRNA analysis and quantification

Numerous approaches have been developed to analyze and quantify the expression of miRNAs. A commonly adopted strategy is to perform mass scale expression profiling/signature of miRNAs on a small cohort of patients to identify most significantly dysregulated miRNAs. Expression profiling is usually followed by a validation of selected miRNAs on an independent cohort by using QRT-PCR. Expression profiling has been performed using Hybridization-Microarray, Real Time Polymerase Chain Reaction (QRT-PCR) Array and most recently Deep-Sequencing (Meyer, et al, 2010). Most of these approaches are developed against the gold standard ‘Northern Blotting’. Each has its unique advantages and disadvantages, such as throughput, sensitivity, ease of use and cost. QRT-PCR can detect very low concentrations of molecules with much superior sensitivity and expenditure of time and money (Chen, et al, 2005). Microarray-based techniques have the advantage of being relatively cost-effective, quick and simple to utilize (Pradervand, et al, 2010). Ultra high throughput miRNA sequencing allows de-novo detection and relative quantification of miRNAs, but requires a considerable amount of time and cost for data generation and data analysis (Wang, et al, 2007). A key issue of miRNA detection and quantification is the selection of endogenous controls for relative quantification. In QRT-PCR based detection systems, several small nuclear and small nucleolar RNAs (e.g. RNU6B) are recommended for normalising miRNA expression signature/profiles in tissues, cell lines, and human body fluids. However, RNU6B is heat unstable and rapidly degrades resulting in poor reproducibility of experiments. That’s why many researchers have used the invariant and most stable miRNAs as endogenous controls (Meyer, et al, 2010). In order to overcome this problem of normalization in QRT-PCR and other detection systems, researchers have used different statistical strategies including: global mean expression; quantile; scaling; and normalizing factor. However, some normalization methods have been challenged whereas others were adapted to the specific nature of miRNA profiling experiments. At present, there is no generally agreed normalization strategy for any of the known detection approaches. Table 2 shows the comparison of different detection systems by practical application, throughput, cost and time expenditure.
8. Role of MicroRNA in colorectal cancer development

MiRNAs have been shown to play an important role in colorectal cancer oncogenesis, progression, angiogenesis, invasion and metastasis (Lee, et al, 2007, Huang, et al, 2008 & Liu, et al, 2011). Esquela-Kerscher & Slack in their review have suggested that the dysregulation of miRNA genes that target mRNAs for tumour suppressor or oncogenes can influence tumourigenesis (Esquela-Kerscher, et al, 2006). The miRNA expression profiling studies on colonic tumour and adjacent normal tissue have identified several differentially expressed miRNAs in cancerous tissue. Table 1 summarizes the relatively over-expressed and under-expressed miRNAs studied in CRC tissue from different studies. Studies focussing on the functional and mechanistic involvement of miRNAs in colon cancers have reported that selected groups of distinct miRNAs are commonly and concurrently upregulated or downregulated in colon cancer tissues and are often associated with distinct cytogenetic abnormalities (Xi, et al, 2006, Schepeler, et al, 2008 & Schetter, et al, 2008). Table 3 shows the summary of dysregulated miRNAs in colorectal tumour tissue compared to adjacent normal colonic mucosa. Over expressed or under expressed miRNAs identified by two or more studies are underlined and the miRNAs with conflicting expression levels in different studies are identified in Bold.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Downregulated miRNAs in CRC tissue</th>
<th>Upregulated miRNAs in CRC tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michael, et al, 2003</td>
<td>let-7, <strong>miR-16</strong>, <strong>miR-24</strong>, <strong>miR-26a</strong>, <strong>miR-102</strong>, <strong>miR-143</strong>, <strong>miR-145</strong>, <strong>miR-200b</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th>Detection Systems</th>
<th>MicroRNA QRT-PCR Expression Profiling</th>
<th>MicroRNA-Array</th>
<th>MicroRNA-Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>PCR</td>
<td>Hybridization</td>
<td>Deep Sequencing</td>
</tr>
<tr>
<td>Initial RNA Concentration</td>
<td>10ng</td>
<td>100 ng</td>
<td>250ng</td>
</tr>
<tr>
<td>Time Required</td>
<td>&lt; 24 hours</td>
<td>24-48 hours</td>
<td>&gt;1 week</td>
</tr>
<tr>
<td>Cost</td>
<td>Low-medium for Pool Profiling. Even lower for custom designed individual assays.</td>
<td>Low-medium for Pool Profiling</td>
<td>High</td>
</tr>
<tr>
<td>Throughput</td>
<td>Medium-high</td>
<td>High</td>
<td>Ultra-high</td>
</tr>
<tr>
<td>Utility</td>
<td>Relative and absolute quantification of miRNAs</td>
<td>Relative and absolute quantification of miRNAs</td>
<td>Relative quantification of known miRNAs. Identification of novel miRNA sequences.</td>
</tr>
<tr>
<td>Studies</td>
<td>Downregulated miRNAs in CRC tissue</td>
<td>Upregulated miRNAs in CRC tissue</td>
<td></td>
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<tr>
<td>-------------------</td>
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<tr>
<td>Volinia, et al, 2006</td>
<td>let-7a-1, miR-9-3, miR-23b, miR-138, miR-218</td>
<td>miR-16, miR-17-5p, miR-20a, miR-21, miR-29b, miR-141, miR-195, miR-199a</td>
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<tr>
<td>Xi, et al, 2006</td>
<td>let-7b, let-7 g, miR-26a, miR-30a-3p, miR-132, miR-181a, miR-181b, miR-296, miR-320, miR-372</td>
<td>miR-10a, miR-15b, miR-23a, miR-25, miR-27a, miR-27b, miR-30c, miR-107, miR-125a, miR-191, miR-200c, miR-339</td>
<td></td>
</tr>
<tr>
<td>Bandrés. et al, 2006</td>
<td>miR-133b, miR-145</td>
<td>miR-31, miR-96, miR-135b, miR-183</td>
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</tr>
<tr>
<td>Akao, et al, 2006</td>
<td>miR-143, miR-145, let-7</td>
<td>let-7 g, miR-181b, miR-200c</td>
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<tr>
<td>Nakajima, et al, 2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanza, et al, 2007</td>
<td>miR-200b, miR-210, miR-224</td>
<td>miR-17-5p, miR-20, miR-25, miR-92, miR-93-1, miR-106a</td>
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<tr>
<td>Rossi, et al, 2007</td>
<td>miR-145</td>
<td>miR-19a, miR-20, miR-21, miR-23a, miR-25, miR-27a, miR-27b, miR-29a, miR-30e, miR-124b, miR-132, miR-133a, miR-135b, miR-141, miR-147, miR-151, miR-152, miR-182, miR-185</td>
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</tr>
<tr>
<td>Slaby, et al, 2007</td>
<td>miR-31, miR-143, miR-145</td>
<td>miR-21</td>
<td></td>
</tr>
<tr>
<td>Monzo, et al, 2008</td>
<td>miR-145</td>
<td>miR-17-5p, miR-21, miR-30c, miR-106a, miR-107, miR-191, miR-221</td>
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</tr>
<tr>
<td>Schepeler, et al, 2008</td>
<td>miR-101, miR-145, miR-455, miR-484</td>
<td>miR-20a, miR-92, miR-510, miR-513</td>
<td></td>
</tr>
<tr>
<td>Schetter, et al, 2008</td>
<td></td>
<td>miR-20a, miR-21, miR-106a, miR-181b, miR-203</td>
<td></td>
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<tr>
<td>Arndt, et al, 2009</td>
<td>miR-1, miR-10b, miR-30a-3p, miR-30a-5p, miR-30c, miR-125a, miR-133a, miR-139, miR-143, miR-145, miR-195, miR-378*, miR-422a, miR-422b, miR-497</td>
<td>miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-25, miR-29a, miR-29b, miR-31, miR-34a, miR-93, miR-95, miR-96, miR-106a, miR-106b, miR-130b, miR-181b, miR-182, miR-183, miR-203, miR-224</td>
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</tr>
<tr>
<td>Slattery, et al, 2011</td>
<td>miR-143, miR-145, miR-192, miR-215</td>
<td>miR-21, miR-21*, miR-183, miR-92a, miR-17, miR-18a, miR-19a, miR-34a</td>
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</tr>
</tbody>
</table>

Table 3.
9. The use of circulating satellite MicroRNA for colorectal cancer detection

Recent work by Mitchell & Gilad (Mitchell, et al, 2008 & Gilad, et al, 2008) has identified the presence of cancer related miRNAs in the body fluids of patients with different body organ cancers. These tumour-derived miRNAs are present in human serum or plasma in a remarkably stable form and are protected from endogenous ribonuclease activity. Given that aberrantly expressed miRNAs in CRC tissue are secreted into blood, circulating miRNAs can potentially serve as non-invasive markers for CRC detection. In 2008, Chen and colleagues used high-throughput sequencing technique and compared the miRNA expression profiles of patient with CRC and healthy controls (Chen, et al, 2008). MiRNA expression profiles of CRC and healthy controls were significantly different. However, more than 75% of the aberrantly expressed miRNAs, detected in the serum of CRC patients were also present in the serum of patients with lung cancer. A similar trend was also observed in another study where expression profiles generated from plasma of breast cancer patients were compared with colorectal cancer and other solid organ cancers (Heneghan, et al, 2010).

Identification and quantification of cancer related circulating miRNAs are associated with challenges in terms of sample preparation, experimental design, and pre-analytic variation, selection of diagnostic miRNAs, data normalization and data analysis. Meyer & Kroch (Meyer, et al, 2010 & Kroh, et al, 2010) have recently addressed many of these obstacles and provided a guide for effective strategies to overcome these issues.

Preliminary studies (Ng, et al, 2009, Pu, et al, 2010 & Cheng, et al, 2011) suggest that colorectal tumour derived miRNAs are present in the circulation at detectable levels and can used as potential biomarkers for colorectal neoplasia detection. These studies used either whole plasma or total RNA extracted from a defined amount of plasma samples collected from healthy controls and diseased patients. QRT-PCR based detection systems were applied to detect selected circulating miRNAs. Selection of miRNAs was based either on results of plasma miRNA expression profiling experiments performed on relatively small cohorts of healthy and diseased patients or highly up regulated miRNAs in CRC tissue. Table 4 summarizes the sensitivity and specificity of different miRNAs investigated for their utility as biomarkers. Results of these studies are very encouraging due to the high sensitivity for detection of CRCs and adenomas. The accuracy of miRNA based detection modalities is much higher than stool based detection modalities and may be comparable with endoscopic modalities. Furthermore, the ability to detect adenomas highlights the potential role of circulating miRNAs in bowel cancer screening. Therefore, in addition to a stand alone blood test for CRC, a miRNA based blood assay can be used as a replacement of FOBT in bowel cancer screening programmes. With its higher sensitivity and specificity, it may prove cost effective and help reduce the need for unnecessary colonic investigations. Table 4 shows the comparison of sensitivity and specificity of different miRNAs for their utility as biomarkers. For detection of adenocarcinoma and adenoma, QRT-PCR based quantification of miRNAs has been the preferred method of study in the majority of these studies.

Though the analysis of circulating miRNAs in CRC patients has identified several diagnostic miRNAs, their diagnostic accuracy is still questionable. This is due to overlapping miRNA expression with other cancers, non-cancerous conditions and variability of individual miRNA expression with stage and grade of tumour. It is possible that common carcinogenesis-related miRNAs are shared by different types of tumours and investigators...
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<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Studies</th>
<th>Participants</th>
<th>Target MiRNAs</th>
<th>Diagnostic Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sensitivity %</td>
</tr>
<tr>
<td>Whole Plasma</td>
<td>Pu, et al, 2010</td>
<td>CRC (n=103)</td>
<td>miR-221</td>
<td>86</td>
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<tr>
<td></td>
<td></td>
<td>Controls (n=37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cheng, et al, 2011</td>
<td>CRC I-IV (n=102)</td>
<td>miR-141</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls (n=48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma RNA</td>
<td>Ng, et al, 2009</td>
<td>CRC (n=90)</td>
<td>miR-17-3p</td>
<td>64</td>
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<tr>
<td></td>
<td></td>
<td>Controls (n=40)</td>
<td>miR-92</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Huang, et al, 2010</td>
<td>CRC (n=100)</td>
<td>miR-29</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenomas* (n=37)</td>
<td>miR-92a</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls (n=59)</td>
<td></td>
<td>64.9*</td>
</tr>
</tbody>
</table>

Table 4.

are detecting cancer-related but not tissue specific miRNAs. Another explanation of the findings is that the detection of miRNAs released into the circulation originates in immune cells which occur as a result of a systemic immune response generated by the tumour causing abnormal proliferation of colonic cells (Dong, et al, 2011). This might also explain the finding of commonly dysregulated miRNAs in patients with CRC and Ulcerative Colitis (Pekow, et al, 2011). Furthermore, studies to date have focused on measuring the circulating levels of either single miRNAs or a subset of the known miRNAs. Due to the above reasons, a single miRNA based detection strategy would be rather ineffective whereas a CRC tissue specific expression signature generated from plasma or serum of patients with CRC and adenoma could be more informative and accurate.

The recent discovery of exosome mediated transport of cancer related miRNAs into the circulation, has shifted the focus of miRNA studies towards the isolation of tissue specific circulating exosomes and their encompassed miRNAs. Exosomes are membrane bound small vesicles (20 to 100 nm in diameter) of endocytic origin and are released by a variety of cells in both healthy and disease conditions (Théry, et al, 2002 & Keller, et al, 2006). Exosomes correspond to the internal vesicles of multivesicular bodies (MVBs) and are released in the extracellular environment upon fusion of MVBs with the plasma membrane (Théry, et al, 2002 & Cocucci, et al, 2009). Since exosome formation includes two inward budding processes, exosomes maintain the same topological orientation as the cell, with membrane proteins on the outside and some cytosol on the inside. Exosomes contain cytoplasmic proteins, miRNAs and mRNA transcripts (Valadi, et al, 2007).

The topical orientation of exosomal membrane may help in identification of their source by using surface antigen directed antibodies e.g. anti-MHCII. One drawback of this isolation method is that unless all the exosomes contain the specific surface antigen used for the
isolation, only a fraction of the exosomes will be isolated. Circulating exosomes can also be isolated based on their size, density and surface proteins. A commonly used method of purifying exosomes involves removal of cells and debris with either a filtration process or by a series of centrifugations (differential centrifugation), followed by a final high speed centrifugation (ultracentrifugation) to pellet the exosomes. Exosomes have a specific density and can be purified by floatation in a sucrose density gradient or by sucrosedeuterium oxide (D2O) cushions. Another purification method is based on exosome size and utilizes chromatography. The size and characterisation of exosomes is performed by using transmission electron microscopy, immune-electron microscopy, flow cytometry and dynamic light scattering. Table 5 summarizes the exosome isolation and characterisation methods used by different groups to analyse exosomes specific to colorectal cancer cells and methods of isolation of circulating exosomes for miRNAs analysis for other cancers (Simpson, et al, 2009). There is, however, a growing need for a fast and reliable method that yields a highly purified exosome fraction.

Based on this immunoaffinity strategy, several groups have isolated exosomes from the blood of patients with different cancers and have performed miRNA expression profiles on the total RNA isolated from these purified and probably tumour specific exosomes (Taylor, et al, 2008, Logozzi, et al, 2009 & Rabinowits, et al, 2009). Patients with cancer are found to have relatively higher quantities of exosome and encompassed miRNAs in the circulation (Rabinowits, et al, 2009). The analysis of miRNAs extracted from circulating exosomes in patients with ovarian cancer, has been proven to be equivalent to ovarian tissue biopsies (Taylor, et al, 2008). By using a similar approach of isolation and analysis, exosomal miRNAs in colorectal cancer can be evaluated for their diagnostic accuracy and may prove a breakthrough diagnostic modality.
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Isolation and Characterisation of Colorectal Cancer Cell line Exosomes

<table>
<thead>
<tr>
<th>Studies</th>
<th>Colorectal Cancer Cell lines</th>
<th>Isolation method</th>
<th>Characterisation and Validation of Exosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mathivanan, et al, 2010&lt;sup&gt;70&lt;/sup&gt;</td>
<td>LIM1215</td>
<td>Filtration, Ultracentrifugation, Immunoaffinity</td>
<td>Transmission Electron Microscopy Immune Electron Microscopy Western Blotting</td>
</tr>
<tr>
<td>Choi, et al, 2007&lt;sup&gt;71&lt;/sup&gt;</td>
<td>HT29</td>
<td>Differential Centrifugation and filtration(100k) Density Gradient</td>
<td>Transmission Electron Microscopy, Western Blotting</td>
</tr>
<tr>
<td>van Nigel, et al, 2001&lt;sup&gt;72&lt;/sup&gt;</td>
<td>HT29-19A T84 DRB1*0401/ CIITA</td>
<td>Differential Centrifugation and filtration(100k) Density Gradient</td>
<td>Transmission Electron Microscopy, Immune Electron Microscopy Western Blotting</td>
</tr>
</tbody>
</table>

Isolation and Characterisation of Circulating Exosomes for MicroRNA Analysis

<table>
<thead>
<tr>
<th>Studies</th>
<th>Cancer Type</th>
<th>Isolation Method</th>
<th>Specific Method/ Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logozzi, et al, 2009&lt;sup&gt;73&lt;/sup&gt;</td>
<td>Malignant Melanoma</td>
<td>Ultracentrifugation and filtration</td>
<td>400x g 20 min isolate plasma 1,200x g20 min 10,000x g 30 min and filter through 0.22um filter 1,00,000x g 60 min</td>
</tr>
<tr>
<td>Rabinowits, et al, 2009&lt;sup&gt;74&lt;/sup&gt;</td>
<td>Lung Cancer</td>
<td>Immunoaffinity Ultracentrifugation</td>
<td>anti-EpCAM coated Immunobead</td>
</tr>
<tr>
<td>Taylor , et al, 2008&lt;sup&gt;75&lt;/sup&gt;</td>
<td>Ovarian Cancer</td>
<td>Immunoaffinity Ultracentrifugation</td>
<td>anti-EpCAM antibody coated Immunobead</td>
</tr>
</tbody>
</table>

Table 5.

10. The use of stool MicroRNAs for detection of colorectal neoplasia

Colonic epithelium is the most dynamic cell population of the human organism. Highly differentiated colonocytes are continuously shed into the colon of healthy individuals and
patients with CRC (Brittan, et al, 2004 & Loktionov, et al, 2007). It is presumed that 
exfoliated colonocytes from healthy colon and neoplastic lesions carry important genetic 
and epigenetic information that could be utilized for subsequent testing, such as the 
detection of mutant genes or dysregulated mRNAs, proteins and miRNAs (Loktionov, et al, 
2009). It is proposed that even small neoplastic loci can alter colonic cell exfoliation rate and 
may lead to early detection of these lesions (Loktionov, et al, 2007). The effectiveness of an 
exfoliated colonocyte based detection system requires an efficient isolation of colonocytes 
while minimizing the amount of background faecal debris. In order to achieve maximum 
retrieval of colonocytes, strategies that have been employed include density gradient 
centrifugation and/or immunoaffinity on either homogenized stool samples or scrapings 
from the stool surface (Loktionov, et al, 2007). However, cell yields are generally very low, 
often with conspicuous background debris, which makes cell identification difficult and 
time consuming (Deuter, et al, 1995). Consequently, such preparations would be unsuitable 
for high-throughput population screening programs (White, et al, 2009). Furthermore, 
colonocytes shed from a proximal colonic region travel a longer distance and are more 
exposed to cytolytic agents, thus making them less likely to be preserved and sampled. If 
this does prove to be a common problem, stool miRNA markers for right-sided CRC will be 
less effective. There is evidence, from the work of Koga and Colleagues (Koga, et al, 2010) 
that this is indeed the case. In this study immunomagnetic beads were conjugated with 
EpCAM monoclonal antibody to isolate colonocytes from stool. Despite the selection of two 
highly up regulated miRNAs in CRC cells, the sensitivity of detection was approximately 
70% as shown in table 6. However, the detection rate for left sided colonic and rectal tumour 
was significantly higher, suggesting the potential utility of exfoliated colonocytes based 
miRNA assay as an alternative to flexible sigmoidoscopy. It is well established that 
profound deregulation of apoptosis is a characteristic feature of cancer. As a result of 
apoptosis, tumour specific proteins and genetic information i.e. DNA, RNA and miRNA are 
released into the lumen of colon (Ahlquist, et al, 2010). Stool environment is much more 
complex and hostile than plasma, and human RNA are rapidly degraded and only 
constitute <1% of total stool RNA (Ahlquist, et al, 2010). In contrast with the fast 
degradation of mRNA, human miRNAs are packed in micro vesicles and are well protected 
from degradation. The available data indicates that stool miRNA analysis can distinguish

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Studies</th>
<th>Participants</th>
<th>Target MiRNAs</th>
<th>Diagnostic Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Exfoliated Colonocytes</td>
<td>Koga, et al, 2010</td>
<td>CRC (n=197) / Control (n=119)</td>
<td>miR-17-92</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-135</td>
<td>46.2</td>
</tr>
<tr>
<td>Faeces</td>
<td>Link, et al, 2010</td>
<td>CRC (n=10) / Adenoma (n=9) / Control (n=10)</td>
<td>miR-21</td>
<td>Distinguished adenomas and carcinomas from healthy controls P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-106</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.
MicroRNAs are Novel Biomarkers for Detection of Colorectal Cancer

adenoma and carcinoma from healthy controls (Link, et al, 2010). The detection of miRNAs in stool specimens requires efficient protocols for stool preparation, stool miRNA extraction and quantitative analysis (Ahmed, et al, 2009). The utility of stool miRNAs as a biomarker is still in its infancy; further studies of stool miRNA are needed on larger cohorts to validate its diagnostic accuracy.

In summary, systemic and faecal miRNAs can accurately correlate with disease status and can potentially be used for colorectal cancer detection and screening. Detection of colorectal cancer based on miRNA expression analysis requires extensive pre analytical considerations for sample selection & processing, isolation of miRNAs, the method of expression analysis, selection of endogenous controls for normalisation and data analysis. Studies performed so far have shown great promise for miRNA based detection of colorectal carcinoma and adenoma. There is, however, a further need to develop and evaluate miRNA based assays before their clinical application.

11. References

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Pu XX, Huang GL, Guo HQ, et al. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. J Gastroenterol Hepatol. 2010;25(10):1674-80


Clinicians, scientists, and health care professionals use biomarkers or biological markers as a measure of a person’s present health condition or response to interventions. An ideal biomarker should have the following criteria: (I) ability to detect fundamental features of the disease, (II) ability to differentiate from other closely related diseases, (III) ability to detect early stages and stages of progression, (IV) the method should be highly reliable, easy to perform and inexpensive, and (V) sample sources should be easily accessible from body. Most of the chapters in this book follow the basic principle of biomarkers.

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