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MicroRNAs are Novel Biomarkers for

Detection of Colorectal Cancer Muhammad Imran Aslam^{1,2}, Maleene Patel^{1,2}, Baljit Singh^{1,2},

John Stuart Jameson² and James Howard Pringle¹ ¹Department of Cancer Studies and Molecular Medicine, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, ²Department of Colorectal Surgery, University Hospitals of Leicester NHS Trust, Leicester General Hospital, Gwendolen Road, Leicester, United Kingdom

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 guaiacbased 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-neoplasia 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

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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.

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

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 RNAinduced 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.





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

(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 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 denovo 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.

Detection Systems	MicroRNA QRT-PCR	MicroRNA-	MicroRNA-	
	Expression Profiling	Array	Sequencing	
Method	PCR	Hybridization	Deep Sequencing	
Initial RNA	10ng	100 ng	250ng	
Concentration				
Time Required	< 24 hours	24-48 hours	>1 week	
Cost	Low-medium for Pool Profiling. Even lower for custom designed individual assays.	Low-medium for Pool Profiling	High	
Throughput	Medium-high	High	Ultra-high	
Utility	Relative and absolute quantification of miRNAs	Relative and absolute quantification of miRNAs	Relative quantification of known miRNAs. Identification of novel miRNA sequences.	

Table 2.

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 tumourigensis (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 underexpressed 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.

Studies	Downredulated miRNAs in CRC tissue	Upregulated miRNAs in CRC tissue
Michael, et al, 2003	<u>let-7</u> , miR-16 , miR-24, <u>miR-26a,</u> miR-102, <u>miR-143</u> , <u>miR-145</u> , <u>miR-200b</u>	

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Studies Downredulated miRNAs in		Upregulated miRNAs in CRC		
	CRC tissue	tissue		
Volinia, et al, 2006	<u>let-7a-1</u> , miR-9-3, miR-23b, miR- 138, miR-218	miR-16 , <u>miR-17-5p</u> , <u>miR-20a</u> , <u>miR-21</u> , <u>miR-29b</u> , <u>miR-141</u> , miR- 195 , miR-199a		
Xi, et al, 2006	let-7b, let-7 g , <u>miR-26a</u> , <u>miR-30a-3p</u> , miR-132 , miR-181a, miR-181b , miR-296, miR-320, miR-372	miR-10a, miR-15b , <u>miR-23a</u> , <u>miR-25</u> , <u>miR-27a</u> , <u>miR-27b</u> , miR- 30c , miR-107, miR-125a , <u>miR-191</u> , <u>miR-200c</u> , miR-339		
Bandrés. et al, 2006	miR-133b, <u>miR-145</u>	miR-31 , <u>miR-96</u> , <u>miR-135b</u> , <u>miR-</u> <u>183</u>		
Akao, et al, 2006	<u>miR-143</u> , <u>miR-145</u> , <u>let -7</u>			
Nakajima, et al, 2006		let-7 g , miR-181b, <u>miR-200c</u>		
Lanza, et al, 2007		<u>miR-17-5</u> p, <u>miR-20</u> , <u>miR-25</u> , <u>miR-</u> 92, <u>miR-93-1</u> , <u>miR-106a</u>		
Rossi, et al, 2007	<u>miR-200b</u> , miR-210 , miR-224	<u>miR-19a</u> , <u>miR-20</u> , <u>miR-21</u> , <u>miR-23a</u> , <u>miR-25</u> , <u>miR-27a</u> , <u>miR-27b</u> , <u>miR- 29a</u> , miR-30e, miR-124b, miR-132 , miR-133a , <u>miR-135b</u> , <u>miR-141</u> , miR-147, miR-151, miR-152, <u>miR- 182</u> , miR-185		
Slaby, et al, 2007	miR-31 , <u>miR-143</u> , <u>miR-145</u>	<u>miR-21</u>		
Monzo, et al, 2008	<u>miR-145</u>	<u>miR-17-5p</u> , <u>miR-21</u> , miR-30c , <u>miR-</u> <u>106a</u> , miR-107, <u>miR-191</u> , miR-221		
Schepeler, et al, 2008	miR-101, <u>miR-145</u> , miR-455, miR-484	<u>miR-20a</u> , <u>miR-92</u> , miR-510, miR- 513		
Schetter, et al, 2008		<u>miR-20a</u> , <u>miR-21</u> , miR-106a, miR- 181b , miR-203		
Arndt, et al, 2009	miR-1, miR-10b, <u>miR-30a-3p</u> , miR-30a-5p, miR-30c , miR-125a , miR-133a , miR-139, <u>miR-143</u> , <u>miR-145</u> , miR-195 , miR-378*, miR-422a, miR-422b, miR-497	<u>miR-17-5p</u> , miR-18a, <u>miR-19a</u> , miR-19b, <u>miR-20a</u> , <u>miR-21</u> , <u>miR-25</u> , <u>miR-29a</u> , <u>miR-29b</u> , miR-31 , <u>miR-34a</u> , <u>miR-93</u> , miR- 95, <u>miR-96</u> , miR-106a , miR-106b, miR-130b, miR-181b , <u>miR-182</u> , <u>miR-183</u> , miR-203, miR-224		
Slattery, et al, 2011	<u>miR-143</u> , <u>miR-145</u> , miR-192, miR-215	<u>miR-21</u> , miR-21*, <u>miR-183</u> , <u>miR-</u> 92a, <u>miR-17</u> , miR-18a, <u>miR-19a,</u> <u>miR-34a</u>		

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

Tissue	sue Studies Participants		S	Target	Diagnostic Accuracy	
Туре				MiRNAs	Sensitivity	Specificity
					%	%
Whole	Pu, et al, 2010	CRC	(n=103)	miR-221	86	41
Plasma		Controls	(n=37)			
		CRC I-IV	(n=102)	miR-141	66.7	80.8
Plasma RNA	Cheng , et al, 2011	Controls	(n=48)			
	Ng at al	CRC	(n=90)	miR-17-3p	64	70
	2009	Controls	(n=40)	miR-92	89	70
		CRC	(n=100)	miR-29	69	89.1
	Huang, et al ,	Adenomas	* (n=37)		62.2*	84.7*
	2010			miR-92a	84	71
		Controls	(n=59)			
					64.9*	81.4*

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

Biomarker



Fig. 2.

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-electronmicroscopy, 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.

Isolation and Characterisation of Colorectal Cancer Cell line Exosomes						
Studies	Colorectal Cancer Cell lines	Isolation method	Characterisation and Validation of Exosome			
Huber, et al, 2005 ⁶⁹	SW403 1869col CRC28462	Differential Centrifugation	Transmission Electron Microscopy Immune Electron Microscopy Fluorescence-activated cell sorting (FACS) Western Blotting			
Mathivanan, et al, 2010 ⁷⁰	LIM1215	Filtration, Diafiltration (5K) Ultracentrifugation Immuoaffinity	Transmission Electron Microscopy Immune Electron Microscopy Western Blotting			
Choi, et al, 2007 ⁷¹	HT29	Differential Centrifugation Diafiltration(100k) Density Gradient	Transmission Electron Microscopy, Western Blotting			
van Nigel, et al, 2001 ⁷²	HT29-19A T84- DRB1*0401/ CIITA	Differential Centrifugation Density Gradient	Transmission Electron Microscopy, Immune Electron Microscopy Western Blotting			
Isolation and C	haracterisation	of Circulating Exosor	nes for MicroRNA Analysis			
Studies	Cancer Type	Isolation Method	Specific Method/ Technique			
Logozzi, et al, 2009 ⁷³	Malignant Melanoma	Ultracentrifugation and filtration	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			
Rabinowits,	Lung Cancer	Immunoaffinity	anti-EpCAM coated Immunobead			
et al, 2009 ⁷⁴		Ultracentrifugation				
1 aylor , et al, 2008 ⁷⁵	Cancer	Immunoaffinity Ultracentrifugation	anti-EpCAM antibody coated Immunobead			

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

Tissue Type Studies		Participants	Target	Diagnostic Accuracy	
	1976		MiRNAs	Sensitivity %	Specificity %
E. Caliata d	Koga , et al, 2010	CRC (n= 197)	miR-17-92	69.5	81.5
Colonocytes		Control (n=119)	miR-135	46.2	95
Faeces	Link , et al, 2010	CRC (n=10) Adenoma (n=9) Control (n=10)	miR-21 miR-106	Distinguished adenomas and carcinomas from healthy controls P<0.05	

Table 6.

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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.

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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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University Campus STeP Ri Slavka Krautzeka 83/A 51000 Rijeka, Croatia Phone: +385 (51) 770 447 Fax: +385 (51) 686 166 www.intechopen.com

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