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Identification of serum microRNA profiles in colon cancer

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Background: microRNAs (miRNAs) exist in blood in an apparently stable form. We have explored whether serum miRNAs can be used as non-invasive early biomarkers of colon cancer.

Methods: Serum samples from 30 patients with colon cancer stage IV and 10 healthy controls were examined for the expression of 375 cancer-relevant miRNAs. Based on the miRNA profile in this study, 34 selected miRNAs were measured in serum from 40 patients with stage I–II colon cancer and from 10 additional controls.

Results: Twenty miRNAs were differentially expressed in serum from stage IV patients compared with controls ($P<0.01$). Unsupervised clustering revealed four subgroups; one corresponding mostly to the control group and the three others to the patient groups. Of the 34 miRNAs measured in the follow-up study of stage I–II patients, 21 showed concordant expression between stage IV and stage I–II patient. Based on the profiles of these 21 miRNAs, a supervised linear regression analysis (Partial Least Squares Regression) was performed. Using this model we correctly assigned stage I–II colon cancer patients based on miRNA profiles of stage IV patients.

Conclusion: Serum miRNA expression profiling may be utilised in early detection of colon cancer.

Colorectal cancer (CRC) is the second leading cause of cancer-related death in developed countries (Jemal *et al*, 2010; Hrasovec and Glavac, 2012). Early detection improves survival, as 5-year survival rate declines from nearly 90% in early-stage disease (stage I–II) to 12–13% in metastatic disease (stage IV; http://www.kreftregisteret.no). To detect early cancer or adenomas, various population-based screening programmes have been implemented (Geiger and Ricciardi, 2009; Hol *et al*, 2010). Colonoscopy is the gold standard method for early detection of CRC, but widespread use is limited due to its invasive nature and high costs. The most widely used non-invasive screening method, the fecal occult blood test, is compromised by limited diagnostic accuracy. Thus, new non-invasive methods are needed.

MicroRNAs (miRNAs) are small, 19–25 nucleotide noncoding RNAs, which negatively regulate gene transcription at transcriptional or post-transcriptional level (Iorio and Croce, 2012a) and have an important role in the control of biological processes, such as cellular development, differentiation, proliferation and apoptosis. Prior studies have demonstrated the impact of miRNAs in tumour biology and oncogenesis (Stefani and Slack, 2008; Inui

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et al, 2010; Babashah and Soleimani, 2011). MicroRNAs are frequently dysregulated in cancer and have shown great potential as tissue-based markers for cancer classification and prognostication (Paranjape *et al*, 2009; Gandellini *et al*, 2011; Kong *et al*, 2012; Iorio and Croce, 2012b).

In CRC tissue, various miRNAs have been found differentially expressed compared with matched normal tissue (Liu and Chen, 2010; Schee *et al*, 2010; Dong *et al*, 2011; Hrasovec and Glavac, 2012). Some miRNAs, as mir-21 (Schetter *et al*, 2008; Dong *et al*, 2011), miR-31 (Bandres *et al*, 2006; Dong *et al*, 2011) and miR-429 (Li *et al*, 2013), have been characterised as possible prognostic markers of CRC (Dong *et al*, 2011; Menendez *et al*, 2013), whereas others such as miR-126 (Hansen *et al*, 2012) and miR-150 (Ma *et al*, 2012) have been proposed as predictive markers of response to chemotherapy. Specific miRNA signatures have also been reported to predict response to neoadjuvant chemoradiotherapy in rectal cancer (Della Vittoria Scarpati *et al*, 2012; Kheirelseid *et al*, 2012).

MiRNAs are found in serum, plasma and other body fluids (Chen *et al*, 2008; Cortez *et al*, 2011), and exist in an apparently stable extracellular form (Zheng *et al*, 2011). The mechanism underlying their stability in the RNase-rich environment of blood is not well understood, but the current model posits that circulating miRNAs are stabilised by the formation of the Ago2-miRNA complex and/or protected of degradation by encapsulation in exosomes (Valadi *et al*, 2007; Meckes *et al*, 2010; Zomer *et al*, 2010; Russo *et al*, 2012). Exosomes containing miRNA produced by malignant cells may have an important role in metastasis by promoting angiogenesis, cell proliferation and/or tumour cell invasion (Iorio and Croce, 2009; Liu *et al*, 2011; Zheng *et al*, 2011). The current comprehension involves that miRNA profiles in serum from cancer patients may mirror the profiles of the tumours. Several recent studies have characterised miRNA profiles in serum and urine aiming to identify appropriate diagnostic markers of cancer (Huang *et al*, 2010; Ohshima *et al*, 2010; Wittmann and Jack, 2010).

A limited number of studies have been undertaken searching for miRNA expression in blood from CRC patients (Chen *et al*, 2008; Ng *et al*, 2009; Huang *et al*, 2010; Pu *et al*, 2010; Nugent *et al*, 2012). The aim of this study was to identify miRNAs for early diagnosis of colon cancer. Serum samples from patients with newly diagnosed colon cancer and from blood donors were assessed for miRNA expression. We characterise a serum miRNA profile in colon cancer that may serve as a new non-invasive approach in early detection of colon cancer.

MATERIALS AND METHODS

Patients and controls. Newly diagnosed colon cancer patients from two hospitals in Norway (St Olavs Hospital, Trondheim and Hamar Hospital, Hamar) were included, as described in Trano *et al* (2009). Blood, tumour tissue and adjacent normal mucosa were collected from the patients after informed consent had been obtained. The selected groups consisted of 30 patients with metastatic (stage IV) colon cancer, and 40 patients with early-stage colon cancer (7 patients with stage I and 33 with stage II). Serum from 20 blood donors aged $\geqslant 50$ years (10 females and 10 males) were used as controls (Blood bank, St Olavs Hospital, Trondheim).

MiRNA profiling by miRCURY LNA Universal RT miRNA PCR. Isolation of RNA and all real-time quantitative PCR (Q-PCR) experiments were performed by Exiqon Company, Vedbaek, Denmark (www.exiqon.com). RNA was purified from 250 μl serum with the miRNeasy mini kit from Qiagen (Venlo, Holland) according to the manufacturers' protocol. Q-PCR was performed by using the miRCURY LNA Universal RT microRNA PCR system containing 375 miRNA assays in Study 1, and 34 miRNAs in Study 2. In Study 1, there were no replicas, whereas in Study 2 three replicates per sample were polyadenylated and reverse transcribed (RT) into cDNA for all miRNA. One real-time Q-PCR amplification was performed for each RT reaction. Detectable miRNAs were those with a Cp (crossing point) <37, or 5 Cp below the negative control.

Study design. Increasing numbers of studies postulate that colon and rectal cancer differ with respect to molecular and genetic characteristics (Li and Lai, 2009; Koga *et al*, 2010; Slattery *et al*, 2011). To ensure a homogenous patient population, only colonic cancer was included. Initially, we measured 375 cancer-relevant miRNAs in serum from 30 patients with stage IV disease and from 10 control samples (5 females and 5 males; Study 1). Twenty miRNAs were differentially expressed in serum from stage IV patients compared with controls ($P<0.01$). Subsequently, serum from 40 patients with stage I–II colon cancer was analysed for the expression of 34 miRNAs (Study 2). In all, 20 of these miRNAs were selected based on differential expression in Study 1 ($P<0.01$), 10 miRNAs were chosen from review of the miRNA literature and 4 miRNAs were additional reference miRNAs provided by Exiqon.

Statistical analysis. All miRNA profiles from Study 1 and Study 2 were normalised using the geometric mean for each sample over all miRNAs. Profiles of the 20 most differentially expressed miRNAs ($P<0.01$) in Study 1 were subjected to hierarchical clustering to create a heatmap. Missing values were imputed using the K-nearest-neighbour method.

The 21 miRNAs that showed concordant expression in advanced (Study 1) and localised (Study 2) disease were used to construct a Partial Least Squares Regression (PLSR) model. The 21 miRNA profiles from Study 1 were used as model variables, and cancer status ($1/-1$ for cancer/control) for the 30 colon cancer patients and 10 controls in Study 1 was used as a target variable. Root-mean-square error after cross-validation indicated that two principal components were optimal for this model. The two-component PLSR model was then used to investigate whether the difference between cancer and control samples in Study 2 could be recognised using the miRNA profiles of the same 21 miRNAs used to create the model from Study 1. The profiles from Study 2 thus represent a completely independent test set for model evaluation. Results were displayed graphically to determine the optimal threshold separating cancers from controls. The analysis was performed using the statistical scripting language R (http://www.r-project.org/).

RESULTS

MiRNA profile in serum from patients with stage IV colon cancer. To investigate a possible difference in miRNA expression profile between colon cancer stage IV patients and healthy subjects, 375 cancer-relevant miRNAs were assessed in sera from 30 patients and 10 healthy blood donors (Study 1). Patients and tumour characteristics are shown in Table 1. We observed a distinct different expression pattern in cancer *vs* healthy subjects. Twenty miRNAs were significantly differentially expressed with $P<0.01$. A total of 9 miRNAs were upregulated and 11 downregulated. We performed a hierarchical clustering of the 20 miRNAs with highest differential expression between serum from patients and controls (Figure 1). This clustering analysis revealed four subgroups; one corresponding principally to the control group and the three others to the patient groups.

MicroRNA profile in serum from patients with stage I and II colon cancer. To examine whether a corresponding serum miRNA profile as demonstrated in colon cancer stage IV could

Table 1. Patients' characteristics

Characteristic	Patients with metastatic disease ($n=30$), *n* (%)	Patients with localised disease ($n=40$), *n* (%)
Age (years)		
<60	9 (30)	8 (20)
60–75	8 (26.7)	12 (30)
⩾75	13 (43.3)	20 (50)
Median (range)	68.6 (41–86)	72.4 (30–93)
Sex		
Male	14 (46.7)	18 (45)
Female	16 (53.3)	22 (55)
K-RAS mutation		
Yes	8 (26.7)	11 (27.5)
No	22 (73.3)	29 (72.5)
B-RAF mutation		
Yes	13 (43.3)	14 (35)
No	17 (56.7)	26 (65)
Microsatellite instability		
Stable (MSS)	23 (76.7)	15 (37.5)
Unstable (MSI-H)	7 (23.3)	25 (62.5)
Tumour location		
Coecum	9 (30)	9 (22.5)
Ascending colon	4 (13.3)	8 (20)
Right flexure	0 (0)	4 (10)
Transverse colon	7 (23.3)	4 (10)
Left flexure	2 (6.7)	1 (2.5)
Descending colon	0 (0)	0 (0)
Sigmoid colon	8 (26.7)	14 (35)
TNM status		
T1	0 (0)	3 (7.5)
T2	1 (3.3)	4 (10)
T3	19 (63.3)	28 (70)
T4	9 (30)	5 (12.5)
Missing	1 (3.3)	0 (0)
N0	8 (26.7)	40 (100)
N1	7 (23.3)	0 (0)
N2	15 (50)	0 (0)
M0	0 (0)	40 (100)
M1	30 (100)	0 (0)
CEA		
<5	8 (26.7)	
5–100	11 (36.7)	
⩾100	5 (16.7)	
Missing	6 (20)	

Abbreviations: CEA = carcinoembryonal antigen; MSI-Hi = microsatellite instability-high; MSS = microsatellite stable; TNM = TNM staging; .

be found in early stage cancer, 34 miRNAs were further analysed in sera from 40 patients with stage I or II colon cancer and 10 healthy controls (Study 2). Figure 2 shows that 21 of the 26 detected miRNAs displayed the same expression profile as in advanced colon cancer, suggesting that most of these miRNAs are relevant as diagnostic markers. Of these 21 miRNAs, miR-423-5p, miR-210, miR-720, miR-320a and miR-378 showed the highest expression compared with controls, whereas miR-106a, miR-143, miR-103, miR-199a-3p, miR-382 and miR-151-5p showed the lowest expression. The relevance to CRC of these 26 miRNAs according to literature is summarised in Table 2.

The five miRNAs that showed different expression in early- *vs* late-stage cancer were miR-34a, miR-146a, miR-21, miR-484 and mir-425 (Figure 2). MiR-484 and miR-21 were highly expressed in stage IV as compared with controls, but lower expressed than controls in blood samples from early-stage I–II patients. The opposite was found for miR-34a and miR-146a. The expression level of miR-425 was reduced at stage I–II compared with controls, but no significant changes detected in samples from stage IV patients.

The PLSR model correctly assigns stage I–II colon cancer patients based on miRNA profiles of stage IV patients. We generally observed a good correspondence between expression profiles of stage IV (Study 1) and stage I–II cancer patients (Study 2), as 21 out of 26 miRNAs showed the same pattern of expression. This suggests that the miRNAs selected for analysis in Study 2 may be relevant as diagnostic markers. To investigate whether our miRNA expression profiles of stage IV colon cancer could be used to recognise cancer in early-stage I–II patients, we used PLSR, a supervised linear regression method, which is used for prediction and classification in multivariate analyses (Martens, 1989). Patient to patient variation in miRNA expression profiles makes it difficult to create reliable recognition models using individual miRNAs. Utilising information from several miRNA expression profiles simultaneously (multivariate analysis) is a powerful strategy to improve the confidence of such models. We thus used PLSR to model and validate our results. The model was trained using miRNA profiles from the 40 subjects (30 stage IV colon cancer patients and 10 controls) analysed in Study 1, and evaluated using cross-validation. The resulting model was then used to assign the 50 subjects (40 stage I–II cancer patients and 10 controls) from Study 2 to either the cancer or control group. In this setting, the subjects from Study 2 is regarded as a completely independent test set, and is not involved at any stage in the modelling phase. Partial Least Squares Regression can only assign independent data using the same set of miRNAs in the modelling and test set. We thus selected from Study 1 only the miRNAs assessed further in Study 2 to build our PLSR model. As five miRNAs showed clear inconsistencies in expression profiles between Study 1 and Study 2, these were not suited for modelling. The remaining set of 21 miRNAs was thus used in the final modelling and validation. The results are illustrated in Figure 3, showing that 9 out of 10 controls (specificity of 90%) and 35 out of 40 cancer patients (sensitivity of 87.5%) from Study 2 could be correctly assigned using the selected threshold.

DISCUSSION

In this study, we demonstrate distinct differences in the expression profile of miRNA in sera from colon cancer patients *vs* healthy subjects, and identify a 21 miRNA serum colon cancer profile that may be utilised to identify colon cancer patients at an early stage of the disease. The generation of a PLSR model correctly assigned stage I–II patients based on the miRNA profiles of stage IV patients, which mathematically support the trend observed in our data.

The miRNA profile in sera from CRC patients did not show a uniform profile; but showed up to be clustered into three subgroups. These three subgroups may reflect the heterogeneity in gene expression and signalling pathways leading to CRC development. MicroRNA profiling may thus be a valuable supplement to the gene expression profiling of CRC subgroups.

Our demonstration of a difference in serum miRNA expression in colon cancer patients compared with healthy individuals are in

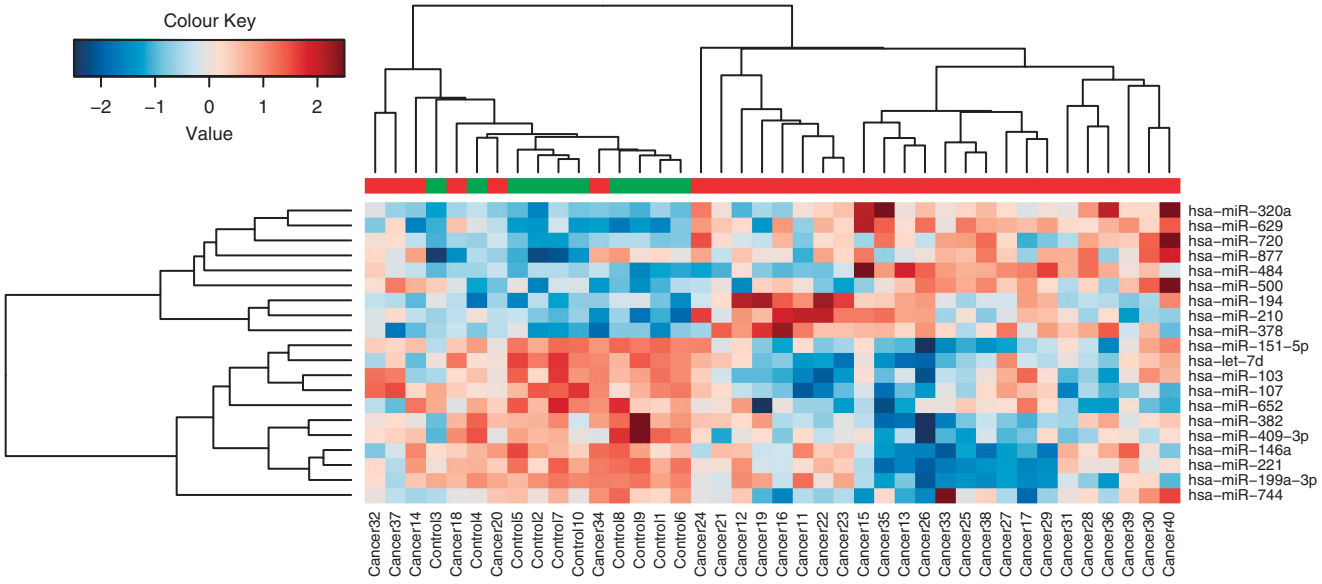

Figure 1. Heat-map diagram of a two-way hierarchical clustering analysis consisting of the 20 most differentially expressed miRNAs in serum from 30 metastatic (stage IV) colon cancer patients as compared with 10 healthy subjects (*P*-value <0.01). Red colour represents an expression level above mean, blue colour represents expression lower than the mean. Upper colour labelling show patient samples in red and controls in green.

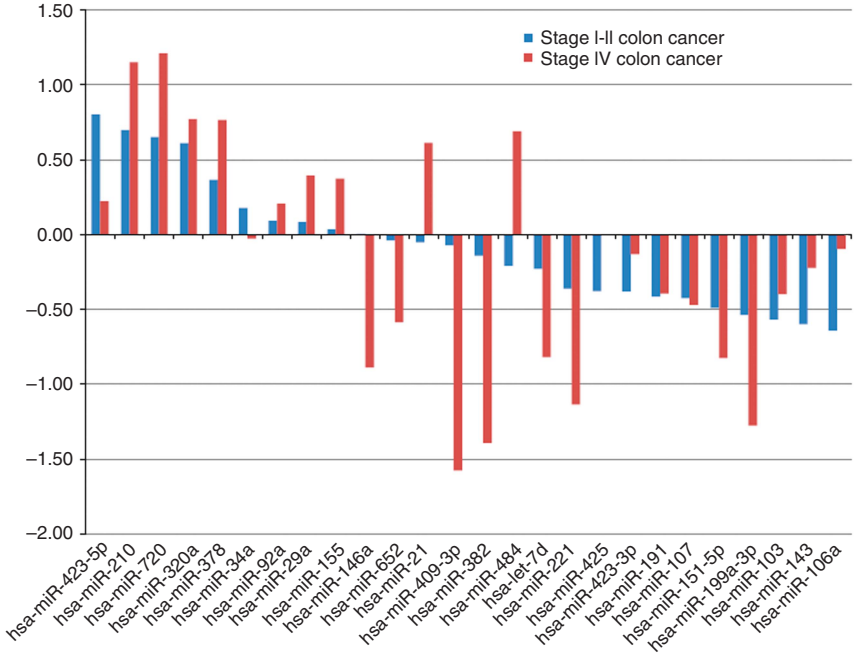

Figure 2. Differentially expressed miRNAs in stage IV (red bars) *vs* stage I–II (blue bars) colon cancer. The expression of 34 miRNAs was compared, and 26 miRNAs were detected. In all, 21 of 26 detected miRNAs showed the same expression profile in early-stage I–II *vs* metastatic stage IV colon cancer.

accordance with the relatively few studies that have assessed miRNA expression in serum of CRC patients (Chen *et al*, 2008; Ng *et al*, 2009; Huang *et al*, 2010; Pu *et al*, 2010; Nugent *et al*, 2012; Menendez *et al*, 2013). However, with regard to single miRNAs that have been reported differentially expressed, these studies present some conflicting results (Ng *et al*, 2009; Huang *et al*, 2010; Cheng *et al*, 2011; Nugent *et al*, 2012). Our finding of an upregulation of miR-92a in sera from colon cancer patients is in accordance with studies by Ng *et al* (2009) and Huang *et al* (2010), but in contrast to studies by Cheng *et al* (2011) and Nugent *et al* (2012). The reasons for opposite results may be several. Whereas most studies have included both colon and rectal cancers in their analyses, our study involves only patients with colon cancer. Thus, the issue of possible differences in miRNA expression in colon *vs* rectal cancer has been omitted in our study. Differential expression of miRNAs by tumour location could not be excluded (Slattery *et al*, 2011; Gaedcke *et al*, 2012), as well as ethnical differences in miRNA expression, which is reflected in studies from Norway (Cekaite *et al*, 2012) and China (Ng *et al*, 2009; Huang *et al*, 2010). Moreover, the detection of miRNAs still involves challenges that are reflected both in the study design and the analytical methods. We therefore suggest that a serum miRNA

Table 2. Examples of relevance to CRC of miRNAs found in sera from patients with metastatic (stage IV) and early stage (stage I-II) CRC

microRNA	Selection criteria	microRNA expression in CRC compared with controls (literature)	Concordance present study and literature	References
Upregulated in present study (stage I–II and IV colon cancer)				
miR-423-5p	Reference	Not reported in CRC	New	
miR-210	$P<0.01$	Upregulated (cells)	Yes	Ota *et al* (2012)
miR-720	$P<0.01$	Upregulated (tissue)	Yes	Ragusa *et al* (2012) Della Vittoria Scarpati *et al* (2012)
miR-320a	$P<0.01$	Downregulated (tissue and cells)	No	Schepeler *et al* (2008) Sun *et al* (2012)
miR-378	$P<0.01$	Downregulated (tissue)	No	Wang *et al* (2010); Faltejskova *et al* (2012)
miR-92a	Literature	Upregulated (plasma)	Yes and no	Ng *et al* (2009)
		Downregulated (plasma)		Huang *et al* (2010) Cheng *et al* (2011)
miR-29a	Literature	Upregulated (plasma)	Yes	Huang *et al* (2010); Weissmann-Brenner *et al* (2012)
miR-155	Literature	Upregulated (tissue)	Yes	Wang *et al* (2012) Valeri *et al* (2010)
Downregulated in present study (stage I–II and IV colon cancer)				
miR-106a	Literature	Upregulated (stool)	No	Link *et al* (2010) Diaz *et al* (2008)
miR-143	Literature	Downregulated in CRC	Yes	Michael *et al* (2003)
miR-103	$P<0.01$	Upregulated (cell lines and tissue)	No	Gottardo *et al* (2007); Li *et al* (2011b); Chen *et al* (2012)
miR-199a-3p	$P<0.01$	Not reported in CRC	New	
miR-151-5p	$P<0.01$	Not reported in CRC	New	
miR-107	$P<0.01$	Upregulated (cell lines and tissue)	No	Chen *et al* (2012)
miR-191	Reference	Upregulated	No	Xi *et al* (2006)
miR-423-3p	Reference	Not reported in CRC	New	
miR221	$P<0.01$	Upregulated (blood)	No	Pu *et al* (2010)
miR-let7d	$P<0.01$	Not reported in CRC	New	
miR-382	$P<0.01$	Downregulated	New	
miR-409-3p	$P<0.01$	Not reported in CRC	New	
miR-652	$P<0.01$	Upregulated (rectal cancer)	No	Lulla *et al* (2011) Gao *et al* (2011)
Different expression in the present study between stage I–II and IV colon cancer				
miR-34a	Literature	Upregulated	Yes (present study: upreg stage I–II; weak downreg stage IV)	Wang *et al* (2012)
		Downregulated (serum)		Nugent *et al* (2012)
miR-146a	$P<0.01$	No difference (serum)	No (present study: downreg stage IV)	Huang *et al* (2010)
miR-21	Literature	Upregulated (tissue and blood)	Yes (present study: upreg stage IV; weak downreg stage I–II)	Schetter *et al* (2008); Pu *et al* (2010); Dong *et al* (2011); Kanaan *et al* (2012)
miR-484	$P<0.01$	Not reported in CRC	New	
miR-425	Reference	No difference (tissue)	No (present study: downreg stage I–II)	Chang *et al* (2010)

Abbreviations: CRC = colorectal cancer; miRNA = microRNA.

profile may be more reliable than the detection of few individual miRNAs.

Several of the 21 miRNAs that constitute our colon cancer serum miRNA profile have been described to be involved in the development of cancer. Our demonstration of an enhanced expression of miR-21 is in accordance with previous reports showing elevated levels of miR-21 both in CRC tissue (Schetter *et al*, 2008; Dong *et al*, 2011) and blood (Pu *et al*, 2010; Kanaan *et al*, 2012). In a recent systematic review, overexpression of miR-21 and a reduced expression of let-7d were the two miRNAs that most frequently were associated with poor outcome across diverse malignancies (Nair *et al*, 2012). Interestingly, a recent large study from Denmark characterised the mir-21 expression as a prognostic factor in stage II colon cancer (Kjaer-Frifeldt *et al*, 2012). Members of the Let-7 family are reduced in a wide range of cancers and target-known oncogenes, like c-myc, RAS and HMGA2, and are therefore considered as to function as tumour suppressor-like miRNAs (Menendez *et al*, 2013). Our demonstration of a reduced level of miR-let-7d in sera from colon cancer patients was therefore not surprisingly, but to our knowledge, no previous assessment of

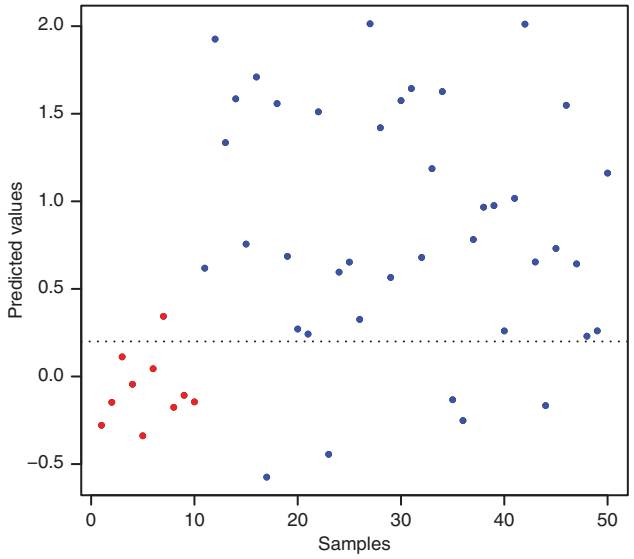

Figure 3. Prediction analysis of early-stage colon cancer patients. Controls are shown in red and cancer samples in blue. 9 out of 10 healthy controls were correctly predicted as true negatives and 35 out of 40 patients with cancer as true positives.

let-7d expression in sera from colon cancer patients has been reported. MiR-378 has been shown to function as an oncogene-like miRNA (Lee *et al*, 2007), to be functionally important for c-Myc-driven transformation (Feng *et al*, 2011) and to correlate with progression of human breast cancer (Eichner *et al*, 2010). However, in other studies, miR-378 has been found to be downregulated in CRC tissue (Wang *et al*, 2010; Faltejskova *et al*, 2012). In accordance with our results, a higher expression of miR-720 has been demonstrated in CRC tissue than in normal colon epithelial tissue controls (Ragusa *et al*, 2012).

Perhaps, somewhat unexpected was our findings of reduced serum levels of miR-103 and miR-107 in colon cancer patients compared with controls. MiR-103 and miR-107 have been found highly expressed in several solid tumours (Gottardo *et al*, 2007; Li *et al*, 2011b; Kim *et al*, 2012), and have been associated with poor prognosis in CRC. (Chen *et al*, 2012). On the other hand, a downregulation of miR-103 has been found in haematological malignancies (Li *et al*, 2011a; Machova Polakova *et al*, 2011). Notably, the miR-103/107 family is located to chromosomal loci prone to deletions or amplifications, and miR-103/107 is consequently reported to be deregulated in various diseases (Finnerty *et al*, 2010). We cannot exclude that the decreased miRNA expression found in our study reflects such genomic alterations. In our opinion, the diagnostic value of these miRNAs in blood should thus be evaluated with caution and at least compared with genomic analysis of the tumour sample.

Although our results have to be confirmed in larger clinical studies, the identification of a miRNA profile in serum of early-stage colon cancer patient that is quite similar to the late-stage cancer patients is promising and suggests that the miRNA profile may be used to identify early-stage cancer. Taken into consideration the complex regulation of miRNAs, we advocate that the miRNA profile – rather than assessment of a single miRNA expression – must be the foundation in establishing potentially new biomarkers.

In conclusion, we have identified a 21 miRNA profile in blood samples from colon cancer patients consisting of miRNAs that have been previously reported as possible markers of CRC, others not. Our study identifying miRNA expression profiles indicates that serum miRNA may be utilised to detect colon cancer in early curative stages.

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