Identification of high-risk Dukes B colorectal cancer by microRNA expression profiling: a preliminary study


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Abstract

Aim MicroRNAs (miRNAs) from tumour tissue and common gene mutations were studied to determine whether they predict the development of metastasis in patients with Dukes B colorectal cancer.

Method Patients who underwent curative resection for Dukes B colorectal cancer who subsequently developed distant metastatic disease at some stage in the following 5 years (‘high-risk B’) were compared with case-matched controls of Dukes A, Dukes B (no metastases, ‘low-risk B’) and Dukes C patients without any detectable metastasis at 5 years of follow-up. MiRNAs from tumour and adjacent normal tissue and common gene mutations (KRAS, BRAF, PIK3CA) in primary cancer tissue were analysed to identify prognostic tissue markers for the development of metastasis in patients with Dukes B colorectal cancer.

Results Expression of miR-15b and miR-135b was significantly downregulated \((P < 0.001)\) in ‘high-risk B’ tumours compared with Dukes A, ‘low-risk B’ and C without metastasis. No significant differences were noted for mutation status and the development of metastasis.

Conclusion The study suggests that the development of metastasis in Dukes B tumours may be predictable based on the miRNA expression of miR-15b and miR-135b. This requires further study on a much larger cohort.

Keywords MicroRNA profiling, KRAS, metastatic disease, miR-135b, miR-15b, colorectal cancer

What does this paper add to the literature?

Prediction of future metastasis in high-risk Dukes B cancers based on radiological and histological features is unreliable. This study emphasizes the role of cancer tissue-based miR-135b and miR-15b as a possible predictive marker.

Introduction

According to the International Agency for Research on Cancer (IARC), approximately 1.24 million new cases of colorectal cancer (CRC) were detected worldwide in 2008 [1]. About a quarter of patients with CRC are diagnosed with Dukes B or Stage II disease and have a 5-year survival of 75–80% when surgically resected [2]. A significant proportion (20–25%) of such patients develop metastatic disease [2]. This has led to uncertainty about the role of postoperative chemotherapy in Dukes B or Stage II disease, as has been highlighted in the third edition (2007) of Guidelines for the management of colorectal cancer from the Association of Coloproctology of Great Britain and Ireland (ACPGBI) [3].

A number of features indicating poor risk can be identified in Dukes B cancers, including serosal involvement (T4a), perforated or obstructed tumour, poorly differentiated or mucinous histology and perineural or extramural vascular invasion (EMVI). A combination of these features may confer a worse prognosis in a node-negative tumour. This has often led oncologists to refer to ‘good Bs’ and ‘bad Bs’. Chemotherapy is frequently offered to patients with Dukes B carcinoma with the adverse risk features of ‘bad Bs’, but patients with Dukes B and no poor-risk histological features may still develop metastatic disease. It is not possible to predict whether or not a patient with a Dukes B tumour will develop metastases based on histopathological examina-
tion of the resected specimen [4]. Molecular markers may possibly be able to identify the high-risk Dukes B patient who may benefit from postoperative chemotherapy.

MicroRNAs (miRNAs) are single-stranded, evolutionarily conserved small (17–25 ribonucleotides) non-coding RNA molecules [5]. They function as negative regulators of target genes by directing cleavage of specific messenger RNA or translational inhibition [6,7]. MiRNAs play an important role in colorectal tumour biology, including oncogenesis, progression, invasion, metastasis and angiogenesis [8–11]. Dysregulation of miRNAs in primary CRC tissue has been associated with the development of recurrence and reduced disease-free survival [12–15]. The initiation and progression of CRC result from sequential accumulation of genetic alterations in oncogenic and tumour suppressor genes in colonic epithelium [16]. Though the frequency of common somatic mutations (KRAS 30–40%, BRAF 15%, PIK3CA ~15% of CRCs) [17,18] is independent of tumour stage [19], mutation-specific gene expression profiles in other cancers have successfully identified aggressive carcinomas [20]. Furthermore, studies have also reported accelerated metastatic progression in patients with KRAS mutation [21,22], but no study to date has looked at miRNA expression in Dukes B tumour tissue to identify markers that may predict the subsequent development of metastases after curative resection. We evaluated the utility of tumour tissue miRNAs and common gene mutations (KRAS, BRAF, PIK3CA) in primary CRC tissue to predict the development of metastasis in patients with Dukes B CRC.

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Method

Ethical permission for the study was obtained from the local research ethics committee (05/Q2502/28 ‘Markers of tumour progression in colorectal cancer’).

Patients and tissue samples

Patients who underwent curative bowel resection for Dukes A, B, ‘low-risk B’ (without distant metastases at 5-year follow-up) and ‘high-risk B’ (developed metastatic disease at some stage during the following 5 years) were identified from the hospital CRC database, a prospectively collected database of patients treated for CRC in University Hospitals of Leicester over the last 11 years. All patients with Dukes C had received postoperative chemotherapy and no patient with a ‘low-risk B’ tumour had postoperative chemotherapy.

Formalin-fixed, paraffin-embedded (FFPE) cancer and adjacent normal tissue specimen blocks were obtained from the Department of Histopathology at the University Hospitals of Leicester NHS Trust. Histological diagnosis and cancer stage were reconfirmed by a consultant histopathologist. In total, matched pairs of surgically removed cancer and adjacent normal tissues for 100 patients were obtained. The yield of DNA from three cancer tissue samples and RNA from eight normal adjacent tissue samples was inadequate and these samples were not used for subsequent analysis. Table 1 shows the basic demographics and clinicopathological variables for all specimens.

**DNA extraction and mutation analysis**

Tissue from micro-dissected sections was dissolved in 300 μl of 0.05 Tris buffer pH 8/0.1% SDS (TrisBase; [296x686]Table 1 [296x698]Table 1 shows the basic demographics and clinicopathological variables for all specimens.**

| Gender (n) | Male 60 | Female 40 |
| Dukes stage (n) | A 13 | B 50 | High risk 15 | Low risk 35 | C1 31 | C2 6 |
| Tumour location (n) | Left 52 | Right 48 |
| Tumour background (n) | Polyp 22 | Diverticulitis 13 | Ulcerated 3 | None 56 |
| Age (years) | Mean 71.6 | Range 37–96 |
| Tumour stage (n) | T1 5 | T2 7 | T3 54 | T4 34 |
| Extramural vascular invasion (n) | Present 57 | Absent 40 |
| Serosal involvement (n) | Present 20 | Absent 77 |
| Tumour type (n) | Mucinous 18 | Adenocarcinoma 79 |
samples were stored at 56 °C. TRI Reagent/chloroform and an RNeasy® Mini Kit (Qiagen, Manchester, UK) were used according to the manufacturer’s protocol to extract total RNA. The concentration and integrity of RNA were measured with the Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA samples were stored at −20 °C.

MicroRNA expression profiling

Twenty age- and gender-matched paired tumour and adjacent normal tissues, five from each of Dukes A, ‘low-risk B’, ‘high-risk B’ and C were used for the miRNA expression profiling as a training cohort. One hundred nanograms of RNA was reverse transcribed to cDNA by using Taqman® Megaplex™ RT Primers Human Pool A v.2.1 and Pool B v.2.0 (Applied Biosystems) according to the manufacturer’s protocol. Five microlitres of cDNA for each sample was preamplified using Taqman® Megaplex™ PreAmp Primers Human Pool A v.2.1 and Pool B v.2.0 (Applied Biosystems). Table S4 gives the concentration of reagents used in each reaction. Reverse transcription (RT) and preamplification reactions were carried out in Veriti® 96-well thermal cycler (Applied Biosystems). Preamplified cDNA (25 μl) was diluted to 100 μl by adding 75 μl of 0.1x Tris EDTA buffer. After dilution, 20 μl of preamplified cDNA from each of five samples in each group was pooled to 100 μl.

MicroRNA expression profiling was performed according to the manufacturer’s protocol by using a 7900HT Fast Real Time PCR system (Applied Biosystems) and TaqMan® MicroRNA Array Card A v.2.1 and Card B v.2.0 (Applied Biosystems). Raw data from the 7900HT Fast Real Time PCR system were exported into Microsoft Office Excel files. Only well-expressed miRNA (CT < 35) were considered for further analysis. MicroRNA expression levels (CT) in the array were normalized to expression levels of the endogenous control RNU6B and relative expression levels (ΔΔCT) were calculated. Comparative expression levels (ΔΔCT) for tumour to normal adjacent tissue were calculated and intergroup comparisons were performed using the Mann–Whitney U-test. Data analysis was carried out with Microsoft Office Excel and Multi Experiment Viewer (MeV v.4.4; Boston, Massachusetts, USA) software.

PCR for validation of arrays

Based on differential expression, highly dysregulated miRNAs for ‘high-risk B’ tumours and two endogenous controls (RNU6B) were selected from the TaqMan® MicroRNA Array Card A v.2.1. Taqman MicroRNA Assays (Applied Biosystems) for individual miRNAs
were used to run PCR reactions for all the 20 cancers and matched adjacent normal tissue used for the miRNA arrays.

**Quantitative RT-PCR for the validation cohort**

The RT reaction for the validation cohort (72 cases of paired tumour and adjacent normal tissue) was set up using a Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems). One hundred nanograms of RNA was reverse transcribed and preamplified using MegaPLEX™ RT and Pre-amplification Primers Pool A v.2.1 (Applied Biosystems) on a Veriti® thermal cycler (Applied Biosystems). For PCR in the 7500 fast Real-Time system (Applied Biosystems) we used 4.5 μl of 1:20 diluted preamplified cDNA.

**Statistical analysis**

The relative expression (ΔCT) for each miRNA was calculated by normalizing the expression levels (CT) with that of RNU6B. Comparative expression levels (ΔΔCT) for tumour to adjacent normal tissue and fold changes were calculated. Intergroup and intragroup comparisons were performed using Student’s t-test, the Mann–Whitney U-test and one-way analysis of variance (ANOVA) with Bonferroni correction. Fisher’s exact test was carried out to identify the significant frequency of mutations. All the analysis was performed on SPSS Software v.18.0 (IBM, New York, USA). Expression graphs were created in GraphPad Prism 5 (GraphPad Software Inc, San Diego, California, USA).

**Results**

**Mutation analysis**

Ninety-seven cancer tissue specimens were analysed for KRAS, BRAF and PIK3CA mutation status; three cancer tissue specimens could not be analysed due to poor-quality DNA. The KRAS oncogene was found to be mutated in 28, BRAF in 19 and PIK3CA in nine cases. Mutation analysis for all three oncogenes revealed no significant difference for ‘low-risk B’ and ‘high-risk B’ cancer tissue. KRAS mutations were significantly higher in Dukes A than in Dukes C disease (P = 0.011). BRAF and KRAS were mutually exclusive, except in two subjects where a complex mutation was observed. Similarly, in two patients, mutations were observed in KRAS codons 12 and 13. BRAF mutation was significantly higher for right-sided cancers (P = 0.0001) and cancer tissue resected from female patients (P = 0.02).

The complete analysis of the mutation status with clinical variables is shown in Table 2.

**MicroRNA expression signature**

Not all the miRNAs in the array were expressed in colorectal tissue. MiRNAs with expression levels of CT < 35 and present in at least one pooled sample of normal or cancer tissue were included for further analysis. The comparison of RNU6B-normalized data showed significantly different miRNAs for ‘low-risk B’ and ‘high-risk B’ tumours. These differentially expressed miRNAs were selected and further compared for paired pools of adjacent normal and cancer tissue to identify dysregulated miRNAs in tumour tissue (paired Student’s t-test, P < 0.05). The miRNA expression signature derived from MeV (Multiexperiment Viewer v.4.4) software showed miRNAs dysregulated in tumour tissue and Dukes B (Fig. 1). MiRNAs dysregulated in tumour tissue compared with adjacent normal tissue and significantly different for ‘high-risk B’ tumours were selected from TaqMan® MicroRNA Array Card A v.2.1 for further validation (hsa-miR-15b, hsa-miR-21, hsa-miR-32, hsa-miR-125a-5p, hsa-miR-135b, hsa-miR-182, hsa-miR-302b, hsa-miR-330-3p, hsa-miR-330-5p, hsa-miR-381, hsa-miR-483, hsa-miR-508 and hsa-miR-708). hsa-miR-34a was selected due to its strong relation with P53 and hsa-miR-184 and RNU6B were selected as endogenous controls for normalization purposes.

**Validation of selected miRNAs on a second cohort**

**Tumour vs normal tissue miRNA**

Comparison of RNU6B-normalized miRNA expression (ΔCT) in cancer and adjacent normal tissue (non-matched pair analysis with Student’s t-test) showed significant (P < 0.05) upregulation of hsa-miR-21, hsa-miR-34a, hsa-miR-135b, hsa-miR-182 and hsa-miR-708 in tumour tissue. Matched pair analysis of miRNA (paired Student’s t-test) in cancer and adjacent normal tissue showed downregulation of hsa-miR-125a-5p and upregulation of hsa-miR-15b, hsa-miR-21, hsa-miR-34a, hsa-miR-135b, hsa-miR-182 and hsa-miR-708 in tumour tissue (Fig. 2).

**Dukes ‘low-risk B’ vs ‘high-risk B’**

RNU6B-normalized expression (ΔCT) of miRNAs in tumour tissue of different Dukes stages were compared with analysis of variance with Bonferroni correction. For ‘low-risk B’ and ‘high-risk B’ tumours there were no significant difference in expression levels (ΔCT) of hsa-miR-21, hsa-miR-34a, hsa-miR-125a, hsa-miR-508 and
hsa-miR-708 in tumour tissue. The expression level of hsa-miR-135b was significantly lower in ‘high-risk B’ tumour compared with Dukes A, C ($P = 0.0001$) and ‘low-risk B’ ($P = 0.0003$) tumours. Similarly, expression levels of hsa-miR-15b were significantly lower in ‘high-risk B’ tumour tissue compared with Dukes A, C and ‘low-risk B’ ($P < 0.001$) (Fig. 3). The expression levels of hsa-miR-135b in tumour and adjacent normal tissue were relatively much lower in comparison with Dukes C and ‘low-risk B’ (Fig. 4).

**Correlation with other clinicopathological variables**

No significance was observed for miRNA expression in correlation with age, gender, background, tumour location, **BRAF** and **PIK3CA** mutation status. The Mann–Whitney test showed significantly higher levels of expression of hsa-miR-34a ($P = 0.028$) and hsa-miR-708 ($P = 0.023$) for **KRAS**-mutated tumour tissues compared with wild-type tumours. Differential expression of miR-34a (mean difference in ΔCT $-1.13$, 99% CI $-1.42$ to $-0.84$) for **KRAS** mutant tumours were higher in comparison with tumours with the wild-type **KRAS** gene. The expression levels of hsa-miR-34a increased with higher tumour T stage. One-way ANOVA with Bonferroni correction showed significantly higher expression levels for miR-34a in **KRAS**-mutant tumour tissues compared with wild-type tumours. Differential expression of miR-34a (mean difference in ΔCT $0.885$, 99% CI $0.661$ to $1.11$) and miR-708 (mean difference in ΔCT $-0.84$ to $-1.1$) for **KRAS**-mutated tumour tissues showed a progressive increase of expression from

**Table 2** Comparative analysis of mutation status for **KRAS**, **BRAF** and **PIK3CA**. The table compares the mutation status for **KRAS**, **BRAF**, **PIK3CA** with tumour Dukes stage, location, background, T-stage and gender of patients.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Subgroups</th>
<th>Number of samples</th>
<th>Number of mutations</th>
<th>P-value</th>
<th>Number of mutations</th>
<th>P-value</th>
<th>Number of mutations</th>
<th>P-value</th>
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<td>Dukes stage</td>
<td>A</td>
<td>7</td>
<td>1</td>
<td></td>
<td>5</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low-risk B</td>
<td>39</td>
<td>8</td>
<td></td>
<td>12</td>
<td></td>
<td>3</td>
<td></td>
</tr>
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<td></td>
<td>C</td>
<td>36</td>
<td>3</td>
<td></td>
<td>5</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High-risk B</td>
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<td>1</td>
<td></td>
<td>6</td>
<td></td>
<td>1</td>
<td></td>
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<tr>
<td>A vs low-risk B</td>
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<td>0.075</td>
<td></td>
<td>0.309</td>
<td></td>
<td>0.324</td>
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<tr>
<td>A vs C</td>
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<td>0.52</td>
<td>0.01</td>
<td></td>
<td>0.298</td>
<td></td>
<td>0.92</td>
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<tr>
<td>A vs high-risk B</td>
<td></td>
<td>0.641</td>
<td>0.198</td>
<td></td>
<td>0.989</td>
<td></td>
<td>0.989</td>
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<tr>
<td>Low-risk B vs C</td>
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<td>0.649</td>
<td>0.08</td>
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<tr>
<td>Low-risk B vs high-risk B</td>
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<td></td>
<td></td>
<td>0.989</td>
<td></td>
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<tr>
<td>High-risk B vs C</td>
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<td>0.839</td>
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<td>59</td>
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<td>$0.002$</td>
<td>16</td>
<td>0.644</td>
<td>3</td>
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<tr>
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<td>14</td>
<td></td>
<td>12</td>
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<tr>
<td>Location</td>
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<td>52</td>
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<td>$0.0001$</td>
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<td>76</td>
<td>15</td>
<td></td>
<td>21</td>
<td>0.631</td>
<td>7</td>
<td>0.966</td>
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<td>4</td>
<td>0.945</td>
<td>7</td>
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<tr>
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<td>2</td>
<td></td>
<td>6</td>
<td></td>
<td>2</td>
<td></td>
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<tr>
<td></td>
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<td>51</td>
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<td></td>
<td>12</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
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<td></td>
<td>10</td>
<td></td>
<td>4</td>
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</tr>
<tr>
<td>T2 vs T3</td>
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<td></td>
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<tr>
<td>T3 vs T4</td>
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<td></td>
<td>0.371</td>
<td></td>
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<tr>
<td>T4 vs T2</td>
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<td>0.47</td>
<td>0.068</td>
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<td>0.412</td>
<td></td>
<td>0.412</td>
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</table>

The P-values were calculated based on ANOVA and a P-value $\leq 0.01$ was deemed significant (bold).
T2 staged tumours to T3 and T4 staged tumours ($P = 0.038$). Furthermore, in the presence of EMVI, levels of expression of miR-125a-5p, miR-135b, miR-182 and miR-708 were significantly different ($P < 0.05$) in tumours without EMVI (Fig. 5). The levels of expression of miR-21 in both high-risk Dukes B
and Dukes C were significantly higher especially for left-sided tumours ($P < 0.05$).

**Discussion**

This study shows higher levels of hsa-miR-135b in micro-dissected CRC tissue compared with adjacent normal mucosa. This finding is in line with previously published studies [23–26], but none of these have correlated the levels of hsa-miR-135b to cancer stage or identified the prognostic significance of hsa-miR-135b for Dukes stage. The present study has shown no difference in expression of hsa-miR-135b for Dukes B and C, but the lower levels of expression of hsa-miR-135b can...
significantly differentiate ‘high-risk B’ from the ‘low-risk B’ cancer. Inactivation of the adenomatous polyposis coli (APC) gene is a major initiating event in colorectal carcinogenesis [27] occurring in more than 60% of colorectal adenomas and carcinomas and leading to stimulation of the Wnt pathway via free β-catenin [28]. Inactivation of APC has been found in more than 60% of colonic tumours [16] and such inactivation is associated with upregulation of hsa-miR-135b in CRC cells [29]. Nagel et al. [29] have also shown that hsa-miR-135b levels are found to be upregulated in colorectal adenomas and carcinomas and correlate with low APC levels. These observations suggest that alteration in the hsa-miR-135 family can be one of the early events in molecular pathogenesis of CRC. On matched pair analysis it was observed that in ‘high-risk’ Dukes B cancers the levels of expression of hsa-miR-135b were not significantly different for tumour and adjacent normal mucosa, which suggests an alternative mechanism of tumour initiation in ‘high-risk’ Dukes B tumours.

For the validation cohort, no differences in levels of hsa-miR-15b were noted for cancer and adjacent normal tissue. A previously published expression profiling study by Xi et al. [30] has shown similar results for hsa-miR-15b in CRC. We report that the levels of hsa-miR-15b are significantly lower for patients who developed metastatic disease after surgical resection of a Dukes B tumour. A prognostic study of hsa-miR-15b in hepatocellular carcinoma has shown a similar trend in which lower levels of expression of hsa-miR-15b are associated with a higher risk of recurrence [31]. In another study hsa-miR-15b was found to be downregulated in CRC tissue but did not correlate with the presence of lymph node metastasis and disease-free survival [32]. The exact role of hsa-miR-15b in the development and progression of CRC is yet not fully understood, but mechanistic studies have shown that hsa-miR-15b correlates with E2F-regulated genes and appears to be part of the E2F-regulatory network [33,34]. E2F1, hsa-miR-15b and Cyclin E constitute a feed-forward loop that modulates E2F activity and cell-cycle progression where an inhibition of hsa-miR-15b expression results in an enhanced E2F1-induced Cyclin E and G(1)/S transition [33]. Bcl-2 has also been shown to be a target of hsa-miR-15b in gastric cancer, but in vitro experiments to knock down hsa-miR-15b in CRC cell lines did not show any change in expression levels of Bcl-2 [35].

The present study also reports higher levels of expression of hsa-miR-21 in tumour tissue than in adjacent normal healthy tissue. These findings are in line with previous studies showing that miR-21 is upregulated in CRC and contributes to tumour progression and drug resistance [36,37].

Figure 5 Comparison of levels of miRNA expression for tumours with histological evidence of extramural vascular invasion (EMVI; Mann–Whitney test) showing significantly higher levels for miR-125a, miR-135b, miR-182 and lower levels for miR-708 (P < 0.05): (a) levels of expression of miR-125a for EMVI-positive and EMVI-negative tumours (mean difference in ΔCT −1.56, 99% CI −1.354 to −1.699); (b) levels of expression of miR-135b for EMVI-positive and EMVI-negative tumours (mean difference in ΔCT −1.15, 99% CI −1.269 to −1.036); (c) levels of expression of miR-182 for EMVI-positive and EMVI-negative tumours (mean difference in ΔCT −2.96, 99% CI −2.75 to −3.14); (d) levels of expression of miR-708 for EMVI-positive and EMVI-negative tumours (mean difference in ΔCT 0.96, 99% CI 0.7–1.21).
with previously published studies. It has already been established that the elevated hsa-miR-21 expression leads to reduced apoptosis and increased cell proliferation, cell migration, vascular invasion and metastasis by targeting several tumour suppressor genes such as programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), tropomyosin 1 (TPM1), cell division cycle 25 homolog A (Cdc25a), reversion-inducing-cysteine-rich protein with kazal motifs (RECK), TIMP3, maspin, nuclear factor 1 B-type (NEIB), sprouty 2 (SPRT2) and Ras homolog gene family member B (RHOB) [36]. A higher expression of hsa-miR-21 in CRC tissue samples has been linked to worse prognosis and the therapeutic outcome, highlighting its potential use as a prognostic and predictive biomarker for CRC [13,37–39]. It has also been proposed that when used in combination with other prognostic parameters such as staging, microsatellite instability status and genotyping, mRNA profiling may provide a much improved risk stratification to help guide the right treatment strategies and may result in much improved survival [36]. The association of elevated miR-21 with worse prognosis has now been reported in other haematological and solid organ malignancies. In this study, though, ΔCT for hsa-miR-21 levels were not significantly different for low-risk and high-risk Dukes B CRC, but the levels of expression were higher in high-risk Dukes B and C and especially left-sided tumours, suggesting that hsa-miR-21 is significantly involved in tumour metastasis.

The levels of hsa-miR-34a, hsa-miR-125a-5p, hsa-miR-708 and hsa-miR-182 were significantly higher in cancer tissue; specifically, levels of hsa-miR-34a were significantly higher in KRAS mutant cancer tissue. Such overexpression was also identified by Slattery et al. [15]. In line with the previous study by Zhang et al. [40], hsa-miR-125a-5p has been shown to be downregulated in CRC tissue and did not correlate with any clinical parameters. Studies in lung cancer have shown that hsa-miR-125a-5p acts as a tumour suppressor by inducing apoptosis mediated by p53 [41], but its exact role in CRC is still unknown. Higher levels of expression of hsa-miR-34a and hsa-miR-125a-5p suggest an alternative, TP53-independent mechanism of overexpression of both of these miRNAs. The upregulation of miR-708 in APC knockout tumours and colitis-associated tumours in mice highlights its role in tumour initiation and transformation via known cancer signalling pathways [42].

In line with recently published studies [43,44] hsa-miR-182 expression is increased in colonic tumours compared with the adjacent normal healthy tissue. Other in vitro studies have identified hsa-miR-182 as an inhibitor of apoptosis, resulting in increased cancer cell survival and cancer progression [45]. More importantly, miR-183-96-182 gene clustering is located in the 7q32 genomic region. Researchers have identified its amplification in 26% of primary solid organ tumours and 30% of liver metastases [46]. The higher expression of hsa-miR-182 in T3 tumours compared with T1/T2, EMVI-positive tumours and Dukes C suggests that hsa-miR-182 is a driving force of tumour progression.

There is a widespread belief that the mechanism of tumour spread in the colon varies according to the location of the tumour. For example, 70% of cancers in hereditary nonpolyposis colorectal cancer are on the right side [47]. BRAF mutations are also significantly more frequent in right-sided tumours, and have been shown to drive microsatellite instability [48].

This cohort study has some limitations. The sample size of Dukes A and ‘high-risk’ Dukes B were smaller than that of the ‘low-risk’ Dukes B and C. Although the normal tissue used in the study was referred to as ‘adjacent’ normal, its exact location with respect to the tumour is not known.

To summarize the results of the present study: hsa-miR-135b and hsa-miR-15b are significantly downregulated in patients with Dukes B who developed metastatic disease during follow-up. The downregulation of hsa-miR-15b and hsa-miR-135b in low-risk Dukes B tumours indicates that different molecular pathways may be activated for node-negative metastatic Dukes B tumours. The exact interaction of these miRNAs into cancer pathways is still not known and future studies focusing on such interactions may provide some insight into their role.

Conflict of interest
Nil.

References
4 Petersen VC, Baxter KJ, Love SB et al. Identification of objective pathological prognostic determinants and models...


Supporting Information
Additional Supporting Information may be found in the online version of this article:
Table S1. Primers and probes for mutation analysis.
Table S2. Mutation analysis by qPCR.
Table S3. Thermal profiles for different PCR reaction.
Table S4. Reagent concentrations for reverse transcription, pre-amplification, Array and PCR reactions.