Early Detection and Staging of Colorectal Cancer Using a Panel of Micro RNAs

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Abstract

Purpose: To improve lymph node (LN) staging in patients with colon cancer (CC). The present study describes the selection of CC-specific miRNAs and assesses their utility as a micro metastases detection assay.

Methods: 30 miRNAs have been selected from a microarray assay and 16 miRNAs from database mining for their specific upregulation in colon cancer tissues as compared to normal adjacent tissues. Differential expression was validated by RT-qPCR in a larger cohort of samples (n=20) and compared to normal lymphatic tissues (n=6) and normal peripheral blood lymphocytes (PBLs, n=14). The selected miRNA panel was then used for the screening of 84 lymph nodes (LN) obtained from colon cancer patients (n=20).

Results: After validation, a panel of 8 miRNAs was found to be significantly upregulated in CC compared to normal adjacent tissues and to normal lymphatic tissues: miR-96, miR-183, miR-194, miR-200a, miR-200b, miR-200c, miR-203 and miR-429. A total of 84 LNs were analysed: 12 LN metastases were detected by H&E, 18 by CK staining whereas 32 were detected by the CC-specific miRNA analysis. This represents an increase of 40% in the detection rate.

Conclusion: This study demonstrated the ability of a CC-specific 8 miRNA panel in detecting micro metastases in CC patients.

Keywords: Colorectal cancer; miRNA; Staging; Colorectal metastases; Tumor markers

Introduction

Colorectal cancer (CRC) is the fourth most common cause of cancer and second leading cause of cancer-related death in the US. There are over 140,000 new cases diagnosed each year in the US and over one million worldwide [1,2]. The survival and prognosis of colorectal cancer patients depends mainly on the disease stage at the time of detection. Global 5-year survival of patients without lymph node involvement (Stage I and II) is around 80% which drops if positive lymph nodes or distant metastasis are detected (stage 3 and 4) [3,4]. Therefore, precise determination of the regional lymph nodes status is an important diagnostic and prognostic factor in surgically relevant colorectal adenocarcinoma and defines the need for adjuvant chemotherapy [3,5,7]. Indeed, it has been proven that adjuvant chemotherapy treatment for patients detected with lymph nodes metastases (AJCC Stage III), significantly improves patient survival [7]. Interestingly, patients diagnosed with AJCC stage II colon cancer, presented only marginal improvement to adjuvant chemotherapy. Out of this group of patients, approximately 20-25% will develop recurrence of disease within 5 years after surgery [3,4,8,9]. The high rate of recurrence may be attributed to the presence of occult lymph node metastases undetected by conventional histopathology or due to minimal residual disease (MRD) in the form of circulating tumor cells in the blood, lymphatic system or peritoneal cavity [3,5,8,10]. The ability to identify patients liable to relapse and to treat them before the onset of distant metastases may improve their survival [5,10,11].

Routine histopathological lymph node examination is based on paraffin-embedded specimen section (4 mm thick) stained with haematoxylin and eosin (H&E). This technique allows the detection of metastases larger than 2 mm and therefore lack the sensitivity to detect micro metastases (0.2 mm < diameter < 2 mm). The addition of immuno-histochemical cytokeratin analysis (IHC) to standard H&E staining showed significant improvement in staging accuracy for 4-39% of the patients [3,10]. Further methods such as ultra-sectioning and RT-qPCR have shown an increase in detection sensitivity for occult metastases (15-50%).

In recent years, important efforts have been made to find stable and specific markers for cancer detection. Among them, miRNAs have emerged as particularly promising markers because of their implication in the tumorigenesis, progression and prognosis of many cancers [12–19].

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Received June 11, 2018; Accepted July 16, 2018; Published July 18, 2018


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miRNA expression profiling using microarrays: RNA processing, microarray fabrication, array hybridization, and data acquisition were performed by a service provider (LC Sciences, Houston, TX). Ten arrays (Mi Human Chip H8.1) were performed on the RNA extracts from 10 patients’ samples; each of them contained the paired samples tumour tissue/ normal adjacent tissue. Every chip used covered 480 human miRNAs and controls. A transcript to be listed as detectable must meet at least two conditions: signal intensity higher than 3 times (background SD) and spot coefficient of variation less than 0.5. Coefficient of variation was calculated by (SD)/ (signal intensity). Differentially expressed transcripts with P<0.01 along with data processing statistics were considered. The ratio values were presented in log2 scale: a positive log2 value indicates an upper regulation, and a negative log2 value indicates a down-regulation.

Data mining: Scientific publications and databases (http://miracancer.ecu.edu [25,26], http://www.oncomir.umn.edu [27], www.mir2disease.org [28], genome.ewha.ac.kr/miRGator/mirNAProfiling.html [29], www.microrna.org [30], http://mirdb.org [31], www.mirbase.org [32], www.ncbi.nlm.nih.gov/pubmed/ and www.geencards.org) were screened for miRNA corresponding to the aforementioned criteria. All candidate miRNAs, from both sources, were checked in-silico for their expression profile in normal lymphatic tissue and compatibility with placed conditions.

RNA extraction

Total RNA was extracted from tissues using miRvana miRNA isolation kit (Ambion, USA) following the manufacturer’s instructions. The RNA concentration was measured with Nanodrop Spectrophotometer (ND-1000, Nanodrop Technologies, USA) whereas the quality was assessed by gel electrophoresis (0.7% agarose).

RT-qPCR amplification

The real-time qPCR of microRNA expression was performed with TaqMan® MicroRNA Assays (Applied Biosystems, USA). The reverse transcription and real time quantification were carried out on Applied Biosystems 7500 HT Real-Time PCR System (Applied Biosystems, USA). The synthesis of cdNA was performed from 50 ng of total RNA using TaqMan® MicroRNA Reverse Transcription Kit according to manufacturer’s instructions (Applied Biosystems, USA). Real time quantitative PCR was performed using real-time PCR miRNA specific primers and FAM-dye fluorescent probe provided with TaqMan MicroRNA Assay (Applied Biosystems, USA). Each sample was checked in duplicates and the expression levels of microRNA were normalized to endogenous snORU43.

Statistical analysis

Summary statistics were performed according to established methods. Student t-test or Kruskal-Wallis test were used to compare variables as appropriate. Statistical analysis was performed using IBM-SPSS’s statistical package, Version 20 (SPSS Inc. Chicago, IL, USA).

Results

Selection of optimal microRNAs

An overview of the miRNAs selection is illustrated in Figure 1. Selection from miRNA microarray profiling miRNA expression from paired samples of tumour and adjacent normal tissues were analysed on human miRNA chip. Among the miRNA transcripts listed in...
Sanger miRBase Release 8.1, 60 were differentially expressed (p<0.01) between tumour tissue and matching normal adjacent tissue (Suppl. data 1). Only the miRNAs (n=30) overexpressed in tumour tissues were pre-selected for further investigation. An additional selection was then undertaken using electronic databases, targeting miRNAs for their expression in normal lymphatic tissue and normal peripheral blood lymphocytes (PBLs). Five miRNAs were found to be under-expressed/not-expressed in normal lymphatic tissue and PBLs (Suppl. data 2) and were therefore selected.

Selection from data mining

16 miRNAs were selected from the different databases for their specific high expression in colon cancer tumour tissue compared to normal colon tissue and normal PBLs (Suppl. data 2).

Second round selection by RT-qPCR

For the second selection round, the 21 candidate miRNAs were studied by RT-qPCR on tumour colon tissues (n=5), normal adjacent colon tissues (n=5), healthy lymphatic tissues (n=3) and healthy white blood cells (n=3). The expression levels of these candidate miRNA markers are summarized in Suppl. data 2. We selected those miRNAs for which the expression in tumour tissue was higher than the one in adjacent normal tissue and with a very low expression in LN/PBLs. As a result, the following eight candidate markers were chosen: miR-96, miR-183, miR-194, miR-200a, miR-200b, miR-200c, miR-203 and miR-429.

In order to support previous results, a complementary study has been undertaken on additional samples. Thus, samples from 20 tumour colon tissues and normal adjacent, 14 PBLs from healthy patients and 6 normal lymph node tissues, Suppl. data 3. In order to reduce type I error (false positive), only samples with at least 2 upregulated miRNAs were considered as positive.

Ultra-staging of sentinel lymph nodes of CC patients using the miRNA panel

Twenty patients (n=20) presenting sporadic primary tumours at AJCC stages 2-3 (without distant metastases) were included in the study. Patient characteristics are provided in Table 1. There were 40% men and 60% women, and the median patient age was 69.5 (±12.6). Each patient had at least 3 detected SLNs and part of them was determined as pathologically positive. SLNs were bi-halved: one half was subjected to enhanced pathological examination using H&E and immunohistochemistry (CK) staining. The other half was used for RT-qPCR.
for miRNA screening. A total of 84 LNs were analysed and results are summarized on Suppl. data 4. Among the 84 LNs, 12 were detected by H&E, 18 were detected by CK staining whereas 32 were detected by miRNA analysis. This represents an increase of 40% in the detection rate (Figure 3). In addition, three LNs were not detected by the miRNA panel while metastases were detected by standard histopathological analysis (False Negative). At miRNA level, results showed differences between miRNA of the panel with miR-183 and miR-194 presenting higher sensitivity and accuracy in detecting metastases than others (Table 2). Next, the relationship between N stage and the miRNA expression was studied (Suppl. data 5). Interestingly, the number of miRNA necessary for detection was positively correlated to the N stage. Thus, the more the stage is advanced the more the number of miRNA detected increases. Altogether, the miRNA assay had a sensitivity of 83%, a specificity of 74% and an accuracy of 76% (Table 3).

**Figure 2:** Box and Whisker plot showing relative miRNA levels in LNs/PBLs, adjacent normal tissues (Adj.N) and tumor tissues (T) by quantitative RT-PCR. The miRNA expression has been normalized to snoRNU43 expression and results are represented by the log10 of the relative quantity (RQ). In each box the median value is indicated. p values were calculated by student's t-test.

**Figure 3:** Comparison of metastases detection in sentinel lymph nodes by the different techniques. miRNA panel assay has detected 40% more metastases than traditional standard histopathological methods. H&E: haematoxylin and eosin staining; CK: immuno-histochemical cytokeratin analysis; MIR: miRNA panel assay.
Discussion

The presence of lymph node metastases is one of the most important prognostic factors in patients with colon cancer. As such, the decision to administer adjuvant chemotherapy in colon cancer patients is based mainly on the lymph node status [5–7]. Up to 25% patients with node-negative colon cancer staged by standard pathologic techniques ultimately suffer disease recurrence [3,4,8,9]. Several reasons can explain recurrence: inadequate surgery (incomplete nodal resection), extra nodal spread of the disease or occult nodal disease overlooked by conventional techniques. The American Joint Committee on Cancer (AJCC) recommends the assessment of 12 lymph nodes by standard histopathological techniques [33-37]. These techniques consist in the microscopic examination of one or two sections which represents sampling of less than 1% of the lymphatic tissue. Hence, the risk of misdetection of small tumor cell aggregate is important. The ability to use colon cancer-specific molecular markers for a PCR-based lymphatic staging is appealing. Many investigators examined various epithelial markers such as CK-20, MUC2 or cancer specific markers such as CCAT-1 [38] for lymph node staging. Whereas PCR-based method significantly increases the sensitivity of staging, it cannot be applied to all harvested nodes, as processing time, human resource requirement, and cost would be prohibitive.

We have shown before in two multicentre trials [39,40] that enhanced pathological examination of targeted nodes significantly improves macro- as well as micro-metastasis detection. In addition, the mature results of our first prospective randomized trial (The USMCI-G01 trial) showed a survival benefit for the ultra-staged patients [41].

<table>
<thead>
<tr>
<th>Number of SLNs</th>
<th>mir-96</th>
<th>mir-183</th>
<th>mir-194</th>
<th>mir-200a</th>
<th>mir-200b</th>
<th>mir-200c</th>
<th>mir-203</th>
<th>mir-429</th>
</tr>
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<td>TP</td>
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<td>14</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>FP</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>7</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>FN</td>
<td>7</td>
<td>4</td>
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<td>58</td>
<td>56</td>
<td>54</td>
<td>59</td>
<td>49</td>
<td>51</td>
</tr>
</tbody>
</table>

Sensitivity 61% 78% 67% 56% 56% 56% 56% 67%
Specificity 83% 83% 88% 85% 82% 89% 74% 77%
PPV 50% 56% 60% 50% 45% 59% 41% 42%
NPV 89% 93% 91% 88% 87% 88% 89% 88%
Accuracy 79% 82% 83% 79% 76% 82% 73% 74%
Panel sensitivity -- -- -- -- -- 83% -- --
Panel specificity -- -- -- -- -- 74% -- --
Panel PPV -- -- -- -- -- 47% -- --
Panel NPV -- -- -- -- -- 92% -- --
Panel accuracy -- -- -- -- -- 76% -- --

TP: True Positive; FP: False Positive; FN: False Negative; TN: True Negative.

Table 2: Results of the molecular testing on 84 LNs by the miRNA panel assay.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number of SLN</th>
<th>TNM</th>
<th>AJCC Stage</th>
<th>Adjuvant Chemotherapy</th>
<th>Pathological analysis</th>
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<td>Patient 1</td>
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<td>IIIB</td>
<td>Yes</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4 T3N1M0</td>
<td>IIIB</td>
<td>Yes</td>
<td>P</td>
<td>P</td>
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<tr>
<td>Patient 3</td>
<td>6 T3N0M0</td>
<td>IA</td>
<td>No</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Patient 4</td>
<td>3 T3N2M0</td>
<td>IIIC</td>
<td>Yes</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Patient 5</td>
<td>4 T3N1M0</td>
<td>IIIB</td>
<td>Yes</td>
<td>P</td>
<td>P</td>
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<td>Patient 6</td>
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<td>IIIB</td>
<td>Yes</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Patient 7</td>
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<td>Patient 8</td>
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<td>IIIA</td>
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<td>P</td>
<td>P</td>
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<tr>
<td>Patient 9</td>
<td>3 T3N1M0</td>
<td>IIIB</td>
<td>Yes</td>
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<td>Patient 10</td>
<td>7 T3N0M0</td>
<td>IA</td>
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<td>5 T3N0M0</td>
<td>IA</td>
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<td>Patient 12</td>
<td>4 T4N0M0</td>
<td>IA</td>
<td>Yes</td>
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<td>N</td>
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<td>Patient 13</td>
<td>2 T3N1M0</td>
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<td>Yes</td>
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<td>Patient 14</td>
<td>5 T4N0M0</td>
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<td>Patient 15</td>
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<td>N</td>
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<td>Patient 16</td>
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<td>IA</td>
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<td>Patient 17</td>
<td>4 T3N1</td>
<td>IA</td>
<td>Yes</td>
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<td>Patient 18</td>
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<td>Patient 19</td>
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<td>N</td>
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<td>Patient 20</td>
<td>6 T3N0M0</td>
<td>IA</td>
<td>No</td>
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Table 3: Summary of the LNs analysis.
In an attempt to improve lymph node staging, we decided to combine lymph node mapping and RT-qPCR techniques. Because of their implication in the tumorigenesis, progression and prognosis of many cancers [12-22], miRNA was looked as attractive candidates. In the current study, we elected to identify a panel of microRNAs specifically expressed in colon cancer. Using in-silico as well as “wet” expression analysis we were able to identify a panel of 8 microRNAs upregulated in colon cancer, but which were poorly expressed in normal lymphatic tissue.

Interestingly, all the selected miRNAs of the panel have been previously described having a role in different processes of cancer. Indeed, microRNA-96 has been described to contribute to CRC cells growth via regulation of KRAS, TP53INP1, FOXO1 and FOXO3a expressions [42,43]. The miR-200 cluster (comprising miR-200a, miR-200b, miR-200c and mir-429) and miR-194 were reported to regulate the Epithelial-Mesenchymal-Transition (EMT) process in CRC [44-48]. Finally, miR-203 and miR-183 were involved in differentiation and proliferation processes of cancer cells [49-52]. Given that colon cancer can have multiple origins, the fact of possessing a test comprising several markers involved in various processes represents a real advantage. Furthermore, the use of CC-specific microRNAs reduces possibility of contamination by normal colonic cells up taken by the lymphatic system. Using this panel of newly-discovered microRNAs, we analysed lymph nodes obtained from colon cancer patients as part of our previous clinical trial evaluating targeted nodal assessment of lymph nodes.

Conclusion

A total of 84 LNhs have been tested, 12 LN metastases were detected by H&E, 18 by CK staining whereas 32 were detected by the CC-specific miRNA analysis. This represents an increase of 40% in the detection rate. A set of 8 miRNA was identified as significantly and specifically upregulated in colon tissues compared to LN and PBLs. The use of this panel of miRNA improved the detection rate of 40% compared to standard histo-pathological analysis.

Acknowledgements

A.N. acknowledges the Israel Cancer Association and the Office of the Chief Scientist in the Israel Ministry of Economy for their financial support.

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J Cancer Sci Ther, an open access journal
ISSN: 1948-5956