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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 respectable 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–

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22]. The first study of miRNA expression in colorectal tumor tissue compared to normal colonic tissue was reported in 2003 by Michael et al. [23]. In this study, miR-200c was first isolated in normal colonic tissue and miR-143 and -145 were found to be down-regulated in tumor tissue compared to normal colon tissue. Since then, many studies have compared the dysregulation of different miRNAs in CRC [24,25].

The aim of the present study is to identify a set of colon cancer-specific miRNAs that will be suitable for the detection of occult metastases in regional lymph nodes.

Material and Methods

Patient recruitment and tissue specimen collection

Patients over the age of 18 years with histologically confirmed primary adenocarcinoma of the colon were offered participation in the study. Patients who received prior radiation or chemotherapy were excluded from the study. The study protocol was approved by the Institutional Review Board (IRB, Helsinki Committee) of Hadassah-Hebrew University Medical Center. Patients meeting eligibility criteria (n=20) signed an IRB-approved informed consent and were enrolled into the study. Primary cancerous biopsies and their non-cancerous adjacent tissue were collected from patients during standard surgical resection. All specimens were submitted for standard histopathological examination. Formalin-fixed paraffin embedded samples were stained with haematoxylin and eosin (H&E).

LN histopathology analysis

Nearby tumor draining sentinel lymph nodes (SLNs), 4 nodes on average from each patient (a total of 84 nodes), were mapped after subserosal *ex-vivo* around tumour injection of 1-2 ml isosulfan blue dye (Lymphazurin 1%, Ben Venue Labs, Bedford, OH). Sentinel nodes were defined as the first blue staining nodes to appear within 5–10 minutes of dye injection [10]. After the blue-stained SLN harvesting, each node was sectioned on two halves. One piece of each node separate specimen, along with the resected colon and mesentery, were formalin-fixed and submitted for the standard pathologic examination [10,24]. After the diagnosis confirmation and tumour staging, according to AJCC guidelines, each tagged sentinel lymph node was paraffin embedded and underwent four steps sectioning, approximately 4 mm thick. All four sections of each paraffin-embedded specimen were examined by routine H&E staining and cytokeratin immunohistochemistry (IHC). Cytokeratin immunohistochemistry was done with a pan-specific antibody cocktail (AE1/AE3, CAM5.2, 35bH11; Ventana Medical Systems, Tucson. AZ). Detailed sentinel lymph node histopathological evaluation was performed as previously described [10]. Clinical decision regarding adjuvant chemotherapy was based on conventional pathologic determining of SLNs epithelial cells contamination. The remaining half node specimen, together with the collected biopsies, were immediately stored in liquid nitrogen for further molecular examination.

MicroRNA selection

MiRNAs fulfilling the following criteria were chosen for further investigation: significant up-regulation in colon tumour tissue versus normal-adjacent tissue; very low expression in normal lymphatic tissue and lymphocytes; significant up-regulation in colon tumour tissue versus normal lymphatic tissue and PBLs of healthy volunteers. The selection has been done through two different approaches:

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://mircancer.ecu.edu [25,26], http://www.oncomir.umn.edu [27], www.mir2disease.org [28], http://genome.ewha.ac.kr/miRGator/miRNAprofiling.html [29], www.microrna.org [30], http://mirdb.org [31], www.mirbase.org [32], http://www.ncbi.nlm.nih.gov/pubmed/ and www.genecards.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 snoRNU43.

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\ 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

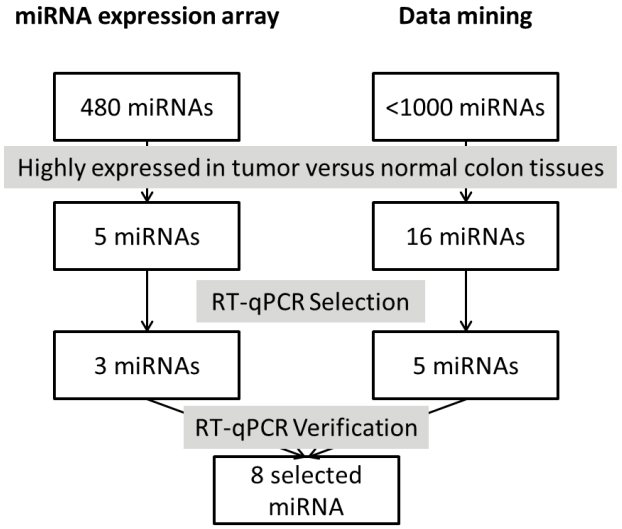

Figure 1: Flow shart showing an overview of the miRNAs selection process.

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 volunteers and 6 lymph nodes obtained from patients undergoing colon resection for benign conditions were studied for their expression of the 8 pre-selected miRNAs (Figure 2). All the 8 miRNAs were significantly upregulated in colon tissues (tumour and normal adjacent) compared to LN and PBLs ($p<0.00001$, Kruskal-Wallis test). In addition, the 8 miRNAs were significantly upregulated in tumour tissues compared to normal adjacent colonic tissues, LN and PBLs ($p<0.0001$, Student's t-test).

The specificity and sensitivity of the miRNAs to differentiate tumour tissues from normal lymphatic tissues were of 100% except for miR-96 (Suppl. data 6). In addition, miR-96, miR-183 and miR203 were presenting the highest specificity and sensitivity to discriminate between tumour tissues and normal adjacent tissues. Altogether, these results pointed miR-183 and miR-203 as the most accurate markers from our panel. Altogether, these results demonstrate the ability of the selected miRNAs to discriminate between tumour tissues and normal tissues making them suitable markers for lymphatic staging.

Determining suitable threshold values for miRNA panel screening

The threshold value of each miRNA was established as the mean RQ value + SD value measured in 20 negative controls (14 PBLs from healthy people 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

Patients Characteristics	No	%
Age	40-78 years	--
Gender		
Male	8	40
Female	12	60
Location of tumor		
Right Colon	7	35
Left Colon	7	35
Transverse	1	5
Sigmoid	2	10
Rectum	1	5
AJCC (T+N)		
T2N1	2	10
T3N0	7	35
T3N1	6	30
T3N2	1	5
T3N3	1	5
T4N0	1	5
T4N1	1	5
Positive SLNs (n=84)	14	16.3
Tumor differentiation		
Moderate/poor	3	15
Moderate	15	75
Moderate/well	2	10
Mucin secretion	5	25

Table 1: Patients' characteristics. A total of 20 patients suffering from colon cancer stage II and III were enrolled in the study.

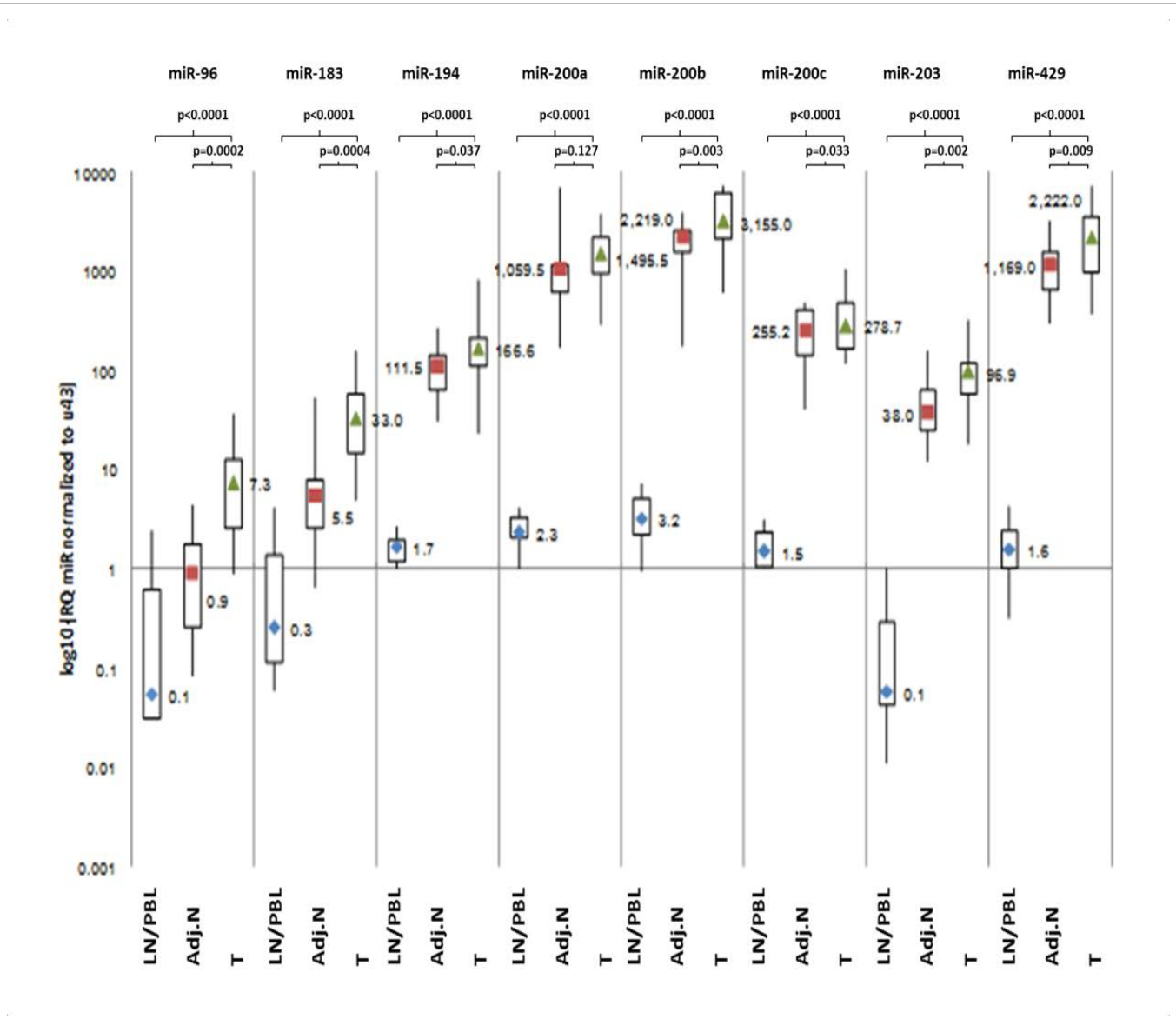

Figure 2: Box and Whisker plot showing relative miRNA levels in LNs/PBLs, adjacent normal tissues (Adj.N) and tumor tissues (T) by quantitative RT-PCR. The miRNA expression has been normalized to snoRNU43 expression and results are represented by the log10 of the relative quantity (RQ). In each box the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

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).

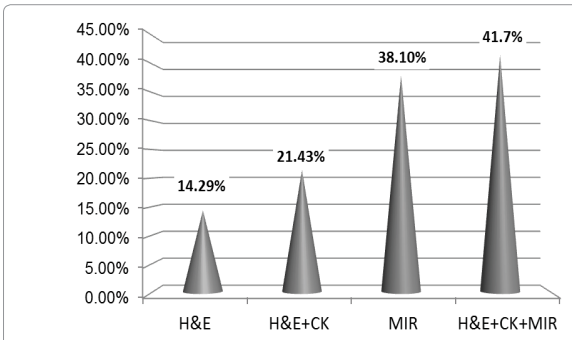

Figure 3: Comparison of metastases detection in sentinel lymph nodes by the different techniques. miRNA panel assay has detected 40% more metastases than traditional standard histopathological methods. H&E: haematoxylin and eosin staining; CK: immuno-histochemical cytokeratin analysis; MIR: miRNA panel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

Number of SLNs	miR-96	miR-183	miR-194	miR-200a	miR-200b	miR-200c	miR-203	miR-429
TP	11	14	12	10	10	10	12	11
FP	11	11	8	10	12	7	17	15
FN	7	4	6	8	8	8	6	7
TN	55	55	58	56	54	59	49	51
Sensitivity	61%	78%	67%	56%	56%	56%	67%	61%
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.

Patients	Number of SLN	TNM	AJCC Stage	Adjuvant Chemotherapy	Pathological analysis		
					H&E	IHC	miRNA panel test
Patient 1	4	T3N1M0	IIIB	Yes	P	P	P
Patient 2	4	T3N1M0	IIIB	Yes	P	P	P
Patient 3	6	T3N0M0	IIA	No	N	P	P
Patient 4	3	T3N2M0	IIIC	Yes	N	N	P
Patient 5	4	T3N1M0	IIIB	Yes	P	P	P
Patient 6	6	T4N1M0	IIIB	Yes	N	N	N
Patient 7	3	T3N0M0	IIA	No	N	N	P
Patient 8	3	T2N1M0	IIIA	Yes	P	P	P
Patient 9	3	T3N1M0	IIIB	Yes	N	N	P
Patient 10	7	T3N0M0	IIA	No	N	N	N
Patient 11	5	T3N0M0	IIA	Yes	N	N	P
Patient 12	4	T4N0M0	IIA	Yes	N	N	P
Patient 13	2	T3N1M0	IIIB	Yes	P	P	P
Patient 14	5	T4N0M0	IIA	Yes	N	N	N
Patient 15	3	T3N0M0	IIA	No	N	N	P
Patient 16	5	T3N3M0	IIIB	Yes	P	P	P
Patient 17	4	T3N1	IIIB	Yes	P	P	P
Patient 18	4	T3N1M0	IIIB	Yes	P	P	P
Patient 19	3	T2N1M0	IIIA	Yes	N	N	N
Patient 20	6	T3N0M0	IIA	No	N	N	P
P: Positive – up regulation, N: Negative – down regulation.							

Table 3: Summary of the LNs analysis.

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].

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 LNs 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% compare to standard histo-pathological analysis.

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