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MicroRNA Expression in Ileal Carcinoid Tumors: Down-regulation of MicroRNA-133a with Tumor Progression

Katharina Ruebel[1], **Alexey A. Leontovich**[2], **Gail A. Stilling**[1], **Shuya Zhang**[1], **Alberto Righi**[1], **Long Jin**[1], and **Ricardo V. Lloyd**[1]

[1] Department of Laboratory Medicine and Pathology, Division of Anatomic Pathology, Mayo Clinic, Rochester, MN

[2] Department of Health Science Research, Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN

Abstract

MicroRNAs are involved in cell proliferation, differentiation, and apoptosis and can function as tumor suppressor genes or oncogenes. The role of microRNAs in neuroendocrine tumors such as ileal carcinoids is largely unknown. We examined the differential expression of 95 microRNAs by RT-PCR using the QuantiMir System in eight matching primary and metastatic carcinoid tumors from the ileum. All microRNAs chosen for the QuantiMir System Array were based on their potential functions related to cancer biology, cell development and apoptosis. The expression of microRNAs for the samples was normalized to microRNA-197, and the matching primary and metastatic tumors were compared. There was down-regulation of microRNA-133a, 145, 146, 222 and 10b in all samples between the primary and matching metastatic tumors and up-regulation of microRNA-183, 488 and 19a + b in six of eight metastatic carcinoids compared to the primary tumors. MicroRNA-133a was further analyzed by TaqMan Real Time RT-PCR and Northern hybridization using six additional matching primary and metastatic samples which supported the PCR Array findings. There were significant differences in microRNA-133a expression with down-regulation in the metastasis compared to the primary in the eight original cases ($p<0.009$) and in the six additional cases used for validation ($p<0.014$). Laser capture microdissection and Real Time RT-PCR analysis using normal ileum found microRNA-133a expression in normal enterochromaffin cells. In situ hybridization in normal ileum showed that some of the mucosal endocrine cells expressed microRNA-133a. Both primary and metastatic ileal carcinoid tumors expressed microRNA-133a by in situ hybridization. These results provide information about novel marker microRNAs that may be used as biomarkers and/or therapeutic targets in intestinal carcinoid tumors.

Keywords

PCR array; carcinoids; enterochromaffin cells; RT-PCR; in situ hybridization

Address Correspondence to: R. V. Lloyd, M.D., Ph.D., Mayo Clinic, Department of Laboratory Medicine and Pathology, 200 First Street SW, Rochester, MN 55905, Fax: 507-284-1875, Tel: 507-284-4022, lloyd.ricardo@mayo.edu.

Introduction

MicroRNAs (miRNAs) are a family of 21 to 25 nucleotide, noncoding small RNAs that function as gene regulators (1–7). MiRNAs are usually excised from 60–110 nucleotide fold back RNA precursor molecules. They are involved in critical biologic processes including development, differentiation, apoptosis and proliferation (1,6,7). MiRNAs regulate post transcriptional gene silencing by inducing mRNA degradation or by binding to the 3′-untranslational region of target RNAs leading to repression of the translational process (1,6,7). Mature miRNAs are processed from stem-100bp precursor molecules which are transcribed as part of longer primary transcripts. The primary miRNAs are processed in the nucleus by the RNase Drosha, and then the precursor miRNAs are exported to the cytoplasm and further processed by the RNase Dicer (1,6,7). MiRNAs are usually deregulated in cancers (1,6,7).

Gastroenteropancreatic neuroendocrine tumors, which include ileal carcinoids, are rare neoplasm which secrete peptides and neuroamines that can cause distinct clinical syndromes including the carcinoid syndrome (8–11). The incidence of carcinoid tumors has been increasing i (8) and recent studies have begun to elucidate the role of growth factors, growth factor receptors and other molecules in the development of these tumors, however, the pathogenesis remains unknown (8–15). Patients with advanced disease often present with metastatic disease to the liver and other sites. Many of these patients have unresectable disease and only a limited number of drugs are available for treatment of these patients (13,16,17).

MiRNAs have not been previously examined in carcinoid tumors. We analyzed the type of miRNAs expressed in normal enterochromaffin cells from the terminal ileum and in primary midgut carcinoid and in metastatic ileal carcinoid tumors to determine if specific miRNAs would have a unique profile in these neuroendocrine tumors and if there were changes in the levels of miRNA expression in primary and metastatic carcinoids and to possibly identify important molecular tests for further functional investigations to develop new diagnostic and therapeutic targets for midgut carcinoid tumors.

Materials and Methods

Tissues and miRNA Extraction

Tissues analyzed included eight fresh frozen matching primary and metastatic human ileal carcinoid tumors containing 70–90% tumor. Another six matching pairs of primary and metastatic ileal carcinoids were also used for validation studies. Approximately 100 mg of each tumor was disrupted using a mechanical homogenizer. Total RNA containing the small RNA fraction was then extracted by acid-pheno1:chloroform using the mirVana miRNA Isolation Kit (Ambion Inc., Austin, TX) or with Trizol (Invitrogen, Carlsbad, CA). RNA was purified by glass-fiber filters included in the kit, quantified by spectrophotometric absorbance at A260 and stored at −70°C. RNA quality was assessed using the Small RNA Kit (Agilent) on the Agilent 2100 Bioanalyzer. IRB approval was obtained for the study.

Real time RT-PCR Array

One microgram total RNA from each carcinoid tumor sample was reverse-transcribed using the QuantiMir Kit (System Biosciences (SMI), Mountain View, CA) in a total volume of 20 μl.

The cDNA sample was analyzed by Real Time PCR using the Cancer MicroRNA qPCR Array Kit (SBI) which provides 95 miRNA-specific and U6-specific forward primers in a 96-well plate format and the 3′ universal reverse primer. SYBR Greener Real Time PCR Super Mix (Invitrogen, La Jolla, CA) was added to a final concentration of 1X. Each cDNA sample was run in triplicate on a 384-well optical plate in a total volume of 15 μl per well. qPCR was performed on the 9700HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following profile: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 sec and 60°C for 1 minutes. A dissociation step was performed following the qPCR amplification for melting curve analysis.

Calculation of Relative Expression Level

Threshold cycle (Ct) values were defined as the cycle number at which the fluorescence exceeded a fixed threshold value above an automatically-set baseline. MiR-197 was selected as the endogenous control because of Ct values that vary less than 1.0 cycle between matching primary and metastatic samples. Ct values of each target miRNA transcript were normalized against the Ct value of miR-197. Relative change of miRNA expression in metastatic compared to its primary carcinoid tumor was then calculated using the ΔΔCt method. Three replicates for each experimental sample were performed and averaged together. Ten individual miRNA targets did not amplify or give a clean melting curve and were excluded. Results were expressed as the mean ±SEM. Melting curve analysis of the qPCR products verified product specificity.

Real Time Rt-PCR TaqMan miRNA Expression Assay

Mi-RNA-133a was selected from the PCR array for further analysis. Mature miRNA expression levels were measured using the TaqMan MicroRNA Assay Kit (Applied Biosystems). 10 ng of total RNA from matching primary and metastatic carcinoids from the eight samples previously analyzed and an additional six material samples were reverse-transcribed in a total volume of 15 μl using the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's instruction. 1.33 μl of each RT reaction was amplified by PCR in 20 μl total containing 10 μl of the TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). PCR was performed on the 7900HT Fast Real-Time PCR System with an initial incubation at 95oC for 15 seconds and 60oC for 60 seconds. Each PCR reaction was performed in triplicate a minimum of 3 times. Agarose gel electrophoresis and densitometric analyses were done. Expression levels were normalized with a small nucleolar RNA, RNU48, which was co-amplified as the endogenous control.

Northern Blotting

The Northern blot assay was performed using the MiRNA Northern Blot Assay Kit (Signosis) according to the manufacturer's protocol. In brief, following a 30-minute pre-run, 20 μg denatured total RNA from each matching tumor case was resolved by electrophoresis

in a 60% TBE-Urea Ready Gel (Bio-Rad). Following the transfer, the membrane was UV cross-linked and baked at 42°C in the hybridization buffer provided by the kit. 10 μl of the RNU48 DIG-labeled probe and 10 μl of hsa-miR-133a biotin-labeled probe were added and incubated overnight at 42°C. The blots were washed for 30 minutes then blocked for 30 minutes in blocking buffer. 20 μl Streptavidin-horse radish peroxidase conjugate was added to the blocking buffer for 45 minutes. The blots were washed three times with detection washing buffer. An equal mixture of Substrate A and Substrate B was then applied to the blot for 5 minutes. The excess liquid was removed, and the blots exposed to Kodak radiography film and developed.

Laser Capture Microdissection of Normal Enterochromaffin Cells of Ileal Mucosa

Laser capture microdissection was combined with immunocytochemical staining using specific antibodies to recognize endocrine cells associated with the crypts. Normal ileal mucosa tissue blocks were cut in 10 μm (cryostat) sections and adhered on uncharged glass slides and single cells were isolated as described previously (18,19). Briefly, the slides were incubated with anti-chromogranin A antibody (1/100 Dako, Santa Barbara, CA), washed and incubated with a secondary horse radish peroxidase-conjugated antibody, washed and counterstained with hematoxylin. All steps were performed at the minimum time required and always in the presence of RNAse inhibitor. We have verified in the past that our anti-chromogranin A antibody can be combined with laser capture microdissection(19). Enterochromaffin cells are located in the crypts within the ileum, and we estimate that one endocrine cell and 3–4 non-endocrine cells were captured with each laser treatment, i.e., approximately 25% of the captured cells are endocrine and the majority of them are enterochromaffin cells. Immunostained cells were isolated using a PixCell II laser-capture microdissection system (Arcturus Bioscience, Mount View, CA, USA).

In Situ Hybridization

In situ hybridization to detect specific miRNAs was performed using normal ileum (n=5), frozen sections of the original 8 matched cases used for miRNA RT-PCR Array and qRT-PCR screening and an additional 6 cases of matched primary and metastatic tumors. In addition, 30 formalin-fixed paraffin-embedded tissue cases of matching primary and metastatic tumors to the liver were also analyzed. Probes for in situ hybridization were purchased from Exiqon (Denmark and Woburn, MA) and consisted of locked nucleic acid oligonucleotide probes labeled at both the 5′ and 3′ ends with digoxigenin. In situ hybridization was performed as previously reported from our laboratory (20) with modifications for the locked nucleic acid probes (21–23). Frozen tissues were fixed in 4% paraformaldehyde and stored at −70°C until used.

Briefly, in situ hybridization was performed by microwave pretreatment in a 700 watt oven in 10 mM citric acid pH 6.0 for 5 minutes (frozen tissues) or 12 minutes (paraffin-embedded tissues) followed by cooling at room temperature for 20 minutes. Tissues were digested in 25 μg/ml proteinase K at 37°C for 5 minutes (frozen tissues) or 10 minutes (formalin-fixed paraffin-embedded tissue) solution. Hybridization was done at 60°C overnight in a humidified chamber using 100 nM for frozen tissues and 200 nM of probe for formalin-

fixed paraffin-embedded tissues. Slides were washed in 2 × sodium chloride-sodium citrate twice for 3 minutes at 55°C and in 0.5 × 55°C twice for 10 minutes.

Slides were rinsed in Tris-buffered saline, pH7.5 (buffer A) for 3 minutes and treated with 300 μl of antidigoxigenin-alkaline phosphatase antibodies at a 1/200 dilution for 3 hours followed by washes in buffer A with 1% normal swine serum and 0.3% triton X-100. After washes in buffer C, pH 9.5, the slides were reacted with nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, pH9.5 in buffer C and developed between 1 to 4 hours and counterstained with 0.1% Nuclear Fast Red for 3 minutes.

Controls consisted of substituting a scrambled probe (Exiqon) at the same concentration as the hybridization probe. As a second control, slides were treated with RNAse A before hybridization. Both the scramble probe and RNAse pretreatment resulted in no hybridization signal.

Slides were scored as 0, negative, 1+ weak staining, 2+ moderate staining and 3+ strong staining.

Statistical Analysis

Gene Spring GX 10.0 software was used to identify miRNAs with a metastatic to primary ratio less than or greater than 1 (up-regulated or down-regulated in metastatic tumors) in all 8 samples. An average of the ratio across all 8 samples was calculated and miRNAs were ranked in order of increasing average.

Densitometric analysis after Real Time RT-PCR and gel electrophoresis was expressed in mean ± standard error of the mean and analyzed with the Student's t test. $P<0.05$ was considered statistically significant.

Results

Clinicopathologic Features

The eight cases consisted of 6 females and 2 males. Metastatic tumor was present in the liver in seven cases and in a mesenteric lymph node in 1 case (Table 1). The six additional case studies included six primary ileal carcinoids with matching metastatic carcinoid to lymph nodes (n=5) and liver (n=1). The 30 formalin-fixed paraffin-embedded tissues used for ISH consisted of primary ileal carcinoids with matching liver metastases.

Expression of miRNAs in Primary and Metastatic Carcinoid Tumors

Analysis of the 95 miRNAs with real time RT-PCR Array showed that 10 miRNAs did not amplify consistency and were excluded form the study. Samples were normalized against the Ct value of miR-197, and the relative usage of miRNA expression was expressed in metastatic compared to the primary carcinoid tumors using the ΔΔCt method. Using Gene Spring Gx 10.0 software, we identified miRNAs with a metastatic/primary ratio less than 1 (up-regulated in primary tumors) in all 8 samples. The ranking of samples that were highest in the primary compared to the metastatic tumor (down-regulated) is shown in Table 2.

MiRNA-133a was ranked as one; while miRNA-145, 146, 222 and 106 were ranked lower (Figures 1A, 1B, 1C and 1D).

The ranking of samples that were highest in the metastatic compared to the primary tumor (up-regulated) are shown in Table 3 (Figures 1A, 1B, 1C and 1D). These included miRNA-183, 488 and 19a + b. The other miRNAs were more variably down-regulated and up-regulated between samples. A notable exception was miRNA122 which appeared to be highly up-regulated in the metastatic tumors. However, since miRNA122 was highly expressed in liver tissue, this was interpreted to represent contaminated liver tissue in the metastatic cases.

Real-time RT-PCR

Taq-Man RT-PCR analysis of miRNA-133a was done with the eight original paired samples and after gel electrophoresis; the results were analyzed by densitometry (Figures 2A, B, C and D). There was a significant down-regulation of miRNA-133a between the primary and metastatic carcinoids when samples were normalized in RNU48 from the same case. Comparison of the entire group (n=8) showed highly significant down-regulation when quantitated by relative densitometry units ($p<0.009$). Two individual cases (case 2 and 4) showed significant down-regulation when the individual cases were evaluated. Analysis of six additional samples showed a similar trend in down-regulation of miRNA-133a between the primary and metastatic tumors ($p<0.014$).

Analysis of Normal Enterochromaffin Cells

After laser capture microdissection and real-time RT-PCR, the enriched enterochromaffin cells were found to express relatively high levels of miRNA-133a when the results were normalized with RNU48 (Figure 3).

Northern Hybridization

Northern hybridization using matching pairs of primary and metastatic carcinoid tumors followed by densitometry analysis showed higher levels of miRNA-133a in primary tumors compared to metastatic tumors in two of the four samples analyzed when comparing miRNA-133a to RNU48 (Figure 4).

In Situ Hybridization

In situ hybridization using normal ileum showed expression of miRNA-133a in some of the enterochromaffin cells in the ileal mucosa (Figure 5A). ISH also showed expression of miRNA-133a in inflammatory cells including plasma cells and lymphocytes in the lamina propria of the ileum. The smooth muscle in the muscularis propria stained weakly, while the liver adjacent to hepatic metastases was negative for miRNA-133a. Analysis of 12 tumors with primary and metastasis using frozen tissue sections showed expression of miRNA-133a in both the primary and metastatic tumors (Figure 5B). Five cases showed higher levels of miRNA-133a in the primary tumors, three cases had higher levels in the metastases, while in four cases the levels were similar. The intensity of labeling between the primary and metastatic tumors was variable (Figure 5B and 5C). ISH analysis of miRNA-133a in 30 formalin-fixed paraffin-embedded tissue sections showed a positive hybridization signal in

all cases (Figure 5C). Similar levels of staining intensity (1+ to 2+) were present in most cases from the formalin-fixed paraffin-embedded sections. In general, the hybridization signal was weaker in the formalin-fixed paraffin-embedded tissues compared to the frozen tissue sections. The control sections with a scramble control probe (Figure 5D) and after RNAse treatment (not shown) resulted in a negative miRNA signal.

Discussion

Analysis of a unique 95 miRNA expression profile in primary and metastatic midgut carcinoid tumors showed consistent down-regulation of miRNAs-133a, 145, 146, 222 and 10b in metastatic compared to primary carcinoids and up-regulation of miRNA-183, 488 and 19a + b in metastatic carcinoid tumors. Validation of miRNA-133a in these neuroendocrine tumors was done by Real-time RT-PCR, Northern and in situ hybridization.

MiRNA-133a has been studied in skeletal and cardiac muscle where it has been found to be down-regulated in hypertrophic muscle tissues (24–27). MiRNA-133a has also been reported to be down-regulated in squamous cell carcinoma of the tongue (28) and miRNA-133a expression has been reduced in hepatocellular and lung carcinomas compared to normal tissues (29,30). MiRNA-133a along with miRNA-1 are on chromosome 18 in the same bicistronic unit and are involved in muscle differentiation and proliferation (31).

A recent study found that miRNA-133a was down-regulated in neutrophils in myeloproliferative disorders (32). These findings would agree with our observation of miRNA-133a expression in inflammatory cells, including plasma cells and lymphocytes, in the lamina propria of the ileum by in situ hybridization. The mechanism of action of miR-133 on gene expression is currently being investigated. Xu et al reported that miRNA-133 and miR-1 had opposing effects on apoptosis, since miRNA-133 repressed caspase-9 expression at both the protein and mRNA level, while miRNA-1 reduced levels of HSP60 and H5P70 proteins without changing their transcription levels.

Several other miRNAs were consistently down-regulated in metastatic carcinoid tumors compared to the primary tumors in this study. MiRNA-145 has been detected in various neoplasms (34,35). It was reported to inhibit cell growth in some lung adenocarcinomas (34) and is associated with decreased expression in gastric cancers (35). MicroRNA-146 has been reported to suppress breast cancer metastasis (36). MiRNA-222 has been associated with various tumors including gliomas, prostate and thyroid carcinomas (37–39). MiRNA-222 has been shown to target the cell cycle protein p27kip1 in various neoplasms (37,38). MiRNA-10b is expressed in invasive gliomas and has also been associated with breast cancer metastasis (40,41).

Of the three miRNAs, that were most consistently up-regulated in metastatic ileal carcinoids compared to the primary tumors, miRNA 183 has recently been shown to be overexpressed in metastatic colon cancer (42). An analysis of various miRNAs that are down-regulated and/or up-regulated in metastatic ileal carcinoid tumors may possibly help to predict the metastatic potential of these tumors.

These studies also show that the normal ileal enterochromaffin cells expresses miRNA-133a in both the primary and metastatic tumor. Densitometric analysis showed that the levels of expression by real-time RT-PCR were similar to that in the primary carcinoid tumors. However, since the laser captured enterochromaffin cells were mixed with ileal epithelial cells and constituted about 25% of the laser captured cells, we can assume that there may be at least 2-to 4-fold more miRNA-133a in the enterochromaffin cells compared to the primary tumor. If this assumption is correct, this would suggest that there is decreased expression of miRNA-133a in the development of primary carcinoids and a further decrease in miRNA-133a during progression from primary to metastatic carcinoid tumors. This suggests that decreasing levels of miRNA-133a has an important role in midgut carcinoid tumor development and progression.

This study also shows that in situ hybridization can be used to validate newly described miRNA in specific tumors and to show the localization of these miRNA in normal tissues as with miRNA-133a in the endocrine cells of the crypt epithelial cells in the ileum. Although in situ hybridization was useful in both frozen tissue sections and formalin-fixed paraffin-embedded tissues, higher probe concentrations and a longer development time to visualize the signal was needed with the formalin-fixed paraffin-embedded samples. We used probes labeled with digoxigenin at both the 3′ and 5′ ends to increase the detection signal, especially with formalin-fixed paraffin-embedded tissues. Although other studies have used locked nucleic acid probes labeled with digoxigenin to localize miRNA in formalin-fixed paraffin-embedded tissues (21–23), the use of probes labeled at both the 3′ and 5′ ends should further increase the sensitivity of the in situ hybridization procedure.

In summary, we have used a combination of RT-PCR Arrays with the QuantiMir System to detect miRNAs in primary and metastatic ileal carcinoid tumors. MiRNA-133a was down-regulated during progression from primary to metastatic carcinoid tumors. We used a combination of TaqMan Real Time RT-PCR, Northern and in situ hybridization to demonstrate expression of miRNA-133a in carcinoid tumors and in enterochromaffin cells from the ileum. These findings suggest that miRNA-133a may have an important role in carcinoid tumor development and progression and that this miRNA may have diagnostic and/or prognostic utility in midgut carcinoid tumors.

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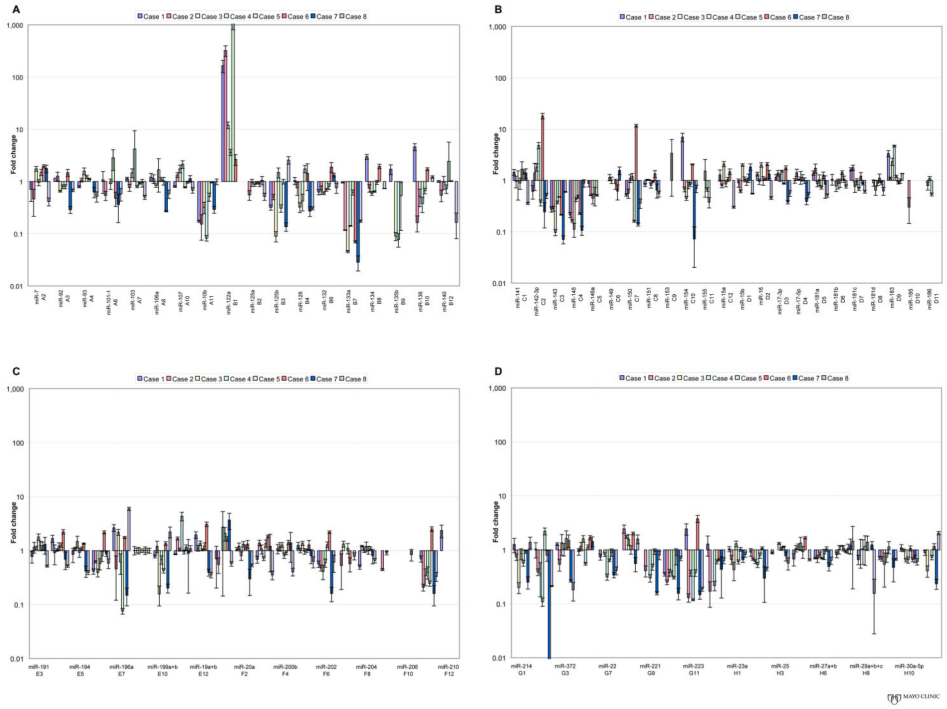

Figure 1.

Distribution of PCR array from eight ileal carcinoid tumors as log of fold change expressed as the ratio of metastatic to primary carcinoid tumors (Figures 1A, 1B, 1C and 1D). One μg log of total RNA was used from each carcinoid tumor using a QuantiMir kit. Results were expressed relative to microRNA-197. Relative change of miRNA expression was calculated by the comparative CT method. Figures 1A, 1B, 1C and 1D show the distribution as fold change for most of the miRNAs analyzed. Sixty-two microRNAs are labeled in the four figures.

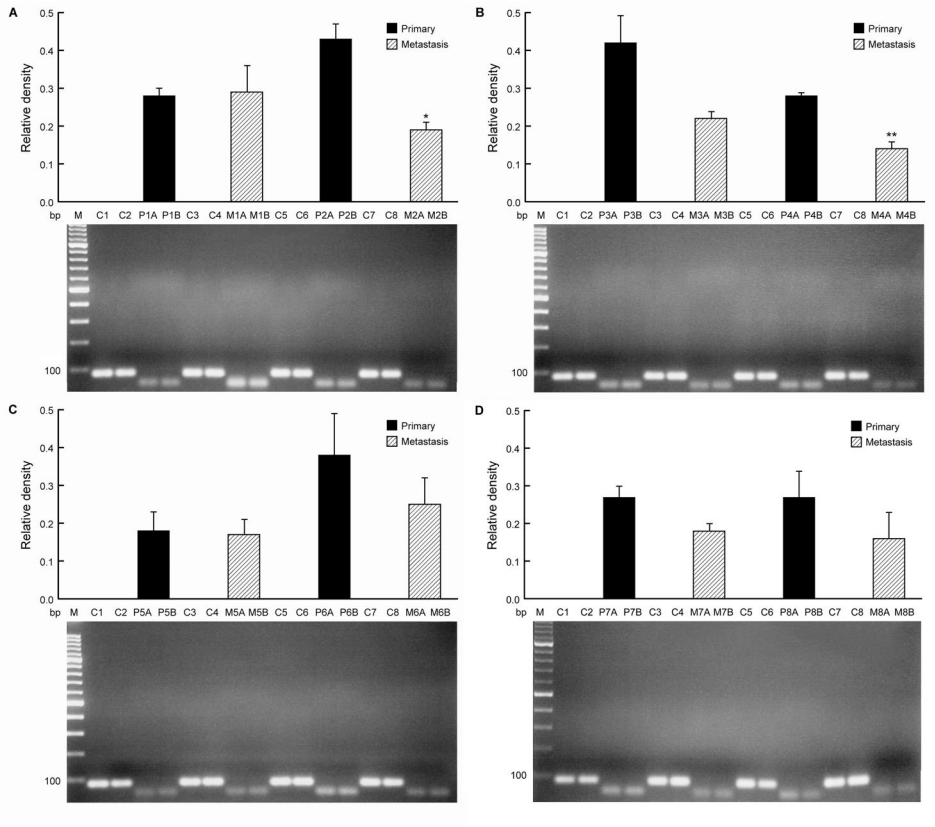

Figure 2.

TaqMan qRT-PCR analysis for miRNA-133a followed by gel electrophoresis and densitometric analysis of eight matching primary and metastatic ileal carcinoid tumors (Figures 2A, 2B, 2C and 2D). The upper panel shows the relative densitometric analysis of miR133a relative to RNU48 for each sample, while the lower panels show the original gel samples. C1 through C8 are the RNU48 samples. P represents the primary tumors and M represents the metastatic samples. Results are mean ± standard error of the mean. *$p<0.05$ ** $p<0.001$.

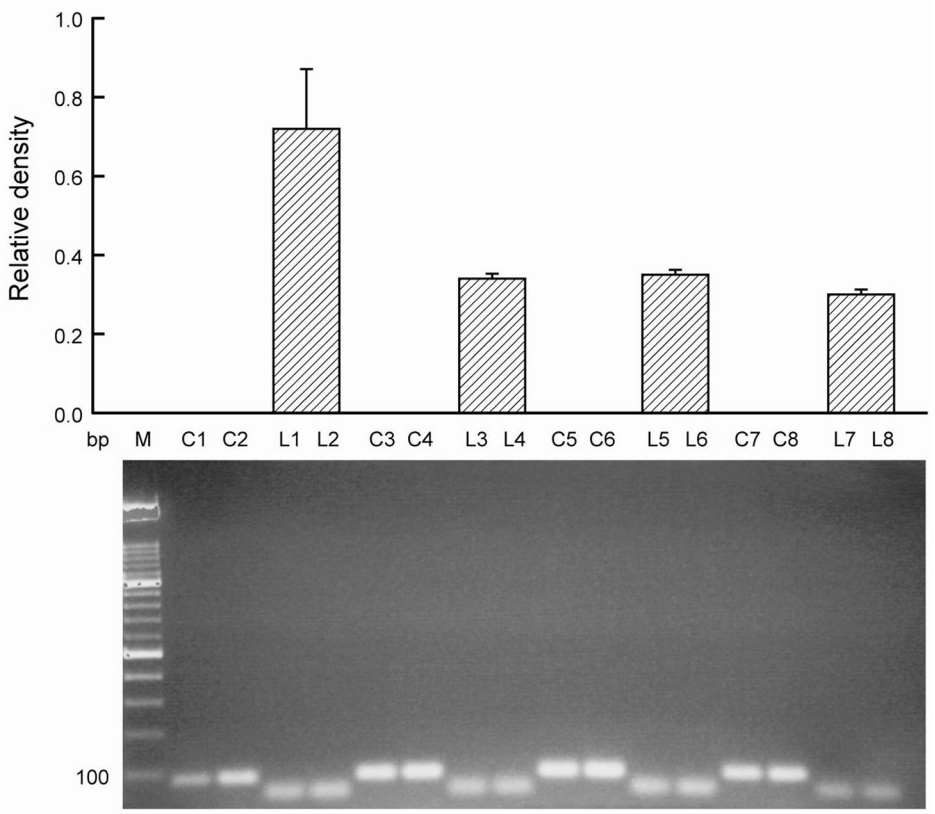

Figure 3.

TaqMan qRT-PCR analysis for miRNA-133a followed by gel electrophoresis and densitometric analysis of four samples of enterochromaffin cells harvested from 5×10^4 enterochromaffin cells. The upper panel shows the relative densitometric analysis of miR133a relative to RNU48 for each of four experiments. C1 through C8 are the RNU48 samples and L represent the laser captured miRNA-133a samples. Results are expressed as mean ± standard error of the mean.

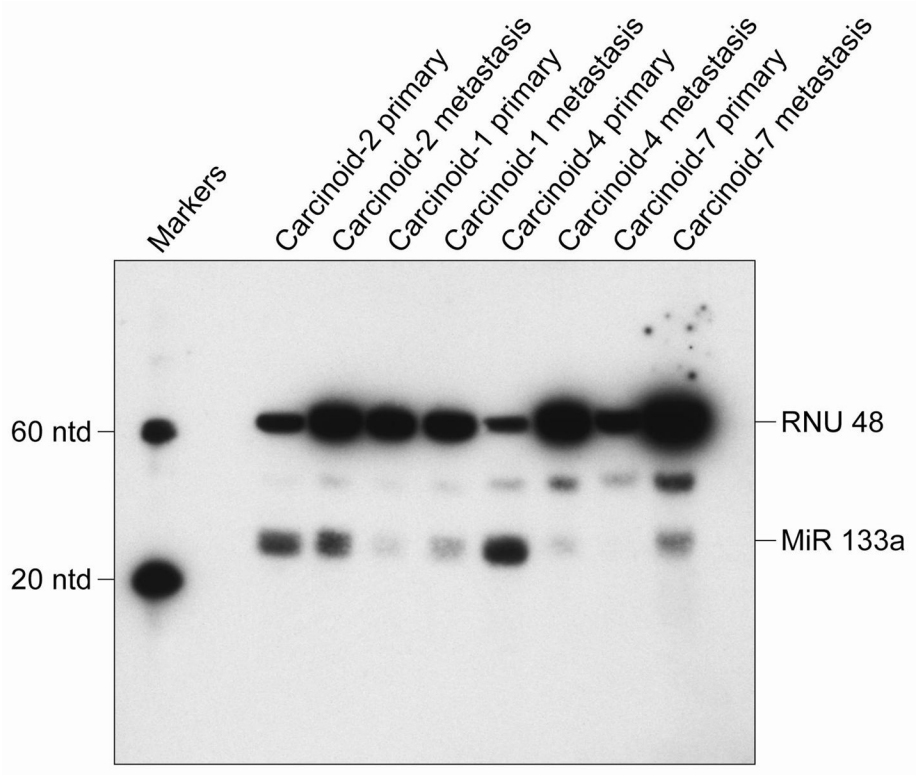

Figure 4.

Northern hybridization analysis of four sets of matching primary and metastatic ileal carcinoid tumors. Twenty μg of total RNA was used for the Northern hybridization with a Signos8is Kit according to the manufacturer's protocol. The results were developed by enhanced chemiluminescence. Results were expressed relative to small nucleolar RNA, RNU48. RNA standards (20 nucleotides and 60 nucleotides) were run with the gel.

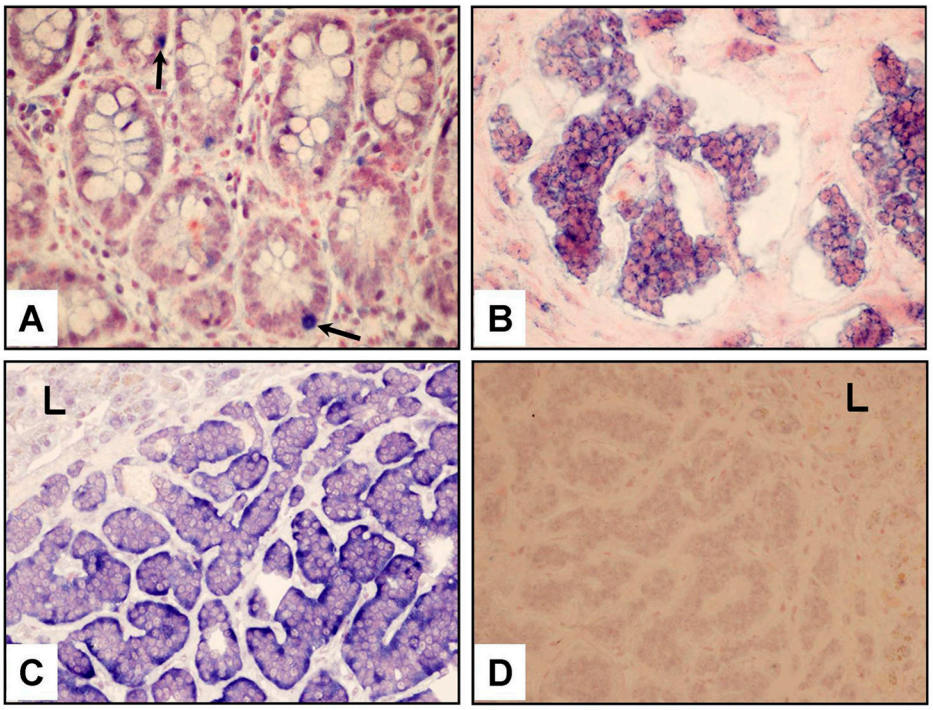

Figure 5.

In situ hybridization analysis using digoxigenin-labeled probes.

A. Normal ileal mucosa showing enterochromaffin cells with a positive hybridization signal for miRNA-133a (Arrows). Formalin-fixed paraffin-embedded tissue sections. Two-hundred nM of the 133a probe labeled at the 5′ and 3′ ends were used.

B. Frozen tissue sections of a primary ileal carcinoid tumor showing positive detection of miRNa-133a in the cytoplasm of tumor cells. The adjacent connective tissue is negative.

C. Paraffin section of a metastatic carcinoid tumor to the liver showing a strong positive hybridization signal for miRNA-133a in the cytoplasm of the tumor cells. The adjacent liver (L) is negative.

D. Paraffin section of hybridization with a scramble probe substituted for miRNA-133a used as a control. All other conditions were identical as with the miRNA-133a probe. There is no detection of miRNA-133a in the tumor or in the adjacent liver (L).

Table 1

Clinicopathologic Features of patients with midgut carcinoid tumors with liver metastases.

Case	Age/Sex	Primary Tumor Size (cm)	Metastatic Site	F/U (yrs)	F/U
1	56 F	1.5	Liver	5	AWD
2	40 F	3.8	Liver	6	AWD
3	61 M	0.7	Liver	6	AWD
4	46 F	4.7	Liver	6	AWD
5	59 F	1.5	Liver	6	AWD
6	54 M	3.0	Lymph Node	3	AWD
7	59 M	2.6	Liver	1	AWD
8	47 F	5.6	Liver	3	AWD

F/U=Follow-up

Table 2

Ranking of the highest miRNA expression in metastatic carcinoids compared to primary carcinoids.

MiRNA	Number of Samples Passed	Metastatic/Primary Ratio (mean value)	Rank
133a	8	0.26787	1
145	8	0.3265	2
146	8	0.359375	3
222	8	0.411375	4
10b	8	0.43575	5

A total of eight matched primary and metastatic samples were analyzed.

Table 3

Ranking of the highest miRNA expression in metastatic carcinoids compared to primary carcinoids.

miRNA	Number of Samples Passed	Metastatic/Primary Ratio (mean value)	Rank
183	6	1.99325	1
488	6	1.55757	2
19 a + b	6	1.31525	3

A total of eight matched primary and metastatic samples were analyzed.