

Supplementary Materials

Supplementary Methods

Nanostring assay and miRNA microarray analysis

100 ng of total RNA from frozen tissues were analyzed using NanoString human miRNA v1 assays (NanoString Technologies, Seattle, USA) according to the manufacturer's instructions (NCBIGEO GSE44121). After background subtraction, data was normalized to the geometric mean of the top 75 miRNAs. Paired Student's *t*-test was used to calculate statistical significance. We also analyzed our miRNA microarray expression data (NCBIGEO GSE7828) (1) for miRNA expression in primary tumors that were associated with distant metastases.

MiRNA isolation and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA (including miRNA) was extracted from FFPE specimens using the Total Nucleic Acid Isolation Kit for FFPE tissues (Ambion, Austin, TX, USA), as previously described (2, 3). RNA was extracted from frozen tissue specimens using standard Trizol protocols (Invitrogen), as previously described (4). Expression of let-7i, miR-10b, miR-30b, miR-221, miR-320a, and miR-885-5p were analyzed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA), and miR-16 expression was used as an endogenous control for data normalization, as previously described (3).

Small RNAs were isolated from 250 μ L serum samples from CRC patients using the Qiagen miRNAeasy Kit (Qiagen, Valencia, CA). For normalization of sample-to-sample variation during RNA isolation, 25 fmol of synthetic *C. elegans* miRNA (cel-miR-39, Applied Biosystems) was added to each serum sample as previously described (5). Expression of miR-885-5p was analyzed using TaqMan miRNA assays (Applied Biosystems) as described previously (3).

The average expression levels of serum and tissue miRNAs were normalized against cel-miR-39 and miR-16 using the $2^{-\Delta C_t}$ method. We selected miR-16 as an endogenous normalization control, because miR-16 expression is often used for normalizing miRNA

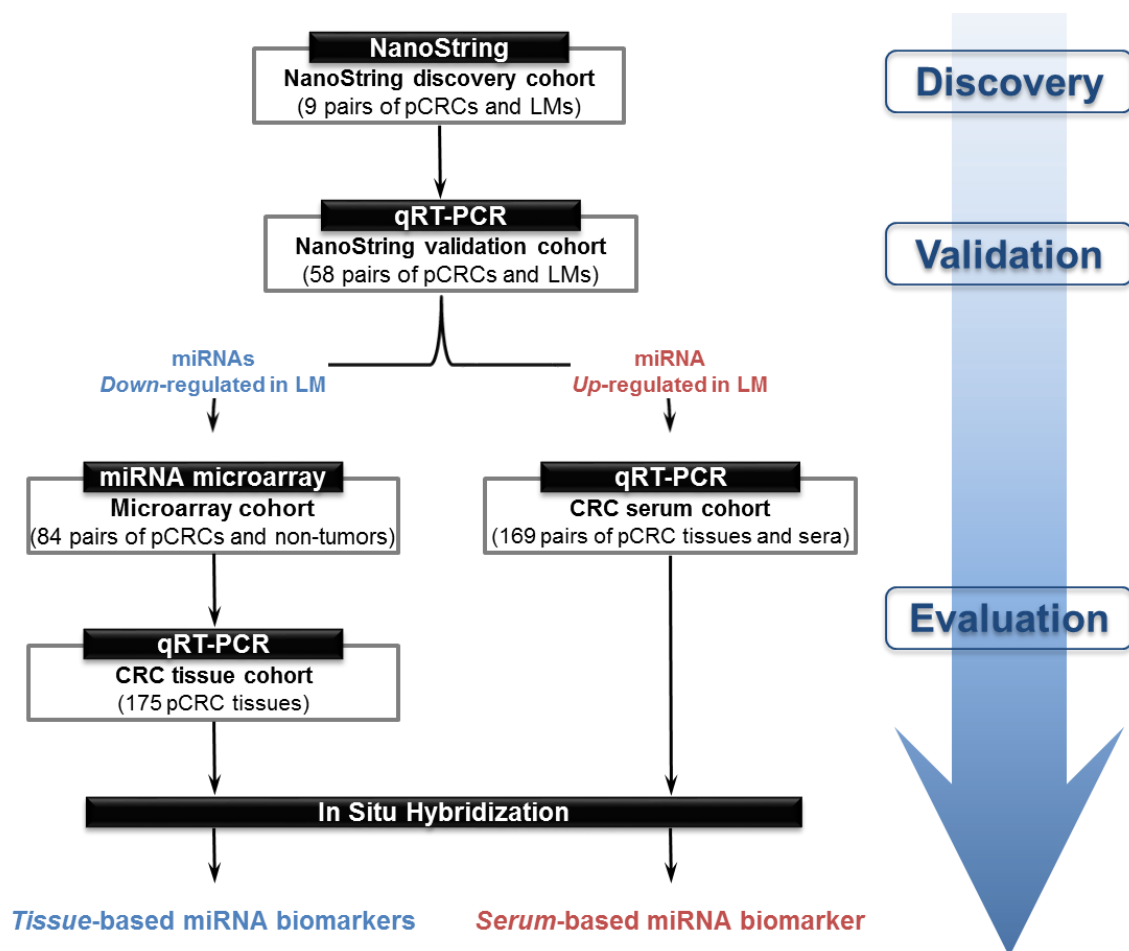
expression in FFPE tissues, as reported previously (3, 5). Differences between groups were presented as ΔC_t , which indicate the difference between C_t values of the miRNA of interest and the C_t value of the normalizer miRNA.

In Situ Hybridization (ISH) analysis

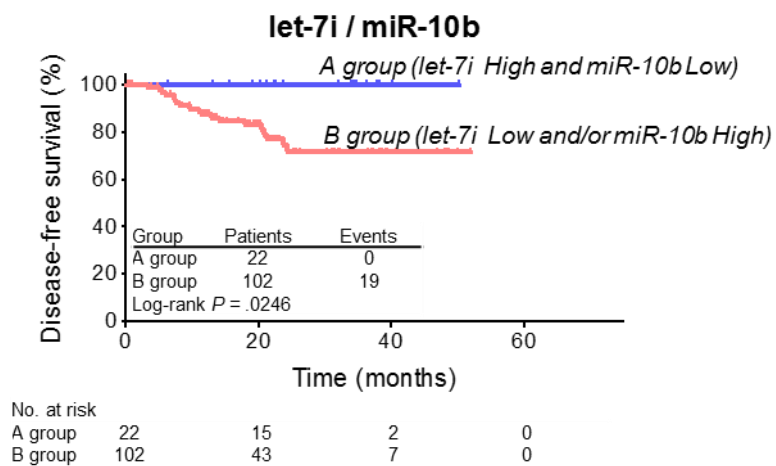
Five micrometer thick FFPE tissue sections were hybridized with the let-7i, miR-10b, and miR-885-5p probes (LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon, Woburn, Massachusetts, USA), respectively, as described previously (3). To ensure a fair comparison between all categories of tissues analyzed by ISH (stage I-III CRC vs. stage IV CRC and CRC vs. LM), all tissue sections were processed at the same time in parallel, to eliminate any batch variations that could be introduced by staining at different time points. Positive controls (U6 snRNA, LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon) and negative controls (scrambled miRNA control, LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon) were included in each hybridization procedure as previously described (3).

Supplementary Figure 1: Study design and outline

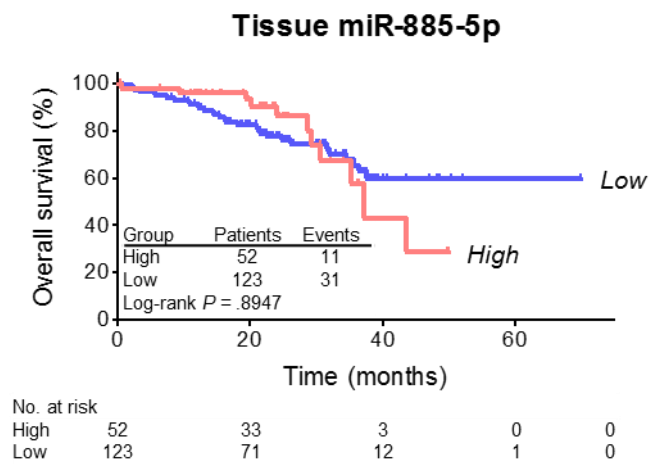
Discovery of CRC metastasis-specific miRNAs was conducted by Nanostring analysis in matching pCRCs and LM tissue cohort. Subsequently, identified miRNAs were quantitatively validated using qRT-PCR in an independent cohort of matching pCRCs and LM tissues. Clinical relevance of prioritized miRNAs was evaluated by miRNA microarray and qRT-PCR in independent matching pCRC and adjacent non-tumor tissue cohorts as well as matching CRC tissue and serum cohorts, respectively. Pathologic expression patterns of miRNAs were confirmed by ISH. LM = liver metastasis; pCRC = primary CRC; ISH = in situ hybridization.



Supplementary Figure 2: Disease-free survival analyses based on combination let-7i and miR-10b in CRC tissue cohort (stage I-III).



Supplementary Figure 3: Kaplan-Meier Overall Survival Analysis based on miR-885-5p in pCRC Tissue Cohort.



Supplementary Table 1: Characteristics of All Patients from each CRC Cohorts

	NanoString discovery cohort	NanoString validation cohort	Microarray cohort	CRC tissue cohort	CRC serum cohort
Characteristics	Patients <i>n</i> =9	Patients <i>n</i> =58	Patients <i>n</i> =84 (Deceased <i>n</i> =36)	Patients <i>n</i> =175 (Deceased <i>n</i> =42)	Patients <i>n</i> =169 (Deceased <i>n</i> =35)
Age, years					
< 40	1	1	1	2	3
40-49	1	6	6	10	8
50-59	2	12	22	19	18
60-69	3	26	24	66	61
≥ 70	2	13	31	78	78
mean±SD	59.3±13.6	62.3±10.3	64.6±10.7	67.5±10.3	67.8±10.6
median (max-min)	60.0 (37-78)	64 (32-88)	66.5 (32-87)	68.0 (37-92)	68.0 (34-92)
Sex					
Male	4	32	66	102	96
Female	5	26	18	73	73
Adenocarcinoma histology					
Adenocarcinoma*	-	58	75	162	152
Mucinous	-	0	8	10	10
Tumor location					
Proximal	-	12	34	58	54
Distal	-	18	48	49	44
Rectum	-	28		68	71
TNM stage					
I	0	0	8	38	37
II	0	7	29	53	58
III	0	17	36	44	42
IV	9	34	10	40	30

* Adenocarcinoma includes well, moderately, and poorly differentiated.

Supplementary Table 2: Clinical Relevance (normalized expression) of 4 miRNAs (let-7i, miR-10b, miR-221, and miR-320a) in the miRNA Microarray Cohort (84 pairs of frozen pCRC and adjacent non-tumor tissues; One-way ANOVA Test)

	hsa-let-7i			hsa-miR-10b			hsa-miR-221			hsa-miR-320a		
	<i>n</i>	<i>mean</i> ± <i>SD</i>	<i>P value</i>	<i>n</i>	<i>mean</i> ± <i>SD</i>	<i>P value</i>	<i>n</i>	<i>mean</i> ± <i>SD</i>	<i>P value</i>	<i>n</i>	<i>mean</i> ± <i>SD</i>	<i>P value</i>
Sex			0.18			0.579			0.629			0.085
Male	66	0.02407±0.2635		65	-0.3092±1.1328		66	0.2713±0.8388		66	0.04704±0.3012	
Female	18	-0.06910±0.2418		18	-0.4715±0.9354		18	0.3753±0.6618		18	-0.10260±0.3935	
Age (Years)			0.729			0.984			0.796			0.856
≤50	7	-0.028790±0.2321		7	-0.3526±1.0560		7	0.3691±0.7193		7	0.03658±0.1627	
>50	77	0.007094±0.2641		76	-0.3436±1.0995		77	0.2867±0.8126		77	0.01302±0.3380	
T stage			0.616			0.279			0.815			0.015
T1	0	-		0	-		0	-		0	-	
T2	12	0.071440±0.2190		12	-0.8053±1.0168		12	0.3131±0.7537		12	0.09735±0.2206	
T3	67	-0.005201±0.2716		66	-0.2572±1.1060		67	0.2738±0.7934		67	0.02956±0.3294	
T4	5	-0.032800±0.2058		5	-0.3897±0.9246		5	0.5115±1.1478		5	-0.37810±0.2654	
N stage			0.44			0.175			0.136			0.587
N0	38	0.03021±0.2648		38	-0.5572±1.2224		38	0.1865±0.7953		38	0.06200±0.2960	
N1	34	-0.01718±0.2761		33	-0.2638±0.9682		34	0.2464±0.7730		34	-0.03233±0.3229	
N2	10	-0.06630±0.1928		10	0.2659±0.7966		10	0.8431±0.7864		10	-0.02785±0.4452	
N3	2	0.22200±0.01820		2	-0.6827±0.6934		2	0.3823±1.0487		2	0.14000±0.4133	
M stage			0.01			0.02			0.121			0.066
M0	74	0.03081±0.2563		73	-0.4470±1.0561		74	0.2436±0.7895		74	0.03904±0.3240	
M1	10	-0.19350±0.2090		10	0.4043±1.0896		10	0.6632±0.8350		10	-0.16300±0.3022	
TNM stage			0.073			0.032			0.418			0.201
I	8	0.08200±0.2585		8	-1.0788±1.1221		8	0.1901±0.8263		8	0.1268000±0.2359	
II	29	0.02817±0.2658		29	-0.4334±1.2465		29	0.1778±0.8144		29	0.0640100±0.2979	
III	37	0.02180±0.2541		36	-0.3174±0.8312		37	0.3067±0.7792		37	0.0004827±0.3596	
IV	10	-0.19350±0.2090		10	0.4043±1.0896		10	0.6632±0.8350		10	-0.1630000±0.3022	

Supplementary Table 3: Interaction P tests between tumor suppressor-miRNAs (let-7i and miR-320a) as well as oncogenic-miRNAs (miR-10b and miR-221) in miRNA microarray discovery cohort.

Variable	<i>Tumor suppressor-miRNAs</i> (let-7i and miR-320a)	<i>Oncogenic-miRNAs</i> (miR-10b and miR-221)
	$P_{\text{interaction}}$	$P_{\text{interaction}}$
Overall survival	0.3927	0.3537
Distant metastasis	0.3011	0.187

Supplementary Table 4: Clinical Relevance (normalized expression) of 3 miRNAs (let-7i, miR-10b, and miR-885-5p) in CRC Tissue Cohort (175 FFPE pCRC tissues; Kruskal-Wallis Test)

	hsa-let-7i			hsa-miR-10b			hsa-miR-885-5p		
	<i>n</i>	<i>mean±SD</i>	<i>P value</i>	<i>n</i>	<i>mean±SD</i>	<i>P value</i>	<i>n</i>	<i>mean±SD</i>	<i>P value</i>
Sex			0.9441			0.1311			0.5311
Male	102	0.7862±0.2881		102	0.05870±0.02865		102	0.0006554±0.0007500	
Female	73	0.7956±0.2960		73	0.05391±0.03159		73	0.0005863±0.0004942	
Age (Years)			0.6466			0.5857			0.4619
≤68	86	0.7938±0.2804		86	0.05588±0.03143		86	0.0005798±0.0004904	
>68	89	0.7875±0.3012		89	0.05734±0.02864		89	0.0006717±0.0007817	
T stage			0.6927			0.0183			0.3976
T1/T2	50	0.7441±0.1990		50	0.04823±0.02788		50	0.0005246±0.0003865	
T3/T4	125	0.8081±0.3183		125	0.06036±0.02983		125	0.0006675±0.0007339	
N stage			0.0407			0.6505			0.3597
N0	95	0.8375±0.3025		95	0.05571±0.03017		95	0.0006975±0.0008064	
N1/N2/N3	80	0.7235±0.2608		80	0.05808±0.02981		80	0.0005445±0.0003985	
M stage			<0.0001			0.0486			0.3175
M0	135	0.8517±0.3013		135	0.05443±0.02938		135	0.0006501±0.0006979	
M1	40	0.6133±0.1562		40	0.06701±0.03105		40	0.0005515±0.0004856	
TNM stage			0.0002			0.0208			0.7954
I	38	0.7513±0.2090		38	0.04884±0.03021		38	0.0005713±0.0004014	
II	53	0.8889±0.3261		53	0.06255±0.03029		53	0.0007243±0.0009604	
III	44	0.8896±0.3245		44	0.05029±0.02540		44	0.0006289±0.0005012	
IV	40	0.6133±0.1562		40	0.06701±0.03105		40	0.0005515±0.0004856	

Supplementary Table 5: Clinical Relevance of Tissue- and Serum-miR-885-5p Expression in Matched CRC Tissue and Serum Cohorts (175 FFPE pCRC tissues and 169 sera)*

	Tissue miR-885-5p			Serum miR-885-5p		
	Low (n = 123)	High (n = 52)	P value (χ^2 test)	Low (n = 94)	High (n = 75)	P value (χ^2 test)
Sex			0.4623			0.5533
Male	69	33		51	45	
Female	54	19		43	30	
Age (Years)			0.9857			0.0011
≤68	61	25		34	47	
>68	62	27		60	28	
Histological Grade			0.2475			0.6393
well/mod.	110	50		85	66	
poor/mucin.	13	2		8	9	
T stage			0.2783			0.0733
T1/T2	37	11		31	15	
T3/T4	84	41		60	59	
N stage			0.1717			0.0327
N0	62	33		61	36	
N1/N2/N3	60	19		31	38	
M stage			0.3921			0.0069
M0	92	43		83	54	
M1	30	9		9	20	
TNM stage			0.4479			0.0235
I	28	10		26	11	
II	33	20		34	24	
III	31	13		23	19	
IV	30	9		10	20	
Liver Metastasis			0.473			0.0011
Absent	101	46		89	59	
Present	21	6		3	15	
Lymphatic Invasion			0.8579			0.0141
Absent	25	12		30	11	
Present	97	40		62	63	

*All statistical tests were two-sided.

Supplementary Table 6: Interaction tests between tissue miR-885-5p and serum miR-885-5p in the matched CRC Tissue and Serum cohorts.

<i>Tissue-miR-885-5p and Serum-miR-885-5p</i>	
Variable	$P_{\text{interaction}}$
Overall survival	0.2315
Lymph node metastasis	0.7469
Distant metastasis	0.3128

Supplementary References

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