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miRNA expression profiles in head and neck squamous cell carcinoma and adjacent normal tissue

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Abstract

Background—The expression of miRNA in head and neck squamous cell carcinomas (HNSCCs) that had been classified as high risk by surgical pathologic features and validated by trial outcome for disease recurrence was determined and compared with matched adjacent normal tissues.

Methods—miRNA and corresponding gene expression were determined using miRNA bioarrays and gene expression arrays.

Results—Twenty miRNAs were determined to be differentially regulated in the HNSCC samples when compared to their normal tissue counterparts. Quantitative RT-PCR (QRT-PCR) confirmed differential regulation of miRNA expression and gene expression analysis on these same-paired samples confirmed the loss of putative mRNA targets including genes such as adenomatous polyposis coli (APC), programmed cell death protein 4 (PDCD4) and TGF beta receptor 3 (TGFBR3) in the tumor samples.

Conclusions—These data suggest a role for the upregulation of specific miRNAs in high risk HNSCC. Furthermore, upregulation of these miRNAs may be responsible for the elimination of mRNAs that lead to the growth and progression of HNSCC.

Keywords

HNSCC; miRNA; miRNA targets; gene expression

INTRODUCTION

MicroRNAs (miRNAs) are small, highly conserved recently discovered, 18-25 nucleotide length single strand RNA molecules. miRNAs are involved in the post-transcriptional

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regulation of gene expression through the binding to 3′-untranslated mRNAs which are then degraded or translationally inhibited(1-3). Coding sequences for many miRNAs are conserved between very distantly related organisms, suggesting that they do indeed play important roles in cellular processes (4) and for several miRNAs there is specific evidence for their regulatory roles (2). Hence it is not surprising to find that miRNAs have a role in cancer development through the control of aspects of cellular growth and differentiation (5-8). MiRNA expression profiles in tumor samples have been described as phenotypic signatures for particular cancers and like mRNA expression profiles, miRNA expression signatures may ultimately be informative cancer diagnostic tools (5,9,10).

Like mRNA expression profiles, miRNA expression profiles vary from tissue to tissue, however, they are similar for the same type of tissue taken from different individuals (11). Thus, a comparative miRNA analysis between a diseased tissue and the normal tissues could reveal diagnostic markers or perhaps even therapeutic targets.

According to Calin and Croce (7), every type of tumor analyzed by miRNA profiling has shown a differential miRNA expression profile when compared with normal cells from the same tissue and the data described herein are consistent with that notion.. In addition, Volinia et al (11) described a common signature composed of 21 miRNAs differentially expressed in at least three tumor types from 540 samples including lung, breast, stomach, prostate, colon and pancreatic tumors. miR-21 was found to be overexpressed in all six types of cancers while miR-17-5p and miR-191 were overexpressed in five. Based on the predicted miRNA targets, they identified many well-known cancer associated genes as targets for the signature miRNA and concluded that miRNAs are involved in the pathogenesis of solid tumors.

Head and neck cancer is the term given to a variety of malignant tumors that develop in the oral cavity, larynx, pharynx and salivary glands, and are predominantly squamous cell carcinomas (HNSCC). Molecular signatures using gene expression analysis have been identified that describe these tumors by location. However, HNSCC of the oral cavity is noted for its heterogeneity and has defied substantive molecular classification (12). In contrast to gene expression analysis of tumors, a relatively small number of miRNAs can be used to classify tumors (9) and it is our notion that less heterogeneity will exist in miRNA expression profiles of various HNSCC although that is not the point of this study. In this study we have examined the miRNA expression of high-risk head and neck squamous cell carcinomas and their corresponding normal tissues in order to develop a miRNA signature of HNSCC and to explore the potential role that miRNA may play in the development of head and neck cancer.

METHODS

Tumor and normal tissue samples

Frozen HNSCCs and the corresponding adjacent normal tissues were obtained from five patients treated at MD Anderson Cancer Center as part of a randomized trial that included surgical resection of the primary tumor and postoperative radiotherapy (13). The patients had histologically proven locally advanced squamous cell carcinoma of the oral cavity or laryngopharynx and were classified as having a high-risk for recurrence based upon surgical and pathologic criteria (see Table 1) and for whom no subsequent recurrence was seen. Tumor and normal tissue were obtain as follows: 1)Grossly normal mucosa from the farthest resection margin was carefully excised and subjected to frozen section evalution to exclude dysplasia and the presence of inflammatory cells. The surface squamous epithelium of an adjacent area was then carfully peeled off the submucosa and placed immediately in liquid nitrogen. This process insured more than 90% epithelial cells in each “normal” sample.

2)Tumor tissue was also harvested from the periphery of the tumor mass and a thin slice was used for frozen section evaluation to assess the quality and quantity of the specimen. All of the samples used contained an excess of 80% viable tumor cells.

MicroRNA isolation

Approximately 100mg of frozen tissue was lysed in approximately 2ml of Trizol (Invitrogen, Carlsbad, CA) using a motorized Pro200 homogenizer (Pro Scientific, Oxford, CT). Extreme care was taken to avoid any partial thawing of the frozen sample prior to lysis. The Trizol lysate was subjected to organic extraction as described in the manufacturer's protocol. The aqueous phase that contained the total RNA was carefully removed and used to enrich for small RNAs (< 200 nt). The enrichment was accomplished by first immobilizing large RNAs on a glass fiber filter (Ambion, Austin, TX), then eluting with a relatively low ethanol concentration and collecting the flow-through that contained mostly small RNA species. More ethanol was added to this flow-through, and the mixture was passed through the second glass filter where the small RNAs were immobilized. The second filter was then washed several times and the small RNA enriched sample eluted with a low ionic strength solution. Large RNAs (>200 nt) from the first filter were also eluted after a few washes and used to check the integrity of the RNA source by running an aliquot on an Agilent 2100 Bioanalyzer automated electrophoresis platform (Agilent Technologies, Santa Clara CA). (Total RNA collected for gene expression analysis was also examined on this platform.) Approximately 100μg of the small RNA enriched samples were fractionated by passing through a Flash PAGE fractionator (Ambion, Austin, TX). The flash PAGE™ Fractionators can fractionate mature miRNAs from other small RNA species in just 12 minutes. This system has been designed and optimized to separate mature miRNA (19–23 nt) away from longer precursor molecules and provides an efficient alternative to standard, time-consuming polyacrylamide gel electrophoresis and subsequent gel elution. The fraction containing the miRNAs (<40nt) was recovered and cleaned using Ambion reagents following the manufacturer's protocol. miRNA samples were quantified by placing 1μl of the samples in the ND-1000 Nanodrop (NanodropTech Wilmington, DE).

miRNA labeling and hybridization

To determine the amount of enriched miRNA needed to produce sufficient fluorescence signal intensity, preliminary hybridizations were performed using 1, 2, 5, and 10μl of enriched miRNA (68ng/μl). From these results, 600ng was chosen as the amount of enriched miRNA to be used for subsequent hybridizations. The *mir*Vana miRNA labeling system (Applied Biosystems, Foster City, CA) was used to add a 3′ amine-modified tail to each miRNA target sample that was then labeled with NHS-esters of Cy5 fluorescent dye (GE Healthcare Piscataway, NJ) according to the manufacturer's protocol.

The mirVana miRNA bioarray system from Ambion (Austin, TX) was used to determine miRNA expression. The preprinted mirVana miRNA bioarray V1 has probes targeting a selection of human (213), mouse (51), and rat (30) miRNAs from the miRBase Version 8.0 Sanger miRNA Registry, as well as Ambi-miRs (105) that are exclusive to Ambion. Twenty-six controls are also present on each array. Each target sample was applied to two arrays and the arrays were sealed in hybridization chambers (Corning Acton, MA). The arrays were incubated overnight at 42°C in a rocking hybridization oven. The arrays were then washed in a low stringency wash buffer twice for 30s, followed by a high stringency wash buffer twice for 30s, then quickly rinsed twice in water and dried by centrifugation.

Data Analysis

The dried arrays were scanned using the ArrayWorx Auto^{e} scanner (Applied Precision, Issaquah, WA) and the fluorescence quantified using ArrayVision software (Imaging

Research, St. Catherines, Ontario, Canada). The quantified data were processed using the statistical package Splus 6 (Insightful, Seattle, WA). Local estimates of the background signal were subtracted from raw signal intensities for each array feature. Based on a histogram of the maximum spot intensity and maximum S/N, the features were filtered for a maximum S/N>4 as being trusted features. Of these, 249 features were included as trusted and the median intensities of these features were used to normalize the data on each array. The normalized data was logarithm transformed to base two and the mean data of the replicates was determined. The log ratio values were calculated for the tumor and corresponding paired normal samples. Differentially regulated miRNA between the two samples were identified using a paired t test.

Quantitative RT-PCR of miRNA

Quantitative RT-PCR was performed using the TaqMan MiRNA assay system (Applied Biosystems). The miRNA enriched fraction was used instead of the total RNA so as to include the normalization control miRNA Rnu-44, which is longer than the regular miRNAs. Briefly, about 2 ng of the miRNA enriched fraction (< 200 nt) was subjected to a RT reaction using a miRNA-specific looped primer according to the manufacturer's protocol in order to make cDNA. Subsequent PCR used miRNA specific forward and reverse primers along with an appropriate quantity of RT cDNA product and Taqman universal mix. The PCR reaction for each miRNA-cDNA, was run in quadruplicate. A negative control without template was included in parallel to assess the specificity of the PCR reaction. PCR was carried out in AB7900 (Applied Biosystems) in a 20μl volume with the following thermal cycling parameters: enzyme activation step at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15sec; and annealing/extension at 60°C for 60 sec. All other conditions used the manufacturer's values.

Data acquired from the PCR reactions was analyzed using SDS2.3 software (Applied Biosystems), which is based on the comparative C_T (threshold cycle) – $\Delta\Delta C_T$ method. Rnu-44 normalized $\Delta\Delta C_T$ values obtained for each miRNA from the normal samples was compared with the corresponding miRNA from the corresponding tumor sample. Comparisons are described as log values of the ratio of miRNA expression in tumor samples vs adjacent normal tissue.

mRNA gene expression profiling

mRNA hybridization of the tumor and normal tissues were performed as described in (14) using the spotted Oligonucleotide microarray chips produced by the Wiegand Radiation Oncology Core at MDACC. These arrays contain approximately 18,000 human oligonucleotides (SigmaGenosys, St.Louis, MO). A 22.5μg aliquot of the >200nt RNA fraction from either the tumor or normal tissue was compared against a reference Human Universal RNA (Stratagene Biocrest La Jolla, CA). All samples were labeled using the SuperScript indirect labeling kit (Invitrogen Corporation Carlsbad, CA) to produce cDNA for each slide. The cDNA was labeled with either a Cy3 (tumor or normal tissue) or Cy5 (Universal Reference) monofunctional reactive dyes (GE Healthcare Piscataway, NJ). Labeled Cy-cDNA was then mixed 1:1 with HybIt Hybridization buffer (Telechem Inc, Sunnyvale, CA). The target samples were injected onto the oligo array chips that had been loaded into a Lucidea SlidePro automated hybridization station (GE Healthcare, Piscataway, NJ). The arrays were incubated for 16 h at 42°C. The arrays were first washed at 50°C first with 4ml of 1 X SSC and 0.2% Sarkosyl, then with 2ml of 0.1 X SSC and 0.2% Sarkosyl, followed by a final Wash at room temperature with 2 ml of 0.1 X SSC. The arrays were then rinsed with isopropanol and air dried before being scanned with the Array Worx scanner.

The initial data analysis was carried out as described in (14). A simple two-sample t-test was used to score the differential gene expression between tumor samples and the corresponding normal tissues using a beta-uniform mixture model (15) and false discovery rate for feature cutoff.

RESULTS

Differential expression of miRNA in HNSCC tissues

The miRNA expression for the tumor samples and the matched normal tissues were analyzed using a paired t test analysis. As described in the methods section, a global median normalization for the trusted array features provided the best fit to the data. With a false discovery rate of 10% and a p-value cutoff of <0.05 for all trusted features and all human probes on the array, 58 features were determined to be differentially expressed. These 58 features represent 20 human miRNA's that were differentially regulated across most of the five HNSCC tumor samples compared with the adjacent normal tissue samples, and included 14 miRNAs that were differentially regulated across all samples. Differential expression values were as high as 13-fold (see Table 2). Figure 1 depicts the differential expression of all 20 differentially expressed miRNAs as box plots; figure 1a describing miRNAs up-regulated in tumors and figure1b describing miRNAs that were down-regulated in tumor samples when compared to their matched adjacent normal tissue samples. Log ratio values for the ratio of miRNA expression between tumor and normal tissues are shown in figure 2 which highlights the distinct differential regulation of these miRNAs.

QRTPCR verification

Quantitative RT-PCR was used to validate the expression of miR-21, miR-155, miR-103, miR-107, miR-93, miR-23b, miR-125b and let7i miRNAs in both tumor and adjacent normal tissue samples. The log ratio expression values for the tumor samples vs normal samples determined from the RT-PCR data were compared to that of the miRNA microarray data and are represented in Figure 3a. Only four discrepancies were noted out of 40 measurements and they were unique to specific paired samples and were not seen across all samples. These discrepancies were typically where the expression values for the arrays were low and likely less reliable. Furthermore, given the discrepancies often seen between absolute values when array data are compared to QRTPCR data, that the two data types trend in the same direction suggests that the array data are at least reliable. The exception is miR103 in the T4/N4 pair data. Figure 3b shows the mean log ratio data for all five tumors as determined by both QRT-PCR and miRNA arrays. Only miR107 is contradictory, however, again, the relative values are quite low and hence have limited reliability.

Putative miRNA target gene expression analysis

Expression analysis of mRNA was performed on all samples (see methods). A list of genes differentially expressed between tumor and corresponding normal tissues was generated using a two-group t-test. This list was interrogated using a list of putative mRNA targets that are available in several public databases including the MIRANDA (3), TargetScan (16) and PICTAR (17) databases in order to probe putative mRNA targets of differentially expressed miRNAs. Because the role of miRNA is to interact with target mRNAs at specific sites to either induce cleavage of the message or inhibit translation (3) there are predicted interaction sites on the human genome transcript for almost all known miRNAs. Table 3 lists putative and known target genes listed in the public miRNA databases that have been determined to be regulated by the miRNA found to be differentially expressed in this study. In order to reduce false positives, only those genes from this list that satisfied the p-value criteria set for the identification of differentially expressed genes in the gene expression analysis (beta uniform method, false discovery rate 10%) were used. Using this approach 66 unique genes

were identified. Thirty-eight of these 66 genes were identified as targets of miR-21, miR-155, miR-103, miR-107, and let7i. All of these 38 genes were found to be down-regulated by gene expression analysis. In addition, 28 of the 66 genes, identified as targets of the downregulated mi-RNAs miR-125a, miR-125b and miR-23b, were found to be upregulated when compared to the normal tissue counterparts. This expression pattern is represented by Figure 4 where a heat map of the up- and down-regulated genes in tumor compared to normal tissue samples for the cluster of miRNA targeted genes is shown.

Two of the more differentially expressed miRNa were ambi_miR_7083 (13 fold relative increase in tumors) and ambi_miR_7029 (4-fold relative reduction in tumors). These miRNA are exclusive to the Ambion (Austin, TX) platform and were bioinformatically predicted to be miRNA. Mature sequences were obtained from the company and a computational method of predicting miRNA targets was used for potential target identification. This process predicted 32 target genes for each of these putative miRNA, however, as would be expected, of these 64 putative target mRNAs only a handful of genes were found on the array platform used here and even fewer were identified as differentially expressed at the mRNA level (Table 4). For ambi_miR_7083, lymphotactin XCL1 (chemokine (c-motif) ligand 1) was significantly downregulated in 3 of the 5 tumor samples while peroxiredoxin 4 (PRDX4), an antioxidant enzyme associated with metastatic colon carcinoma (18), a putative target for ambi_miR_7029, was found to be consistently upregulated in all five tumor samples. Interestingly, PKD1 was found to be down-regulated 10-fold in tumor samples. PKD1, Polycystic Kidney Disease 1, is suggested to be a tumor suppressor gene through the inhibition of cancer cell migration and invasion via the wingless (WNT) gene signaling (19). Down-regulation of PKD1 via the specific down-regulation of ambi7029 would seem counterintuitive given the mechanism of miRNA action and as such this result, while interesting may only be correlative.

miRNAs as regulators of oncogenes or tumor suppressor genes

Based upon evidence from other cancer sites such as glioblastoma, breast, and lung, those miRNAs whose expression increased in the tumor tissues compared to their normal tissue counterpart could be considered as equivalents of oncogenes. These miRNAs appear to be regulators of genes that are probably involved in the promotion of tumor development via the inhibiting of tumor suppressor genes and/or other genes that control cell differentiation or apoptosis (20). In that regard, miR-21 was one of the more abundantly expressed miRNA in tumor tissues (>3-fold) and was seen upregulated in all tumor samples when compared to their normal tissue counterpart. miR-21, as well as miR-103, was also determined to be up-regulated in a series of nine head and neck cell lines, however, because this was a comparison between tumor cell lines and not tumor against adjacent normal tissue direct comparisons are hard to make (21). mRNA expression analysis identified several putative targets for this miRNA that were downregulated including programmed cell death protein 4 (PDCD4) whose upregulation facilitates apoptosis, inhibits pathways important for cell proliferation and suppresses pathways involved in tumor angiogenesis (22). PDCD4 is also a putative target for miR-103 and miR-107, as identified by PicTar, each of which were overexpressed in tumor as compared to adjacent normal tissue suggesting PDCD4 is targeted by multiple miRNAs.

Mir-155 was upregulated on average six-fold in the tumor samples and has also been shown to be upregulated in breast, colon and lung cancer tissues. Upon analysis for putative targets from the gene expression data, APC was found to be downregulated in tumor samples which is consistent with the notion that loss of APC function likely plays role in colon cancer progression (23). The same may be true for high risk HNSCC.

Another highly suppressed gene, identified as a target for miR-103 and mir107, was TGFBR3 which was down-regulated a minimum of 3.7 fold in tumor samples. TGFBR3 down regulation is an important step in prostrate tumorigenesis (24) and Dong et al also showed that the loss of TGFBR3 through allelic imbalance is a frequent genetic event during human breast cancer development that increases metastatic potential and is a poor prognosis factor (25).

DISCUSSION

This is the first report of differentially transcribed miRNA in HNSCC tumor samples. Tissue samples from five surgically excised HNSCC tumors from four discrete anatomical locations designated as high risk for recurrence of disease or poor survival, were analyzed for miRNA expression and compared to miRNA expression within their respective adjacent normal tissue. Similar to the observations of He et al (26) and others (9) for other tumor sites, many miRNA transcripts were differentially upregulated in the HNSCC tumor tissues. Our study extends the commonality of miRNA expression in tumor samples to head and neck squamous cell carcinomas.

As summarized by Zhang (20) miRNAs can function as a novel class of oncogenes or tumor suppressor genes and there were a number of miRNAs previsously identified in other tumors seen differentially regulated in these HNSCC samples. For example, the up regulation of miR-21 and miR-155 has been seen in breast cancer and in diffuse large B-cell lymphoma, while miR-21 was shown to be an anti apoptotic factor in human glioma (27) and other solid tumors. MiR-21 is over expressed in at least six types of solid tumors (7) and phosphatase and tensin homolog (PTEN) was shown to be a direct target of mir21 within cholangiocarcinoma cells (28). Similar to the findings for mir21 in other tumor types, mir21 expression in the HNSCC samples was high in both tumor as well as normal tissues but the expression of mir21 was twice as high in tumor samples. Similarly, one other target gene to miR-21, and to miR-103/107, that was found to be down regulated in these HNSCC samples, was programmed cell death 4 (PDCD4) gene. This gene was recently identified as a novel tumor suppressor gene and loss of expression was found in several types of human cancer cell lines. Gao et al showed that in many glioma samples loss of PDCD4 expression partially contributed to the development of the tumor (29). Thus, the role of miR-21 would be predicted to be associated with the inhibition of apoptosis and thus have a pro survival role. On the otherhand, MiR_155, an oncogenic miRNA, over expressed in B-cell lymphomas (30), in DLBCL lymphomas (31) , in breast cancers (32) and lung cancers (33), was over expressed in the HNSCC tumor tissues compared to the normal tissues. Of the numerous predicted targets to mir-155, down regulation of APC was confirmed by mRNA expression profiling (see Figure 4) and by qRTPCR (data not shown).

In summary, in this study we observed the differential regulation of twenty miRNA in either tumor tissues or in samples of matched adjacent normal tissues. The expression of these 20 miRNas was used to stratify tumors from adjacent normal tissue, and gene expression associated with these miRNAs were also used to stratify tumor from adjacent normal tissue. A number of these miRNA have been characterized as oncogenic in other cancers. Developing a miRNA expression profile in HNSCC could lead to a better understanding of the genetic basis of this heterogeneous and complex disease and potentially be used as diagnostic and prognostic markers. Furthermore, some of the miRNA, because they can target multiple genes, might be considered as targets for interventional therapies. These results, even though from a very small sample set suggest that further classification of HNSCC by specific site and by therapeutic outcome is a rational strategy to pursue in order to determine whether miRNA expression has clinical value for the management of this disease.

Acknowledgments

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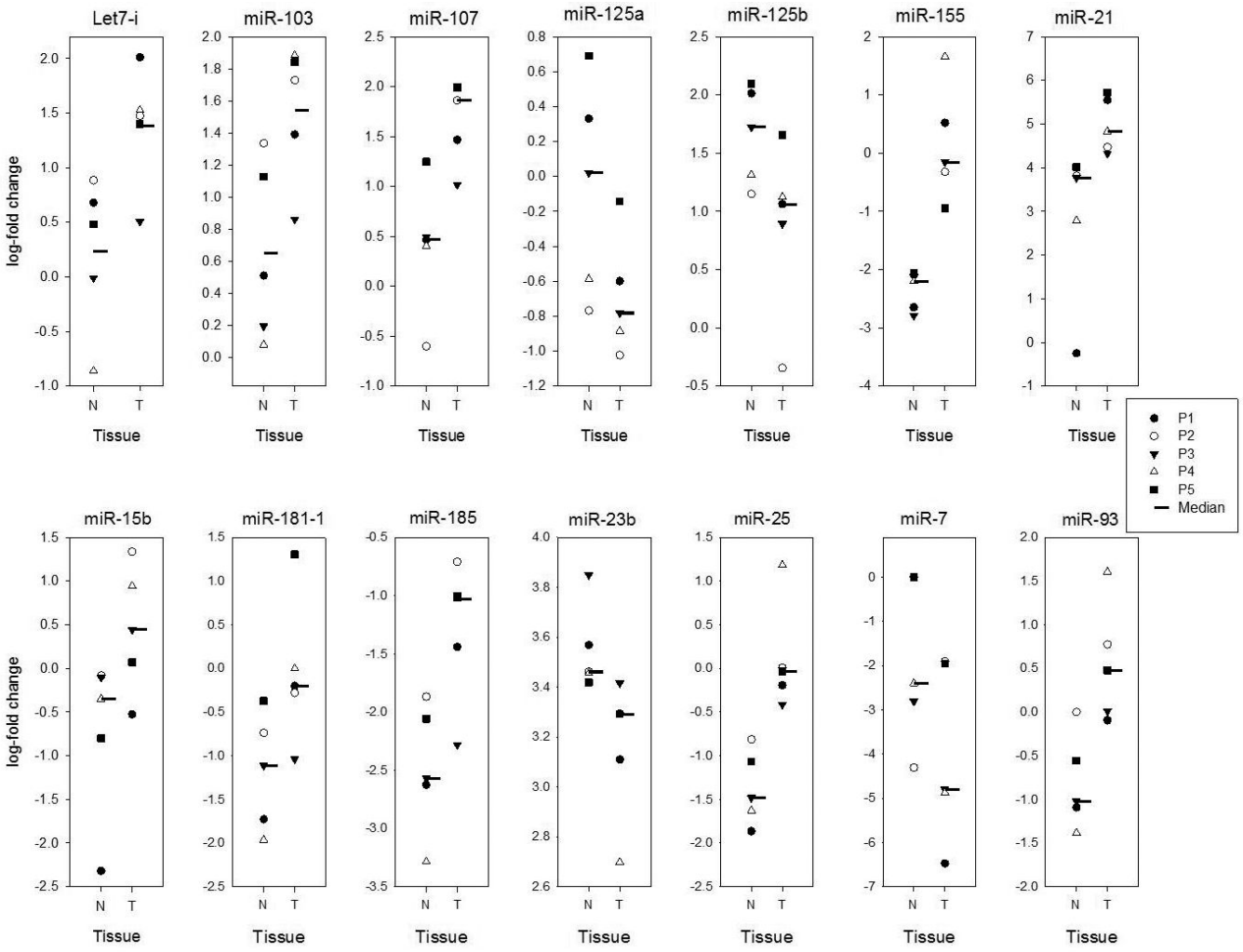


Figure 1.
Differential expression of miRNA for each patient sample, both normal tissue (N) and tumor (T) are shown. The horizontal bar represents the median value for all 5 samples in each tissue type.

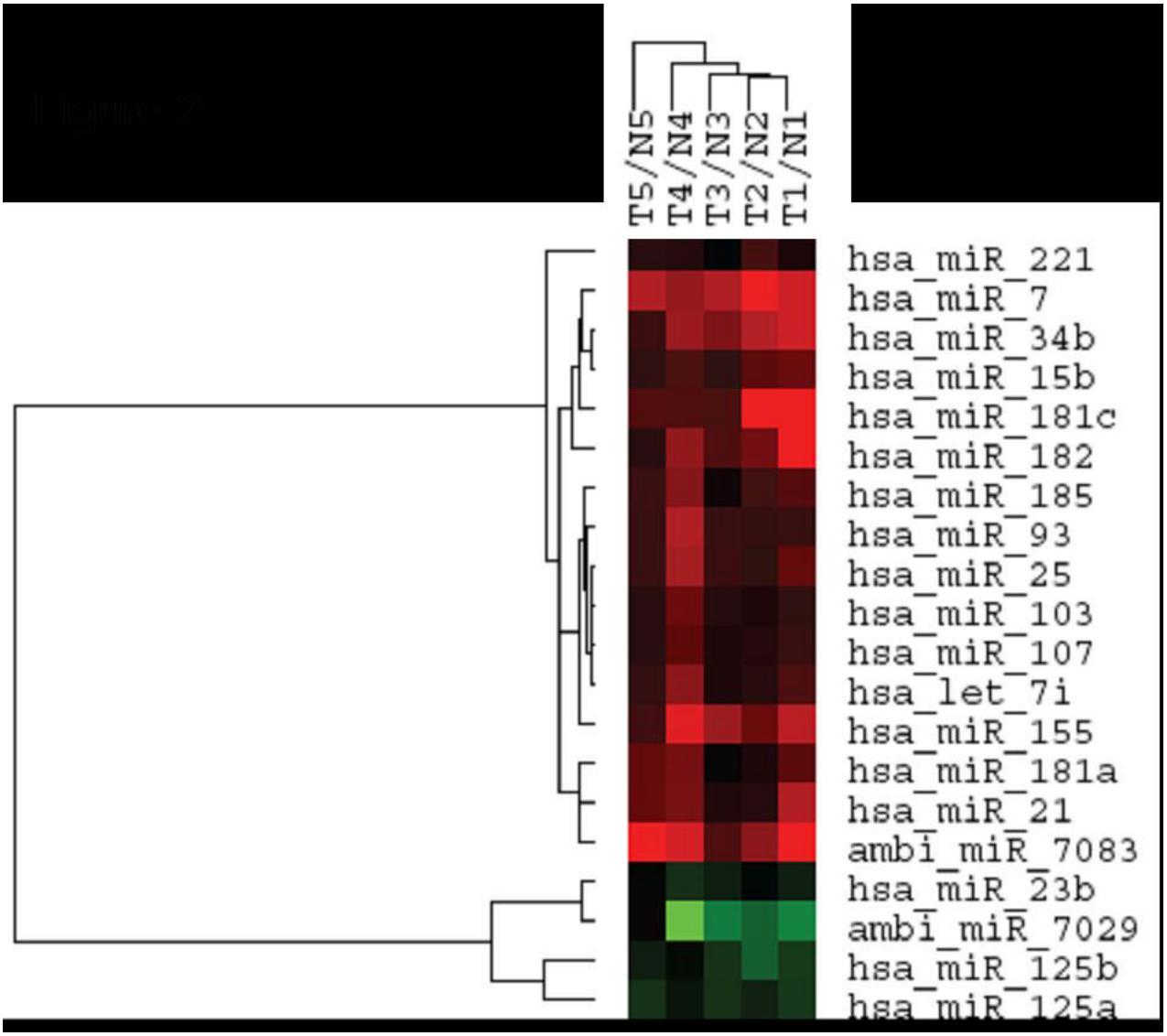
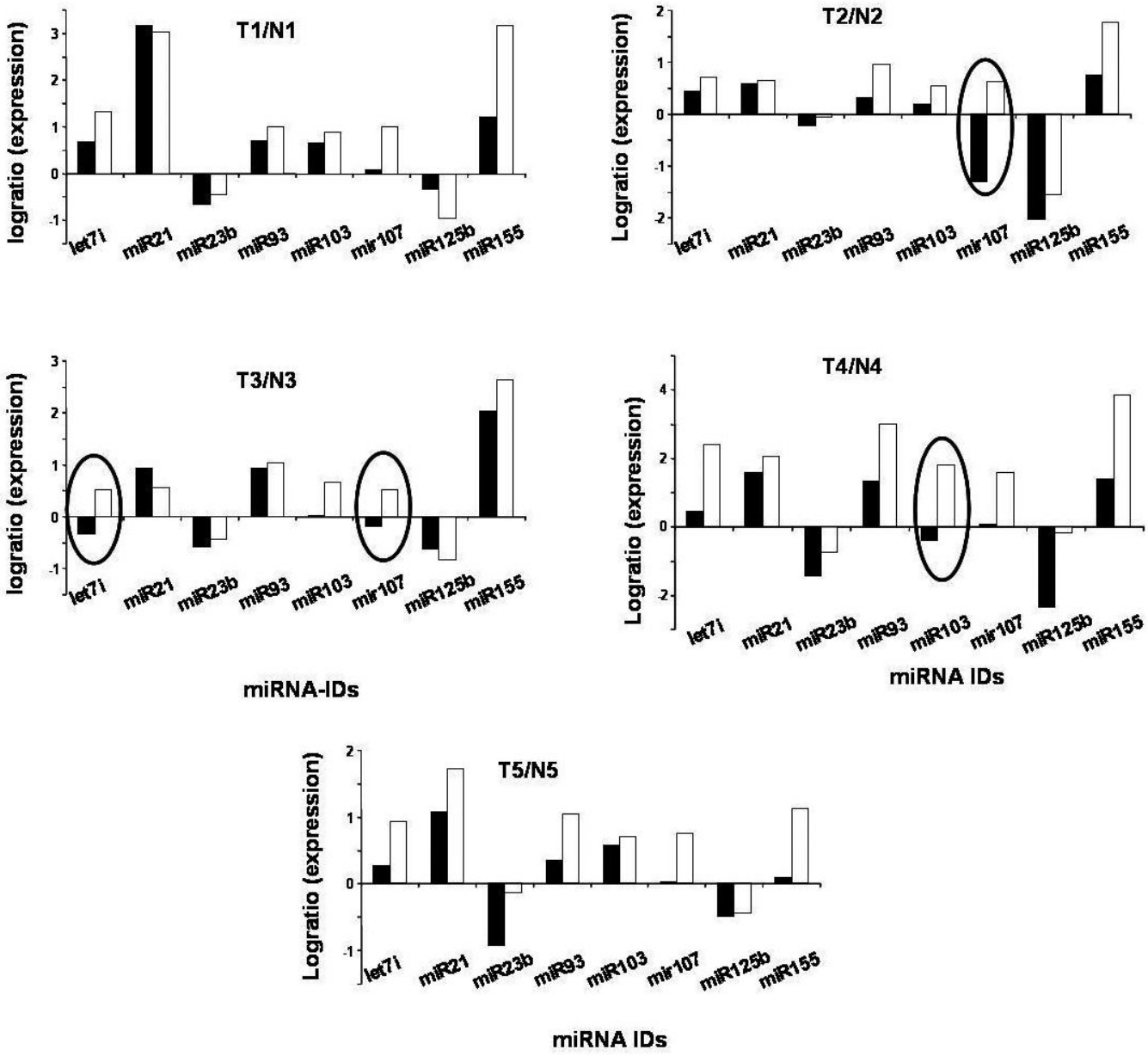


Figure 2.
Cluster analysis showing differential expression of miRNA in paired tumor-normal HNSCC samples. Logfold values range from −2.08 – 13.18-fold.



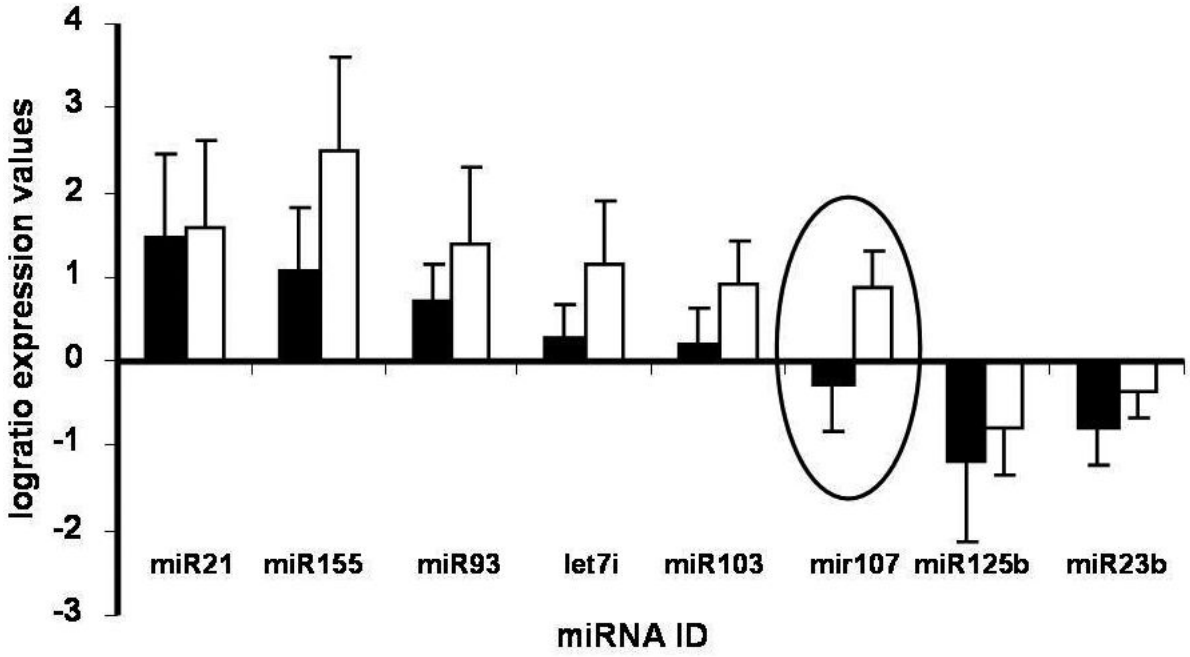


Figure 3.

Comparison of the mean log ratio for QRTPCR and miRNA array data for selected miRNA. The solid bars represent values from QRT-PCR analysis while the open bars represent the miRNA array data. The circled miRNA indicate discrepancies between the array data and QRTPCR data.

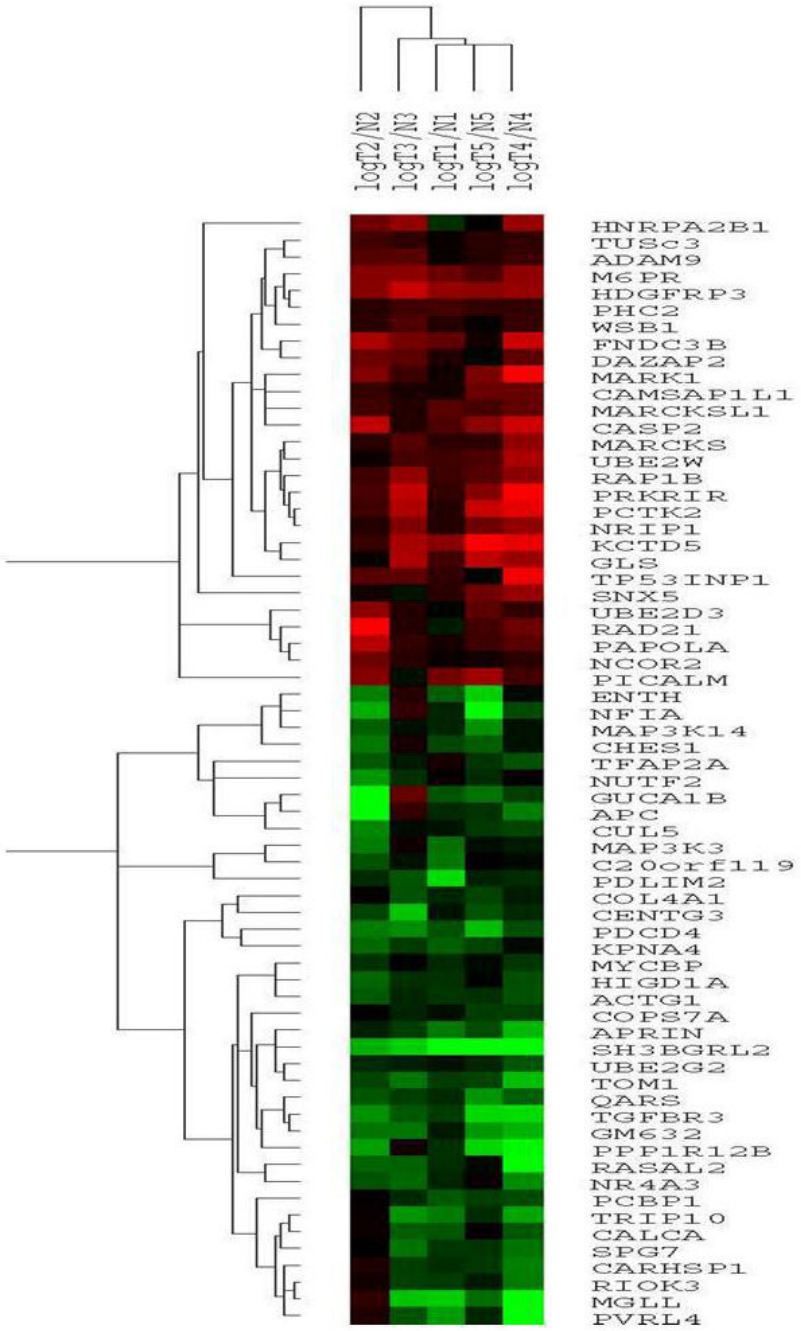


Figure 4.

Cluster of 66 putative target genes whose expression is inversely correlated with the differential expression of miRNA. A cluster of 38 target genes, correlate inversely with the over expression of miR-21, miR-155, miR-103, miR-107 and let7i, while a cluster of 28 upregulated target genes are inversely correlated with the reduced expression of miR-23b, miR-125a and miR-125b.

Table 1

Tissue sample information.

sample ID	Tissue type	Anatomical Site	
N1	Normal	tongue, NOS	
T1	Tumor	tongue, NOS	
N2	Normal	tongue, NOS	
T2	Tumor	tongue, NOS	
N3	Normal	base of tongue	
T3	Tumor	base of tongue	
N4	Normal	laryngopharynx	
T4	Tumor	laryngopharynx	
N5	Normal	floor of mouth	
T5	Tumor	floor of mouth	

Table 2

Differential expression of miRNA in HNSCC tissues compared to normal tissue. Positive log ratio values are miRNA with higher expression in tumor samples and negative log ratio values are for those miRNA having lower expression in tumor samples.

miRNA Probe Name	p values	Log ratio	Fold change
* ambi_miR_7083	1.82E-02	3.49	13.18
hsa_miR_7	7.42E-03	3.31	10.03
hsa_miR_34b	2.40E-02	2.67	6.44
* hsa_miR_155	7.17E-03	2.64	6.24
* hsa_miR_182	3.37E-02	1.80	3.49
* hsa_miR_21	2.55E-02	1.71	3.27
* hsa_miR_181c	2.43E-02	1.62	3.08
hsa_miR_181a	3.43E-02	1.53	2.88
* hsa_miR_15b	2.44E-03	1.29	2.47
* hsa_miR_185	2.49E-02	1.17	2.26
* hsa_miR_25	1.60E-02	1.07	2.10
* hsa_miR_93	2.50E-02	1.04	2.05
* hsa_let_7i	2.49E-02	0.92	1.89
* hsa_miR_107	1.05E-02	0.75	1.68
* hsa_miR_103	1.62E-02	0.73	1.65
hsa_miR_221	3.77E-02	0.67	1.59
hsa_miR_23b	4.50E-02	-0.40	0.76
* hsa_miR_125a	5.25E-03	−0.79	0.58
* hsa_miR_125b	2.82E-02	−0.83	0.56
ambi miR 7029	4.81E-02	−2.08	0.24

* Differentially expressed across all sample pairs.

Table 3

Differentially expressed putative target genes (gene expression data) for the specified differentially expressed miRNA. The negative log ratio values depict genes that are down regulated in tumor samples (the potential regulating miRNA has higher expression in tumor samples) and the genes with positive log ratio values are those that are upregulated in tumor samples (the potential regulating miRNA shows lower expression).

MiRNA	Target Information					
miRNA	**Symbol**	**Name**	**UGRepAcc**	**logratio**	**Fold change**	**p-value**
let7i	MGLL	Monoglyceride lipase	NM_007283	−1.901	0.268	1.30E-02
	GM632	KIAA1196 protein	NM_020713	−1.385	0.383	1.68E-02
	QARS	Glutaminyl-tRNA synthetase	AK222510	−1.021	0.493	4.08E-03
	PDLIM2	PDZ and LIM domain 2 (mystique)	NM_176871	−0.993	0.502	3.17E-02
	SPG7	Spastic paraplegia 7, paraplegin (pure and complicated autosomal recessive)	NM_003119	−0.779	0.583	1.39E-02
	CHES1	Checkpoint suppressor 1	NM_005197	−0.641	0.641	6.73E-02
	RIOK3	RIO kinase 3 (yeast)	NM145906	−0.597	0.661	9.41E-02
	CALCA	Calcitonin/calcitonin-related polypeptide, alpha	X02330	−0.586	0.666	4.71E-02
	NUTF2	Nuclear transport factor 2	CR621534	−0.527	0.694	9.36E-02
	UBE2G2	Ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)	AL834129	−0.509	0.703	5.20E-03
	COL4A1	Collagen, type IV, alpha 1	NM_001845	−0.484	0.715	1.63E-02
	MYCBP	C-myc binding protein	AK123051	−0.380	0.768	5.21E-02
miR103, 107	SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2	AF340151	−4.745	0.037	3.28E-02
	TGFBR3	Transforming growth factor, beta receptor III (betaglycan, 300kDa)	L07594	−1.703	0.307	5.37E-03
	RASAL2	RAS protein activator like 2	NM_170692	−1.345	0.394	6.98E-02
	NFIA	Nuclear factor I/A	AB007954	−1.219	0.429	6.22E-02
	GUCA1B	Guanylate cyclase activator 1B (retina)	BX537393	−1.184	0.440	9.95E-02
	TOM1	Target of myb1 (chicken)	AK097926	−1.169	0.445	8.91E-04
	TRIP10	Thyroid hormone receptor interactor 10	AF502289	−1.090	0.470	3.07E-02
	CENTG3	Centaurin, gamma 3	AL442089	−0.945	0.519	4.13E-02
	NR4A3	Nuclear receptor subfamily 4, group A, member 3	NM_173198	−0.817	0.568	9.00E-02

MiRNA		Target Information				
miRNA	**Symbol**	**Name**	**UGRepAcc**	**logratio**	**Fold change**	**p-value**
	HIGD1A	HIG1 domain family, member 1A	BQ924153	−0.661	0.632	1.13E-02
	C20orf119	Similar to embryonic poly(A) binding protein	AK124047	−0.540	0.688	5.48E-02
	COPS7A	Ubiquinol-cytochrome c reductase core protein II	AK094006	−0.397	0.759	3.52E-02
miR103, 107, let7i	PPP1R12B	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	NM_032105	−1.516	0.350	7.38E-02
miR107	PVRL4	Poliovirus receptor-related 4	AK027753	−1.204	0.434	4.95E-02
	APRIN	Androgen-induced proliferation inhibitor	AL137201	−1.113	0.462	1.06E-02
	ACTG1	Actin, gamma 1	CR606241	−0.732	0.602	1.24E-03
	CUL5	Cullin 5	NM_003478	−0.695	0.618	1.28E-02
	TFAP2A	Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	NM_001032280	−0.524	0.695	5.14E-02
miR107, let7i	MAP3K3	Mitogen-activated protein kinase kinase kinase 3	NM_203351	−0.611	0.655	8.88E-02
miR125a-b	NCOR2	Nuclear receptor co-repressor 2	NM_006312	0.527	1.441	4.54E-02
	PHC2	Polyhomeotic-like 2 (Drosophila)	NM198040	0.639	1.558	1.35E-02
	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	NM_003816	0.672	1.593	2.67E-02
	UBE2W	Ubiquitin-conjugating enzyme E2W (putative)	NM_001001481	0.915	1.886	7.09E-03
	PAPOLA	Poly(A) polymerase alpha	NM_032632	0.955	1.939	6.28E-03
	NRIP1	Nuclear receptor interacting protein 1	NM_003489	1.069	2.098	4.98E-03
	M6PR	Mannose-6-phosphate receptor (cation dependent)	BC024206	1.392	2.625	1.03E-03
	FNDC3B	Fibronectin type III domain containing 3B	NM_022763	1.422	2.680	8.89E-03
	CASP2	Caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally down-regulated 2)	AB209640	1.524	2.876	1.41E-03
	KCTD5	Potassium channel tetramerisation domain containing 5	AK000047	2.195	4.580	1.38E-03

MiRNA		Target Information				
miRNA	**Symbol**	**Name**	**UGRepAcc**	**logratio**	**Fold change**	**p-value**
miR125a -b, 23b	TP53INP1	Tumor protein p53 inducible nuclear protein 1	AK125880	1.101	2.145	4.94E-02
	GLS	Glutaminase	CR749593	1.393	2.627	6.01E-03
	DAZAP2	DAZ associated protein 2	AK125855	0.743	1.673	9.40E-03
miR155	APC	Adenomatosis polyposis coli	NM_000038	−1.106	0.465	1.72E-01
	ENTH	Enthoprotin	AK128594	−0.942	0.521	6.93E-02
	MAP3K14	Mitogen-activated protein kinase kinase kinase 14	NM_003954	−0.730	0.603	2.47E-02
	CARHSP1	Calcium regulated heat stable protein 1, 24kDa	BC108283	−0.601	0.659	6.87E-02
miR155, 103, 107, let7i	KPNA4	Karyopherin alpha 4 (importin alpha 3)	NM_002268	−0.790	0.578	2.01E-02
miR21	PCBP1	Poly(rC) binding protein 1	CR624156	−0.620	0.651	9.32E-02
miR21, 103, 107	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	BX537500	−1.428	0.372	4.40E-04
miR23b	TUSC3	Tumor suppressor candidate 3	BX641112	0.508	1.422	8.96E-03
	WSB1	WD repeat and SOCS box-containing 1	NM_015626	0.531	1.445	6.06E-03
	SNX5	Sorting nexin 5	AK054634	0.713	1.639	3.95E-02
	CAMSAP1L1	Calmodulin regulated spectrin-associated protein 1-like 1	NM_203459	0.733	1.663	1.48E-02
	UBE2D3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	AK127304	0.734	1.664	1.24E-02
	MARCKSL1	MARCKS-like 1	BG911975	0.833	1.781	2.14E-02
	HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	BX537494	0.835	1.784	4.39E-02
	MARCKS	Myristoylated alanine-rich protein kinase C substrate	NM_002356	0.855	1.809	2.18E-02
	PICALM	Phosphatidylinositol binding clathrin assembly protein	NM_007166	1.079	2.113	1.14E-02
	RAP1B	RAP1B, member of RAS oncogene family	NM015646	1.167	2.246	7.99E-04
	RAD21	RAD21 homolog (S. pombe)	NM_006265	1.168	2.247	5.04E-02
	PRKRIR	Protein-kinase, interferon-inducible double stranded RNA dependent	BX641144	1.651	3.141	7.26E-03

MiRNA		Target Information				
miRNA	Symbol	Name	UGRepAcc	logratio	Fold change	p-value
		inhibitor, repressor of (P58 repressor)				
	PCTK2	PCTAIRE protein kinase 2	NM_002595	1.654	3.148	7.28E-03
	HDGFRP 3	Hepatoma-derived growth factor, related protein 3	AL133102	1.804	3.492	2.51E-04
miR125a -b	MARK1	MAP/microtubule affinity-regulating kinase 1	NM_018650	1.443	2.718	2.92E-02

Table 4

Differential expression ratios of putative target genes for ambi_miRNA7029 (top) and ambi_miRNA7083 (bottom). The ratios expressed are tumor vs adjacent normal tissue. The regulating miRNA had higher expression in tumor samples where the gene expression log ratios are negative and the genes with positive log ratio values are those where the miRNA showed relatively lower expression in tumor samples.

Symbol	**Name**	**UGRepAcc**	**logratio**	**foldchange**
PRDX4	Peroxiredoxin 4	CD579519	1.017	2.024
CLEC2D	“C-type lectin domain family 2, member D”	BC063128	0.429	1.346
TMED2	Transmembrane emp24 domain trafficking protein 2	BU956289	0.339	1.264
GABARAP	GABA(A) receptor-associated protein	AK094586	0.123	1.089
NFIB	Nuclear factor I/B	BX537698	−0.531	0.746
CLEC4E	“C-type lectin domain family 4, member E”	AB024718	0.0265	1.019
FLJ41603	FLJ41603 protein	NM 001001669	−3.053	0.120
PKD1	Polycystic kidney disease 1 (autosomal dominant)	NM 001009944	−3.219	0.108
Symbol	Name	UGRepAcc	logratio	foldchange
XCL1	Chemokine (C motif) ligand 1	NM 002995	−0.389	0.764
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85α)	NM_181523	−0.057	0.961
CLEC2D	“C-type lectin domain family 2, member D”	BC063128	0.429	1.346
AZIN1	Antizyme inhibitor 1	NM 015878	1.110	2.158
SMAD2	“SMAD, mothers against DPP homolog 2 (Drosophila)”	NM 005901	1.831	3.557