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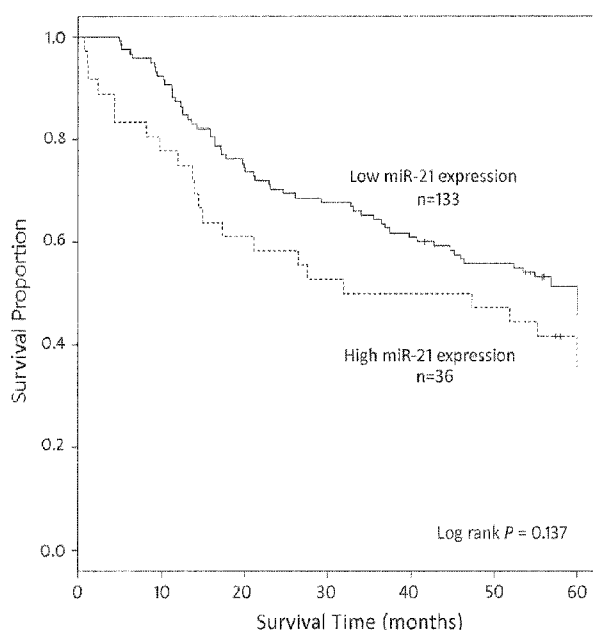
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[Continued on next page]

(54) Title: METHODS OF DIAGNOSING AND TREATING CARCINOMAS

Figure 5



(57) Abstract: Methods of diagnosing a carcinoma include comparing the expression of miRNAs in a sample with a control and determining ratios of expression of miRNAs. Methods of treatment include administering a nucleic acid encoding a miR-375 gene product. Methods of optimizing treatment in a subject include determining expression of an miR21-gene product.



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## METHODS OF DIAGNOSING AND TREATING CARCINOMAS

## RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application No. 61/209,320, filed on March 5, 2009. The entire teachings of the above application  
5 are incorporated herein by reference.

## GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by grants R01CA078609, R01CA100679, and T32ES007272 from the National Institutes of Health, National Cancer Institute and National Institute of Environmental Health Sciences. The  
10 Government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

Carcinomas can account for about 80-90% of all cancers in humans. The underlying causes of many types of carcinomas, including head and neck squamous cell carcinoma (HNSCC), are poorly understood. Early detection of carcinomas can  
15 be important in determining the course of treatment and in increasing survival rates. Thus, there is a need to develop new, improved and effective methods of diagnosing a carcinoma.

## SUMMARY OF THE INVENTION

The present invention generally relates to methods of diagnosing a  
20 carcinoma.

In an embodiment, the invention is a method of diagnosing a carcinoma in a subject, comprising the step of determining at least one expression ratio selected from the group consisting of a miR-21/miR-375 expression ratio, a miR-181d/miR-375 expression ratio, a miR-181b/miR-375 expression ratio, a miR-491/miR-375  
25 expression ratio, a miR-455/miR-375 expression ratio, a miR-18a/miR-375 expression ratio, a miR-130b/miR-375 expression ratio, a miR-221/miR-375 expression ratio, a miR-193b/miR-375 expression ratio, a miR-181a/miR-375 expression ratio, and a miR-18b/miR-375 expression ratio in a sample, wherein a

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ratio greater than about 1.0 is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma.

In another embodiment, the invention is a method of diagnosing a carcinoma in a subject, comprising the step of comparing an expression level of at least two  
5 microRNAs selected from the group consisting of miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a  
10 subject that would potentially benefit from a therapy to treat the carcinoma.

In a further embodiment, the invention is a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma in a subject, comprising the step of comparing an expression level of at least one microRNA selected from the group consisting of  
15 miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNA in the sample relative to the control expression level is diagnostic of the head squamous cell carcinoma and the neck squamous cell carcinoma and  
20 identifies a subject that would potentially benefit from a therapy to treat the head squamous cell carcinoma and the neck squamous cell carcinoma.

In an additional embodiment, the invention is a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma in a subject, comprising the step of comparing  
25 an expression level of at least one microRNA selected from the group consisting of miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample to a corresponding control expression level, wherein a difference in the expression level of the microRNA in the sample relative to the control expression level is diagnostic of the head  
30 squamous cell carcinoma and the neck squamous cell carcinoma and identifies a subject that would potentially benefit from a therapy to treat the head squamous cell

carcinoma and the neck squamous cell carcinoma and wherein the sample is not an established cell line.

In another embodiment, the invention is a method of treating a squamous cell carcinoma in a subject, comprising the step of administering a nucleic acid encoding  
5 a miR-375 gene product to the subject.

In a further embodiment, the invention is a method of optimizing treatment of a subject having a squamous cell carcinoma, comprising the step of determining an expression level of a miR-21 gene product in a sample from the subject, wherein  
10 over expression level of the miR-21 gene product in the sample compared to expression of a reference miR21-gene product identifies a subject that has an aggressive squamous cell carcinoma that would potentially benefit from a therapy to treat the aggressive squamous cell carcinoma.

The methods of the invention can be employed to diagnose a carcinoma in a subject. Advantages of the claimed invention include, for example, improved  
15 sensitivity and specificity in discriminating a carcinoma from a normal, non-carcinoma tissue to detect a carcinoma at an early stage.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts global normalized signal of six miRNAs (also referred to herein as “miR”) found to be significantly differentially expressed by microarray  
20 analysis using the SAM method.

Figure 1B depicts quantitative real-time PCR analysis showing the relative expression of the same six miRNAs shown in Figure 2A and confirming differential expression of four of the miRNAs identified as aberrantly expressed in the microarray. \* indicates  $P < 0.01$ .

25 Figure 2A depicts validation of differential expression of miR-21 by quantitative realtime PCR in tumor (n=99) and normal (n=14) samples. Boxes denote distribution of expression values from the 25th to 75th percentile. Horizontal lines in boxes represent median values, whiskers represent 5th and 95th percentiles and outliers are denoted by dots.  $P < 0.0001$ .

30 Figure 2B depicts validation of differential expression of miR-375 by quantitative realtime PCR in tumor (n=99) and normal (n=14) samples. Boxes

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denote distribution of expression values from the 25th to 75th percentile. Horizontal lines in boxes represent median values, whiskers represent 5th and 95th percentiles and outliers are denoted by dots.  $P < 0.0001$ .

Figure 2C depicts validation of differential expression of miR-18a by quantitative realtime PCR in tumor (n=99) and normal (n=14) samples. Boxes denote distribution of expression values from the 25th to 75th percentile. Horizontal lines in boxes represent median values, whiskers represent 5th and 95th percentiles and outliers are denoted by dots.  $P < 0.0001$ .

Figure 2D depicts validation of differential expression of miR-221 by quantitative realtime PCR in tumor (n=99) and normal (n=14) samples. Boxes denote distribution of expression values from the 25th to 75th percentile. Horizontal lines in boxes represent median values, whiskers represent 5th and 95th percentiles and outliers are denoted by dots.  $P < 0.0001$ .

Figure 3A depicts receiver operating curve (ROC) analysis of an expression ratio of miR-221 to miR-375 for differentiation of HNSCC tumors and normal tissues. The curve was constructed using ratio value cut-offs ranging from 0.75 to 1.25 for the ratio. Area under the curve (AUC) value is indicated.

Figure 3B depicts receiver operating curve (ROC) analysis of an expression ratio of miR-21 to miR-375 for differentiation of HNSCC tumors and normal tissues. The curve was constructed using ratio value cut-offs ranging from 0.75 to 1.25 for the ratio. Area under the curve (AUC) value is indicated.

Figure 3C depicts receiver operating curve (ROC) analysis of an expression ratio of miR-18a to miR-375 for differentiation of HNSCC tumors and normal tissues. The curve was constructed using ratio value cut-offs ranging from 0.75 to 1.25 for the ratio. Area under the curve (AUC) value is indicated.

Figure 4A depicts a class prediction analysis using Prediction Analysis of Microarray (PAM) showing misclassification error graphed as a function of the threshold parameter. Threshold was set to include all miRNA predicted by both SAM and ANOVA. Cross-validation was used to calculate misclassification error at each threshold.

Figure 4B depicts a class prediction analysis using Prediction Analysis of Microarray (PAM) showing cross-validated probabilities for each sample.

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Threshold was set to include all miRNA predicted by both SAM and ANOVA. Cross-validation was used to calculate misclassification error at each threshold.

Figure 4C depicts a class prediction analysis using Prediction Analysis of Microarray (PAM) showing tumor and normal scores for each miRNA and  
5 confusion matrix showing error associated with class predictions. Threshold was set to include all miRNA predicted by both SAM and ANOVA. Cross-validation was used to calculate misclassification error at each threshold.

Figure 5 depicts Kaplan-Meier curves showing differences in survival  
between patients with miR-21 expression in the highest 25th %tile (dotted line)  
10 compared to all other patients (solid line). Vertical hatch marks represent censored data.

Figure 6A depicts precursor *miR-375* transfection in FaDu cells. Increase in fold change expression of *miR-375* compared to negative control-transfected cells. Expression was normalized to RNU48 expression and was determined as fold-  
15 change above negative-control transfected cells by the  $2^{-(\Delta\Delta C_T)}$  calculation.

Figure 6B depicts precursor *miR-375* transfection in FaDu cells. Increasing colony formation with increasing concentration of *miR-375* precursor.

Figure 6C depicts precursor *miR-375* transfection in FaDu cells. Change in wound healing ability in cells transfected with 5 nM *miR-375* precursor compared to  
20 control-transfected cells.

Figure 7A depicts precursor *miR-375* transfection in FaDu cells. Increased proliferation rate in *miR-375*-transfected cells compared to control as measured by proliferation assay.

Figure 7B depicts precursor *miR-375* transfection in FaDu cells. Increased  
25 resistance to 0.5  $\mu$ M CDDP in *miR-375*-transfected cells compared to negative control as assessed by colony formation assay.

Figure 8 depicts quantitative real-time PCR showing *miR-375* expression following both methods of overexpression. Expression was normalized to RNU48 expression and was determined as fold-change above negative-control transfected  
30 cells by the  $2^{-(\Delta\Delta C_T)}$  calculation.

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Figure 9A depicts *miR-375* expression vector transfection in FaDu cells. Decreased proliferation rate in both *miR-375*-transfected clones compared to control-transfected cells as measured by proliferation assay.

- Figure 9B depicts *miR-375* expression vector transfection in FaDu cells.
- 5 Increased sensitivity to 0.5  $\mu$ M CDDP in *miR-375*-transfected clones compared to negative control as assessed by colony formation assay.

#### DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention, either as steps of the invention or as combinations of parts of the invention, will now be more particularly  
10 described and pointed out in the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention.

In an embodiment, the invention is a method of diagnosing a carcinoma in a  
15 subject, comprising the step of determining at least one expression ratio selected from the group consisting of a miR-21/miR-375 expression ratio, a miR-181d/miR-375 expression ratio, a miR-181b/miR-375 expression ratio, a miR-491/miR-375 expression ratio, a miR-455/miR-375 expression ratio, a miR-18a/miR-375 expression ratio, a miR-130b/miR-375 expression ratio, a miR-221/miR-375  
20 expression ratio, a miR-193b/miR-375 expression ratio, a miR-181a/miR-375 expression ratio, and a miR-18b/miR-375 expression ratio in a sample, wherein a ratio greater than about 1.0 is diagnostic of the carcinoma.

Nucleic acid sequences encoding miRNA gene products and the nucleic acid sequences of premature (also referred to as "mature stem loop") miRNA gene  
25 products and mature miRNA gene products for use in the methods of the invention are known to one of skill in the art. For example, hsa-miR-21 (SEQ ID NO: 27) (HGNC:MIR21) is encoded by SEQ ID NO: 1; hsa-miR-18a (SEQ ID NO: 28) HGNC:MIR18A is encoded by SEQ ID NO: 2; hsa-miR-375 (SEQ ID NO: 29) HGNC:MIR375 is encoded by SEQ ID NO: 3; and hsa-miR-218 (SEQ ID NO: 30)  
30 HGNC:218-1 is encoded by SEQ ID NO: 4.

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Exemplary miR gene products (SEQ ID NOs: 27-39), nucleic acids encoding the miR gene products (e.g., SEQ ID NOs: 1-13) and mature stem loop miR gene products (also referred to herein as “premature miR gene product”) (SEQ ID NOs: 14-26) can include the following:

5

hsa-miR-21 (HGNC:MIR21) ref|NT\_010783.15|:23192778-23192849 Homo sapiens chromosome 17 genomic contig, GRCh37 reference primary assembly  
TTGTCGGGTAGCTTATCAGACTGATGTTGACTGTTGAATCTCATGGCAAC  
ACCAGTCGATGGGCTGTCTGAC (SEQ ID NO: 1);

10

hsa-miR-18a (HGNC:MIR18A) >ref|NT\_009952.14|:5092680-5092750 Homo sapiens chromosome 13 genomic contig, GRCh37 reference primary assembly  
TTGTTCTAAGGTGCATCTAGTGCAGATAGTGAAGTAGATTAGCATCTACT  
GCCCTAAGTGCTCCTTCTGGC (SEQ ID NO: 2);

15

hsa-miR-375 (HGNC:MIR375) >ref|NT\_005403.17|:c70075847-70075784 Homo sapiens chromosome 2 genomic contig, GRCh37 reference primary assembly  
CCCGCGACGAGCCCCTCGCACAAACCGGACCTGAGCGTTTTGTTC  
GTTTCGGCTCGCGTGAGGCA (SEQ ID NO: 3);

20

hsa-mir-181d (HGNC:MIR181D) ref|NT\_011295.11|:5248490-5248626 Homo sapiens chromosome 19 genomic contig, GRCh37 reference primary assembly  
AGTGATAATG TAGCGAGATT TTCTGTTGTG CTTGATCTAA  
CCATGTGGTT GCGAGGTATG  
AGTAAAACATGGTTCCGTCAAGCACCATGG AACGTCACGC  
AGCTTTCTAC (SEQ ID NO: 4);

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hsa-mir-181d (HGNC:MIR181D) ref|NT\_011295.11|:5248490-5248626 Homo sapiens chromosome 19 genomic contig, GRCh37 reference primary assembly  
CGTCCCCTCCCCTAGGCCACAGCCGAGGTCACAATCAACATTCATTGTTG  
TCGGTGGGTTGTGAGGACTGAGGCCAGACCCACCGGGGGATGAATGTCA  
5 CTGTGGCTGGGCCAGACACGGCTTAAGGGGAATGGGGA (SEQ ID NO: 5);

hsa-mir-181b (HGNC:MIR181B1) ref|NT\_004487.19|:c50316752-50316643 Homo sapiens chromosome 1 genomic contig, GRCh37 reference primary assembly  
CTGTGCAGAGATTATTTTTTAAAAGGTCACAATCAACATTCATTGCTGTC  
GGTGGGTTGAACTGTGTGGACAAGCTCACTGAACAATGAATGCAACTGT  
10 GGCCCCGCTTT (SEQ ID NO: 6);

hsa-mir-491 (HGNC:MIR491) GRCh37 reference primary assembly  
ATTGACTTAGCTGGGTAGTGGGGAACCCTTCCATGAGGAGTAGAACACT  
CCTTATGCAAGATTCCCTTCTACCTGGCTGGGTTG (SEQ ID NO: 7);

hsa-mir-455 (HGNC:MIR455) ref|NT\_008470.19|:46136245-46136340 Homo sapiens chromosome 9 genomic contig, GRCh37 reference primary assembly  
15 TTCCCTGGCGTGAGGGTATGTGCCTTTGGACTACATCGTGGAAGCCAGCA  
CCATGCAGTCCATGGGCATATACACTTGCCTCAAGGCCTATGTCAT (SEQ  
ID NO: 8);

hsa-mir-130b (HGNC:MIR130B) ref|NT\_011520.12|:1398161-1398242 Homo sapiens chromosome 22 genomic contig, GRCh37 reference primary assembly  
20 AGGCCTGCCCCGACACTCTTCCCTGTTGCACTACTATAGGCCGCTGGGAA  
GCAGTGCAATGATGAAAGGGCATCGGTCAGGT (SEQ ID NO: 9);

hsa-mir-221 (HGNC:MIR221) ref|NT\_079573.4|:c8457437-8457328 Homo sapiens chromosome X genomic contig, GRCh37 reference primary assembly  
25 GAACATCCAGGTCTGGGGCATGAACCTGGCATAACAATGTAGATTTCTGT  
GTTTCGTTAGGCAACAGCTACATTGTCTGCTGGGTTTCAGGCTACCTGGAA  
ACATGTTCTCC (SEQ ID NO: 10);

hsa-mir-193b (HGNC:MIR193B) ref|NT\_010393.16|:14337823-14337905 Homo sapiens chromosome 16 genomic contig, GRCh37 reference primary assembly

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TGTGGTCTCAGAATCGGGGTTTTGAGGGCGAGATGAGTTTATGTTTTATC  
CAACTGGCCCTCAAAGTCCCGCTTTTGGGGTCA (SEQ ID NO: 11);

hsa-mir-181a (HGNC:MIR181A1) ref|NT\_004487.19|:c50316923-50316814

Homo sapiens chromosome 1 genomic contig, GRCh37 reference primary assembly

5 GAGTTTTGAGGTTGCTTCAGTGAACATTCAACGCTGTCGGTGAGTTTGA  
ATTAATAATCAAAACCATCGACCGTTGATTGTACCCTATGGCTAACCATCA  
TCTACTCCAT (SEQ ID NO: 12);

hsa-mir-18b (HGNC:MIR18B) ref|NT\_011786.16|:c17571850-17571780 Homo  
sapiens chromosome X genomic contig, GRCh37 reference primary assembly

10 GTGTTAAGGTGCATCTAGTGCAGTTAGTGAAGCAGCTTAGAATCTACTGC  
CCTAAATGCCCCCTTCTGGCAC (SEQ ID NO: 13);

hsa-miR-21 (HGNC:MIR21) Mature Stem Loop

UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCA  
ACACCAGUCGAUGGGCUGUCUGACA (SEQ ID NO: 14);

15 hsa-miR-18a (HGNC:MIR18A) Mature Stem Loop

UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGAUUAGCAUCUA  
CUGCCCUAAGUGCUCCUUCUGGCA (SEQ ID NO: 15);

hsa-miR-375 (HGNC:MIR375) Mature Stem Loop

20 CCCC GCGACGAGCCCCUCGCACAAACCGGACCUGAGCGUUUUGUUCGU  
UCGGCUCGCGUGAGGC (SEQ ID NO: 16);

hsa-mir-181d (HGNC:MIR181D) Mature Stem Loop

GUGAUAAUGU AGCGAGAUUU UCUGUUGUGC UUGAUCUAAC  
CAUGUGGUUG CGAGGUAUGA GUAAAACAUG GUUCCGUCAA  
GCACCAUGGA ACGUCACGCA GCUUUCUACA (SEQ ID NO: 17);

25 hsa-mir-181d (HGNC:MIR181D) Mature Stem Loop

GUCCCCUCCCCUAGGCCACAGCCGAGGUCACAAUCAACAUUCAUUGUU  
GUCGGUGGGUUGUGAGGACUGAGGCCAGACCCACCGGGGGAUGAAUG

- 10 -

UCACUGUGGCUGGGCCAGACACGGCUUAAGGGGAUGGGGAC (SEQ ID NO: 18);

hsa-mir-181b (HGNC:MIR181B1) Mature Stem Loop

CCUGUGCAGAGAUUAUUUUUAAAAGGUCACAAUCAACAUUCAUUGC  
5 UGUCGGUGGGUUGAACUGUGUGGACAAGCUCACUGAACAAUGAAUGC  
AACUGUGGCCCCGCUU (SEQ ID NO: 19);

hsa-mir-491 (HGNC:MIR491) Mature Stem Loop

UUGACUUAGCUGGGUAGUGGGGAACCCUCCAUGAGGAGUAGAACAC  
UCCUUAUGCAAGAUUCCCUUCUACCUGGCUGGGUUGG (SEQ ID NO: 20);

10 hsa-mir-455 (HGNC:MIR455) Mature Stem Loop

UCCCUGGCGUGAGGGUAUGUGCCUUUGGACUACAUCGUGGAAGCCAGC  
ACCAUGCAGUCCAUGGGCAUAUACACUUGCCUCAAGGCCUAUGUCAUC  
(SEQ ID NO: 21);

hsa-mir-130b (HGNC:MIR130B) Mature Stem Loop

15 GGCCUGCCCGACACUCUUUCCCUGUUGCACUACUAUAGGCCGCUGGGA  
AGCAGUGCAAUGAUGAAAGGGCAUCGGUCAGGUC (SEQ ID NO: 22);

hsa-mir-221 (HGNC:MIR221) Mature Stem Loop

UGAACAUCCAGGUCUGGGGCAUGAACCUGGCAUACAAUGUAGAUUUC  
UGUGUUCGUUAGGCAACAGCUACAUUGUCUGCUGGGUUUCAGGCUAC  
20 CUGGAAACAUGUUCUC (SEQ ID NO: 23);

hsa-mir-193b (HGNC:MIR193B) Mature Stem Loop

GUGGUCUCAGAAUCGGGGUUUUGAGGGCGAGAUGAGUUUAUGUUUUA  
UCCAACUGGCCCUCAAAGUCCCGCUUUUGGGGUCAU (SEQ ID NO: 24);

hsa-mir-181a (HGNC:MIR181A1) Mature Stem Loop

25 UGAGUUUUGAGGUUGCUUCAGUGAACAUUCAACGCUGUCGGUGAGUU  
UGGAAUUAAAAUCAAAACCAUCGACCGUUGAUUGUACCCUAUGGCUA  
ACCAUCAUCUACUCCA (SEQ ID NO: 25);

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hsa-mir-18b (HGNC:MIR18B) Mature Stem Loop

UGUGUUAAGGUGCAUCUAGUGCAGUUAGUGAAGCAGCUUAGAAUCUA  
CUGCCCUAAAUGCCCCUUCUGGCA (SEQ ID NO: 26);

hsa-miR-21 (HGNC:MIR21) UAGCUUAUCAGACUGAUGUUGA

5 (SEQ ID NO: 27);

hsa-miR-18a (HGNC:MIR18A) UAAGGUGCAUCUAGUGCAGAUAG

(SEQ ID NO: 28);

hsa-miR-375 (HGNC:MIR375) UUUGUUCGUUCGGCUCGCGUGA

(SEQ ID NO: 29);

10 hsa-mir-181d (HGNC:MIR181D) UUGUGCUUGAUCUAACCAUGU

(SEQ ID NO: 30);

hsa-mir-181d (HGNC:MIR181D) AACAUUCAUUGUUGUCGGUGGGU

(SEQ ID NO: 31);

hsa-mir-181b (HGNC:MIR181B1) AACAUUCAUUGCUGUCGGUGGGU

15 (SEQ ID NO: 32);

hsa-mir-491 (HGNC:MIR491) AGUGGGGAACCCUCCAUGAGG

(SEQ ID NO: 33);

hsa-mir-455 (HGNC:MIR455) UAUGUGCCUUGGACUACAUCG

(SEQ ID NO: 34);

20 hsa-mir-130b (HGNC:MIR130B) CAGUGCAAUGAUGAAAGGGCAU

(SEQ ID NO: 35);

hsa-mir-221 (HGNC:MIR221) AGCUACAUUGUCUGCUGGGUUUC

(SEQ ID NO: 36);

hsa-mir-193b (HGNC:MIR193B) AACUGGCCCUCAAAGUCCCGCU

25 (SEQ ID NO: 37);

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hsa-mir-181a (HGNC:MIR181A1) AACAUUCAACGCUGUCGGUGAGU  
(SEQ ID NO: 38);.

hsa-mir-18b (HGNC:MIR18B) UAAGGUGCAUCUAGUGCAGUUAG  
(SEQ ID NO: 39).

5           “Expression ratio,” as used herein, refers to the value of one microRNA (miR) relative to another miR. For example, a miR-21/miR-375 expression ratio is a value obtained when an expression level of miR-21 is divided by an expression level of miR-375.

Exemplary techniques for determining microRNA expression are well  
10 known in the art and include, for example, microarray-based methods, reverse-transcriptase polymerase chain reaction (RT-PCR) (e.g., quantitative RT-PCR), Northern-blot analysis and in situ hybridization.

The subject diagnosed by the methods described herein can be a human subject or a non-human subject (e.g., monkey, rat, mouse).

15           In an embodiment, the carcinoma diagnosed by the methods of the invention includes a carcinoma of non-glandular origin, such as at least one member selected from the group consisting of a squamous cell carcinoma, a basal cell carcinoma, a transitional cell carcinoma, and an undifferentiated carcinoma. Exemplary squamous cell carcinomas include at least one member selected from the group  
20 consisting of a head squamous cell carcinoma, a neck squamous cell carcinoma, a skin squamous cell carcinoma, a prostate squamous cell carcinoma, a lung squamous cell carcinoma, a vaginal squamous cell carcinoma and a cervical squamous cell carcinoma.

Exemplary head squamous cell carcinomas that can be diagnosed by the  
25 methods described herein can include at least one member selected from the group consisting of an oral cavity squamous cell carcinoma (e.g., tongue squamous cell carcinoma, squamous cell carcinoma of floor of mouth, squamous cell carcinoma of the wall of mouth, gingivae squamous cell carcinoma, hard palate squamous cell carcinoma, soft palate squamous cell carcinoma), a nasal cavity squamous cell  
30 carcinoma, a carcinoma of the paranasal sinuses and a nasopharyngeal squamous cell carcinoma. The oral cavity squamous cell carcinoma can include at least one

member selected from the group consisting of a tongue squamous cell carcinoma, a squamous cell carcinoma of floor of mouth, a squamous cell carcinoma of the wall of mouth, a gingivae squamous cell carcinoma, a hard palate squamous cell carcinoma and a soft palate squamous cell carcinoma.

5           The squamous cell carcinoma can be a neck squamous cell carcinoma. Exemplary neck squamous cell carcinomas include a pharyngeal squamous cell carcinoma (e.g., an oropharyngeal squamous cell carcinoma, a hypopharyngeal squamous cell carcinoma), a laryngeal squamous cell carcinoma and a tracheal squamous cell carcinoma. The pharyngeal squamous cell carcinoma can include at  
10   least one member selected from the group consisting of an oropharyngeal squamous cell carcinoma and a hypopharyngeal squamous cell carcinoma.

          The carcinoma diagnosed by the methods of the invention can also be at least one member selected from the group consisting of an adenocarcinoma and a carcinoma that includes cells of glandular and non-glandular origin, such as an  
15   adenosquamous carcinoma).

          Exemplary adenocarcinomas that can be diagnosed by the methods of the invention can include at least one member selected from the group consisting of an adenocarcinoma of the colon, an adenocarcinoma of the lung, an adenocarcinoma of the ovary, an adenocarcinoma of the breast, an adenocarcinoma of the pancreas, an  
20   adenocarcinoma of the prostate, an adenocarcinoma of the stomach, an adenocarcinoma of the urachus, an adenocarcinoma of the vagina, an adenocarcinoma not otherwise specified (NOS), a cholangiocarcinoma, an adenoid cystic carcinoma, a hepatocellular carcinoma, a renal cell carcinoma, an adrenocorticol carcinoma and an esophageal adenocarcinoma.

25           The sample employed in the methods described herein can include at least one member selected from the group consisting of a cell sample, a tissue sample and a fluid sample. The sample can be from a subject (e.g., a biopsy, a swab, a saliva sample, a sputum sample, a mouth rinse, a blood serum sample, a blood plasma sample). Alternatively, the sample can be a cell line or cultured cell sample, such as  
30   a cell line and a cultured cell sample prepared from a sample from a subject.

          Exemplary tissue samples for use in the methods of the invention include at least one member selected from the group consisting of an oral cavity sample, a

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laryngeal tissue sample, an esophageal tissue sample, a uvula tissue sample, a skin tissue sample, a lip tissue sample, a rectal tissue sample, a renal tissue sample, a bladder tissue sample, a prostate tissue sample, a lung tissue sample and a cervical tissue sample.

5           The tissue sample employed in the methods of the invention can include at least a portion of a tumor. "At least a portion," as used herein in reference to a sample, means any part or the entirety of a sample. The tumor can be a malignant tumor, a pre-malignant tumor or a benign tumor. The tumor can be a primary tumor or a metastatic tumor. The tumor can be of any stage, for example a Stage 0 tumor,  
10   a Stage I tumor, a Stage II tumor, a Stage III tumor, or a Stage IV tumor, according to an appropriate staging system (e.g., the TNM Classification of Malignant Tumors Staging System). The type and class of tumor, and tumor stage, can be readily determined by one of ordinary skill in the art.

          The sample employed in the methods of the invention can be obtained from  
15   the subject.

          In an embodiment, the tumor is an early-stage tumor. An "early-stage tumor," as used herein, refers to a tumor that includes at least one member selected from the group consisting of a Stage 0 tumor, a Stage I tumor and a Stage II tumor. An early stage tumor can be classified based on, for example, the TNM  
20   Classification of Malignant Tumors Staging System.

          Human papilloma virus (HPV) infection is a major risk factor for certain types of carcinoma, such as head and neck squamous cell carcinoma). In an embodiment, the tissue sample includes at least a portion of an HPV-positive tumor. In another embodiment, the tissue sample includes at least a portion of an HPV-  
25   negative tumor.

          The tissue sample employed in the methods of the invention can include at least a portion of a preneoplastic lesion. Preneoplastic lesions diagnosed by the methods of the invention can include at least one member selected from the group consisting of an actinic keratosis, an atypical adenomatous hyperplasia, a cutaneous  
30   horn, a squamous cell carcinoma in situ, a keratoacanthoma, an oral leukoplakia and a pharyngeal leukoplakia.

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In another embodiment, the invention is a method of diagnosing a carcinoma in a subject, comprising the step of comparing an expression level of at least two microRNAs selected from the group consisting of miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma.

An expression level of at least two microRNAs that are over-expressed in a carcinoma (e.g., miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, and miR-18b) can be compared to a microRNA that is underexpressed in the carcinoma (e.g., miR-375) to thereby diagnose the carcinoma. The comparison can be a relative comparison, such as comparing the levels of miRNA that are over and underexpressed, or the comparison can be an expression ratio.

Expression levels of microRNAs can be readily determined by quantitative methods as described herein, such as nucleic acid amplification assays. The methods described herein can identify over-expression (increases) or under-expression (decreases) of microRNAs (miRNAs) compared to a control or a reference miRNA. Over-expression or under-expression can be correlated with subject characteristics (e.g., age, risk factors, such as alcohol consumption and smoking) and carcinoma characteristics (e.g., grade, stage, aggressive, less aggressive, invasive).

Over and under expression of genes described herein can be assessed by determining the Hazard Ratio (HR) by the methods described herein. The Hazard Ratio is derived from a survival analysis and describes the effect of an explanatory variable on the risk of an event. It is similar in concept to an Odds Ratio, except it is based on time-dependent data. A Hazard Ratio is calculated from a Cox Proportional Hazards Model that allows adjustment for potential confounders in the analysis of an association between some variable (e.g., miRNA expression) and risk of death. A Hazard Ratio greater than one (1) suggests an increased risk of death or morbidity, while a Hazard Ratio less than one (1) suggests a decreased risk of death

(e.g., a protective factor). For example, an HR of 1.68 for high miR-21 expression is interpreted to mean that if a subject has head and neck squamous cell carcinoma and high miR-21 expression, the subject is 68% more likely to die at any given time, compared to a person with head and neck squamous cell carcinoma and low miR-21 expression, controlled for age, gender and tumor stage. When those factors are controlled, and the miR expression is still significant, the miR expression is considered to be an independent predictor (e.g., independent of other known risk factors) of patient survival.

In yet another embodiment, the invention is a method of diagnosing a carcinoma in a subject, comprising the step of comparing an expression level of at least two microRNAs selected from the group consisting of miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and wherein the sample is not an established cell line.

“An established cell line,” as used herein, refers to a cell line that is commercially available. The established cell line is a cell line that has the ability to proliferate indefinitely consequent to a mutation. For example, established cell lines can include a FaDu established cell line (hypopharyngeal carcinoma), HN6 established cell line (base of tongue primary carcinoma), HN13 established cell line (tongue primary carcinoma), UM-SCC9 established cell line (tongue primary carcinoma), UM-SCC47 established cell line (tongue primary carcinoma), UM-SCC10A established cell line (larynx primary carcinoma), UM-SCC11A established cell line (larynx primary carcinoma), UM-SCC38 established cell line (tonsil primary carcinoma), UM-SCC4 established cell line (tonsil primary carcinoma), JHU-011 established cell line (laryngeal carcinoma), JHU-012 established cell line (neck node metastasis), JHU-019 established cell line (base of tongue carcinoma) and OKF6 established cell line (oral keratinocyte carcinoma). Cell lines that are obtained or immortalized from samples obtained from the subjects that are diagnosed by the methods of the invention are not established cell lines.

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“Corresponding control expression level,” as used herein, refers to an expression level of a microRNA observed in a non-carcinoma sample of the same microRNA whose expression level is being evaluated in the sample from the subject. For example, if the expression level of miR-181d is being evaluated in the subject

5 the corresponding miR-181d would be a control or normal sample of miR181d. The corresponding control expression level can be an expression level of a microRNA in a non-carcinoma sample (e.g., a non-carcinoma sample from the same subject, a non-carcinoma sample from a different subject). Alternatively, the corresponding control expression level can be a reference standard for a typical expression level of

10 a microRNA in a non-carcinoma sample.

A difference in the expression level of the miRNA in the sample compared to the corresponding control sample is any difference in the level of a microRNA in a sample from a subject relative to a corresponding control expression level. For example, if the level of microRNA in the sample from the subject is different

15 (greater or less) than the corresponding control level, the subject has a diagnosis of a high probability of having a carcinoma (i.e., a positive prediction that the subject has a carcinoma). If the level of one or more selected microRNAs in the sample from the subject is identical to, or essentially the same as, the corresponding control level, it is unlikely that the subject has a carcinoma (i.e., a negative prediction that the

20 subject has a carcinoma).

The expression level of the microRNA in the sample from the subject can be diagnostic of a carcinoma when it is either greater or less than a corresponding control expression level. For example, in an embodiment, the expression level of a particular microRNA in the sample from the subject can be diagnostic of a

25 carcinoma when it is at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 3.5-fold, at least about 4-fold, at least about 4.5-fold, or at least about 5-fold greater than a corresponding control expression level.

The expression level of miR-18a can be determined in a sample. When the expression level of miR-18a in the sample is greater (e.g., at least about 2.5-fold

30 greater) than a corresponding control, miR-18a expression level is predictive of a carcinoma in the subject.

The expression level of miR-21 can be determined in a sample. When the expression level of miR-21 is greater (e.g., at least about 3.5-fold greater) than a corresponding control, miR-21 expression level is predictive of a carcinoma in the subject.

5           The expression level of miR-221 can be determined in a sample. When the expression level of miR-221 is greater (e.g., at least about 2-fold greater) than a corresponding control, miR-21 expression level is predictive of a carcinoma in the subject.

10           In another embodiment, the expression level of miR-375 can be determined in a sample. When the expression level of miR-375 is less (e.g., at least about 20-fold less) than a corresponding control, miR-375 expression level is predictive of a carcinoma in the subject.

15           In a further embodiment, the invention is a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma in a subject, comprising the step of comparing an expression level of at least one microRNA selected from the group consisting of miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level  
20           of the microRNA in the sample relative to the control expression level is diagnostic of the head squamous cell carcinoma or the neck squamous cell carcinoma and identifies a subject that would potentially benefit from a therapy to treat the head squamous cell carcinoma and the neck squamous cell carcinoma.

25           An expression level of at least one microRNAs that is over-expressed in a carcinoma (e.g., miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, and miR-18b) can be compared to a microRNA that is underexpressed in the carcinoma (e.g., miR-375) to thereby diagnose the carcinoma. The comparison can be a relative comparison, such as comparing the levels of miRNA that are over and underexpressed, or the comparison can be an  
30           expression ratio.

          The method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma in a subject by

comparing an expression level of at least one microRNA selected from the group consisting of miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNA in the sample relative to the control expression level is diagnostic of the head squamous cell carcinoma or the neck squamous cell carcinoma can further include the step of comparing the expression level of miR-21 in the sample from the subject to a corresponding miR-21 control expression level.

In still another embodiment, the invention is a method of treating a squamous cell carcinoma in a subject, comprising the step of administering a nucleic acid encoding a miR-375 gene product to the subject. The squamous cell carcinoma treated by the methods of the invention can be at least one member selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma. The nucleic acid encoding the miR-375 gene product can include an expression vector. The expression vector can include a promoter, such as at least one member selected from the group consisting of an RNA polymerase II promoter (e.g., a cytomegalovirus (CMV) promoter, an elongation factor 1 (EF-1) promoter, an hPGK promoter) and an RNA polymerase III promoter (e.g., a U6 promoter, an H1 promoter). The RNA polymerase II promoter can include a constitutive promoter. The constitutive promoter can include a human cytomegalovirus immediate early promoter. The nucleic acid encoding the miR-375 gene product can include a nucleic acid that encodes a premature miR-375 gene product. The expression vector can include at least one member selected from the group consisting of a bacterial vector and a lentiviral vector. Suitable expression vectors for use in the methods of the invention include, for example, the BLOCK-iT<sup>TM</sup> Pol II miR RNAi vector (Invitrogen) and BLOCK-iT<sup>TM</sup> shRNA vectors for RNAi (Invitrogen), the miRNASelect<sup>TM</sup> pEP mir Cloning and Expression Vector (Cell Biolabs, Inc.), pLKO.1 vectors (Sigma-Aldrich), BLOCK-iT<sup>TM</sup> Lentiviral Pol II or III miR RNAi Expression System (Invitrogen) and miR-express Human Lentiviral microRNA Vectors (Thermo Fisher).

Exemplary vectors and promoters can include the following:

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BLOCK-iT<sup>TM</sup> Pol II miR RNAi vector can be used with at least one member selected from the group consisting of an RNA Polymerase II expression system (RNA Pol II promoters are promoters used to transcribe most protein coding genes of a mammalian cell), an EF-1a and an alpha-1-anti-trypsin promoters (normal  
5 mammalian gene promoters). In particular, CMV (cytomegalovirus) immediate early Pol II promoter can be employed to drive transcription.

BLOCK-iT<sup>TM</sup> shRNA vectors for RNAi can employ an RNA Polymerase Type III promoter for expression (Pol III promoters are promoter which drive transcription of many constitutive non-coding RNAs in the cell, like ribosomal  
10 RNAs), such as the U6 promoter or the H1 promoter.

miRNASelect<sup>TM</sup> pEP mir Cloning and Expression Vector can employ a Pol II promoter, such as an EF-1 promoter.

pLKO.1 vectors can employ Pol III (U6) and Pol II (CMV, hPGK) promoters.

15 BLOCK-iT<sup>TM</sup> Lentiviral Pol II or III miR RNAi Expression System can employ a Pol II promoter (e.g., CMV, EF-1) or Pol III (e.g., U6) and inserts the promoter and miR construct into lentiviral production vector to generate replication-incompetent Lentivirus that can transduce dividing and non-dividing mammalian cells.

20 miR-express Human Lentiviral microRNA Vectors can employ an RNA Pol II CMV promoter.

The use of siRNA as therapeutics agents is growing in interest, and similarly growing are methods for delivery of therapeutic siRNAs to appropriate target tissues and organs. MicroRNAs, which share functional similarity to siRNA, but are  
25 produced from an initial hairpin structure, may also be suitable as therapeutic agents. The miR-375 gene product can be employed as a therapeutic agent in human cancer cell lines, to determine the utility of overexpression of this miRNA on growth of the cells, and their response to chemotherapeutic agents.

Two different systems were used for overexpression of miR-375 in the  
30 invasive HNSCC cell line FaDu. The first system involves transient transfection with a synthetic double-stranded RNA molecule designed to mimic the mature miRNA (Pre-miR<sup>TM</sup> miRNA Precursor Molecules from Ambion). Functioning like

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small interfering RNAs (siRNAs), these miRNA precursors are chemically modified to ensure that the appropriate strand becomes incorporated into the RISC. A Pre-miR<sup>TM</sup> of miR-375 was commercially available and purchased. This system has been used in-vitro and in animal models as a method for delivery of an siRNA. In  
5 the case of animal delivery, the synthetic oligos would be administered systemically and taken up by the cells, wherein they can act on their target mRNA. Studies such as Song et al (Nature Medicine 9, 347 - 351 (2003)) demonstrated that injection of synthetic siRNA duplexes in the tail vein of mice led to their uptake by the liver, although the uptake by other tissues was not reported.

10 The second method utilizes an expression vector-based system to allow expression of an engineered miRNA sequence from a Pol II promoter (Block-iT<sup>TM</sup> Pol II miR RNAi Expression Vector from Invitrogen). This expression vector contains the human cytomegalovirus (CMV) immediate early promoter, allowing for constitutive miRNA expression in mammalian cells. Double stranded oligos which  
15 were inserted into a vector to produce the mature miRNA within the cells. This type of vector could also be systemically administered, but numerous studies have shown poor uptake of this type of vector. This vector, though, utilizes Invitrogen's Gateway<sup>®</sup>-adapted expression vector, which is designed to allow for recombination into other tissue-specific, regulated, or lentiviral vector systems. Thus, this vector  
20 can be recombined to allow for specific viral delivery of the expression plasmid to target cells, which could be a more specific and efficacious method of therapeutic delivery.

In a prefer embodiment, the invention is a method of optimizing treatment of a subject having a squamous cell carcinoma (e.g., at least one member selected from  
25 the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma), comprising the step of determining an expression level of a miR-21 gene product in a sample from the subject, wherein the overexpression level of the miR-21 gene product in the sample compared to expression in a reference miR21-gene product identifies a subject that has an aggressive squamous cell carcinoma  
30 that would potentially benefit from a therapy to treat the aggressive squamous cell carcinoma.

“Reference,” as used herein with respect to expression of a miR gene product, such as miR-21, refers to expression of an miR gene product in a sample obtained from a subject that has a carcinoma with a relatively favorable prognosis.

“Aggressive,” as used herein with respect to a squamous cell carcinoma, refers to a carcinoma that is associated with an increased morbidity. As described herein, miRNA expression can be predictive of an aggressive carcinoma, which may or may not correlate with the stage (I, II, III or IV) of the carcinoma. For example, miRNA expression in a sample of a carcinoma (e.g., squamous cell carcinoma such as a head and neck squamous cell carcinoma) that is greater than about 2.0 fold to about 4.0 fold (e.g., about 2.5 fold, about 2.6 fold, about 3.0 fold, about 3.5 fold) of that observed in a control (e.g., normal or noncancerous sample) reference (e.g., sample from a carcinoma that is known not be aggressive) sample can indicate a more aggressive carcinoma. In general, early stage disease (non-metastatic disease) is treated with wide surgical excision or curative radiation therapy alone or in combination. In combination, radiation therapy is often used first in order to reduce the size of the tumor in hopes of improving the cosmetic and functional results from surgery. Chemotherapy is generally used in organ preservation protocols for laryngeal and hypopharyngeal tumors, and often plays a role in palliative care of recurrent disease. Identification of primary tumors that will respond to curative radiation therapy (likely considered the lesser aggressive of the therapy regimens) would be ideal, as this could avoid often disfiguring and function-limiting surgeries to the head and neck. Chemotherapeutic regimens are also under investigation for this disease, including front-line therapies, as targeted therapeutics, and so identifying patients who may benefit from such strategies would be an advantage. Exemplary therapies to treat aggressive squamous cell carcinomas could include, for example, a combination of surgery, post-surgical radiation and chemotherapy. Therapies to treat non-aggressive carcinomas generally employ a single (e.g., radiation therapy alone, surgery alone) therapy and generally do not include chemotherapy.

“Therapeutically effective,” as used herein refers to an amount of an miRNA gene product, nucleic acid encoding an miRNA gene product, chemotherapeutic

agent or other suitable therapy, such as radiation therapy that can produce a measurable positive effect in a subject, such as a regression in a carcinoma.

## EXEMPLIFICATION

### 5 EXAMPLE 1

Head and neck squamous cell carcinoma includes carcinomas arising from the epithelium of the oral cavity, pharynx, and larynx, and is the sixth most common malignancy worldwide (1). The major risk factors for the disease are tobacco and alcohol use, and human papillomavirus (HPV) infection (2-4). Despite advances in  
10 detection, as well as surgical and chemotherapeutic treatments over recent decades, the five year survival rate for head and neck squamous cell carcinoma has remained around 50%, one of the lowest of the major cancers (5). Frequent late stage diagnosis, formation of additional primary tumors and regional and distant metastases all contribute to this poor survival rate (2).

15 A better understanding of the molecular pathways that give rise to head and neck squamous cell carcinoma is essential in the identification of novel molecular biomarkers that have clinical utility in predicting prognosis and therapeutic efficacy, as well as in designing targeted therapy for this disease. In recent years, gene expression profiling technologies have become increasingly sophisticated, allowing  
20 investigators to explore their diagnostic and therapeutic potential as biomarkers in cancers (6-8). These biomarkers, however, have had limited success in the clinical setting and, to date, limited utility in further elucidating mechanisms of head and neck squamous cell carcinoma carcinogenesis.

The discovery of microRNAs (miRNAs), about 22 nucleotide long, non-  
25 coding RNA molecules, has revolutionized our understanding of the modulation of gene expression. Nearly 700 miRNAs have been identified in humans, a number that is rapidly growing and expected to reach 1,000 or higher (9). Highly ubiquitous and largely conserved across species, miRNAs regulate gene expression post-transcriptionally by base-pairing, usually imperfectly, to the 3'-untranslated region  
30 (10) of a cognate messenger RNA (11). The interaction of a miRNA with a target mRNA transcript results either in translational repression of the mRNA or in its direct degradation (11). Due to the partial complementarity between miRNAs and

their target transcripts, a single miRNA is capable of simultaneously regulating up to hundreds of genes, giving rise to an enormous modulatory potential (12).

Through their targets, miRNAs are known to play important roles in cell differentiation, proliferation, and apoptosis (13). Different cancer types have been associated with miRNA expression profiles that vary between the tumor tissues and the corresponding normal tissue (19-21). Moreover, some studies have identified miRNA expression profiles that can distinguish different tumor subtypes or developmental lineages, which may have clinical applications in diagnostics and tumor staging (16, 22).

As described herein, the expression of miRNAs in normal head and neck epithelia were compared with primary head and neck squamous cell carcinoma tumors and cultured head and neck squamous cell carcinoma cell lines. Differences in expression are described herein, which may be important in differentiating disease and as markers that diagnosis head and neck squamous cell carcinoma. Upon identifying miRNAs specifically altered in head and neck cancer, a subset of these miRNAs were validated in a larger population of tumors to identify a clinically-applicable diagnostic tool.

The data described herein shows that a microRNA expression ratio can distinguish between non-diseased tissue and tumor tissue with great accuracy in the context of head and neck squamous cell carcinoma, an important public health concern worldwide. The ratio of miR-221:miR-375 showed high discriminatory potential, with a sensitivity of about 92% and specificity of about 93% in distinguishing tumor from normal tissue, which may be a simple molecular marker for diagnosing head and neck squamous cell carcinoma.

#### Purpose:

miRNAs altered in head and neck squamous cell carcinoma were identified to determine whether miRNA expression is predictive of disease.

#### Experimental Design:

RNA was isolated from fresh frozen primary tumors, fresh frozen non-diseased head and neck epithelial tissues, and head and neck squamous cell carcinoma cell lines, and profiled for the expression of 662 miRNAs by microarray. The miRNAs that were both differentially expressed on the array and by qRT-PCR

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were subsequently validated by qRT-PCR using a total of 99 head and neck squamous cell carcinoma samples and 14 normal epithelia.

#### Results:

5 A marked difference in miRNA expression pattern was observed between tumors and cell lines. Eighteen miRNAs were significantly altered in their expression between normal tissues and tumors. Four of these miRNAs were validated in the larger sample series, and each showed significant differential expression ( $P < 0.0001$ ). Further, an expression ratio of miR-221:miR-375 demonstrated a high sensitivity (0.92) and specificity (0.93) for disease prediction.

#### 10 Conclusions:

These data suggest that cultured tumor cell lines are inappropriate for miRNA biomarker identification, and that the pattern of miRNA expression in primary head and neck tissues is reflective of disease status, with certain miRNAs exhibiting strong predictive potential. These results show that miR-221 and miR-  
15 375 may be important diagnostic biomarkers.

#### Materials and Methods

head and neck squamous cell carcinoma samples and cell lines.

Non-diseased head and neck epithelial tissue were obtained from the National Disease Research Interchange and consisted of fresh-frozen tongue, larynx  
20 and uvula samples. All fresh-frozen head and neck squamous cell carcinoma samples were obtained, with informed consent after Institutional Review Board approval at participating hospitals, as part of a population-based case-control study of head and neck squamous cell carcinoma spanning December 1999 to December 2003 in the Greater Boston Metropolitan area. The fresh-frozen tumors originated  
25 from uvula, larynx, floor of mouth, and tongue resections. Details of this study have been described previously (23). Study pathologists confirmed greater than about 75% tumor in all head and neck squamous cell carcinoma samples tested. FaDu and Cal27, head and neck squamous cell carcinoma cell lines, were obtained from American Type Culture Collection (ATCC) and maintained in Eagle's Minimum  
30 Essential Medium and Dulbecco's Modified Eagle's Medium, respectively, both supplemented with fetal bovine serum to a final concentration of 10%.

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RNA isolation and microarray profiling.

Total RNA was isolated from normal tissues, tumors, and cell lines using the mirVANA™ RNA Isolation Kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. RNA was quantified using the NanoDrop™ ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), aliquoted, and stored at -80°C briefly until needed. Total RNA (about 5 µg) was evaluated for miRNA profiling studies at Asuragen Services using the mirVANA™ miRNA Bioarrays platform v2 (Ambion, Inc., Catalog No. AM1566V2) as single channel format according to the standard operating procedures of the company, including pre-array qualitative Bioanalyzer (Agilent, Santa Clara, CA) RNA analysis, as previously described (24). The Bioarrays platform v2 contains probes specific to miRNA identified in human, mouse, and rat, as well as additional miRNAs identified through cloning at Ambion, Inc. The Cy5 fluorescence on the arrays was scanned at an excitation wavelength of 635 nm using a GenePix® 4200AL scanner (Molecular Devices, Union City, CA). The fluorescent signal associated with the probes and local background was extracted using GenePix® Pro (version 6.0, Molecular Devices). Raw signal data were normalized by first log2 transformation of signal intensity followed by global Variance Stabilization Normalization (25) of all the arrays within the project. Normalized data were submitted to the GEO archive (accession #GSE11163).

#### 20 Quantitative reverse transcription-PCR.

TaqMan® miRNA Assays (Applied Biosystems) were used to quantify mature miRNA. cDNA was synthesized by priming with a pool of gene-specific looped primers including the primers of the miRNAs of interest and RNU48, as a universally-expressed endogenous control (Applied Biosystems). 10 µl of total RNA diluted to a final concentration of about 5 ng/µl was used for each reverse transcription (RT) reaction along with other RT components, per manufacturer's specifications. Reactions (about 40µl) were incubated in an Applied Biosystems GeneAmp® PCR system 9700 for about 30 min at about 16°C, about 30 min at about 42°C, about 5 min at about 85°C, and held at about 4°C. qRT-PCR was performed as previously described (26) with the following exception: all reactions, excluding no-template controls and non-reverse-transcribed controls, were run in triplicate on an ABI 7500 Fast Real Time PCR Detection System. All real-time

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PCR data were analyzed using the comparative CT method, normalizing against expression of RNU48.

Statistical analysis.

Differential expression of miRNAs by microarray was determined with  
5 Significance Analysis of Microarray (SAM) software (Stanford University Labs) using 1000 permutations of the data and with delta adjusted to minimize false discovery rate. All hierarchical clustering analyses were carried out using Cluster 3.0 (Stanford University) with Euclidian distance as the distance metric and centroid linkage between clusters. A two-tailed Student's t test was used to compare miRNA  
10 expression levels determined by real-time PCR. miRNA expression ratios were calculated following the methods of Gordon et al. (27), and receiver operating curve (ROC) analyses were used to assess the predictive power of the miRNA quotients.

Unsupervised hierarchical clustering analysis

A microarray platform was used to determine miRNA expression of 662  
15 miRNAs in 16 fresh frozen head and neck squamous cell carcinoma tumors, 5 non-diseased head and neck epithelial tissues, and 2 individual head and neck squamous cell carcinoma cell lines. Unsupervised hierarchical clustering based on all the miRNAs spotted on the chip revealed a marked, very distinct separation of the cell line miRNA profiles compared to those of primary tissues. Additionally, hierarchical  
20 clustering based on the limited set of 18 miRNAs determined by SAM analysis to be differentially expressed between tumor and normal showed a clear separation of these two tissue types.

## RESULTS

miRNA expression patterns differentiate head and neck squamous cell  
25 carcinoma cell lines from primary tissues.

A microarray platform was used to determine miRNA expression of 662  
miRNAs in 16 fresh frozen head and neck squamous cell carcinoma tumors, 5 non-diseased head and neck epithelial tissues, and 2 individual head and neck squamous cell carcinoma cell lines. The normalized data has been deposited in the GEO  
30 archive (accession #GSE11163).

Unsupervised hierarchical clustering based on all the miRNAs spotted on the chip revealed a marked, very distinct separation of the cell line miRNA profiles compared to those of primary tissues. SAM identified 67 significantly differentially expressed miRNAs ( $Q < 0.0001$ ) between cell lines and primary tissues, consistently showing lower expression of miRNAs in cell lines compared to tumors (Table 1).

Table 1. Differentially expressed miRNA in cell lines vs. tumors as determined by SAM method ( $Q < .001$ ). (hsa: Homo sapiens; mmu: Mus musculus; ambi: Ambion; rno: Rattus norvegicus)

| miRNA           | fold change |
|-----------------|-------------|
| hsa_miR_143     | -232.76     |
| hsa_miR_145     | -244.22     |
| hsa_miR_199a    | -88.23      |
| hsa_miR_199a_AS | -127.06     |
| hsa_miR_146b    | -38.49      |
| hsa_miR_223     | -81.81      |
| hsa_miR_214     | -34.36      |
| hsa_miR_126     | -37.12      |
| hsa_miR_34a     | -11.68      |
| ambi_miR_3046   | -29.63      |
| ambi_miR_13258  | -9.50       |
| ambi_miR_2660   | -9.35       |
| mmu_miR_10b     | -11.09      |
| hsa_miR_10b     | -9.04       |
| hsa_miR_142_5p  | -9.74       |
| ambi_miR_10411  | -6.13       |

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|                |        |
|----------------|--------|
| mmu_miR_451    | -32.32 |
| ambi_miR_7029  | -29.44 |
| hsa_miR_152    | -5.09  |
| mmu_miR_140_AS | -4.53  |
| hsa_miR__451   | -25.01 |
| hsa_miR_199b   | -6.69  |
| ambi_miR_13268 | -3.28  |
| hsa_miR_34b    | -3.30  |
| rno_miR_140_AS | -3.43  |
| mmu_miR_99a    | -10.28 |
| hsa_miR_368    | -6.22  |
| hsa_miR_99a    | -9.55  |
| hsa_miR_24     | -2.60  |
| hsa_miR_497    | -4.70  |
| hsa_miR_26b    | -2.94  |
| hsa_miR_342    | -3.37  |
| rno_miR_497    | -5.08  |
| hsa_miR_195    | -5.03  |
| ambi_miR_2837  | -4.39  |
| mmu_miR_199b   | -4.20  |
| mmu_miR_203    | -22.71 |
| ambi_miR_13205 | -2.90  |
| hsa_miR_22     | -2.41  |
| hsa_miR_203    | -15.66 |
| hsa_miR_150    | -7.22  |

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|                |       |
|----------------|-------|
| hsa_miR_146a   | -9.06 |
| rno_miR_409_3p | -2.92 |
| hsa_miR_27b    | -2.45 |
| ambi_miR_444   | -2.29 |
| ambi_miR_13232 | -2.18 |
| ambi_miR_9651  | -2.70 |
| hsa_miR_27a    | -2.09 |
| mmu_miR_379    | -2.49 |
| hsa_miR_125b   | -3.76 |
| hsa_miR_455    | -2.02 |
| hsa_miR_452    | -3.79 |
| hsa_miR_29c    | -2.98 |
| ambi_miR_9451  | -2.40 |
| hsa_miR_210    | -3.61 |
| ambi_miR_12061 | -2.28 |
| hsa_miR_483    | -1.87 |
| hsa_miR_126_AS | -2.90 |
| ambi_miR_13260 | -2.32 |
| rno_miR_29c_AS | -2.08 |
| mmu_miR_341    | -3.14 |
| hsa_miR_139    | -2.59 |
| rno_miR_382    | -2.24 |
| hsa_miR_189    | -2.07 |
| hsa_miR_326    | -2.02 |
| mmu_miR-487b   | -2.45 |

18 miRNAs are differentially expressed in head and neck squamous cell carcinoma tumor tissue compared to normal head and neck epithelia.

SAM analysis identified 18 miRNAs to be significantly altered in their expression between non-diseased tissues and primary head and neck squamous cell carcinoma tumors, with 17 being up-modulated and 1 down-modulated in tumors ( $Q < 0.0001$ ) (Table 2). Of the 18 differentially expressed miRNAs, 12 were human miRNAs, four were miRNAs identified on mirVANA™ miRNA Bioarray platform v2 (Ambion Inc. Catalog No. AM1566V2) and two were mouse orthologues (mmu\_miR\_503 and mmu\_miR\_221), both of which have known human counterparts with identical mature sequences. Hierarchical clustering based on this limited set of miRNAs showed clear separation of tumors from normal tissues.

Table 2. Differentially expressed miRNAs in tumor vs normal as determined by SAM method ( $Q < .001$ ). (hsa: Homo sapiens; mmu: Mus musculus; ambi: Ambion)

| miRNA          | fold change |
|----------------|-------------|
| ambi_miR_562   | 5.24        |
| hsa_miR_21     | 3.67        |
| ambi_miR_7083  | 5.45        |
| hsa_miR_181d   | 2.86        |
| hsa_miR_181b   | 2.97        |
| hsa_miR_491    | 5.08        |
| hsa_miR_455    | 2.58        |
| ambi_miR_13258 | 2.56        |

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|                |        |
|----------------|--------|
| hsa_miR_18a    | 2.78   |
| ambi_miR_11541 | 2.28   |
| hsa_miR_130b   | 4.17   |
| mmu_miR_503    | 2.63   |
| hsa_miR_221    | 2.26   |
| hsa_miR_193b   | 3.44   |
| hsa_miR_181a   | 2.11   |
| mmu_miR_221    | 2.17   |
| hsa_miR_18b    | 2.50   |
| hsa_miR_375    | -21.88 |

All human miRNAs identified by SAM method showed greater than two-fold difference between normal and tumor tissue (Table 3). Of the human miRNAs, six (miR-21, miR-181d, miR-181b, miR-18a, miR-221, and miR-375) were chosen for confirmation of the microarray results using stem-loop based RT-PCR followed by conventional Taqman real-time PCR using miRNA-specific probes, as validated assays were available for these six miRNA and they constituted one primer pool, allowing for examination using a single reverse transcription step. Confirmation assays utilized only the samples screened on the array. Of the six miRNAs tested, four were reliably confirmed (miR-21, miR-18a, miR-221, and miR-375), showing significant differential expression between tumor and normal ( $P < 0.01$ , Figures 1A and B).

Table 3. Annotated human miRNAs differentially expressed in Tumor vs Normal by SMA ( $Q < 0.001$ )

| miRNAs   | Fold change |
|----------|-------------|
| miR-21   | 3.67        |
| miR-181d | 2.86        |
| miR-181b | 2.97        |
| miR-491  | 5.08        |
| miR-455  | 2358        |
| miR-18a  | 2.78        |
| miR-130b | 4.17        |
| miR-221  | 2.26        |
| miR-193b | 3.44        |
| miR-181a | 2.11        |
| miR-18b  | 2.5         |
| miR-375  | -21.88      |

miRNA expression ratio demonstrates high specificity and sensitivity in predicting disease.

Validation of the four confirmed miRNAs was performed in nine additional normal tissue samples (total  $n = 14$ ), and 83 additional tumor samples (total  $n = 99$ ). Quantification of miRNA expression in this large set of samples showed strong validation of the results seen in the smaller population. miR-21, miR-18a, and miR-221 showed significant upregulation in tumors ( $P < .0001$ ; Figures 2A, 2C, and 2D, respectively), whereas miR-375 was significantly downregulated in tumors ( $P < .0001$ ; Figure 2B).

As miR-21, miR-18a, and miR-221 demonstrated consistent upregulation, and miR-375 consistent downregulation, expression ratios were constructed between these miRNAs to determine whether the ratios could improve predictive potential for differentiating head and neck squamous cell carcinoma tumor from non-diseased epithelia. Following the methods of Gordon et al. (27), miRNA expression ratios were calculated by dividing the relative expression value of each of the three miRNAs showing upregulation in tumors by the expression value of the only

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downregulated miRNA, miR-375. ROC analysis was performed to determine which of these ratios demonstrated the greatest predictive power (Figures 3A, 3B and 3C). Table 4 lists the representative sensitivity and specificity of ratios using the cutoff value of 1 for each of the upregulated miRNA to the downregulated miR-375 in

5 differentiating between non-diseased tissue and head and neck squamous cell carcinoma, using the validation series. miR-21:miR-375 ratios above 1.0 exhibited high specificity (0.99) but low sensitivity (0.14) whereas the relationship of miR-18a:miR-375 showed high sensitivity (1.00) but low specificity (0.52) (Table 4). However, the ratio of miR-221:miR-375 exhibited the strongest predictive ability

10 with both high sensitivity and specificity (about 0.92 and about 0.93 respectively; Table 4).

| Table 4. Examination of sensitivity and specificity of disease prediction using a miRNA expression ratio greater than 1.0 |             |             |
|---------------------------------------------------------------------------------------------------------------------------|-------------|-------------|
| Ratio                                                                                                                     | Sensitivity | Specificity |
| miR-21/miR-375                                                                                                            | 0.99        | 0.14        |
| miR-18a/miR-375                                                                                                           | 0.52        | 1           |
| miR-221/miR-375                                                                                                           | 0.92        | 0.93        |

## DISCUSSION

The present study revealed a number of miRNAs to be aberrantly expressed

15 in head and neck squamous cell carcinoma tumors, including an extensively validated subset that may hold utility as clinical biomarkers of disease. Microarray profiling of over 600 miRNAs identified 18 miRNAs that were significantly differentially expressed in tumor tissues compared to analogous non-diseased head and neck epithelia. Of the 12 human miRNAs in this group, 4 miRNA which were

20 validated by qRT-PCR were used for in-depth examination of a larger population of fresh frozen head and neck squamous cell carcinoma tumors and normal head and neck tissue.

The permutation-based software SAM was used to identify differentially expressed genes by pair-wise comparisons between groups of interest. It should be

25 noted that SAM was designed (and may be better suited) for identification of important genes from high density microarrays, as it allows the user to control the

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number of findings based on a desired false discovery rate (FDR) while avoiding parametric assumptions about the data, inherent in tests such as the Analysis of Variance (ANOVA) (28).

The microarray data revealed that head and neck squamous cell carcinoma cell lines show a distinct pattern of miRNA expression compared to primary tumors. This is consistent with past reports demonstrating a clear segregation of cell lines away from primary tumors upon high-throughput analysis of their miRNA expression (22, 29), suggesting that cell lines have limited utility as a model system for the identification of clinically relevant miRNA biomarkers. There remains, though, significant utility in utilizing in-vitro approaches for defining the biological mechanisms of these miRNAs, with appropriate understanding that the pattern of their expression is markedly different from that observed in the parent primary tissue.

Some of the miRNAs identified as differentially expressed in head and neck squamous cell carcinoma compared to normal tissues have been characterized in past reports, particularly in relation to cancer. Relative levels of miR-21 and other miRNAs have been reported to have prognostic relevance for predicting patient survival in lung cancer (31). Targets of miR-21 may include the tumor suppressor genes tropomyosin 1 (TPM1) and programmed cell death 4 (PDCD4) (32, 33). Additionally, miR-221 and miR-18a, both shown to be upregulated in head and neck squamous cell carcinoma tumors, have previously been implicated in hepatocellular, prostate, and other cancers (34-37).

As described herein, miR-375 was the only downregulated miRNA found when comparing tumors and normal tissues, showing about a 22 fold decrease in tumors. Validation experiments also showed it to be sharply and significantly downregulated in tumors relative to normal tissues. miR-375 has been found to regulate insulin secretion in mice and its downregulation has been implicated in aberrant morphology of pancreatic islet cells in zebrafish (38, 39). Recently, miR-375 downregulation has also been associated with  $\beta$ -catenin mutation in hepatocellular adenoma (40). The downregulation of miR-375 in head and neck squamous cell carcinoma tumors, as shown in this work, is consistent with a

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possible role for miR-375 in the transcriptional repression of oncogenes, analogous to the regulation of oncogenic KRAS by the let-7 miRNA (41).

The ratio of miR-221:miR-375 demonstrated high discriminatory potential, with a sensitivity of about 92% and specificity of about 93% in distinguishing tumor  
5 from normal tissue. These data suggest that the ratio of these miRNAs may hold significant clinical potential to diagnose carcinomas.

The identification of an head and neck squamous cell carcinoma -specific miRNA signature indicates a plausible role for miRNAs in the development or progression of this disease. Finding abnormally expressed miRNAs may prove to be  
10 an important step in identifying the specific mechanisms of head and neck squamous cell carcinoma carcinogenesis, as these aberrations may constitute early events in initiation or progression of the disease (42). As such, the utilization of a miRNA expression ratio that distinguishes disease tissue from non-diseased tissue holds potential as a simple, early diagnostic for head and neck squamous cell carcinoma.  
15 An examination of these miRNA expression ratios in preneoplastic lesions, early stage tumors, and samples obtained for screening, such as saliva and mouthwash can be done. The expression or the miRNA described herein can also be examined with respect to clinical criteria, such as tumor stage, metastasis and prognosis in a larger series of tumors than what has been examined here. The miRNAs identified for  
20 diagnostics described herein may be useful in further understanding the diagnostic potential of the miR-221:miR-375 expression ratio.

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## EXAMPLE 2

5 Quantitative real-time PCR was used to determine the relative expression of four of the miRNAs that were identified in Example 1 as being differentially expressed in head and neck squamous cell carcinomas compared to non-diseased epithelia in a larger, independent case-series of head and neck squamous cell carcinoma tumors (n=169), and examine associations of miRNA expression with  
10 exposures and clinical features associated with head and neck squamous cell carcinoma. In multivariate analyses, expression of miR-375 was shown to increase with alcohol consumption ( $P = 0.001$ ), and showed higher expression in tumors of pharyngeal and laryngeal origin compared with oral tumors ( $P < 0.05$  and  $P < 0.01$ , respectively). Additionally, high miR-21 expression was associated with  
15 significantly decreased 5-year survival in patients (HR, 1.68; 95% CI 1.03-2.74). Together, these data suggest that alterations in miRNA expression are related to exposures causal in head and neck cancer and may be useful biomarkers of patient outcome.

### Introduction

20 Head and neck squamous cell carcinoma, the sixth most common cancer worldwide, arises from various sites in the upper aerodigestive tract (1,2). The intricate anatomy of the primary tumor sites bring about complex patterns of invasion and locoregional spread that have proven difficult to treat (1). These features, along with the frequent occurrence of late-stage diagnosis and second  
25 primary tumor formation have contributed to a relatively poor 5-year survival rate that has shown only modest improvement over the last three decades (1,3). The major risk factors for head and neck squamous cell carcinoma include tobacco and alcohol, which can act both independently and synergistically, as well as human papilloma virus (HPV) infection, which is an independent risk factor (4). A  
30 complete understanding of how these exposures alter cellular functions and the molecular basis for their risk remains elusive. Understanding the molecular nature of head and neck squamous cell carcinoma carcinogenesis is indispensable for

improving early diagnosis, predicting prognosis, and establishing effective therapeutics. While several attempts have been made at defining genetic biomarkers for head and neck squamous cell carcinoma (5-7), epigenetic biomarkers likely contribute considerable clinical and biological utility to treating and understanding this disease as it is clear that epigenetics plays a pivotal role in its development and progression.

The field of epigenetic regulation is being intensely researched and in recent years, important roles for epigenetic alterations in head and neck squamous cell carcinoma carcinogenesis have been revealed (8-10). MicroRNAs (miRNAs), a class of non protein-coding RNAs, are now recognized as critical products of the epigenome, orchestrating events ranging from organogenesis to immunity and they are known to be critical in the development of many diseases, including cancer (11,12). By binding to partially complementary sites in the 3' untranslated regions of their mRNA targets, miRNAs interfere with mRNA translation or cause mRNA degradation thereby repressing gene expression post-transcriptionally (13).

Dysregulation of miRNAs in cancer has been shown to associate with various tumor characteristics and prognosis in a variety of tumor types (14-17). Though aberrations in miRNA expression in primary head and neck squamous cell carcinoma tumors have recently been defined in several reports (18-21), little is known about how these differences associate with clinical features and disease risk factors. In-vitro studies and animal models have suggested that the expression of miRNAs are altered in response to various toxicant exposures (22-24). Determining such associations in human populations may be vital to better understanding the molecular mechanism through which exposures and the environment contribute to head and neck carcinogenesis. The expression of four miRNAs, previously found to be differentially expressed in head and neck squamous cell carcinoma tumors compared to normal tissues (18), was studied and associations with clinicopathologic features of tumors were examined to determine if these miRNA alterations are useful as prognostic biomarkers. Likewise, associations of expression of these miRNAs with patient carcinogen exposure were investigated in order to better understand if these exposures act via alterations to miRNA.

## Materials and Methods

### Study Population.

A total of 169 fresh-frozen head and neck squamous cell carcinoma tumor samples were obtained, with informed consent after Institutional Review Board approval at participating hospitals, as part of a previously described study of head and neck cancer in the Greater Boston Metropolitan area (25,26). A study pathologist confirmed >75% tumor content in each of the head and neck squamous cell carcinoma samples used in these analyses. HPV-16 DNA status was previously determined (26,27).

### 10 RNA isolation and microarray profiling.

Total RNA was isolated from tumors using the mirVANA RNA Isolation Kit (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), aliquoted, and stored at -80°C briefly until used in laboratory analysis.

### 15 Quantitative reverse transcription-PCR.

TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) were used to quantify mature miRNA of miR-21, miR-18a, miR-375, and miR-218. cDNA was synthesized by priming with a pool of gene-specific looped primers including the primers of the miRNAs of interest and RNU48, a universally-expressed endogenous control (Applied Biosystems, Foster City, CA). 10 µl of total RNA diluted to a final concentration of 5 ng/µl was used for each reverse transcription (RT) reaction along with other RT components, per manufacturer's specifications. 40 µl reactions were incubated in an Applied Biosystems GeneAmp PCR system 9700 for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and held at 4°C. All reactions, excluding no-template controls and non-reverse-transcribed controls, were run in triplicate on an ABI 7500 Fast Real Time PCR Detection System. All real-time PCR data were quantified by calculating fold change using the  $\Delta\Delta CT$  method, normalizing miRNA expression of cases to expression data from a pooled set of non-diseased head and neck epithelium samples.

### Statistical Analysis.

All analyses were carried out using SAS 9.1 (SAS Institute, Cary, North Carolina). Fold change expression values for all miRNAs were log transformed to create a normal distribution for parametric analysis. Categories of smoking and drinking were created with never smokers/drinkers as referents and low and high categories dichotomized at the median value for each variable. For univariate analyses, student's t-tests or one-way analysis of variance (ANOVA) were used for discrete variables of two or greater than two categories, respectively, except in the case of tumor site, where t-tests were conducted to compare pharyngeal vs oral and laryngeal vs oral tumors. Spearman's rank correlation was used for continuous variables. Linear regression analysis was used for multivariate testing for associations with exposures and clinicopathologic features. Using the Kaplan–Meier method and the log-rank test to determine significance, overall 5-year survival rates were compared across strata of miRNA expression using log-transformed miRNA expression values stratified around the top 25th percentile of miRNA expression. A multivariate Cox proportional hazards regression analysis was used to confirm predictors of case fatality. P-values of <0.05 were considered significant. For best parsimony in multivariate models, all variables were initially included in models but non-significant variables were removed if their removal did not result in greater than 15% change in the effect estimates of other variables (i.e. the variables were not considered confounders).

### Results

#### Quantification of miRNA expression.

A subset of 169 patients for which fresh-frozen tumor was available and covariate data were well-annotated was used for this study (Table I). The population consisted of patients with a mean age of  $61.5 \pm 11.9$ , 68% of which were males. Greater than half of the participants had smoked more than or equal to 33 pack-years and drank more than or equal to 14 alcoholic drinks per week (51.1% and 51.8%, respectively). The majority of the patients had oral tumors, high stage disease, and were HPV-16 negative (64%, 72%, and 90% respectively).

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Table I : Patient Demographics and clinicopathological characteristics  
(n=169)

|                                         | n (%)       |
|-----------------------------------------|-------------|
| Gender                                  |             |
| Female                                  | 54 (32.0)   |
| Male                                    | 115 (68.0)  |
| Age, mean (SD)                          | 61.5 (11.9) |
| Lifetime pack-years smoked <sup>a</sup> |             |
| 0 (none)                                | 22 (15.5)   |
| >0 to <36.75                            | 59 (41.5)   |
| ≥36.75                                  | 61 (43.0)   |
| Drinks per week <sup>b</sup>            |             |
| none(0)                                 | 16 (11.5)   |
| >0 to <18                               | 61 (43.9)   |
| ≥18                                     | 62 (44.6)   |
| Tumor Site <sup>c</sup>                 |             |
| Oral                                    | 94 (64.0)   |
| Pharyngeal                              | 31 (21.1)   |
| Laryngeal                               | 22 (15.0)   |
| Stage <sup>d</sup>                      |             |
| Low (I, II)                             | 46 (28.0)   |
| High (III, IV)                          | 118 (72.0)  |
| HPV-16 tumor DNA status <sup>e</sup>    |             |
| negative                                | 90 (82.6)   |
| positive                                | 19 (17.4)   |

<sup>a</sup>Data missing in 27 samples<sup>b</sup>Data missing in 30 samples<sup>c</sup>Data missing in 22 samples<sup>d</sup>Data missing in 5 samples<sup>e</sup>Data missing in 60 samples

- Four miRNAs were selected for analysis in this population based on the results from the study described in Example 1 herein, which identified these miRNAs as differentially expressed between head and neck squamous cell carcinoma tumor and normal head and neck epithelia (18). Quantitative real-time PCR was performed on 169 head and neck squamous cell carcinoma tumors and a fold change expression values of each miRNA was determined by normalizing its expression to a pool of non-diseased samples. The average log-transformed expression values for each of the four miRNAs evaluated are listed in Table Ia.

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miR-375 expression is associated with tumor site, stage, and alcohol consumption.

In a univariate comparison, miR-375 was expressed at a significantly greater level in laryngeal tumors compared with those of the oral cavity ( $P = 0.004$ ; Table Ia). Tobacco smoking (measured as pack-years smoked, duration (years smoked), or intensity (packs per day)) and HPV status were not associated with miR-375 or any other miRNA's expression. However, univariate analyses showed that the expression of miR-375 increased significantly with alcohol consumption ( $P = 0.009$ ; Table Ia). To control for potential confounders, a multivariate linear regression analysis was employed, which demonstrated that both categories of drinkers,  $>0$ -14 and  $\geq 14$  drinks per week, were associated with higher miR-375 expression compared to nondrinkers ( $P = 0.052$  and  $P = 0.001$ , respectively; Table II). Further, miR-375 expression was greater in both pharyngeal and laryngeal tumors compared with oral tumors ( $P = 0.047$  and  $P = 0.004$ , respectively; Table II) in a model controlling for age, gender, and tobacco smoking.

15

Table IA. Log-transformed normalized fold-change expression of miRNAs

| microRNA | Mean expression (range) |
|----------|-------------------------|
| miR-21   | 1.66 (-2.15, 5.74)      |
| miR-375  | -2.3 (-7.76, 2.82)      |
| miR-221  | 1.36 (-2.36, 6.44)      |
| miR-18a  | -0.3 (-3.01, 3.53)      |

Table II. Univariate analysis of miRNA expression with etiological factors and clinicopathological characteristics<sup>a</sup>

|                            | n (%)       | log miR21     |      |               | log miR375 |                |      | log miR18a    |      |               | log miR221 |               |      |
|----------------------------|-------------|---------------|------|---------------|------------|----------------|------|---------------|------|---------------|------------|---------------|------|
|                            |             | Mean Exp (SD) | p    | Mean Exp (SD) | p          | Mean Exp (SD)  | p    | Mean Exp (SD) | p    | Mean Exp (SD) | p          | Mean Exp (SD) | p    |
| Gender                     |             |               |      |               |            |                |      |               |      |               |            |               |      |
| Female                     | 54 (32.0)   | 1.76 (1.37)   |      | -2.49 (2.04)  |            | -0.2 (1.14)    |      | 1.5 (1.49)    |      |               |            |               |      |
| Male                       | 115 (68.0)  | 1.64 (1.47)   | 0.61 | -2.20 (2.14)  | 0.41       | -0.33 (0.94)   | 0.41 | 1.31 (1.40)   | 0.41 |               |            |               | 0.42 |
| Age, mean (SD)             | 61.5 (11.9) | 1.68 (1.44)   | 0.21 | -2.3 (2.10)   | 0.63       | -0.29 (1.01)   | 0.29 | 1.37 (1.42)   | 0.12 |               |            |               |      |
| Lifetime pack-years smoked |             |               |      |               |            |                |      |               |      |               |            |               |      |
| 0 (none)                   | 22 (15.5)   | 1.54 (1.69)   |      | -2.47 (2.1)   |            | -0.20 (1.3)    |      | 1.66 (1.80)   |      |               |            |               |      |
| >0 to <36.75               | 59 (41.5)   | 1.63 (1.40)   |      | -2.67 (2.07)  |            | -0.20 (0.87)   |      | 1.35 (1.29)   |      |               |            |               |      |
| ≥36.75                     | 61 (43.0)   | 1.64 (1.28)   | 0.96 | -1.98 (2.27)  | 0.21       | -0.44 (1.00)   | 0.37 | 1.28 (1.39)   | 0.55 |               |            |               |      |
| Drinks per week            |             |               |      |               |            |                |      |               |      |               |            |               |      |
| none(0)                    | 16 (11.5)   | 1.62 (1.27)   |      | -3.45 (1.56)  |            | -0.21 (1.00)   |      | 1.51 (1.16)   |      |               |            |               |      |
| >0 to <18                  | 61 (43.9)   | 1.59 (1.47)   |      | -2.53 (2.38)  |            | -0.26 (1.09)   |      | 1.21 (1.62)   |      |               |            |               |      |
| ≥18                        | 62 (44.6)   | 1.68 (1.39)   | 0.94 | -1.81 (1.93)  | 0.014      | -0.32 (0.95)   | 0.9  | 1.51 (1.31)   | 0.49 |               |            |               |      |
| Tumor Site <sup>b</sup>    |             |               |      |               |            |                |      |               |      |               |            |               |      |
| Oral                       | 94 (64.0)   | 1.62 (1.49)   |      | -2.58 (2.06)  |            | -0.32 (1.11)   |      | 1.37 (1.54)   |      |               |            |               |      |
| Pharyngeal                 | 31 (21.1)   | 1.46 (1.36)   | 0.11 | -1.78 (2.52)  | 0.12       | -0.36 (0.82)   | 0.81 | 1.14 (1.25)   | 0.41 |               |            |               |      |
| Laryngeal                  | 22 (15.0)   | 2.18 (1.39)   | 0.56 | -1.23 (1.71)  | 0.004      | -0.0006 (0.91) | 0.17 | 1.71 (1.38)   | 0.31 |               |            |               |      |
| Stage                      |             |               |      |               |            |                |      |               |      |               |            |               |      |
| Low (I, II)                | 46 (28.0)   | 1.74 (1.36)   |      | -2.46 (1.98)  |            | -0.42 (1.23)   |      | 1.02 (1.3)    |      |               |            |               |      |
| High (III, IV)             | 118 (72.0)  | 1.60 (1.47)   | 0.58 | -2.36 (2.11)  | 0.77       | -0.38 (0.98)   | 0.78 | 1.15 (1.24)   | 0.31 |               |            |               |      |
| HPV status                 |             |               |      |               |            |                |      |               |      |               |            |               |      |
| negative                   | 90 (82.6)   | 1.73 (1.33)   |      | -2.27 (2.16)  |            | -0.26 (1.02)   |      | 1.53 (1.29)   |      |               |            |               |      |
| positive                   | 19 (17.4)   | 1.45 (1.62)   | 0.43 | -2.42 (2.11)  | 0.79       | -0.32 (1.00)   | 0.9  | 1.28 (1.48)   | 0.61 |               |            |               |      |

<sup>a</sup>Expression values represent log-transformed values obtained by using the ΔΔCT method to normalize data to a pooled referent of non-diseased head and neck epithelium samples.<sup>b</sup>T-test results for tumor site compared pharyngeal vs oral and laryngeal vs oral

miR-21 expression is associated with poorer patient survival.

Kaplan-Meier survival analysis demonstrated that patients with miR-21 expression in the highest quartile showed a trend toward worse survival than those with lower miR-21 expression (Figure 5,  $P = 0.137$ , log-rank test). However, in a multivariate Cox proportional-hazards model controlling for age, gender and tumor stage, high expression of miR-21 was shown to be associated with significantly decreased 5-year survival of patients (HR = 1.68; 95% CI, 1.04-2.77;  $P = 0.034$ ; Table III).

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Table III: Correlation of miR-375 expression with etiological factors and tumor site in a multivariate linear regression model (Total  $n=133$ )

|                              | n (%)       | Reg. Coeff. | <i>p</i> |
|------------------------------|-------------|-------------|----------|
| Gender                       |             |             |          |
| Female                       | 42 (31.6)   | reference   |          |
| Male                         | 91 (68.4)   | -0.41       | 0.33     |
| Age, mean (SD)               | 61.5 (11.3) | -0.004      | 0.806    |
| Pack-years smoked            |             |             |          |
| none(0)                      | 22 (16.5)   | reference   |          |
| <36.75                       | 43 (32.3)   | -0.87       | 0.129    |
| ≥36.75                       | 68 (51.1)   | -0.5        | 0.375    |
| Drinks per week <sup>a</sup> |             |             |          |
| none(0)                      | 13 (9.8)    | reference   |          |
| <18                          | 50 (37.6)   | 1.3         | 0.039    |
| ≥18                          | 70 (52.6)   | 2.26        | 0.002    |
| Tumor Site                   |             |             |          |
| Oral                         | 83 (62.4)   | reference   |          |
| Pharyngeal                   | 31 (23.3)   | 0.89        | 0.049    |
| Laryngeal                    | 19 (14.3)   | 1.7         | 0.005    |

<sup>a</sup> Trend test  $P = 0.002$

Table IV. Cox Proportional Hazards Multivariate Model (n = 150)<sup>a</sup>

|                       | n (%)      | Hazard Ratio | 95% CI |       | p      |
|-----------------------|------------|--------------|--------|-------|--------|
|                       |            |              | Lower  | Upper |        |
| Gender                |            |              |        |       |        |
|                       |            | 1.0          |        |       |        |
| Female                | 49 (32.7)  | (reference)  |        |       |        |
| Male                  | 101 (67.3) | 1.24         | 0.76   | 2.01  | 0.388  |
| Age, mean (SD)        |            | 1.03         | 1.02   | 1.05  | <0.001 |
| log miR-21 expression |            |              |        |       |        |
|                       |            | 1.0          |        |       |        |
| < 2.58 (n = 117)      | 117        | (reference)  |        |       |        |
| ≥ 2.58 (n = 33)       | 33         | 1.68         | 1.04   | 2.77  | 0.034  |
| Tumor stage           |            |              |        |       |        |
|                       |            | 1.0          |        |       |        |
| I, II (n = 43)        | 43         | (reference)  |        |       |        |
| III, IV (n = 107)     | 107        | 1.56         | 0.94   | 2.6   | 0.096  |

<sup>a</sup>Survival data missing in 15 samples

## Discussion

The potential utility in assessing miRNA expression as a useful biomarker in cancer diagnostics, prognostics and therapeutics, is becoming increasingly apparent.

- 5 Many studies have reported significant associations between miRNA profiles and important clinical features of tumors as well as patient survival (14,17,28-30). Here, miRNA expression in head and neck squamous cell carcinoma tumors was analyzed and a significant correlation with alcohol consumption, a major head and neck squamous cell carcinoma risk factor, was found, as well as associations with tumor site and overall
- 10 patient survival.

- Four miRNAs were validated as significantly differentially expressed between primary head and neck squamous cell carcinoma tumors and analogous normal tissue (Example 1). These miRNAs may play a role in head and neck squamous cell carcinoma carcinogenesis and that analysis of a large set of tumors would reveal
- 15 associations between expression of these miRNAs and various clinicopathologic and etiologic variables amongst tumors. Though miR-18a and miR-221 were not found to

associate with any covariates in this study, there is good reason to believe that they play a role head and neck squamous cell carcinoma carcinogenesis. miR-18a is a member of the miR-17-92 cluster, found to be overexpressed in gastric, colorectal, and ovarian cancers and is thought to inhibit expression of estrogen receptor- $\alpha$  in hepatocellular carcinoma (31-34). miR-221 expression is also increased in many cancers and its inhibition, along with that of miR-21 has been shown to induce cell-cycle arrest and apoptosis as well as sensitizing cells to chemotherapeutic agents (35,36). Several significant associations with miR-21 and miR-375 were identified in this study. miR-21 is one of the best studied miRNA (37,38). Though miR-375 has mainly been studied in the context of diabetes, as it influences beta-cell mass and insulin levels (39,40), its expression has been shown to be decreased in a number of malignancies including pancreatic adenocarcinomas and esophageal squamous cell and adenocarcinomas (41,42). Additionally, the recent identification of a target for miR-375, phosphoinositide-dependent protein kinase-1 (PDPK1), suggests a feasible role for miR-375 as a tumor suppressor since PDPK1 is crucial for the activation of anti-apoptotic AKT (43).

Alcohol consumption has been associated with altered miRNA expression in hepatocellular tumors and alcohol treatment has been shown to affect miRNA levels in rat neurons and fetal mouse brains (44-46). miR-375 expression was shown to increase with increasing alcohol consumption independent from tobacco smoking. While it is known that alcohol is a carcinogen and an independent risk factor for head and neck squamous cell carcinoma, the mechanism for this association is poorly understood. One possibility is that alcohol acts as an irritant and the resultant inflammation contributes to carcinogenesis (47). The oxidation of ethanol in the saliva by mucosal and microbial alcohol dehydrogenases results in the production of acetaldehyde, which is a known carcinogen in animals and possible carcinogen in humans (48,49). Additionally, it is well established that alcohol interferes with the absorption of folate, a methyl-donor critical for maintenance of normal DNA methylation patterns (50). Although miR-375 is down-regulated in tumors compared to normal tissues, alcohol consumption could contribute to its altered expression in the tumor microenvironment. The regulation of

miRNAs is complex and perturbations of the normal homeostatic mechanisms responsible for overall epigenetic stability could play a crucial role in potentially carcinogenic gene expression.

Higher expression of miR-375 was also found in pharyngeal and laryngeal tumors compared with tumors of the oral cavity. This observation is consistent with findings indicating that miRNA profiles are tumor and cell-type specific and can even precisely differentiate tumor subtypes (51,52). Moreover, the proclivity for differential expression of miR-375 in tissues might reflect etiology. The significant association observed between drinking and miR-375 expression coupled with its tendency for higher expression in pharyngeal and laryngeal tumors may suggest that the dysregulation of miRNA by exposures occurs preferentially in certain tissues.

This study has identified a significant association between high miR-21 expression in tumors and poor patient survival, the same relationship that has been demonstrated in cancers of the breast and colon as well as in non-small cell lung cancer (15,16,53). Several targets of miR-21 have been experimentally validated, many of which are tumor-suppressor genes (54-56). One of these targets, programmed cell death 4 (PDCD4) is known to be down-regulated in head and neck squamous cell carcinoma and in two recent studies of miRNA profiles in head and neck squamous cell carcinoma, its expression was shown to be inversely related to miR-21 in tumors (20,21). Another important target which shows reduced expression in head and neck squamous cell carcinoma is PTEN, a gene whose product inhibits growth and cell survival through antagonism of the AKT/PI3K pathway (57). Thus, it is likely that miR-21 functions through several targets to contribute to head and neck squamous cell carcinoma malignancy, thereby modifying risk associated with the disease.

The results in this Example suggest that miRNAs, such as miR-375, may modulate the carcinogenic response associated with exposure to risk factors for the disease. Further, this modulation may be differentially regulated in tumors depending on the tissue, as the expression of miR-375 was shown to differ amongst tumor sites. More in-depth study of miR-375 may prove invaluable for understanding of how exposures modify risk or progression of head and neck squamous cell carcinoma.

Additionally, high miR-21 expression correlated with poor prognosis in head and neck squamous cell carcinoma patients. As miR-21 seems to be a significant indicator of prognosis for this and other cancers, it should be considered as a potential therapeutic target for these diseases. The results in this Example also suggest there may be  
 5 significant prognostic utility in examining these specific miRNA expression signatures.

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## EXAMPLE 3

MicroRNAs (miRNA) are small non-coding RNA molecules, which influence biological functions through their interactions with multiple targets. Overexpression studies have been indispensable for the validation of miRNA targets and determination of cellular functions. The study described in Example 1 herein shows that *miR-375* expression is down-regulated in head and neck squamous cell carcinoma. *miR-375* may function as a tumor suppressor in head and neck squamous cell carcinoma carcinogenesis. The effects on proliferation, migration and sensitivity to cisplatin (CDDP), were assessed in cells transfected with mature *miR-375* mimic molecules and those transfected with a plasmid vector carrying the premature *miR-375* sequence under the control of a CMV promoter. *miR-375* mimic transfection resulted in >2,000 fold upregulation in *miR-375* levels, faster proliferation and migration and increased resistance to CDDP in *miR-375* transfected cells. On the other hand, the *miR-375* expression vector-based transfection only increased *miR-375* expression by ~4 fold and caused slower proliferation and increased sensitivity to CDDP. These results have shown that different methods of miRNA overexpression vary considerably in their resultant biological effects perhaps due to disparities in the extent to which they analogize endogenous miRNAs. As such, the caveats of their use in such studies must be carefully considered.

## 20 Introduction

MicroRNAs (miRNA) are now known to be important post-transcriptional regulators of gene expression, orchestrating diverse molecular functions through their mRNA targets (1). Primary miRNA transcripts (pri-miRNA) of 100s to 1000s of base pairs in length are transcribed from intergenic regions or intronic sequences into large stem-loop structures which are sequentially processed first generating a ~70 bp hairpin, which is exported out of the nucleus, and then a mature ~22 bp duplex (2). The active strand of the duplex is incorporated into a ribonucleoprotein effector complex known as RNA-induced silencing complex (RISC) in the cytoplasm. The miRNA/RISC can then

target a range of partially or fully complementary mRNA transcripts, resulting either in their translational repression or degradation, respectively (2).

An involvement of miRNAs has been identified for almost all major cancers (3). Many of the miRNAs significantly altered in cancer tend to target genes that regulate cell proliferation, differentiation and death, disruptions in which are classically associated with malignancy. Previous work showed *miR-375*, a miRNA primarily associated with pancreatic islet cells (4), to be down-regulated in head and neck squamous cell carcinoma compared to non-diseased tissue (5). *miR-375* has been shown to target 3'-phosphoinositide-dependent protein kinase-1 (PDPK-1) in pancreatic beta cells (6). As PDPK-1 is a major activator of anti-apoptotic AKT (7), it is possible that *miR-375* down-regulation in head and neck squamous cell carcinoma potentiates a pro-survival carcinogenic phenotype.

The growing interest in miRNAs' involvement in disease has spurred the development of myriad systems designed to study their expression levels, identify targets, and define their pleiotropic roles in vitro as well as in vivo (8). Given their diminutive size, and the fact that they do not code for proteins, the detection of miRNAs has required customized technologies including the development of specialized miRNA arrays and adaptation of traditional real-time PCR techniques for their quantification. The examination of miRNA functions in cultured cell lines carries several caveats as their expression is generally lower in cell lines compared to analogous primary tissues (9,10).

Here several experiments were performed to test changes in cell proliferation, migration and sensitivity to cisplatin, a chemotherapeutic agent commonly used in the treatment of head and neck squamous cell carcinoma (HNSCC). Two different systems were used for overexpression of *miR-375* in the invasive head and neck squamous cell carcinoma cell line FaDu. The first system involves transient transfection with a synthetic double-stranded RNA molecule designed to mimic the mature miRNA. Functioning much like small interfering RNAs (siRNAs), these miRNA precursors are chemically modified to ensure that the appropriate strand becomes incorporated into the RISC. The second method utilizes a vector-based system to allow expression of an

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engineered miRNA sequence from a Pol II promoter. This expression vector contains the human cytomegalovirus (CMV) immediate early promoter, allowing for constitutive miRNA expression in mammalian cells. Both systems have advantages caveats to their use and this study aims to investigate which system is better suited for determination of miRNA function and which most closely reproduces miRNAs generated endogenously.

## Materials and Methods

### Cell lines

FaDu cells were obtained from ATCC (HTB-43) and cultured in Eagles's Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

### Reverse transfection of miRNA mimics

Pre-miR<sup>TM</sup> miRNA Precursor Molecules (Ambion) were transfected into cells according to manufacturer's instructions. Briefly, 0.24  $\mu$ l or 2.4  $\mu$ l of precursor and .45 or 4.5  $\mu$ l of NeoFX Transfection Agent (Ambion) were diluted in Opti-MEM Reduced Serum Medium, combined and applied to cells while passaging. Normal growth medium was replaced after about 24 hours and cells were used for experiments after another 24 hours.

### Creation and transfection of *miR-375* expression constructs

Single stranded oligonucleotides containing the premature *miR-375* sequence were designed as follows: sense, 5'-TGCTGCCCCGCGACGAGCCCC-TCGCACAAACCGGACCTGAGCGTTTTGTTCGTTTCGGCTCGCGTGAGGC-3' (SEQ ID NO: 40); and antisense, 5'-CCTGGCCTCACGCGAGCCGAACGAACAAAACGCTCAGGTCCGGTTTGTGCGAGGGGCTCGTCGCGGGGC-3' (SEQ ID NO: 41). The oligos were annealed and the double-stranded oligo was ligated into the Block-iT<sup>TM</sup> Pol II miR RNAi Expression Vector according to the manufacturer's instructions (Invitrogen). Both *miR-375* and negative control plasmids were transformed into TOP10 Competent E.coli using One Shot TOP10 Transformation Protocol (Invitrogen). Transformants were analyzed by sequencing and successful clones expanded, and their plasmids purified by maxi-prep

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(Qiagen). FaDu cells were seeded to about 90% confluency in 6-well plates the day before transfection. A vector containing either *miR-375* or the negative control miRNA plasmid was transfected into cells by applying 10 µl Lipofectamine-2000 (Invitrogen) and 4 µg plasmid DNA, each diluted in Opti-MEM Reduced Serum Medium (Invitrogen) and combined. Medium was changed to normal growth medium after 4 hours incubation. Vectors contained the coding sequence of EmGFP (Emerald Green Fluorescent Protein) such that the pre-miRNA insertion site is in the 3' untranslated (3'UTR) region of the fluorescent protein mRNA, allowing for determination of transfection efficiency. Cells were used for experiments about 24 hours after transfection.

#### RNA isolation and quantitative real-time PCR

Total RNA was extracted using mirVana miRNA Isolation Kit (Ambion Inc.) according to manufacturer's protocol. Reverse Transcription PCR using a GeneAmp PCR System 9700 (Applied Biosystems) was performed using 5X primers for *hsa-miR-375* and RNU48 as an endogenous control (Applied Biosystems). An ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) was used for quantitative real-time PCR analysis using 20X probes for *hsa-miR-375* and RNU48. The expression levels of *miR-375* were normalized to levels of RNU48. Technical triplicates were performed.

#### Wound healing assay

Six-well plates were seeded with 1.2 million cells per well and transfected using the precursor molecules as above. Forty-eight hours after transfection, the wells were scratched lightly with 200 µl pipette tips. The rate of wound closure was recorded at 12-hour intervals for 60 hours using 20X magnified microscope photographs. Wound closure was then measured using a fixed angle ruler tool in Adobe Photoshop CS3. Experimental and control conditions were performed in duplicate.

#### Proliferation assay

96-well plates were seeded with 5000 cells per well. Cells were stained with 20 µL per well of CellTiter 96 Aqueous One Solution (Promega) and incubated for one hour at 37°C before plates were read using a SpectraMax M2 Microtiter Plate Reader.

Plates were then analyzed at 6 hours to establish a baseline reading, followed by readings at 24, 48, and 72 hours. SoftMax Pro software was used to record absorbance at the indicated time points. Experimental and control conditions were performed in 12 technical replicates.

## 5 Clonogenic assay and CDDP treatment

For clonogenic survival studies, 10 mm dishes were seeded with 3000 transfected cells and either allowed to grow for 14 days to examine basal growth of transfected cells or allowed to adhere overnight before treating with 0.5  $\mu$ M cisplatin (CDDP) or dimethyl formamide (DMF) control for 24 hrs before allowing to grow for  
10 14 days. Experimental and control conditions were performed in triplicate.

## Results

Transfection of cells with *miR-375* miRNA mimic leads to strong overexpression of *miR-375*, increased colony growth and migration

Precursor molecules mimicking the mature form of *miR-375* or a control  
15 sequence with no known gene target were transfected into the invasive head and neck squamous cell carcinoma cell line, FaDu, at increasing concentrations. Real-time PCR analysis showed strong upregulation of *miR-375*, from ~2000 fold to >10,000 fold overexpression compared to control-transfected cells (Figure 6A).

Effects of increasing *miR-375* expression on FaDu cells were assessed by  
20 clonogenic assay, which showed an overall increase in colony formation across transfection groups (Figure 6B;  $P = 0.002$ , ANOVA). A wound healing assay was performed following transfection with 5 nM *miR-375* precursor or negative control. *miR-375*-transfected cells tended to show faster wound closure compared to control (Figure 6C).

25 Transfection of cells with *miR-375* miRNA mimic leads to increased proliferation and resistance to CDDP

A cell proliferation assay was used to test the effects of *miR-375* mimic-transfection on proliferation of FaDu cells. Results showed that *miR-375*-transfected cells proliferated slightly faster than control-transfected cells (Figure 7A). To test the

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effect of *miR-375* mimic-transfection on sensitivity to CDDP, a commonly used chemotherapeutic drug used in the treatment of head and neck squamous cell carcinoma (11), a clonogenic assay was performed following 0.5  $\mu$ M CDDP exposure. *miR-375*-transfected cells showed greater survival following CDDP treatment than negative control-transfected cells (Figure 7B).

Transient transfection of cells with *miR-375* expression constructs leads to modest overexpression of *miR-375* decreased proliferation and increased sensitivity to CDDP

*miR-375* expression constructs were created by inserting the premature *miR-375* sequence into a Pol II expression vector and two transformants were selected for purification. Transient transfection efficiency was determined to be ~40%, determined by counting the GFP-positive cells (data not shown). Following transfection of expression plasmids into FaDu cells, *miR-375* expression was assessed using real-time PCR. *miR-375* expression in both constructs showed modest overexpression (~4 fold) compared to cells transfected with even a low concentration (0.5 nM) of *miR-375* precursor molecules (Figure 8). Contrary to the results seen following transfection with the miRNA mimic, cells transfected with *miR-375* expression constructs exhibited slower rates of proliferation and greater sensitivity to CDDP compared to negative control-transfected cells (Figure 9A-B).

#### Discussion

In vitro miRNA gain-of-function studies are important for identifying targets and determining biological function. This work has demonstrated two different methods of overexpressing *miR-375*, a miRNA that was found to be down-regulated in head and neck squamous cell carcinoma. *miR-375* may act as a tumor-suppressor miRNA, normally targeting genes that are upregulated in cancer, such as its known target, PDPK-1 (6). Increasing *miR-375* expression in cancer cells may rescue some of their malignant characteristics, such as increased proliferation, migration and resistance to chemotherapeutic agents. Though both types of transfections tested are commonly used in overexpression studies of miRNA, they produce drastically different results,

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both in the level of upregulation of the miRNA and the resultant phenotype seen in transfected cells.

The first method used was the more simplistic of the two, involving transfection, via a lipid-based transfection agent, of a small double stranded miRNA molecule, analogous to the miRNA in its mature form. The duplex is chemically modified in such a way as to ensure strand specificity and the details of this modification are likely based on the current comprehension of strand bias in small RNAs. In siRNA, the strand incorporated into RISC is the one with the 5' end that is less tightly paired to its complement (12). In human miRNA, it is known that the active strand shows U enrichment while the alternate strand shows C enrichment at its 5' end (13). Additionally the purine/pyrimidine content of the two strands is markedly different. Once inside cells, the active strand, having an identical sequence to the mature miRNA of interest, binds the RISC in the cytoplasm and carries out its function of targeting its cognate mRNAs.

The second transfection method involved the generation of a *miR-375* expression vector which contains the premature sequence of *miR-375* under the control of a constitutive CMV immediate early promoter. Upon transfection, the plasmid enters the nucleus, the promoter is recognized by Pol II, and the *pre-miR-375* sequence is transcribed. From this point, the premature sequence likely forms a hairpin structure and is exported and further processed alongside endogenous pre-miRNAs.

Overexpression of *miR-375* using the miRNA precursor molecule system resulted in massive upregulation of the mature miRNA, increases in colony formation, proliferation, and migration, as measured by wound healing assay. Consistent with this, *miR-375* mimic-transfected cells were also more resistant CDDP, a chemotherapeutic agent commonly used to treat head and neck squamous cell carcinoma (11). These results seemed to oppose the belief that *miR-375* acts as a tumor-suppressor miRNA. However, the extremely high level of *miR-375* upregulation seen when using this transfection method calls into question the reliability of the results.

Previous work showed that in primary tissues, there was a 22-fold reduction in *miR-375* expression in head and neck squamous cell carcinoma tumors compared to

normal epithelia(5). Endogenous miRNAs are a tightly regulated class of molecules which, in their mature form, are quite stable(14). Due to their wide regulatory scope, even small changes in miRNA expression, on the order of 2 or 3 fold-change, have been shown to influence biological function significantly (15,16). In the precursor  
5 transfection system, a large number of these mature miRNA analogs are transfected into cells and function at the level of the mature miRNA. It is possible that oversaturation of the enzymatic effector complex, RISC, with these more stable, nonendogenous mimics may be interfering with the targeting functions of the many other miRNA that depend on RISC, resulting in an artifactual result related to inhibition of the functional pathway.

10 Using the expression vector system to increase *miR-375* levels resulted in a much more modest ~4 fold upregulation of the miRNA. Experiments also showed results opposite to the previous system, including slower proliferation and increased sensitivity to CDDP in *miR-375*-transfected cells compared to control. This system requires far more steps and is more complex than the previous system, as plasmid DNA  
15 must enter the nucleus and be recognized, transcribed and processed by nuclear enzymes. This is likely to result in far fewer mature miRNA being generated, as the processing rate is limited by endogenous cell machinery. Additionally, since a transient transfection only results in ~40% transfection efficiency, the population of cells in which miRNA expression is measured is quite heterogeneous, contributing to a lower  
20 expression change and a more muted phenotypic change when examining an entire population of cells.

Several experiments could be performed to confirm or address the potential problems inherent in the systems used to overexpress *miR-375*. A RISC activity assay, as described by Liu et al. (17), can be performed to confirm the that miRNA mimics  
25 overwhelm the RISC. Additionally, the miRNA/RISC complexes can be isolated to determine the proportion of functional *miR-375* vs other miRNAs bound to RISC(18). Lysates of transfected cells would be subjected to immunoprecipitation using antibodies against Ago-2 proteins which interact with miRNAs in the RISC.

The expression vector system allows for generation of a stable line of cells  
30 overexpressing *miR-375*. Stable transfection may yield a more homogeneous population

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of cells with higher expression of *miR-375* and a more dramatic phenotypic change. Though this may have the desired effect on *miR-375* level, the observed phenotype may be influenced by compensatory mechanisms exerted by the cells in response to the vector's integration into the genome and the resultant constitutive expression of the  
5 miRNA.

The use of a luciferase reporter assay based on the *mir-375* target sequence would help to elucidate the relative levels of *miR-375* function in the two systems used (19). A construct bearing the firefly luciferase mRNA with the 3' UTR sequence of a known target of *miR-375*, PDPK-1, or a perfect complement of *miR-375* could be  
10 designed and co-transfected with either the *miR-375* mimic or expression construct and luciferase activity could be assayed to determine whether the transfected *miRNAs* are functioning as they do endogenously, inhibiting their target mRNA.

Compared to the *miRNA* precursor system, the expression vector system for *miRNA* overexpression seems to be a more relevant method for increasing *miRNA* expression  
15 as it more closely mirrors true *miRNA* processing and results in expression changes on the order of magnitude of physiologic *miRNA* alterations. To determine which system is best suited for *miRNA* functional analysis, additional work must be done to determine which allows for proper *miRNA* function without disruption of endogenous *miRNA* machinery.

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The teachings of all of the references cited herein are hereby incorporated by reference in their entirety.

## EQUIVALENTS

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

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## CLAIMS

What is claimed is:

1. A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio selected from the group consisting of a miR-21/miR-375  
5 expression ratio, a miR-181d/miR-375 expression ratio, a miR-181b/miR-375 expression ratio, a miR-491/miR-375 expression ratio, a miR-455/miR-375 expression ratio, a miR-18a/miR-375 expression ratio, a miR-130b/miR-375 expression ratio, a miR-221/miR-375 expression ratio, a miR-193b/miR-375 expression ratio, a miR-181a/miR-375 expression ratio, and a miR-18b/miR-375  
10 expression ratio in a sample, wherein a ratio greater than about 1.0 is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma.
2. The method of Claim 1, wherein the expression ratio is the miR-221/miR-375 expression ratio.
- 15 3. The method of Claim 1, wherein the expression ratio is the miR-21/miR-375 expression ratio.
4. The method of Claim 1, wherein the expression ratio is the miR-18a/miR-375 expression ratio.
5. The method of Claim 1, wherein the carcinoma is a squamous cell carcinoma.
- 20 6. The method of Claim 5, wherein the squamous cell carcinoma is at least one member selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma.
7. The method of Claim 6, wherein the head squamous cell carcinoma is an oral cavity squamous cell carcinoma.

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8. The method of Claim 6, wherein the neck squamous cell carcinoma includes at least one member selected from the group consisting of a pharyngeal squamous cell carcinoma and a laryngeal squamous cell carcinoma.
9. The method of Claim 1, wherein the carcinoma is an adenocarcinoma.
- 5 10. The method of Claim 9, wherein the adenocarcinoma is an esophageal adenocarcinoma.
11. The method of Claim 1, wherein the sample includes a tissue sample.
12. The method of Claim 11, wherein the tissue sample includes at least one member selected from the group consisting of an esophageal tissue sample, a laryngeal tissue sample, a pharyngeal tissue sample and an oral tissue sample.
- 10 13. The method of Claim 11, wherein the tissue sample includes at least a portion of a tumor.
14. The method of Claim 13, wherein the tumor is an early-stage tumor.
15. The method of Claim 13, wherein the tumor is a human papilloma virus positive tumor.
- 15 16. The method of Claim 13, wherein the tumor is a human papilloma virus negative tumor.
17. The method of Claim 11, wherein the tissue sample includes a preneoplastic lesion.
- 20 18. The method of Claim 1, wherein the sample includes a saliva sample.
19. A method of diagnosing a carcinoma, comprising the step of comparing an expression level of at least two microRNAs selected from the group consisting of miR-21, miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b,

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- miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma.
- 5
20. The method of Claim 19, wherein the miR-375 expression level in the sample is less than the control miR-375 control expression level.
21. The method of Claim 19, wherein the carcinoma is a squamous cell carcinoma.
22. The method of Claim 19, wherein the squamous cell carcinoma is at least one member selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma.
- 10
23. The method of Claim 21, wherein the squamous cell carcinoma is at least one member selected from the group consisting of an oral cavity squamous cell carcinoma, a pharyngeal squamous cell carcinoma and a laryngeal squamous cell carcinoma.
- 15
24. The method of Claim 1, wherein the carcinoma is an adenocarcinoma.
25. The method of Claim 24, wherein the adenocarcinoma is an esophageal adenocarcinoma.
26. The method of Claim 19, wherein the sample includes a tissue sample.
- 20 27. The method of Claim 26, wherein the tissue includes at least one member selected from the group consisting of an esophageal tissue sample, a laryngeal tissue sample, a pharyngeal tissue sample and an oral tissue sample.
28. The method of Claim 26, wherein the tissue sample includes at least a portion of a tumor.

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29. The method of Claim 28, wherein the tumor is an early-stage tumor.
30. The method of Claim 28, wherein the tumor is a human papilloma virus positive tumor.
31. The method of Claim 28, wherein the tumor is a human papilloma virus negative  
5 tumor.
32. The method of Claim 26, wherein the tissue sample includes a preneoplastic lesion.
33. The method of Claim 17, wherein the sample includes a saliva sample.
34. A method of diagnosing a carcinoma selected from the group consisting of a  
10 head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of at least one microRNA selected from the group consisting of miR-181d, miR-181b, miR-491, miR-455, miR-18a, miR-130b, miR-221, miR-193b, miR-181a, miR-18b and miR-375 in a  
15 sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNA in the sample relative to the control expression level is diagnostic of the head squamous cell carcinoma and the neck squamous cell carcinoma and identifies a subject that would potentially benefit from a therapy to treat the head squamous cell carcinoma and the neck squamous cell carcinoma.
- 20 35. The method of Claim 34, wherein the squamous cell carcinoma is the head squamous cell carcinoma.
36. The method of Claim 34, wherein the squamous cell carcinoma is the neck squamous cell carcinoma.
37. The method of Claim 34, wherein the expression level of the microRNA in the  
25 sample is at least about two-fold greater than the control expression level.

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38. The method of Claim 34, wherein the expression level of the microRNA in the sample is at least about twenty-fold less than the control expression level.
39. The method of Claim 34, further comprising the step of comparing the expression level of miR-21 in the sample to a miR-21 control expression level.
- 5 40. The method of Claim 34, wherein the sample includes a tissue sample.
41. The method of Claim 40, wherein the tissue sample is at least one member selected from the group consisting of an esophageal tissue sample, a laryngeal tissue sample, a pharyngeal tissue sample and an oral tissue sample.
42. The method of Claim 40, wherein the tissue sample includes at least a portion of  
10 a tumor.
43. The method of Claim 42, wherein the tumor is an early-stage tumor.
44. The method of Claim 42, wherein the tumor is a human papilloma virus positive tumor.
45. The method of Claim 42, wherein the tumor is a human papilloma virus negative  
15 tumor.
46. The method of Claim 40, wherein the tissue sample includes a preneoplastic lesion.
47. The method of Claim 34, wherein the sample includes a saliva sample.
48. A method of treating a squamous cell carcinoma in a subject, comprising the  
20 step of administering a nucleic acid encoding a miR-375 gene product to the subject.

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49. The method of Claim 48, wherein the squamous cell carcinoma is selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma.
50. The method of Claim 48, wherein the nucleic acid encoding the miR-375 gene  
5 product includes an expression vector.
51. The method of Claim 50, wherein the expression vector includes an RNA polymerase II promoter.
52. The method of Claim 51, wherein the RNA polymerase II promoter includes a constitutive promoter.
- 10 53. The method of Claim 52, wherein the constitutive promoter includes a human cytomegalovirus immediate early promoter.
54. The method of Claim 48, wherein the nucleic acid encoding the miR-375 gene product includes a nucleic acid that encodes a premature miR-375 gene product.
55. A method of optimizing treatment of a subject having a squamous cell  
15 carcinoma, comprising the step of determining an expression level of a miR-21 gene product in a sample from the subject, wherein overexpression level of the miR-21 gene product in the sample compared to expression in a reference miR21-gene product identifies a subject that has an aggressive squamous cell carcinoma that would potentially benefit from a therapy to treat the aggressive  
20 squamous cell carcinoma.
56. The method of Claim 55, wherein the squamous cell carcinoma is selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma.

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Figure 1A

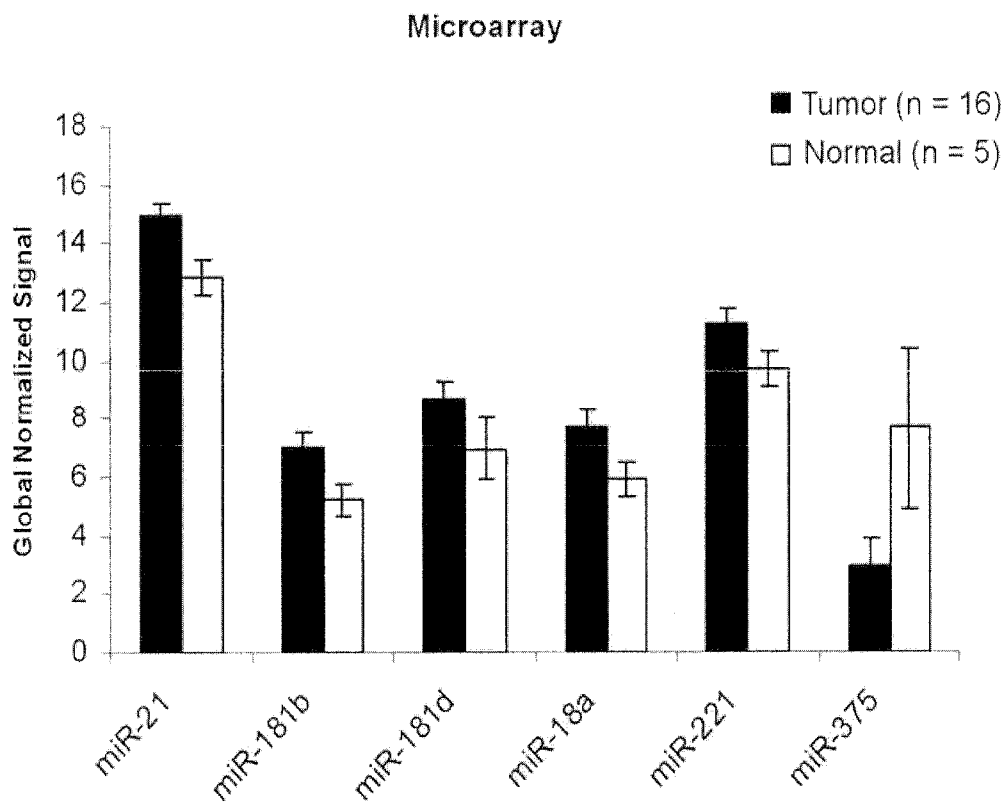
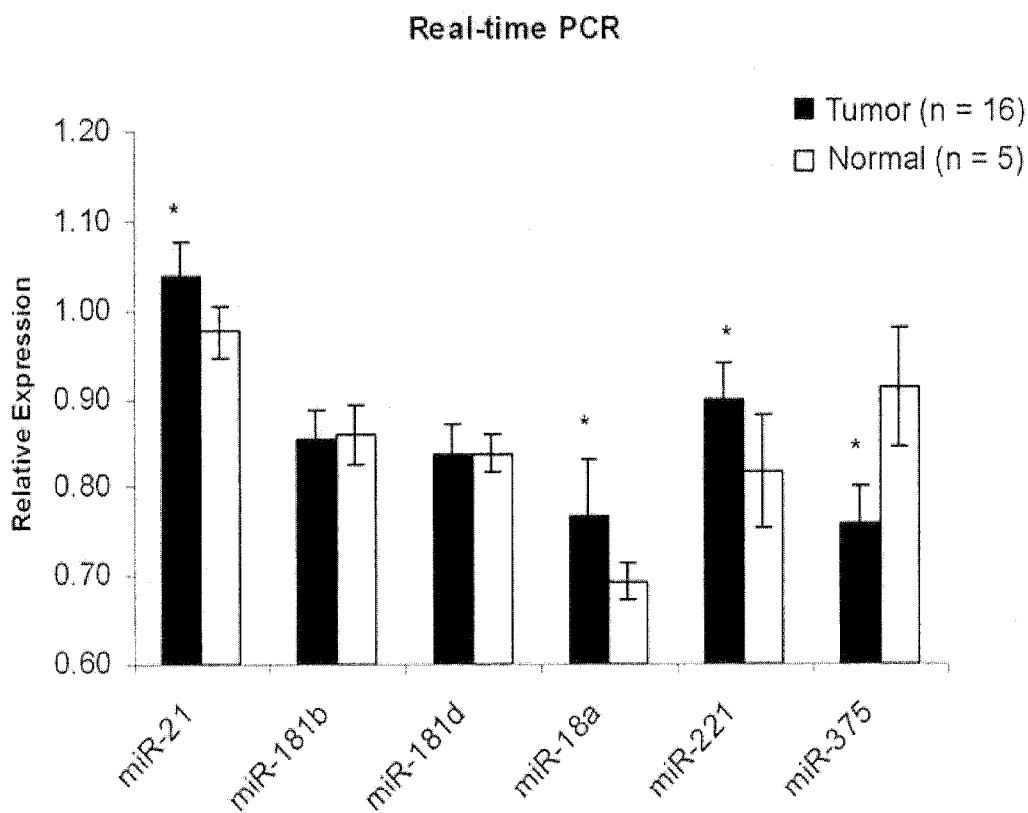


Figure 1B



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Figure 2A

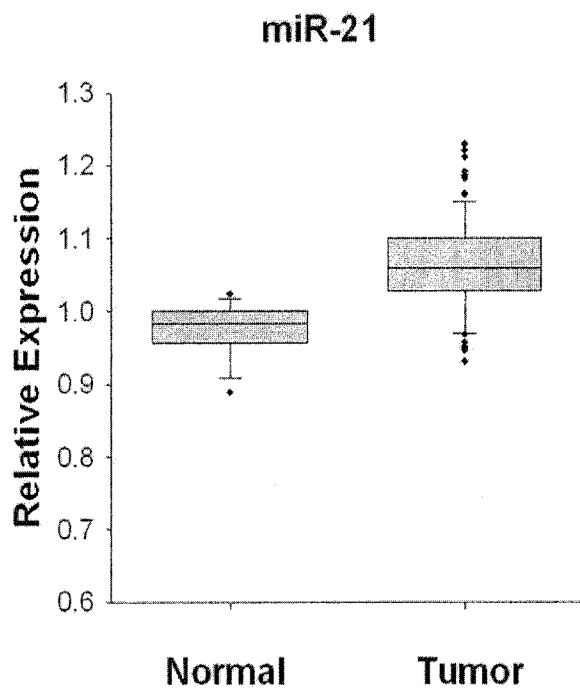


Figure 2B

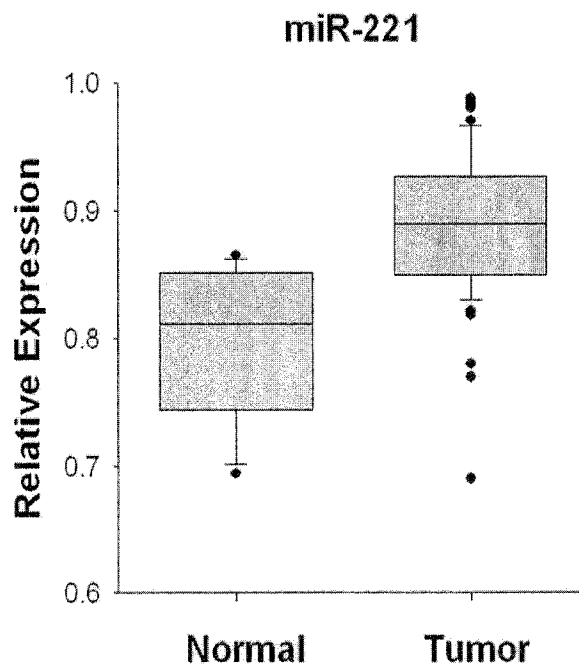
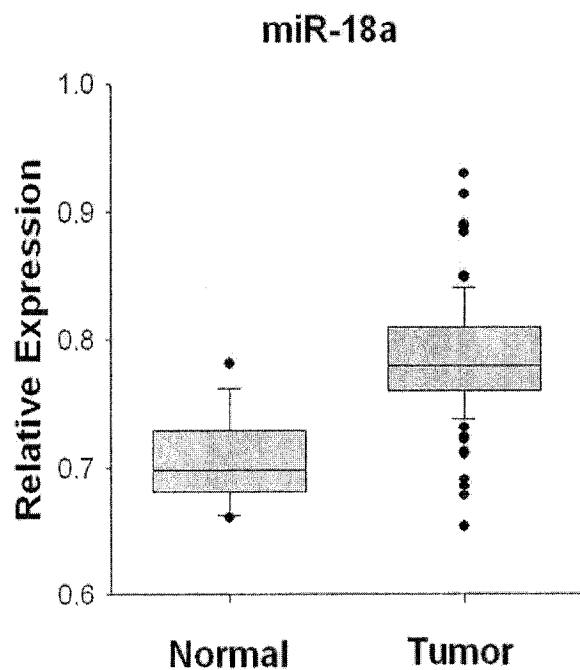
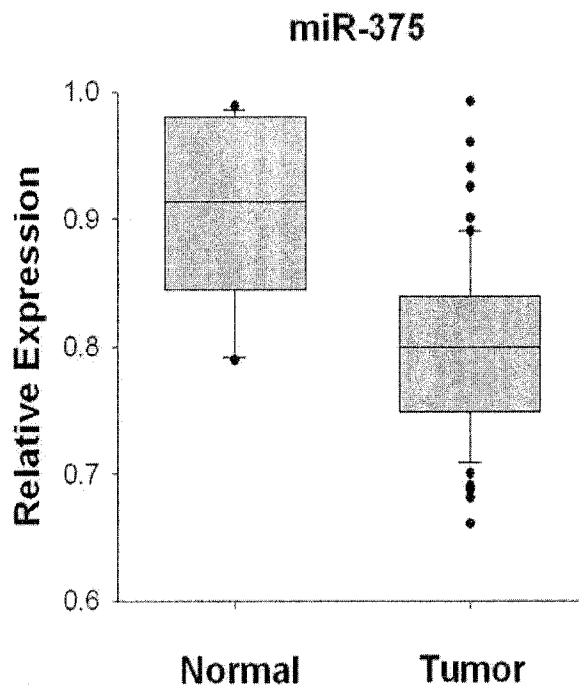
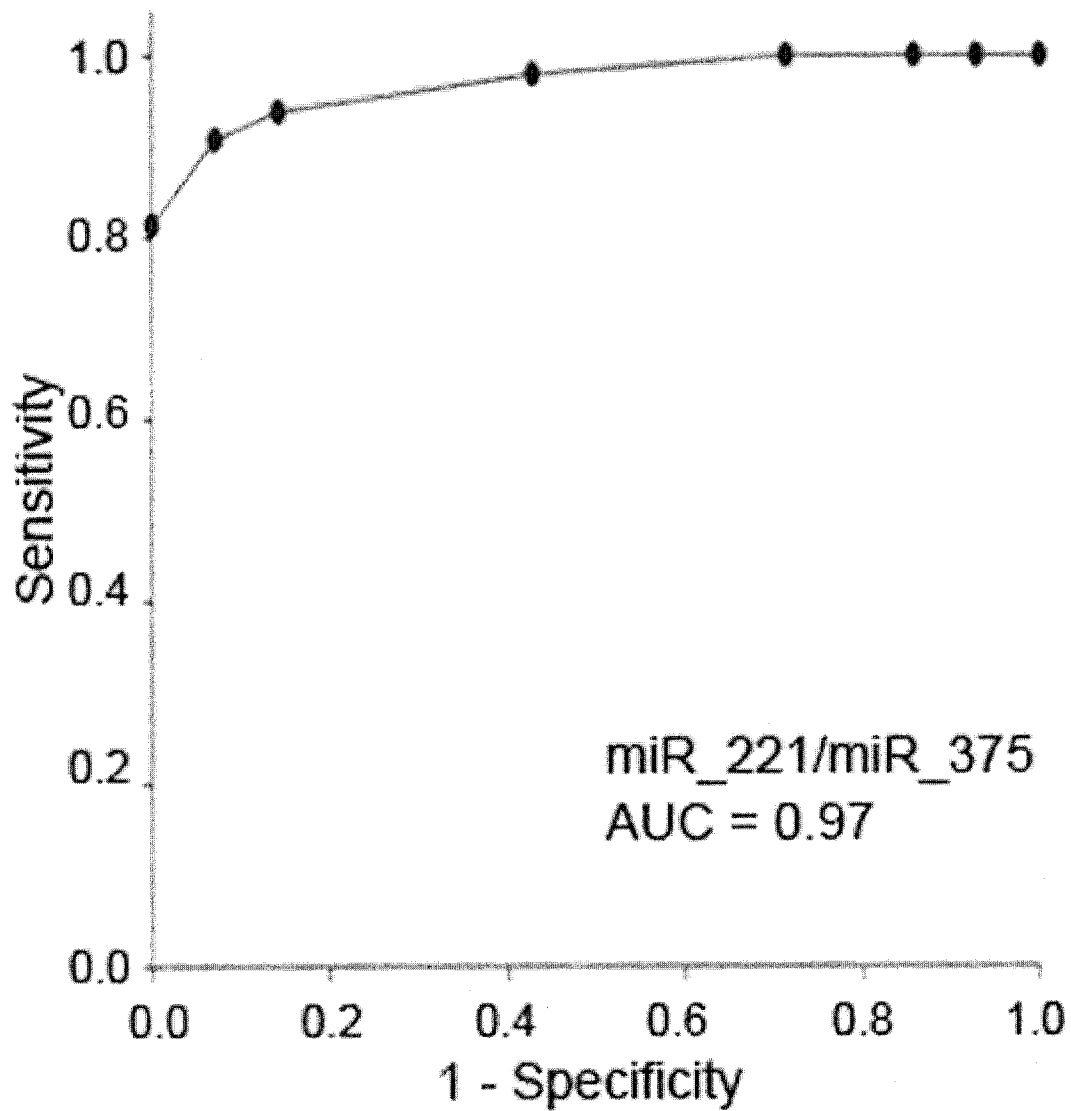


Figure 2C

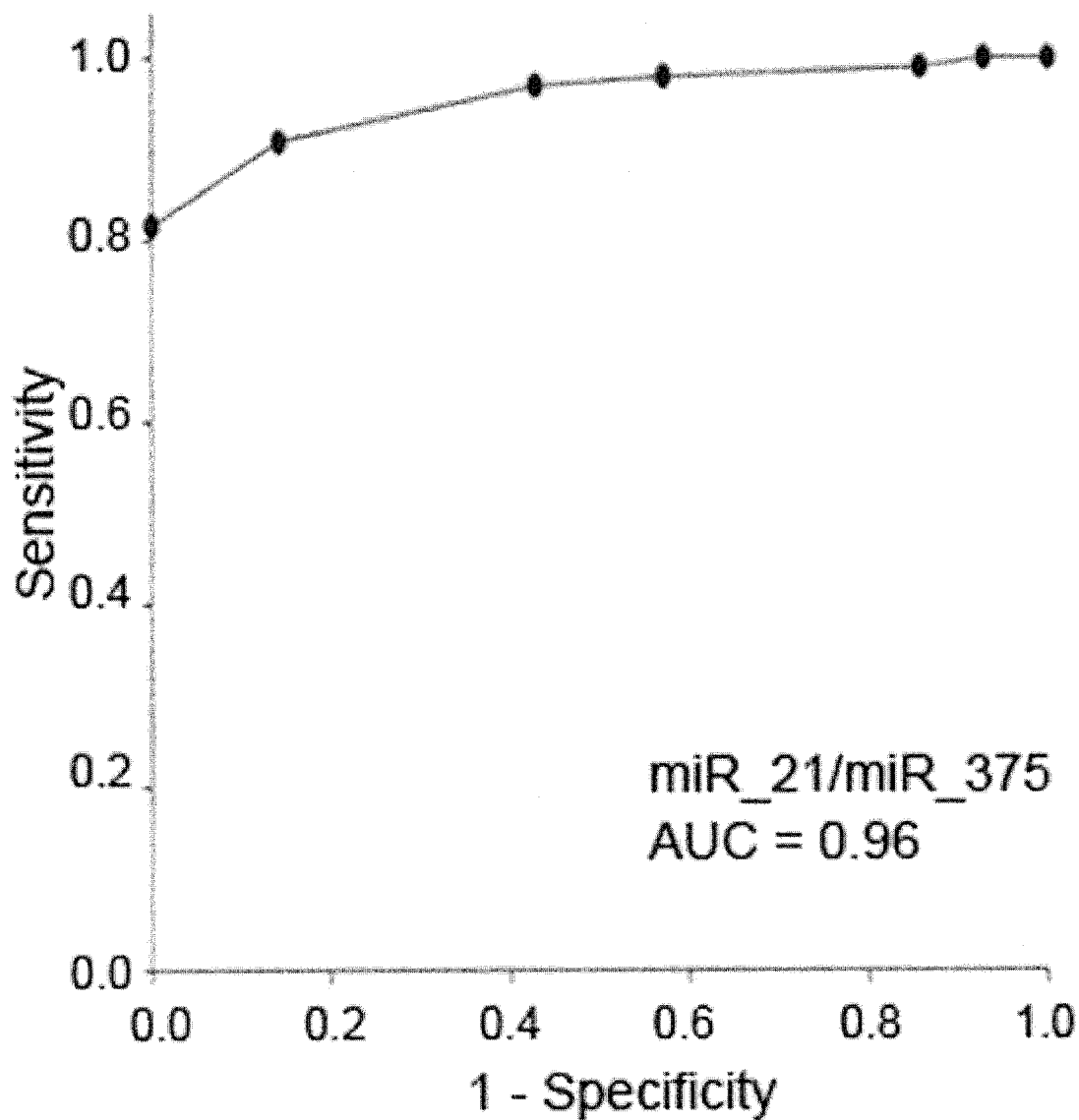
Figure 2D

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Figure 3A



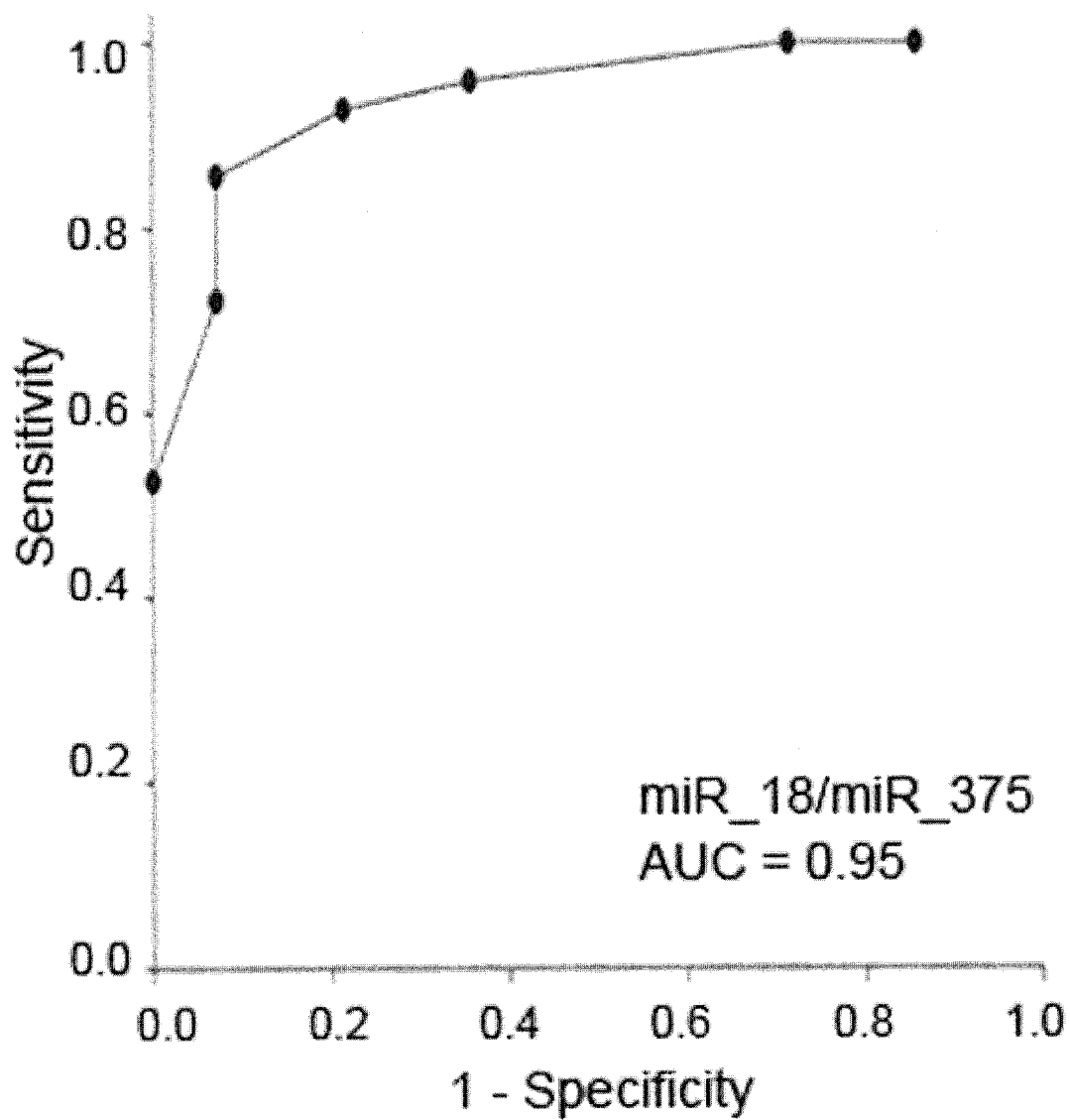
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# Figure 3B



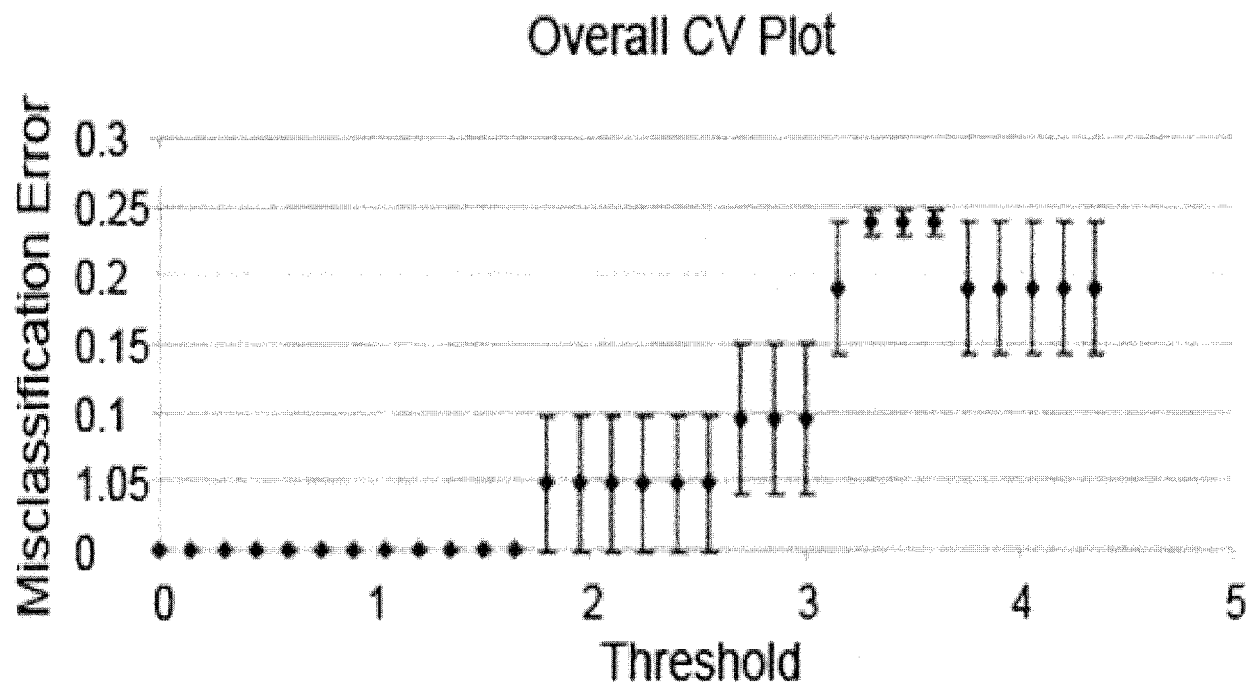
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## Figure 3C



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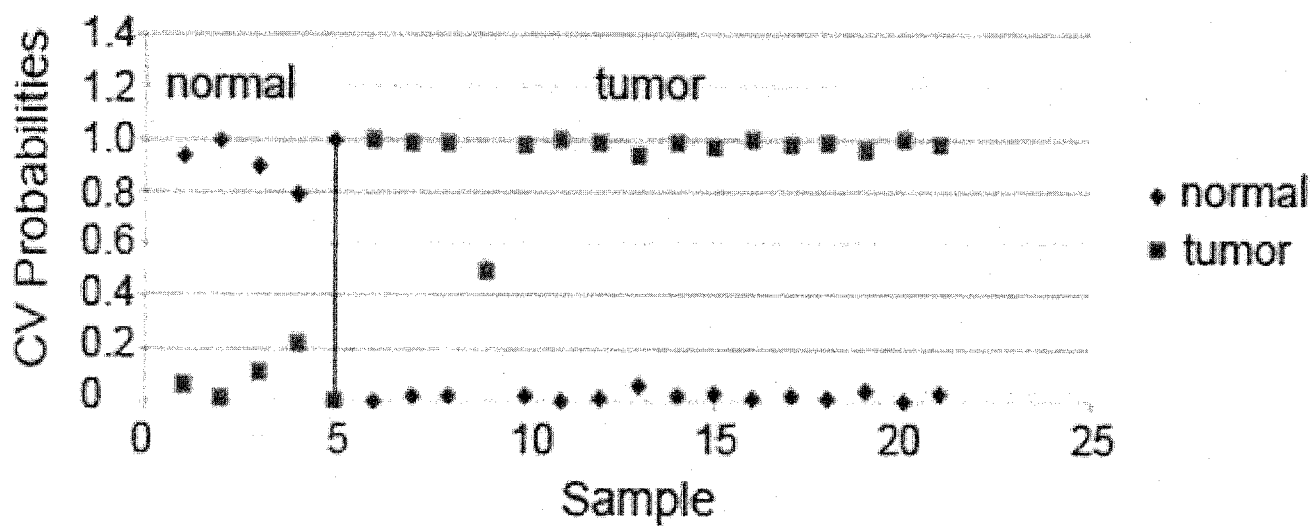
# Figure 4A



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# Figure 4B

Cross-validated Probabilities (Threshold=1.65138)



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# Figure 4C

| miRNA          | Tumor<br>Score | Normal<br>Score |
|----------------|----------------|-----------------|
| hsa_miR_375    | -0.33          | 1.06            |
| ambi_miR_562   | 0.22           | -0.69           |
| ambi_miR_7083  | 0.20           | -0.65           |
| hsa_miR_21     | 0.20           | -0.63           |
| hsa_miR_181d   | 0.09           | -0.30           |
| hsa_miR_181b   | 0.09           | -0.27           |
| hsa_miR_455    | 0.07           | -0.21           |
| ambi_miR_13258 | 0.06           | -0.19           |
| ambi_miR_11541 | 0.05           | -0.15           |

CV Confusion Matrix (Threshold = 1.50512)

| True/Predicted | normal | tumor | Class Error Rate |
|----------------|--------|-------|------------------|
| normal         | 5      | 0     | 0                |
| tumor          | 0      | 16    | 0                |

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Figure 5

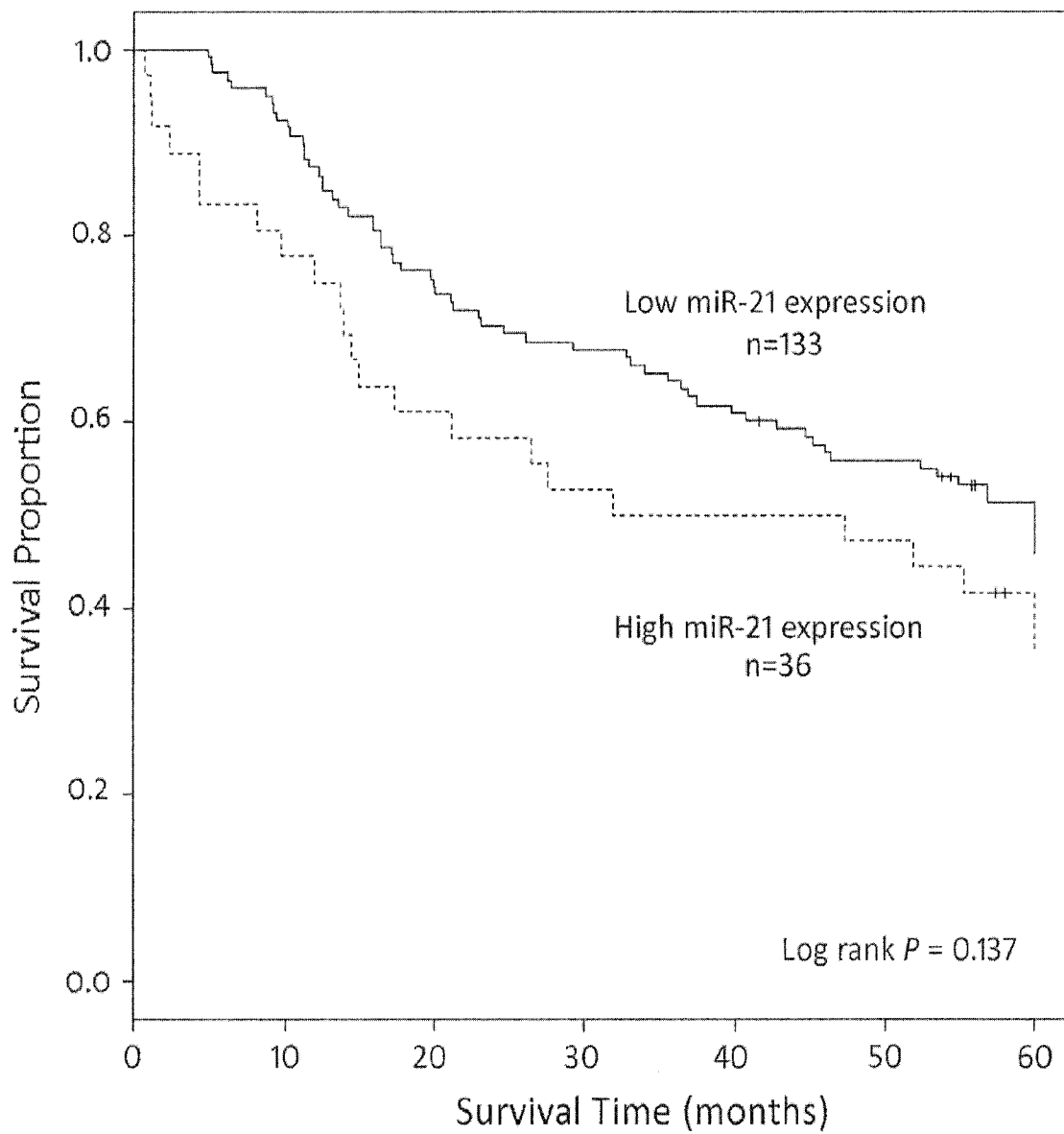


Figure 6A

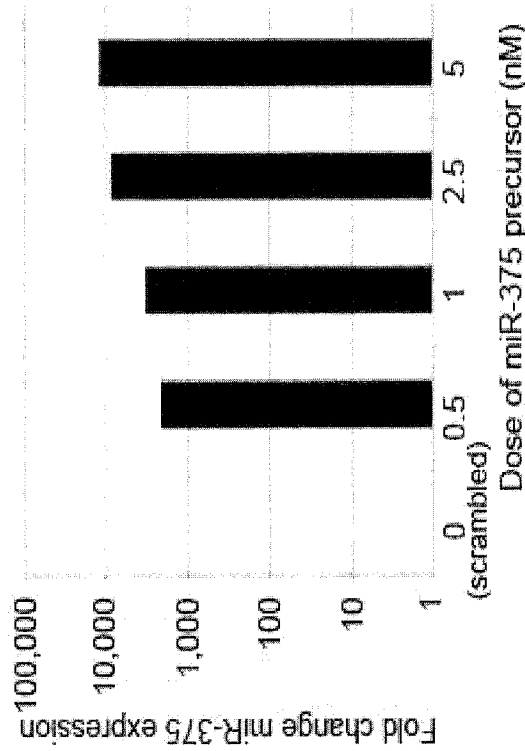


Figure 6B

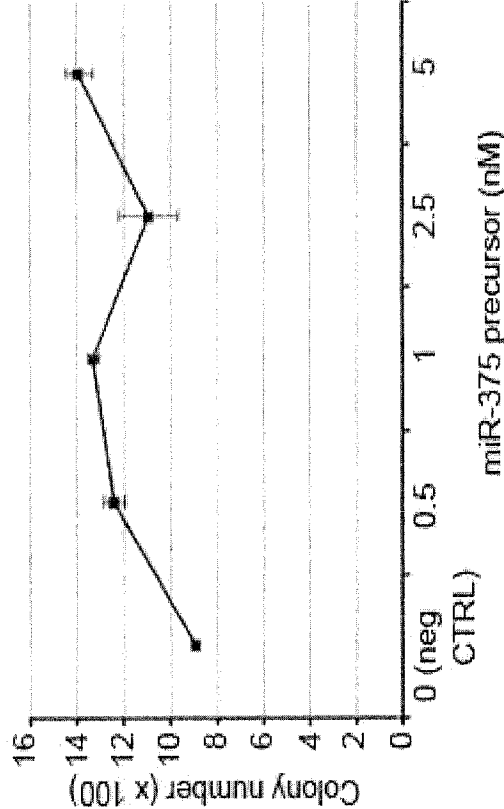
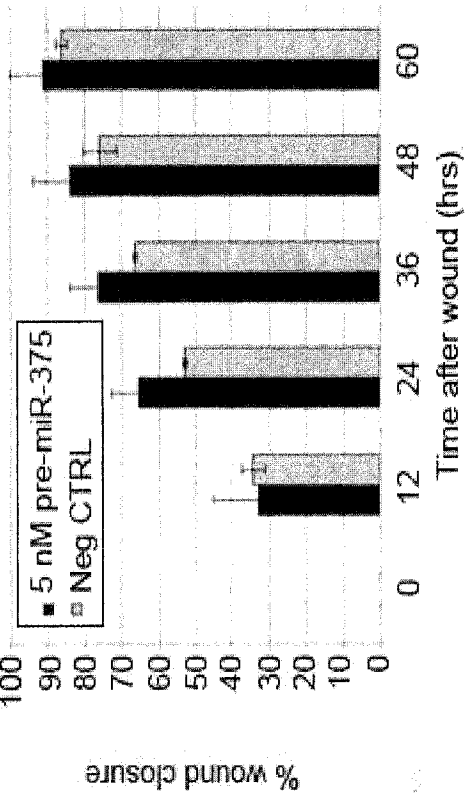


Figure 6C



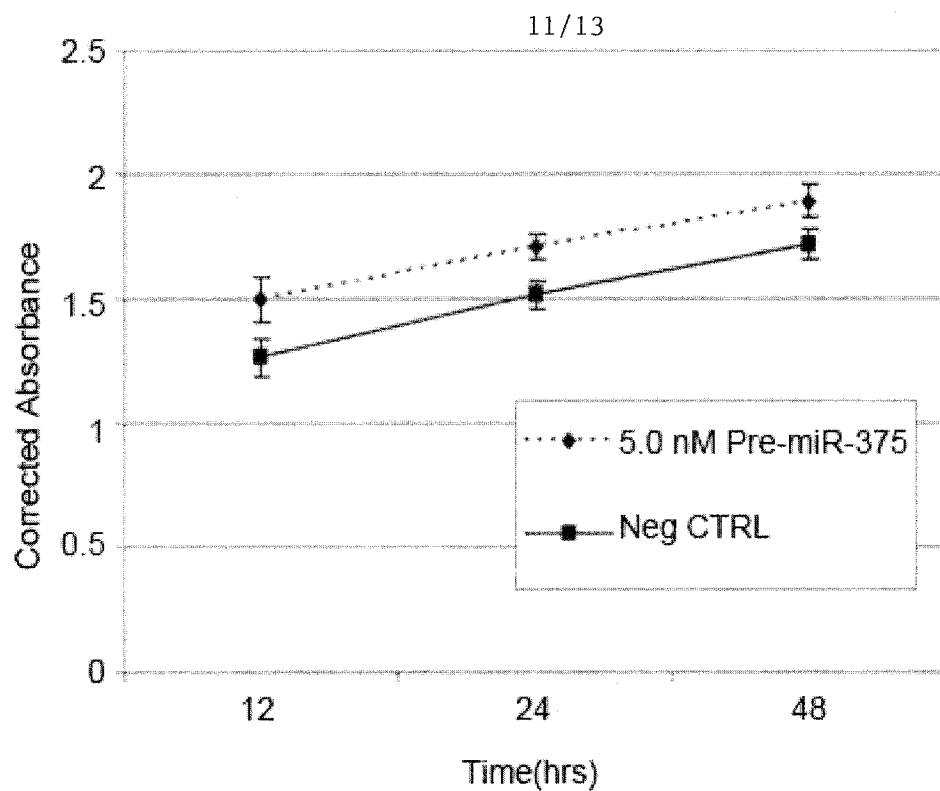


Figure 7A

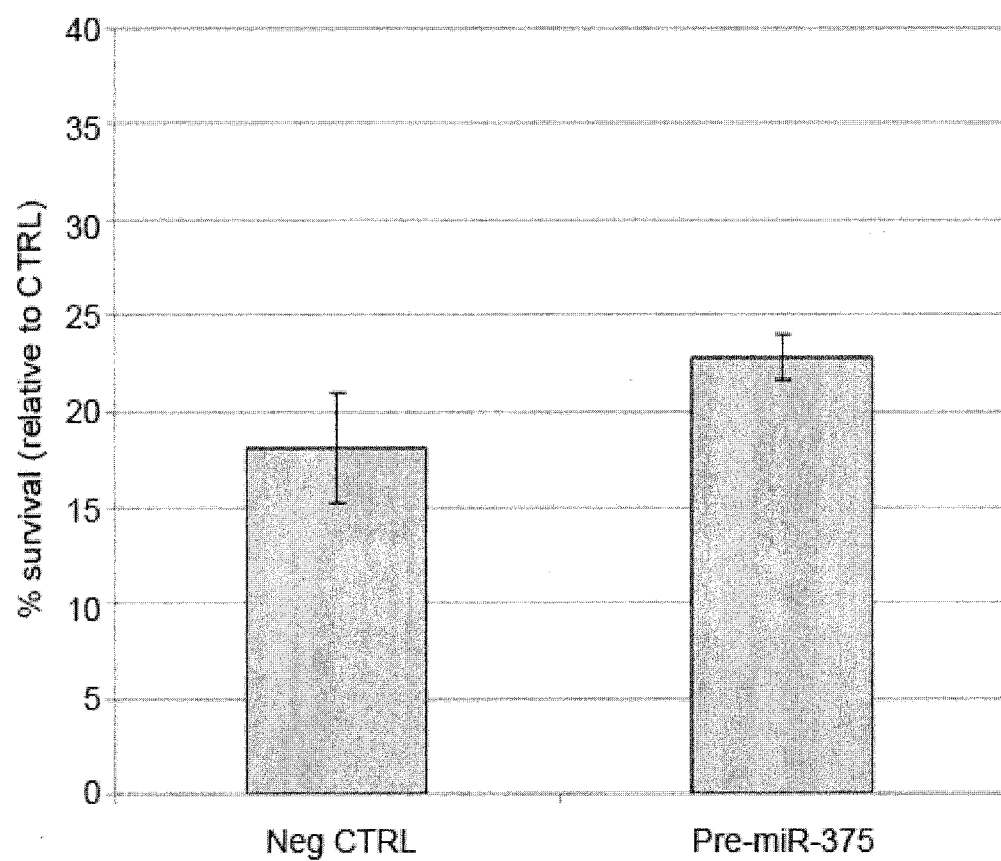
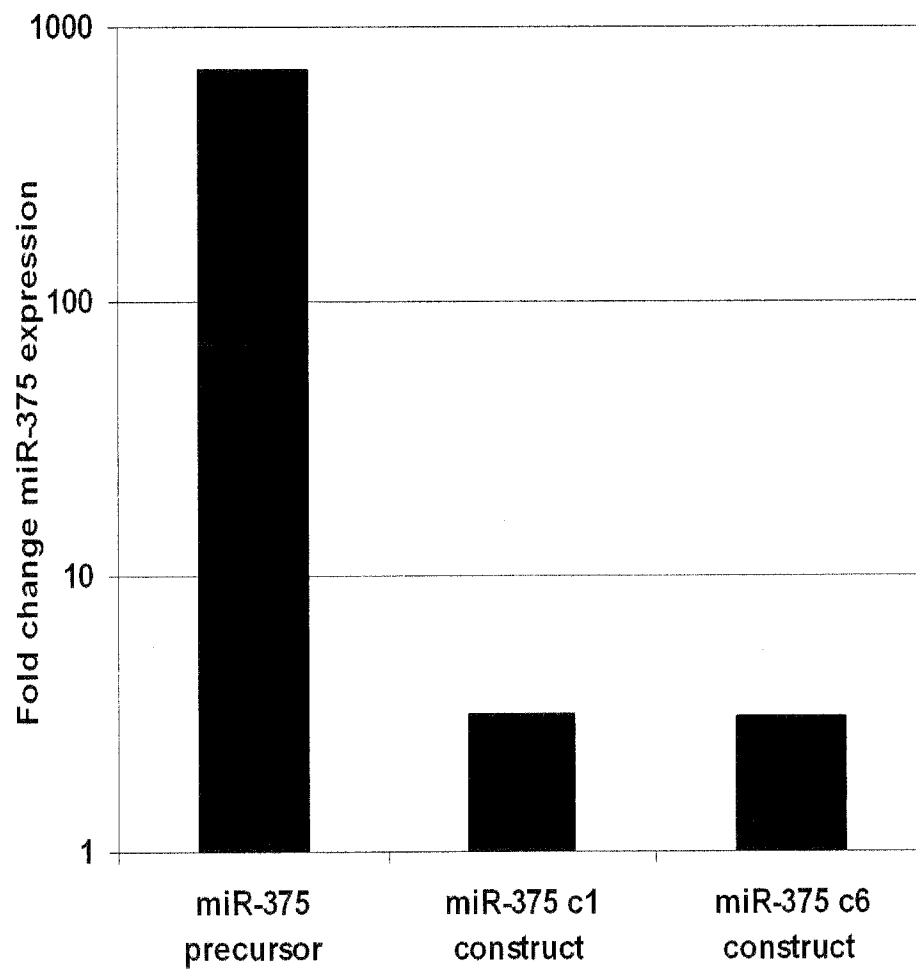


Figure 7B

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Figure 8



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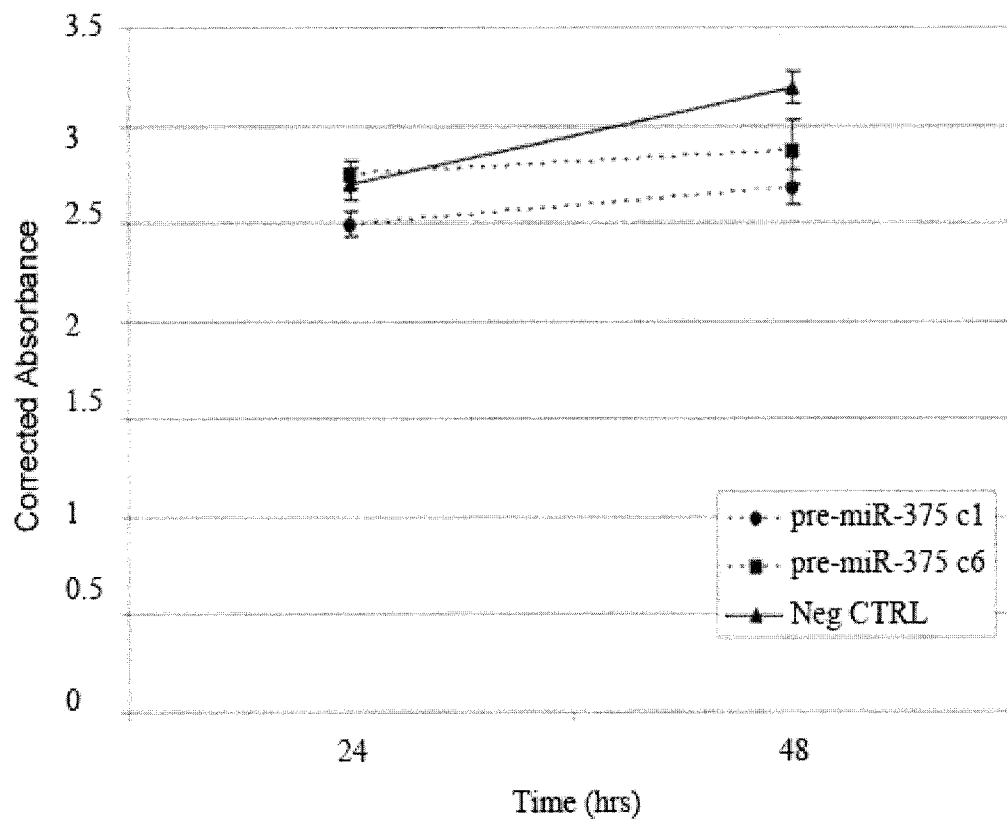


Figure 9A

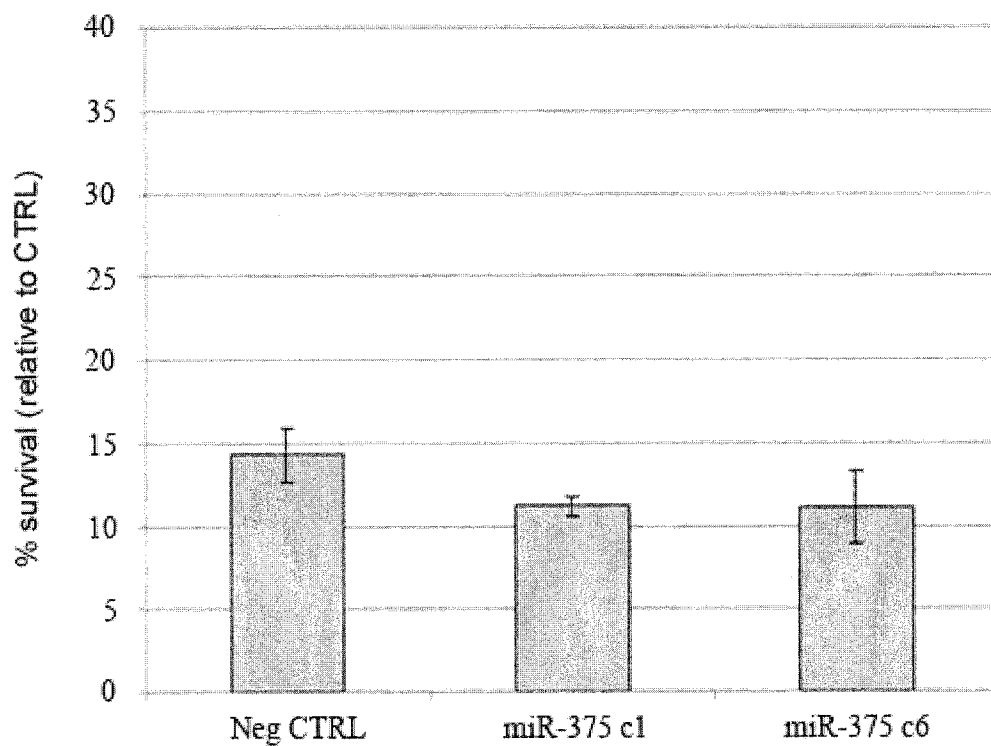


Figure 9B

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/026388

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, BIOSIS, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                     | Relevant to claim No.           |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
| X         | <p>MCLEMORE M S ET AL: "miR-21 upregulation and loss of PTEN expression in head &amp; neck squamous cell carcinoma" LABORATORY INVESTIGATION, vol. 88, no. Suppl. 1, January 2008 (2008-01), page 239A, XP008121337 &amp; 97TH ANNUAL MEETING OF THE UNITED-STATES-AND-CANADIAN-ACADEMY-OF-PATHOLOGY; DENVER, CO, USA; MARCH 01 -07, 2008 ISSN: 0023-6837 the whole document</p> <p>-----<br/>-/--</p> | <p>19,<br/>21-23,<br/>26,28</p> |

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

5 August 2010

Date of mailing of the international search report

11/08/2010

Name and mailing address of the ISA/

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Authorized officer

Botz, Jürgen

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/026388

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                 | Relevant to claim No.                      |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|
| X         | <p>CHANG STEVEN S ET AL: "MicroRNA alterations in head and neck squamous cell carcinoma"</p> <p>INTERNATIONAL JOURNAL OF CANCER, vol. 123, no. 12, December 2008 (2008-12), pages 2791-2797, XP002577189</p> <p>ISSN: 0020-7136</p> <p>the whole document</p>                                                                                                                                                                      | <p>19,<br/>21-23,<br/>26,28</p>            |
| X         | <p>TRAN ET AL: "MicroRNA expression profiles in head and neck cancer cell lines"</p> <p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US LNKD-DOI:10.1016/J.BBRC.2007.03.201, vol. 358, no. 1, 12 May 2007 (2007-05-12), pages 12-17, XP022077599</p> <p>ISSN: 0006-291X</p> <p>the whole document</p>                                                                                     | <p>19,<br/>21-23,<br/>26,28,<br/>55,56</p> |
| X         | <p>MORI ETAL.: "MicroRNA-21 induces cell proliferation and invasion in esophageal squamous cell carcinoma"</p> <p>MOLECULAR MEDICINE REPORTS, vol. 2, 1 March 2009 (2009-03-01), pages 235-239, XP002577203</p> <p>the whole document</p>                                                                                                                                                                                          | <p>9,10,19,<br/>21-26,28</p>               |
| X         | <p>LADEIRO YANNICK ET AL: "MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations"</p> <p>HEPATOLOGY, vol. 47, no. 6, June 2008 (2008-06), pages 1955-1963, XP002577191</p> <p>ISSN: 0270-9139</p> <p>the whole document</p>                                                                                                                                | <p>19,26,28</p>                            |
| X         | <p>LIU J J ET AL: "Esophageal Adenocarcinomas Overexpress MicroRNA-21: A Tissue Microarray Study of 64 Cases Using In-Situ Hybridization"</p> <p>LABORATORY INVESTIGATION, vol. 89, no. Suppl. 1, January 2009 (2009-01), page 138A, XP008121338</p> <p>&amp; 98TH ANNUAL MEETING OF THE UNITED-STATES-AND-CANADIAN-ACADEMY-OF-PATHOLOGY; BOSTON, MA, USA; MARCH 07 -13, 2009</p> <p>ISSN: 0023-6837</p> <p>the whole document</p> | <p>9,10</p>                                |

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## INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/026388

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                      | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | NEELY L A ET AL: "A microRNA expression ratio defining the invasive phenotype in bladder tumors"<br>UROLOGIC ONCOLOGY, ELSEVIER, NEW YORK, NY, US,<br>vol. 28, no. 1,<br>16 September 2008 (2008-09-16), pages<br>39-48, XP026820985<br>ISSN: 1078-1439 [retrieved on 2008-09-16]<br>the whole document | 1-56                  |
| A         | WO 2008/153692 A2 (BRIGHAM & WOMENS HOSPITAL [US]; KRICHEVSKY ANNA [US]; MOLLENHAUER BRIT)<br>18 December 2008 (2008-12-18)<br>the whole document                                                                                                                                                       | 1-56                  |
| X,P       | WO 2009/049129 A1 (UNIV OHIO STATE RES FOUND [US]; GOVERNMENT OF THE US SECRETARY [US]; CR) 16 April 2009 (2009-04-16)<br>the whole document                                                                                                                                                            | 1-56                  |
| X,P       | WO 2009/147525 A1 (KAROLINSKA INST INNOVATIONS AB [SE]; PIVARCSI ANDOR [SE]; SONKOLY ENIK)<br>10 December 2009 (2009-12-10)<br>the whole document                                                                                                                                                       | 1-56                  |

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US2010/026388

### Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

48-56(completely); 1-47(partially)

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 48-56(completely); 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-21/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-21 and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-375, a method of treating a squamous cell carcinoma in a subject, comprising the step of administering a nucleic acid encoding a miR-375 gene product to the subject as well as a method of optimizing treatment of a subject having a squamous cell carcinoma, comprising the step of determining an expression level of a miR-21 gene product in a sample from the subject, wherein overexpression level of the miR-21 gene product in the sample compared to expression in a reference miR-21-gene product identifies a subject that has an aggressive squamous cell carcinoma that would potentially benefit from a therapy to treat the aggressive squamous cell carcinoma.

---

2. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-181d/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-181d and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-181d.

---

3. claims: 1-47(partially)

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-181b/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-181b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-181b.

---

## 4. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-491/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-491 and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-491.

---

## 5. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-455/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-455 and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-455.

---

## 6. claims: 1-47(partially)

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-18a/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-18a and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-18a.

---

## 7. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-130b/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-130b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-130b.

---

## 8. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-221/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-221 and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-221.

---

## 9. claims: 1-47(partially)

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-193b/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-193b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-193b.

---

## 10. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-181a/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-181a and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-181a.

---

## 11. claims: 1-47(partially)

A method of diagnosing a carcinoma, comprising the step of determining at least one expression ratio, namely miR-18b/miR-375, a method of diagnosing a carcinoma, comprising the step of comparing an expression level of miR-18b and miR-375 in a sample from the subject to a corresponding control expression level, wherein a difference in the expression level of the microRNAs in the sample relative to the control expression level is diagnostic of the carcinoma and identifies a subject that would potentially benefit from a therapy to treat the carcinoma, a method of diagnosing a carcinoma selected from the group consisting of a head squamous cell carcinoma and a neck squamous cell carcinoma, comprising the step of comparing an expression level of miR-18b.

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# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/026388

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 2008153692 A2                          | 18-12-2008          | US 2010167948 A1           | 01-07-2010          |
| WO 2009049129 A1                          | 16-04-2009          | AU 2008310704 A1           | 16-04-2009          |
|                                           |                     | CA 2702241 A1              | 16-04-2009          |
|                                           |                     | EP 2212440 A1              | 04-08-2010          |
| WO 2009147525 A1                          | 10-12-2009          | NONE                       |                     |