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Decreased Expression of miR-125b and miR-100 in Oral Cancer Cells Contributes to Malignancy

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Abstract

Altered microRNA (miRNA) expression profiles have been observed in numerous malignancies, including oral squamous cell carcinoma (OSCC). However, their role in disease is not entirely clear. Several genetic aberrations are characteristic of OSCC, with amplification of chromosomal band 11q13 and loss of distal 11q being amongst the most prevalent. It is not known if the expression levels of miRNAs in these regions are altered or whether they play a role in disease. We hypothesize that the expression of miRNAs mapping to 11q are altered in OSCC due to loss or amplification of chromosomal material, and that this contributes to the development and progression of OSCC. We found that miR-125b and miR-100 are downregulated in OSCC tumors and cell lines and that transfecting cells with exogenous miR-125b and miR-100 significantly reduced cell proliferation and modified the expression of target and non-target genes, including some that are overexpressed in radioresistant OSCC cells. In conclusion, the downregulation of miR-125b and miR-100 in OSCC appears to play an important role in the development and/or progression of disease and may contribute to the loss of sensitivity to ionizing radiation.

INTRODUCTION

MicroRNAs are a class of small non-protein encoding RNAs that can inhibit translation. Therefore, they play an important role in gene regulation. MiRNAs are involved in numerous biological processes and have been conserved throughout evolution (Pasquinelli et al., 2000). They are transcribed individually or in combination (Mattick and Makunin, 2005) into long primary transcripts (pri-miRNAs) by RNA polymerase II or III (Cai et al., 2004; Lee et al., 2004; Borchert et al., 2006). Drosha cleaves pri-miRNAs into 70–100 nucleotide (nt) pre-miRNAs that form specific secondary hairpin-loop structures (Denli et al., 2004; Zhang et al., 2007a). Pre-miRNAs are translocated to the cytoplasm (Bohnsack et al., 2004; Zhang et al., 2007a) and modified by Dicer into small double-stranded RNA molecules that contain the mature miRNA, 16–29 nt in length (Wiemer, 2007), and its antisense strand (Hutvagner et al., 2001; Wiemer, 2007). This duplex is then unwound by a

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helicase and the mature miRNA is loaded onto the RNA-induced silencing complex (RISC), while the antisense strand is degraded (Gregory et al., 2005). Once associated with RISC, miRNAs bind to the 3' UTR (untranslated regions) of target mRNAs and inhibit translation by either sequestering the transcript, mRNA cleavage, or mRNA degradation (Zhang et al., 2007b). To date, more than 650 miRNAs have been annotated in the Human genome (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006) and as many as 1,000 have been predicted through bioinformatics (Berezikov et al., 2005). The miRNA expression profiles of many cancers have been shown to be significantly different than non-cancerous tissue (Wiemer, 2007), and in some cases, they can be used to differentiate particular malignancies (Calin et al., 2004; Lu et al., 2005; Calin and Croce, 2006; Volinia et al., 2006). Although miRNAs are located throughout the genome, many are clustered near fragile sites and as such, can be affected by chromosomal losses and gains (Calin et al., 2004). As with protein encoding genes, loss or gain of chromosomal material can lead to loss of heterozygosity, haploinsufficiency, copy number gain, or amplification, all of which can affect miRNA expression (Calin et al., 2004).

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and includes tumors within the oral cavity, larynx, and pharynx (Jemal et al., 2008). Causes of HNSCC include tobacco and alcohol use as well as human papillomavirus infection. Although diagnosis and treatment of HNSCC have improved, the survival rate has not increased substantially in 40 years. In this study, we focus on those HNSCC occurring within the oral cavity, otherwise known as oral squamous cell carcinomas (OSCC).

Genetic aberrations are common in OSCC, with alterations of chromosome 11 being amongst the most common. Loss of distal 11q from the fragile site, *FRA11F* (11q14.2) to 11qter occurs in over 50% of OSCC (Jin et al., 1998; 2006; Martin et al., 2007; Parikh et al., 2007), and amplification of chromosomal band 11q13 occurs in ~45% of OSCC (Gollin, 2001; Jin et al., 2006; Martin et al., 2007; Parikh et al., 2007). Amplification of 11q13 contributes to the poor prognosis of OSCC (Akervall et al., 1997; Huang et al., 2002; 2006; Parikh et al., 2007), but how the loss of distal 11q impacts OSCC is just now being elucidated (Jin et al., 1998; 2006; Parikh et al., 2007).

Here we examined the expression levels of selected miRNAs mapping to 11q (Fig. 1) in OSCC primary tumors and cell lines compared to normal human oral keratinocytes (NHOK). We hypothesize that miRNAs mapping to 11q have altered expression levels due to the loss or amplification of chromosomal material and that this affects the translation of target genes, thereby ultimately contributing to the disease. We found that both miR-125b and miR-100 levels were substantially lower in OSCC cell lines and tumors than in NHOK controls. We also found that increasing levels of both miR-125b and miR-100 reduces cell proliferation and alters global gene expression.

MATERIALS AND METHODS

Cell Culture

Ten OSCC cell lines were chosen from our UPCI:SCC collection (Table 1) (Martin et al., 2007; White et al., 2007) and cultured in Minimal Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with gentamicin, L-glutamine, nonessential amino acids, and 10% fetal bovine serum. NHOK controls were derived from five individual uvulopalatopharyngoplasty (UP3) specimens (Table 1), and were cultured in Keratinocyte Serum-free medium (Ker-sfm) (Invitrogen), supplemented with hEGF in 0.1% bovine serum albumin, penicillin/streptomycin, L-glutamine, and bovine pituitary extract. NHOK cells were grown to high density in 50% DMEM / 50% F12 (DFK) with the same supplements v/

as Ker-sfm. OSCC primary tumor samples and NHOK controls were obtained from the University of Pittsburgh Head & Neck SPORE Tissue Bank.

RNA Isolation

RNA was isolated using either Trizol® (Invitrogen) followed by the Phase Lock Gel™ system (Eppendorf, Westbury, NY) or the miRNeasy miRNA isolation kit (Qiagen, Valencia CA). RNA quality was assessed by gel electrophoresis or the 2100 Bioanalyzer (Agilent, Foster City, CA), and quantification was done with the DU®800 UV/Vis Spectrophotometer (Beckman Coulter, Fullerton, CA).

MiRNA Quantitative Reverse Transcriptase PCR

TaqMan® MicroRNA Assays were used to quantify the expression of miRNAs as well as the control, RNU43 (Applied Biosystems, Foster City, CA). Reverse transcription reactions were done using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) at 10 ng and 20 ng of total RNA. Three biological replicates of each OSCC cell line were examined. Quantitative PCR (QPCR) was done with the TaqMan® 2x Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) in triplicate, with no reverse transcriptase and no template controls included. The data were analyzed using the comparative CT method.

Fluorescence In Situ Hybridization

Our FISH experiments were performed following previously established protocols in our lab (Parikh et al., 2007; Reshmi et al., 2007). To prepare mitotic cells for fluorescence in situ hybridization (FISH), cells were treated for 5 h in 0.1 µg/ml Colcemid™ (Irvine Scientific, Santa Ana, CA), 18 min hypotonic KCl (0.075 M), and fixed in 3:1 methanol:glacial acetic acid. Cells were harvested and dropped onto slides, treated with RNase/2XSSC at 37°C, and dehydrated using a graded series of ethanol washes. Chromatin was denatured with 70% formamide at 75°C for 5 min followed by dehydration in a second graded series of ethanol washes.

Deidentified OSCC primary tumors were obtained from the Head and Neck SPORE Tissue Bank at the University of Pittsburgh. These were provided as 4–5 µm thick sections mounted on charged slides. Tissue sections were treated in 0.8% sodium citrate and fixed in Carnoy's (3:1 methanol:glacial acetic acid), then digested with pepsin (0.005% in 0.02 N HCl) at 37°C for 30–60 sec, rinsed in 70% ethanol for 30 sec, and pretreated in 2xSSC (pH 7) at 37°C for 60 min. The slides were then dehydrated in a graded series of ethanol washes, denatured in 70% formamide/2xSSC (pH 7) at 75°C for 5 min and dehydrated again in a graded series of ethanol washes. The probes were denatured and added to the slides. The slides were coverslipped, sealed with rubber cement, and hybridized in humidified chamber at 37°C for at least 12 h.

All FISH analyses were carried out using an Olympus BX-61 epifluorescence microscope (Olympus Microscopes, Melville, NY). The Applied Imaging CytoVision workstation with Genus v3.6 software was used for image capture and analysis (Applied Imaging, San Jose, CA). BAC probes were obtained from the Children's Hospital of Oakland Research Institute (CHORI, Oakland, CA). For this study, we used BAC clone RP11-241D13 (11q22.3), BAC clone RP11-100D20 (21q21.1), as well as the commercially available centromeric probe for chromosome 11 (CEP11) (Abbott Molecular Inc., Des Plaines, IL). All probes were labeled with either Spectrum Orange or Spectrum Green using the Abbott Molecular Nick Translation kit (Abbott Molecular Inc., Des Plaines, IL). Slides were counterstained with DAPI (100 ng/ml) prior to analysis.

For detecting the loss of distal 11q, we performed FISH with RP11-241D13, which is distal to *FRA11F* yet proximal to miR-125b-1 and miR-100, and compared its copy number to the copy number of CEP11. For determining the relative copy number of chromosome 21, we independently compared the copy number of CEP11 to RP11-100D20, which is a chromosome 21 probe that encompasses miR-125b-2.

Transfections

For all transfections, we used the UPCI:SCC029 cell line. To establish optimal transfection efficiencies, cells were transfected with Alexa Fluor 488 labeled AllStars Negative Control siRNA (Qiagen) at a variety of conditions followed by fluorescence microscopy. For 96 well plates, 2.5×10^4 cells were seeded into each well, and for six well plates 1×10^6 cells were seeded into each well in antibiotic-free MEM the night before transfection. Transfections were carried out in Opti-MEM® I (Invitrogen) with Lipofectamine™ 2000 (Invitrogen) (diluted 1:100). Twenty-four h after transfection, the cells were either harvested or the medium was replaced. Cells were transfected with Pre-miR™ miR-125b, Pre-miR™ miR-100 (Ambion, Austin, TX), or Pre-miR™ Negative Control 1.

Cell Proliferation Assays

Cell proliferation was determined using the MTT Cell Growth Assay Kit (Millipore, Billerica, MA) following the manufacturer's instructions. We examined three experimental groups: 1) cells transfected with miR-125b, 2) cells transfected with miR-100, and 3) cells co-transfected with both miR-125b and miR-100 at equal concentrations. Within each of these groups, cells were transfected with 50 nM, 100 nM, and 150 nM of the corresponding Pre-miR™ precursor molecule(s). In addition, we studied two control groups: 1) cells transfected with 50 nM, 100 nM, and 150 nM of the negative control, and 2) cells that were mock-transfected with only Opti-MEM® I and Lipofectamine™ 2000. Each of the experimental and control groups were examined at 0 h, 24 h, 48 h, and 72 h after transfection in triplicate within three biological replicates. All experimental groups and the negative control were normalized to the mock transfection control to correct for any affect Lipofectamine™ 2000 had on proliferation. The results from the negative control at all concentrations (50 nM, 100 nM, and 150 nM) were averaged together at each time point.

Microarray Analysis

UPCI:SCC029 cells were transfected with 100 nM of either the negative control, miR-125b, miR-100, or miR-125b and miR-100 in three biological replicates, and harvested at 48 h. 700 ng of purified RNA was reverse transcribed to synthesize cDNA using the Low Input Linear Amplification Kit (Agilent). cDNA was then used to generate Cy-3-labeled cRNA that was recovered using RNeasy columns (Qiagen), and fragmented for hybridization according to the manufacturer's instructions. Cy-3-labeled cRNA was hybridized to 4x44K Whole Human Genome Microarrays (Agilent), which were scanned using a GenePix 4000B scanner (Agilent), and individual hybridization intensities were acquired with the Feature Extraction software v9.1 (Agilent).

To analyze the data, expression values were log2 transformed and then normalized using cyclic-loess with three iterations. Normalized values were validated using MA-plots and by comparing the observed and expected fold changes of the spike-in controls. Out of the 45,016 probes, 1,092 control probes were dropped, leaving 43,294 probes for further analysis. For each gene, we fitted a two-way repeated measures ANOVA model with interaction to the expression values observed across the 12 arrays to determine if gene expression was modified by individual treatment with miR-125b, miR-100, or if expression was modified by a unique interaction caused by the co-treatment of miR-125b and miR-100. The obtained P-values were corrected for multiple testing errors by generating False

Discovery Rate (FDR) adjusted P-values using the Benjaminii and Hochberg method (Benjamini and Hochberg, 1995). However, since our sample size was small, an FDR correction p-value cutoff of 0.05 may be too stringent. Therefore, we used an FDR adjusted p-value of 0.1 as our cutoff for statistical significance, and selected genes that were modified by 1.8-fold or more for further study. However, we did make an exception for those predicted target genes that had significantly different expression values, but were below the 1.8-fold threshold.

Microarray results for selected genes were validated using RT-PCR and Western blotting. Reverse transcription reactions were performed as previously described (Huang et al., 2002) with two total RNA inputs (400 ng and 100 ng) for each sample using MMLV Reverse Transcriptase (Epicentre, Madison, WI) and random hexamers. For Quantitative PCR (QPCR), we used TaqMan® Gene Expression Assays (Applied Biosystems) for all of our genes including the 18s RNA control, and the TaqMan® 2x Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). All QPCR samples were performed in triplicate and each QPCR plate included no reverse transcriptase and no template controls. The data were analyzed using the comparative CT method.

For Western blotting, UPCI:SCC029 cells were transfected with 100 ng of miR-125b, miR-100, and the negative control and cells were collected at 24 h, 48 h, and 72 h. Cells were washed with cold PBS and lysed (on ice) with a solution containing 50 mM Tris, 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 10 µg/ml leupeptin, 0.1% sodium dodecyl sulfate, 10 µg/ml pepstatin, and 1 nM phenyl methyl sulfonyl fluoride. Protein concentrations were established using the Bio-Rad Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with the SmartSpec 3000 (Bio-Rad Laboratories). Normalized cell lysates were resolved by sodium dodecyl sulfate PAGE (SDS-PAGE) polyacrylamide gel electrophoresis. Proteins were transferred to an Immobilon-P membranes (Millipore Corporation, Billerica, MA). Membranes were hybridized in primary antibody for 2–4 h at room temperature. Antibodies for KLF13 (RFLAT-1, C-13), CXCL11 (I-TAC, FL-94), MMP13 (D-17), and FGFR3 (C-15) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a 1:1,000 dilution. To verify equal protein loading, membranes were sectioned and probed for α -actinin (H-2) or β -tubulin (H-235), both of which were obtained from Santa Cruz Biotechnology and used at a dilution of 1:1,000. Membranes were incubated with the corresponding secondary antibody (1:4,000) for 1.5 h at room temperature. Proteins were visualized using the Western Lightning™ Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, MA) following the manufacturer's suggestions. Densitometric analyses were carried out using Un-Scan-It Gel™ software (Silk Scientific, Orem, UT) following the manufacturer's recommendations.

Gene Ontology and miRNA Databases

To determine the biological function of genes with altered expression, we used GoMiner™ (<http://discover.nci.nih.gov/gominer/>) (Zeeberg et al., 2003). Databases used to obtain predicted target genes for miR-125b and miR-100 include <http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl>, <http://microrna.sanger.ac.uk/sequences/>, and <http://mirnamap.mbc.nctu.edu.tw/>.

Statistical Analysis

Data presented here are the mean $\pm$ standard error. Assessment of statistical significance in all of our experiments (other than the microarray studies) was done using two-sided *t*-tests, with *P*-values < 0.05 being considered statistically significant.

RESULTS

MiR-125b and miR-100 Levels are Significantly Lower in OSCC Cell Lines and Tumors

At this time, 18 miRNAs have been mapped to chromosome 11, 13 of which are located on 11q (Fig. 1). Previous FISH and QUMA (quantitative microsatellite analysis) data (Huang et al., 2002;2006;Parikh et al., 2007;Reshmi et al., 2007) indicate that miR-130a, miR-611, miR-192, miR-194-2, and miR-612 map proximal to the 11q13 amplicon core in OSCC; therefore, they were not examined. FISH and QUMA results show that miR-139 and miR-326 are located near the distal end of the 11q13 amplicon and may be amplified in some of our OSCC cell lines and tumors (Huang et al., 2002;2006;Parikh et al., 2007;Reshmi et al., 2007). The miRNAs, miR-34b, miR-34c, miR-125b-1, let-7a-2, and miR-100 are distal to the *FRA11F* fragile site, and FISH and QUMA results indicate that they are lost (or have a reduced copy number) in a substantial proportion of our OSCC cell lines and tumors (Huang et al., 2002;2006;Parikh et al., 2007;Reshmi et al., 2007). Because of the possibility of either being lost or amplified, we chose to examine the expression of miR-139, miR-326, miR-34b, miR-34c, miR-125b-1, let-7a-2, and miR-100 in 10 OSCC cell lines and nine OSCC tumors and compare those results to five independent NHOK samples that were pooled together.

We found no significant difference in the expression levels of miR-139, miR-326, miR-34b, miR-34c, and let-7a between cell lines, tumors, and NHOK controls (data not shown). Expression studies revealed that levels of miR-125b and miR-100 were substantially lower in cell lines and tumors than in NHOK controls (Figure 2A and B).

Loss of Chromosomes 11q and 21 in OSCC Cell Lines and Tumors

MiR-125b-1, let-7a-2, and miR-100 map to distal 11q and are likely expressed as a single transcript (miRNA operon). Two similar operons exist elsewhere in the genome, possibly arising through duplication during evolution. These are the miR-125b-2, let-7c, miR-99a operon on chromosome 21 and the miR-125a, let-7e, miR-99b operon on chromosome 19. Expression of miR-125b-1 and miR-125b-2 produces identical mature miRNAs (miR-125b). The expression assays used here quantitate mature miRNAs and as such, cannot distinguish between expression of miR-125b-1 and miR-125b-2. Therefore, we determined the copy number of chromosomes 21 and 11q in our cell lines and tumors using FISH. The copy number of 11q has been determined at a comparable passage in our cell lines (Martin et al., 2007; Parikh et al., 2007; Reshmi et al., 2007). We add to these data by determining the copy number of 11q in our tumors and the copy number of chromosome 21 in both our cell lines and tumors. We found that 11q and chromosome 21 have a reduced copy number compared to the centromere region of chromosome 11 in the majority of our cell lines and tumors (Table 1). This is consistent with previously published FISH and karyotype data (Jin et al., 1998; 2006; Martin et al., 2007; Parikh et al., 2007; Reshmi et al., 2007).

Increasing miR-125b and miR-100 Levels Reduces Cell Proliferation

The reduced expression of miR-125b and miR-100 in OSCC cell lines and tumors prompted us to determine whether or not increasing their levels would affect cell proliferation. We carried out cytotoxicity assays (MTT) on UPCI:SCC029 cells that had been transfected with miR-125b and miR-100 mimics (individually and in combination) and compared the results to cells that had been transfected with a negative control. The miRNA mimics for miR-125b and miR-100 (referred to as miR-125b and miR-100 henceforth) are synthetic small double stranded molecules that are processed by RISC into molecules that are identical to endogenously expressed miRNAs. The negative control is also processed by RISC, but has no known target genes. We found that individually transfecting cells with miR-125b and miR-100 led to a statistically significant decrease in proliferation (Fig. 3A and B,

respectively). To determine if treating cells with both miR-125b and miR-100 elicited a synergistic effect on proliferation, we co-transfected UPCI:SCC029 cells with miR-125b and miR-100. This led to a statistically significant decrease in proliferation that was slightly larger than transfection with either miRNA individually.

Increasing miR-125b and miR-100 Levels Alters Global Gene Expression

Given the effects that miR-125b and miR-100 have on proliferation, we sought to determine their effect on global gene expression in OSCC, and hypothesized that increasing their cellular levels would alter the expression of both target genes (direct effect) and non-target genes (indirect effect). Similar studies have demonstrated that miRNAs impact gene expression by affecting both target and non-target genes (Bagga et al., 2005; Lim et al., 2005; Johnson et al., 2007). In addition, we wanted to determine if the decreased proliferation seen with co-transfection of miR-125b and miR-100 was due to an additive effect of the two miRNAs or a unique synergistic effect of the two in combination. We addressed these questions by doing microarray analysis on cells treated with miR-125b and miR-100 (individually and in combination) and comparing the results to cells treated with the negative control (48 h post transfection).

Transfecting cells with miR-125b significantly altered the expression of more than 50 genes, with 19 being altered by 1.8-fold or more (Supplemental Table 1). Transfecting cells with miR-100 significantly modified the expression of 700 genes, of which more than 130 were modified by 1.8-fold or more (Supplemental Table 2). In addition to those genes that are modified by 1.8-fold or more, the expression of a number of miR-125b and miR-100 predicted target genes were significantly altered, but were below the 1.8-fold threshold; and these genes are included in Supplemental Tables 1 and 2. We chose to include these genes because miRNA-mediated translational inhibition can in some instances lead to mRNA degradation and in other cases have no effect on transcript levels (Bagga et al., 2005; Lim et al., 2005; Johnson et al., 2007; Zhang et al., 2007b). Reduced expression of these genes suggests that they are repressed by miR-125b or miR-100. Co-transfection of cells with miR-125b and miR-100 did not elicit a synergistic change in gene expression, but appeared to be additive.

The biological functions of the genes differentially expressed in response to miR-125b and miR-100 transfection include cell cycle progression, immune response, proliferation, differentiation, and development among others (Supplemental Tables 3 and 4).

We validated our microarray results by doing RT-PCR analysis of selected genes (both predicted target genes and non-target genes) that were affected by transfection with miR-125b and miR-100. For cells transfected with miR-125b we chose *KLF13*, *CXCL11*, and *FOXA1*; and for cells transfected with miR-100 we chose *ID1*, *EGR2*, *MMP13*, and *FGFR3*. The results of which were compared to cells transfected with the negative control. RT-PCR confirmed the down-regulation of *KLF13* and the up-regulation of *CXCL11* and *FOXA1* in cells transfected with miR-125b (Fig. 4A). RT-PCR also validated the down-regulation of *ID1*, *EGR2*, *MMP13*, and *FGFR3* in cells transfected with miR-100 (Fig. 4B).

In addition, we sought to determine whether the alteration in gene expression seen in the microarray and RT-PCR data extended to the protein level. Western blotting was used to compare levels of KLF13 and CXCL11 in cells transfected with miR-125b to cells treated with the negative control (Fig. 5A). We found that levels of CXCL11 increased by as much as 2.3-fold and KLF13 levels were reduced by as much as 2-fold. Western blotting was also used to compare levels of MMP13 and FGFR3 in cells treated with miR-100 to cells treated with the negative control (Fig. 5B). MMP13 protein levels were decreased by more than 2-fold and FGFR3 levels were decreased by as much as 1.8-fold. Our western blot data

support the microarray and RT-PCR results, confirming that miR-125b and miR-100 play a role in gene expression in OSCC cells.

DISCUSSION

The miR-125b and miR-100 levels are lower in our OSCC tumors and cell lines than in our NHOK controls. Several other groups have examined miRNA expression in HNSCC/OSCC. Tran et al. (2007) carried out miRNA arrays and found that miR-100 is downregulated in HNSCC. However, they did not find reduced expression of miR-125b. Chang et al. (2008) also did miRNA arrays and elegantly showed that miR-21 can affect proliferation and apoptosis in HNSCC. They did not find dysregulation of miR-125b or miR-100 in HNSCC. Wong et al. (2008), using QPCR, found that miR-125b and miR-100 are downregulated in OSCC. The differences in these studies could be due to the different methodologies used to obtain expression data or it could be due the origin sites of the tumors and cell lines used. Our group and Wong et al. (2008) selected tumors and cell lines derived from the oral cavity; however, Tran et al. (2007) and Chang et al. (2008) used samples derived from the oropharynx, hypopharynx, larynx, and tonsils in addition to the oral cavity.

The decreased expression of miR-125b and miR-100 in our OSCC tumors and cell lines in combination with our FISH data suggest that the copy number losses of 11q and chromosome 21 contribute to the reduced expression of these miRNAs. Cell lines with loss of 11q have lower levels of miR-100 expression than those without loss. The expression of miR-125b is not as clearcut, since it is expressed from both chromosome 21 and 11q, but miR-125b expression was higher in UPCI:SCC116, which does not have loss of 11q than it was in cell lines with loss.

The association between the loss of 11q and the decreased expression of miR-125b and miR-100 is further substantiated by a recent report in breast cancer (Smirnov and Cheung, 2008) showing that transcription of miR-125b and miR-100 is repressed by CDX2, which is a transcription factor that is negatively regulated by ATM. The *ATM* gene is located on distal 11q at 11q23.3 and has a reduced copy number and lower expression levels in OSCC cells with loss of 11q (Parikh et al., 2007). This suggests that the lower ATM protein levels could further decrease the expression of miR-125b and miR-100. The interrelationship between protein coding genes and miRNAs on 11q merits further investigation.

Individually transfecting OSCC cells with miR-125b and miR-100 significantly decreased cell proliferation; however co-transfection had a greater effect on proliferation than either did individually. The reduced proliferation in cells co-transfected with miR-125b and miR-100, although slight, was statistically significant. Cells co-transfected with 50 nm and 100 nm of miR-125b and miR-100 had significantly lower proliferation values at 24 h and 48 h post transfection (P-values ranging from 0.001 to 0.05) than did cells that were transfected with either miRNA individually. The proliferation assays led us to question whether transfecting cells with miR-125b and miR-100 (individually and in combination) would affect gene expression and if co-transfection would induce a unique change in gene expression. We found that transfecting OSCC cells with miR-125b and miR-100 modified the expression of a number of genes, including both predicted target genes and non-target genes. Predicted target genes that are downregulated in response to transfection with miR-125b or miR-100 suggest that they are actual targets of these miRNAs. Given the number of predicted miR-125b and miR-100 target genes in public databases, it is surprising that so few were modified after transfection with these miRNAs. However, it is possible that a number of miR-125b and miR-100 target genes were translationally repressed without affecting mRNA levels, preventing detection by microarray analysis. The ability of a miRNA to inhibit the translation of a gene without significantly altering transcript levels has

been demonstrated previously (Zhang et al., 2007b). We found little evidence to suggest that co-transfecting cells with miR-125b and miR-100 resulted in a unique synergistic effect on gene expression, suggesting that the reduced proliferation seen in cells co-transfected with miR-125b and miR-100 was due to an additive effect of the two miRNAs on gene expression.

MiR-125b is involved in neuronal development and differentiation (Sempere et al., 2004; Smirnova et al., 2005; Wu and Belasco 2005). It negatively affects proliferation (Mizuno et al., 2008), and can modify the immune response by regulating TNF-alpha levels (Tili et al., 2007). Altered miR-125b expression occurs in OSCC (Wong et al., 2008), ovarian cancer (Nam et al., 2008; Yang et al. 2008), breast cancer (Iorio et al., 2005), and prostate cancer (Ozen et al., 2008). Transfecting OSCC cells with miR-125b significantly reduced cell proliferation and modified the expression of numerous genes including *KLF13*, *CXCL11*, and *FOXA1*; all of which have important biological functions. *KLF13* is a transcription factor and predicted miR-125b target that is involved in the proliferation and differentiation of the heart (Lavallee et al., 2006; Nemer and Horb, 2007), as well as being required for B-cell and T-cell development (Outram et al., 2008). *KLF13* is also required for the expression of cyclin D1 (Nemer and Horb, 2007) and *CCL5* (*RANTES*) (Song et al., 1999; Krensky and Ahn, 2007). This is of interest since cyclin D1 is known to be overexpressed in OSCC (Izzo et al., 1998), and *CCL5* has been shown to be involved in lung cancer (Borczuk et al., 2008), breast cancer (Karnoub et al., 2007), prostate cancer (Robinson et al., 2003; Vaday et al., 2006), melanoma (Karnoub et al., 1999), T-cell leukemia (Mori et al., 2004), and ovarian cancer (Negus et al., 1997). *CXCL11* is a chemokine and prominent ligand for *CXCR3*, which is involved in immunity, inflammation, and angiostasis; and has been implicated in a variety of cancers (Romagnani et al., 2004; Chu et al., 2007; Balestrieri et al., 2008). Forced *CXCL11* expression increases survival rates and reduces metastasis in mice (Hensbergen et al., 2005; Berencsi et al., 2007; Chu et al., 2007). *FOXA1* is a transcription factor that can both simulate and repress growth (Habashy et al., 2008), and is responsible for expression of estrogen responsive genes (Laganieri et al., 2005). *FOXA1* overexpression blocks metastasis (Liu et al., 2005; Williamson et al., 2006) and inhibits proliferation in prostate cancer (Lee et al., 2008). The ability of miR-125b to alter the expression of *KLF13*, *CXCL11*, and *FOXA1* could have therapeutic implications, given the functions of these genes. Furthermore, the ability of miR-125b to modify the expression on non-target genes suggests that it indirectly contributes to OSCC development.

MiR-100 is downregulated in ovarian cancer (Nam et al., 2008; Yang et al., 2008), hepatocellular carcinomas (Varnholt et al., 2008), and HNSCC/OSCC (Tran et al., 2007; Wong et al., 2008). Transfecting OSCC cells with miR-100 modified the expression of a number of genes including *ID1*, *EGR2*, *MMP13*, and *FGFR3*, all of which are overexpressed in a variety of malignancies. *FGFR3* is a fibroblast growth factor receptor and predicted miR-100 target that is mutated in a number of cancers (Shotelersuk et al., 2001) and involved in development and proliferation of urinary bladder carcinomas (Gomez-Roman et al., 2005). *ID1* is a helix-loop-helix protein that inhibits transcription and is overexpressed in a variety of malignancies (Perk et al., 2005), including OSCC (Nishimine et al., 2003; Ishigami et al., 2007) and esophageal squamous cell carcinoma (ESCC) (Yuen et al., 2007). Furthermore, *ID1* has also been associated with metastasis in breast cancer (Fong et al., 2003). *MMP13* is a member of the matrix metalloproteinase (MMP) family of proteins and is responsible for the activation of other MMP proteins (Leeman et al., 2002; Zhang et al., 2008). The expression of *MMP13* is altered in breast cancer (Zhang et al., 2008), ESCC (Chiang et al., 2006), and OSCC (Chiang et al., 2006; Vairaktaris et al., 2007). *EGR2* is a transcription factor that is responsible for myelin development (LeBlanc et al., 2007), and is overexpressed in OSCC (Liu et al., 2006; 2008). The ability of miR-100 to modify the

expression on non-target genes suggests that it contributes to OSCC development via an indirect mechanism.

The miR-100 induced modification of *ID1*, *MMP13*, and *FGFR3* expression becomes even more interesting in light of the report by Ishigami et al. (2007) that identified a number of genes that are overexpressed in radioresistant OSCC cells. Within this list are *ID1*, *MMP13*, *FGFR3*, and *FGFBP1*, all of which are downregulated in OSCC cells transfected with miR-100 (Supplemental Table 2) (Ishigami et al., 2007). In our study, the downregulation of these genes in response to transfection with miR-100 suggests that miR-100 could be used therapeutically to increase the sensitivity of OSCC cells to ionizing radiation by decreasing the expression of the genes that confer radioresistance.

The data presented here suggest that the down-regulation of miR-125b and miR-100 contribute to the development and or progression of OSCC and that modifying their expression may have therapeutic applications. Transfection of OSCC cells with miR-125b and miR-100 reduced cell proliferation and affected the expression of a number of genes known to be involved in cancer. Furthering our understanding of miR-125b and miR-100 induced gene regulation will increase our knowledge of the molecular mechanisms underlying OSCC as well as the role miRNAs play in cancer. The data presented here may also apply in other malignancies with down-regulation of miR-125b and miR-100. In conclusion, the downregulation of miR-125b and miR-100 in OSCC appears to play an important role in the development and or progression of disease and further, may contribute to the loss of sensitivity to ionizing radiation in tumors with distal chromosome 11q loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An ideogram of chromosome 11 with the locations of the 11q13 amplicon core, *FRA11F* fragile site, and the miRNAs on 11q noted.

Figure 2. RT-PCR results of (A) miR-125b and (B) miR-100 in OSCC cell lines, OSCC tumors, and NHOK controls, with standard error indicated. NHOK (composed of five independent samples) is represented by a black bar, OSCC tumors are represented by white bars, and OSCC cell lines are represented by black bars with white hatch marks.

Figure 3.

Results of MTT assays of cells transfected with (A) miR-125b and (B) miR-100, and miR-125b and miR-100), with standard error indicated. Cells transfected with 50 nm are represented by diamonds (◆), 100 nm by circles (●), 150 nm by triangles (▲), and the negative control by squares (■). All experimental groups and the negative control are normalized to the mock-transfection control in all three graphs.

Figure 4. Comparison of microarray and RT-PCR results for cells transfected with (A) miR-125b and (B) miR-100, with standard error indicated. Fold change values determined from microarray data are represented by black bars and the fold change values determined from RT-PCR data are represented by white bars.

Figure 5.

Western blot confirmation of microarray results. Cells were transfected with 100 nm miR-125b and examined for changes in (A) CXCL11 and (B) KLF13 protein levels at 24, 48, and 72 hours after transfection. Cells were transfected with 100 nm miR-100 and examined for changes in (C) MMP13 and (D) FGFR3 at 24, 48, and 72 hours after transfection. Lanes are labeled C for the pooled negative control.

TABLE 1

The OSCC Cell Lines, OSCC Tumors, and NHOK Samples Used in This Study.

Cell line	Sex	Race	Age	Smoking	Alcohol	Family history	Site	Stage	11q loss	21 loss
UPCI:SCC084	M	Cau	53	Yes	Yes	No	RMT	T2N2B	Yes	Yes
UPCI:SCC078	M	Cau	60	No	Yes	Yes	FOM	T2N0	Yes	Yes
UPCI:SCC131	M	Cau	73	No	No	No	FOM	T2N2	Yes	Yes
UPCI:SCC040	M	Cau	51	No	Yes	Yes	Tong	T2N2	Yes	Yes
UPCI:SCC029	M	Cau	85	Yes	Yes	No	Buc	T4N2	Yes	Yes
UPCI:SCC032	M	Cau	60	Yes	Yes	Yes	RMT	T2N2B	Yes	Yes
UPCI:SCC104	M	Cau	58	No	No	No	FOM	T4NX	Yes	Yes
UPCI:SCC142	M	Cau	58	No	No	No	FOM	T4NX	Yes	No
UPCI:SCC116	M	Cau	58	Yes	Yes	Yes	Alv	T2N0	No	Yes
UPCI:SCC066	F	Cau	75	Yes	Yes	Yes	Alv	T1N0	No	Yes
Tumor										
05-1797	M	Cau	42	Yes	Yes	Yes	Buc	T2N0	Yes	Yes
05-1872	M	Cau	79	Yes	No	No	Buc	T4N0	Unk	Unk
04-1555	M	Cau	65	Yes	Yes	Yes	Tong	T2N1	No	Yes
04-1756	M	Cau	32	Yes	No	No	Tong	T3N1	Yes	Yes
05-1742	M	Cau	54	No	Yes	Yes	RMT	T4N1	Yes	Yes
05-1902	M	Cau	68	Yes	No	Yes	Tong	T3N2B	Yes	Yes
05-1980	M	Cau	41	No	Yes	Yes	Tong	T3N2	Unk	Unk
05-2006	M	Cau	90	Yes	Yes	Unk	Alv	T4N0	Unk	Unk
05-1789	M	Cau	59	Yes	Yes	Yes	Tong	T3N0	Unk	Unk
NHOK										
06-2665	M	n/a	35	No	No	Unk	UP3	nl	n/a	n/a
07-2729	F	Cau	19	No	No	Unk	UP3	nl	n/a	n/a
05-2015	M	Cau	78	Yes	Yes	Unk	UP3	nl	n/a	n/a
06-2205	M	Cau	74	No	No	Unk	UP3	nl	n/a	n/a
06-2260	M	AA	42	No	No	Unk	UP3	nl	n/a	n/a

AA, African American; Alv, alveolar ridge; Buc, buccal mucosa; Cau, Caucasian; FOM, floor of mouth; n/a, data not acquired; nl, normal histopathology; RMT, retromolar trigone; Tong, tongue; Unk, data unknown; UP3, uvula, palate, and/or pharynx.