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Identification and experimental validation of G protein alpha inhibiting activity polypeptide 2 (GNAI2) as a microRNA-138 target in tongue squamous cell carcinoma

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Abstract

MicroRNA deregulation is a critical event in tumor initiation and progression. The down-regulation of microRNA-138 has been frequently observed in various cancers, including tongue squamous cell carcinoma (TSCC). Our previous studies suggest that deregulation of miR-138 is associated with the enhanced proliferation and invasion in TSCC cells. Here, we seek to identify the targets of miR-138 in TSCC, and explore their functional relevance in tumorigenesis. Our genome-wide expression profiling experiments identified a panel of 194 unique transcripts that were significantly down-regulated in TSCC cells transfected with miR-138. A comprehensive screening using six different sequence-based microRNA target prediction algorithms revealed that 51 out of these 194 down-regulated transcripts are potential direct targets for miR-138. These targets include: chloride channel, nucleo-tide-sensitive, 1A (CLNS1A), G protein alpha inhibiting activity polypeptide 2 (GNAI2), solute carrier family 20, member 1 (SLC20A1), eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), and Rho-related GTP-binding protein C (RhoC). GNAI2 is a known proto-oncogene that is involved in the initiation and progression of several different types of tumors. Direct targeting of miR-138 to two candidate binding sequences located in the 3′-untranslated region of GNAI2 mRNA was confirmed using luciferase reporter gene assays. Knockdown of miR-138 in TSCC cells enhanced the expression of GNAI2 at both mRNA and protein levels. In contrast, ectopic trans-fection of miR-138 reduced the expression of GNAI2, which, in consequence, led to reduced proliferation, cell cycle arrest and apoptosis. In summary, we identified a number of high-confident miR-138 target genes, including proto-oncogene GNAI2, which may play an important role in TSCC initiation and progression.

Introduction

Oral squamous cell carcinoma (OSCC) is a complex disease that arises in various sub-sites. Tumors from these different sub-sites have distinct clinical presentations and outcomes, and are associated with different genetic characteristics (Timar et al. 2005). In this study, we focused on oral tongue SCC (TSCC), one of the most common types of OSCCs. TSCC is significantly more aggressive than other forms of OSCCs, with a propensity for rapid local invasion and metastatic spread (Franceschi et al. 1993). The incidence of TSCC is actually increasing in young and middle age populations (Annertz et al. 2002; Mackenzie et al. 2000; Schantz and Yu 2002). While attempts have been made to identify genomic alterations that contribute to initiation and progression of TSCC, most efforts are focused on protein coding genes. Our knowledge of genomic aberrations associated with non-coding genes (e.g., microRNA) and their contributions to the onset and propagation of TSCC is relatively limited.

MicroRNAs are not directly involved in protein coding, but are able to control the expression of their target genes at post-transcriptional levels. Several microRNAs have been functionally classified as proto-oncogenes or tumor suppressors and are aberrantly expressed in different cancer types including leukemia (Calin et al. 2002; Calin et al. 2004), lymphoma (Metzler et al. 2004), breast cancer (Bhaumik et al. 2008; Kondo et al. 2008), colorectal cancer (Michael et al. 2003), lung cancer (Takamizawa et al. 2004; Yanaihara et al. 2006), liver cancer (Murakami et al. 2006; Wang et al. 2008), and OSCC (Hebert et al. 2007; Kozaki et al. 2008; Tran et al. 2007; Wong et al. 2008b). Deregulation (e.g., overexpression or loss of expression) of these “cancerous” microRNAs can figure prominently in tumor initiation and progression (Calin and Croce 2006; Esquela-Kerscher and Slack 2006) by facilitating an inappropriate cellular program that promotes uncontrolled proliferation, favors survival, inhibits differentiation and/or promotes invasive behavior.

The deregulation of miR-138 has been frequently observed in a number of cancer types, including OSCC (Kozaki et al. 2008; Wong et al. 2008b), thyroid cancer (Mitomo et al.

2008), and lung cancer (Seike et al. 2009). Two miR-138 precursor genes, termed pre-miR-138-1 and pre-miR-138-2, were recently identified in the mouse genome (Obernosterer et al. 2006), and their human homologs were mapped to chromosome 3p21.33 and 16q13, respectively. Interestingly, loss of heterozygosity (LOH) at both chromosome loci has been frequently detected in OSCC (Hogg et al. 2002; Piccinin et al. 1998; Wang et al. 1999). Our recent study demonstrated that reduced miR-138 level is associated with enhanced cell growth and invasion in OSCC (Liu et al. 2009b). However, the molecular mechanism(s) underlying the effect of miR-138 on the initiation and progression of TSCC is poorly understood. This study seeks to identify the potential miR-138 targets and investigate the molecular mechanism(s) that underlie the potential tumor suppressor effect of miR-138 in TSCC.

Materials and methods

Tumor procurement and RNA extraction

Tissue samples were obtained from 15 cases of TSCC patients after tumor resection (including 5 stage T1 cases and 10 stage T2 cases). The demographics of the patients were as follows: 9 male, 6 female and average age = 53.2. This study was approved by Institutional Review Boards. These tissues were snap frozen. Cancer tissues containing more than 80% tumor cells on H&E pathological examination were selectively microdissected. The total RNA was isolated using miRNeasy Mini kit (Qiagen), and quantified by the RiboGreen RNA quantitation reagent (Molecular Probes).

Cell culture and transfection

The TSCC cell lines (SCC4, SCC9, SCC15, SCC25, Tca8113, UM1 and UM2) used in this study were maintained at 37°C in a humidified incubator containing 5% CO_2 in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO). Primary normal human oral keratinocytes (NHOK) were prepared and cultured in OKM medium (ScienCell Research Laboratory) as previously described (Park et al. 1991). Human oral keratinocyte primary culture (HOK, Cat. No. 2610) was purchased from ScienCell Research Laboratory. For functional analysis, miR-138 mimics and non-targeting miRNA mimics (Dharmacon), anti-miR-138 peptide nucleic acid (PNA) and negative control PNA (Panagene), and gene specific siRNA (On-TargetPlus SMARTpool, Dharmacon) were transfected into cells using Dharma-FECT Transfection Reagent 1 as described previously (Liu et al. 2009b).

Real-time RT-PCR analysis

The relative expression level of miR-138 was determined using mirVana™ qRT-PCR microRNA Detection Kit (Ambion) as described previously (Liu et al. 2009b). The relative mRNA levels of CLNS1A, GNAI2, SLC20A1, EIF4EBP1, and RhoC were examined using a quantitative 2-step RT-PCR assay with gene specific primer sets (Ori-Gene) as described previously (Zhou et al. 2006). The relative expression level was computed using the $2^{-\Delta\Delta Ct}$ analysis method, where actin was used as an internal reference (Livak and Schmittgen 2001).

Microarray analysis

The UM1 cells were transfected with miR-138 mimic and non-targeting miRNA mimic in triplicates as described above. Cells were harvested 48 h post transfection, and the total RNA was isolated, labeled, and hybridized to the Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays according to the standard protocols described previously (Ye et al. 2008; Zhou et al. 2006). The arrays were scanned with a GeneChip Scanner 3000. The scanned

array images were processed with GeneChip Operating software (GCOS). The microarray data were pre-processed using Robust Multi-array Analysis (RMA) (Irizarry et al. 2003). The differentially expressed genes were defined as fold difference <0.67 (down-regulated) or >1.5 (up-regulated), and *p* value <0.05.

MicroRNA target prediction

The candidate targets of miR-138 were identified using miRGen (Megraw et al. 2007), an integrated online database which contains a collection of 5 bioinformatics tools, including 4-way PicTar, 5-way PicTar, TargetScanS, miRanda at microrna.org, and miRanda at miRBase. In addition, TargetScanHuman 5.1 (Friedman et al. 2009) was also used for predicting the miR-138 targets. As such, the miR-138 targets are predicted by 3 different methods (PicTar, TargetScan, and miRanda) with 2 different versions for each method. For our study, genes that were predicted by at least one method were defined as potential miR-138 targets. Genes that were predicted by all 3 methods were identified as high confidence candidate miR-138 targets.

Western blotting analysis

Western blots were performed as described previously (Liu et al. 2009c) using antibodies specific against GNAI2 (Cell Signaling) and beta-actin (Sigma).

Dual luciferase reporter assay

A 73-bp fragment from the 3′-untranslated region (3′-UTR) of GNAI2 gene (position 26807 to 26879, NM_002070, containing the miRNA-138 binding site E1) and a 68-bp fragment from the 3′-UTR of GNAI2 (position 27054 to 27121, NM_002070, containing the miRNA-138 binding site E2) were cloned into the Xba I site of the pGL3 firefly luciferase reporter vector (Promega). The corresponding mutant constructs were created by mutating the seed regions of the miR-138 binding sites. The constructs were then verified by sequencing. Cells were transfected with the reporter contructs containing the targeting sequence from the GNAI2 3′-UTR (named pGL-GNAI2-E1 and pGL-GNAI2-E2) or its mutant (named pGL-GNAI2-E1m and pGL-GNAI2-E2m) using lipofectamine 2000 (Invitrogen). The pRL-TK vector (Promega) was co-transfected as internal control for normalization of the transfection efficiency. The luciferase activities were then determined as described previously (Liu et al. 2009c) using a Lumat LB 9507 Luminometer (Berthold Technologies).

Proliferation assay

Cell proliferation was measured by MTT assay as described previously. In brief, 48 h post transfection, transfectional medium in each well was replaced by 100 μl of fresh serum-free medium with 0.5 g/L MTT. After incubation at 37°C for 4 h, the MTT medium was aspirated out, and 50 μl of DMSO was added to each well. After incubation at 37°C for another 10 min, the absorbance value of each well was measured using a plate reader at a wavelength of 540 nm.

Apoptosis assay

Cells were grown in 6-well plates to about 60% confluence and transiently transfected with the desired miRNA reagents at a final concentration of 100 nM. The cells were digested and collected 48 h post-transfection, and twice washed with PBS. For cell apoptosis measurement, the cells were resuspended in 1 × Binding Buffer and 5 μl of Annexin FITC Conjugate, and 10 μl of propidium iodide solution were added to each cell suspension, separately. The stained cells (1×10^5) were then analyzed with a flow cytometer (FACScalibur, Becton–Dickinson).

Results

Previous studies have reported that miR-138 is down-regulated in OSCC (Kozaki et al. 2008; Wong et al. 2008a; Wong et al. 2008b). Here, we confirmed that miR-138 is also frequently down-regulated in TSCC. As shown in Fig. 1a, reduced miR-138 levels were observed in 13 out of 15 TSCC tissue samples as compared to the control samples (adjacent histologically normal tissue samples) from the same patients. The significantly lower miR-138 levels were also observed in a panel of TSCC cell lines as compared to the normal human oral keratinocyte primary cultures (NHOK and HOK) (Fig. 1b). Among these TSCC cells, UM1 and UM2 are paired cell lines that were previously established from a single patient (Nakayama et al. 1998). Significantly lower miR-138 level was observed in UM1 as compared to UM2.

MicroRNA can have multiple targets. We performed microarray-based differential expression analysis on UM1 cells transfected with miR-138 mimic and negative control mimic, with three experimental replicates for each condition. As shown in Fig. 2a, ectopic transfection of the miR-138 mimic to the UM1 cells led to an increase of the miR-138 level, as measured by quantitative RT-PCR. Microarray analysis revealed that the levels of 225 transcripts were significantly altered after miR-138 treatment (fold difference >1.5 and $p < 0.05$). These include 194 down-regulated transcripts and 31 up-regulated transcripts (Fig. 2b and Supplementary Table 1). The set of down-regulated transcripts was of particular interest, given its potential to contain direct targets of miR-138. To identify the potential targets of miR-138 in this set of down-regulated transcripts, a bioinformatics-based analysis was carried out based on a combination of 6 different sequence-based microRNA target prediction algorithms. Our analysis revealed that the set of down-regulated transcripts was significantly enriched with the predicted targets of miR-138 ($p = 5.25 \times 10^{-19}$, Supplementary Table 2), while no significant enrichment of the predicted miR-138 targets was observed in the set of up-regulated transcripts. Fifty-one out of these 194 down-regulated transcripts are potential direct targets of miR-138 (Fig. 2c, Table 1, and Supplementary Table 3). Among these 51 potential targets, we identified 5 highly confidence candidate targets (fold difference <0.67, p value <0.01, and consistently predicted by all 3 methods: TargetScan, PicTar, and miRanda), including chloride channel, nucleotide-sensitive, 1A (CLNS1A), G protein alpha inhibiting activity polypeptide 2 (GNAI2), solute carrier family 20, member 1 (SLC20A1), eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), and Rho-related GTP-binding protein C (RhoC). The miR-138 mediated down-regulations of these five candidate genes in UM1 cells were validated by quantitative RT-PCR analysis (Supplementary Figure 1).

GNAI2 (also known as Gi alpha 2) is a known proto-oncogene (Dhanasekaran et al. 1998). A reverse correlation between miR-138 and GNAI2 levels was observed in the TSCC cell line panel (Pearson's correlation coefficient = −0.94, $p < 0.001$, Supplementary Figure 2). Based on the bioinformatics prediction, two miR-138 targeting sequences were identified in the 3′-untranslated region of GNAI2 mRNA (Fig. 3a). The first targeting sequence (E1, located at position 26837 to 26859) is a highly conserved targeting site for miR-138, and the second targeting sequence (E2, located at 27084 to 27106) is a poorly conserved targeting site for miR-138 (Supplementary Figure 3). To confirm that miR-138 directly targets these sequences, dual luciferase reporter assays were performed using constructs in which these targeting sites were cloned into the 3′-UTR of the reporter gene (pGL-GNAI2-E1 and pGL-GNAI2-E2). As illustrated in Fig. 3b, when cells were transfected with miR-138, the luciferase activities were significantly reduced as compared to the cells transfected with negative control for both constructs. When the seed regions of these targeting sites were mutated (pGL-GNAI2-E1m and pGL-GNAI2-E2m), the miR-138 effects on luciferase were abolished. As shown in Fig. 3c with quantitative RT-PCR analysis, a significant decrease of

GNAI2 mRNA level was detected in UM1 cells that were transfected with the miR-138 mimic. An apparent increase in GNAI2 mRNA was observed when UM2 cells were treated with anti-miR-138 PNA. These results suggest that miR-138 regulates GNAI2 gene expression, at least in part, by regulating the stability of GNAI2 mRNA. Furthermore, as shown in Fig. 3d, ectopic transfection of miR-138 reduced the protein level of GNAI2 in UM1 cells. Knockdown of miR-138 using anti-miR-138 PNA increased the protein level of GNAI2 in UM2 cells.

To confirm that the miR-138 mediated reduction of GNAI2 has a functional relevance in TSCC, we knocked-down GNAI2 in UM1 cells using specific siRNA, and demonstrated that the reduced GNAI2 level is associated with reduced cell proliferation (Fig. 4a) and enhanced apoptosis (Fig. 4b). Similar effects on proliferation (Fig. 4a) and apoptosis (Fig. 4b) were observed when cells were treated with miR-138 mimic, which is in agreement with our previous observations (Liu et al. 2009b). As shown in Fig. 4c and d, knockdown of miR-138 in UM2 cells using anti-miR-138 PNA enhanced cell proliferation and suppressed apoptosis. When cells were pre-treated with siRNA against GNAI2, the miR-138 effects on proliferation and apoptosis were diminished (Fig. 4c, d). These results suggest that miR-138 regulates TSCC cell growth, at least in part, by targeting GNAI2.

Discussion

It is currently estimated that the human genome may have about 1,000 microRNAs. Although they account for only a minor fraction of the expressed genome, microRNAs are essential regulators of diverse cellular processes including proliferation, differentiation, apoptosis, survival, and motility. Our study focused on miR-138, a microRNA that has been shown to be frequently down-regulated in OSCC (Liu et al. 2009a), and is associated with enhanced cell proliferation and invasion (Liu et al. 2009b). We confirmed that miR-138 is also down-regulated in TSCC, one of the major sub-sites of OSCC. The deregulation of miR-138 has also been implied in other cancers, including thyroid cancer (Mitomo et al. 2008), lung cancer (Seike et al. 2009), and leukemia (Zhao et al. 2010). In addition to its role(s) in tumorigenesis, miR-138 has also been thought to play a role in the development of mammary gland (Wang and Li 2007), regulating dendritic spine morphogenesis (Siegel et al. 2009), and modulating cardiac patterning during embryonic development (Morton et al. 2008). The precise regulation of these diversified biological processes is dependent on the ability of miR-138 to regulate multiple target genes in the specific physiological/pathological settings. In our study, we combined the differential expression experiments with the sequence-based target prediction approaches to identify the miR-138 targets in TSCC. Our microarray-based experiments measure the differential expression at mRNA levels, and are only sensitive to the targets that are regulated by microRNA mediated degradation, but not to the targets that are regulated by micro-RNA mediated translational inhibition. We anticipate that a portion of true miR-138 targets will not be detected by our approach. Nevertheless, our study identified a panel of genes regulated by miR-138, including the experimentally confirmed miR-138 target gene, RhoC (Jiang et al. 2010). As a complementary approach to our differential expression analysis, we also used 6 different target prediction tools to refine our list of target genes for miR-138. Each tool utilizes a different model to define targeting sequences that are associated with functionality. Consequently, the predictions will differ when applied to the same micro-RNAs, with each method having different levels of coverage and false positive prediction (Dai and Zhou 2010). These differences reflect the varying biological attributes that each mathematical model highlights. In order to achieve better sensitivity for identifying miR-138 targets in our differential expression gene set, we utilized three common microRNA target prediction methods, each with two different versions (4-way PicTar, 5-way PicTar, TargetScanS, miRanda with microrna.org, and miRanda with miRBase, and TargetScanHuman 5.1). With

our comprehensive approach, a number of important candidate targets for miR-138 have been identified, including chloride channel, nucleotide-sensitive, 1A (CLNS1A), G protein alpha inhibiting activity polypeptide 2 (GNAI2), solute carrier family 20, member 1 (SLC20A1), eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), and Rho-related GTP-binding protein C (RhoC). Among these candidates, RhoC has been experimentally confirmed to be a functional target of miR-138 (Jiang et al. 2010).

GNAI2 belongs to the family of Gi alpha proteins that includes three polypeptides: Gi alpha 1 (GNAI1), Gi alpha 2 (GNAI2), and Gi alpha 3 (GNAI3). They form heterotrimers with beta and gamma subunits, and are involved in a wide variety of signaling events mediated by G-protein-coupled receptor (GPCR). Among the Gi alpha family members, GNAI2 gene is the only one contains conserved miR-138 targeting sequence. GNAI2 is a known proto-oncogene that is involved in the onset and the propagation of many different types of tumors (Dhanasekaran et al. 1998). Gain-of-function mutations that led to constant activation of GNAI2 have been identified in a number of cancers which prompt growth and survival of cancer cells (Lyons et al. 1990). Over-expression of GNAI2 has been observed previously in ovarian cancer (Peters et al. 2005), and hepatocellular carcinoma (McKillop et al. 1999; McKillop et al. 1998). It has been reported that the Gi alpha can activate the Ras-ERK/MAPK mitogenic pathway by membrane recruitment of Rap1 GTPase-activating protein and reduction of GTP-bound Rap1 (Mochizuki et al. 1999). It has also been suggested that Gi aipha enhances cell survival by activation of AKT (Mizutani et al. 2009), and suppresses apoptosis by regulating Bcl-2 expression (Seo et al. 2009). Our data suggests that miR-138 directly targets the 3′-UTR of the GNAI2 mRNA. The down-regulation of miR-138 in TSCC resulted in increased expression of GNAI2 which led to enhanced cell proliferation and suppressed apoptosis in TSCC. Interestingly, a recent report showed that GNAI2 is also a functional target of miR-30d in hepatocellular carcinoma cells (Yao et al. 2010). It is worth knowing that down-regulation of miR-30d has also been observed in OSCC (Kozaki et al. 2008). Taken together, this evidence suggests a novel paradigm in which microRNAs regulate GPCR signaling by targeting GNAI2 mRNA and suppressing its expression at post-transcriptional levels.

In addition to miR-138 mediated post-transcriptional regulation, GNAI2 is also regulated by alternative splicing. Alternative splicing has been shown to produce two GNAI2 transcript variants. These two transcript variants differ in the 5′-UTRs and the coding sequences, resulting in two protein products with distinct N-terminus (Montmayeur and Borrelli 1994). Both splicing variants have identical 3′-UTR containing the miR-138 targeting sequences E1 and E2, which implies that miR-138 regulates both splicing variants. However, transcript variant 2 is not expressed to any significant extent and was not detectable in our cell lines based on our qRT-PCR tests (data not shown). Nevertheless, it remains possible that miR-138 may functionally target this alternative splicing variant in other cell types or different biological systems. It is worth knowing that the protein product from GNAI2 transcript variant 2 has a unique sub-cellular localization (Golgi and other intracellular membranes) and has been suggested to have a functional role in vesicular transport (Montmayeur and Borrelli 1994).

In summary, miR-138 is a multi-functional molecule regulator that regulates a variety of biological processes. Our study identified a number of high-confident miR-138 target genes, including proto-oncogene GNAI2, which may play an important role in TSCC initiation and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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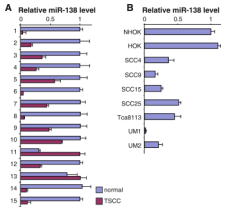

Fig. 1.

Reduced miR-138 level in TSCC. **a** The qRT-PCR was performed to assess the miR-138 levels in paired TSCC tissue samples and normal control tissue samples ($n = 15$). Statistically significant reductions in the miR-138 level were observed in 13 out of 15 TSCC samples tested as compared to their matching control samples ($p < 0.05$). **b** The miR-138 levels were also assessed in 7 TSCC cell lines, and normal oral keratinocyte cell cultures (NHOK and HOK). Statistically significant reductions of miR-138 were observed in all 7 TSCC cell lines compare to both NHOK and NOK ($p < 0.05$)

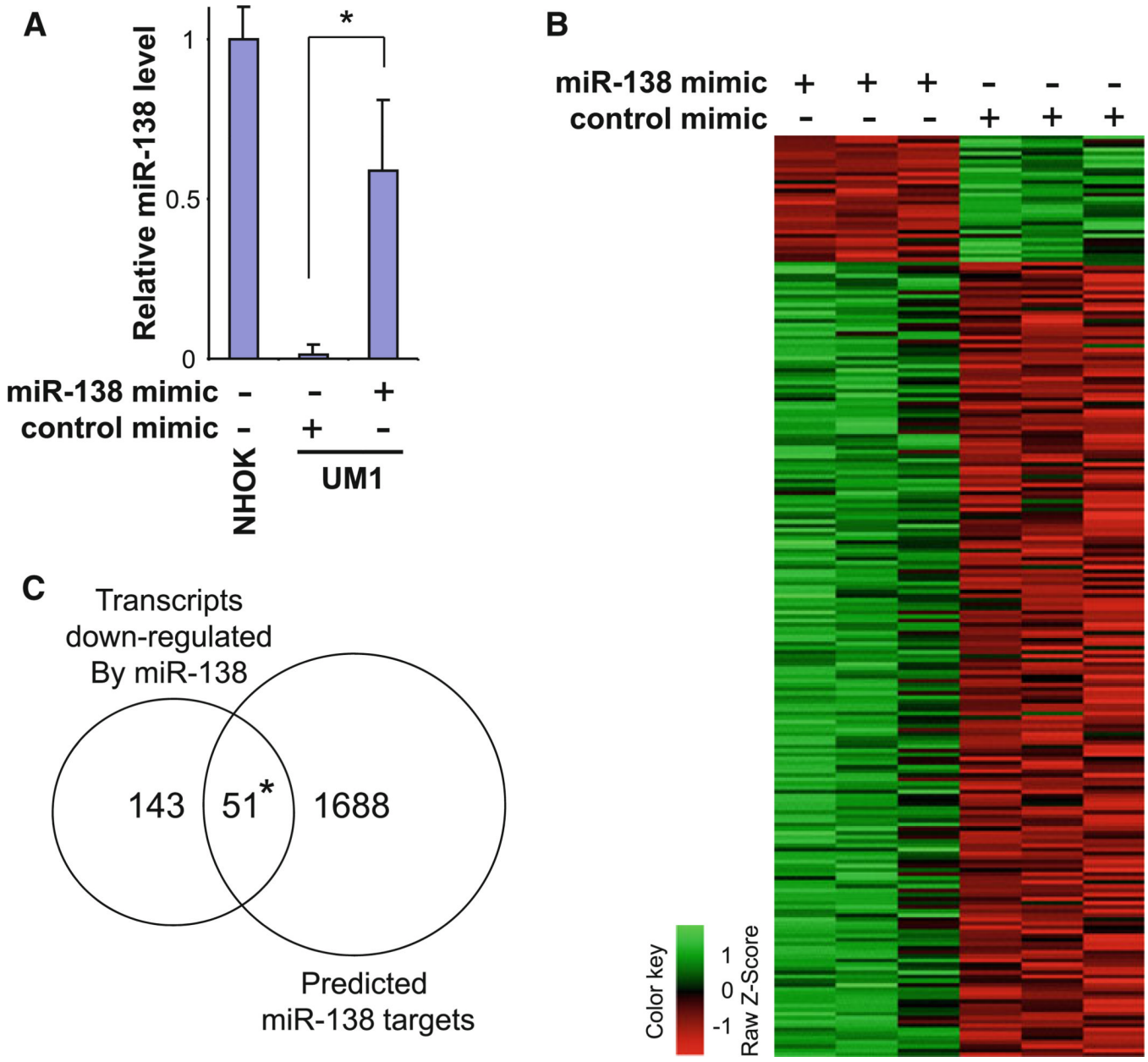

Fig. 2.

Identification of candidate miR-138 targets. **a** The UM1 cells were transfected with miR-138 mimic, and the increased miR-138 level was achieved as compared to the control mimic transfected cells as measured by qRT-PCR. **b** A total of 225 transcripts were differentially expressed (194 down-regulated and 31 up-regulated) based on the microarray analysis of the miR-138 mimic transfected cells and control mimic transfected cells. **c** Using a combination of 6 bioinformatics tools, a total of 1,739 candidate miR-138 targets were predicted. Fifty-one out of 1,739 candidate targets were also presented in the set of down-regulated transcripts, representing a significant enrichment of the down-regulated transcripts ($p = 5.25 \times 10^{-19}$, Supplementary Table 2)

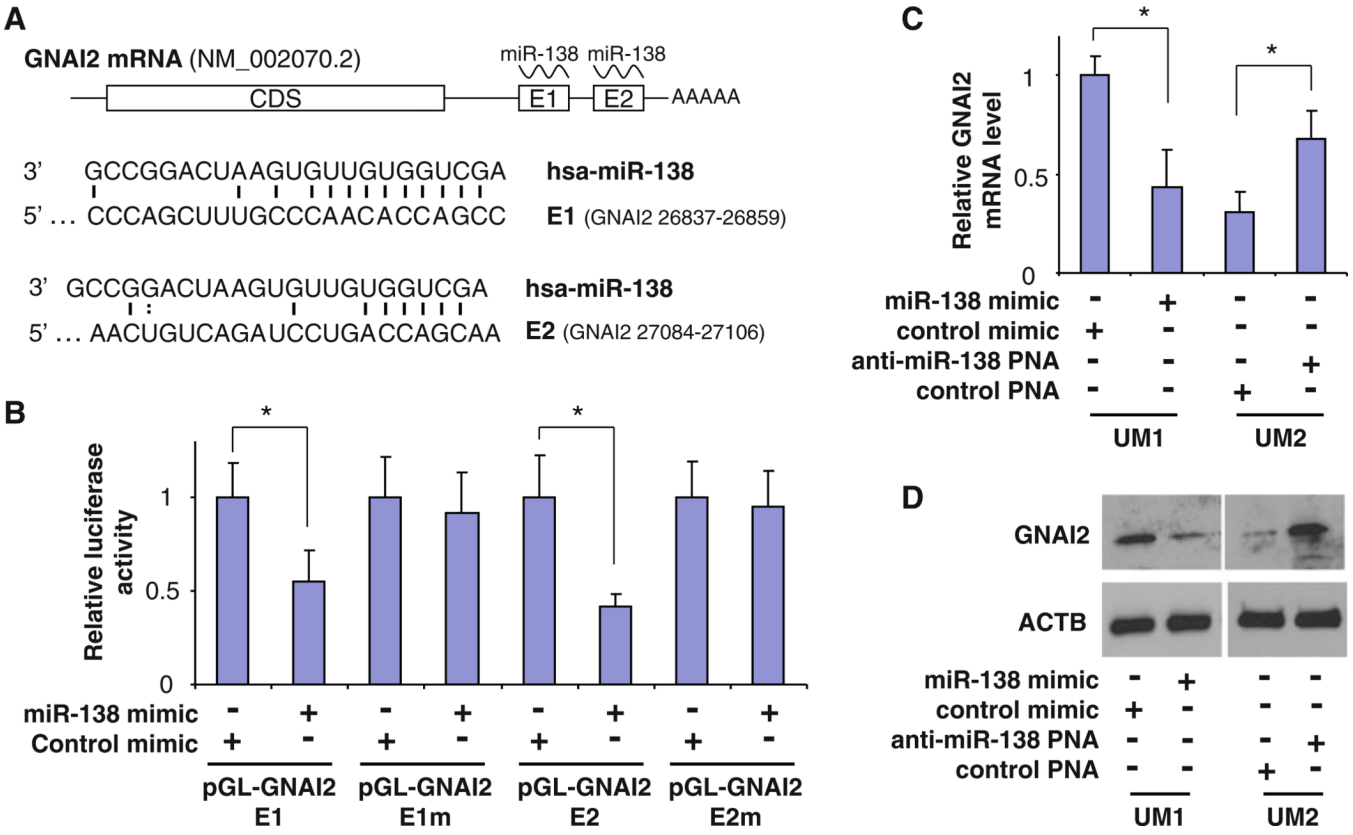

Fig. 3.

MiR-138 direct targeting GNAI2 mRNA. **a** The predicted miR-138 targeting sequences (E1 and E2) located in the 3′-untranslated region (3′-UTR) of GNAI2 mRNA. **b** Dual luciferase reporter assays were performed to test the interaction of miR-138 and its targeting sequences in the GNAI2 3′ -UTR using constructs containing the predicted targeting sequences (pGL-GNAI2-E1 and pGL-GNAI2-E2) and mutated targeting sequences (pGL-GNAI2-E1m and pGL-GNAI2-E2m) cloned into the 3′-UTR of the reporter gene. **c** Quantitative RT-PCR assays were performed to examine the effects of miR-138 on GNAI2 gene expression at mRNA level. **d** Western blot analyses were performed to examine the effects of miR-138 on GNAI2 gene expression at the protein level

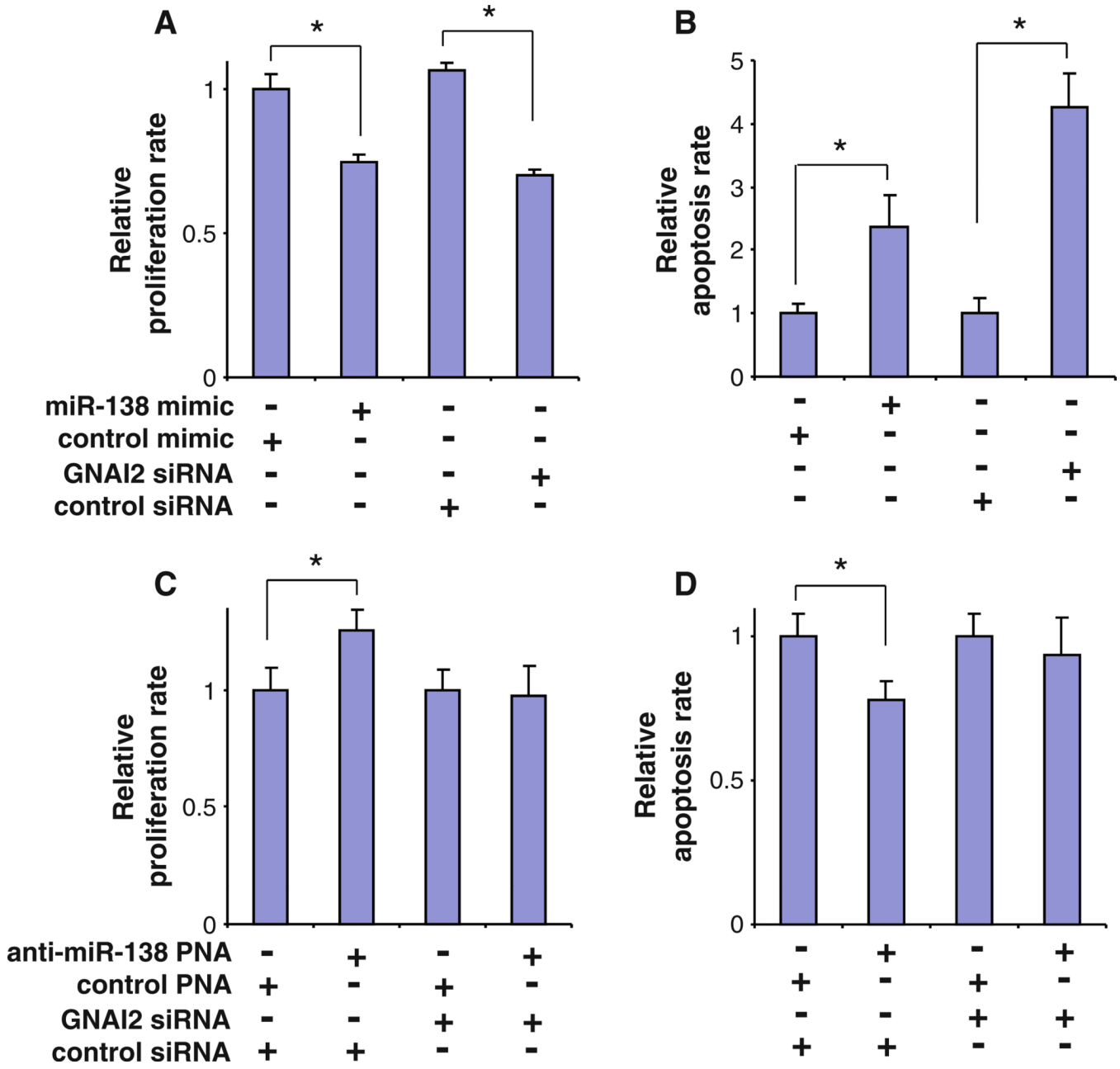

Fig. 4.

The effect of miR-138 and GNAI2 on TSCC cell growth. UM1 cells were treated with either miR-138 mimic or control mimic, or GNAI2 specific siRNA or control siRNA. The proliferation assay (**a**) and the apoptosis assay (**b**) were performed on these cells. UM2 cells were pre-treated with GNAI2 specific siRNA or control siRNA. There cells were then treated with anti-miR-138 PNA or control PNA. The effects on cell proliferation (**c**) and apoptosis (**d**) were then measured. Data represents at least 3 independent experiments with similar results. *$p < 0.05$

Table 1

miR-138 targets identified by microarray analysis and bioinformatics prediction

Transcripts down-regulated by miR-138 treatment (microarray analysis)[a]			**miR-138 targets predicted by bioinformatic tools**[b]					
Gene name[c]	**Fold diff. (miR-138/control)**	***p* value**	**PicTar 4-way**	**PicTar 5-way**	**TargetScanS**	**TargetScan Human 5.1**	**miRanda (microrna.org)**	**miRanda (miRBase)**
FRMD4A	0.597037	0.000101			1	1		
CLNS1A	0.573031	0.000601	1		1	1	1	1
RAVER1	0.518763	0.000753	1		1	1		
CCND3	0.532693	0.001684				1	1	
ZHX2	0.553348	0.002026	1		1			
GNAI2	0.605883	0.003401	1		1	1	1	1
LAPTM4A	0.613127	0.003654	1	1			1	1
EIF2C1	0.556826	0.003783	1		1	1		
SLC20A1	0.562802	0.004338	1		1		1	
EIF4EBP1	0.278416	0.004424	1			1	1	
SH2B3	0.548043	0.006015	1			1		
MAP2K7	0.633192	0.00712			1	1		
C20orf117	0.629688	0.007575	1		1	1		
VIM	0.475594	0.008976			1	1	1	
RHOC[d]	0.66474	0.042956	1		1	1	1	

Complete gene list consists of 51 candidate miR-138 targets is presented in Supplementary Table 3

[a] Fold difference <0.67, and *p* value < 0.01

[b] Genes that were predicted to be candidate targets of miR-138 were identified by 1 s

[c] Gene names in bold font were predicted to be miR-138 targets by all 3 methods (TargetScan, PicTar, miRanda)

[d] While did not reach the statistical cut-off indicated in[a], the down-regulation of RHOC gene by miR-138 has been experimentally confirmed in our recently study (Jiang et al. 2010)