Identification of novel molecular targets regulated by tumor suppressive *miR-1*/*miR-133a* in maxillary sinus squamous cell carcinoma

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Abstract. Based on our microRNA (miRNA) expression signature analysis of maxillary sinus squamous cell carcinoma (MSSCC), we found that *miR-1* and *miR-133a* were significantly reduced in tumor tissues. Quantitative real-time RT-PCR revealed that the expression levels of *miR-1* and *miR-133a* were significantly downregulated in clinical MSSCC tumor tissues compared with normal tissues. We focused on the functional significance of *miR-1* and *miR-133a* in cancer cells and identification of the novel cancer networks regulated by these miRNAs in MSSCC. Restoration of downregulated miRNAs (*miR-1* or *miR-133a*) in cancer cells revealed that both miRNAs significantly inhibited cancer cell proliferation and induced cell apoptosis. Molecular target identification of these miRNAs showed that transgelin 2 (*TAGLN2*) and purine nucleoside phosphorylase (*PNP*) were regulated by *miR-1* and *miR-133a*. Both *TAGLN2* and *PNP* mRNA expression levels were significantly upregulated in clinical MSSCC tumor tissues. Silencing studies of target genes demonstrated that both genes inhibited cancer cell proliferation. The identification of novel *miR-1*/*miR-133a*-regulated cancer pathways could provide new insights into potential molecular mechanisms of MSSCC oncogenesis.

Introduction

The maxillary sinuses are single pyramidal cavities in the bodies of the maxillae. Squamous cell carcinoma is the most common cancer of the maxillary sinus (60-70%), followed by adenoid cystic carcinoma (1). Maxillary sinus squamous cell carcinoma (MSSCC) comprises 2-3% of all head and neck tumors and the annual incidence is 0.5-1.0 per 100,000 people (2,3). Clinical symptoms of MSSCC present insidiously, and sometimes resemble those of chronic sinusitis. Although presentation of lymph node and/or distant metastasis is uncommon, primary tumors are often diagnosed as advanced disease. The five-year survival rate of T4 tumors is approximately 50%. Local recurrence is the most common cause of treatment failure and death (2-4).

From an epidemiologic standpoint, occupational exposures to leather, wood dust, nickel, arsenic and formaldehyde have been implicated in the etiology of MSSCC (5,6). In contrast, tobacco, a major risk factor for head and neck squamous cell carcinoma (HNSCC), does not play an important role in MSSCC (7,8). It has been suggested that the molecular mechanism of carcinogenesis differ for these two cancers. Thus far, there have been few genome-wide gene expression analyses of MSSCC. Furthermore, analysis of microRNA (miRNA) has not been conducted for this disease.

miRNAs are a class of small non-coding RNA molecules consisting of 19-22 nucleotides that have an important role in a variety of biological processes including development, differentiation, apoptosis, and cell proliferation. They regulate gene expression through translational repression and mRNA cleavage (9). Bioinformatic predictions indicate that miRNAs regulate more than 30% of protein coding genes (10). So far, 1048 human miRNAs have been registered at miRBase release 16.0 (http://microrna.sanger.ac.uk/). Although biological functions of miRNAs remain mostly unknown, recent studies suggest that miRNAs contribute to the initiation and development of various types of cancer (11). miRNAs can be separated into two classes: those which are oncogenic and those which are tumor suppressive. Overexpressed miRNAs could act as oncogenes by repressing tumor suppressor genes, whereas under-expressed miRNAs could function as tumor suppressors by negatively regulating oncogenes (12).

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Our previous studies of miRNA expression signatures showed that *miR-1* and *miR-133a* were frequently down-regulated in various cancer cells (13,14). Interestingly, *miR-1-1*/*miR-133a-2* and *miR-1-2*/*miR-133a-1* are clustered on different chromosomal regions in the human genome, 20q13.33 and 18q11.2, respectively. It is well known that several miRNAs form clusters in the human genome such as *miR-17-92*, *miR-106a-363* and *miR-106b-25* (15). Furthermore, the *miR-15a-16-1* cluster is known to act as a tumor suppressor by targeting multiple oncogenes, such as *BCL2*, *MCL1*, *CCND1* and *WINT3A* (16). We have focused on the function of clustered miRNAs and the identification of target genes (14). The functional analysis of clustered miRNAs is important in the field of cancer research.

The aim of this study was to clarify the functional significance of *miR-1* and *miR-133a* in MSSCC and to identify cancer pathways regulated by those miRNAs. For target gene searches of *miR-1* and *miR-133a* in MSSCC cells, we performed genome-wide gene expression analysis. We focused on two genes as candidate common targets of *miR-1* and *miR-133a* in this study, as those were among the most down-regulated genes. Insights into the association between tumor suppressive miRNAs and their target oncogene networks could enhance our understanding of the molecular mechanism of MSSCC carcinogenesis.

Materials and methods

Clinical MSSCC specimens. Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Chiba University. Twenty pairs of primary tumor tissues and corresponding normal epithelial tissues were obtained from patients with MSSCC in Chiba University Hospital (Chiba, Japan) from 2007 to 2010. The normal tissue was confirmed to be free of cancer cells by pathologic examination. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at -20˚C until RNA was extracted.

MSSCC cell culture. The human MSSCC cell line, IMC-3 (17), was used. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37˚C.

RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically, and molecular integrity was checked by gel electrophoresis. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Mature miRNA transfection and small interfering RNA treatment. The following RNA species were used in this study: mature miRNAs, Pre-miR™ miRNA Precursors (*hsa-miR-1*; Pre-miR ID: PM10633, *hsa-miR-133a*; Pre-miR ID: PM10413), negative control miRNA (P/N: AM17111) (Applied Biosystems, Foster City, CA, USA), small interfering RNA (Stealth Select RNAi™ siRNA; si-transgelin 2 (*TAGLN2)* Cat#; HSS144746, si-purine nucleoside phosphorylase (*PNP)* Cat#; HSS107264) (Invitrogen) and negative control siRNA (Stealth™ RNAi Negative Control Medium GC Duplex; 12935-300) (Invitrogen). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine™ RNAiMax reagent (Invitrogen) as described previously (18). The transfection efficiency of Pre-miR™ in the cell line was confirmed based on down-regulation of *TWF1* (*PTK9*) mRNA following transfection with *miR-1* as previously reported (13,19).

Cell proliferation assays. Cells were transfected with 10 nM miRNA and siRNA by reverse transfection and plated in 96-well plates at $3x10^3$ cells per well. After 72 or 96 h, cell proliferation was determined by the XTT assay, using the Cell Proliferation kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported (13,19). Triplicate wells were measured for cell viability in each treatment group.

Flow cytometry. IMC-3 cells were transiently transfected with miRNA-control, *miR-1* or *miR-133a* and were harvested 72 h later by trypsinization. After double staining with FITC-annexin V and propidium iodide (PI) [FITC Annexin V Apoptosis Detection kit (BD Biosciences)] according to the manufacturer's recommendations, the cells were analyzed by flow cytometry (FACScan®; BD Biosciences) equipped with CellQuest software (BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and then the relative ratio of early apoptotic cells to mock cultures from each experiment was compared. Experiments were done in triplicate.

Target gene search for miR-1 and miR-133a. A genome-wide screen using *miR-1* or *miR-133a* transfectants was performed to identify their target genes in the IMC-3 cells. Oligo-microarray human 44K (Agilent Technologies) was used for expression profiling of the transfectants in comparison with a miRNA-negative-control transfectant. Hybridization and wash steps were performed as previously described (20). The arrays were scanned using a Packard GSI Lumonics ScanArray 4000 (Perkin Elmer, Boston, MA, USA). The data were analyzed by means of DNASIS array software (Hitachi Software Engineering, Tokyo, Japan), which converted the signal intensity for each spot into text format. The log2 ratios of the median subtracted background intensities were analyzed. Data from each microarray study were treated by a global normalization method. Predicted target genes and their target miRNA binding site seed regions were investigated using TargetScan (release 5.1, http://www.targetscan.org/). The sequences of the predicted mature miRNAs were confirmed using miRBase (release 16.0, http://microrna.sanger.ac.uk/).

Quantitative real-time RT-PCR. First-strand cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene-specific PCR products were assayed continuously using a 7900-HT Real-Time PCR System according to the manufacturer's protocol. The initial PCR step consisted of a 10-min hold at 95˚C, followed by 40 cycles consisting of a 15 sec denaturation at 95˚C and a 1 min annealing/extension at 63˚C. TaqMan® probes and primers for *TAGLN2* (P/N: Hs00761239_s1), *PNP* (P/N: Hs00165367_m1) and *GUSB* (P/N: Hs99999908_m1) internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of *miR-1* (Assay ID: 002222) and *miR-133a* (Assay ID: 002246) were analyzed

Table I. Summary of clinical information obtained from twenty patients with MSSCC.

No.	Age	Gender	Differentiation	T	N	M	Stage
1	68	Male	Well	4b	0	0	IVB
2	77	Male	Poor	3	0	0	III
3	76	Male	Moderate	3	0	0	III
4	61	Male	Well	3	0	0	III
5	54	Male	Poor	3	0	0	III
6	65	Female	Poor	4b	0	0	IVB
7	65	Male	Moderate	4a	0	0	IVA
8	64	Male	Poor	4b	0	0	IVB
9	74	Male	Well	4a	0	0	IVA
10	71	Male	Moderate	3	1	0	III
11	64	Male	Moderate	4a	0	0	IVA
12	80	Male	Moderate	4a	0	0	IVA
13	66	Female	Poor	4a	2c	0	IVA
14	67	Male	Moderate	4a	0	0	IVA
15	60	Male	Poor	4a	0	0	IVA
16	66	Female	Moderate	4a	0	0	IVA
17	85	Male	Poor	4a	0	0	IVA
18	69	Male	Well	4a	0	0	IVA
19	57	Male	Poor	4a	0	0	IVA
20	69	Male	Poor	4a	2b	0	IVA

by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to *RNU48* (Assay ID: 001006). All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

Immunoblotting. Cells were harvested 72 h after transfection and lysates were prepared. Each cell lysate (50 μg of protein) was separated by NuPAGE on 4-12% bis-tris gels (Invitrogen) and transferred to PVDF membranes. Immunoblotting was performed with diluted (1:250) polyclonal *TAGLN2* antibody (HPA001925) and polyclonal *PNP* antibody (HPA001625) (Sigma-Aldrich, St. Louis, MO, USA), with β-actin antibody (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA) used as an internal control. The membrane was washed and incubated with goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized by echochemiluminescence (GE Healthcare Bio-Sciences, Princeton, NJ, USA), and the expression levels of these proteins were evaluated by ImageJ software (ver.1.43; http://rsbweb.nih.gov/ij/index.html).

Statistical analysis. The relationships between two groups and the numerical values obtained by real-time RT-PCR were analyzed using the non-parametric Mann-Whitney U test or the paired t-test. The relationship among more than three variables and numerical values was analyzed using the Bonferroni adjusted Mann-Whitney U test. Spearman's rank test was used to evaluate the relationships among the relative expression levels of *miR-1*, *miR-133a*, *TAGLN2* and *PNP* mRNA. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA).

Results

miR-1 and miR-133a are down-regulated in clinical MSSCC specimens. Data characterizing 20 MSSCC patients are presented in Table I. We first confirmed that expression levels of *miR-1* and *miR-133a* were significantly down-regulated in clinical MSSCC tumor tissues compared with normal tissues ($p=0.0023$ and $p=0.0382$, respectively, Fig. 1A). A significant positive correlation was shown between expression of *miR-1* and *miR-133a* (Fig. 1B).

Effect of miR-1 and miR-133a on cell proliferation and apoptosis in IMC-3 cells. The XTT assay showed statistically significant inhibition of cell proliferation in *miR-1* and *miR-133a* transfectants compared with miRNA controls after 72 and 96 h. For example, after 72 h, *miR-1* and *miR-133a* transfected IMC-3 cultures grew 59% and 90%, respectively, as much as mock cultures ($p<0.0001$ and $p=0.0051$, respectively), moreover after 96 h, proliferation cell to 27% and 62% of mock, respectively (both $p<0.0001$, Fig. 2A).

Cell apoptosis in *miR-1* and *miR-133a* transfected cells was assessed by flow cytometry. The fraction of early apoptotic cells significantly increased in *miR-1* transfectants approximately 6.4-fold and in *miR-133a* transfectants approximately 2.7-fold compared with mocks (both $p<0.0001$, Fig. 2B).

Gene expression profiling identifies down-regulated genes in miR-1 and miR-133a transfectants. To investigate candidate molecular targets of *miR-1* and *miR-133a* in MSSCC cells, we examined the effects of *miR-1* and *miR-133a* on protein coding genes. Mature *miR-1* or *miR-133a* was transiently transfected

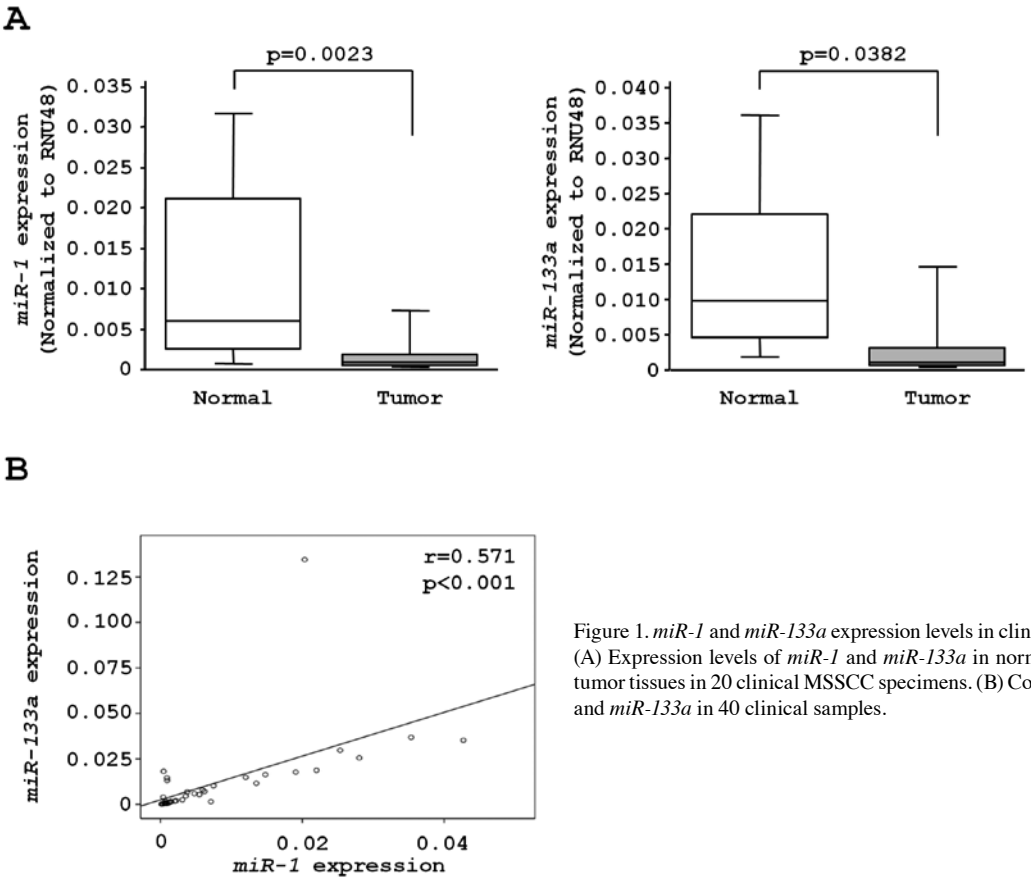

Figure 1. *miR-1* and *miR-133a* expression levels in clinical MSSCC specimens. (A) Expression levels of *miR-1* and *miR-133a* in normal adjacent tissues and tumor tissues in 20 clinical MSSCC specimens. (B) Correlation between *miR-1* and *miR-133a* in 40 clinical samples.

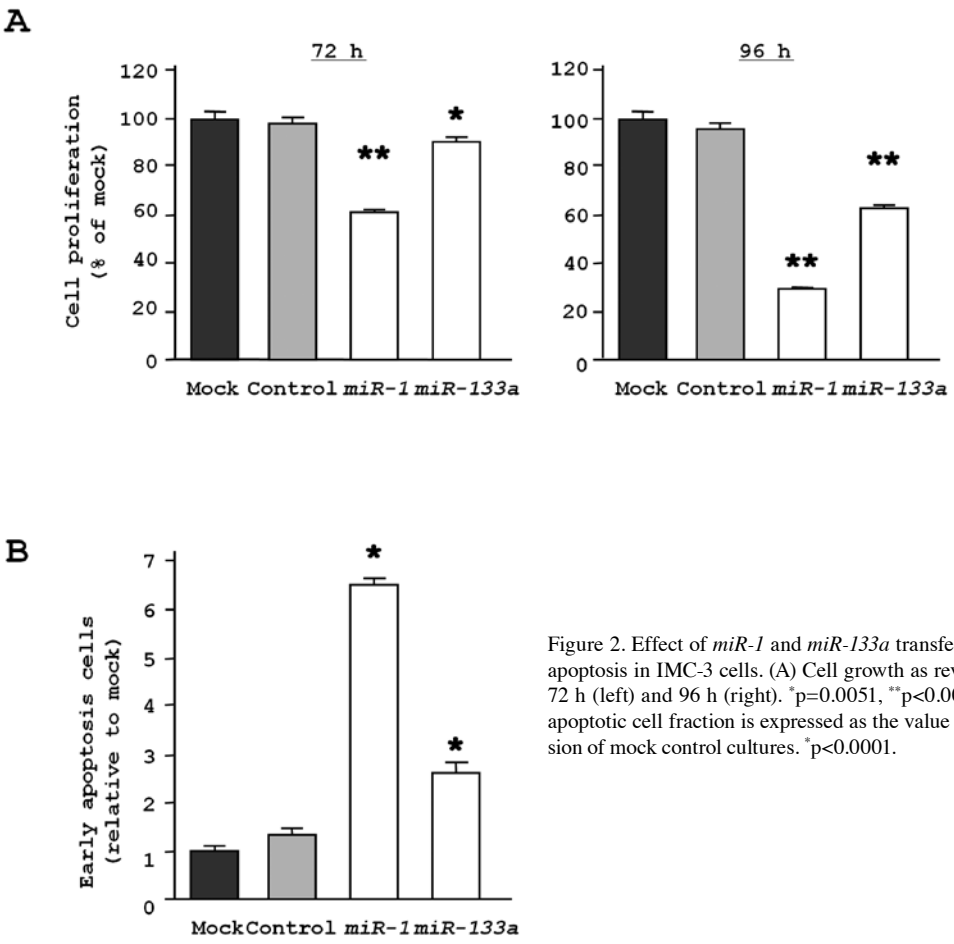

Figure 2. Effect of *miR-1* and *miR-133a* transfection on cell proliferation and apoptosis in IMC-3 cells. (A) Cell growth as revealed by the XTT assay after 72 h (left) and 96 h (right). *p=0.0051, **p<0.0001. (B) The size of the early apoptotic cell fraction is expressed as the value relative to the average expression of mock control cultures. *p<0.0001.

Table II. Top 23 genes that were down-regulated less than -1.50 (Log2 ratio) in *miR-1* and *miR-133a* transfected IMC-3 cells are shown in ascending order of average.

No.	Entrez gene ID	Symbol	Gene name	Log2 ratio miR-1	Log2 ratio miR-133a	Average	miR-1 target site	miR-133a target site
1	8407	TAGLN2	Transgelin 2	-2.78	-1.98	-2.38	3	2
2	5819	PVRL2	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	-2.7	-1.9	-2.3	-	-
3	201895	C4orf34	Chromosome 4 open reading frame 34	-3.01	-1.51	-2.26	2	1
4	83473	KATNAL2	Katanin p60 subunit A-like 2	-2.65	-1.69	-2.17	-	-
5	1081	CGA	Glycoprotein hormones, α polypeptide	-2.38	-1.92	-2.15	-	-
6	255758	TCTEX1D2	Tctex1 domain containing 2	-2.12	-2.14	-2.13	-	-
7	100130691	hCG_1817306	Hypothetical LOC100130691	-1.79	-2.22	-2.0	-	-
8	56062	KLHL4	Kelch-like 4 (*Drosophila*)	-1.83	-2.08	-1.96	-	-
9	2200	FBN1	Fibrillin 1	-1.78	-1.98	-1.88	-	-
10	7837	PXDN	Peroxidasin homolog (*Drosophila*)	-1.74	-1.99	-1.87	1	2
11	9120	SLC16A6	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	-2.01	-1.7	-1.86	1	1
12	81704	DOCK8	Dedicator of cytokinesis 8	-2.16	-1.55	-1.86	-	-
13	79974	C7orf58	Chromosome 7 open reading frame 58	-2.07	-1.62	-1.84	-	-
14	3641	INSL4	Insulin-like 4 (placenta)	-1.53	-2.12	-1.83	-	-
15	7784	ZP3	Zona pellucida glycoprotein 3 (sperm receptor)	-1.76	-1.89	-1.83	-	-
16	441054	C4orf47	Chromosome 4 open reading frame 47	-1.67	-1.97	-1.82	-	-
17	29070	CCDC113	Coiled-coil domain containing 113	-1.75	-1.83	-1.79	-	-
18	252983	STXBP4	Syntaxin binding protein 4	-1.64	-1.83	-1.74	3	2
19	4860	PNP	Purine nucleoside phosphorylase	-1.66	-1.67	-1.67	1	1
20	3920	LAMP2	Lysosomal-associated membrane protein 2	-1.69	-1.61	-1.65	-	-
21	6446	SGK1	Serum/glucocorticoid regulated kinase 1	-1.74	-1.56	-1.65	-	1
22	582	BBS1	Bardet-Biedl syndrome 1	-1.68	-1.59	-1.64	-	-
23	89122	TRIM4	Tripartite motif-contaning 4	-1.55	-1.6	-1.58	-	1

into IMC-3 cells, with negative miRNA transfection used as a control. Comprehensive gene expression analysis (see Methods) clearly showed changes in gene expression patterns between both *miR-1* and control transfectants, and *miR-133a* and controls. To identify common candidate target genes of *miR-1* and *miR-133a*, a cut-off of fold-change value more than -1.5 (Log2 ratio) was applied to the array data. This filter resulted in the identification of 23 genes that were significantly down-regulated upon *miR-1* or *miR-133a* transfection in IMC-3 cells (Table II). Entries from the microarray data were approved by the Gene Expression Omnibus (GEO), and were assigned GEO accession number GSE26032. The 3' UTR of these down-regulated genes were examined for *miR-1* and *miR-133a* target sites using the TargetScan database. Of the 23 putative gene targets, six genes contained *miR-1* and *miR-133a* common target sites.

Expression levels of candidate target genes of miR-1 and miR-133a in MSSCC clinical specimens. We measured the mRNA expression levels of six candidate genes in 20 of clinical MSSCC specimens by quantitative real-time RT-PCR. Two genes, *TAGLN2* and *PNP* were significantly up-regulated in MSSCC tumor tissues (p=0.0321 and p=0.0089, respectively, Fig. 3A and B, left). The other four genes (*C4orf34*, *PXDN*, *SLC16A6* and *STXBP4*) were not up-regulated in MSSCC tumor tissues (data not shown). There were significant inverse correlations between *TAGLN2* - *miR-1*, *TAGLN2* - *miR-133a* and *PNP* - *miR-1*. There was a trend but no significant inverse correlation between *PNP* - *miR-133a* (Fig. 3A and B, middle and right).

TAGLN2 and PNP mRNA and protein levels are repressed by miR-1 and miR-133a. The qRT-PCR analysis showed that the mRNA expression levels of *TAGLN2* and *PNP* in the IMC-3 cell line were 2.7-fold and 1.5-fold higher, respectively, than those in normal epithelia (data not shown). We performed gain-of-function studies using *miR-1* and *miR-133a* transfected IMC-3 cells, and the mRNA and protein expression levels of *TAGLN2* and *PNP* were markedly down-regulated in the transfectants in comparison with the mock controls (Fig. 4A and B).

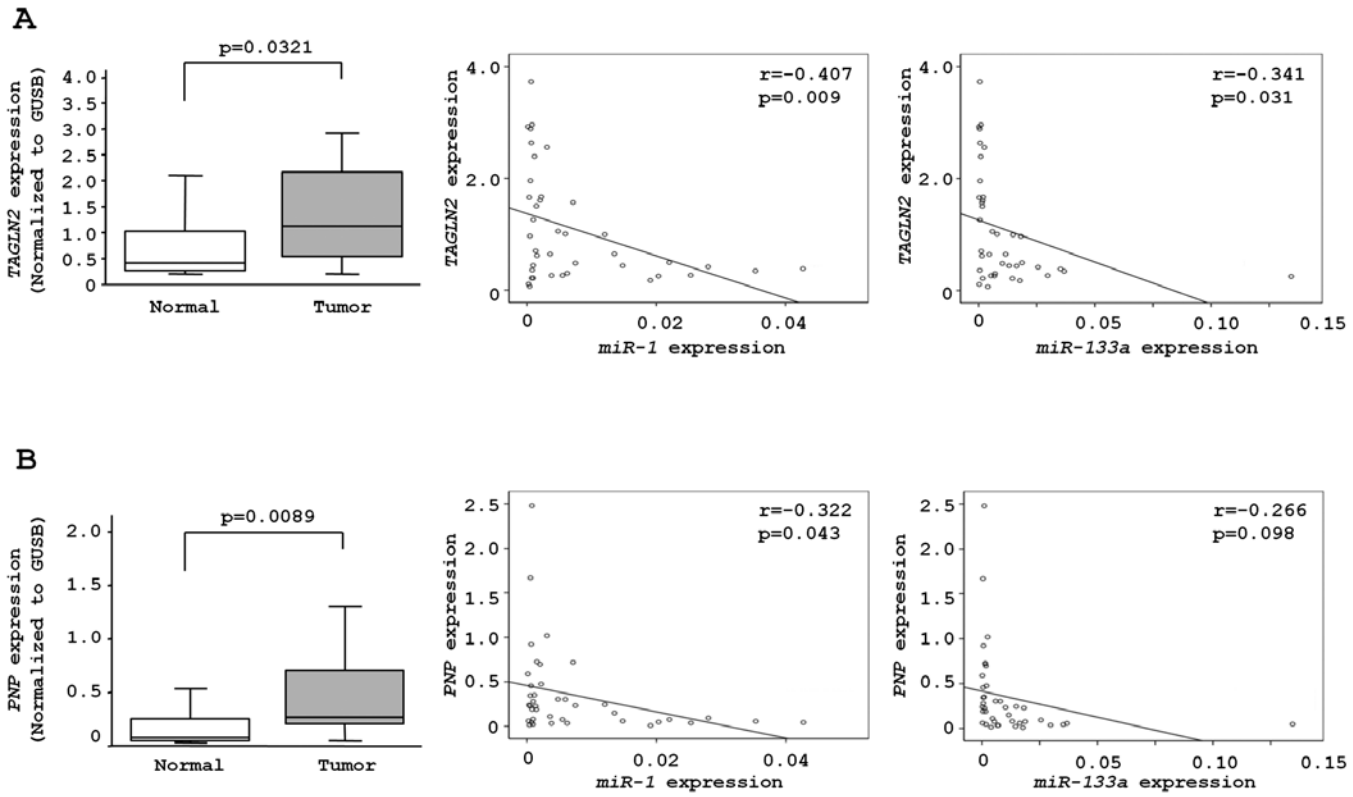

Figure 3. *TAGLN2* and *PNP* expression levels in clinical MSSCC specimens and IMC-3 cells. (A) Expression levels of *TAGLN2* in normal tissues and tumor tissues in 20 clinical MSSCC specimens (left). The correlation between *TAGLN2* and *miR-1* (middle) and the correlation between *TAGLN2* and *miR-133a* in 40 clinical samples (right). (B) Expression levels of *PNP* in normal tissues and tumor tissues in 20 clinical MSSCC specimens (left). Correlation between *PNP* and *miR-1* (middle) and correlation between *PNP* and *miR-133a* in 40 clinical samples (right).

Silencing TAGLN2 and PNP inhibits cell proliferation of IMC-3 cells. We performed loss-of-function studies using si-*TAGLN2* and si-*PNP* transfected IMC-3 cells. The expression levels of the mRNAs and proteins were repressed by si-*TAGLN2* and si-*PNP* (Fig. 5A and B, left and middle). XTT assays revealed that cell proliferation was only 59% of mock control levels in si-*TAGLN2* transfectants and 75% of mock controls in si-*PNP* transfectants (Fig. 5A and B, right).

Discussion

In the field of cancer research, evidence has been accumulating that aberrant expression of miRNAs and uncontrolled gene expression of tumor suppressors and oncogenes contribute to the development of human cancers (21). We performed the first miRNA expression signature analysis of MSSCC using clinical specimens. Our data showed that *miR-1* and *miR-133a* were significantly down-regulated in cancer cells. We validated the reduction of both *miR-1* and *miR-133a* in 20 specimens of MSSCC in this study. It is suggested that this event is a key step in oncogenesis of MSSCC. To confirm the importance of this observation, we investigated the functional significance of *miR-1* and *miR-133a* in IMC-3 cells. Our present data revealed that restoration of *miR-1* or *miR-133a* expression suppressed cancer cell proliferation and promoted apoptosis in IMC-3 cells.

As for the relation of human cancers and *miR-1*, recent articles revealed that *miR-1* induced apoptosis through repression of Mcl-1 in lung cancer (22). *miR-1* also targets c-Met in rhabdomyosarcoma (23). Consistent with these reports, our previous studies revealed that *miR-1* was down-regulated in cancer and acts as a tumor suppressor (14,24).

With regard to *miR-133a*, several reports showed that *miR-133a* was under-expressed in pancreatic ductal adenocarcinoma, colorectal carcinoma, tongue squamous cell carcinoma (SCC), and rhabdomyosarcoma (25-28). *miR-133a* inhibited cell proliferation and induced apoptosis and directly bound to oncogenic *PKM2* in tongue SCC (27). Our research also revealed that *miR-133a* was repressed in the tumor and had tumor suppressive functions (13,29). Taken together, *miR-1* and *miR-133a* are important miRNAs acting as tumor suppressors in human cancers.

Of particular interest is the fact that one miRNA regulates many protein-coding genes. However, the nature of mRNA-microRNA networks in the human genome is unclear. The elucidation of new regulatory networks in cancer is important for understanding carcinogenesis. Therefore, we are continuing our investigation of tumor suppressive miRNA-regulated oncogenic targets in various cancer cells (13,14,19,24,29). In this study, we adopted a method of genome-wide gene expression analysis in IMC-3 cells, using *miR-1* or *miR-133a* transfectants to identify targets. We identified genes regulated by *miR-1* or *miR-133a* by analyzing the expression profiles. This approach led us to investigate the mRNA expression levels of six candidate genes (*TAGLN2*, C4orf34, PXDN, SLC16A6, STXBP4 and *PNP*)

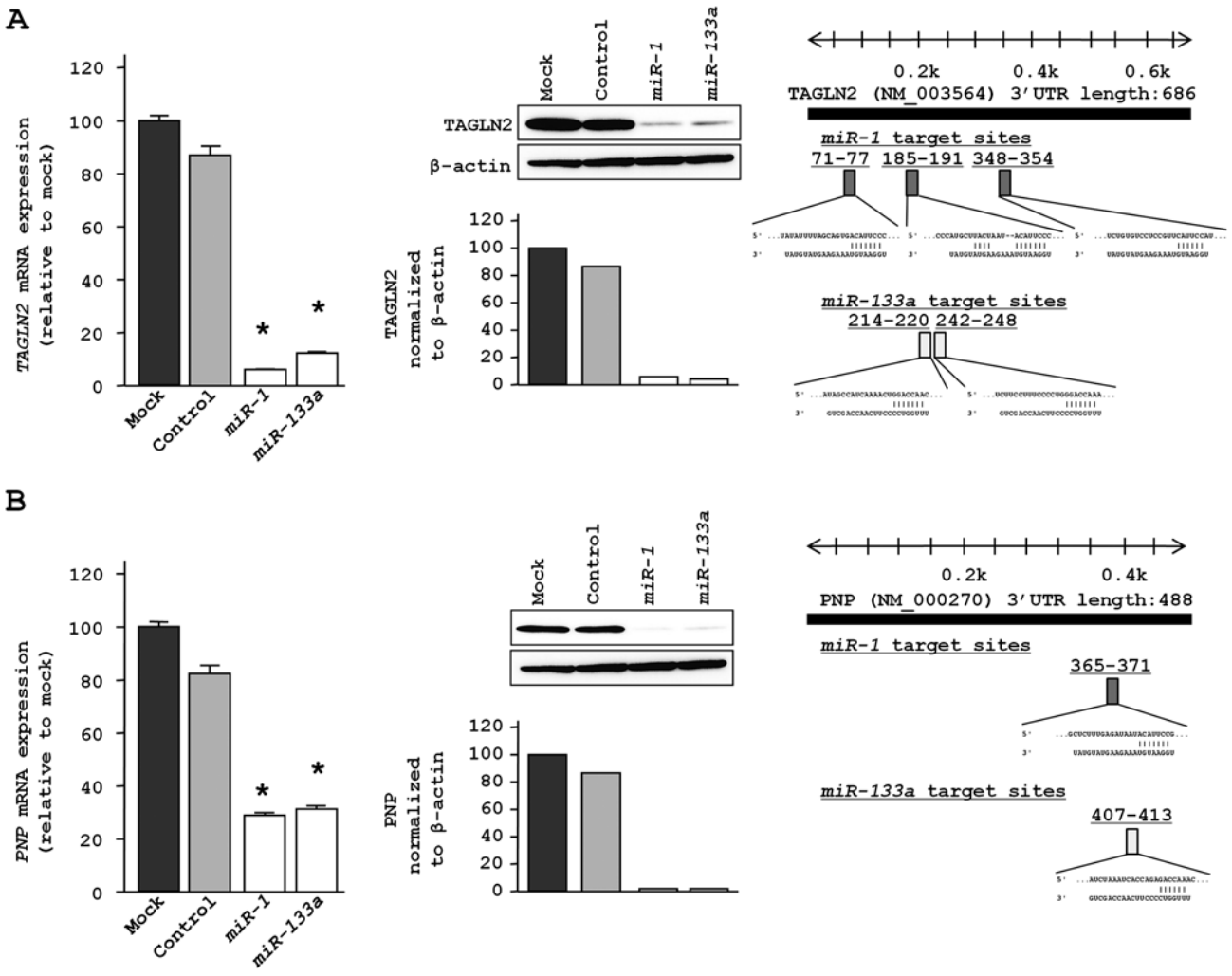

Figure 4. Regulation of *TAGLN2* and *PNP* in *miR-1* and *miR-133a* transfected IMC-3 cells. (A) RT-PCR revealed that *TAGLN2* mRNA was markedly repressed in both *miR-1* and *miR-133a* transfectants compared with mock controls (left). Western blots showed that TAGLN2 protein was also decreased in both *miR-1* and *miR-133a* transfectants (middle). Predicted target sites of *TAGLN2* 3'UTR with *miR-1* or *miR-133a* are shown (right). *p<0.0001. (B) *PNP* mRNA was markedly repressed in both *miR-1* and *miR-133a* transfected IMC-3 cells (left). Western blots revealed that PNP protein was also decreased in both *miR-1* and *miR-133a* transfectants (middle). Predicted target sites of *PNP* 3'UTR with *miR-1* or *miR-133a* are shown (right). *p<0.0001.

using MSSCC clinical specimens. Our criteria for selection in this analysis were that candidate genes had to be up-regulated in cancer cells. Two genes (*TAGLN2* and *PNP*) were chosen according to this standard.

TAGLN2 is a member of the calponin family of actin-binding proteins. TAGLN and TAGLN3 are homologues of TAGLN2, and TAGLN3 is a novel neuron-specific protein (30,31). The protein encoded by the *TAGLN* gene is an actin-binding protein like *TAGLN2*, found in fibroblasts and smooth muscle (32). Overexpression of TAGLN2 was observed in hepatocellular carcinoma, lung adenocarcinoma, and pancreatic cancer (33-36). More recently, we also found overexpression of TAGLN2 in HNSCC and bladder cancer (14,24). Furthermore, we proved that *miR-1* and/or *miR-133a* function as tumor suppressors and directly regulate *TAGLN2* in cancer cells (14,24). The results of our MSSCC study are in accord with past reports. Reduction of *miR-1* and/or *miR-133a* expression levels and the increase of *TAGLN2* appear causally related, suggesting that novel pathways in cancer cells play important roles in human carcinogenesis. However, it is still unknown how TAGLN2, an actin-binding protein, contributes to cell proliferation, which should be investigated by further analysis.

Our analysis suggests that overexpression of *PNP* has an important role in MSSCC carcinogenesis. PNP is an enzyme involved in purine metabolism. PNP catalyzes the conversion of adenosine to adenine, inosine to hypoxanthine, and guanosine to guanine, creating ribose phosphate in each case. PNP is considered a therapeutic target in malignant lymphoproliferative diseases. This is based on studies of genetic PNP-deficiency syndrome in which 2'-deoxyguanosine accumulates in plasma and deoxyguanosine triphosphate (dGTP) in lymphocytes, accordingly leading to dGTP-directed inhibition of DNA synthesis and cell death (37). Forodesine (BCX-1777) is a novel PNP inhibitor which induces apoptosis of chronic lymphocytic leukemia (CLL) cells (38). Phase II clinical trials, conducted from 2005 until 2009, used Forodesine in patients with

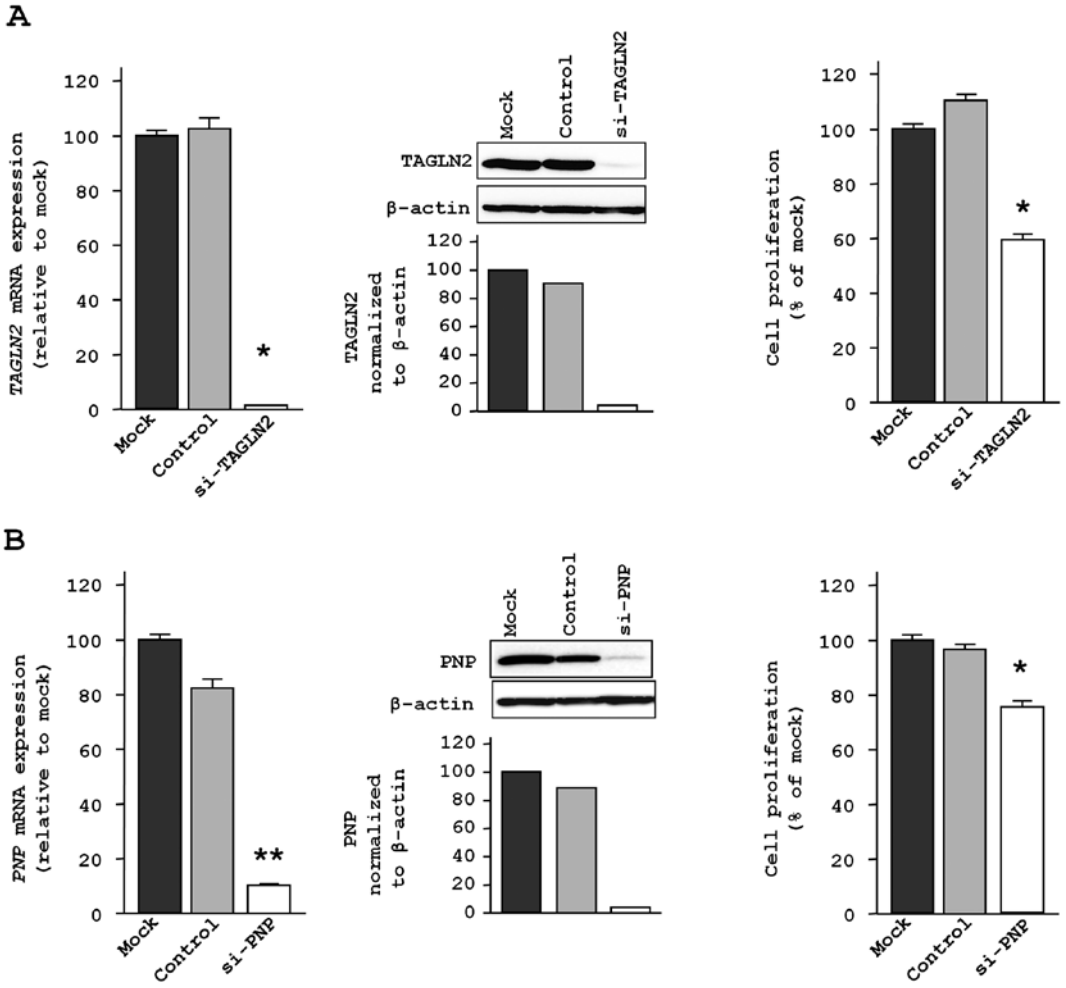

Figure 5. Effect of si-TAGLN2 and si-PNP transfection on IMC-3 cells. (A) RT-PCR revealed that *TAGLN2* mRNA was markedly repressed in si-TAGLN2 (left). Western blots revealed that TAGLN2 protein was also decreased in si-TAGLN2 (middle). Cell growth as revealed by the XTT assay after 72 h (right). $^{*}p<0.0001$ (B) RT-PCR revealed that PNP mRNA was markedly repressed in si-PNP (left). Western blots revealed that PNP protein was also decreased in si-PNP (middle). Cell growth as revealed by the XTT assay after 72 h (right). $^{*}p=0.0140$, $^{**}p<0.0001$.

advanced, Fludarabine-refractory CLL (39). Silencing *PNP* inhibited proliferation of IMC-3 cells in our present study. PNP inhibition might be a potential strategy for novel treatment of MSSCC.

In conclusion, *miR-1* and *miR-133a* were frequently reduced in MSSCC cancer cells. Both of the miRNAs may function as tumor suppressors regulating several oncogenic genes such as *TAGLN2* and *PNP* in MSSCC. Both *TAGLN2* and *PNP* were up-regulated in MSSCC specimens. The *miR-1/miR-133a* cluster regulates novel cancer pathways and as such could provide new insights into molecular mechanisms of MSSCC. Further studies could contribute to the development of new therapeutic strategies for this disease.

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References

1. Million RR and Cassisi NJ: Management of Head and Neck Cancer: A Multidisciplinary Approach. 2nd edition. In: Nasal Vestibule, Nasal Cavity, and Paranasal Sinuses. Parsons JT, Striger SP, Mancuso AA and Million RR (ed.) J.B. Lippincott Company, Philadelphia, pp556-559, 1994.
2. Tiwari R, Hardillo JA, Mehta D, Slotman B, Tobi H, Croonenburg E, van der Waal I and Snow GB: Squamous cell carcinoma of maxillary sinus. Head Neck 22: 164-169, 2000.
3. Dulguerov P, Jacobsen MS, Allal AS, Lehmann W and Calcaterra T: Nasal and paranasal sinus carcinoma: are we making progress? A series of 220 patients and a systematic review. Cancer 92: 3012-3029, 2001.
4. Konno A, Ishikawa K, Terada N, Numata T, Nagata H and Okamoto Y: Analysis of long-term results of our combination therapy for squamous cell cancer of the maxillary sinus. Acta Otolaryngol (Suppl) 537: 57-66, 1998.
5. Bornholdt J, Hansen J, Steiniche T, Dictor M, Antonsen A, Wolff H, Schlunssen V, Holmila R, Luce D, Vogel U, Husgafvel-Pursiainen K and Wallin H: K-ras mutations in sinonasal cancers in relation to wood dust exposure. BMC Cancer 8: 53, 2008.
6. Luce D, Leclerc A, Begin D, Demers PA, Gerin M, Orlowski E, Kogevinas M, Belli S, Bugel I, Bolm-Audorff U, Brinton LA, Comba P, Hardell L, Hayes RB, Magnani C, Merler E, Preston-Martin S, Vaughan TL, Zheng W and Boffetta P: Sinonasal cancer and occupational exposures: a pooled analysis of 12 case-control studies. Cancer Causes Control 13: 147-157, 2002.

7. Holt GR: Sinonasal neoplasms and inhaled air toxics. Otolaryngol Head Neck Surg 111: 12-14, 1994.
8. Zheng W, McLaughlin JK, Chow WH, Chien HT and Blot WJ: Risk factors for cancers of the nasal cavity and paranasal sinuses among white men in the United States. Am J Epidemiol 138: 965-972, 1993.
9. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism and function. Cell 116: 281-297, 2004.
10. Filipowicz W, Bhattacharyya SN and Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9: 102-114, 2008.
11. Calin GA and Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866, 2006.
12. Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
13. Kano M, Seki N, Kikkawa N, Fujimura L, Hoshino I, Akutsu Y, Chiyomaru T, Enokida H, Nakagawa M and Matsubara H: miR-145, miR-133a and miR-133b: Tumor suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. Int J Cancer 127: 2804-2814, 2010.
14. Yoshino H, Chiyomaru T, Enokida H, Kawakami K, Tatarano S, Nishiyama K, Nohata N, Seki N and Nakagawa M: The tumour-suppressive function of miR-1 and miR-133a targeting TAGLN2 in bladder cancer. Br J Cancer 104: 808-818, 2011.
15. Mendell JT: miRiad roles for the *miR-17-92* cluster in development and disease. Cell 133: 217-222, 2008.
16. Aqeilan RI, Calin GA and Croce CM: miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death Differ 17: 215-220, 2010.
17. Mizoguchi H, Komiyama S, Matsui K, Hamanaka R, Ono M, Kiue A, Kobayashi M, Shimizu N, Welgus HG and Kuwano M: The response to epidermal growth factor of human maxillary tumor cells in terms of tumor growth, invasion and expression of proteinase inhibitors. Int J Cancer 49: 738-743, 1991.
18. Ichimi T, Enokida H, Okuno Y, Kunimoto R, Chiyomaru T, Kawamoto K, Kawahara K, Toki K, Kawakami K, Nishiyama K, Tsujimoto G, Nakagawa M and Seki N: Identification of novel microRNA targets based on microRNA signatures in bladder cancer. Int J Cancer 125: 345-352, 2009.
19. Kikkawa N, Hanazawa T, Fujimura L, Nohata N, Suzuki H, Chazono H, Sakurai D, Horiguchi S, Okamoto Y and Seki N: miR-489 is a tumour-suppressive miRNA target PTPN11 in hypopharyngeal squamous cell carcinoma (HSCC). Br J Cancer 103: 877-884, 2010.
20. Sugimoto T, Seki N, Shimizu S, Kikkawa N, Tsukada J, Shimada H, Sasaki K, Hanazawa T, Okamoto Y and Hata A: The galanin signaling cascade is a candidate pathway regulating oncogenesis in human squamous cell carcinoma. Genes Chromosomes Cancer 48: 132-142, 2009.
21. Nelson KM and Weiss GJ: MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7: 3655-3660, 2008.
22. Nasser MW, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, Wang B, Suster S, Jacob ST and Ghoshal K: Down-regulation of micro-RNA-1 (*miR-1*) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by *miR-1*. J Biol Chem 283: 33394-33405, 2008.
23. Yan D, Dong Xda E, Chen X, Wang L, Lu C, Wang J, Qu J and Tu L: MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J Biol Chem 284: 29596-29604, 2009.
24. Nohata N, Sone Y, Hanazawa T, Fuse M, Kikkawa N, Yoshino H, Chiyomaru T, Kawakami K, Enokida H, Nakagawa M, Shozu M, Okamoto T and Seki N: *miR-1* as a tumor suppressive microRNA targeting TAGLN2 in head and neck squamous cell carcinoma. Oncotarget 2: 29-44, 2011.
25. Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, Labourier E and Hahn SA: MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene 26: 4442-4452, 2007.
26. Arndt GM, Dossey L, Cullen LM, Lai A, Druker R, Eisbacher M, Zhang C, Tran N, Fan H, Retzlaff K, Bittner A and Raponi M: Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC Cancer 9: 374, 2009.
27. Wong TS, Liu XB, Chung-Wai Ho A, Po-Wing Yuen A, Wai-Man Ng R and Ignace Wei W: Identification of pyruvate kinase type M2 as potential oncoprotein in squamous cell carcinoma of tongue through microRNA profiling. Int J Cancer 123: 251-257, 2008.
28. Rao PK, Missiaglia E, Shields L, Hyde G, Yuan B, Shepherd CJ, Shipley J and Lodish HF: Distinct roles for miR-1 and miR-133a in the proliferation and differentiation of rhabdomyosarcoma cells. FASEB J 24: 3427-3437, 2010.
29. Chiyomaru T, Enokida H, Tatarano S, Kawahara K, Uchida Y, Nishiyama K, Fujimura L, Kikkawa N, Seki N and Nakagawa M: miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer 102: 883-891, 2010.
30. Prinjha RK, Shapland CE, Hsuan JJ, Totty NF, Mason IJ and Lawson D: Cloning and sequencing of cDNAs encoding the actin cross-linking protein transgelin defines a new family of actin-associated proteins. Cell Motil Cytoskeleton 28: 243-255, 1994.
31. Ito M, Depaz I, Wilce P, Suzuki T, Niwa S and Matsumoto I: Expression of human neuronal protein 22, a novel cytoskeleton-associated protein, was decreased in the anterior cingulate cortex of schizophrenia. Neurosci Lett 378: 125-130, 2005.
32. Lawson D, Harrison M and Shapland C: Fibroblast transgelin and smooth muscle SM22alpha are the same protein, the expression of which is down-regulated in many cell lines. Cell Motil Cytoskeleton 38: 250-257, 1997.
33. Huang J, Sheng HH, Shen T, Hu YJ, Xiao HS, Zhang Q, Zhang QH and Han ZG: Correlation between genomic DNA copy number alterations and transcriptional expression in hepatitis B virus-associated hepatocellular carcinoma. FEBS Lett 580: 3571-3581, 2006.
34. Shi YY, Wang HC, Yin YH, Sun WS, Li Y, Zhang CQ, Wang Y, Wang S and Chen WF: Identification and analysis of tumour-associated antigens in hepatocellular carcinoma. Br J Cancer 92: 929-934, 2005.
35. Rho JH, Roehrl MH and Wang JY: Tissue proteomics reveals differential and compartment-specific expression of the homologs transgelin and transgelin-2 in lung adenocarcinoma and its stroma. J Proteome Res 8: 5610-5618, 2009.
36. Chen R, Yi EC, Donohoe S, Pan S, Eng J, Cooke K, Crispin DA, Lane Z, Goodlett DR, Bronner MP, Aebersold R and Brentnall TA: Pancreatic cancer proteome: the proteins that underlie invasion, metastasis, and immunologic escape. Gastroenterology 129: 1187-1197, 2005.
37. Bzowska A, Kulikowska E and Shugar D: Purine nucleoside phosphorylases: properties, functions, and clinical aspects. Pharmacol Ther 88: 349-425, 2000.
38. Alonso R, Lopez-Guerra M, Upshaw R, Bantia S, Smal C, Bontemps F, Manz C, Mehrling T, Villamor N, Campo E, Montserrat E and Colomer D: Forodesine has high antitumor activity in chronic lymphocytic leukemia and activates p53-independent mitochondrial apoptosis by induction of p73 and BIM. Blood 114: 1563-1575, 2009.
39. Balakrishnan K, Verma D, O'Brien S, Kilpatrick JM, Chen Y, Tyler BF, Bickel S, Bantia S, Keating MJ, Kantarjian H, Gandhi V and Ravandi F: Phase 2 and pharmacodynamic study of oral forodesine in patients with advanced, fludarabine-treated chronic lymphocytic leukemia. Blood 116: 886-892, 2010.