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Identification of tumour suppressive *microRNA-451a* in hypopharyngeal squamous cell carcinoma based on microRNA expression signature

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Background: Hypopharyngeal squamous cell carcinoma (HSCC) has a very poor prognosis because of its high rates of regional and distant metastasis. Identification of differentially expressed miRNAs and their regulated molecular targets in tumour cells might enhance our understanding of the molecular mechanisms of metastasis in human cancers.

Methods: A HSCC miRNA signature was constructed by array-based methods. Functional studies of *microRNA-451a* (*miR-451a*) and target genes were performed to investigate cell proliferation, migration and invasion by cancer cell lines. To identify *miR-451a*-regulated molecular targets, we adopted gene expression analysis and *in silico* database analysis.

Results: Our miRNA signature revealed that *miR-451a* was significantly downregulated in HSCC. Restoration of *miR-451a* in cancer cell lines revealed that this miRNA significantly inhibited cancer cell migration and invasion. Our data demonstrated that the gene coding for endothelial and smooth muscle cell-derived neuropilin-like molecule (*ESDN/DCBLD2*) was a direct target of *miR-451a* regulation. Silencing of *ESDN* inhibited cell migration and invasion by cancer cells.

Conclusions: Loss of tumour suppressive *miR-451a* enhanced cancer cell migration and invasion in HSCC through direct regulation of *ESDN*. Our miRNA signature and functional analysis of targets regulated by tumour suppressive *miR-451a* provide new insights into the potential mechanisms of HSCC oncogenesis and metastasis.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and ~500 000 cases are diagnosed every year (Jemal *et al*, 2010). Hypopharyngeal squamous cell carcinoma (HSCC) occurs less frequently and accounts for 3–5% of all HNSCC (Hall *et al*, 2008; Cooper *et al*, 2009). In spite of considerable advances in multimodality therapy, including surgery, radiotherapy and chemotherapy, the overall survival rate for patients with HSCC is only 15–45% (Takes *et al*, 2012; Chan & Wei, 2013). Patients with HSCC are usually diagnosed at a late stage and local tumour recurrence and distant metastasis occur after conventional therapies (Takes *et al*, 2012; Chan and Wei, 2013). Recurrence is the primary cause for poor survival of HSCC. Therefore, understanding the molecular mechanisms of metastatic pathways underlying HSCC using currently available genomic approaches might improve therapies for and prevention of the disease.

The discovery of non-coding RNAs (ncRNAs) in the human genome was an important conceptual breakthrough in

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the post-genome sequencing era (Carthew and Sontheimer, 2009). Further improving our understanding of ncRNAs is necessary for continued progress in cancer research. MicroRNAs (miRNAs) are endogenous small ncRNA molecules (19–22 bases in length) that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (Bartel, 2004). Numerous studies have shown that miRNAs are aberrantly expressed in many human cancers and that they have significant roles in the initiation, development and metastasis of those cancers (Filipowicz *et al*, 2008; Hobert, 2008; Friedman *et al*, 2009; Iorio and Croce, 2009). Moreover, normal regulatory mechanisms can be disrupted by the aberrant expression of tumour suppressive or oncogenic miRNAs in cancer cells (Esquela-Kerscher and Slack, 2006; Kwak *et al*, 2010). Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating miRNA-mediated oncogenic pathways.

With those points in mind, we have constructed miRNA expression signatures of HSCC using clinical specimens. Using those data, we have investigated the specific roles of miRNAs in HSCC metastasis by examining the differentially expressed miRNAs. Data from our present HSCC signature showed that *miR-451a* is significantly downregulated in cancer tissues, suggesting that *miR-451a* might act as a tumour suppressor. Several studies reported aberrant expression of *miR-451a* in several types of human cancers (Pan *et al*, 2013). Furthermore, the expression levels of *miR-451a* are associated with the clinical outcome in several types of cancer, including lung cancer, oesophageal cancer and nasopharyngeal cancer (Wang *et al*, 2011, 2013; Liu *et al*, 2013). These data indicated that *miR-451a* might have critical roles in cancer cells and oncogenesis. However, the functional role of *miR-451a* in HSCC is unknown.

The aim of the present study was to investigate the functional significance of *miR-451a* and to identify the molecular targets regulated by the miRNAs in HSCC cells. Our data demonstrated that restoration of mature *miR-451a* inhibited cancer cell migration and invasion. Moreover, gene expression data and *in silico* database analysis showed that the gene coding for endothelial and smooth muscle cell-derived neuropilin-like molecule (*ESDN/DCBLD2*), a novel neuropilin-like membrane protein, was a potential target of *miR-451a*-mediated regulation. Silencing studies of the *ESDN/DCBLD2* gene significantly inhibited cell migration and invasion of cancer cells. The discovery of tumour suppressive *miR-451a*-regulated targets provides new insight into the potential mechanisms of HSCC oncogenesis and suggests novel therapeutic strategies for the treatment of the disease.

MATERIALS AND METHODS

Clinical HSCC specimens and RNA isolation. A total of 22 pairs of primary tumours and corresponding normal epithelial specimens were obtained from patients with HSCC at Chiba University Hospital (Chiba, Japan) from 2007 to 2013. The fresh specimens were immediately immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA was extracted. The noncancerous specimens were macroscopically normal and were confirmed to be free of cancer cells by microscopic pathological examination. The patients' backgrounds and clinicopathological characteristics are summarised in Table 1. The patients were classified according to the 2002 Union for International Cancer TNM staging criteria before treatment.

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.

Table 1. Clinical features of 22 patients with HSCC

No	Age	Sex	T	N	M	Stage	Differentiaion
1	66	Male	2	2c	0	IVA	Moderate
2	64	Female	4a	1	0	IVA	Poor
3	53	Male	2	2b	0	IVA	Moderate
4	59	Male	3	2b	0	IVA	Poor
5	66	Male	4a	2c	0	IVA	Well
6	58	Female	4a	2c	0	IVA	Moderate
7	52	Male	4a	1	1	IVC	Moderate
8	74	Male	4a	2c	0	IVA	Poor
9	45	Male	4a	2c	0	IVA	Moderate
10	64	Female	4a	0	0	IVA	Well
11	75	Male	4a	2c	0	IVA	Well
12	76	Male	4a	1	0	IVA	Well
13	68	Male	4a	0	0	IVA	Moderate
14	73	Male	3	1	0	III	Poor
15	65	Male	3	0	0	III	Moderate
16	71	Male	2	2b	0	IVA	Poor
17	64	Male	2	0	0	II	Moderate
18	55	Male	3	2b	0	IVA	Moderate
19	71	Male	4a	2b	0	IVA	Moderate
20	66	Male	4b	2c	0	IVB	Moderate
21	64	Male	2	0	0	II	Moderate
22	58	Male	4a	0	0	IVA	Well

Total RNA was isolated using the TRIzol regimen (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA).

Construction of the miRNA expression signature of HSCC. To identify differentially expressed miRNAs in HSCC, 250 ng of total RNA was subjected to microarray analysis using a miRCURY LNA microRNA Array (Exiqon, Vedbaek, Denmark). Labelling, hybridisation, washing and scanning of the microarray were performed by Cosmo Bio (Tokyo, Japan) following the instructions of the manufacturer. Hy3 (sample) signals were normalised by division using the Hy5 (Universal reference) signal. Then, each signal value of the unique probe ID was calculated from the average of the Hy3/Hy5 normalised signal intensities of quadruplicate probes.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The procedure for PCR quantification was carried out as previously described (Kikkawa *et al*, 2010; Nohata *et al*, 2011a; Kinoshita *et al*, 2013). The expression levels of *miR-451a* (Assay ID: 001141; Applied Biosystems, Foster City, CA, USA) were analysed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalised to *RNU6B* (Assay ID: 001093). TaqMan probes and primers for *ESDN/DCBLD2* (P/N: Hs00294635_m1) and *GUSB* (P/N: Hs00939627_m1; an internal control) were used for expression level determination of HSCC clinical specimens. All reactions were performed in triplicate, and each assay included negative control reactions that lacked cDNA.

Transfections with mature miRNA and small-interfering RNA (siRNA). The following mature miRNAs species were used in this study: mirVana miRNA mimic for hsa-miR-451a (Product ID: MC10286) (Applied Biosystems). The following siRNAs were used: Stealth Select RNAisiRNA; si-*ESDN*/*DCBLD2* (Cat no.HSS134348)

and negative control miRNA/siRNA (P/N: AM17111, Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen) as described previously. The transfection efficiency of miRNA in cell lines was confirmed based on downregulation of *TWF1* (*PTK9*) mRNA following transfection with *miR-1* as previously reported(Kikkawa *et al*, 2010).

Cell proliferation, migration and invasion assays. To investigate the functional significance of *miR-451a or si-ESDN/DCBLD2*, we performed cell proliferation, migration and invasion assays using FaDu and SAS cells. The experimental procedures were performed as described in our previous studies (Kinoshita *et al*, 2013).

Genome-wide gene expression and *in silico* analysis for the identification of genes regulated by *miR-451a*. The TargetScan database (Release 6.2; http://www.targetscan.org/) was used for *in silico* identification of candidate target genes that contained *miR-451a* targets sites in their 3′-untranslated (3′-UTR) region. To identify *miR-451a*-regulated genes, we used genome-wide gene expression analysis of FaDu cells transfected with *miR-451a*. SurePrint G3 Human GE 60K Microarray (Agilent Technologies) was used for expression profiling of *miR-451a* transfectants in comparison with negative control miRNA transfectants.

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. Fifty micrograms of protein from each lysate was separated on Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with rabbit anti-DCBLD2 antibodies (1 : 100; HPA016909; Sigma-Aldrich, St Louis, MO, USA), and anti-GAPDH antibodies (1 : 1000; ab8245, Abcam, Cambridge, UK) were used as an internal loading control.

Plasmid construction and dual-luciferase reporter assays. The partial wild-type sequences of the *ESDN/DCBLD2* 3′-UTR or those with deleted *miR-451a* target sites (positions 2756–2762 of *ESDN/DCBLD2* 3′-UTR) were inserted between the XhoI-Pmel restriction sites in the 3′-UTR of the hRluc gene in the psiCHECK-2 vector (C8021, Promega, Madison, WI, USA). The sequences of the oligonucleotides are described in Supplementary Document 1. SAS cells were transfected with 50 ng of vector and 10 nM of *miR-451a* using Lipofectamine 2000 (Invitrogen). The activities of firefly and *Renilla* luciferases in cell lysates were determined with dual-luciferase assay systems (E1910; Promega) as described previously (Kikkawa *et al*, 2010; Nohata *et al*, 2011a; Kinoshita *et al*, 2013).

Statistical analysis. The relationships between two groups and the numerical values obtained by real-time PCR were analysed using the paired *t*-test. Spearman's rank test was used to evaluate the correlation between the expression of *miR-451a* and target genes. The relationship among more than three variables and numerical values were analysed using the Bonferroni adjusted Mann–Whitney *U*-test. All analyses were performed using Expert Stat View (version 4, SAS Institute Inc., Cary, NC, USA).

RESULTS

Differentially expressed miRNAs in HSCC clinical specimens identified by miRNA expression signature analysis. We conducted miRNA expression signature analysis in HSCC clinical specimens (11 pairs of HSCC tissues and corresponding normal tissues, patient numbers 1–11) (Table 1). Expression signatures revealed that 3 miRNAs were upregulated and 23 miRNAs were downregulated in HSCC tissues (Table 2). Entries from the gene expression data in this analysis were approved by GEO, and were assigned GEO accession number GSE51129.

Our expression signature showed that three miRNAs (*miR-21-5p*, *miR-4732-5p* and *miR-4776-3p*) were upregulated (Fold-change >2.0) in HSCC tissues.

As for downregulated miRNAs, the top four (*miR-1, miR-133a*, *miR-133b* and *miR-29c*) were described in our previous reports as tumour suppressive miRNAs in various types of cancers including HNSCC (Table 2) (Nohata *et al*, 2011b, 2012; Kinoshita *et al*, 2012b, 2013). In this study, we focused on *miR-451a* and functional studies were performed.

Expression levels of *miR-451a* in HSCC clinical specimens and cell lines. To validate our miRNA signature results, we evaluated the expression levels of *miR-451a* in 22 HSCC specimens (patient numbers 1–22)(Table 1). Quantitative stem-loop RT-PCR demonstrated that *miR-451a* expression was significantly lower in clinical HSCC specimens and cell lines (FaDu and SAS) compared with noncancerous specimens (Figure 1A). There was no significant correlation between *miR-451a* expression and various tested clinicopathological parameters of HSCC (data not shown).

Effect of restoring *miR-451a* expression on cell proliferation, migration and invasion activities in cancer cell lines. To investigate the functional effects of *miR-451a*, we performed gain-of-function studies using miRNA transfection of FaDu and SAS cell lines.

The XTT assay demonstrated that cell proliferation was not inhibited in FaDu cells transfected with *miR-451a,* whereas SAS cells were significantly slowed by transfection in comparison with the mock or miR-control transfectants (Figure 1B). However, cell migration activity was significantly inhibited by *miR-451a* transfection of both cell lines compared with mock- or miR-control-transfected cells (Figure 1C). Moreover, in the Matrigel invasion assays, transfection with *miR-451a* significantly inhibited invasion compared with mock- or miR-control-transfected cells (Figure 1D).

Identification of candidate genes targeted by *miR-451a* in HSCC. To identify genes targeted by *miR-451a*, we analysed *in silico* and gene expression data in *miR-451a* transfectants. First, we selected *miR-451a* target genes that contained putative binding sites in their 3′UTR regions as determined by the TargetScan database. A total of 397 genes were identified in this selection. Next, we performed genome-wide gene expression analysis using a cancer cell line (FaDu) and selected downregulated genes ($\log_2$ ratio < -1.0) by *miR-451a* transfection compared with miR-control transfection. Among 397 putative candidate genes, 5 genes (*SPC25*, *MIF*, *ESDN/DCBLD2*, *C4orf46 and AKR1B1*) were downregulated by *miR-451a* transfection in cancer cells (Table 3). Entries from the gene expression data of *miR-451a* transfection into FaDu cells was approved by GEO, and was assigned GEO accession number GSE56243.

We further investigated the expression levels of these five genes in HSCC clinical specimens. Our data showed that three genes (*SPC25*, *ESDN/DCBLD2 and AKR1B1*) were significantly upregulated in cancer tissues compared with normal tissues (Figure 2). Spearman's rank test showed a negative correlation between the expression of *miR-451a* and those of the three genes (Figure 3).

Among these candidates, we focused on the *ESDN/DCBLD2* gene because it was the most significantly upregulated in cancer tissues. Thus, we examined the functions and characteristics of *ESDN/DCBLD2* in further analyses.

***ESDN/DCBLD2* was a direct target of *miR-451a* in HSCC cells.** We performed quantitative real-time RT-PCR and western blotting in FaDu and SAS cells to investigate whether restoration of *miR-451a* altered *ESDN/DCBLD2* gene and protein expression. The mRNA and protein expression levels of ESDN/DCBLD2 were significantly repressed in *miR-451a* transfectants compared with mock- or miR-control-transfected cells (Figure 4A and B).

Table 2. Differentially expressed miRNAs in HNSCC

MicroRNA	Accession no.	Location	Fold-change (tumour/normal)	Normal	Tumour	*P*-value
Downregulated miRNAs in HSCC						
hsa-miR-1	MIMAT0000416	20q13.33, 18q11.2	0.12	2.31	0.27	0.066
hsa-miR-133a	MIMAT0000427	18q11.2, 20q13.33	0.17	3.87	0.66	0.091
hsa-miR-133b	MIMAT0000770	6p12.2	0.18	4.49	0.79	0.069
hsa-miR-29c-3p	MIMAT0000681	1q32.2	0.25	1.66	0.42	0.004
hsa-miR-451a	MIMAT0001631	17q11.2	0.26	4.66	1.23	0.070
hsa-miR-206	MIMAT0000462	6p12.2	0.32	1.20	0.38	0.073
hsa-miR-378a-3p	MIMAT0000732	5q32	0.33	1.74	0.57	0.017
hsa-miR-29a-3p	MIMAT0000086	7q32.3	0.33	1.70	0.57	0.000
hsa-miR-378d	MIMAT0018926	8q22.1	0.36	0.20	0.07	0.062
hsa-miR-125b-5p	MIMAT0000423	11q24.1, 21q21.1	0.39	6.08	2.39	0.001
hsa-miR-101-3p	MIMAT0000099	1p31.3, 9p24.1	0.40	2.12	0.84	0.000
hsa-miR-1184	MIMAT0005829	Xq28	0.41	3.53	1.45	0.030
hsa-miR-4328	MIMAT0016926	Xq21.1	0.43	0.83	0.36	0.092
hsa-miR-126-3p	MIMAT0000445	9q34.3	0.44	2.91	1.28	0.001
hsa-miR-145-5p	MIMAT0000437	5q32	0.45	1.33	0.59	0.089
hsa-let-7c	MIMAT0000064	21q21.1	0.45	4.43	2.00	0.001
hsa-miR-4324	MIMAT0016876	19q13.33	0.46	1.28	0.59	0.000
hsa-miR-203a	MIMAT0000264	14q32.33	0.46	4.58	2.11	0.023
hsa-miR-4462	MIMAT0018986	6p21.2	0.47	0.21	0.10	0.012
hsa-miR-29b-3p	MIMAT0000100	7q32.3, 1q32.2	0.48	1.01	0.48	0.006
hsa-miR-659-5p	MIMAT0022710	22q13.1	0.48	0.19	0.09	0.073
hsa-miR-5000-3p	MIMAT0021020	2p12	0.48	0.95	0.46	0.005
hsa-miR-4638-5p	MIMAT0019695	5q35.3	0.50	0.41	0.21	0.009
Upregulated miRNAs in HSCC						
hsa-miR-21-5p	MIMAT0000076	17q23.1	3.83	2.49	9.54	0.001
hsa-miR-4732-5p	MIMAT0019855	17q11.2	2.58	0.58	1.49	0.065
hsa-miR-4776-3p	MIMAT0019933	2q34	2.00	0.13	0.26	0.084

Therefore, we performed luciferase reporter assays in SAS cells to determine whether *ESDN/DCBLD2* mRNA carried a target site for *miR-451a*. The TargetScan database predicted that one putative *miR-451a* binding site existed in the 3′-UTR of the gene (position 2756–2762). We used vectors encoding either a partial wild-type sequence (including the predicted *miR-451a* target site) or deletion-type sequence of the 3′-UTR of *ESDN/DCBLD2* mRNA. We found that the luminescence intensity was significantly reduced by co-transfection with *miR-451a a*nd the vector carrying the wild-type 3′-UTR of *ESDN/DCBLD2* (Figure 4C).

Effects of *ESDN/DCBLD2* silencing on cell proliferation, migration and invasion in cancer cell lines. To investigate the functional role of *ESDN/DCBLD2* in HSCC cells, we performed loss-of-function studies using *si-ESDN/DCBLD2* transfectants. First, we evaluated the knockdown efficiency of *si-ESDN/DCBLD2* transfection in FaDu and SAS cells. Quantitative real-time RT-PCR and western blotting indicated that the siRNA effectively down-regulated ESDN/DCBLD2 expression in both cell lines (Figure 5A).

In our functional analyses, XTT assays demonstrated that cell proliferation was not inhibited in FaDu *si-ESDN/DCBLD2* transfectants compared with mock- or si-control-transfected cells (Figure 5B). In contrast, growth of SAS cell transfectants was slowed significantly. Transfection with *si-ESDN/DCBLD2* inhibited both cell migration and invasion compared with mock- or si-control-transfected cells (Figure 5C and D), similar to the results observed for restoration of *miR-451a*.

DISCUSSION

Aberrant expression of miRNAs can upset tightly regulated RNA networks in normal cells, thereby promoting the development and progression of human cancers. Therefore, the first step in defining the contribution of miRNAs to oncogenesis is to identify the miRNAs that are differentially expressed in cancer cells. Our group has constructed miRNA expression signatures in various cancers, allowing us to identify tumour suppressive miRNAs and their targeted cancer pathways (Kikkawa *et al*, 2010; Nohata *et al*, 2011a; Yoshino *et al*, 2011).

In this study, we constructed a new HSCC miRNA expression signature because new human miRNAs had been added to the public databases after we undertook our initial analysis. In both our original signature of HSCC and the current one, *miR-1/133a* and *miR-206/133b* clusters and the *miR-29s*-family were found to be downregulated. These findings demonstrate that our method of deriving an HSCC signature is reproducible. Moreover, our recent studies showed that the aforementioned miRNAs function as tumour suppressors via targeting several oncogenic pathways

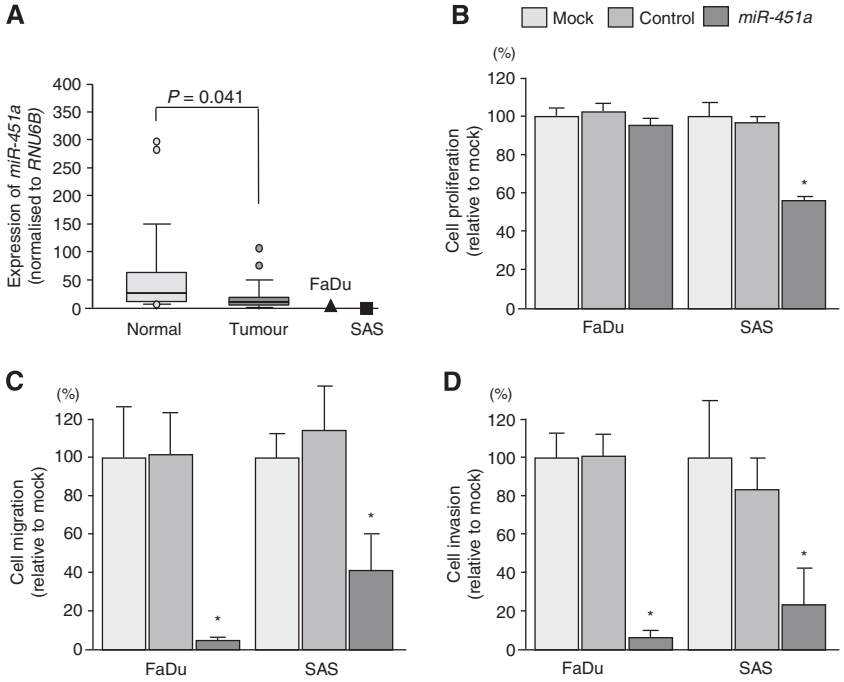

Figure 1. The functional significance of *miR-451a* in HSCC cells. (**A**) Expression levels of *miR-451a* in HSCC clinical specimens and cell lines. *RNU6B* was used as the internal control. (**B**–**D**) Effects of transfection of mature *miR-451a* in cancer cell lines. (**B**) Cell proliferation was determined with XTT assays in FaDu and SAS cell lines 72 h after transfection with 10 nM of *miR-451a*, miR-control or mock transfection. (**C**) Cell migration activity determined with the migration assay. (**D**) Cell invasion activity was determined with the Matrigel invasion assay. *$P<0.0001$.

Table 3. Candidate target gene for miR-451a

Entrez gene ID	Gene symbol	Gene name	Location	Log_2 ratio (*miR-451a*/miR-control)
57405	*SPC25*	SPC25, NDC80 kinetochore complex component, homologue (S. cerevisiae)	2q31.1	−3.55
4282	*MIF*	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	22q11.23	−2.34
131566	*ESDN/DCBLD2*	Endothelial and smooth muscle cell-derived neuropilin-like molecule/discoidin, CUB and LCCL domain containing 2	3q12.1	−1.81
201725	*C4orf46*	Chromosome 4 open reading frame 46	4q32.1	−1.43
231	*AKR1B1*	Aldo-keto reductase family 1, member B1(aldose reductase)	7q33	−1.25

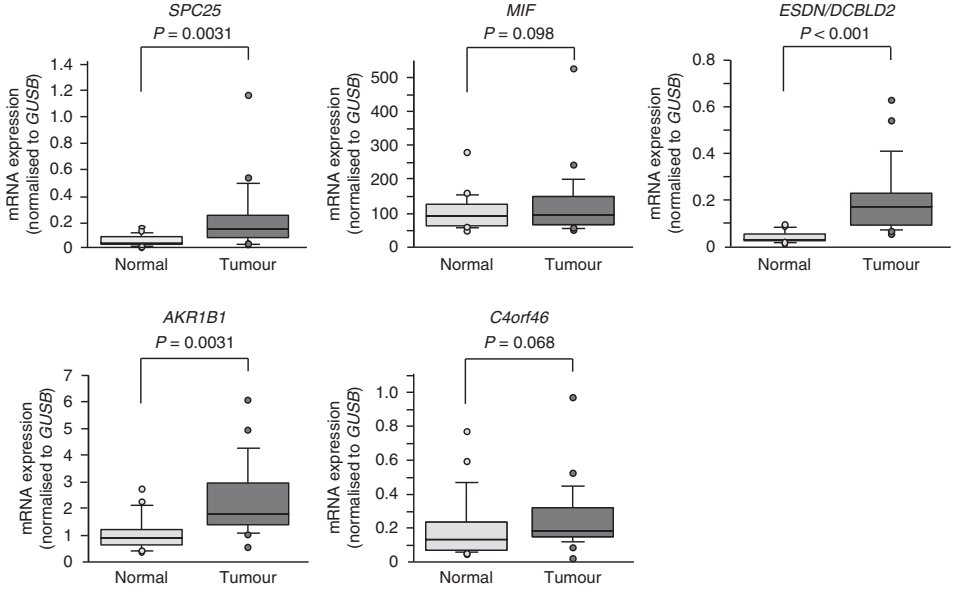

Figure 2. Expression levels of putative candidate target genes of *miR-451a*. The expression levels of five candidate genes (*SPC25*, *MIF*, *ESDN/DCBLD2*, *AKR1B1* and *C4orf46*) were measured in HSCC clinical tissues by RT-PCR. *GUSB* was used as the internal control.

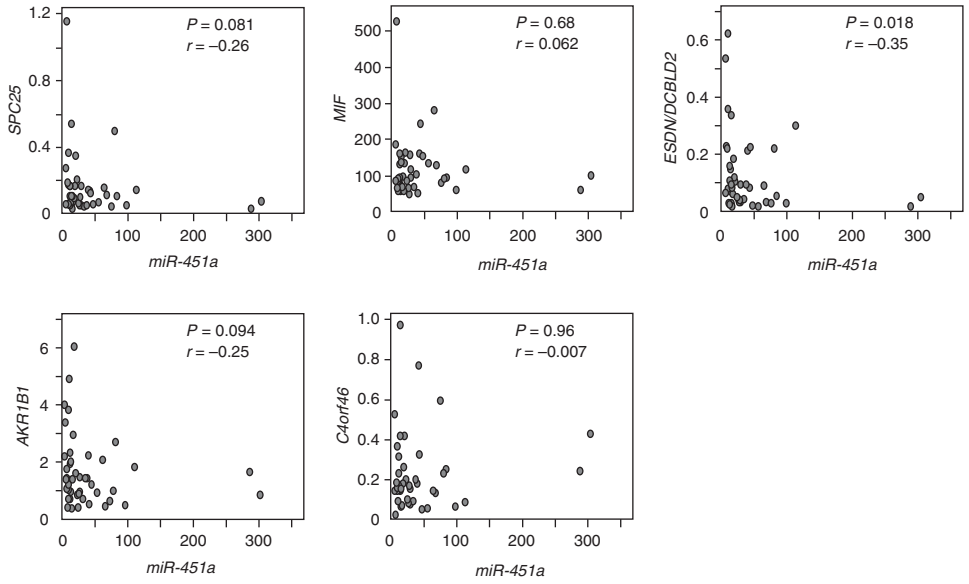

Figure 3. Correlation between the relative expression levels of *miR-451a* and target genes. Correlation between the relative expression levels of five candidate genes (y-axis; *SPC25*, *MIF*, *ESDN/DCBLD2*, *AKR1B1* and *C4orf46*) and that of *miR-451a* (x-axis) was plotted in scatter diagrams. Spearman's rank test was used to evaluate the correlation between the expression of *miR-451a* and the target genes.

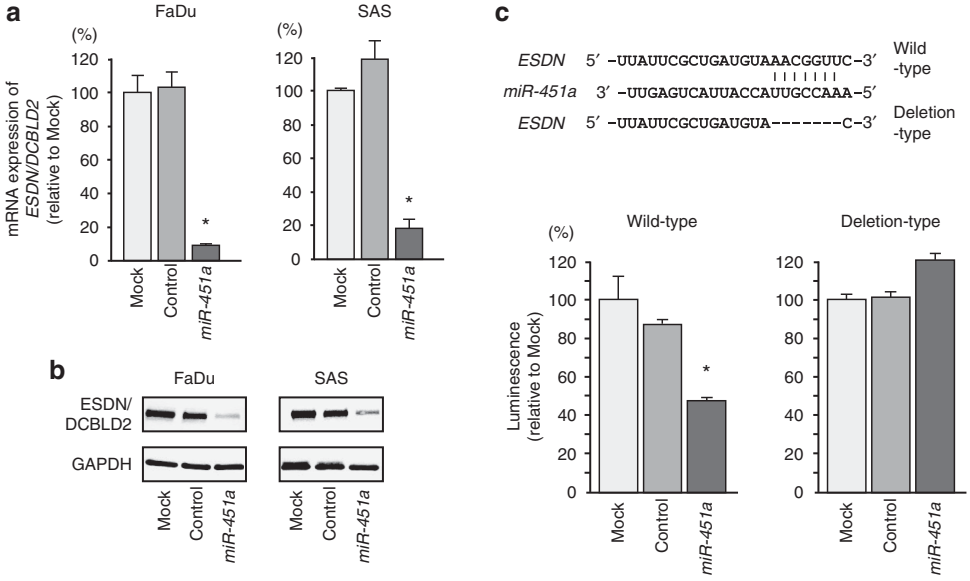

Figure 4. *ESDN/DCBLD2* expression was directly regulated by *miR-451a* in cancer cells. (**A**) *ESDN/DCBLD2* mRNA expression 72 h after transfection with *miR-451a*. *GUSB* expression was used for normalisation. (**B**) ESDN/DCBLD2 protein expression 72 h after transfection with *miR-451a*. GAPDH was used as a loading control. (**C**)The *miR-451a* binding site in the 3′-UTR of *ESDN/DCBLD2* mRNA. Luciferase reporter assays used vectors that included (WT) or lacked (DEL) wild-type sequences of the putative *miR-451a* target site at position 2756–2762. *Renilla* luciferase values were normalised to firefly luciferase values. *$P<0.01$.

(Nohata *et al*, 2012; Kinoshita *et al*, 2013). In our signature, *miR-21* was the most upregulated miRNA in HSCC tissues. Overexpression of *miR-21* was reported in several types of human cancers including HNSCC and *miR-21* is a key regulator of human oncogenic and metastatic signalling pathways (Pan *et al*, 2010; Fu *et al*, 2011). Therefore, elucidation of *miR-21*-regulated molecular targets and cancer pathways is an important study theme of the HSCC research fields.

Here, we focused on *miR-451a*. In this study, *miR-451a* was downregulated in HSCC clinical specimens. Several studies showed that epigenetic alterations such as DNA hypermethylation and histonmodification caused aberrant expression of miRNAs in cancer cells (Baer *et al*, 2013). Our preliminary study showed that expression of *miR-451a* was not recovered by treatments of inhibitors of DNA methyltransferase or histone deacetylase in HNSCC cell lines (FaDu, SAS and HSC3). In contrast, the expression of *miR-451a* was significantly increased by treatment with 5-aza-2′-deoxycitidine or sodium plenylbutyrate in non-small cell lung cancer (Wang *et al*, 2011). Promoter sequence of *miR-451a* includes several transcription factor-binding sites (Pan *et al*, 2013), suggesting that *miR-451a* is affected by various transcription factors. Future studies of chromatin immunoprecipitation assays are needed in HNSCC cells.

Analysis of miRNA signatures previously revealed that several miRNAs form clusters in the human genome and are down-regulated together (Nohata *et al*, 2012; Kojima *et al*, 2013). Indeed,

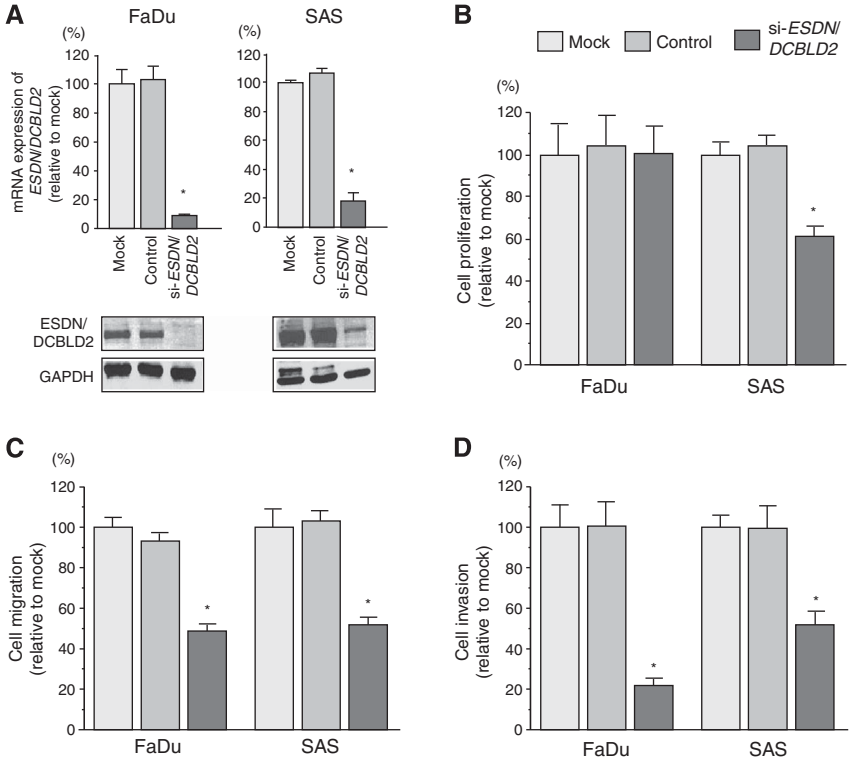

Figure 5. Effects of *ESDN/DCBLD2* silencing by si-*ESDN/DCBLD2* transfection on cancer cell lines. (**A** upper) *ESDN/DCBLD2* mRNA expression 72 h after transfection with si-*ESDN/DCBLD2*. *GUSB* expression was used for normalisation. (**A** lower) ESDN/DCBLD2 protein expression 72 h after transfection with si-*ESDN/DCBLD2*. GAPDH was used as a loading control. (**B**–**D**) Effects of *ESDN/DCBLD2* silencing on cancer cell lines. (**B**) Cell proliferation was determined with XTT assays in FaDu and SAS cell lines 72 h after transfection with 10 nM of si-*ESDN/DCBLD2*, si-control or mock transfection. (**C**) Cell migration activity determined by the migration assay. (**D**) Cell invasion activity determined by the Matrigel invasion assay. *$P<0.0001$.

247 human miRNAs have been found to be located in 64 clusters at inter-miRNA distances of <5000 bp in the human genome (Yoshino *et al*, 2013). As demonstrated in our previous reports, while the seed sequences of *miR-1* and *miR-133a* are different, the *miR-1*/*miR-133a* cluster regulates common oncogenic genes, such as transgelin-2 and purine nucleoside phosphorylase in cancer cells (Yoshino *et al*, 2011; Nohata *et al*, 2011b; Yamasaki *et al*, 2012). In the human genome, *miR-451a* is located on chromosome 17q11.2, close to *miR-144*, indicating that these miRNAs form a clustered group. We investigate the expression levels of *miR-144* and *miR-451a* and found that both were downregulated in cancer tissues. In fact, *miR-451a* expression was highly correlated with that of *miR-144* (Supplementary Figure 1). These data suggest that expression of the *miR-451a/144* cluster is regulated as a single transcriptional unit in the human genome. In future studies, we will perform functional analysis of *miR-144* in HSCC to find novel networks in HSCC oncogenesis.

Our present data demonstrated that restoration of *miR-451a* expression inhibited cancer cell migration and invasion, suggesting *miR-451a* functions as a tumour suppressor in HSCC. Down-regulation of *miR-451a* was reported in other types of cancers, such as lung cancer, breast cancer, gastric cancer and brain tumours (Bandres *et al*, 2009; Wang *et al*, 2011; Bergamaschi and Katzenellenbogen, 2012; Tian *et al*, 2012). In lung cancer, ectopic *miR-451a* expression suppressed cancer cell proliferation and enhanced apoptosis via targeting of ras-related protein 14 (*RAB14*) and AKT signalling pathways (Wang *et al*, 2011). Inhibition of PI3K/AKT pathways by *miR-451a* was also reported in glioma and colorectal carcinoma cells(Tian *et al*, 2012; Li *et al*, 2013).

miRNAs are unique in their ability to regulate multiple protein-coding genes. Bioinformatic predictions indicate that miRNAs regulate $>60\%$ of the protein-coding genes in the human genome. Full understanding of the targets and signalling pathways in HSCC that are regulated by the *miR-451a* family might contribute to our knowledge of HSCC metastasis. To identify the targets of tumour suppressive miRNAs and their pathways, we used gene expression data and *in silico* analyses in recent publications (Nohata *et al*, 2011a; Kinoshita *et al*, 2013). Using this approach, we successfully identified several tumour suppressive microRNA-mediated cancer pathways (Kinoshita *et al*, 2012a; Kojima *et al*, 2013). In this study, we found five genes that are putative *miR-451a* targets (*SPC25*, *MIF*, *ESDN/DCBLD2*, *C4orf46 and AKR1B1*). A recent study demonstrated that macrophage migration inhibitory factor (*MIF*) was a direct target of *miR-451a* in nasopharyngeal carcinoma and that *MIF* controlled cancer cell growth and invasion (Liu *et al*, 2013). Our data also showed that expression of *AKR1B1* (aldo-keto reductase family 1, member B1) and *SPC25* (NDC80 kinetochore complex component) were upregulated in HSCC tissues. When we check the previous studies of these genes, overexpression of *AKR1B1* was reported in several types of cancers and promising drug target in solid cancer and leukaemias (Laffin and Petrash, 2012). Therefore, it is needed to investigate the *miR-451a-AKR1B1*-mediated novel pathways to elucidate HSCC oncogenesis and metastasis. In contrast, there are few reports about expression of *SPC25* and human cancers. Examination will be necessary about the functional role of *SPC25* in human cancers in future.

Here, we focused on the *ESDN/DCBLD2* gene as a tumour suppressive *miR-451a* target in HSCC because expression of the gene was most significantly upregulated in HSCC clinical specimens. We investigated tissue distribution of ESDN/DCBLD2 by immunohistochemical staining, and confirmed the expression of the protein in cancer lesions (Supplementary Figure 2). *ESDN* was initially cloned from human coronary artery and lung metastatic cancer cells (Kobuke *et al*, 2001). ESDN is a transmembrane

protein and it contains discoidin and CUB domains similar to neuropilins (Kobuke *et al*, 2001). Recent study showed that ESDN promotes endothelial VEGF signalling and controls angiogenesis (Nie *et al*, 2013). However, no report has shown that *ESDN* modulates migration and invasion in cancer cells. Thus, our data are the first to report that *ESDN* was directly regulated by *miR-451 a*nd contributed to cancer cell migration and invasion. Furthermore, to reinforce our data of HSCC, we measured expression status of *miR-451a* and *ESDN/DCBLD2* in other sites of HNSCC. Downregulation of *miR-451a* and upregulation of *ESDN/DCBLD2* were confirmed in this analysis (Supplementary Figures 3 and 4). As such, tumour suppressive *miR-451a* and *ESDN* might serve as therapeutic targets for cancer metastasis. Confirmation of these data using an *in vivo* mouse model is essential to support the conclusions of our *in vitro* results within the context of HSCC oncogenesis and metastasis.

CONCLUSIONS

Downregulation of *miR-451a* was observed in a miRNA expression signature of HSCC. Restoration of *miR-451a* significantly inhibited cancer cell migration and invasion, suggesting that *miR-451a* functioned as a tumour suppressor in HSCC. To the best of our knowledge, this is the first report demonstrating that tumour suppressive *miR-451a* directly regulated *ESDN/DCBLD2* in HSCC cells. Moreover, *ESDN/DCBLD2* was upregulated in HSCC clinical specimens and contributed to cancer cell migration and invasion, indicating that it functioned as an oncogene. The identification of novel target oncogenes regulated by *miR-451a* might lead to a better understanding of HSCC metastasis and the development of new therapeutic strategies to treat this disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Baer C, Claus R, Plass C (2013) Genome-wide epigenetic regulation of miRNAs in cancer. *Cancer Res* **73**: 473–477.

Bandres E, Bitarte N, Arias F, Agorreta J, Fortes P, Agirre X, Zarate R, Diaz-Gonzalez JA, Ramirez N, Sola JJ, Jimenez P, Rodriguez J, Garcia-Foncillas J (2009) microRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells. *Clin Cancer Res* **15**: 2281–2290.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–297.

Bergamaschi A, Katzenellenbogen BS (2012) Tamoxifen downregulation of miR-451 increases 14-3-3zeta and promotes breast cancer cell survival and endocrine resistance. *Oncogene* **31**: 39–47.

Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**: 642–655.

Chan JY, Wei WI (2013) Current management strategy of hypopharyngeal carcinoma. *Auris Nasus Larynx* **40**: 2–6.

Cooper JS, Porter K, Mallin K, Hoffman HT, Weber RS, Ang KK, Gay EG, Langer CJ (2009) National Cancer Database report on cancer of the head and neck: 10-year update. *Head Neck* **31**: 748–758.

Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* **6**: 259–269.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**: 102–114.

Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**: 92–105.

Fu X, Han Y, Wu Y, Zhu X, Lu X, Mao F, Wang X, He X, Zhao Y, Zhao Y (2011) Prognostic role of microRNA-21 in various carcinomas: a systematic review and meta-analysis. *Eur J Clin Invest* **41**: 1245–1253.

Hall SF, Groome PA, Irish J, O'Sullivan B (2008) The natural history of patients with squamous cell carcinoma of the hypopharynx. *Laryngoscope* **118**: 1362–1371.

Hobert O (2008) Gene regulation by transcription factors and microRNAs. *Science* **319**: 1785–1786.

Iorio MV, Croce CM (2009) MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol.* **27**: 5848–5856.

Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. *CA Cancer J Clin* **60**: 277–300.

Kikkawa N, Hanazawa T, Fujimura L, Nohata N, Suzuki H, Chazono H, Sakurai D, Horiguchi S, Okamoto Y, Seki N (2010) miR-489 is a tumour-suppressive miRNA target PTPN11 in hypopharyngeal squamous cell carcinoma (HSCC). *Br J Cancer* **103**: 877–884.

Kinoshita T, Hanazawa T, Nohata N, Kikkawa N, Enokida H, Yoshino H, Yamasaki T, Hidaka H, Nakagawa M, Okamoto Y, Seki N (2012a) Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma. *Oncotarget* **3**: 1386–1400.

Kinoshita T, Nohata N, Fuse M, Hanazawa T, Kikkawa N, Fujimura L, Watanabe-Takano H, Yamada Y, Yoshino H, Enokida H, Nakagawa M, Okamoto Y, Seki N (2012b) Tumor suppressive microRNA-133a regulates novel targets: moesin contributes to cancer cell proliferation and invasion in head and neck squamous cell carcinoma. *Biochem Biophys Res Commun* **418**: 378–383.

Kinoshita T, Nohata N, Hanazawa T, Kikkawa N, Yamamoto N, Yoshino H, Itesako T, Enokida H, Nakagawa M, Okamoto Y, Seki N (2013) Tumour-suppressive microRNA-29s inhibit cancer cell migration and invasion by targeting laminin-integrin signalling in head and neck squamous cell carcinoma. *Br J Cancer* **109**: 2636–2645.

Kobuke K, Furukawa Y, Sugai M, Tanigaki K, Ohashi N, Matsumori A, Sasayama S, Honjo T, Tashiro K (2001) ESDN, a novel neuropilin-like membrane protein cloned from vascular cells with the longest secretory signal sequence among eukaryotes, is up-regulated after vascular injury. *J Biol Chem* **276**: 34105–34114.

Kojima S, Enokida H, Yoshino H, Itesako T, Chiyomaru T, Kinoshita T, Fuse M, Nishikawa R, Goto Y, Naya Y, Nakagawa M, Seki N (2013) The tumor-suppressive microRNA-143/145 cluster inhibits cell migration and invasion by targeting GOLM1 in prostate cancer. *J Hum Genet.* **59**: 78–87.

Kwak PB, Iwasaki S, Tomari Y (2010) The microRNA pathway and cancer. *Cancer Sci* **101**: 2309–2315.

Laffin B, Petrash JM (2012) Expression of the aldo-ketoreductases AKR1B1 and AKR1B10 in human cancers. *Front Pharmacol* **3**: 104.

Li H-Y, Zhang Y, Cai J-H, Bian H-L (2013) MicroRNA-451 inhibits growth of human colorectal carcinoma cells via downregulation of Pi3k/Akt pathway. *Asian Pac J Cancer Prev* **14**: 3631–3634.

Liu N, Jiang N, Guo R, Jiang W, He QM, Xu YF, Li YQ, Tang LL, Mao YP, Sun Y, Ma J (2013) MiR-451 inhibits cell growth and invasion by targeting MIF and is associated with survival in nasopharyngeal carcinoma. *Mol Cancer* **12**: 123.

Nie L, Guo X, Esmailzadeh L, Zhang J, Asadi A, Collinge M, Li X, Kim JD, Woolls M, Jin SW, Dubrac A, Eichmann A, Simons M, Bender JR, Sadeghi MM (2013) Transmembrane protein ESDN promotes endothelial VEGF signaling and regulates angiogenesis. *J Clin Invest* **123**: 5082–5097.

Nohata N, Hanazawa T, Enokida H, Seki N (2012) microRNA-1/133a and microRNA-206/133b clusters: dysregulation and functional roles in human cancers. *Oncotarget* **3**: 9–21.

Nohata N, Hanazawa T, Kikkawa N, Sakurai D, Fujimura L, Chiyomaru T, Kawakami K, Yoshino H, Enokida H, Nakagawa M, Katayama A, Harabuchi Y, Okamoto Y, Seki N (2011a) Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. *Br J Cancer* **105**: 833–841.

Nohata N, Sone Y, Hanazawa T, Fuse M, Kikkawa N, Yoshino H, Chiyomaru T, Kawakami K, Enokida H, Nakagawa M, Shozu M, Okamoto Y, Seki N (2011b) miR-1 as a tumor suppressive microRNA targeting TAGLN2 in head and neck squamous cell carcinoma. *Oncotarget* **2**: 29–42.

Pan X, Wang R, Wang ZX (2013) The potential role of miR-451 in cancer diagnosis, prognosis, and therapy. *Mol Cancer Ther* **12**: 1153–1162.

Pan X, Wang Z-X, Wang R (2010) MicroRNA-21: A novel therapeutic target in human cancer. *Cancer Biol Ther* **10**: 1224–1232.

Takes RP, Strojan P, Silver CE, Bradley PJ, Haigentz Jr. M, Wolf GT, Shaha AR, Hartl DM, Olofsson J, Langendijk JA, Rinaldo A, Ferlito A. International H, Neck Scientific G (2012) Current trends in initial management of hypopharyngeal cancer: the declining use of open surgery. *Head Neck* **34**: 270–281.

Tian Y, Nan Y, Han L, Zhang A, Wang G, Jia Z, Hao J, Pu P, Zhong Y, Kang C (2012) MicroRNA miR-451 downregulates the PI3K/AKT pathway through CAB39 in human glioma. *Int J Oncol* **40**: 1105–1112.

Wang R, Wang ZX, Yang JS, Pan X, De W, Chen LB (2011) MicroRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting ras-related protein 14 (RAB14). *Oncogene* **30**: 2644–2658.

Wang T, Zang WQ, Li M, Wang N, Zheng YL, Zhao GQ (2013) Effect of miR-451 on the biological behavior of the esophageal carcinoma cell line EC9706. *Dig Dis Sci* **58**: 706–714.

Yamasaki T, Yoshino H, Enokida H, Hidaka H, Chiyomaru T, Nohata N, Kinoshita T, Fuse M, Seki N, Nakagawa M (2012) Novel molecular targets regulated by tumor suppressors microRNA-1 and microRNA-133a in bladder cancer. *Int J Oncol* **40**: 1821–1830.

Yoshino H, Chiyomaru T, Enokida H, Kawakami K, Tatarano S, Nishiyama K, Nohata N, Seki N, Nakagawa M (2011) The tumour-suppressive function of miR-1 and miR-133a targeting TAGLN2 in bladder cancer. *Br J Cancer* **104**: 808–818.

Yoshino H, Enokida H, Itesako T, Kojima S, Kinoshita T, Tatarano S, Chiyomaru T, Nakagawa M, Seki N (2013) Tumor-suppressive microRNA-143/145 cluster targets hexokinase-2 in renal cell carcinoma. *Cancer Sci* **104**: 1567–1574.

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