Journal of Human Genetics (2011) 56, 595–601

www.nature.com/jhg

ORIGINAL ARTICLE

Tumor suppressive *microRNA-375* regulates oncogene *AEG-1/MTDH* in head and neck squamous cell carcinoma (HNSCC)

Nijiro Nohata[1,2], Toyoyuki Hanazawa[2], Naoko Kikkawa[1,2], Muradil Mutallip[1,2], Daiju Sakurai[2], Lisa Fujimura[3], Kazumori Kawakami[4], Takeshi Chiyomaru[4], Hirofumi Yoshino[4], Hideki Enokida[4], Masayuki Nakagawa[4], Yoshitaka Okamoto[2] and Naohiko Seki[1]

Our microRNA (miRNA) expression signatures of hypopharyngeal squamous cell carcinoma, maxillary sinus squamous cell carcinoma and esophageal squamous cell carcinoma revealed that *miR-375* was significantly reduced in cancer tissues compared with normal epithelium. In this study, we focused on the functional significance of *miR-375* in cancer cells and identification of *miR-375*-regulated novel cancer networks in head and neck squamous cell carcinoma (HNSCC). Restoration of *miR-375* showed significant inhibition of cell proliferation and induction of cell apoptosis in SAS and FaDu cell lines, suggesting that *miR-375* functions as a tumor suppressor. We adopted genome-wide gene expression analysis to search for *miR-375*-regulated molecular targets. Gene expression data and luciferase reporter assays revealed that *AEG-1/MTDH* was directly regulated by *miR-375*. Cancer cell proliferation was significantly inhibited in HNSCC cells transfected with si-*AEG-1/MTDH*. In addition, expression levels of *AEG-1/MTDH* were significantly upregulated in cancer tissues. Therefore, *AEG-1/MTDH* may function as an oncogene in HNSCC. The identification of novel tumor suppressive miRNA and its regulated cancer pathways could provide new insights into potential molecular mechanisms of HNSCC oncogenesis.
Journal of Human Genetics (2011) **56,** 595–601; doi:10.1038/jhg.2011.66; published online 14 July 2011

Keywords: *AEG-1/MTDH*; HNSCC; microRNA; *miR-375*; tumor suppressor

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), the sixth most common malignancy worldwide, arises in the oral cavity, oropharynx, larynx or hypopharynx.[1] In spite of considerable advances in multimodality therapy including surgery, radiotherapy and chemotherapy, the overall 5-year survival rate for patients with HNSCC is only 40–50%. It is among the lowest of the major cancer types and has not improved dramatically for decades.[2] HNSCC is a disease characterized by genetic and biological heterogeneity. Aberrant signal pathways have been identified in HNSCC and inhibition of epidermal growth factor receptor has proven to be a successful therapeutic strategy.[3] However, most studies on the cancer genome have focused mainly on protein-coding genes and our understanding of alterations of non-protein-coding sequences in cancer is largely unclear.

MicroRNAs (miRNAs) constitute a class of small non-protein-coding RNA molecules, 19–22 nucleotides in length.[4] They negatively regulate gene expression at the post-transcriptional level through translational repression and/or mRNA cleavage. They have important roles in a variety of biological processes including proliferation, differentiation and apoptosis.[4] They bind to the 3′-untranslated region (UTR) of target mRNAs by imperfect 7–8 mer base pairing, so one miRNA can regulate multiple target genes. Bioinformatic predictions indicate that miRNAs regulate more than 30% of the protein-coding genes.[5] miRNAs contribute to the initiation and development of various types of cancers.[6] miRNAs are aberrantly expressed in many human cancers, and can function either as tumor suppressors or oncogenes. Downregulated miRNAs in cancer cells could function as tumor suppressors by negatively regulating oncogenes, alternatively overexpressed miRNAs could function as oncogenes by repressing tumor suppressor genes.[7] We previously reported aberrantly expressed miRNAs based on miRNA expression signatures in several cancers, and identified tumor suppressive miRNAs and their target oncogenes.[8–12] In HNSCC, we found that miRNAs such as *miR-489*, *miR-1* and *miR-133a* were downregulated in cancer cells and potentially had tumor suppressive function through direct repression of several oncogenes.[11,13–15]

[1]Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba, Japan; [2]Department of Otorhinolaryngology/Head and Neck Surgery, Chiba University Graduate School of Medicine, Chiba, Japan; [3]Biomedical Research Center, Chiba University, Chiba, Japan and [4]Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan
Correspondence: Dr N Seki, Associate Professor of Functional Genomics, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan.
E-mail: naoseki@faculty.chiba-u.jp
Received 9 May 2011; revised 22 May 2011; accepted 23 May 2011; published online 14 July 2011

miR-375 was significantly downregulated in hypopharyngeal squamous cell carcinoma, esophageal squamous cell carcinoma and maxillary sinus squamous cell carcinoma (unpublished data) in our miRNA expression signatures.[10,11] Interestingly, several reports found that *miR-375* is one of the most downregulated miRNAs in HNSCC.[16,17] In other cancers, *miR-375* is also known to function as a tumor suppressor by targeting certain oncogenes, including *PDK1*, *YWHAZ*/*14-3-3ζ*, *YAP* and *JAK2*.[18–21] Thus *miR-375* is a potential tumor suppressor candidate in HNSCC. However, the function of *miR-375* and its target genes in HNSCC remains uncertain.

The aim of this study was to clarify the functional significance of *miR-375* in HNSCC and to identify cancer pathways regulated by *miR-375*. We analyzed the effect of *miR-375* on cell proliferation and cell apoptosis in HNSCC cell lines. For target genes searches of *miR-375*, we performed genome-wide gene expression analysis. We focused on *AEG-1*/*MTDH* as a candidate target oncogene of *miR-375*. The identification of a novel tumor suppressive *miR-375*-regulated oncogene pathway could further unravel the molecular mechanism of HNSCC oncogenesis.

MATERIALS AND METHODS

HNSCC cell culture

Human HNSCC cell lines (SAS, derived from a primary lesion of tongue squamous cell carcinoma; and FaDu, derived from a primary lesion of hypopharyngeal squamous cell carcinoma) were used. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) 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-375*; Pre-miR ID: PM10327), miRNA-control (P/N: AM17111) (Applied Biosystems, Foster City, CA, USA), small interfering RNA (Stealth Select RNAi siRNA; si-*AEG-1*/*MTDH* Cat#; HSS150644 and HSS150645) (Invitrogen) and siRNA-control (Stealth RNAi Negative Control Medium GC Duplex; 12935-300). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen) as described previously.[9] Transfection efficiency of Pre-miR in cell lines was confirmed based on downregulation of *TWF1* (*PTK9*) mRNA following transfection with *miR-1* as previously reported.[8,10]

Cell proliferation and cell apoptosis assays

Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 96-well plates at 3×10^3 cells per well. After 72 h, cell proliferation was determined by the XTT assay, using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany).[10,11] Triplicate wells were measured for cell viability in each treatment group.

SAS and FaDu cells were transiently transfected with miRNA-control or *miR-375* and were harvested after 72 h by trypsinization. Double staining with FITC-annexin V and propidium iodide (PI) was carried out using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer's recommendations. 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 ratios of early apoptotic cells to miRNA-control transfectants from each experiment were compared. Experiments were conducted in triplicate.

Target gene search for *miR-375*

Genome-wide screens using *miR-375* transfectants were performed to identify target genes of *miR-375* in two HNSCC cell lines, SAS and FaDu. Oligo-microarray human 44 K (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.[22] 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 $\log_2$ ratios of the median-subtracted background intensities were analyzed. Data from each microarray study were normalized by a global normalization method.[22]

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 17.0, http://microrna.sanger.ac.uk/).

Real-time quantitative reverse-transcription PCR

First-strand of complementary DNA was synthesized from 1 μg of total RNA using a High Capacity complementary DNA 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 *HERPUD1* (P/N: Hs00206652_m1), *LDHB* (P/N: Hs00929956_m1), *PSIP1* (P/N: Hs00253515_m1), *AEG-1*/*MTDH* (P/N: Hs00757841_m1), *SLC7A11* (Hs00204928_m1) and *GAPDH* (A/N: NM_002046) internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of *miR-375* (Assay ID: 000564) were analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to *RNU48* (A/N: X96648). All reactions were performed in triplicate, and included negative control reactions that lacked complementary DNA.

Western blot

Cells were harvested 72 h after transfection and lysates were prepared. A measure of 50 μg protein from each lysate was separated by NuPAGE on 4–12% bis-tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed with diluted (1:200) polyclonal AEG-1/MTDH antibody (HPA015104; 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 level of each protein was evaluated by ImageJ software (ver.1.44; http://rsbweb.nih.gov/ij/index.html).

Plasmid construction and dual-luciferase assay

The wild-type sequences of *AEG-1*/*MTDH* 3′-UTR (WT-3′-UTR) and those with deleted *miR-375* target sites (DEL-3′-UTR) were inserted between the *Xho*I and *Pme*I restriction sites in the 3′-UTR of the *hRluc* gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). Sequences of oligonucleotides were described in Supplementary Information. The synthesized DNA was cloned into the psiCHECK-2 vector. FaDu cells were then transfected with 5 ng vector, 10 nM mature miRNA molecules, Pre-miRNA *miR-375* (Applied Biosystems), and 1 μl Lipofectamine 2000 (Invitrogen) in 100 μl Opti-MEM (Invitrogen). Firefly and *Renilla* luciferase activities in cell lysates were determined using a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the quotient of *Renilla*/firefly luciferase activities.

Clinical HNSCC specimens

A total of 20 pairs of primary HNSCC (oral cavity, six; larynx, three; oropharynx, five; hypopharynx, six) and corresponding normal epithelial samples were obtained from patients at Chiba University Hospital (Chiba, Japan) from 2007 to 2011. All tissue specimens were obtained from patients undergoing surgical treatment. Normal tissues were obtained far from the

center of the cancer in surgical specimens. No cancer cells were detected in neighboring formalin-fixed paraffin-embedded tissues. 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. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA was extracted.

Statistical analysis

The relationships between two groups and the numerical values obtained by real-time reverse-transcription PCR were analyzed using the non-parametric Mann–Whitney *U* test or the paired *t*-test. The relationships among more than three variables and numerical values were analyzed using the Bonferroni's-adjusted Mann–Whitney *U* test. All analyses were carried out using Expert StatView (version 4, SAS Institute, Cary, NC, USA).

RESULTS

Expression of *miR-375* in HNSCC clinical specimens

Data characterizing 20 HNSCC patients are presented in Table 1. The expression levels of *miR-375* were significantly downregulated in clinical HNSCC specimen compared with adjacent normal tissues (Figure 1a).

Effect of *miR-375* transfection of HNSCC cell lines

To determine the function of *miR-375*, we performed gain-of-function analysis using *miR-375* or miRNA-control transfectants. The XTT assay showed statistically significant inhibition of cell proliferation in *miR-375* transfectants in comparison with mock controls after 72 h. Both *miR-375*-transfected cultures grew only 70.5% in SAS and 74.0% in FaDu as much as mock cultures (both $P<0.0001$, Figure 1b).

Cell apoptosis in *miR-375* transfected cells was assessed by flow cytometry. The fraction of early apoptotic cells significantly increased in *miR-375* transfectants, ~1.7-fold in SAS and 2.0-fold in FaDu compared with mock controls (both $P<0.0001$; Figure 1c).

Gene expression profiling identifies downregulated genes in *miR-375* transfectants

To investigate candidate molecular targets of *miR-375* in HNSCC cells, we examined the effect of *miR-375* on protein-coding genes. Mature *miR-375* was transiently transfected into SAS and FaDu cells, with miRNA-control transfectants. Comprehensive gene expression analysis (see Methods section) clearly showed changes in gene expression patterns between *miR-375* and negative-control transfectants. To iden-

Table 1 Clinical features of 20 patients with HNSCC

No.	*Gender*	*Age*	*Location*	*Differentiation*	*T*	*N*	*M*	*Stage*
1	Male	82	Tongue	Well	1	0	0	I
2	Male	64	Tongue	Moderate	3	2b	0	IVA
3	Male	66	Tongue	Well	2	0	0	II
4	Male	70	Tongue	Well	1	0	0	I
5	Male	67	Oral floor	Moderate	3	2b	0	IVA
6	Male	47	Oral floor	Moderate	1	2a	0	IVA
7	Male	69	Larynx	Well	3	0	0	III
8	Male	73	Larynx	Well	2	0	0	II
9	Male	80	Larynx	Poor	3	2b	0	IVA
10	Male	59	Oropharynx	Poor	4a	0	0	IVA
11	Male	76	Oropharynx	Poor	2	0	0	II
12	Male	55	Oropharynx	Moderate	3	2c	0	IVA
13	Female	83	Oropharynx	Moderate	1	0	0	I
14	Male	74	Oropharynx	Well	2	0	0	II
15	Male	66	Hypopharynx	Moderate	2	2c	0	IVA
16	Male	71	Hypopharynx	Poor	2	2b	0	IVA
17	Male	49	Hypopharynx	Moderate	2	2b	0	IVA
18	Male	71	Hypopharynx	Poor	4a	2b	0	IVA
19	Male	66	Hypopharynx	Well	4a	2c	0	IVA
20	Male	66	Hypopharynx	Moderate	3	2c	0	IVA

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

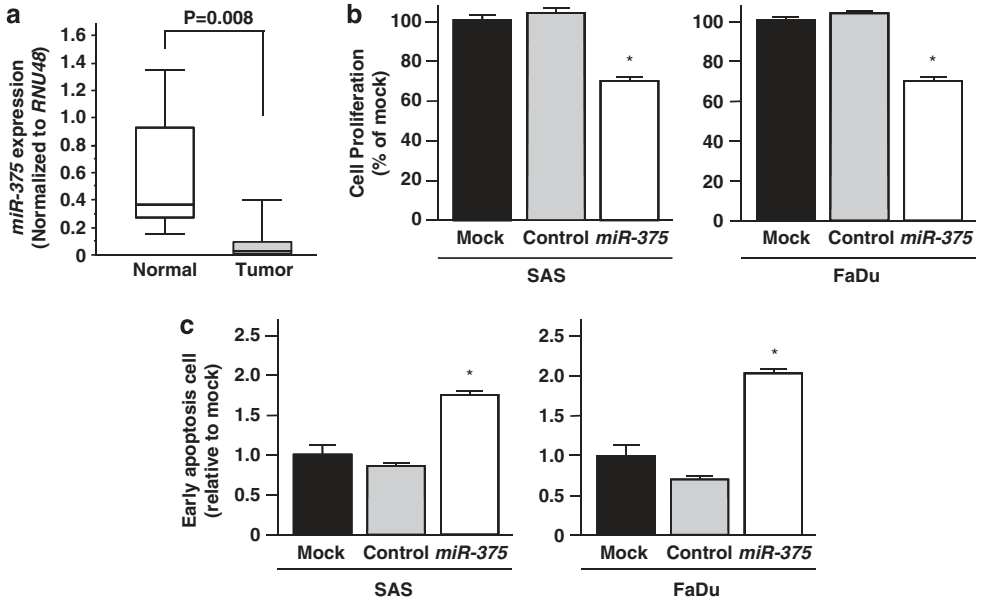

Figure 1 miR-375 function as a tumor supressor in HNSCC. (**a**) The expression levels of *miR-375* were significantly downregulated in tumor tissues compared with normal tissues. *RNU48* was used as an internal control. (**b**) Cell proliferation was determined by the XTT assay in the SAS and FaDu cell lines 72 h after transfection of 10 nM *miR-375* or miRNA-control. The XTT assay showed significant inhibition of cell proliferation in *miR-375* transfectants in comparison with mock cultures. (**c**) The data for the early apoptotic cell fraction at 72 h after transfection are expressed as the relative value of the average expression of the mock cultures. The apoptosis assay showed significant promotion of early cell apoptosis in *miR-375* transfectants in comparison with mock cultures. *$P<0.0001$.

tify candidate *miR-375* target genes, first, signal values of raw data in miR-control transfectants less than 5000 were cut off. Thereafter, a cut-off of value <-2.00-fold was applied to the array data. This filter resulted in the identification of 55 genes that were significantly downregulated upon *miR-375* transfection in both SAS and FaDu cells (55 genes are shown in Table 2). 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 downregulated genes were examined for *miR-375* target sites using the TargetScan database. Of the 55 putative gene targets, sixteen genes contained *miR-375* target sites. We selected five genes that were downregulated more than *YWHAZ/14-3-3ζ*, a reported target of *miR-375*.[18]

Expression levels of candidate target genes of *miR-375* in HNSCC clinical specimens

We measured the mRNA expression levels of five candidate genes in HNSCC clinical specimens by quantitative real-time reverse-transcription-PCR. Two genes, *lactate dehydrogenase B* (*LDHB*) and *astrocyte elevated gene-1/metadherin* (*AEG-1/MTDH*) were significantly upregulated in cancer tissues ($P=0.0143$ and $P=0.0154$, respectively, Figure 2). The other three genes (*HERPUD1*, *PSIP1* and *SLC7A11*) were not significantly upregulated in the tumor tissues of HNSCC (Supplementary Figure 1).

AEG-1/MTDH is directly regulated by *miR-375*

We focused on *AEG-1/MTDH*, because *AEG-1/MTDH* has been reported to be an oncogene in several cancers.[23,24] The expression levels of *AEG-1/MTDH* mRNA were significantly decreased in both HNSCC cell lines (SAS and FaDu) transfected with *miR-375* compared with mocks (Figure 3a). The protein expression levels were also markedly reduced in *miR-375* transfectants compared with mocks (Figure 3b).

FaDu cells were used to determine the mechanism of *miR-375* suppression of *AEG-1/MTDH* expression. The TargetScan database identified one putative target site in the 3′-UTR of *AEG-1/MTDH* (Figure 4, upper). We performed a luciferase reporter assay to determine whether *AEG-1/MTDH* mRNA had a target site for *miR-375*. We used a vector encoding either the partial sequence of the 3′-UTR of *AEG-1/MTDH* mRNA, including the predicted *miR-375* target site, positions 1454–1460 (WT-3′-UTR) or a vector lacking the *miR-375* target site (DEL-3′-UTR). We found that the luminescence intensity was significantly reduced by transfection of the WT-3′-UTR while DEL-3′-UTR blocked the decrease in luminescence (Figure 4, lower).

Effects of *AEG-1/MTDH* silencing in HNSCC cell lines

Loss-of-function assays using si-*AEG-1/MTDH* were performed to examine the oncogenic function of *AEG-1/MTDH*. We asked whether si-*AEG-1/MTDH* reduced both mRNA and protein expression levels of AEG-1/MTDH in SAS and FaDu. Both AEG-1/MTDH mRNA and protein were reduced in two si-*AEG-1/MTDH* transfectants (Figure 5). The XTT assay revealed significant cell growth inhibition in two si-*AEG-1/MTDH*-transfected cells at 72 h after transfection. Specifically, with si-*AEG-1/MTDH*_1 and si-*AEG-1/MTDH*_2, SAS grew only 38.7 and 39.8%, respectively, while FaDu proliferated 75.1 and 78.1%, respectively, compared with the mock cultures (Figure 6).

DISCUSSION

Numerical articles have reported that miRNAs are aberrantly expressed in many types of human cancers.[25] Moreover, miRNA expression signatures of HNSCC have been reported by many researchers.[26,27] Recently, we also reported differentially expressed miRNAs in hypopharyngeal squamous cell carcinoma and esophageal squamous cell carcinoma based on expression signatures.[8–12] When we compared our expression data with past reports from other researchers, *miR-375* was frequently observed to be downregulated in many types of cancers including HNSCC.[16–20] DNA methylation and histone deacetylation might be involved in downregulation of *miR-375* in gastric cancer.[18] According to one of those studies of HNSCC, an expression ratio of *miR-221*:*miR-375* provided a useful biomarker (92% sensitivity and 93% specificity) in distinguishing tumor from normal tissues.[16] Another report of 51 formalin-fixed HNSCC specimens showed that *miR-375* was significantly reduced, and exogenous overexpression of *miR-375* in HNSCC cell lines reduced both proliferation and clonogenicity.[17]

In this study, we asked whether *miR-375* had a tumor suppressive function in HNSCC cells. Our data revealed that restoration of *miR-375* suppressed cancer cell proliferation and induced cell apoptosis in SAS and FaDu cells, confirming past results. Tumor suppressive function of *miR-375* was reported with other cancer cells, such as esophageal, gastric and liver cancer.[18–21] Our data and those in past articles strongly indicate that *miR-375* is an important miRNA functioning as a tumor suppressor 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 oncogenesis. For that reason, we are continuing our investigation of tumor suppressive miRNA-regulated oncogenic targets in various cancer cells.[13–15,28–30] In this study, we adopted a method of genome-wide gene expression analysis of SAS and FaDu cells, using *miR-375* transfectants to search target genes regulated by *miR-375*. Our *miR-375*-regulated gene expression profile showed that the expression of several genes was reduced in *miR-375* transfectants. Among them was *YWHAZ/14-3-3ζ*, a potent antiapoptotic gene. It had already been reported that this gene was repressed by ectopic expression of *miR-375*, and luciferase reporter assays demonstrated that it was a direct target of *miR-375* in gastric cancer.[18] Because this previously known target of *miR-375* appeared in our expression list, our profile appeared to be an effective approach to identify new targets of *miR-375*.

We focused on five downregulated genes (*HERPUD1*, *LDHB*, *PSIP1*, *AEG-1/MTDH* and *SLC7A11*), which contained *miR-375* target sites and were significantly downregulated more than *YWHAZ/14-3-3ζ* in our expression profile. Our criterion for selection in this analysis was that candidate *miR-375*-regulated oncogenes should be upregulated in cancer tissues. We validated the expression levels of candidate genes using HNSCC clinical specimens, and two genes (*LDHB* and *AEG-1/MTDH*) were overexpressed in cancer tissues compared with adjacent non-cancerous tissues. Among them, we focused on *AEG-1/MTDH* to study the functional significance of HNSCC. Although *AEG-1/MTDH* has been interpreted as an oncogene in several cancers, its functional role in HNSCC is not understood. Interestingly, in breast cancer, *miR-26a* directly controlled *AEG-1/MTDH* in the cell line MCF7.[31] However, no relationship between *AEG-1/MTDH* and *miR-375* was known.

AEG-1/MTDH was initially cloned as a gene induced by HIV-1 infection or tumor necrosis factor-alpha treatment in human fetal astrocytes.[32] Overexpression of *AEG-1/MTDH* was observed in many types of cancers, such as malignant astrocytoma,[33] neuroblastoma,[34] and cancers of esophagus,[35] breast,[36] prostate[37] and liver.[38] Many reports have indicated that *AEG-1/MTDH* contributes to several steps in human oncogenesis, including cancer-cell progression, invasion, metastasis and chemoresistance. Thus, *AEG-1/MTDH* can be considered an oncogene.[23,24] Our findings in this study support the oncogenic

Table 2 A total of 55 downregulated genes by *miR-375*

Number	Entrez gene ID	Gene symbol	Gene name	Log_2 ratio			miR-375 target sites
				SAS	FaDu	Average	
1	9709	*HERPUD1*	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	−1.71	−4.12	−2.92	+
2	9601	*PDIA4*	Protein disulfide isomerase family A, member 4	−1.79	−3.04	−2.41	−
3	3309	*HSPA5*	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	−1.44	−3.22	−2.33	−
4	3945	*LDHB*	Lactate dehydrogenase B	−2.19	−2.28	−2.24	+
5	4925	*NUCB2*	Nucleobindin 2	−1.80	−2.57	−2.18	−
6	11 168	*PSIP1*	PC4 and SFRS1 interacting protein 1	−1.80	−2.53	−2.17	+
7	57 007	*CXCR7*	Chemokine (C-X-C motif) receptor 7	−2.56	−1.63	−2.10	−
8	92 140	*AEG-1/MTDH*	Astrocyte-elevated gene-1/metadherin	−1.71	−2.44	−2.07	+
9	23 657	*SLC7A11*	Solute-carrier family 7, (cationic amino-acid transporter, y+ system) member 11	−1.23	−2.81	−2.02	+
10	3852	*KRT5*	Keratin 5	−1.93	−2.10	−2.01	−
11	7534	*YWHAZ/14-3-3ζ*	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide/14-3-3zeta	−1.90	−2.08	−1.99	+
12	51 726	*DNAJB11*	Dnaj (Hsp40) homolog, subfamily B, member 11	−1.40	−2.22	−1.81	−
13	3856	*KRT8*	Keratin 8	−1.78	−1.76	−1.77	−
14	343 477	*HSP90B3P*	Heat shock protein 90 kDa beta (*Grp94*), member 3 (pseudogene)	−1.21	−2.32	−1.77	−
15	84 650	*EBPL*	Emopamil binding protein-like	−1.78	−1.67	−1.73	+
16	154 467	*C6orf129*	Chromosome 6 open reading frame 129	−1.48	−1.95	−1.72	−
17	145 567	*TTC7B*	Tetratricopeptide repeat domain 7B	−1.44	−1.98	−1.71	+
18	7184	*HSP90B1*	Heat shock protein 90 kDa beta (Grp94), member 1	−1.08	−2.30	−1.69	−
19	401 152	*C4orf3*	Chromosome 4 open reading frame 3	−1.49	−1.88	−1.69	−
20	23 753	*SDF2L1*	Stromal cell-derived factor 2-like 1	−1.14	−2.15	−1.65	−
21	27 230	*SERP1*	Stress-associated endoplasmic reticulum protein 1	−1.04	−2.18	−1.61	−
22	5052	*PRDX1*	Peroxiredoxin 1	−1.17	−2.00	−1.59	+
23	50 999	*TMED5*	Transmembrane emp24 protein transport domain-containing 5	−1.27	−1.88	−1.58	+
24	7873	*MANF*	Mesencephalic astrocyte-derived neurotrophic factor	−1.00	−2.12	−1.56	−
25	54 541	*DDIT4*	DNA-damage-inducible transcript 4	−1.03	−2.00	−1.52	−
26	442 249	*LOC442249*	Similar to keratin 18	−1.62	−1.34	−1.48	−
27	10 857	*PGRMC1*	Progesterone receptor membrane component 1	−1.31	−1.66	−1.48	−
28	7056	*THBD*	Thrombomodulin	−1.58	−1.37	−1.47	−
29	57 179	*KIAA1191*	KIAA1191	−1.28	−1.65	−1.46	+
30	5955	*RCN2*	Reticulocalbin 2, EF-hand calcium-binding domain	−1.33	−1.58	−1.45	−
31	284 085	*FLJ40504*	Keratin 18 pseudogene	−1.59	−1.31	−1.45	−
32	374	*AREG*	Amphiregulin	−1.82	−1.06	−1.44	−
33	8140	*SLC7A5*	Solute-carrier family 7 (cationic amino-acid transporter, y+ system), member 5	−1.26	−1.60	−1.43	−
34	708	*C1QBP*	Complement component 1, q subcomponent-binding protein	−1.19	−1.65	−1.42	+
35	6520	*SLC3A2*	Solute-carrier family 3 (activators of dibasic and neutral amino-acid transport), member 2	−1.08	−1.76	−1.42	−
36	114 569	*MAL2*	mal, T-cell differentiation protein 2	−1.37	−1.46	−1.42	+
37	5328	*PLAU*	Plasminogen activator, urokinase	−1.45	−1.27	−1.36	−
38	10 960	*LMAN2*	Lectin, mannose-binding 2	−1.26	−1.41	−1.33	−
39	641 746	*LOC641746*	Glycine cleavage system protein H pseudogene	−1.38	−1.26	−1.32	−
40	6418	*SET*	*SET* nuclear oncogene	−1.23	−1.40	−1.31	+
41	3875	*KRT18*	Keratin 18	−1.40	−1.20	−1.30	−
42	5048	*PAFAH1B1*	Platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45 kDa)	−1.31	−1.24	−1.27	+
43	4697	*NDUFA4*	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9 kDa	−1.27	−1.27	−1.27	−
44	8932	*MBD2*	Methyl-CpG-binding domain protein 2	−1.35	−1.09	−1.22	+
45	6185	*RPN2*	Ribophorin II	−1.18	−1.26	−1.22	−
46	3914	*LAMB3*	Laminin, beta 3	−1.31	−1.05	−1.18	−
47	83 955	*NACAP1*	Nascent-polypeptide-associated complex alpha polypeptide pseudogene 1	−1.18	−1.17	−1.18	−
48	5699	*PSMB10*	Proteasome (prosome, macropain) subunit, beta type, 10	−1.06	−1.29	−1.18	−
49	9343	*EFTUD2*	Elongation factor Tu GTP-binding domain containing 2	−1.05	−1.23	−1.14	−
50	2653	*GCSH*	Glycine cleavage system protein H (aminomethyl carrier)	−1.07	−1.14	−1.10	−
51	4666	*NACA*	Nascent polypeptide-associated complex alpha subunit	−1.11	−1.08	−1.09	−
52	342 538	*NACA2*	Nascent polypeptide-associated complex alpha subunit 2	−1.10	−1.07	−1.09	−
53	7347	*UCHL3*	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	−1.03	−1.12	−1.08	−
54	481	*ATP1B1*	ATPase, Na+/K+ transporting, beta 1 polypeptide	−1.00	−1.07	−1.03	−
55	205	*AK3L1*	Adenylate kinase 3-like 1	−1.02	−1.03	−1.02	−

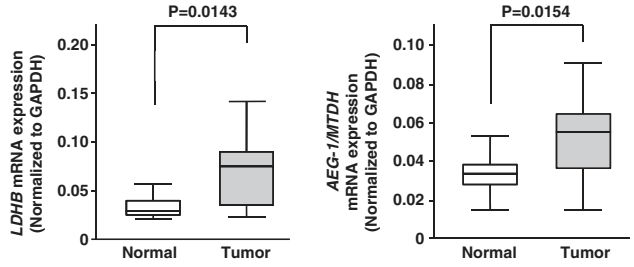

Figure 2 Expression levels of LDHB and AEG-1/MTDH mRNA in HNSCC clinical specimens. The expression levels of *LDHB* and *AEG-1/MTDH* mRNA were significantly upregulated in tumor tissues compared with normal tissues. *GAPDH* was used as an internal control.

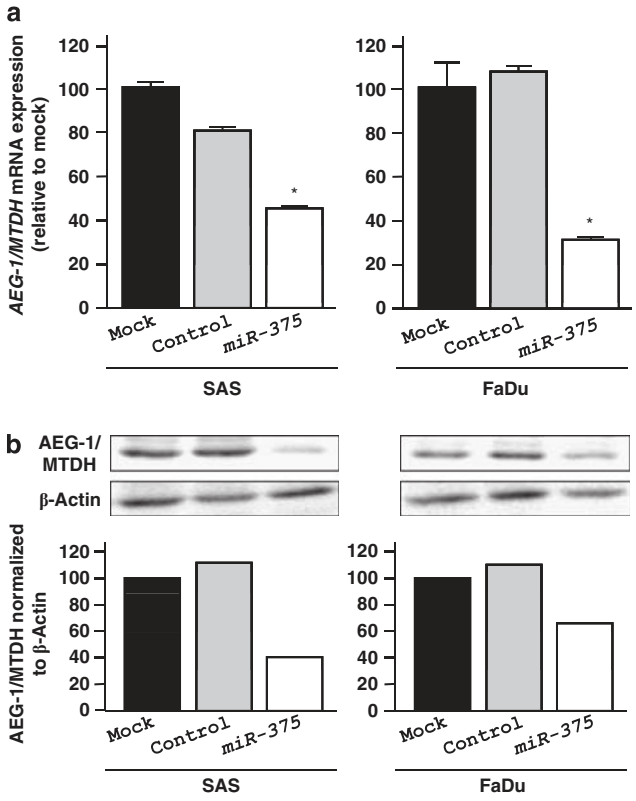

Figure 3 AEG-1/MTDH mRNA and protein expression in the HNSCC cell lines. (**a**) *AEG-1/MTDH* mRNA expression 48 h after transfection with 10 nM *miR-375*. *AEG-1/MTDH* mRNA expression was significantly repressed in *miR-375* transfectants. *GAPDH* was used as an internal control. $^*P<0.0001$ (**b**) AEG-1/MTDH protein expression 72 h after transfection with *miR-375*. β-Actin was used as a loading control. The protein expression level of AEG-1/MTDH was also repressed in *miR-375* transfectants.

nature of *AEG-1/MTDH* in HNSCC. It is well known that *AEG-1/MTDH* functions as a downstream mediator in signaling pathways such as PI3K/AKT,[39] NFκB[40] and Wnt/β-catenin.[38] We hypothesized that tumor suppressor *miR-375* may regulate these oncogenic pathways, and its downregulation is a key step in human HNSCC oncogenesis.

It is reported that AEG-1/MTDH expression indicated prognosis of survival of patients with esophageal squamous cell carcinoma and breast cancer.[35,36] However, in our cohort, there was no significant relationship between *miR-375* or *AEG-1/MTDH* expression and clinico-pathological parameters such as lymph node metastasis, distant metastasis and the TNM classification. Our sample number was too small to test for a relationship. The cohort analysis is an important

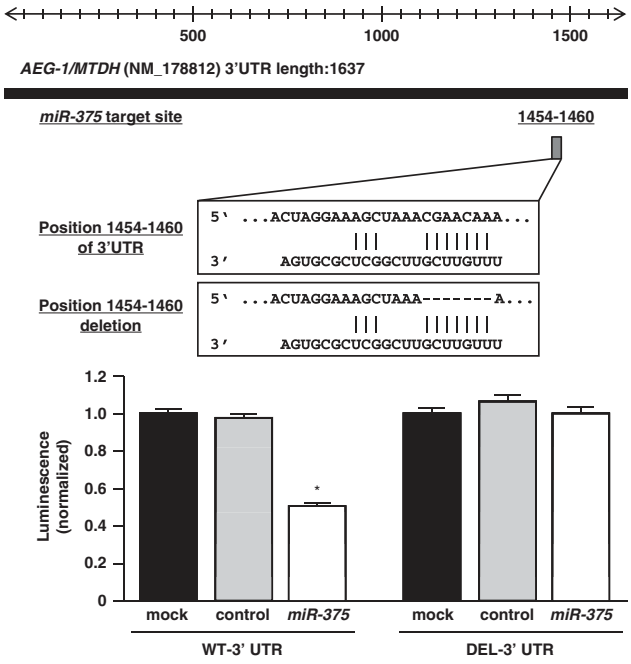

Figure 4 Interaction of miR-375 with AEG-1/MTDH 3′-UTR. At 24 h after transfection with 10 nM *miR-375*, miRNA-control or mocks, a reporter plasmid containing *AEG-1/MTDH* WT-3′-UTR or DEL-3′-UTR and a plasmid expressing *Renilla* luciferase (hRluc) were co-transfected into FaDu cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Relative luciferase activity in *miR-375* transfectants was compared with that in mock cultures, which was set at 1, in cells transfected with WT-3′-UTR or DEL-3′-UTR. $^*P<0.0001$.

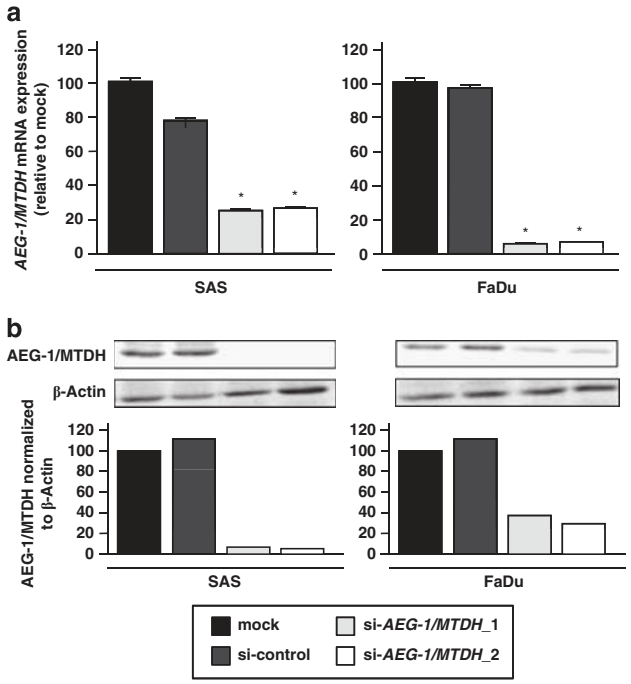

Figure 5 Knockdown of AEG-1/MTDH in HNSCC cell lines by siRNAs. (**a**) *AEG-1/MTDH* mRNA expression 48 h after transfection with 10 nM si-*AEG-1/MTDH*_1, si-*AEG-1/MTDH*_2 or si-control. *AEG-1/MTDH* mRNA expression was repressed in si-*AEG-1/MTDH*_1 and si-*AEG-1/MTDH*_2 transfectants. *GAPDH* was used as an internal control. $^*P<0.0001$. (**b**) AEG-1/MTDH protein expression 72 h after transfection of the siRNAs. β-Actin was used a loading control. The protein expression level of AEG-1/MTDH was also repressed in si-*AEG-1/MTDH*_1 and si-*AEG-1/MTDH*_2 transfectants.

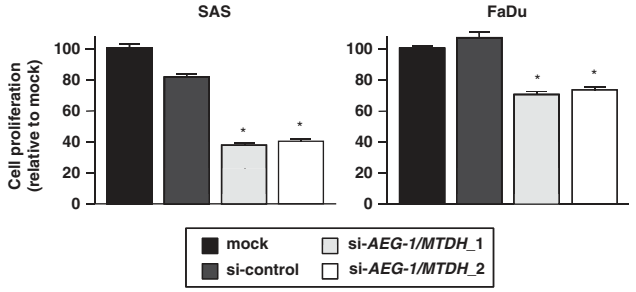

Figure 6 Effect of si-AEG-1/MTDH on cell proliferation in HNSCC cell lines. Cell proliferation was determined with the XTT assay in the SAS and FaDu cell lines 72 h after transfection of 10 nM si-*AEG-1/MTDH*_1, si-*AEG-1/MTDH*_2 or si-control. The XTT assay showed significant inhibition of cell proliferation in si-*AEG-1/MTDH*_1 and si-*AEG-1/MTDH*_2 transfectants in comparison with mock cultures. $*P<0.0001$.

problem, which should be prepared for the large number of clinical specimens. It will be necessary to challenge this problem in future.

In conclusion, the reduction of *miR-375* and the increased expression of *AEG-1/MTDH* are frequent events in HNSCC cancer cells. *miR-375* may function as a tumor suppressor and may directly regulate *AEG-1/MTDH*. *miR-375* regulates novel cancer pathways and could provide new insights into molecular mechanisms in HNSCC and might contribute to the development of new therapeutic strategies for the disease.

ACKNOWLEDGEMENTS

This study was supported by KAKENHI (C), 21592187.

1 Jemal, A., Siegel, R., Xu, J. & Ward, E. Cancer statistics, 2010. *CA Cancer J. Clin.* **60,** 277–300 (2010).
2 Hardisson, D. Molecular pathogenesis of head and neck squamous cell carcinoma. *Eur. Arch. Otorhinolaryngol.* **260,** 502–508 (2003).
3 Leemans, C. R., Braakhuis, B. J. & Brakenhoff, R. H. The molecular biology of head and neck cancer. *Nat. Rev. Cancer* **11,** 9–22 (2011).
4 Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–297 (2004).
5 Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* **9,** 102–114 (2008).
6 Calin, G. A. & Croce, C. M. MicroRNA signatures in human cancers. *Nat. Rev. Cancer* **6,** 857–866 (2006).
7 Esquela-Kerscher, A. & Slack, F. J. Oncomirs—microRNAs with a role in cancer. *Nat. Rev. Cancer* **6,** 259–269 (2006).
8 Chiyomaru, T., Enokida, H., Tatarano, S., Kawahara, K., Uchida, Y., Nishiyama, K. *et al.* miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. *Br. J. Cancer* **102,** 883–891 (2010).
9 Ichimi, T., Enokida, H., Okuno, Y., Kunimoto, R., Chiyomaru, T., Kawamoto, K. *et al.* Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int. J. Cancer* **125,** 345–352 (2009).
10 Kano, M., Seki, N., Kikkawa, N., Fujimura, L., Hoshino, I., Akutsu, Y. *et al.* miR-145, miR-133a and miR-133b: tumor suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. *Int. J. Cancer* **127,** 2804–2814 (2010).
11 Kikkawa, N., Hanazawa, T., Fujimura, L., Nohata, N., Suzuki, H., Chazono, H. *et al.* miR-489 is a tumour-suppressive miRNA target PTPN11 in hypopharyngeal squamous cell carcinoma (HSCC). *Br. J. Cancer* **103,** 877–884 (2010).
12 Yoshino, H., Chiyomaru, T., Enokida, H., Kawakami, K., Tatarano, S., Nishiyama, K. *et al.* The tumour-suppressive function of miR-1 and miR-133a targeting TAGLN2 in bladder cancer. *Br. J. Cancer* **104,** 808–818 (2011).
13 Mutallip, M., Nohata, N., Hanazawa, T., Kikkawa, N., Horiguchi, S., Fujimura, L. *et al.* Glutathione S-transferase P1 (GSTP1) suppresses cell apoptosis and its regulation by miR-133alpha in head and neck squamous cell carcinoma (HNSCC). *Int. J. Mol. Med.* **27,** 345–352 (2011).
14 Nohata, N., Hanazawa, T., Kikkawa, N., Mutallip, M., Fujimura, L., Yoshino, H. *et al.* Caveolin-1 mediates tumor cell migration and invasion and its regulation by miR-133a in head and neck squamous cell carcinoma. *Int. J. Oncol.* **38,** 209–217 (2011).
15 Nohata, N., Sone, Y., Hanazawa, T., Fuse, M., Kikkawa, N., Yoshino, H. *et al.* miR-1 as a tumor suppressive microRNA targeting TAGLN2 in head and neck squamous cell carcinoma. *Oncotarget* **2,** 29–44 (2011).
16 Avissar, M., Christensen, B. C., Kelsey, K. T. & Marsit, C. J. MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. *Clin. Cancer Res.* **15,** 2850–2855 (2009).
17 Hui, A. B., Lenarduzzi, M., Krushel, T., Waldron, L., Pintilie, M., Shi, W. *et al.* Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. *Clin. Cancer Res.* **16,** 1129–1139 (2010).
18 Tsukamoto, Y., Nakada, C., Noguchi, T., Tanigawa, M., Nguyen, L. T., Uchida, T. *et al.* MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. *Cancer Res.* **70,** 2339–2349 (2010).
19 Liu, A. M., Poon, R. T. & Luk, J. M. MicroRNA-375 targets Hippo-signaling effector YAP in liver cancer and inhibits tumor properties. *Biochem. Biophys. Res. Commun.* **394,** 623–627 (2010).
20 Ding, L., Xu, Y., Zhang, W., Deng, Y., Si, M., Du, Y. *et al.* MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. *Cell Res.* **20,** 784–793 (2010).
21 Li, X., Lin, R. & Li, J. Epigenetic silencing of microRNA-375 regulates PDK1 expression in esophageal cancer. *Dig. Dis. Sci.* (e-pub ahead of print 30 April 2011; PMID: 21533613).
22 Sugimoto, T., Seki, N., Shimizu, S., Kikkawa, N., Tsukada, J., Shimada, H. *et al.* The galanin signaling cascade is a candidate pathway regulating oncogenesis in human squamous cell carcinoma. *Genes Chromosomes Cancer* **48,** 132–142 (2009).
23 Hu, G., Wei, Y. & Kang, Y. The multifaceted role of MTDH/AEG-1 in cancer progression. *Clin. Cancer Res.* **15,** 5615–5620 (2009).
24 Sarkar, D., Emdad, L., Lee, S. G., Yoo, B. K., Su, Z. Z. & Fisher, P. B. Astrocyte elevated gene-1: far more than just a gene regulated in astrocytes. *Cancer Res.* **69,** 8529–8535 (2009).
25 Almeida, M. I., Reis, R. M. & Calin, G. A. MicroRNA history: discovery, recent applications, and next frontiers. *Mutat. Res.* (e-pub ahead of print 30 March 2011; PMID: 21458467).
26 Tran, N., O'Brien, C. J., Clark, J. & Rose, B. Potential role of micro-RNAs in head and neck tumorigenesis. *Head Neck* **32,** 1099–1111 (2010).
27 Glazer, C. A., Chang, S. S., Ha, P. K. & Califano, J. A. Applying the molecular biology and epigenetics of head and neck cancer in everyday clinical practice. *Oral Oncol.* **45,** 440–446 (2009).
28 Chiyomaru, T., Enokida, H., Kawakami, K., Tatarano, S., Uchida, Y., Kawahara, K. *et al.* Functional role of LASP1 in cell viability and its regulation by microRNAs in bladder cancer. *Urol. Oncol.* (e-pub ahead of print 14 September 2010; PMID: 20843712).
29 Chiyomaru, T., Tatarano, S., Kawakami, K., Enokida, H., Yoshino, H., Nohata, N. *et al.* SWAP70, actin-binding protein, function as an oncogene targeting tumor-suppressive miR-145 in prostate cancer. *Prostate* (e-pub ahead of print 25 February 2011; PMID: 21360565).
30 Fuse, M., Nohata, N., Kojima, S., Sakamoto, S., Chiyomaru, T., Kawakami, K. *et al.* Restoration of miR-145 expression suppresses cell proliferation, migration and invasion in prostate cancer by targeting FSCN1. *Int. J. Oncol.* **38,** 1093–1101 (2011).
31 Zhang, B., Liu, X. X., He, J. R., Zhou, C. X., Guo, M., He, M. *et al.* Pathologically decreased miR-26a antagonizes apoptosis and facilitates carcinogenesis by targeting MTDH and EZH2 in breast cancer. *Carcinogenesis* **32,** 2–9 (2011).
32 Su, Z. Z., Kang, D. C., Chen, Y., Pekarskaya, O., Chao, W., Volsky, D. J. *et al.* Identification and cloning of human astrocyte genes displaying elevated expression after infection with HIV-1 or exposure to HIV-1 envelope glycoprotein by rapid subtraction hybridization, RaSH. *Oncogene* **21,** 3592–3602 (2002).
33 Warr, T., Ward, S., Burrows, J., Harding, B., Wilkins, P., Harkness, W. *et al.* Identification of extensive genomic loss and gain by comparative genomic hybridisation in malignant astrocytoma in children and young adults. *Genes Chromosomes Cancer* **31,** 15–22 (2001).
34 Lee, S. G., Jeon, H. Y., Su, Z. Z., Richards, J. E., Vozhilla, N., Sarkar, D. *et al.* Astrocyte elevated gene-1 contributes to the pathogenesis of neuroblastoma. *Oncogene* **28,** 2476–2484 (2009).
35 Yu, C., Chen, K., Zheng, H., Guo, X., Jia, W., Li, M. *et al.* Overexpression of astrocyte elevated gene-1 (AEG-1) is associated with esophageal squamous cell carcinoma (ESCC) progression and pathogenesis. *Carcinogenesis* **30,** 894–901 (2009).
36 Hu, G., Chong, R. A., Yang, Q., Wei, Y., Blanco, M. A., Li, F. *et al.* MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell* **15,** 9–20 (2009).
37 Kikuno, N., Shiina, H., Urakami, S., Kawamoto, K., Hirata, H., Tanaka, Y. *et al.* Knockdown of astrocyte-elevated gene-1 inhibits prostate cancer progression through upregulation of FOXO3a activity. *Oncogene* **26,** 7647–7655 (2007).
38 Yoo, B. K., Emdad, L., Su, Z. Z., Villanueva, A., Chiang, D. Y., Mukhopadhyay, N. D. *et al.* Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. *J. Clin. Invest.* **119,** 465–477 (2009).
39 Lee, S. G., Su, Z. Z., Emdad, L., Sarkar, D., Franke, T. F. & Fisher, P. B. Astrocyte elevated gene-1 activates cell survival pathways through PI3K-Akt signaling. *Oncogene* **27,** 1114–1121 (2008).
40 Sarkar, D., Park, E. S., Emdad, L., Lee, S. G., Su, Z. Z. & Fisher, P. B. Molecular basis of nuclear factor-kappaB activation by astrocyte elevated gene-1. *Cancer Res.* **68,** 1478–1484 (2008).

Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)