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# Tumour-suppressive *microRNA-874* contributes to cell proliferation through targeting of *histone deacetylase 1* in head and neck squamous cell carcinoma

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**Background:** Our recent studies of microRNA (miRNA) expression signature demonstrated that *microRNA-874* (*miR-874*) was significantly downregulated in maxillary sinus squamous cell carcinoma (MSSCC), and a putative tumour-suppressive miRNA in human cancers. Our aim of this study was to investigate the functional significance of *miR-874* in cancer cells and to identify novel *miR-874*-mediated cancer pathways and responsible genes in head and neck squamous cell carcinoma (HNSCC).

**Methods:** Gain-of-function studies using mature *miR-874* were performed to investigate cell proliferation and cell cycle distribution in HNSCC cell lines (SAS and FaDu). To identify *miR-874*-mediated molecular pathways and targets, we utilised gene expression analysis and *in silico* database analysis. Loss-of-function assays were performed to investigate the functional significance of *miR-874* target genes.

**Results:** Expression levels of *miR-874* were significantly downregulated in HNSCC tissues (including oral, pharyngeal and laryngeal SCCs) compared with normal counterpart epithelia. Restoration of *miR-874* in SAS and FaDu cell lines revealed significant inhibition of cell proliferation and induction of G2/M arrest and cell apoptosis. Our expression data and *in silico* analysis demonstrated that *miR-874* modulated the cell cycle pathway. Moreover, *histone deacetylase 1* (*HDAC1*) was a candidate target of *miR-874* regulation. Luciferase reporter assays showed that *miR-874* directly regulated *HDAC1*. Silencing of the HDAC1 gene significantly inhibited cell proliferation and induced G2/M arrest and cell apoptosis in SAS cells.

**Conclusions:** Downregulation of *miR-874* was a frequent event in HNSCC. *miR-874* acted as a tumour suppressor and directly targeted *HDAC1*. Recognition of tumour-suppressive miRNA-mediated cancer pathways provides new insights into the potential mechanisms of HNSCC oncogenesis and suggests novel therapeutic strategies for the disease.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and approximately about 500 000 cases are diagnosed every year (Jemal *et al*, 2010). 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% (Haddad and Shin, 2008). Local tumour recurrence and distant metastasis after conventional therapy appear to be major contributing factors for restricted survival of HNSCC patients (Leemans *et al*, 2011). Therefore, understanding the molecular

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cancer pathways underlying HNSCC would help to improve diagnosis, approaches to therapy and prevention of the disease.

The discovery of non-coding RNA in the human genome was an important conceptual breakthrough in the post-genome sequencing era (Mattick, 2004). Improved understanding of non-coding RNA is necessary for continued progress in cancer research. MicroRNAs (miRNAs) constitute a class of small, non-coding RNA molecules, 19–22 nucleotides in length, which modulate gene expression. This is achieved through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes and transcriptional or post-transcriptional regulation of their expression (Bartel, 2004). Currently, 2042 human mature miRNAs are registered at miRBase release 19.0 (http://microrna.sanger.ac.uk/).

A growing body of evidence indicates that miRNAs also contribute to the initiation and development of various types of cancers (Esquela-Kerscher and Slack, 2006). Many human cancers show aberrant expression of miRNAs that can function either as tumour suppressors or oncogenes (Esquela-Kerscher and Slack, 2006). In cancer pathways, normal regulatory mechanisms are disrupted by altered expression of tumour-suppressive or oncogenic miRNAs. Therefore, identification of differentially expressed miRNAs is an important step to understanding human oncogenesis.

Recently, a maxillary sinus squamous cell carcinoma (MSSCC) miRNA expression signature revealed that *microRNA-874* (*miR-874*) was significantly downregulated. Moreover, restoration of *miR-874* inhibited cell proliferation in a MSSCC cell line, IMC-3 (Nohata *et al*, 2011c). To the best of our knowledge, this is the only investigation of *miR-874* in cancer biology. The aim of this study was to investigate the functional significance of *miR-874* and identify the molecular pathways and responsible genes it regulated in HNSCC cells. Genome-wide gene expression analysis of *miR-874* transfectants and *in silico* database analysis showed that the cell cycle was a promising candidate target of *miR-874*. Furthermore, *histone deacetylase 1* (*HDAC1*) was a candidate target of *miR-874* regulation.

*Histone deacetylase 1* belongs to the family of HDACs and is a component of the HDAC complex. It also interacts with retinoblastoma tumour-suppressor protein and this complex is essential for cell proliferation and differentiation (Giacinti and Giordano, 2006). Several studies indicated that HDAC inhibitors (HDACis) are a new class of cytostatic agents that inhibit the proliferation of tumour cells in culture and *in vivo* by inducing cell cycle arrest, differentiation and/or apoptosis (Cruz and Matushansky, 2012; Popovic and Licht, 2012). We focused on *HDAC1* as a putative *miR-874 target* and investigated the functional significance of *HDAC1* in HNSCC. Tumour-suppressive miRNA-modulated cancer pathways provide new insights into the potential mechanisms of HNSCC oncogenesis and suggest novel therapeutic strategies for treatment of the disease.

## MATERIALS AND METHODS

**Clinical HNSCC specimens.** Twenty-three pairs of primary HNSCC and corresponding normal epithelial samples were obtained from patients with HNSCC in Chiba University Hospital (Chiba, Japan) from 2005 to 2011. The samples considered normal were free of cancer cells by pathologic examination. The patient's backgrounds and clinicopathological characteristics are summarised in Table 1. The patients were classified according to the 2002 Union for International Cancer Control TNM staging criteria (Sobin and Wittekind, 2002) before treatment. Written consent of tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the

Table 1. Patient's characteristics

| No. | Location | Age | Sex | T | N | M | Differentiation | Stage |
|---|---|---|---|---|---|---|---|---|
| 1 | Tongue | 68 | M | 2 | 0 | 0 | Well | II |
| 2 | Tongue | 66 | M | 2 | 1 | 0 | Moderate | III |
| 3 | Tongue | 76 | F | 1 | 0 | 0 | Well | I |
| 4 | Tongue | 69 | M | 1 | 0 | 0 | Well | I |
| 5 | Tongue | 76 | F | 1 | 0 | 0 | Well | I |
| 6 | Tongue | 67 | M | 4a | 2c | 0 | Moderate | IVa |
| 7 | Tongue | 36 | F | 3 | 1 | 0 | Moderate | III |
| 8 | Tongue | 64 | M | 1 | 0 | 0 | Well | I |
| 9 | Oral floor | 65 | M | 2 | 2c | 0 | Moderate | IVa |
| 10 | Oropharynx | 67 | M | 3 | 2c | 0 | Poor | IVa |
| 11 | Oropharynx | 52 | M | 3 | 2a | 0 | Moderate | IVa |
| 12 | Oropharynx | 76 | M | 2 | 0 | 0 | Poor | II |
| 13 | Larynx | 63 | M | 3 | 0 | 0 | Moderate | III |
| 14 | Larynx | 69 | M | 3 | 0 | 0 | Well | III |
| 15 | Larynx | 66 | M | 4a | 0 | 0 | Moderate | IVa |
| 16 | Larynx | 82 | M | 3 | 0 | 0 | Poor | III |
| 17 | Hypopharynx | 68 | M | 4a | 1 | 0 | Moderate | IVa |
| 18 | Hypopharynx | 73 | M | 3 | 1 | 0 | Well | III |
| 19 | Hypopharynx | 66 | M | 2 | 2c | 0 | Moderate | IVa |
| 20 | Hypopharynx | 68 | M | 2 | 2b | 0 | Poor | IVa |
| 21 | Hypopharynx | 65 | M | 1 | 2b | 0 | Moderate | IVa |
| 22 | Hypopharynx | 64 | M | 3 | 0 | 0 | Moderate | III |
| 23 | Hypopharynx | 55 | M | 3 | 2b | 0 | Poor | IVa |

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.

**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).

**Cell lines and cell culture.** The following cell lines were used: human HNSCC; SAS (derived from a primary lesion of tongue SCC), FaDu (derived from a primary lesion of hypopharyngeal SCC), HSC3 (derived from a metastatic lymph node of tongue SCC), IMC-3 (derived from a primary lesion of maxillary sinus SCC), human fibroblast; MRC-5. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% $CO_2$ at 37 °C.

**5-Aza-2′-deoxycytidine (5-Aza-dC) treatment.** We investigated the effects of the demethylation agent (5-Aza-dC) treatment (Sigma-Aldrich, St Louis, MO, USA) on HNSCC and fibroblast cell lines (SAS, FaDu, HSC3, IMC-3 and MRC-5). Cells were treated with 5 μM of the 5-Aza-dC, and cells harvested after 72- or 120-h treatment. Experiment manner was as described previously (Enokida *et al*, 2004). The difference expression status of *miR-874* and *HDAC1* was evaluated by real-time PCR methods as follows using before and after 5-Aza-dC treatments.

**Mature miRNA transfection and small interfering RNA treatment.** The following mature miRNAs species were used in this study: hsa-*miR-874*, Ambion Pre-miR, PM12355 (Applied Biosystems, Foster City, CA, USA) and miRIDIAN Mimics, MIMAT0004911 (Thermo Scientific Dharmacon, Waltham, MA, USA). The following siRNAs were used: Stealth Select RNAi siRNA; si-*HDAC1*_1 Cat# HSS104726 and si-*HDAC1*_2 Cat# HSS179192 (Invitrogen), and negative control miRNA/siRNA (Applied Biosystems, P/N: AM17111). RNAs were incubated with OPTI-MEM (Invitrogen) and LipofectamineTM RNAiMax reagent (Invitrogen) as described previously (Ichimi *et al*, 2009). Transfection efficiency of miRNA in cell lines was confirmed based on downregulation of *TWF1* (*PTK9*) mRNA following transfection with *miR-1* as previously reported (Ichimi *et al*, 2009).

**Cell proliferation assays.** Cells were transfected with 10 nM miRNA and siRNA by reverse transfection and plated in 96-well plates at $3 \times 10^3$ cells per well. After 72 h, cell proliferation was determined with the XTT assay, using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported (Chiyomaru *et al*, 2010). Triplicate wells were measured for cell viability in each treatment group.

**Flow cytometry.** Cell cycle status and apoptosis were examined using an APC BrdU Flow kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocol. SAS and FaDu cells transiently transfected with miR-control, *miR-874*, si-control or si-*HDAC1* were harvested 72 h after transfection by trypsinisation. Experiments were done in triplicate.

**Genome-wide gene expression analysis and *in silico* analysis for *miR-874*-modulated pathways.** To gain further insight into which genes were affected by *miR-874*, we performed genome-wide gene expression analysis using *miR-874* transfectants of SAS and FaDu cells. Oligo-microarray human 4 × 44K (GPL10332) (Agilent Technologies) was used for gene expression analysis; the experimental procedure was described previously (Sugimoto *et al*, 2009). To identify the biological processes or pathways potentially regulated by *mi-874*, we performed GENECODIS analysis (Carmona-Saez *et al*, 2007; Nogales-Cadenas *et al*, 2009; http://genecodis.cnb.csic.es/) with our microarray results. Then, to assess the networks regulated by *miR-874* and their target genes, we analysed and characterised those genes in KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Database (Kanehisa *et al*, 2012).

**Quantitative real-time RT–PCR.** First-strand cDNA was synthesised 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-s denaturation at 95 °C and a 1-min annealing/extension at 63 °C. TaqMan probes and primers for, *BUB3* (P/N: Hs00190920_m1), *CCNE1* (P/N: Hs01026536_m1), *CDC23* (P/N: Hs00946641_m1), *CDC25B* (P/N: Hs01550934_m1), *HDAC1* (P/N: Hs02621185_s1) and *GAPDH* (P/N: Hs02758991_g1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of *miR-874* (assay ID: 002268) were analysed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalised to *RNU48* (assay ID: 001006). The delta delta Ct method was adopted and applied to calculate the relative quantities of subject genes. All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

**Western blots.** Cells were harvested 72 h after transfection and lysates were prepared. In all, 50 μg of protein lysate was separated by Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with diluted (1 : 1000) rabbit HDAC1 antibody (600-401-879; Rockland, Gilbertsville, PA, USA), with GAPDH antibody (ab8245, Abcam, Cambridge, UK) used as an internal control. The membrane was washed and incubated with a goat anti-rabbit IgG (H + L)–HRP conjugate (Bio-Rad). Complexes were visualised with an Immun-Star WesternC Chemiluminescence Kit (Bio-Rad), and the expression levels of these genes were evaluated by ImageJ software (ver.1.44; http://rsbweb.nih.gov/ij/).

**Plasmid construction and dual-luciferase reporter assay.** Partial wild-type sequences of *HDAC1* 3′-UTR and those with deleted *miR-874* target sites (positions 149–155) were inserted between the *Xho*I–*Pme*I restriction sites in the 3′-UTR of the hRluc gene in psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Sequences of oligonucleotides were described in Supplementary document 1. The synthesised DNA was cloned into the psiCHECK-2 vector. SAS cells were transfected with 15 ng of vector, 10 nM of *miR-874* (Applied Biosystems), and 1 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of Opti-MEM (Invitrogen). The activities of firefly and Renilla luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalised data were calculated as the quotient of *Renilla*/firefly luciferase activities.

**Statistical analysis.** The relationships between two groups and the numerical values obtained by real-time RT–PCR were analysed using the paired *t*-test. The relationship among three variables and numerical values was analysed using the Bonferroni adjusted Mann–Whitney *U*-test. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA).

## RESULTS

**Expression levels of *miR-874* in HNSCC clinical specimens and cell lines.** The expression levels of *miR-874* were significantly lower in 23 clinical HNSCC specimens than those in corresponding adjacent non-cancerous specimens ($P = 0.0012$). We also

evaluated the expression levels of *miR-874* in two HNSCC cell lines (SAS and FaDu). The expression levels of *miR-874* in these two cell lines were much lower than those in non-cancerous epithelial cells (Figure 1A).

**Effects of *miR-874* restoration on cell proliferation in HNSCC cell lines.** To investigate the functional effects of *miR-874*, we performed gain-of-function studies using miRNA transfection of SAS and FaDu cell lines. We utilised two sources of mature *miR-874* (Ambion and Thermo Scientific Dharmacon) to ensure reproducibility of the data. The XTT assay demonstrated that cell proliferation was significantly inhibited in *miR-874* transfectants in comparison with the mock or miR-control transfectant cells. Specifically, we observed the following growth, expressed as a percentage of the control: (1) SAS – mock, 100.0 ± 1.5; miR-control, 103.5 ± 1.5; *miR-874* (Ambion), 20.2 ± 2.6; *miR-874* (Thermo Scientific Dharmacon), 20.5 ± 0.6 and (2) FaDu – mock, 100.0 ± 1.7; miR-control, 107.3 ± 1.1; *miR-874*, 36.8 ± 2.5; *miR-874* (Thermo Scientific Dharmacon), 36.6 ± 2.3, with $P<0.01$ for both (Figure 1B).

**Effect of *miR-874* restoration on cell cycle and apoptosis.** As *miR-874* restoration significantly inhibited cell proliferation in SAS and FaDu cells, we hypothesised that their restoration induced cell cycle arrest and/or apoptosis. This hypothesis was tested by flow cytometry. With regard to the cell cycle distribution, the fraction of cells in the G2/M phase was significantly larger in *miR-874* transfectants in comparison with the mock (both $P<0.0167$, Figure 2A). These results suggested that *miR-874* restoration induced G2/M arrest in both cell lines. The sub-G0/G1 apoptotic

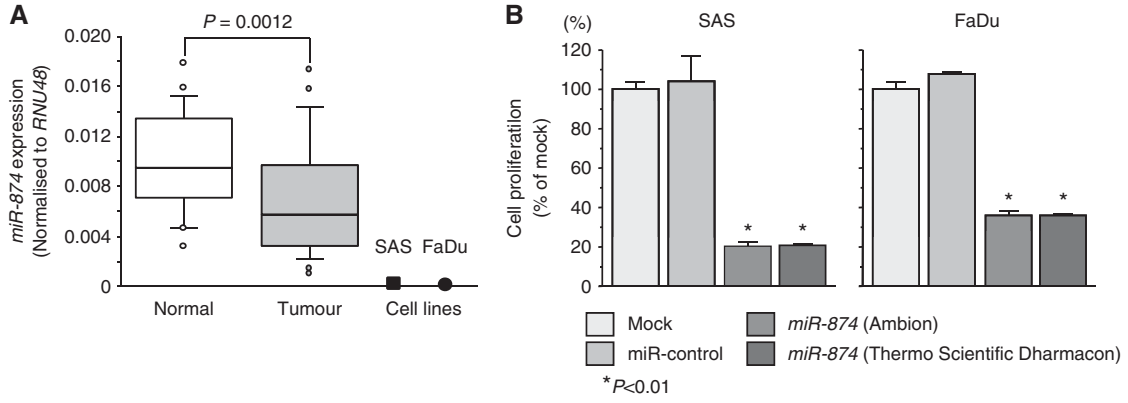


Figure 1. Expression levels of *miR-874* in HNSCC clinical specimens and effect of *miR-874* transfection on SAS and FaDu cells. (**A**) Real-time RT–PCR showed that *miR-874* was significantly downregulated in HNSCC clinical specimens compared with normal specimens ($n=23$). (**B**) Cell proliferation activity after transfection of *miR-874* was determined by XTT assay. Suppression of cell proliferation was similarly observed in both Ambion and Thermo Scientific Dharmacon *miR-874*, taking into consideration the off-target effects of dsRNAs. Trials were independently conducted three times. *$P<0.01$.

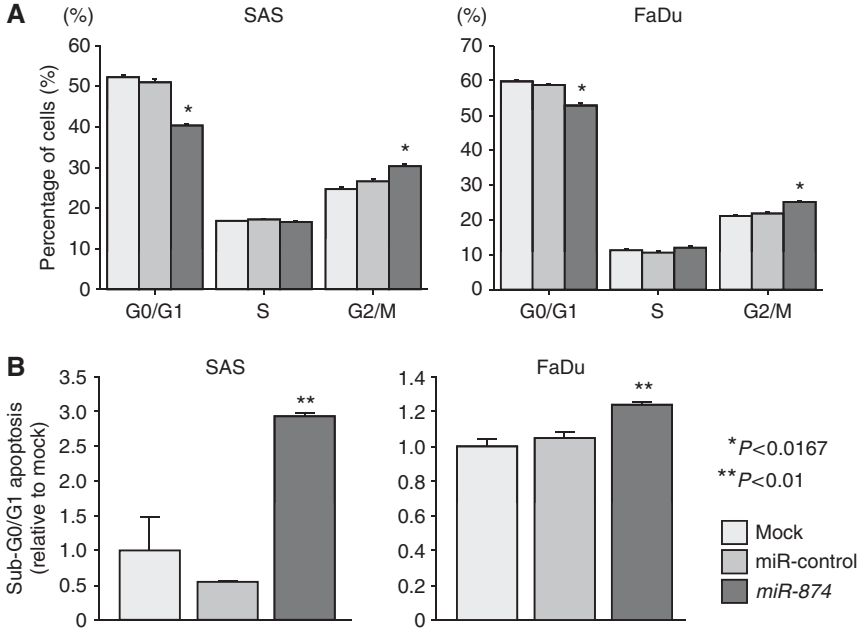


Figure 2. Effect of *miR-874* transfection on cell cycle and apoptosis in SAS and FaDu cells determined by flow cytometry. (**A**) Typical results of cell cycle analysis of mock, miR-control and *miR-874* transfectants. The bar chart represents the percentage of the cells in the G0/G1, S or G2/M phase of the cell cycle. (**B**) Typical results of sub-G0/G1 apoptosis analysis of mock, miR-control and *miR-874* transfectants. Induction of cell apoptosis was shown after transfection of *miR-874*. Trials were independently conducted three times. *$P<0.0167$, **$P<0.01$.

Table 2. (a) Fifty-nine significantly enriched pathways in SAS; (b) Thirteen significantly enriched pathways in FaDu

| KEGG pathway | Genes | *P*-value |
|---|---|---|
| **(a) SAS** | | |
| Protein processing in endoplasmic reticulum | 30 | 4.67E–13 |
| Pathways in cancer | 40 | 1.75E–11 |
| Endocytosis | 29 | 1.90E–10 |
| Focal adhesion | 29 | 2.40E–10 |
| Small cell lung cancer | 19 | 4.29E–10 |
| Regulation of actin cytoskeleton | 29 | 7.09E–10 |
| Lysosome | 21 | 5.25E–09 |
| Axon guidance | 21 | 1.35E–08 |
| ECM–receptor interaction | 15 | 1.07E–06 |
| Insulin signalling pathway | 17 | 1.91E–05 |
| Amoebiasis | 14 | 6.64E–05 |
| p53 signalling pathway | 11 | 1.15E–04 |
| Valine, leucine and isoleucine degradation | 9 | 1.05E–04 |
| Vasopressin-regulated water reabsorption | 9 | 1.05E–04 |
| Prostate cancer | 12 | 2.55E–04 |
| Bacterial invasion of epithelial cells | 10 | 7.80E–04 |
| Notch signalling pathway | 8 | 9.55E–04 |
| ErbB signalling pathway | 11 | 9.49E–04 |
| Phagosome | 14 | 1.11E–03 |
| Cell cycle | 13 | 1.31E–03 |
| Glioma | 9 | 1.37E–03 |
| mTOR signalling pathway | 8 | 1.80E–03 |
| Tight junction | 13 | 1.99E–03 |
| HCM | 10 | 2.00E–03 |
| Pancreatic cancer | 9 | 2.61E–03 |
| Chronic myeloid leukaemia | 9 | 3.45E–03 |
| Dilated cardiomyopathy | 10 | 3.47E–03 |
| Prion diseases | 6 | 4.19E–03 |
| MAPK signalling pathway | 19 | 4.51E–03 |
| Melanogenesis | 10 | 6.69E–03 |
| Calcium signalling pathway | 14 | 8.25E–03 |
| Galactose metabolism | 5 | 8.43E–03 |
| Nucleotide excision repair | 6 | 9.87E–03 |
| Bladder cancer | 6 | 1.09E–02 |
| NOD-like receptor signalling pathway | 7 | 1.08E–02 |
| Inositol phosphate metabolism | 7 | 1.08E–02 |
| ARVC | 8 | 1.08E–02 |
| Glycosaminoglycan degradation | 4 | 1.33E–02 |
| Vascular smooth muscle contraction | 10 | 1.50E–02 |
| Ubiquitin-mediated proteolysis | 11 | 1.77E–02 |
| Amino sugar and nucleotide sugar metabolism | 6 | 1.80E–02 |
| SNARE interactions in vesicular transport | 5 | 2.10E–02 |
| RNA degradation | 7 | 2.09E–02 |
| Starch and sucrose metabolism | 6 | 2.28E–02 |
| Long-term potentiation | 7 | 2.36E–02 |
| Endometrial cancer | 6 | 2.40E–02 |
| CAMs | 10 | 2.55E–02 |
| Non-small cell lung cancer | 6 | 2.47E–02 |
| Vibrio cholerae infection | 6 | 2.47E–02 |
| Melanoma | 7 | 2.44E–02 |
| PPAR signalling pathway | 7 | 2.44E–02 |
| Adherens junction | 7 | 2.58E–02 |
| Oocyte meiosis | 9 | 2.91E–02 |
| Sphingolipid metabolism | 5 | 2.91E–02 |
| Fatty acid metabolism | 5 | 3.18E–02 |
| B-cell receptor signalling pathway | 7 | 3.22E–02 |
| Phosphatidylinositol signalling system | 7 | 3.39E–02 |
| Glycosphingolipid biosynthesis – ganglio series | 3 | 3.60E–02 |
| Protein digestion and absorption | 7 | 3.75E–02 |
| **(b) FaDu** | | |
| Protein processing in endoplasmic reticulum | 17 | 4.67E–07 |
| Systemic lupus erythematosus | 13 | 3.33E–07 |
| Pathways in cancer | 17 | 3.24E–03 |
| Lysosome | 9 | 1.11E–02 |
| Endocytosis | 11 | 1.94E–02 |
| Valine, leucine and isoleucine degradation | 5 | 2.65E–02 |
| Cell cycle | 8 | 3.32E–02 |
| Bile secretion | 6 | 3.03E–02 |
| Neurotrophin signalling pathway | 8 | 2.72E–02 |
| Insulin signalling pathway | 8 | 3.80E–02 |
| Ubiquitin-mediated proteolysis | 8 | 3.79E–02 |
| Inositol phosphate metabolism | 5 | 4.26E–02 |
| Oocyte meiosis | 7 | 3.97E–02 |

Abbreviations: ARVC = arrhythmogenic right ventricular cardiomyopathy; CAMs = cell adhesion molecules; ECM = extracellular matrix; HCM = hypertrophic cardiomyopathy; KEGG = Kyoto Encyclopedia of Genes and Genomes; MAPK = mitogen-activated protein kinase; mTOR = mammalian target of rapamycin; NOD = nucleotide-binding oligomerization domain; PPAR = peroxisome proliferator-activated receptor; SNARE = soluble NSF attachment protein receptor..

cell fraction was significantly larger in *miR-874* transfectants (2.9-fold in SAS and 1.3-fold in FaDu) than in the mock and miR-control (both $P<0.01$, Figure 2B). Flow cytometric data are shown in Supplementary Figure 1.

**Identification of *miR-874*-modulated molecular pathways and putative target genes in HNSCC.** To gain further insight into molecular mechanisms and pathways regulated by tumour-suppressive *miR-874* in HNSCC, we performed genome-wide gene expression analysis using *miR-874* transfected cells, *miR-874*-SAS and *miR-874*-FaDu. Entries from the microarray data were approved by the Gene Expression Omnibus (GEO) and were assigned GEO accession number GSE37119. A total of 1071 and 591 genes were downregulated $< -0.5$ ($\log_2$ ratio) in *miR-874*-SAS and *miR-874*-FaDu transfectants, respectively, compared with control cells. These genes were analysed and characterised in KEGG pathway categories by the GENECODIS 3.0 program.

The GENECODIS analysis categorised 59 and 13 significantly enriched signalling pathways in the *miR-874*-SAS and *miR-874*-FaDu transfectants, respectively (Table 2a and b). We focused on the 'cell cycle' pathway and the 15 genes listed within this pathway (Table 3). Of those 15 genes, five (budding uninhibited by benzimidazoles 3 homolog: *BUB3*, cyclin E1: *CCNE1*, cell division cycle protein 23 homolog: *CDC23*, cell division cycle protein 25 homolog B: *CDC25B* and HDAC 1: *HDAC1*) were downregulated

Table 3. Cell cycle-related genes regulated by *miR-874*

| No. | Symbol | SAS | FaDu | *miR-874* site |
|---|---|---|---|---|
| 1 | *ANAPC11* | − | + | − |
| 2 | *BUB3* | + | + | 2 |
| 3 | *CCNE1* | + | + | 1 |
| 4 | *CDC23* | + | + | 2 |
| 5 | *CDC25B* | + | + | 1 |
| 6 | *CDK6* | + | − | 1 |
| 7 | *CDKN1B* | + | − | − |
| 8 | *CDKN2D* | + | + | − |
| 9 | *GSK3B* | + | − | 1 |
| 10 | *HDAC1* | + | + | 1 |
| 11 | *RAD21* | − | + | 1 |
| 12 | *RB1* | + | − | − |
| 13 | *RBL2* | + | − | − |
| 14 | *SMAD3* | + | − | 4 |
| 15 | *TGFB2* | + | − | − |

Abbreviation: miR-874 = microRNA-874.

by transfection of *miR-874* in both SAS and FaDu cells, and contained putative binding sites of *miR-874* in their 3′-UTR by TargetScan database (Table 3). Our strategy for selection of *miR-874* target genes is shown in Figure 3.

**Expression levels of five candidate genes regulated by *miR-874* in HNSCC clinical specimens.** In cancer pathways, normal regulatory mechanisms can be disrupted by altered expression of tumour-suppressive or oncogenic miRNAs. We hypothesised that downregulation of *miR-874* led to overexpression of oncogenic genes in HNSCC clinical specimens. To support this hypothesis, we investigated the two independent gene expression analyses, one is deposited in GEO database, accession number is GSE9844, and the other is our own expression data by using real-time RT–PCR.

First, we screened the expression of those 5 candidates and other 10 genes in HNSCC clinical samples using data deposited in GEO (accession number GSE9844) by another research group. We noted that *CCNE1*, *HDAC1*, *CDC25B*, *CDC23* and *BUB3* were upregulated in cancer tissues compared with normal epithelia (Table 4 and Supplementary Figure 2). Next, we used real-time RT–PCR to measure the mRNA expression levels of five candidate genes in HNSCC clinical specimens. Four genes (*BUB3*, *CCNE1*, *CDC23* and *CDC25B*) were not upregulated in cancer tissues (Figure 4). In contrast, *HDAC1* was significantly upregulated in cancer tissues compared with adjacent normal epithelia ($P = 0.0077$, Figure 4). Therefore, we focused on the *HDAC1* gene as a possible target gene regulated by *miR-874* in HNSCC and subjected it to further experiments.

***HDAC1* is a direct target of *miR-874*.** We performed quantitative real-time RT–PCR and western blotting to investigate whether HDAC1 expression was downregulated by restoration of *miR-874*. The mRNA and protein expression levels of HDAC1 were significantly repressed in *miR-874*-transfectants in comparison with mock or miR-control transfectants ($P < 0.01$, Figures 5A and B).

We performed a luciferase reporter assay to determine whether *HDAC1* mRNA had a target site for *miR-874*. We used a vector encoding either the partial sequence of the 3′-UTR of *HDAC1* mRNA, including the predicted *miR-874* target site (positions 149–155) or a vector lacking the *miR-874* target site. We found that the luminescence intensity was significantly reduced by transfection of the wild type 3′-UTR of *HDAC1* while deletion of positions 149–155 blocked the decrease in luminescence ($P < 0.01$, Figure 5C).

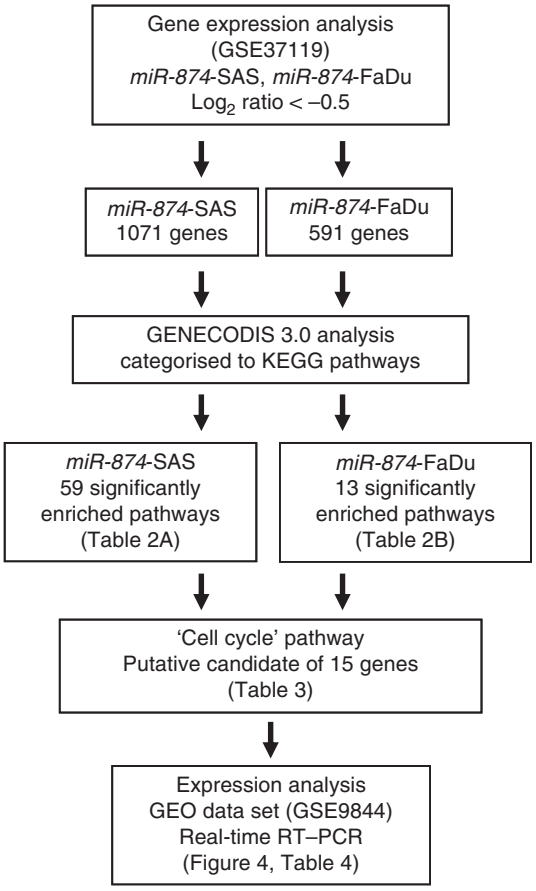


Figure 3. Workflow for the *in silico* analysis of *miR-874*-regulated pathways. miR-control transfectants that produced raw signal values of < 1000 were excluded before comparisons were made. Downregulated genes in *miR-874* transfectants with $< -0.5$ ($log_2$ ratio) downregulation compared with the control transfectants were chosen from the results of microarray analysis (GSE37119). A total of 1071 and 591 genes were identified as genes downregulated by *miR-874* in SAS and FaDu cells, respectively. The gene sets were then analysed and categorised with the KEGG Pathway Database using the GENECODIS 3.0 program. In all, 59 and 13 significantly enriched signalling pathways were derived from the gene sets in SAS and FaDu cells, respectively. In total, 13 and 8 genes were included in the 'cell cycle' pathway in SAS and FaDu cells, respectively.

HNSCC and fibroblast cells were subjected to real-time RT–PCR to evaluate the expression levels of *miR-874* and *HDAC1*. At baseline, expression levels of the *miR-874* were negative in all cell lines. However, after 5-Aza-dC treatment, the expression levels of *miR-874* were significantly increased in all cell lines. Conversely, the expression levels of *HDAC1* mRNA were significantly decreased by 5-Aza-dC treatment. Inverse correlation was recognised between *miR-874* and *HDAC1* expression after 5-Aza-dC treatment (Supplementary Figure 3). This data suggested that demethylation restored the expression of *miR-874* in HNSCC and fibroblast cells and that reduction of the *HDAC1* expression was caused by recovery for expression of *miR-874* in cancer cells.

**Effects of *HDAC1* silencing on cell proliferation, cell cycle and apoptosis in HNSCC cell lines.** To investigate the functional role of *HDAC1*, we performed loss-of-function studies using si-*HDAC1*

transfectants. First, we evaluated the knockdown efficiency of si-*HDAC1* treatments. The expression levels of *HDAC1* mRNA were repressed in two si-*HDAC1* transfectants in comparison with mock and si-control transfectants ($P<0.01$, Figure 6A). Moreover, the expression levels of HDAC1 protein were repressed in si-*HDAC1*-1 and the si-*HDAC1*-2 transfectants in comparison with mock and si-control transfectants (Figure 6B). These results showed that the two siRNAs were effective for loss-of-function assays in this study.

**Table 4.** Expression levels of 15 genes downregulated by *miR-874* in 'cell cycle' pathway (GSE9844)

| Entrez gene | Symbol | Fold change (tumour/normal) | *P*-value |
|---|---|---|---|
| 898 | *CCNE1* | 2.262 | 0.0012 |
| 3065 | *HDAC1* | 1.539 | 0.0012 |
| 994 | *CDC25B* | 1.782 | 0.0023 |
| 8697 | *CDC23* | 1.331 | 0.0095 |
| 1021 | *CDK6* | 1.932 | 0.0118 |
| 1027 | *CDKN1B* | −1.569 | 0.0644 |
| 5885 | *RAD21* | 1.567 | 0.0897 |
| 5934 | *RBL2* | −1.622 | 0.0945 |
| 2932 | *GSK3B* | 1.276 | 0.1100 |
| 7042 | *TGFB2* | 1.405 | 0.1158 |
| 9184 | *BUB3* | 1.162 | 0.1239 |
| 51 529 | *ANAPC11* | 1.264 | 0.2215 |
| 5925 | *RB1* | 1.076 | 0.7581 |
| 4088 | *SMAD3* | −1.076 | 0.8110 |
| 1032 | *CDKN2D* | 1.089 | 0.9277 |

Abbreviation: miR-874 = microRNA-874.

The XTT assay revealed significant inhibition of cell proliferation in the two different si-*HDAC1* transfectants in comparison with growth of the mock and si-control transfectants. The following was observed, expressed as a percentage of control proliferation: mock, 100 ± 4.2; si-control, 93.7 ± 5.6; si-*HDAC1*_1, 74.7 ± 6.4; si-*HDAC1*_2, 38.4 ± 0.9, $P<0.01$; Figure 7A. As for the cell cycle distribution, the fractions of cells in the G1/G0 and G2/M phases were significantly larger in si-*HDAC1* transfectants in comparison with the si-control ($P<0.0167$, Figure 7B), and the sub-G0/G1 apoptotic cell fraction was significantly larger (3.0-fold) in si-*HDAC1* transfectants than in the si-control in SAS cells ($P<0.01$, Figure 7C). Flow cytometric data are shown in Supplementary Figure 4.

## DISCUSSION

Despite considerable advances in cancer treatment, the overall survival rate of HNSCC patients has not markedly improved. Therefore, it is necessary to pursue new concepts to enhance treatment results and to develop new therapeutic strategies. For elucidation of the molecular mechanisms underlying HNSCC, we examined miRNA expression, focusing on its regulated molecular targets and novel cancer pathways based on HNSCC expression signatures (Kikkawa *et al*, 2010; Nohata *et al*, 2011c). Our recent studies demonstrated that *miR-1*, *miR-133a*, *miR-218*, *miR-375* and *miR-489* were significantly reduced in HNSCC clinical specimens. Moreover, they functioned as tumour suppressors through their targeting of several oncogenic genes in HNSCC cells (Kikkawa *et al*, 2010; Mutallip *et al*, 2011; Nohata *et al*, 2011a,b,c,d,e; Kinoshita *et al*, 2012a,b,c,d). Our previous study of MSSCC miRNA expression signature showed that *miR-874* was the most downregulated miRNA and significantly inhibited cancer cell proliferation in IMC-3 cells derived from MSSCC. We also identified putative targets of *miR-874* by genome-wide gene

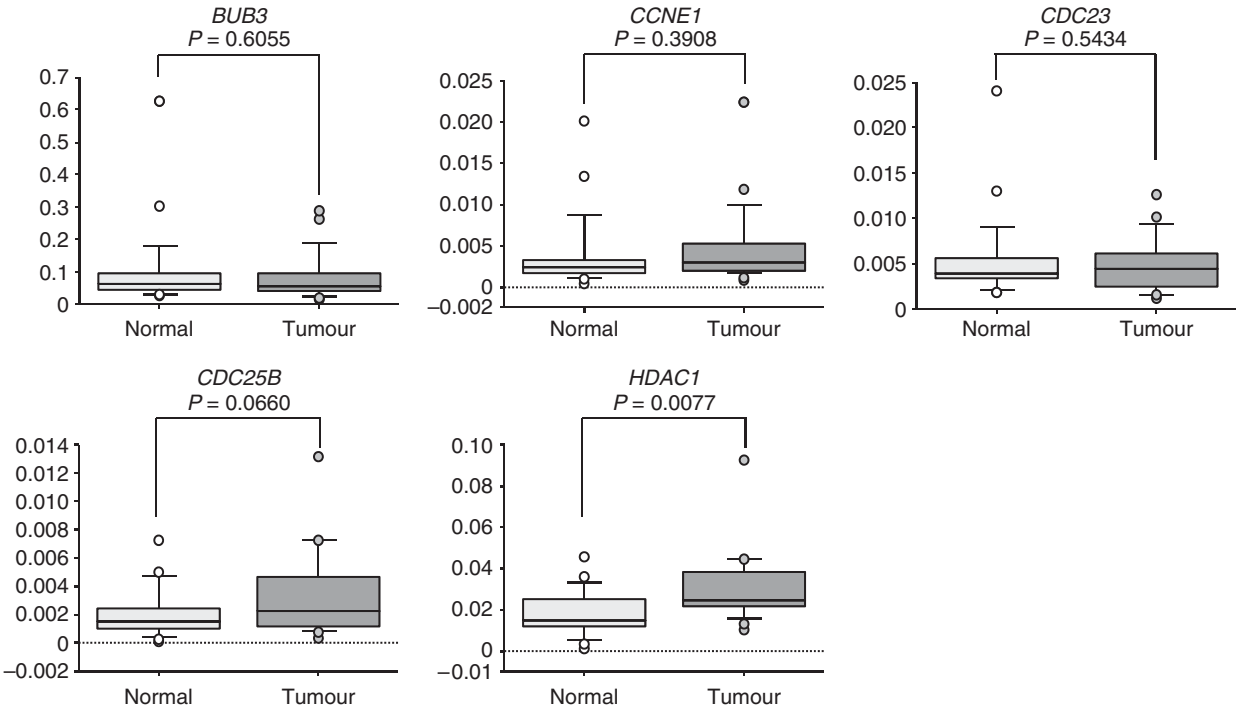


**Figure 4. Expression levels of five candidate genes of *miR-874* target.** *BUB3*, *CCNE1*, *CDC23*, *CDC25B* and *HDAC1* mRNA expression levels in HNSCC clinical specimens were measured by quantitative real-time RT–PCR. *GAPDH* was used for normalisation. All reactions were performed in triplicate.

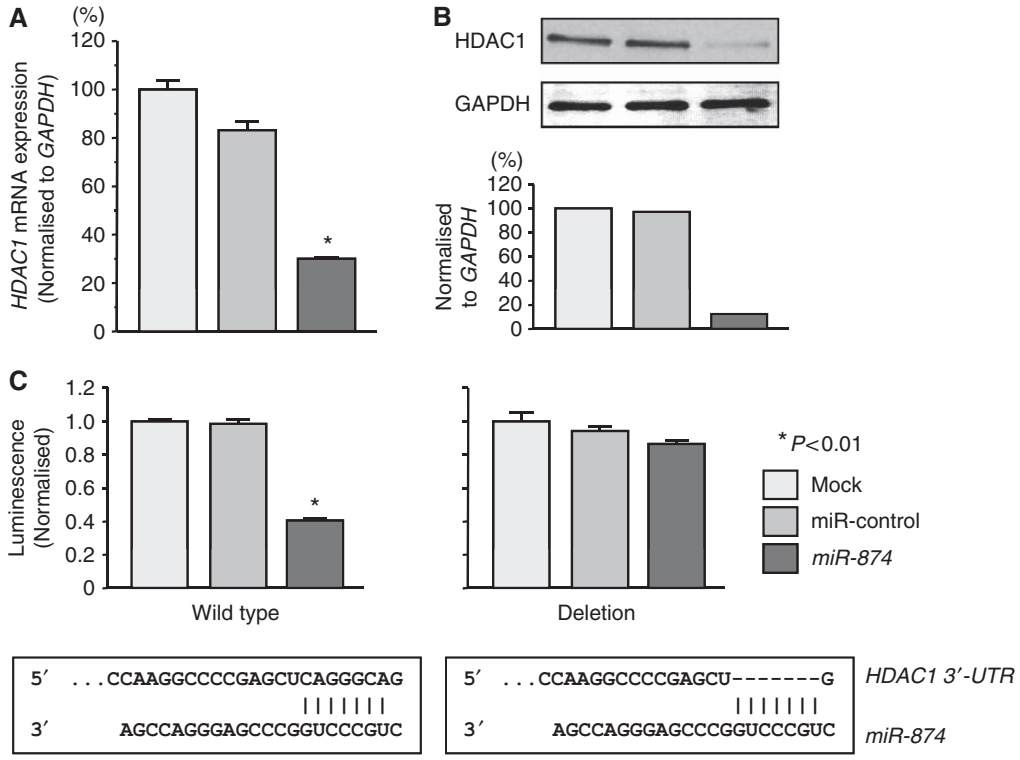


**Figure 5. Effect of *miR-874* on HDAC1 mRNA and protein expression in SAS and FaDu cells.** (**A**) *HDAC1* mRNA expression 72 h after transfection with 10 nM *miR-874*. *HDAC1* mRNA expression was significantly repressed in *miR-874* transfectants. *GAPDH* was used as an internal control. (**B**) HDAC1 protein expression 72 h after transfection with *miR-874*. GAPDH was used as a loading control. The protein expression level of HDAC1 was also repressed in *miR-874* transfectants. (**C**) Interaction of *miR-874* with *HDAC1* 3′-UTR. After 24-h transfection with 10 nM *miR-874*, miR-control or mocks, a reporter plasmid containing *HDAC1* wild type-3′-UTR or deletion-3′-UTR and a plasmid expressing *Renilla* luciferase (hRluc) were co-transfected into SAS cells. Firefly luciferase activity was normalised to *Renilla* luciferase activity. Relative luciferase activity in *miR-874* transfectants was compared with that in mock cultures, which was set at 1, in cells transfected with wild type-3′-UTR or deletion-3′-UTR. Trials were independently conducted three times. *$P<0.01$.

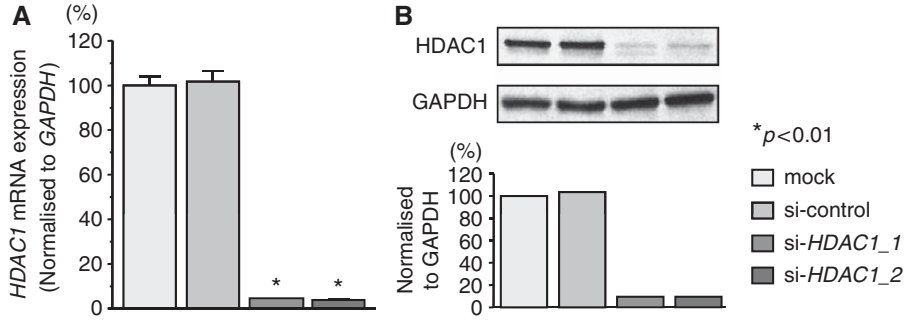


**Figure 6. Silencing of *HDAC1* in HNSCC cell lines by siRNAs.** (**A**) *HDAC1* mRNA expression 72 h after transfection with 10 nM si-*HDAC1*_1, si-*HDAC1*_2 or si-control. *HDAC1* mRNA expression was repressed in si-*HDAC1*_1 and si-*HDAC1*_2 transfectants. *GAPDH* was used as an internal control. (**B**) HDAC1 protein expression 72 h after transfection of the siRNAs. GAPDH was used a loading control. The protein expression level of HDAC1 was also repressed in si-*HDAC1*_1 and si-*HDAC1*_2 transfectants. Trials were independently conducted three times. *$P<0.01$.

expression analysis and *PPP1CA* was a direct target of *miR-874* in MSSCC cells (Nohata *et al*, 2011c). The overexpression of *PPP1CA* was observed in oral cancer cell lines (Hsu *et al*, 2006) and this protein may act as an oncogene since dephosphorylated BRCA1 as breast and ovarian tumour suppressor is considered to be inactive form (Liu *et al*, 2002). The identification of tumour-suppressive miRNA-mediated novel cancer pathways is the first step to consider the effective and promising strategy for miRNA-based therapeutics for the treatment of human cancers. Except for our previous report, there are no data to suggest a relationship between *miR-874* and human cancers. Thus, we believed that analysis of *miR-874* could lead to discoveries of new molecular mechanisms in HNSCC.

First, we evaluated the expression level of *miR-874* in HNSCC clinical specimens that were obtained from the oral cavity, larynx and pharynx. We confirmed that *miR-874* was significantly downregulated in tumour tissues. The elucidation of silencing mechanisms of tumour-suppressive miRNAs will contribute to miRNA-based cancer therapies in near future. We performed preliminary analysis of molecular mechanism of *miR-874* silencing in HNSCC cells. Our data demonstrated that *miR-874* was re-expressed by treatment of 5-Aza-dC, suggesting that silencing of

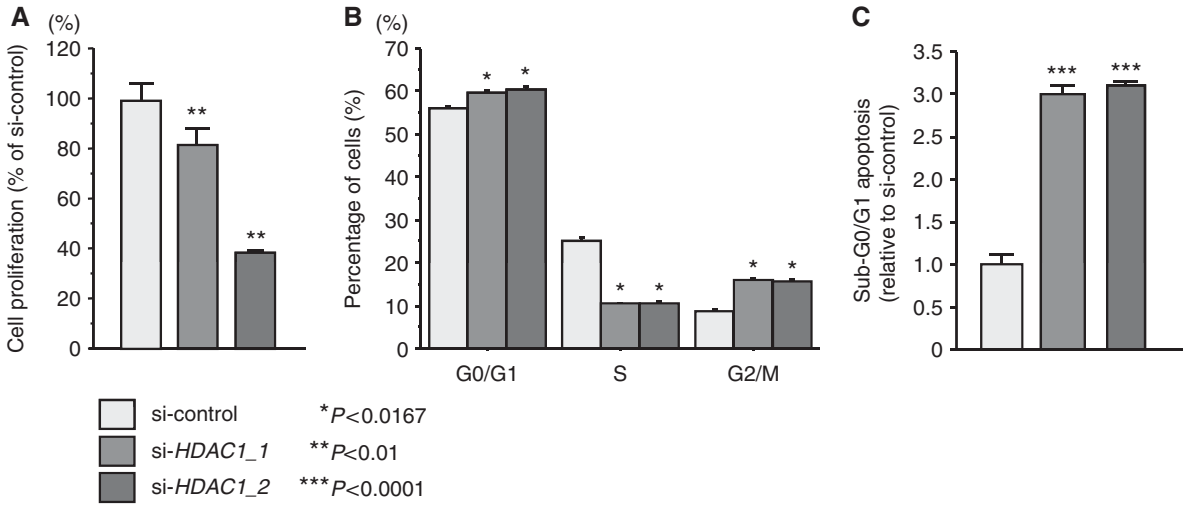


Figure 7. Effect of si-*HDAC1* transfectants on cell proliferation, cell cycle and apoptosis in SAS cells. (**A**) Suppression of cell proliferation after transfection of si-*HDAC1*_1 and si-*HDAC1*_2 into SAS cells as determined by XTT assay. (**B**) Typical results of cell cycle analysis of si-control, si-*HDAC1*_1 and si-*HDAC1*_2 transfectants. The bar chart represents the percentage of the cells in the G0/G1, S or G2/M phase. (**C**) Typical results of cell apoptosis analysis of mock, si-control, si-*HDAC1*_1 and si-*HDAC1*_2 transfectants. Trials were independently conducted three times. *$P<0.0167$, **$P<0.01$, ***$P<0.0001$.

*miR-874* in cancer cells was caused by epigenetic modification especially methylation of CpG island located on the upstream of *miR-874* genomic locus (Supplementary Figure 3). Further detailed analysis is necessary to understand the molecular mechanism of *miR-874* silencing in cancer cells. Next, we investigated the functional significance of *miR-874* in HNSCC using two cell lines, SAS and FaDu. In this study, we used two kinds of miRNA to evaluate the function of *miR-874*. Restoration of mature *miR-874* in cancer cells revealed significant inhibition of cancer cell proliferation, suggesting *miR-874* is indeed a new tumour suppressor in HNSCC. It is likely that further analysis will provide added insight into HNSCC oncogenesis.

miRNAs are unique in their ability to regulate many protein-coding genes. Bioinformatic predictions indicate that miRNAs regulate >30% of protein-coding genes (Filipowicz *et al*, 2008). We have taken the position that identification of novel cancer pathways and responsible genes regulated by tumour-suppressive *miR-874* is an important first step in understanding HNSCC oncogenesis. Based on this view, we performed genome-wide gene expression analysis of *miR-874* transfectants (*miR-874*-SAS and *miR-874*-FaDu) and *in silico* analysis. A single miRNA is capable of targeting a number of genes to regulate biological processes globally. In fact, 1060 and 587 genes were downregulated in *miR-874*-SAS and *miR-874*-FaDu transfectants, respectively. We used GENECODIS analysis to reveal the functional significance of the genes potentially regulated by *miR-874* in HNSCC. GENECODIS analysis applies many genes to known pathways in the KEGG Pathway Database and these data facilitate understanding of tumour-suppressive miRNA-mediated molecular pathways in human cancer. We devised this method of analysis and found that tumour-suppressive miRNA could efficiently regulate cancer-associated pathways (Fuse *et al*, 2012; Kinoshita *et al*, 2012a). In this study, we focused on the 'cell cycle' pathway because restoration of *miR-874* inhibited cancer cell proliferation in HNSCC cell lines.

The expression signatures of *miR-874* transfectants in HNSCC cells and *in silico* analysis revealed that five genes (*BUB3*, *CCNE1*, *CDC23*, *CDC25B* and *HDAC1*) were involved in the 'cell cycle' pathway and that they were candidate targets of *miR-874* regulation. Out of the five genes, *HDAC1* was significantly upregulated in HNSCC clinical specimens as assessed by both our real-time RT–PCR analysis and HNSCC gene expression data deposited in GEO. We further focused on *HDAC1* as a responsible gene in HNSCC and investigated the functional significance of the gene.

Our luciferase reporter assay demonstrated that *miR-874* directly regulated *HDAC1* in HNSCC cells. It has been shown that *HDAC1* is directly regulated by *miR-449a* in prostate cancer (Noonan *et al*, 2009) and lung cancer (Jeon *et al*, 2012). The seed sequence of mature *miR-449a* is identical to that of *miR-34* families, which are well known as p53-responsive genes and tumour suppressors through regulating HDAC *SIRT1*, cyclin-dependent kinases, cyclins and E2Fs (Hermeking, 2010). Recent miRNA researches suggest that one of the reasons why HDACs is overexpressed in various cancers might be downregulation of miRNAs acting as endogenous HDACis (Datta *et al*, 2008; Nasser *et al*, 2008; Noonan *et al*, 2009; Jeon *et al*, 2012).

Histone deacetylases remove acetyl groups from histones and other nuclear proteins that induce chromatin condensation and transcriptional repression (Haberland *et al*, 2009). HDACs have important roles in aberrant epigenetic changes related to human malignant diseases (Marks, 2010). *HDAC1* is upregulated in several types of cancer, such as lung (Sasaki *et al*, 2004), gastric (Choi *et al*, 2001), breast (Zhang *et al*, 2005), prostate (Weichert *et al*, 2008a), liver (Rikimaru *et al*, 2007) and colorectal cancers(Weichert *et al*, 2008b), and it correlates with poor prognosis. HDACis have been watched with keen interest in cancer therapy because aberrant expressions of HDACs are frequent events (Glozak and Seto, 2007; Marks, 2010). Vorinostat and romidepsin have been approved recently for the treatment of refractory cutaneous T-cell lymphoma, and clinical trials of other HDACis are in progress in other lymphomas (Grant *et al*, 2007).

This is the first report that upregulation of *HDAC1* is caused by downregulation of tumour-suppressive *miR-874* in HNSCC cells. Future work on *miR-874* involving its targeting of oncogenic pathways and its targeting of oncogenes may lead to better understanding of HNSCC oncogenesis and development of new therapeutic strategies for HNSCC.

## CONCLUSIONS

Downregulation of *miR-874* was a frequent event in HNSCC. *miR-874* inhibited cancer cell proliferation through direct targeting

of *HDAC1*, suggesting that *miR-874* is a new tumour-suppressive miRNA. Tumour-suppressive *miR-874* appears to modulate cancer-associated pathways. The recognition that *miR-874* target oncogenes may lead to better understanding of HNSCC oncogenesis and the development of new therapeutic strategies for HNSCC.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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