Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma

N Nohata1,2, T Hanazawa2, N Kikkawa1,2, D Sakurai3, L Fujimura2, T Chiyomaru4, K Kawakami4, H Yoshino4, H Enokida4, M Nakagawa3, A Katayama3, Y Harabuchi3, Y Okamoto2 and N Seki*,1

1Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan; 2Department of Otorhinolaryngology/Head and Neck Surgery, Chiba University Graduate School of Medicine, Chiba, Japan; 3Biomedical Research Center, Chiba University, Chiba, Japan; 4Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan; 5Department of Otorhinolaryngology/Head and Neck Surgery, Ashikawa Medical University, Ashikawa, Japan

BACKGROUND: On the basis of the microRNA (miRNA) expression signature of maxillary sinus squamous cell carcinoma (MSSCC), we found that miR-874 was significantly reduced in cancer cells. We focused on the functional significance of miR-874 in cancer cells and identification of miR-874-regulated novel cancer networks in MSSCC.

METHODS: We used PCR-based methods to investigate the downregulated miRNAs in clinical specimens of MSSCC. Our signature analyses identified 23 miRNAs that were significantly reduced in cancer cells, such as miR-874, miR-133a, miR-375, and miR-1. We focused on miR-874 as the most downregulated novel miRNA in our analysis.

RESULTS: We found potential tumour suppressive functions such as inhibition of cancer cell proliferation and invasion. A molecular target search of miR-874 revealed that PPP1CA was directly regulated by miR-874. Overexpression of PPP1CA was observed in MSSCC clinical specimens. Silencing of the PPP1CA gene significantly inhibited cancer cell proliferation and invasion.

CONCLUSION: The downregulation of miR-874 was a frequent event in MSSCC, which suggests that miR-874 functions as a tumour suppressive miRNA, directly regulating PPP1CA that has a potential role of an oncogene. The identification of novel miR-874-regulated cancer pathways could provide new insights into potential molecular mechanisms of MSSCC oncogenesis.

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The maxillary sinuses are single pyramidal cavities in the body of the maxilla. Squamous cell carcinoma is the most common cancer of the maxillary sinus (60–70%), followed by adenoid cystic carcinoma (Million and Cassisi, 1994). Maxillary sinus squamous cell carcinoma (MSSCC) comprises 2–3% of all head and neck tumours and the annual incidence is 0.5–1.0 per 100 000 people (Tiwari et al, 2000; Dulguerov and Allal, 2006). Clinical symptoms of MSSCC present insidiously, and sometimes resemble those of chronic sinusitis. Although presentation of lymph node and distant metastasis were uncommon, primary tumours are often diagnosed as advanced disease. The 5-year survival rate of T4 tumours is ~50%. Local recurrence is the most common cause of treatment failure and death (Konno et al, 1998; Tiwari et al, 2000; Dulguerov and Allal, 2006).

From an epidemiological standpoint, occupational exposures to leather, wood dust, nickel, arsenic, and formaldehyde have been implicated in the aetiology of MSSCC (Luce et al, 2002; Bornholdt et al, 2008). In contrast, tobacco, a major risk factor for head and neck squamous cell carcinoma (HNSCC) does not have an important role in MSSCC (Zheng et al, 1993; Holt, 1994). It has been suggested that molecular mechanisms of carcinogenesis might be different for these cancers (Lopez et al, 2011). Although analyses of major cancer-related genes, such as TP53 and K-ras, mutation were reported (Bornholdt et al, 2008; Holmiela et al, 2010), relatively few genome-wide gene expression analyses of MSSCC have been conducted and no analyses of microRNAs (miRNAs) have been performed for this disease.

MicroRNAs are small non-coding RNAs of 20–22 nucleotides, and are involved in crucial biological processes, including development, differentiation, apoptosis, and proliferation through imperfect pairing with target mRNAs of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression (Barlet, 2004). Bioinformatic predictions indicate that miRNAs regulate >30% of the protein coding genes (Filipowicz et al, 2008). Currently, 1424 human miRNAs are registered at miRBase release 17.0 (http://mirorna.sanger.ac.uk/).

Recent studies suggest that miRNAs contribute to the initiation and development of various types of cancer (Calin and Croce, 2006). Some highly expressed miRNAs could function as oncogenes by repressing tumour suppressor genes, whereas low-expressed miRNAs could function as tumour suppressors by negatively regulating oncogenes (Esquela-Kerscher and Slack, 2006). We have conducted searches of tumour suppressive miRNAs based on analyses of expression signatures of various cancers (Ichimi et al, 2009; Kano et al, 2010; Kikkawa et al, 2010; Yoshino et al, 2011). These studies successfully identified several tumour suppressive miRNAs such as miR-1, miR-133a, and mir-145 (Chiyomaru et al, 2010b; Kano et al, 2010; Yoshino...
**MATERIALS AND METHODS**

**Clinical MSSCC specimens**

In all, 20 pairs of primary MSSCC and corresponding normal epithelial samples were obtained from patients with MSSCC in Chiba University Hospital (Chiba, Japan) from 2005 to 2010. The fresh specimens were immediately immersed in RINalater (Qiagen, Valencia, CA, USA) and stored at −20 ºC until RNA was extracted. The samples macroscopically considered normal were confirmed 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 2002 Union for International Cancer Control TNM staging criteria (Sobin and Wittekind, 2002). Written consent of patients was obtained before treatment. The aim of this study was to identify new tumour suppressive miRNAs revealed in our MSSCC expression analysis. We focused on miR-874, which was the most significantly downregulated miRNA in our signature. We found that it functioned as a tumour suppressor based on our findings of inhibited cancer cell proliferation and invasion. Genome-wide expression analysis identified several candidate target genes such as PPP1CA (protein phosphatase 1, catalytic subunit, alpha isoform), PAAFL1 (proteasomal ATPase-associated factor 1), and TGOLN2 (trans-Golgi network (TGN) protein 2). Insights into the association between tumour suppressive miR-874 and their target oncogene networks could enhance our understanding of the molecular mechanism of MSSCC carcinogenesis.

**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).

**MicroRNA expression signatures and data normalisation**

Tissue specimens for miRNA screening using a low density array (LDA) were from five MSSCC patients at Chiba University Hospital between 2005 and 2007 (Table 1; #1–#5). The miRNA expression patterns were evaluated using the TaqMan LDA Human microRNA Panel v2.0 (Applied Biosystems, Foster City, CA, USA). The assay was composed of two steps: generation of cDNA by reverse transcription (RT) and a TaqMan real-time PCR assay. Description of real-time PCR and the list of human miRNAs can be found on the company’s website (http://www.appliedbiosystems.com). Analysis of relative miRNA expression data was performed using GeneSpring GX version 7.3.1 software (Agilent Technologies) according to the manufacturer’s instructions. A cutoff P-value of <0.05 was used to narrow down the candidates after global normalisation of the raw data. After global normalisation, additional normalisation was carried out with RNU48.

**Maxillary sinus squamous cell carcinoma cell culture**

The human MSSCC cell line IMC-3 (Mizoguchi et al, 1991) was used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37 ºC.

**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-874; pre-miR ID: PM12355), negative control miRNA (P/N: AM17111) (Applied Biosystems), small-interfering RNA (Stealth Select RNAi siRNA; si-PPP1CA_1 Cat#: HSS143413, si-PPP1CA_2 Cat#: HSS143414) (Invitrogen), and negative control siRNA (Stealth RNAi Negative Control Medium GC Duplex; 12935–300) (Invitrogen). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen) as described previously (Ichimi et al, 2009). Transfection efficiency of pre-miR in cell lines was confirmed based on downregulation of TWFI (PTK9) miRNA 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 RT and plated in 96-well plates at 3 × 104 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) as previously reported (Ichimi et al, 2009; Chiyomaru et al, 2010b). Triplicate wells were measured for cell viability in each treatment group. Furthermore, we also conducted counting number of cells with each treatment. Cells (1 × 105) were incubated in a 24-well plate for 24, 48, and 72 h. They were then treated with trypsin and stained with trypan blue. Viable cells, which excluded trypan blue dye, were counted in quadruplicate with a Countess (Invitrogen).

**Cell invasion assays**

A cell invasion assay was carried out using modified Boyden chambers containing transwell precoated Matrigel membrane filter inserts with 8 μm pores in 24-well tissue cultureplates at 1 × 105 cells per well (BD Biosciences, Bedford, MA, USA; Kano et al, 2010;
Chiyomaru et al., 2010b). Triplicate wells were measured for cell invasion in each treatment group.

**Target gene search for miR-874**

Genome-wide screens using miR-874 transfectants were performed to identify target genes of miR-874 in IMC-3. Oligo-microarray human 44K (Agilent Technologies) was used for expression profiling of the transfectants in comparison with a miRNA-negative control transfectant. Hybridisation and wash steps were performed as previously described (Sugimoto et al., 2009). The arrays were scanned using a Packard GSI Lumonics Scan Array 4000 (Perkin Elmer, Boston, MA, USA). The data were analysed by means of DNASIS array software (Hitachi Software Engineering, Tokyo, Japan), which converted the signal intensity for each spot into text format. The log2 ratios of the median subtracted background intensities were analysed. Data from each microarray study were normalised by a global normalisation method. Predicted target genes and their target mRNA 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/).

**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 a cycle consisting of a 15 s denaturation at 95 °C and a 1 min annealing/extension at 63 °C. TaqMan probes and primers for PPIICA (P/N: Hs00267568_m1), PAAFI (P/N: Hs00228523_m1), TGOLN2 (P/N: Hs00197728_m1) and GUSB (P/N: Hs99999908_m1) internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The expression levels of miR-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 ΔΔCt method was adopted and applied to calculate relative quantity of subject genes. All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

**Western blots**

Cells were harvested at 72 h after transfection and lysates were prepared. A 50 μg of protein lysate was separated by NuPAGE on 4–12% bis-tris gels (Invitrogen) and transferred to PVDF membranes. Immunoblotting was performed with diluted (1:200) monoclonal protein phosphatase 1 (P1ph) antibody (sc-7482; Santa Cruz Biotechnology, Santa Cruz, CA, USA), with β-actin antibody (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA), with mouse IgG (H + L)–HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualised by echochemiluminescence (GE Healthcare Bio-Sciences, Princeton, NJ, USA), and the expression levels of these genes were evaluated by ImageJ software (version 1.44; http://rsbweb.nih.gov/ij/).

**Plasmid construction and dual-luciferase reporter assay**

The wild-type sequences of PPIICA 3’-UTR and those with deleted miR-874 target sites (position 237–243) were inserted between the XhoI–PmeI restriction sites in the 3-UTR of the hRluc gene in pSfiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Sequences of oligonucleotides are described in the Supplementary Information. The synthesised DNA was cloned into the psiCHECK-2 vector. The IMC-3 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.

**Table 2** Downregulated microRNAs in maxillary sinus squamous cell carcinoma (normalised to RNU48)

| MicroRNA     | Accession no. | Fold change | Normalised ratio |
|--------------|---------------|-------------|-----------------|
|                                      | Normal      | Tumour      |  P-value       |
| miR-874      | MMAT0004911   | 0.011       | 3.05E-04       | 3.36E-06       | 0.0043 |
| miR-133a     | MMAT0000427   | 0.017       | 1.89E-02       | 3.14E-04       | 0.0033 |
| miR-375      | MMAT0000728   | 0.035       | 3.95E-02       | 1.36E-03       | 0.0161 |
| miR-204      | MMAT0000265   | 0.045       | 3.26E-02       | 1.47E-04       | 0.0055 |
| miR-1        | MMAT0000416   | 0.054       | 1.88E-03       | 1.02E-04       | 0.004 |
| miR-139-5p   | MMAT0000250   | 0.132       | 3.61E-02       | 4.75E-03       | 0.0059 |
| miR-145      | MMAT0000457   | 0.167       | 6.57E-01       | 1.10E-01       | 0.0367 |
| miR-143      | MMAT0000435   | 0.177       | 2.35E-01       | 4.57E-02       | 0.019 |
| miR-486-3p   | MMAT0000742   | 0.183       | 2.26E-03       | 4.13E-04       | 0.0407 |
| miR-146a     | MMAT0000449   | 0.253       | 1.09E+00       | 2.76E-01       | 0.0041 |
| miR-410      | MMAT0002171   | 0.254       | 4.92E-04       | 1.25E-04       | 0.002 |
| miR-126      | MMAT0000445   | 0.265       | 3.16E+00       | 8.28E-01       | 0.0037 |
| miR-539      | MMAT00003163  | 0.278       | 4.00E-03       | 1.11E-03       | 0.005 |
| miR-134      | MMAT0000447   | 0.308       | 1.69E-03       | 5.19E-04       | 0.0158 |
| miR-218      | MMAT0000275   | 0.316       | 1.62E-02       | 5.13E-03       | 0.0329 |
| miR-146b-5p  | MMAT0002809   | 0.337       | 5.18E-01       | 1.75E-01       | 0.0012 |
| miR-140-3p   | MMAT0004597   | 0.341       | 2.04E-02       | 6.94E-03       | 0.0111 |
| miR-30a-3p   | MMAT0000888   | 0.431       | 6.37E-02       | 2.75E-02       | 0.0089 |
| miR-191      | MMAT0000440   | 0.489       | 6.22E-01       | 3.04E-01       | 0.0251 |
| miR-186      | MMAT0000456   | 0.51        | 1.75E-01       | 8.90E-02       | 0.0309 |
| miR-148b     | MMAT0002243   | 0.523       | 3.30E-02       | 1.73E-02       | 0.0334 |
| miR-30e-3p   | MMAT000693    | 0.525       | 4.95E-02       | 2.60E-02       | 0.0048 |
| miR-29c      | MMAT000681    | 0.552       | 7.94E-02       | 4.39E-02       | 0.0305 |

**RESULTS**

Identification of downregulated miRNAs in MSSCC by miRNA expression signature: expression of miR-874 in MSSCC clinical specimens

We evaluated mature miRNA expression levels of five pairs of normal epithelia and MSSCC by miRNA expression signature analysis. In all, 23 significantly downregulated miRNAs were selected after normalisation to RNU48 (Table 2). The miR-874, the most downregulated miRNA in the list, was selected for further study. Quantitative stem–loop RT–PCR demonstrated that the expression levels of miR-874 were significantly lower in 20 MSSCC specimens in comparison with normal tissues (P = 0.0307, Figure 1A).

**Table 2** Downregulated microRNAs in maxillary sinus squamous cell carcinoma (normalised to RNU48)
Effect of miR-874 transfection on the proliferation and invasion of IMC-3

To investigate the functional roles of miR-874, we performed gain-of-function studies using miRNA transfection of IMC-3.

Figure 1  Expression of miR-874 in MSSCC clinical specimens and gain-of-function study using miR-874 in the IMC-3 cell line. (A) The miR-874 expression levels in clinical specimens. Real-time RT-PCR showed that miRNA expression in tumour tissues was lower than that of normal tissues. RNU48 was used as an internal control. (B) Cell proliferation determined by the XTT assay in the IMC-3 cell line transfected with 10 nm of miR-874 or miR-control. (C) Cell number was counted after transfection with 10 nm of miR-874 or miR-control at 24, 48, and 72 h. (D) Cell invasion activity determined by the Matrigel invasion assay in IMC-3 cell lines transfected with 10 nm of miR-874 or miR-control. *P<0.05.

Table 3  The 18 downregulated genes in miR-874 transfectants

| Entrez gene ID | Gene name                                                       | Gene symbol | Log_{2} ratio | miR-874 target |
|---------------|----------------------------------------------------------------|-------------|---------------|----------------|
| 1373          | Carbamoyl-phosphate synthetase 1, mitochondrial                 | CPS1        | −1.85         | 2               |
| 3939          | Lactate dehydrogenase A                                        | LDHA        | −1.75         | —              |
| 1915          | Eukaryotic translation elongation factor 1, X isoform           | EEF1A1      | −1.45         | —              |
| 5499          | Protein phosphatase 1, catalytic subunit, X isoform             | PPP1CA      | −1.25         | 1              |
| 5660          | Proapoptosis                                                   | PSAP        | −1.23         | —              |
| 80227         | Proteasomal ATPase-associated factor 1                          | PAAF1       | −1.22         | 1              |
| 567           | β-2-microglobulin                                               | B2M         | −1.21         | —              |
| 303           | Annexin A2 pseudogene                                          | ANXA2P1     | −1.18         | —              |
| 5223          | Phosphoglycerate mutase 1                                       | PGAM1       | −1.15         | —              |
| 1303          | Collagen, type XII, X isoform                                   | COL12A1     | −1.09         | 1              |
| 3486          | Insulin-like growth factor binding protein 3                    | IGFBP3      | −1.08         | —              |
| 2778          | GNAS complex locus                                              | GNAS        | −1.08         | —              |
| 55536         | Cell division cycle associated 7-likel                          | CDC47       | −1.06         | —              |
| 8667          | Eukaryotic translation initiation factor 3, subunit H           | EIF3H       | −1.05         | 1              |
| 10916         | Melanoma antigen family D, 2                                    | MAGED2      | −1.05         | —              |
| 10618         | Trans-Golgi network protein 2                                   | TGNOL2      | −1.03         | 2              |
| 4077          | Neighbor of BRCA1 gene                                          | NBR1        | −1.02         | 1              |
| 343477        | Heat shock protein 90kDa β (Gpr94), member 3 (pseudogene)       | HSP90B3P    | −1.00         | —              |

The XTT assay showed significant inhibition of cell proliferation in miR-874 transfectants in comparison with the miR-control transfectants (% of cell proliferation, 69.6 ± 0.8 and 100.0 ± 3.3, respectively; P<0.05; Figure 1B). This result was also confirmed by performing cell counting assay (cell......
Expression levels of candidate target genes of miR-874 in MSSCC clinical specimens

We measured the mRNA expression levels of seven candidate genes in MSSCC clinical specimens by quantitative real-time RT–PCR. Three genes, PPP1CA, PAAF1, and TGOLN2 were significantly upregulated in cancer tissues (P = 0.0154, P = 0.0298, and P = 0.0312 respectively; Figure 2A, B and C, upper panel). The other four genes (CPST1, COL12A1, EIF3H, and NBR1) were not upregulated in the tumour region of MSSCC (Supplementary Figure). There were significant inverse correlations between each of the genes and the level of miR-874 expression (Figure 2A, B and C, lower panel).

PPP1CA is directly regulated by miR-874

PPP1CA mRNA and PP1z protein expression levels were markedly downregulated in the miR-874 transfectants in comparison with the controls (Figure 3A and B). We performed a luciferase reporter assay to determine whether PPP1CA mRNA had a target site for miR-874. We used a vector encoding either the total sequence of the 3′-UTR of PPP1CA mRNA, including the predicted miR-874 target site (positions 237–243), or a vector lacking the miR-874 target site. We found that the luminescence intensity was significantly reduced by transfection of the entire 3′-UTR of PPP1CA, whereas deletion of positions 237–243 blocked the decrease in luminescence (Figure 4).

Figure 2  Expression levels of three candidate genes of miR-874 target were measured by real-time RT–PCR. (A, upper) PPP1CA mRNA expression levels in MSSCC clinical specimens. (B, upper) PAAF1 mRNA expression levels in MSSCC clinical specimens. (C, upper) TGOLN2 mRNA expression levels in MSSCC clinical specimens. Real-time RT–PCR showed that each of the three genes in tumour tissues was expressed at higher levels than that in the normal tissues. GUSB was used as an internal control. (A, B, and C, lower) Significant inverse correlations between each of the genes and the level of miR-874 expression were shown.

Figure 3  PPP1CA mRNA and PP1z protein expression in the IMC-3 cell line. (A) PPP1CA mRNA expression 48 h after transfection with 10 nM with miR-874. PPP1CA mRNA expression was significantly repressed in miR-874 transfectants. GUSB was used as an internal control. (B) PP1z protein expression 72 h after transfection with miR-874. β-Actin was used as a loading control. The protein expression level of PP1z was also repressed in miR-874 transfectants.

Expression levels of candidate target genes of miR-874 in MSSCC clinical specimens

To gain further insight into which genes were affected by miR-874 transfection, we performed gene expression analysis with miR-874 transfectants and the controls in IMC-3 cells. Signal values of raw data in miR-control transfectants < 5000 were cutoff. A total of 18 genes were downregulated less than −1.0 (log2 ratio) in miR-874 transfectants compared with the controls. The TargetScan programme showed that seven of the genes had putative target sites of miR-874 in their 3′-UTR (Table 3). Entries from the microarray data were approved by the Gene Expression Omnibus (GEO) and were assigned GEO accession number GSE19714.
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**FIGURE 4** Schematic of conserved binding sites for miR-874 (upper). Putative conserved target site in the PPP1CA 3’-UTR was identified with the TargetScan database: the one miR-874 target site is indicated. IMC-3 cells were transfected with 15 ng of PPP1CA 3’-UTR in a vector construct and 10 nM of miR-874 or miR-control. Vectors were used with encoding the entire sequence of 3’-UTR of PPP1CA mRNA or that with a deletion of the miR-874 target (position 237–243). Renilla luciferase activity was measured after a 24 h transfection. The results were normalised against firefly luciferase values (lower). *P < 0.05.

**Effect of PPP1CA silencing on cell proliferation and invasion in IMC-3**

To examine the functional role of PPP1CA, we performed loss-of-function studies using two different si-PPP1CA transfectants into the IMC-3 cell line. The PPP1CA mRNA and PP1z protein expression levels were markedly reduced by the two different si-PPP1CA transfectants (Figure 5A and B).

The XTT assay revealed significant inhibition of cell proliferation in the two different si-PPP1CA transfectants in comparison with growth of the si-control transfectants (% of cell proliferation: 60.4 ± 1.2, 73.2 ± 1.0, and 100.0 ± 5.0, respectively; *P < 0.0001; Figure 5C). This result was also confirmed by performing cell counting assay (cell number, 8.0 × 10^5 ± 1.2 × 10^5, 1.4 × 10^5 ± 4.6 × 10^4, and 3.2 × 10^5 ± 6.0 × 10^4, respectively; *P = 0.0005 and *P = 0.0018, respectively; Figure 5D).

The Matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in the two different si-PPP1CA transfectants compared with their counterparts (% of cell invasion, 11.5 ± 2.8, 8.1 ± 1.4, and 100.0 ± 11.7, respectively; *P < 0.0001; Figure 5E).

**DISCUSSION**

This is the first article to investigate aberrant miRNA expression in MSSCC clinical specimens. The miR-133a and miR-1 were among the top five downregulated miRNAs in our expression analysis. Interestingly, miR-1-1/miR-133a-2, and miR-1-2/miR-133a-1 are clustered on different chromosomal regions in the human genome, 20q13.33 and 18q11.2, respectively. Recently, our analyses of oesophageal cancer and bladder cancer expression signatures confirmed downregulation of both miRNAs (Kano et al, 2010; Chiyomaru et al, 2010b), and we demonstrated that miR-1 and miR-133a function as tumour suppressors in many types of cancers regulating several oncopgenes (Chiyomaru et al, 2010a,b; Kano et al, 2010; Mutallip et al, 2011; Nohata et al, 2011a,b; Uchida et al, 2011; Yoshino et al, 2011). When we consider other miRNAs in this signature, miR-145 downregulation has frequently been reported in cancers, including prostate, bladder, colon, ovarian, and oesophageal cancers as well as B-cell malignancies (Aka et al, 2007; Iorio et al, 2007; Arndt et al, 2009; Kano et al, 2010; Chiyomaru et al, 2010b; Zaman et al, 2010). The miR-145 is located on chromosome 5q32–33 within a 4.09 kb region (http://microrna.sanger.ac.uk/). Of interest, 5q31.1 is a well-known fragile site in the human genome (http://www.genenames.org/) and is often deleted in cancers. Increasing evidence and our data indicate that miR-145 functions as a tumour suppressive miRNA and inhibits cell growth, invasion, and migration in cancer cells (Kano et al, 2010; Chiyomaru et al, 2010b; Fuse et al, 2011). Our present analysis generated a list of sequences that could be involved in the pathology of MSSCC. Analysis of miRNAs included in this signature could enhance our understanding of MSSCC carcinogenesis.
In this study, we focused on the functional significance of miR-874, because it was the most downregulated miRNA in our signature and functional analysis of miR-874 had not yet been reported. The miR-874 was recently identified based on small RNA library sequencing and is conserved across most, but not all mammals (Landgraf et al., 2007; Lui et al., 2007). Our results showed that miR-874 was downregulated in MSSCC cells and ectopic expression of miR-874 significantly inhibited cell proliferation and invasion in IMC-3 cells. These results indicated that miR-874 might function as a tumour suppressor in IMC-3 cells. Further studies are required to elucidate the precise mechanisms of miR-874 regulation for initiation and development of MSSCC oncogenesis.

We performed a genome-wide analysis using miR-874 transfected IMC-3 cells to elucidate the target genes regulated by miR-874. From the microarray analysis, we identified seven candidate genes (CPS1, PPP1CA, PAAF1, COL12A1, EIF3H, TGOLN2, and NBR1) containing miR-874 target sites. To validate the mRNA expression levels of seven candidate genes in MSSCC clinical specimens by quantitative real-time RT–PCR, we narrowed down to three genes (PPP1CA, PAAF1, and TGOLN2) whose expression levels were significantly upregulated in MSSCC clinical specimens compared with normal tissues.

PAAF1 inhibits proteasome 26S assembly and proteolytic activity by impairing the association of the 19S regulatory complex with the 20S core. The 26S proteasome consists of a 20S proteolytic core particle and 19S regulatory complexes. The 26S proteasome has an important role in ubiquitin-dependent proteolysis, which regulates many biological processes, such as cell cycle progression and signal transduction (Park et al., 2005). TGOLN2, TGN protein 2, is a cargo protein of retrograde transport, in which proteins and lipids are moved between endosomes and the TGN. Although the functions of several cargo proteins have been elucidated in retrograde transport, the role of TGOLN2 remains unknown (Johannes and Popoff, 2008; Pfeffer, 2009). These two genes currently have little association with cancer development. Therefore, we focused on PPP1CA as a subject of further experiment.

PPP1CA encodes the catalytic subunit of PP1z. The PP1z catalytic subunit can form complexes with many regulatory subunits, which regulate various cellular activities such as the cell cycle, apoptosis, and signal transduction (Cohen, 2002; Ceulemans and Bollen, 2004). Previous analysis of the protein showed that PP1z dephosphorylates the BRCA1 protein, coded by the tumour suppressor BRCA1, in breast and ovarian cancer. Those findings indicate that PP1z may have an oncogenic role (Liu et al., 2002). In addition, it has been reported that overexpression of PP1z is observed in pre-malignant hepatic cells and oral squamous cell carcinoma (Saadat et al., 1995; Imai et al., 1999; Hsu et al., 2006). In contrast, PP1z may function as a tumour suppressor by activating tumour suppressor protein pRB (Alberts et al., 1993). Our present data suggest that the PPP1CA gene functions as an oncogene in MSSCC. A molecular network search for downstream targets of PPP1CA in MSSCC will be necessary. Unfortunately, there was no significant relationship between miR-874 or PPP1CA expression and clinicopathological parameters in this study. Our cohort was too small to evaluate this relationship. In addition, our samples are mostly at the late stage. Hence, a large-scale clinical test including the early stage samples will be necessary.

It is also of interest that PPP1CA is located at chromosomal region 11q13. Amplification of the chromosomal region on 11q13...
mimR-874 as a tumour suppressor in MSSC

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is frequently observed in human cancers including HNSCC and breast cancer, and it is well known that CCND1, encoding cyclin D1, is a putative oncogene in the 11q13 amplon (Schuuring, 1995; Schwab, 1998; Gollin, 2001). We recently demonstrated that when chromosomal region 11q13 was gained, the expression levels of several genes were elevated (Sugimoto et al., 2009). The oncogenic function of PP1CA and amplification of this region may be related closely, so it will be important to examine the structural changes in this region in MSSC.

In conclusion, the reduction of mimR-874 and increase of PP1CA were frequent events in MSSC cancer cells. The mimR-874 may function as a tumour suppressor and may directly regulate PP1CA. The mimR-874 regulates novel cancer pathways and could provide new insights into molecular mechanisms in MSSC and might contribute to the development of new therapeutic strategies for the disease.

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Conflict of interest

The authors declare no conflict of interest.

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