miR-3941: A novel microRNA that controls IGBP1 expression and is associated with malignant progression of lung adenocarcinoma

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Lung adenocarcinoma is a leading cause of death in Japan, the USA and Europe.1,2 Even for patients with pathological stage I lung cancer, the 5-year survival rate is 66–83%, which is far from satisfactory. However, Noguchi et al.3,4 have indicated that very early-stage adenocarcinomas classified into Noguchi types A and B, and type C’, show a very favorable prognosis. Recently, Travis et al.5,6 defined these two distinct groups of early-stage lung adenocarcinomas as adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA), respectively, and this concept was accepted in the latest (4th) edition of the WHO classification of lung tumors. Although the biology of AIS and MIA is still unclear, with the exception of EGFR mutation, early-stage adenocarcinomas are known to have a very low frequency of gene abnormalities such as mutations of k-ras and p53, amplifications of PIK3CA, BRAF and HER2, and translocations of ALK, ROS and RET that have been reported in advanced lung adenocarcinomas.7–10 Therefore, it would be very informative and pertinent to clarify the specific genetic, epigenetic and phenotypic abnormalities that occur during the progression of very early-stage adenocarcinoma to early but invasive adenocarcinoma. We have been focusing on such abnormalities at the early stage of lung adenocarcinogenesis and reported several, such as amplification of ECT2, demethylation of SFN, and overexpression of OCIAD2 and immunoglobulin (CD79a) binding protein 1 (IGBP1).11–14

Among them, IGBP1 was initially identified as a signal transduction molecule coprecipitating with MB1 (Iga) of the B-cell antigen receptor (BCR) complex, and was later found to be broadly expressed in various organs. IGBP1 has been characterized as an associated and regulatory component of the catalytic subunits of protein phosphatase (PP) 2, PP4, and PP6. Protein phosphatase 2A (PP2A) is the most abundant phosphatase in mammalian cells, playing important roles in cell growth and cell cycle control.15–25 PP2Ac is one of the catalytic subunits of PP2A, and interaction between IGBP1 and PP2Ac enhances the catalytic activity of the latter and alters its substrate specificity. Binding of PP2Ac to IGBP1 also leads to acquisition of anti-apoptosis function.26 Li et al.27 have found that lactoferrin binds specifically to IGBP1 in a lung adenocarcinoma cell line, thus triggering apoptosis. Subsequently, Sakashita et al.14 reported that the level of IGBP1 protein expression increases along with lung adenocarcinoma progression, being significantly associated with patient
outcome. Therefore, clarification of the molecular mechanism of IGBP1 overexpression would shed important light on the early progression of lung adenocarcinoma and might indicate suitable candidate molecules for targeted therapy.

Generally, gene amplification, point mutation, and methylation of the promoter region have been reported as causes of dysregulated gene expression. However, few reports have focused on such alterations in IGBP1. In addition to these genetic and epigenetic alterations, microRNAs (miRNAs) are reported to be involved in the abnormal expression of oncogenes or anti-oncogenes. miRNAs are endogenous non-coding small RNAs (20–22 nucleotides) that can also regulate gene expression by binding to target messenger RNAs (mRNAs). miRNAs are associated with various cell processes such as cell development, differentiation, proliferation, carcinogenesis and apoptosis. Dysregulation of miRNAs has been especially associated with various human cancers, such as lymphoma, colon carcinoma, gastric carcinoma, lung carcinoma, breast carcinoma, and brain tumors.12–33 Thus, many miRNAs appear to play oncogenic or anti-oncogenic roles in the development and progression of cancers. Chen et al.34 have reported that down-regulation of miR-34b leads to high expression of IGBP1, suggesting that miRNA is an essential regulator of IGBP1 expression.

In the present study using microRNA array analysis and in silico analysis with TargetScan Human, we focused on several candidate miRNAs that influence the expression of IGBP1.

Materials and Methods

Tissue samples and cell lines. Three fresh specimens of human early lung adenocarcinoma, seven frozen specimens of human lung adenocarcinoma in situ, and six frozen specimens of human early lung adenocarcinoma, together with paired samples of adjacent normal lung tissue, were obtained from patients who had undergone surgical resection at Tsukuba University Hospital (Ibaraki, Japan). Informed consent for study of their materials had been obtained from all of the patients. The A549 and PC-9 human lung adenocarcinoma cell lines were purchased from RIKEN Cell Bank (Ibaraki, Japan). A549 was maintained in Dulbecco’s modified Eagle medium/F12 (DMEM/F12) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, NY, USA). PC-9 was maintained in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS). All cells were cultured in a 5% CO2 incubator at 37°C.

microRNA array. Total RNA was extracted from three fresh tumor cells of early lung adenocarcinoma (two cases of small invasive adenocarcinoma and one case of minimally invasive adenocarcinoma), which were collected by scratching directly from the cut surface of freshly resected material, as the tumor employed was very small (<2.0 cm in diameter). The samples were then subjected to microRNA array analysis using miRCURY LNA (Exiqon, Vedbaek, Denmark). The threshold for identifying the dysregulated miRNAs was a twofold change (either down or up).

Immunohistochemistry. The 4-μm-thick sections were deparaffinized and rehydrated, then pretreated by autoclaving at 120°C for 5 min in 10 mM citrate buffer (pH 6.0). The slides were treated with chemMate POD Blocking Solution (Dako, Copenhagen, Denmark) at room temperature for 5 min to block any endogenous peroxidase activity before incubation with the anti-IGBP1 antibody (1:250; Sigma-Aldrich Co., St. Louis, MO, USA). The antibody was applied for 30 min, followed by incubation with the secondary antibody (EnVision+ DualLink; Dako) for 30 min. The slides were then incubated in DAB/Tris solution and finally counterstained with hematoxylin.

Total RNA isolation and real-time reverse transcription PCR. Total RNA was extracted using a mirVana miRNA isolation kit (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. For analysis of each mature miRNA, total RNA was reverse-transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) along with each miRNA-specific primer (Thermo Fisher Scientific). U6 snRNA was used as an endogenous control. Real-time reverse transcription PCR (real-time RT-PCR) for miRNA was performed with TaqMan Universal PCR Master Mix II (Thermo Fisher Scientific) on a GeneAmpVR 7300 Sequence Detection System (Thermo Fisher Scientific) in accordance with the manufacturer’s protocol.

For detection of IGBP1 mRNA, reverse transcription and qRT-PCR were performed using a High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and Power SYBR Green PCR Master mix (Thermo Fisher Scientific) on a GeneAmpVR 7300 Sequence Detection System (Thermo Fisher Scientific) in accordance with the manufacturer’s protocol. The primers used for real-time RT-PCR of IGBP1 were:

| forward | reverse |
|---------|---------|
| 5'-GCCATCAACTTCTAACTCATCTCG-3' | 5'-CTCATACAGTACTCAAGCTCATCAT-3' |

The primers used for real-time RT-PCR of 18S were:

| forward | reverse |
|---------|---------|
| 5'-ACTCAACAGGGGAAACCTCA-3' | 5'-AACCGACAAATCGGTCACCACAC-3' |

Transfection using miRNA mimic and IGBP1-specific siRNA. miRNA mimic (Thermo Fisher Scientific), IGBP1-specific siRNA (siIGBP1), or scrambled RNA (Stealth RNAi Negative Control Medium GC Duplex; Thermo Fisher Scientific), and a nucleic acid transferring agent (lipofectamine RNAiMAX; Thermo Fisher Scientific), were incubated together in Opti-MEM/ reduced serum medium (Thermo Fisher Scientific) in six-well plates for 20 min at room temperature. Antibiotic-free medium containing PC-9 or A549 cells at a density of 6.0 × 10⁴/mL was added to each well to give a final miRNA concentration of 30 nM. The cells were then incubated at 37°C in a CO2 incubator for 24–72 h. The sequence of siIGBP1 was: 5'-GAUCUCGAGAGAAAGAGACUUC-3'.

Luciferase reporter assay. A firefly luciferase reporter plasmid containing the IGBP1 3’ untranslated region (UTR) (pMirTarget-IGBP1-3’ UTR) was purchased from Origene Technologies (Rockville, MD, USA). To generate pMirTarget-IGBP1-3’ UTR-Mut, which carried the mutation in the target region of miR-3941, the WT clone served as template for amplifying the IGBP1 3’ UTR mutant using primers harboring the mutation, and was cloned into the pSGI and MluI sites in the pMirTarget plasmid. A549 cells seeded in 96-well plates were co-transfected with pMirTaget-IGBP1-3’ UTR or pMirTaget-IGBP1-3’ UTR-Mut and the miRNA mimic or negative control RNA duplex (NC duplex: Thermo Fisher Scientific) using FuGene HD transfection reagent (Promega, Madison, WI, USA) and lipofectamine RNAiMAX (Thermo Fisher Scientific), respectively. The pRL-TK (Promega) was transfected simultaneously as a normalization control. Cells were collected 48 h after transfection, and

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luciferase activity was measured using a dual-luciferase reporter assay kit (Promega) and microplate reader (Varioskan LUX, Thermo Fisher Scientific).

**Western blotting.** Total cell lysates were prepared on ice with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The lysates were centrifuged for 10 min at 4°C, and the insoluble fraction was discarded. The total protein in the soluble lysates was measured using a BCA protein assay kit (Thermo Fisher Scientific) and microplate reader (Varioskan LUX, Thermo Fisher Scientific). The proteins were then transferred to polyvinylidene difluoride membranes using an iBlot gel transfer system (Thermo Fisher Scientific). The blots were blocked and then probed with antibodies against the following proteins: IGBP1 (1:200; Sigma-Aldrich Co), beta-actin (ACTB) (1:5000; Thermo Fisher Scientific), cleaved caspase 3 (1:500; Cell Signaling Technology, Denvers, MA, USA), PARP (1:1000; Cell Signaling Technology), and poly ADP ribose polymerase (PARP) (1:1000; Cell Signaling Technology). After extensive washing, immunoreactivity was detected with specific secondary antibodies conjugated to horseradish peroxidase. Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific) and the ChemiDoc Touch Imaging System (Bio-Rad Laboratories).

The positive control for apoptosis detection was a lysate of PC-9 cells treated with camptothecin (Sigma-Aldrich Co, final concentration 10 μM). The negative control was a lysate of non-treated PC-9 cells.

**Cell proliferation assay.** Cells were seeded in six-well plates, cultured for the indicated time periods, and counted with a hematocytometer. Three replicates were prepared for each group. WST-8 assay was carried out using a cell counting kit-8 (DOJINDO, Kumamoto, Japan) in accordance with the manufacturer’s protocol. The IGBP1 ORF sequence was cloned into the pF4A_CMV Flexi vector (Promega) using a Flexi Vector system (Promega). pF4A_CMV_IGBP1 (pIGBP1) was simultaneously transfected with miR-3941 into cells as a rescue experiment. pGFP was used as a negative control for the plasmid.

**Results**

**Six candidate miRNAs targeting IGBP1.** To search for dysregulated miRNAs associated with IGBP1 expression, we performed a microRNA array assay using fresh materials collected from surgically resected lung adenocarcinoma. First, the expression profiles of 1223 miRNAs in two small invasive lung adenocarcinomas and one MIA were compared using miRCURY LNA (Exiqon). The two small invasive adenocarcinomas were positive for IGBP1 expression whereas MIA was IGBP1-negative (Fig. 1a,b). The results indicated that more than 100 miRNAs showed a difference in expression between the small invasive adenocarcinomas and MIA. The results of the microRNA array are available in Gene Expression Omnibus (GEO accession number: GSE89750). On the other hand, TargetScan Human (ver.6.2) analysis of a miRNA database predicted that 79 human miRNAs might target IGBP1. Among the miRNAs highlighted by the microRNA array, six (miR-34b, miR-138, miR-374a, miR-374b, miR-1909, miR-3941) were also included among those highlighted in TargetScan analysis (Fig. 1c). Therefore we considered these six miRNAs to be candidates for association with IGBP1 regulation during progression of adenocarcinoma (Table 1).

Expression of the six candidate miRNAs was then examined by real-time RT-PCR using samples from seven cases of human

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**Table 1. The summary of six candidate microRNAs**

| miRNA   | Fold change (microRNA array) | P-value (real-time RT-PCR) |
|---------|-----------------------------|-----------------------------|
| miR-34b | 2.021424                    | 0.012457753                 |
| miR-138 | 0.40756                     | 0.000816333                 |
| miR-374a| 2.193465                    | 0.010328695                 |
| miR-374b| 0.343731                    | 0.233730387                 |
| miR-1909| 2.435487                    | 0.032925251                 |
| miR-3941| 0.465127                    | 0.03385292                  |

Fold change representing the abundance of miRNA in type C relative to MIA. The P value was calculated for the difference between normal lung tissue and invasive adenocarcinoma.
lungs of adenocarcinoma in situ and six cases of human invasive lung adenocarcinoma in comparison with the corresponding adjacent normal lung tissues. All of the adenocarcinomas in situ were immunohistochemically negative for IGBP1, and the invasive lung adenocarcinomas were immunohistochemically positive for IGBP1 (Fig. 2a). Among the six miRNAs, expression of miR-34b, miR-138, miR-374a, miR-1909, and miR-3941 was significantly lower in invasive adenocarcinoma than in adjacent normal lung tissue (Fig. 2b, Table 1). miR-374b also tended to be downregulated in tumor tissue, but not to a significant degree. Expression of miR-34b, miR-138, miR-374a, and miR-3941 tended to become lower during progression of adenocarcinoma (normal > AIS > invasive adenocarcinoma).

**Direct targeting of IGBP1 by miR-34b and miR-3941.** Next, we carried out dual-luciferase reporter assay analysis to identify miRNAs that directly targeted IGBP1. A firefly luciferase reporter plasmid containing the IGBP1 3’UTR was co-transfected into A549 cells along with the candidate miRNAs. The IGBP1 3’UTR sequence contained all of the binding sites of the six candidates. As shown in Figure 3(a), only miR-34b and miR-3941 inhibited luciferase activity (by 20% and 40%, respectively). However, none of the other candidates (miR-138, miR-374a, miR-374b, and miR-1909) inhibited the luciferase activity. In contrast, miR-3941 failed to bind to the mutated 3’UTR and showed no inhibitory effect on luciferase activity (Fig. 3b,c).

Interestingly, transfected miR-3941 suppressed IGBP1 expression by <50% relative to the control, whereas miR-34b had no suppressive effect (Fig. 3d). On the other hand, both miRNAs suppressed IGBP1 protein expression in comparison to the negative control (Fig. 3e,f).

**Inhibition of cell proliferation and induction of apoptosis by miR-3941 in lung adenocarcinoma cell lines.** Based on the results shown in Figure 3, we focused on miR-34b and miR-3941 and evaluated their effects on cell proliferation and apoptosis in lung adenocarcinoma cell lines.

Cell proliferation was evaluated by cell counting after transfection with miR-34b, miR-3941, or siGBP1 RNA. Scrambled RNA was used as a negative control. miR-34b and miR-3941 significantly suppressed the proliferation of both PC-9 and A549 cells (Fig. 4a,b), the effects being more marked in the former than in the latter. Moreover, we carried out WST-8 assay after transfection with miR-3941 and the IGBP1 ORF clone (pIGBP1) to validate whether IGBP1 overexpression abrogated the growth suppression induced by miR-3941. As expected, although miR-3941 reduced cell proliferation similarly to Figure 4a(b), IGBP1 overexpression significantly recovered the cell growth (Fig. 4c).

Although single transfection with pIGBP1 did not lead to an increase of cell proliferation, this might have been because the cell line we used for this experiment, PC-9, originally had a high level of endogenous IGBP1 and proliferative capacity.

Because IGBP1 has an anti-apoptotic action, we examined the apoptotic effect of miR-34b and miR-3941 in PC-9 cells. After transfection with miR-3941 or siGBP1, the level of IGBP1 expression was significantly suppressed, and both cleaved caspase-3 and cleaved PARP were significantly induced (Fig. 4d). However, the effect of miR-34b was less than that of miR-3941.

**Discussion**

In this study, we examined miRNAs that might regulate IGBP1 expression in lung adenocarcinoma at an early stage. microRNA array analysis and in silico screening revealed that six miRNAs (miR-34b, miR-138, miR-374a, miR-374b, miR-1909, miR-3941) might be associated with the regulation of IGBP1 expression (Fig. 1), and that the expression levels of the five miRNAs (miR-34b, miR-138, miR-374a, miR-1909, miR-3941) were significantly suppressed in the invasive adenocarcinoma examined (Fig. 2). Moreover, the expression levels of miR-34b, miR-138, miR-374a, and miR-3941 tended to become lower during progression of adenocarcinoma (normal > AIS > invasive adenocarcinoma). Many previous reports have described the association of the miR-34 family (miR-34a, miR-34b, miR-34c) with malignant tumors, and they appear to act as key regulators of downstream p53 expression. Many of their targets are involved in the cell cycle, proliferation, and apoptosis, and include c-MYC, CDK6, c-MET, and bcl-2. miR-138 is associated with several types of malignancy. In non-small lung carcinoma, miR-138 is known to be a tumor suppressor targeting several genes, such as enhancer of zeste homolog 2 (EZH2) and cyclin D3. Some reports have described the association of miR-1909 and miR-374 with malignant tumors, and patients with breast tumors show no inhibitory effect on luciferase activity (Fig. 3b,c).

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Fig. 2. (a) Representative examples of IGBP1 immunohistochemistry in adjacent normal lung (left side), adenocarcinoma in situ (middle), and invasive adenocarcinoma (right side). Normal lung tissue and all of the adenocarcinomas in situ showed no reactivity with the anti-IGBP1 antibody, whereas all of the invasive adenocarcinomas showed positive reactivity. (b) Real-time RT-PCR analysis of the expression of the six selected miRNAs. All of the miRNAs except miR-374b were significantly suppressed in invasive adenocarcinoma, compared with the normal counterpart tissues. Expression of miR-34b, miR-138, miR-374a, and miR-3941 tended to become lower during progression of adenocarcinoma (normal > AIS > invasive adenocarcinoma).
The growth-inhibitory effects of miR-3941 and miR-34b were greater in PC-9 than in A549 (Fig. 4a,b). Binding of PP2Ac to IGBP1 leads to suppression of PP2A phosphatase activity, resulting in acquisition of anti-apoptosis function through bcl-2 activation. Furthermore, PP2A inhibits Akt downstream of EGFR.\(^{(42)}\) PC-9 has EGFR mutation and A549 has KRAS mutation. Thus, the effects of IGBP1 inhibition might have been more pronounced in PC-9 than in A549 because of collateral inhibition of the EGFR cascade.

Recently, miRNA mimics have emerged as excellent therapeutic agents, and many studies of the therapeutic effects of miRNAs have been reported, not only alone but also in combination with conventional chemotherapeutic agents or other miRNAs.\(^{(43,44)}\) In this study, we demonstrated that miR-3941 is a specific miRNA that targets and regulates IGBP1 expression. As indicated before, IGBP1 is widely expressed in lung adenocarcinoma and its expression is significantly associated with prognosis. Therefore,
miR-3941 could be a promising therapeutic agent for lung adenocarcinoma.

In summary, we have found that miR-3941 targets and down-regulates IGBP1 most specifically in lung adenocarcinoma cell line, and induces apoptosis. Reduced expression of miR-3941 and overexpression of IGBP1 are thought to be associated with anti-apoptotic events during the course of lung adenocarcinoma progression. Therefore, suppression of IGBP1 using a miR-3941 mimic might be very specific and effective therapeutic strategy.

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Disclosure statement
The authors have no conflict of interest.

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