

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

Taiki Sato,^{1*} Aya Shiba-Ishii,^{2*} Yunjung Kim,¹ Tomoko Dai,¹ Ryan Edbert Husni,¹ JeongMin Hong,¹ Junko Kano,² Shingo Sakashita,² Tatsuo Iijima³ and Masayuki Noguchi²

¹Department of Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki; ²Department of Pathology, Faculty of Medicine, University of Tsukuba, Tsukuba-shi, Ibaraki; ³Department of Pathology, Ibaraki Prefectural Central Hospital, Kasama-shi, Ibaraki, Japan

Key words

Carcinogenesis, IGBP1, lung adenocarcinoma, miR-34b, miR-3941

Correspondence

Shingo Sakashita, Department of Pathology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan.
Tel: +81-29-853-3350; Fax: 81-29-853-3150;
E-mail: sakashingo@hotmail.com

*These authors contributed equally to the manuscript.

Funding Information

Japan Society for the Promotion of Science (JP 15K19045).

Received September 14, 2016; Revised November 25, 2016; Accepted December 18, 2016

Cancer Sci 108 (2017) 536–542

doi: 10.1111/cas.13148

Immunoglobulin (CD79a) binding protein 1 (IGBP1) is universally overexpressed in lung adenocarcinoma and exerts an anti-apoptotic effect by binding to PP2Ac. However, the molecular mechanism of IGBP1 overexpression is still unclear. In the present study, we used a microRNA (miRNA) array and TargetScan Human software to detect IGBP1-related miRNAs that regulate IGBP1 expression. The miRNA array analysis revealed more than 100 miRNAs that are dysregulated in early invasive adenocarcinoma. On the other hand, in silico analysis using TargetScan Human revealed 79 miRNAs that are associated with IGBP1 protein expression. Among the miRNAs selected by miRNA array analysis, six (miR-34b, miR-138, miR-374a, miR-374b, miR-1909, miR-3941) were also included among those selected by TargetScan analysis. Real-time reverse transcription PCR (real-time RT-PCR) showed that the six microRNAs were downregulated in invasive adenocarcinoma (IGBP1+) relative to adjacent normal lung tissue (IGBP1–). Among these microRNAs, only miR-34b and miR-3941 depressed luciferase activity by targeting 3'UTR-IGBP1 in the luciferase vector. We transfected miR-34b and miR-3941 into lung adenocarcinoma cell lines (A549, PC-9), and both of them suppressed IGBP1 expression and cell proliferation. Moreover, the transfected miR-34b and miR-3941 induced apoptosis of a lung adenocarcinoma cell line, similarly to the effect of siIGBP1 RNA. As well as miR-34b, we found that miR-3941 targeted IGBP1 specifically and was able to exclusively downregulate IGBP1 expression. These findings indicate that suppression of miR-3941 has an important role in the progression of lung adenocarcinoma at an early stage.

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.^(28–33) 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 invasive 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) supplemented with 10% 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 miR-CURY LNA (Exiqon, Vedbaek, Denmark). The threshold for identifying the dysregulated miRNAs was a twofold change (either down or up).

Immunohistochemistry. The 4- μ m-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 Highcapacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and Power SYBR Green PCR Master mix (Thermo Fisher Scientific) on a GeneAmp 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; 5'-GGCATCAACTTCTAACTCATCTCG-3',
reverse; 5'-CTCATACCAGTCACTCACCGTCAT-3'.

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

forward; 5'-ACTCAACACGGGAAACCTCA-3',
reverse; 5'-AACCAGACAAATCGCTCCAC-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 6.0×10^4 /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'-GAUCCUGAGAGAAAGAGACUCUUCA-3'.

Luciferase reporter assay. A firefly luciferase reporter plasmid containing the IGBP1 3' untranslated region (UTR) (pMirTarget-IGBP1-3'UTR, cat no #SC204862) 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 *SgfI* and *MluI* sites in the pMirTarget plasmid.

A549 cells seeded in 96-well plates were co-transfected with pMirTarget-IGBP1-3'UTR or pMirTarget-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

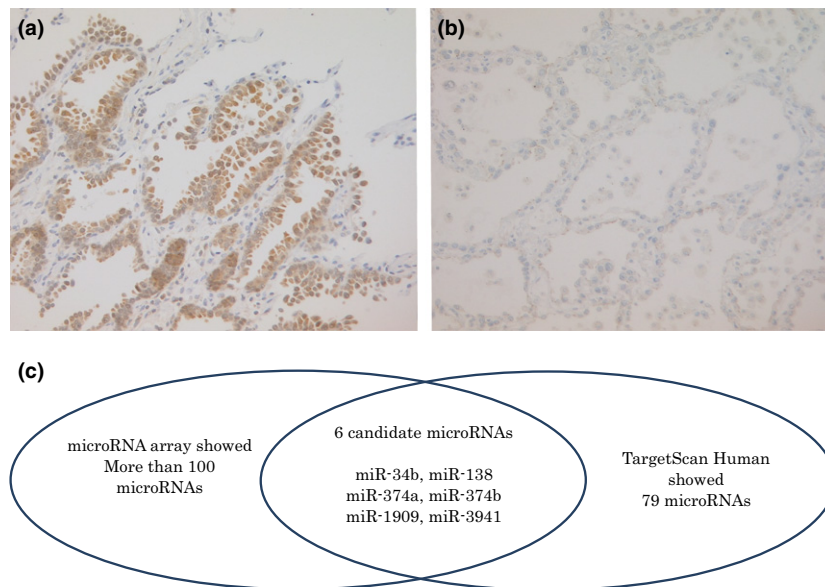


Fig. 1. IGBP1 immunohistochemistry of the tissues used for microRNA array and the number of miRNAs selected by microRNA array analysis and TargetScan Human analysis. (a) Invasive adenocarcinoma, (b) minimally invasive adenocarcinoma. (c) Among the miRNAs highlighted by the microRNA array, six were also included among those highlighted by TargetScan Human analysis.

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). Total protein aliquots (20 µg) were mixed with loading buffer supplemented with DTT, denatured at 95°C for 5 min, and electrophoresed on 10% or 12% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA). 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), caspase 3 (1:500; Cell Signaling Technology, Denvers, MA, USA), cleaved caspase 3 (1:500; 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 hemacytometer. 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

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 microRNA in type C relative to MIA. The P value was calculated for the difference between normal lung tissue and invasive adenocarcinoma.

lung 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 candidate miRNAs. 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 siIGBP1 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 4(a,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 siIGBP1, 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 adenocarcinomas 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.⁽³⁵⁾ 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 (EHZ2) and cyclin D3.^(36,37) Some reports have described the association of miR-1909 and miR-374 with malignant tumors. For example, it has been shown that miR-1909 is included in the specific miRNA signature associated with complete pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer,⁽³⁸⁾ and another report has suggested that several miRNAs including miR-374 are deregulated in small cell lung carcinoma.⁽³⁹⁾ There are also a limited number of reports indicating that miR-3941 is associated with malignant tumors. In acute lymphoblastic leukemia (ALL), Lu *et al.*⁽⁴⁰⁾ have reported that 3' UTR polymorphism of the insulin-like growth factor (IGF) gene influences annealing activity with several miRNAs including miR-3941, and is associated with patient outcome. The present report is a first to have indicated a biological effect of miR-3941 in solid tumors.

Among the six miRNAs we investigated, miR-34b and miR-3941 directly annealed with the IGBP1 3'UTR and the expression of IGBP1 was suppressed by the transfection with both miRNAs. These results indicate that the expression of IGBP1 is directly regulated by these two miRNAs (Figs. 3,4). Until now, miR-34b has been the only miRNA reported to regulate IGBP1 expression.⁽³⁴⁾ As reported previously, miR-34b suppressed the proliferation of a lung adenocarcinoma cell line to a degree equivalent to the effect of miR-3941 (Fig. 4a). However, it did not suppress IGBP1 expression significantly. On the other hand, miR-3941 not only suppressed IGBP1 expression significantly, but also induced apoptosis more effectively than miR-34b (Figs. 3,4). Moreover, IGBP1 overexpression abrogated the cell growth suppression induced by miR-3941 (Fig. 4c). Therefore, although miR-34b might be a strong tumor suppressor, IGBP1 is only one of its many target genes, and its tumor-suppressive role via IGBP1 may only be a secondary, rather than a main effect. On the other hand, miR-3941 is thought to interact with IGBP1 more strongly and more specifically than miR-34b. Hence, miR-3941 is thought to be a specific miRNA that regulates IGBP1 expression.

Real-time RT-PCR was performed to investigate endogenous expression of miR-3941 in PC-9, A549, PL16T (a cell line of atypical adenomatous hyperplasia, a precancerous lesion of lung adenocarcinoma), and PL16B (a normal bronchial epithelium cell line), but expression of miR-3941 in each of these cell lines was below the detection sensitivity (data not shown). IGBP1 is thought not to regulate miR-3941 because siIGBP1 did not increase the expression of miR-3941 (lower than the detection sensitivity). The suppression of miR-3941 in not only PC-9 and A549 but also in PL16T and PL16B may be due to a change in the phenotype of PL16T and PL16B, established in our laboratory, because SV40 large T antigen was induced to immortalize.⁽⁴¹⁾

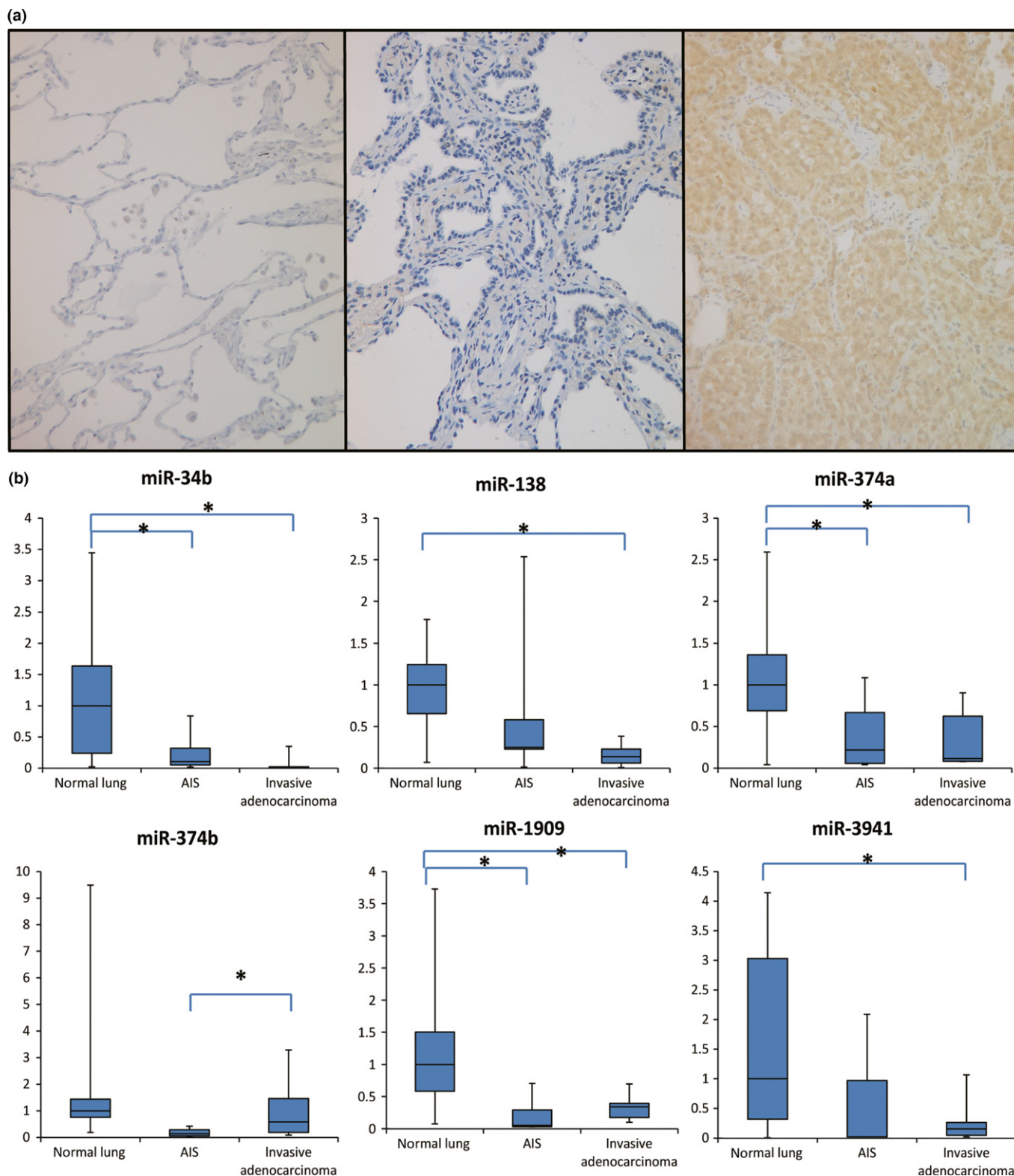


Fig. 2. (a) Representative examples of IGBP1 immunohistochemistry in adjacent normal lung (left side), adenocarcinoma in situ (middle), and invasive adenocarcinoma (right side). Normal lung tissue and all of the adenocarcinomas in situ showed no reactivity with the anti-IGBP1 antibody, whereas all of 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).

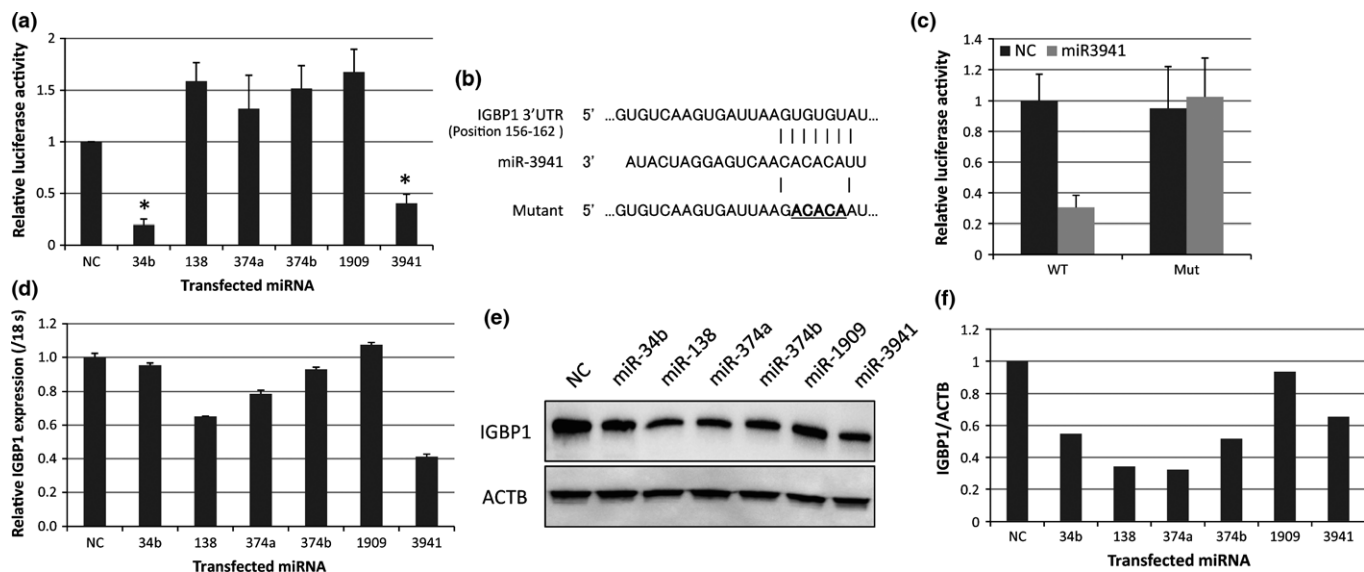


Fig. 3. (a) Luciferase reporter assay. Among the six miRNAs, miR-34b and miR-3941 showed significant inhibition of luciferase activity. (b) The putative target region of miRNA-3941 on the 3'UTR of IGBP1. The mutant generated at the miR-3941 target region in IGBP1 3'UTR is underlined and serves as a control. (c) Luciferase reporter assay using either the wild-type (WT) or mutant (Mut) IGBP1 3'-UTR plasmid. (d) Real-time RT-PCR for IGBP1 using the cells transfected with miRNAs. After transfection of the six miRNAs into A549 cells, miR-3941 suppressed IGBP1 expression to <50% relative to the control, but miR-34b had no suppressive effect. (e) Western blotting using the cells transfected with miRNAs. (f) The density of bands in western blotting (e) were digitalized by Image J software (U.S. National Institutes of Health, Bethesda, Maryland, USA). Each density was normalized by those of ACTB. At the protein level, both miRNAs suppressed IGBP1 protein expression relative to the negative control. NC: negative control RNA duplex.

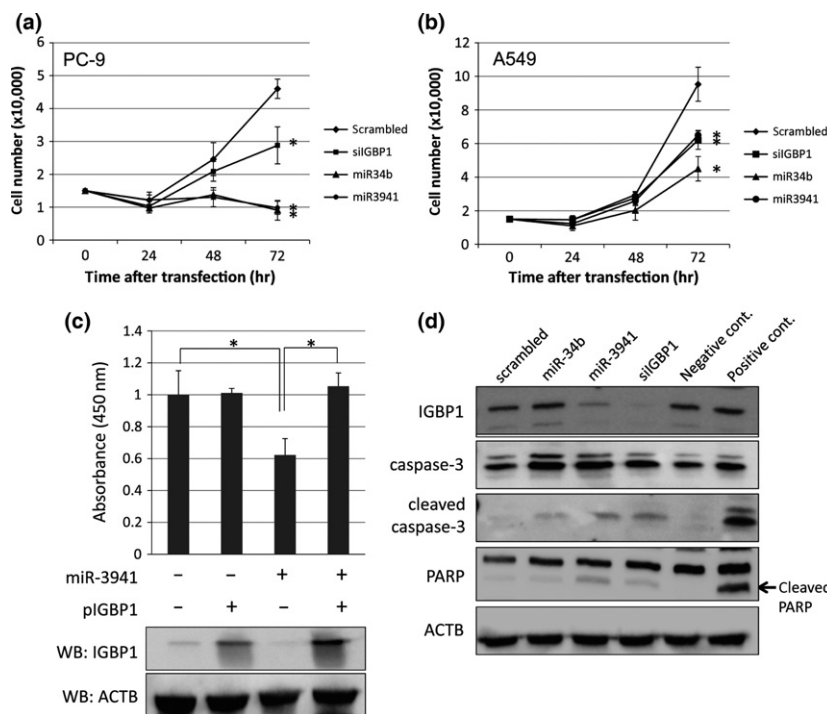


Fig. 4. (a, b) Cell counts were performed after transfection of PC-9 or A549 cells with miR-34b, miR-3941, or siIGBP1 RNA. Both miR-34b and miR-3941 significantly suppressed the proliferation of both cell lines. (c) WST-8 assay after transfection of PC-9 cells with miR-3941 and pIGBP1. IGBP1 overexpression abrogated the cell growth suppression induced by miR-3941. pGFP was used as a negative control for the plasmid. (d) After transfection of miR-34b, miR-3941, or siIGBP1 RNA, miR-3941 significantly suppressed the expression of IGBP1, and induced both cleaved caspase-3 and cleaved PARP. Negative control: PC-9 lysate, Positive control: PC-9 lysate treated with camptothecin (10 μ M).

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.

References

- 1 Youlden DR, Cramb SM, Baade PD. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. *J Thorac Oncol* 2008; **3**: 819–31.
- 2 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87–108.
- 3 Noguchi M, Morikawa A, Kawasaki M *et al*. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995; **75**: 2844–52.
- 4 Minami Y, Matsuno Y, Iijima T *et al*. Prognostication of small-sized primary pulmonary adenocarcinomas with histopathological and karyometric analysis. *Lung Cancer* 2005; **48**: 339–48.
- 5 Travis WD, Brambilla E, Noguchi M *et al*. IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol* 2011; **6**: 244–85.
- 6 Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG. *WHO Classification of Tumors of the Lung, Pleura, Thymus and Heart*, 4th edn. Lyon: International Agency for Research on Cancer, 2015.
- 7 Marchetti A, Buttitta F, Pellegrini S *et al*. Bronchioloalveolar lung carcinomas: K-ras mutations are constant events in the mucinous subtype. *J Pathol* 1996; **179**: 254–9.
- 8 Finberg KE, Sequist LV, Joshi VA *et al*. Mucinous differentiation correlates with absence of EGFR mutation and presence of KRAS mutation in lung adenocarcinomas with bronchioloalveolar features. *J Mol Diagn* 2007; **9**: 320–6.
- 9 Hata A, Katakami N, Fujita S *et al*. Frequency of EGFR and KRAS mutation in Japanese patients with lung adenocarcinoma with features of the mucinous subtype of bronchioloalveolar carcinoma. *J Thorac Oncol* 2010; **5**: 1197–200.
- 10 Soda M, Choi YL, Enomoto M *et al*. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007; **448**: 561–6.
- 11 Murata Y, Minami Y, Iwakawa R *et al*. ECT2 amplification and overexpression as a new prognostic biomarker for early-stage lung adenocarcinoma. *Cancer Sci* 2014; **105**: 490–7.
- 12 Shiba-Ishii A, Noguchi M. Aberrant stratifin overexpression is regulated by tumor-associated CpG demethylation in lung adenocarcinoma. *Am J Pathol* 2012; **180**: 1653–62.
- 13 Ishiyama T, Kano J, Anami Y *et al*. OCIA domain containing 2 is highly expressed in adenocarcinoma mixed subtype with bronchioloalveolar carcinoma component and is associated with better prognosis. *Cancer Sci* 2007; **98**: 50–7.
- 14 Sakashita S, Li D, Nashima N *et al*. Overexpression of immunoglobulin (CD79a) binding protein1 (IGBP-1) in small lung adenocarcinomas and its clinicopathological significance. *Pathol Int* 2011; **61**: 130–7.
- 15 Virshup DM. Protein phosphatase 2A: a panoply of enzymes. *Curr Opin Cell Biol* 2000; **12**: 180–5.
- 16 Murata K, Wu J, Brautigan DL. B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. *Proc Natl Acad Sci USA* 1997; **94**: 10624–9.
- 17 Deng X, Ito T, Carr B, Mumby M, May WS Jr. Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. *J Biol Chem* 1998; **273**: 34157–63.
- 18 Lechward K, Awotunde OS, Swiatek W, Muszynska G. Protein phosphatase 2A. Variety of forms and diversity of functions. *Acta Biochim Pol* 2001; **48**: 921–33.
- 19 Sontag E. Protein phosphatase 2A: the Trojan Horse of cellular signaling. *Cell Signal* 2001; **13**: 7–16.
- 20 Wera S, Hemmings BA. Serine/threonine protein phosphatases. *Biochem J* 1995; **311**: 17–29.
- 21 Ruediger R, Roeckel D, Fait J, Bergqvist A, Magnusson G, Walter G. Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. *Mol Cell Biol* 1992; **12**: 4872–82.
- 22 Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001; **353**: 417–39.

Acknowledgement

The authors are grateful to Tsukuba Human Tissue Biobank for its courtesy in supplying the frozen tissues used in this study.

Disclosure statement

The authors have no conflict of interest.

- 23 Turowski P, Favre B, Campbell KS, Lamb NJ, Hemmings BA. Modulation of the enzymatic properties of protein phosphatase 2A catalytic subunit by the recombinant 65-kDa regulatory subunit PR65alpha. *Eur J Biochem* 1997; **248**: 200–8.
- 24 Agostinis P, Derua R, Sarno S, Goris J, Merlevede W. Specificity of the polycation-stimulated (type-2A) and ATP, Mg dependent (type-1) protein phosphatases toward substrates phosphorylated by P34cdc2 kinase. *Eur J Biochem* 1992; **205**: 241–8.
- 25 Inui S, Sanjo H, Maeda K, Yamamoto H, Miyamoto E, Sakaguchi N. Ig receptor binding protein 1 (alpha4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A. *Blood* 1998; **92**: 539–46.
- 26 Kong M, Fox CJ, Mu J *et al*. The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. *Science* 2004; **306**: 695–8.
- 27 Li D, Sakashita S, Morishita Y *et al*. Binding of lactoferrin to IGBP1 triggers apoptosis in a lung adenocarcinoma cell line. *Anticancer Res* 2011; **31**: 529–34.
- 28 Due H, Svendsen P, Bødker JS *et al*. miR-155 as a biomarker in B-cell malignancies. *Biomed Res Int* 2016; **2016**: 9513037.
- 29 Hollis M, Nair K, Vyas A, Chaturvedi LS, Gambhir S, Vyas D. MicroRNAs potential utility in colon cancer: early detection, prognosis, and chemosensitivity. *World J Gastroenterol* 2015; **21**: 8284–92.
- 30 Kheir TB, Futoma-Kazmierczak E, Jacobsen A *et al*. miR-449 inhibits cell proliferation and is down-regulated in gastric cancer. *Mol Cancer* 2011; **10**: 29.
- 31 Osada H, Takahashi T. let-7 and miR-17-92: small-sized major players in lung cancer development. *Cancer Sci* 2011; **102**: 9–17.
- 32 Wang W, Luo Y. MicroRNAs in breast cancer: oncogene and tumor suppressors with clinical potential. *J Zhejiang Univ Sci B* 2015; **16**: 18–31.
- 33 Lei B, Liu Z, Li Z, Li C, Deng Y. miR-21 induces cell proliferation and suppresses the chemosensitivity in glioblastoma cells via downregulation of FOXO1. *Int J Clin Exp Med* 2014; **7**: 2060–6.
- 34 Chen LP, Lai YD, Li DC *et al*. $\alpha 4$ is highly expressed in carcinogen-transformed human cells and primary human cancers. *Oncogene* 2011; **30**: 2943–53.
- 35 Misso G, Di Martino MT, De Rosa G *et al*. Mir-34: a new weapon against cancer? *Mol Ther Nucleic Acids* 2014; **3**: e194.
- 36 Zhang H, Zhang H, Zhao M *et al*. Mir-138 inhibits tumor growth through repression of EZH2 in non-small cell lung cancer. *Cell Physiol Biochem* 2013; **31**: 56–65.
- 37 Han LP, Fu T, Lin Y, Miao JL, Jiang QF. MicroRNA-138 negatively regulates non-small cell lung cancer cells through the interaction with cyclin D3. *Tumour Biol* 2016; **37**: 291–8.
- 38 Della Vittoria Scarpati G, Falcetta F, Carlomagno C *et al*. A specific miRNA signature correlates with complete pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 2012; **83**: 1113–9.
- 39 Miko E, Czimmerer Z, Csányi E *et al*. Differentially expressed microRNAs in small cell lung cancer. *Exp Lung Res* 2009; **35**: 646–64.
- 40 Lu J, Wang F, He L *et al*. Interaction between IGF1 polymorphisms and the risk of acute lymphoblastic leukemia in Chinese children. *Cell Physiol Biochem* 2015; **36**: 1346–58.
- 41 Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *PNAS* 2000; **97**: 10832–7.
- 42 Shimada A, Kano J, Ishiyama T *et al*. Establishment of an immortalized cell line from a precancerous lesion of lung adenocarcinoma, and genes highly expressed in the early stages of lung adenocarcinoma development. *Cancer Sci* 2005; **96**: 668–75.
- 43 Shi S, Han L, Deng L *et al*. Dual drugs (microRNA-34a and paclitaxel)-loaded functional solid lipid nanoparticles for synergistic cancer cell suppression. *J Control Release* 2014; **194**: 228–37.
- 44 Kashinski AL, Kelnar K, Stahllut C *et al*. A combinatorial microRNA therapeutics approach to suppressing non-small cell lung cancer. *Oncogene* 2015; **34**: 3547–55.