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Expression of HER-2 affects patient survival and paclitaxel sensitivity in endometrial cancer

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BACKGROUND: Disabled phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase signalling is involved in endometrial carcinogenesis, and there is evidence that expression of epidermal growth factor receptor (EGFR) family members has a role in such intracellular signalling pathways. This study analysed the prognostic impact of EGFR family expression in endometrial cancer in relation to PI3K–AKT and MAPK–ERK signalling, as well as drug sensitivity.

METHODS AND RESULTS: Immunohistochemical analysis using 63 surgical specimens of endometrioid-type endometrial cancers revealed that EGFR, human epidermal growth factor receptor (HER)-2 and HER-4 were expressed in 25 (39.7%) of 63, 26 (41.3%) of 63 and 31 (49.2%) of 63 tumours, respectively. Gene amplification of *HER-2* was observed in 2 of 26 patients with high HER-2 expression. Kaplan–Meier analysis revealed that high HER-2 expression was a factor that negatively influenced the progression-free and overall survival rate ($P<0.05$), and multivariate analysis showed high HER-2 expression to be an independent prognostic factor. Subsequently, we performed *in vitro* knockdown analysis to investigate the linkage between HER-2 expression and PI3K–AKT pathways. Short interfering RNA (siRNA)-based knockdown of *HER-2* in endometrial cancer cells led to a significant reduction in phosphorylated AKT (p-AKT) expression, indicating the existence of a HER-2/PI3K-AKT axis. As the PI3K–AKT pathway is known to have crucial roles in anticancer drug sensitivity, we examined the involvement of HER-2 in sensitivity to paclitaxel. Short interfering RNA-based knockdown of *HER-2* conferred increased sensitivity to paclitaxel in endometrial cancer cells, attenuating the induction of p-AKT on paclitaxel stimulation, which was cancelled by inactivating AKT by the introduction of a dominant-negative form.

CONCLUSION: HER-2 is a significant prognostic factor of endometrioid-type endometrial cancer, as well as a key molecule that affects paclitaxel sensitivity by HER-2 interaction with the PI3K–AKT pathway.

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Keywords: HER-2; endometrial cancer; paclitaxel; survival

Endometrial cancer is the second most common gynaecological malignancy in Japan, and its incidence in Japan has increased dramatically over the last decade. Although premalignant lesions of endometrial cancer have been well characterised, the molecular pathways of endometrial carcinogenesis remain unclear. Previous studies have identified various genetic mutations in endometrial cancer, including *PTEN*, *PIK3CA* and *KRAS*. The *PTEN* mutation is the most common genetic abnormality detected in endometrioid-type endometrial cancer (Tashiro *et al*, 1997; Mutter *et al*, 2000; Kanaya *et al*, 2005). The fundamental *in vivo* role of PTEN is to inhibit the phosphatidylinositol 3-kinase (PI3K)–AKT pathway. Mutation of *PTEN* can disable this inhibitory function, inducing the antiapoptotic pathway. Recently, mutation of a novel oncogene, *PIK3CA*, was discovered in multiple human epithelial cancers, including endometrial carcinoma (Oda *et al*, 2005; Hayes *et al*, 2006; Velasco *et al*, 2006); this oncogene encodes the catalytic p110α subunit of PI3K in various cancers (Samuels *et al*, 2004). The mutant proteins have been shown to display enhanced lipid-kinase activity. These findings show the critical roles that the PI3K–AKT pathway has in endometrial carcinogenesis. Another prevalent genetic abnormality in endometrial carcinogenesis is mutation of the *KRAS* gene (Mizuuchi *et al*, 1992; Enomoto *et al*, 1993). The extracellular signal-regulated kinase–mitogen-activated protein kinase (ERK–MAPK) pathway is activated by mitogenic stimuli mediated by receptor-type tyrosine kinases and G-protein-coupled receptors, leading to sequential phosphorylation of RAS, RAF, MEK and ERK1/2. Phosphorylated ERK translocates to the nucleus and regulates a range of substrates that promote cell proliferation, motility, differentiation and survival (Marshall, 1999; Chang and Karin, 2001; Shaw and Cantley, 2006). A *KRAS* mutation can lead to continuous stimulation of its downstream targets, resulting in ERK1/2 activation in the absence of mitogenic stimuli. Thus, most of these prevalent genetic alterations observed in endometrial cancer stimulate the PI3K–AKT and MAPK–ERK pathways, which may have major roles in endometrial carcinogenesis. However, the upstream pathways for their activation remain unclear.

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The EGFR family consists of four members, EGFR (human epidermal growth factor receptor (HER)-1), HER-2, HER-3 and HER-4, which share structural homology consisting of an extracellular domain and a cytoplasmic signal-transduction domain with tyrosine kinase activity (Olayioye *et al*, 2000). Many kinds of tumour cells express multiple EGFR members, which interact to form an array of homodimers and heterodimers. Human epidermal growth factor receptor-2 is predominantly activated by forming a heterodimer with other EGFR members, rather than forming a homodimer (Goldman *et al*, 1990; Wada *et al*, 1990; Gamett *et al*, 1995). Heterodimerisation of HER-2 can result in the activation of intracellular signalling cascades, such as the PI3K–AKT and MAPK–ERK pathways (Dougall *et al*, 1994; Olayioye *et al*, 2000; Olayioye, 2001; Asanuma *et al*, 2005). However, the roles of the EGFR family in endometrial cancer have not been well investigated, particularly in relation to the PI3K–AKT and MAPK–ERK pathways.

In this study, we examined the status of expression of EGFR family members and their involvement in the PI3K–AKT and MAPK–ERK pathways in endometrial cancer. Furthermore, the role of the EGFR family in patient survival and paclitaxel sensitivity was investigated.

MATERIALS AND METHODS

Patients and tissue samples

A total of 63 patients with endometrioid-type endometrial cancer (mean age, 57.5 years; range, 32–78 years) treated at the Department of Obstetrics and Gynecology, Kanazawa University Hospital, from January 1995 to December 2002, were enrolled in the study. All patients underwent a total abdominal or radical hysterectomy plus bilateral salpingo-oophorectomy. Systemic retroperitoneal lymphadenectomy was performed in approximately 70% of patients. Staging was performed for all patients using the International Federation of Gynaecology and Obstetrics (FIGO) surgical staging system: 46 tumours were classified as stage I (substages: Ia, 10 tumours; Ib, 28 tumours; and Ic, eight tumours); six tumours were classified as stage II (substages: IIa, three tumours and IIb, three tumours); nine tumours were classified as stage III (substages: IIIa, three tumours and IIIc, six tumours); and two tumours were classified as stage IV. On the basis of histological examination, 34 tumours were classified as grade (G)1, 14 tumours were classified as G2 and 15 tumours were classified as G3. Patients with deep myometrial invasion, cervical involvement and special histology underwent external radiotherapy, whereas those with positive peritoneal cytology or retroperitoneal lymph-node metastasis were treated with 4–6 cycles of CAP chemotherapy ($90\,mg\,m^{-2}$ cisplatin, $50\,mg\,m^{-2}$ doxorubicin and $500\,mg\,m^{-2}$ cyclophosphamide) or TC chemotherapy ($180\,mg\,m^{-2}$ paclitaxel, and carboplatin, according to Chatelut's formula (area under the curve $= 5\,mg\,ml^{-1}\,min^{-1}$)) as postoperative adjuvant therapy. Patient treatment was followed up with a gynaecological examination, recording of laboratory data, transvaginal and abdominopelvic ultrasonography and a radiological examination. Data from regular follow-up visits to the outpatient department were stored in a database designed specifically for endometrial carcinoma patients. A telephone survey to update the status of all surviving patients was carried out in August 2006. The exact date of disease recurrence was defined as the date when apparent tumours were detected by ultrasonographic or radiological examinations. Tumour samples were collected at the time of surgery, with written informed consent from patients and approval of the Ethics Committee of Kanazawa University. Half of each tissue sample was examined histologically by pathologists, and the remaining portion of each sample was frozen at −80°C until DNA extraction for mutation analysis.

Mutation analysis

Of the 63 endometrial cancers, 45 were available for DNA sequencing and had been evaluated previously for mutations in *PTEN*, *PIK3CA* and *KRAS* (Kanaya *et al*, 2005; Mizumoto *et al*, 2007; Mori *et al*, 2007). All exons of the *PTEN* gene, exons 9 and 20 of the *PIK3CA* gene and exon 1 of the *KRAS* gene (including codons 12 and 13) were amplified by polymerase chain reaction (PCR) using primer sets described previously (Kanaya *et al*, 2005; Mizumoto *et al*, 2007; Mori *et al*, 2007). Polymerase chain reaction products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA), and direct sequencing was performed. Polymerase chain reaction products with suspected mutations were reamplified and subsequently cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany), and sequencing was performed using at least three clones.

Immunohistochemistry

Immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded specimens from the 63 endometrioid-type endometrial cancer samples with a rabbit polyclonal antibody to HER-2 (code number A0485) (DakoCytomation, Carpinteria, CA, USA), a mouse monoclonal antibody to EGFR (clone EGFR.113) (Novocastra, Newcastle upon Tyne, UK) and a rabbit monoclonal antibody to HER-4 (No. 4792; Cell Signaling Technology, Beverly, MA, USA). Of these, 39 cancers had been evaluated previously using mouse monoclonal antibody to MLH1 (clone G168-15, code 13271A) (PharMingen, San Diego, CA, USA) (Kanaya *et al*, 2005). After the specimens were deparaffinised in xylene and a graded alcohol series, epitope retrieval was performed. The sections were heated in a microwave oven at 700 W for 10 min in 1 × antigen retrieval solution (Biogenex, San Ramon, CA, USA). Endogenous peroxidase was then blocked by immersing the sections in 0.3% H_2O_2–methanol for 30 min. After blocking with horse serum, the primary antibodies were diluted at 1:20 (for EGFR) or at 1:400 (for HER-2 and HER-4) and applied for 16 h at 4°C. The reaction was visualised using the EnVision Detection Kit (Dako Cytomation) using diaminobenzidine tetrahydrochloride as the enzyme substrate. All sections were counterstained with GM haematoxylin stain solution (Muto Pure Chemicals, Tokyo, Japan). For negative controls, isotype control immunoglobulin G (IgG) was used. The staining results for HER-2 expression were scored in accordance with the Hercep Test guidelines (Dako Cytomation): score 0, negative, no staining or staining, but without a membranous pattern; score 1, negative, incomplete membranous staining or complete membranous staining in less than 10% of tumour cells; score 2, positive, complete membranous staining in >10% of tumour cells of moderate intensity; and score 3, positive, complete membranous staining in >10% of tumour cells of strong intensity. The staining results for EGFR and HER-4 expression, in which positive staining is defined as any staining of tumour cell membranes above background level, whether it is complete or incomplete circumferential staining, have been classified into three levels (+, ++, and +++) depending on the staining intensity. Staining for MLH1 was previously classified into three levels: − (positive in <25% of tumour cells), + (positive in 25–75% of tumour cells) or as ++ (positive in >75% of tumour cells) (Kanaya *et al*, 2005). For analysis of the relationship with clinicopathological characteristics, the expression of HER-2 was classified into two levels, namely, low (score 0 or 1) and high (score 2 or 3), whereas that of EGFR/HER-4 or MLH1 was also classified into two levels, namely, low (− or +) and high (++ or +++) or low (− or +) and high (++), respectively. Stained sections were evaluated by two observers with no previous knowledge of the clinicopathological parameters. The average staining score was

registered, and there was no statistically significant difference in scoring between observers.

Chromogenic *in situ* hybridisation

Chromogenic *in situ* hybridisation (CISH) for *HER-2* amplification was carried out in patients with high HER-2 expression using a ZytoDot SPEC HER-2 Probe kit (ZytoVision, Bremerhaven, Germany) in accordance with the manufacturer's guidelines, and performed manually. The sections were evaluated with the Olympus BX51 microscope (Olympus Optical Company Ltd., Tokyo, Japan) using a $\times 40$ dry objective. A nonamplified gene copy number was defined as 1–5 signals per nucleus. Amplification was defined as six or more signals per nucleus in more than 50% of cancer cells, or when a large gene copy cluster was seen.

Cell lines and cultures

Human endometrial cancer cells (HEC1A and Ishikawa) were obtained from ATCC (Manassas, VA, USA). Human endometrial epithelial immortalised cells (EM-E6/E7/TERT) were previously established by us (Kyo *et al*, 2003). EM-E6/E7/TERT cells with inactive AKT were established as follows: First, the mutant *AKT* cDNA expressing the dominant-negative form of AKT was excised from vector pUSEamp-Akt1(K179) (Millipore, Billerrica, MA, USA) and subcloned into the retroviral vector pCMSCVpuro, which was then stably transfected into EM-E6/E7/TERT cells, generating EM-E6/E7/TERT/DN-AKT cells. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in an atmosphere of 5% CO_2 at 37°C.

Knockdown study of *human epidermal growth factor receptor-2*

Cells were seeded and transfected with 30 nM of negative control short interfering RNA (siRNA) or human HER-2 siRNA oligonucleotides (Applied Biosystems, Foster City, CA, USA) using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol.

Chemosensitivity assay

A total of 4×10^3 cells (Ishikawa and EM-E6/E7/TERT) or 8×10^3 cells (HEC1A) were seeded in 96-well plates, incubated with siRNA to target *HER-2* for 48 h and then treated with 10 nM paclitaxel (Bristol Pharmaceuticals, Bristol, UK) for different time periods. After each incubation, 1 μg of WST-1 reagent (Roche, Mannheim, Germany) was added to each well, and the cells were further incubated for 2 h at 37°C. Absorbance at wavelengths between 420 and 480 nm was then measured with a microplate reader and WST-1 activity was determined to evaluate chemosensitivity. The experiments were conducted in triplicate.

Western blot analysis

Whole-cell extracts were prepared as described previously (Mizumoto *et al*, 2007; Mori *et al*, 2007) and 50 μg of extracts was then electrophoresed through a sodium dodecylsulphate–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with TBS-T (150 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 0.1% Tween 20) containing 5% nonfat dried milk, and were then incubated with a specific primary antibody against HER-2 (No. 2242; Cell Signaling Technology), or with phosphorylated AKT (p-AKT) (No. 4058; Cell Signaling Technology), AKT (No. 9272; Cell Signaling Technology) or FOXO3a (No. 9467; Cell Signaling Technology), followed by incubation with horseradish peroxidase-linked anti-rabbit IgG. Immunoreactive bands were visualised using the ECL detection system (GE Amersham Bioscience, Freiburg, Germany), according to the manufacturer's instructions. As an internal control for equal protein loading, β-actin expression was examined simultaneously using a specific antibody (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Relative amounts of each protein were quantified using NIH Image software.

Statistical analysis

Statistical analysis was performed using the statistical package StatView version 5.0 (Abacus Concepts, Berkeley, CA, USA). In the *in vivo* study, we used the χ^2-test for 2×2 tables to compare the categorical data. Survival curves were computed using the Kaplan–Meier method, whereas the log-rank test was used to assess statistical significance. Cox's proportional hazards regression model in a stepwise manner was used to analyse the independent prognostic factors. For *in vitro* results, all values represent mean ± s.d. Statistical significance between two groups was determined using a two-tailed *t*-test. A *P*-value <0.05 was considered to indicate statistical significance.

RESULTS

Expression of epidermal growth factor receptor family members and correlation with clinicopathological and genetic characteristics in endometrial cancer

We first investigated the expression of EGFR family members EGFR, HER-2 and HER-4 by immunohistochemistry using 63 surgical specimens of endometrioid-type endometrial cancers, on the basis of the known finding that HER-3 expression is not upregulated in endometrial cancer (Ejskjaer *et al*, 2007). The expression of HER-2 was classified into two levels, namely, low (score 0 or 1) and high (score 2 or 3), whereas that of EGFR/HER-4 was also classified into two levels, namely, low (− or +) and high (++ or +++). High expressions of EGFR, HER-2 and HER-4 were observed in 25 (39.7%) of 63, 26 (41.3%) of 63 and 31 (49.2%) of 63 tumours, in which 48.0% (12 out of 25), 61.5% (16 out of 26) and 54.8% (17 out of 31), respectively, exhibited heterogeneous expression within tumours. There was no preferential expression of these members in any of the specific tumour regions, such as in the invasive front or at the centre of tumours or in areas of squamous differentiation. Representative staining patterns of HER-2 are shown in Figure 1. *Human epidermal growth factor receptor-2* gene amplification was further analysed in 26 patients with a high HER-2 expression by CISH, and two patients (FIGO1b, G1 and FIGO3c, G2, respectively) with score 3 HER-2 expression were found to have *HER-2* gene amplification. We next examined the relationship between these expression patterns and the clinicopathological characteristics of the specimens (Table 1). However, no statistically significant correlation was observed between them.

We then examined the correlation of these expression patterns and the mutation status of *PTEN*, *PIK3CA* and *KRAS*, on the basis of our previous data of the frequency of such mutations in an overlapped population of endometrial cancers (Table 2). Among the 45 patients from whom DNA samples were available, 18 (40%) had a *PTEN* mutation, 7 (15.6%) had a *PIK3CA* mutation and 10 (22.2%) had a *KRAS* mutation. No correlation was observed between the positivity of these factors and the mutation of each gene. However, a high HER-2 expression was likely to have wild-type *PTEN* ($P=0.079$). Notably, strong HER-2 expression with a score of 3 was significantly associated with wild-type *PTEN* ($P=0.032$) (Table 3).

We further examined the correlation of expression of EGFR family members with MLH1, a representative mismatch repair protein, loss of expression of which correlates with mutation of

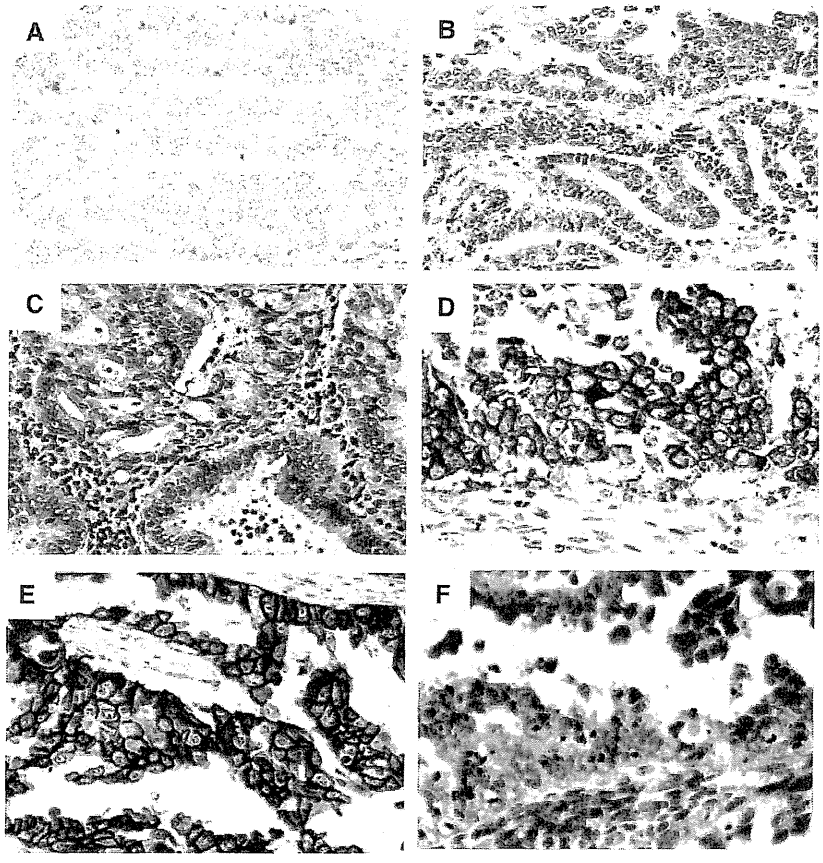

Figure 1 Immunohistochemistry of HER-2 in endometrioid-type endometrial cancer. Representative results of each score are shown (**A**: score 0; **B**: score 1; **C**: score 2 (× 200) and **D**: score 3 (× 400)). The definition of each score is described in Materials and Methods. A breast cancer specimen (**E**) with HER-2 expression was used as the positive control for appropriate staining conditions (× 400). (**F**) CISH analysis of the case (**D**) showing high levels of *HER-2* amplification (× 400). Brown dots show the large gene copy clusters.

PTEN or other genes involved in endometrial carcinogenesis. No significant association was observed between them, but there was a tendency for high HER-2 expression to be associated with high MLH1 expression, although the difference did not reach statistical significance ($P = 0.0746$) (Table 4).

Survival impact of epidermal growth factor receptor family expression in endometrial cancer

Next, the survival impact of EGFR family member expression was examined by Kaplan–Meier analysis (Figure 2). The median follow-up for all patients was 5.16 years (range, 0.58–11.08 years). Among the 63 patients, 11 patients (17.5%) had relapses of endometrial cancer at the time of last follow-up and 12 patients (19.0%) died. When HER-2 staining scores were used as cutoff points to stratify patients into two groups (see Materials and Methods), the progression-free survival (PFS) for low (score 0 or 1) and high (score 2 or 3) HER-2 expression was 91.9 and 69.2%, respectively ($P = 0.016$). The overall survival (OS) for low and high HER-2 expression was 89.2 and 69.2%, respectively ($P = 0.044$). Thus, high HER-2 expression was a factor that negatively influenced PFS and OS rates by univariate analysis. Epidermal growth factor receptor and HER-4 expression levels were not factors that affected PFS or OS by univariate analysis (Supplementary Figure 1). When other known variables for prognosis of endometrial cancer, including FIGO stage, pathological grade and myometrial invasion, were included in a Cox proportional hazard analysis for relapse-free survival, HER-2 expression (hazard ratio 5.31, $P = 0.0180$) and FIGO stage were identified as independent predictive factors of patient survival (Table 5).

The phosphatidylinositol 3-kinase–AKT pathway may be a downstream target of human epidermal growth factor receptor-2 in endometrial cancer

The above findings indicate that HER-2 expression is a critical prognostic factor in endometrial cancer. We next sought to identify the downstream target of HER-2 expression using various endometrial cancer cell lines or immortalised endometrial epithelial cells (EM-E6/E7/TERT). One possible candidate for a downstream target of HER-2 was the PI3K–AKT pathway, based on previous analyses in other tumour types (Knuefermann *et al*, 2003; Qi *et al*, 2009); hence, we focused on this pathway. To examine the linkage between HER-2 and the PI3K–AKT pathway, a knockdown of *HER-2* was performed in the endometrial cancer cell line Ishikawa, using siRNA techniques, and the expression of p-AKT was evaluated by western blot analysis. As shown in Figure 3, knockdown of *HER-2* was confirmed to be efficient, with a more than 50% reduction in expression. Notably, p-AKT expression concomitantly decreased with *HER-2* knockdown. This finding was not limited to Ishikawa cells, as both endometrial cancer HEC1A cells and EM-E6/E7/TERT cells exhibited a similar decrease in p-AKT expression on *HER-2* knockdown (Figure 3).

Table 1 Expressions of EGFR family and clinicopathological characteristics of the patients with endometrial cancer

	EGFR			HER-2			HER-4		
Variables	**High**	**Low**	***P*-value**	**High**	**Low**	***P*-value**	**High**	**Low**	***P*-value**
Age (years)									
<45 ($n=7$)	2	5	0.5239	1	6	0.1240	3	4	0.7215
⩾45 ($n=56$)	23	33		25	31		28	28	
FIGO stage									
I ($n=46$)	18	28	0.8829	18	28	0.5705	24	22	0.4383
II–IV ($n=17$)	7	10		8	9		7	10	
Lymph-node metastasis									
Negative ($n=58$)	24	34	0.3485	25	33	0.3140	28	30	0.6149
Positive ($n=5$)	1	4		1	4		3	2	
Depth (myometrial invasion)									
a, b ($n=47$)	16	31	0.1168	17	30	0.1588	22	25	0.5141
c ($n=16$)	9	7		9	7		9	7	
Histopathological degree of differentiation									
Grades 1 and 2 ($n=48$)	17	31	0.2157	20	28	0.9089	22	26	0.3381
Grade 3 ($n=15$)	8	7		6	9		9	6	
Menopause									
Peri, pre ($n=23$)	6	17	0.0944	6	17	0.0634	10	13	0.4904
Post ($n=40$)	19	21		20	20		21	19	
Body mass index									
<25 ($n=38$)	17	21	0.3120	18	20	0.2254	22	16	0.0890
⩾25 ($n=25$)	8	17		8	17		9	16	

Abbreviations: EGFR = epidermal growth factor receptor; FIGO = International Federation of Gynaecology and Obstetrics.

Table 2 Expressions of EGFR family and genetic mutations of the patients with endometrial cancer

	***PTEN* ($n=45$)**		***PIK3CA* ($n=45$)**		***KRAS* ($n=45$)**	
	MT ($n=18$)	**WT ($n=27$)**	**MT ($n=7$)**	**WT ($n=38$)**	**MT ($n=10$)**	**WT ($n=35$)**
EGFR expression						
Low ($n=26$)	10	16	6	20	7	19
High ($n=19$)	8	11	1	18	3	16
P-value	0.8053		0.1034		0.3749	
HER-2 expression						
Low ($n=28$)	14	14	5	23	7	21
High ($n=17$)	4	13	2	15	3	14
P-value	0.0789		0.5846		0.5651	
HER-4 expression						
Low ($n=28$)	11	17	6	22	7	21
High ($n=17$)	7	10	1	16	3	14
P-value	0.9001		0.1630		0.5651	

Abbreviations: EGFR = epidermal growth factor receptor; HER-2 = human epidermal growth factor receptor-2; MT = mutation; WT = wild type.

Table 3 Expression of *HER-2* and *PTEN* mutation of the patients with endometrial cancer

	***PTEN* ($n=45$)**	
HER-2 expression	**Mutation**	**Wild type**
Score		
0	8	11
1	6	3
2	4	7
3	0	6
P-value	0.0799	

Abbreviation: HER-2 = human epidermal growth factor receptor-2.

Table 4 Expressions of EGFR family and MLH1 of the patients with endometrial cancer

	EGFR ($n=39$)		**HER-2 ($n=39$)**		**HER-4 ($n=39$)**	
MLH1 expression	**Low**	**High**	**Low**	**High**	**Low**	**High**
Low ($n=30$)	16	14	20	10	18	12
High ($n=9$)	7	2	10	6	7	2
Total	23	16	23	16	25	14
P-value	0.1910		0.0746		0.3295	

Abbreviations: EGFR = epidermal growth factor receptor; HER-2 = human epidermal growth factor receptor-2.

Thus, the HER-2 pathway clearly links to the PI3K–AKT pathway in endometrial cancer cells.

We examined the status of mutation in *PTEN*, *PIK3CA* and *KRAS* genes in these cell lines by DNA sequencing, and found a frameshift mutation in exon 8 of the *PTEN* gene (319ACTT del, 289A del) in Ishikawa cells and missense mutations in exon 20 of the *PIK3CA* gene (G3145C), as well as in exon 1 of the *KRAS* gene (G35A) in HEC1A cells as previously reported (Kim *et al*, 2004; Oda *et al*, 2005). We found no mutation of these genes in EM-E6/E7/TERT cells. These findings indicate that the linkage of HER-2 with the PI3K–AKT pathway is maintained irrespective of the mutation status of such genes.

Human epidermal growth factor receptor-2 expression is involved in paclitaxel sensitivity in endometrial cancer cells by regulation of the phosphatidylinositol 3-kinase - AKT pathway

There is accumulating evidence that the PI3K-AKT pathway is critically involved in drug resistance to chemotherapies of various types of cancers by activation of survival signals. Linkage of HER-2 to p-AKT expression prompted us to examine the involvement of HER-2 expression in the chemosensitivity of endometrial cancer cells. We therefore sought to test the sensitivity to paclitaxel, a key drug for endometrial cancer cells. First, preparatory experiments were conducted to test the efficacy of paclitaxel against Ishikawa, HEC1A and immortalised endometrial epithelial cells, in which cells were treated with 1-1000 nM of paclitaxel and cell viability was measured by WST-1 assay. We found that 10 or 100 nM was an ideal concentration to elicit specific effects of paclitaxel in each cell type examined (data not shown). On the basis of these findings, all three cell lines were treated with or without HER-2 siRNA and exposed to 10 nM of paclitaxel for 48 h. Cell viability was then examined by WST-1 assay. As shown in Figures 4 and 5A, *HER-2* knockdown significantly augmented the cytotoxic effect of paclitaxel in each cell type.

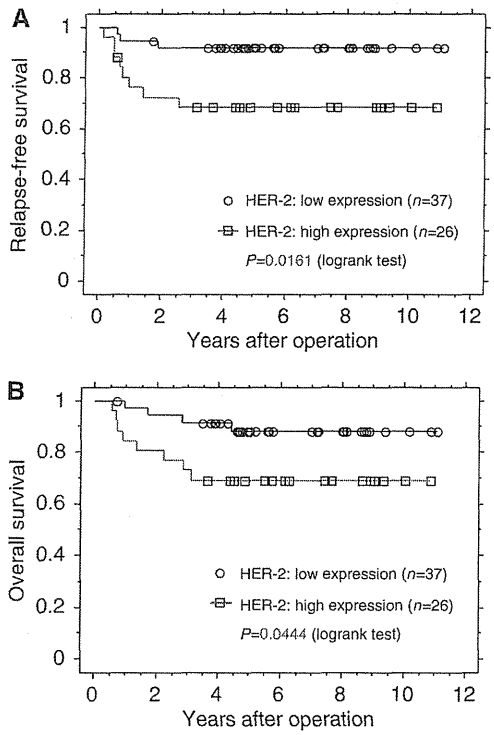

Figure 2 Kaplan-Meier survival curves of 63 patients with endometrial cancer in relation to HER-2 expression. (**A**) Relapse-free survival (**B**) Overall survival.

Next, we monitored the expression of p-AKT during treatment with paclitaxel in each cell type by western blot analysis. In Ishikawa cells, p-AKT expression increased on stimulation of paclitaxel at 24-48 h after treatment (Figure 6A). We also confirmed the expression of FOXO3a, a known downstream target of p-AKT. FOXO3a expression began to increase at 48 h and this upregulation lasted until at least 96 h (data not shown). Thus, we confirmed the functional activation of AKT during treatment with paclitaxel. We also tested whether HER-2 expression affects the induction of p-AKT. Ishikawa cells were treated with 10 nM paclitaxel under knockdown of *HER-2*, and p-AKT expression was then monitored. Figure 6 shows that cells with *HER-2* knockdown led to decreased levels of p-AKT expression, concomitant with increased sensitivity to paclitaxel, as shown in Figure 4. In HEC1A cells, p-AKT expression increased on stimulation with paclitaxel at 12 h after treatment (Figure 6B), and *HER-2* knockdown led to a significant decrease in p-AKT expression. The levels of p-AKT at 24 or 48 h were unstable, but apparently decreased on *HER-2* knockdown at 48 h as well. In EM-E6/E7/TERT cells, p-AKT expression also increased on stimulation with paclitaxel at 12 h or later after treatment (Figure 6C), and *HER-2* knockdown cancelled this elevation at 48 h. Thus, *HER-2* knockdown inhibited paclitaxel-induced activation of p-AKT expression in endometrial cancer or immortalised cell lines. Interestingly, in HEC1A cells, *HER-2* knockdown at 48 h after treatment, when p-AKT induction was no longer observed, enhanced the sensitivity to paclitaxel (Figure 4B), indicating that the basal levels of p-AKT expression have certain roles in inhibiting the cytotoxic effect of paclitaxel.

We further sought to confirm whether the increased sensitivity to paclitaxel by knockdown of *HER-2* is primarily because of the suppression of p-AKT expression. We prepared another immortalised cell in which EM-E6E7/TERT cells were stably transfected with the dominant-negative form of the *AKT* gene (EM-E6/E7/TERT/DN-AKT cells) (see Materials and Methods), so that they

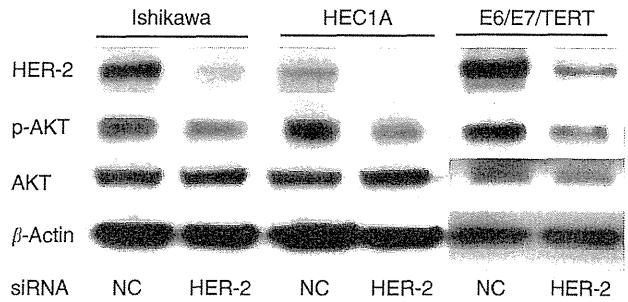

Figure 3 Linkage between HER-2 and p-AKT expressions in endometrial cancer or immortalised cells. Endometrial cancer Ishikawa and HEC1A cells or endometrial immortalised EM-E6/E7/TERT cells were treated with or without siRNA against *HER-2*. After 48 h, cell lysates were prepared and western blot analyses were performed with antibodies against HER-2, p-AKT and total AKT. NC: negative control sample treated with scrambled siRNA.

Table 5 Cox regression hazard model for relapse-free survival

Variable	Risk factor	Univariate (Kaplan-Meier) log-rank test (*P*-value)	Multivariate (Cox regression model) hazard ratio (95% confidence interval)	*P*-value
FIGO stage	⩾II	<0.0001	6.131 (1.525-24.643)	0.0106
Grade	3	0.0042	3.576 (0.978-13.073)	0.0540
Myometrial invasion	⩾1/2	0.0008	2.138 (0.578-7.909)	0.2548
HER-2 expression	High	0.0161	5.308 (1.332-21.156)	0.0180

Abbreviations: FIGO = International Federation of Gynaecology and Obstetrics; HER-2 = human epidermal growth factor receptor-2.

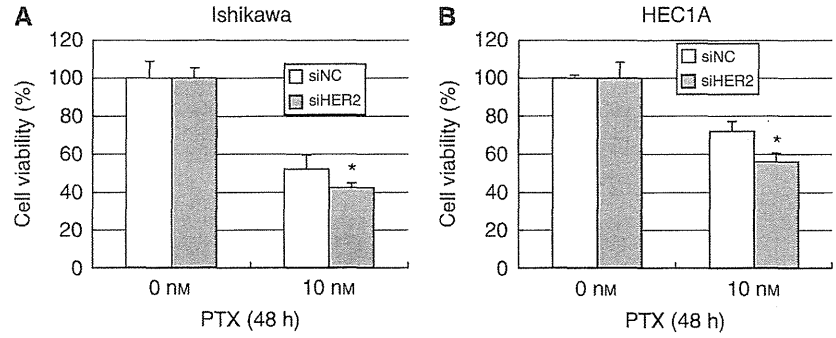

Figure 4 Increased sensitivity to paclitaxel by knockdown of *HER-2*. Endometrial cancer Ishikawa (**A**) and HEC1A cells (**B**) were incubated with siRNA against *HER-2* (siHER-2) or scrambled siRNA (siNC) for 48 h. Cells were then treated with 10 nM of paclitaxel for an additional 48 h, followed by the WST-1 assay to examine cell viability. The viability of untreated cells incubated with siNC was set as the control level (100%) and the percentage of cell viability in each cell type was normalised relative to the untreated control. Each experiment was performed in triplicate in three independent experiments. Columns, mean; bars, ± s.d. $^*P<0.05$.

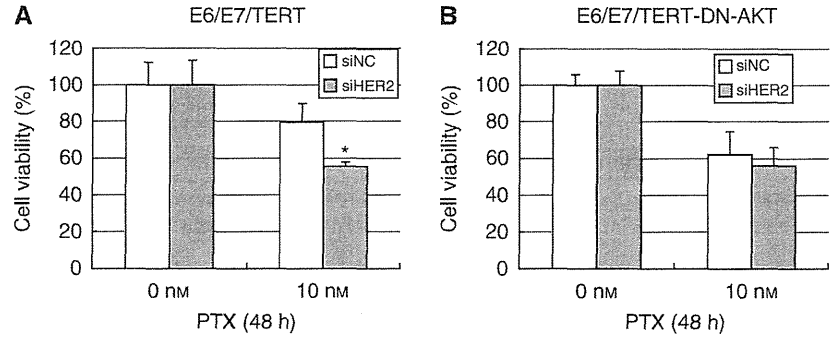

Figure 5 Inactivation of AKT cancels the effect of HER-2 on paclitaxel sensitivity. Endometrial immortalised EM-E6/E7/TERT cells (**A**) or those with the dominant-negative form of AKT (**B**) were incubated with siRNA against *HER-2* (siHER-2) or scrambled siRNA (siNC) for 48 h. Cells were then treated with 10 nM of paclitaxel for an additional 48 h, followed by the WST-1 assay to examine cell viability. The viability of untreated cells incubated with siNC was set as the control level (100%) and the percentage of cell viability in each cell type was normalised relative to the untreated control. Each experiment was performed in triplicate in three independent experiments. Columns, mean; bars, ± s.d. $^*P<0.05$.

have inactivated AKT function. Using these cells, we performed the paclitaxel sensitivity test by the WST-1 assay. As shown in Figure 5B, introduction of dominant-negative AKT largely cancelled the enhanced sensitivity to paclitaxel achieved by the knockdown of *HER-2*. Thus, the effect of HER-2 seems to be primarily dependent on AKT activity. Taken together, we conclude that HER-2 expression has a critical role in the induction of p-AKT expression, thereby affecting the sensitivity of endometrial cancer cells to paclitaxel.

DISCUSSION

In this study, we found frequent expressions of EGFR, HER-2 and HER-4 approximately in 40–50% of endometrioid-type endometrial cancer. There was no significant association between expressions of these three factors and the clinicopathological characteristics of endometrial cancer. These findings are in contrast to a previous study by Morrison *et al* (2006), in which HER-2 expression was associated with high-grade cancers (G3 and nonendometrioid types) or with higher stages (FIGO stages IIIa–IVb). This discrepancy may at least partly be accounted for by the difference in sample population between the studies. Our study examined endometrioid-type cancers, whereas Morrison *et al* (2006) investigated a much larger percentage (39%) of type 2 cancers. Nevertheless, we unexpectedly found that EGFR family expression was not closely associated with the clinicopathological characteristics of endometrioid-type endometrial cancer in Japanese patients. The rate of *HER-2* amplification in our study was low, with only 2 of 26 high-expression patients having *HER-2* amplification. This is in accordance with Morrison's report that endometrioid-type cancers have low frequency of *HER-2* amplification (1% in G1, 3% in G2 and 8% in G3 cancers) (Morrison *et al*, 2006). Owing to the small number of samples with *HER-2* amplification, its relationship with clinicopathological characteristics remains unclear.

The association between the EGFR family and other genetic alterations involved in endometrial carcinogenesis was our next point of focus. We tested the association between mutations of three genes representative of common genetic mutations in endometrial cancers: *PETN*, *PIK3CA* and *KRAS*. Among the factors examined, there was a tendency for HER-2 expression to be associated with wild-type *PTEN* ($P=0.079$). In particular, strong HER-2 expression was closely linked to wild-type *PTEN* ($P=0.032$). These findings suggest that HER-2 signalling is distinct from the *PTEN* mutation pathway. In the present siRNA knockdown study, we found that HER-2 expression was closely associated with p-AKT expression, indicating that HER-2 signalling may be upstream of the PI3K–AKT pathway. These findings suggest that HER-2 signalling activates the PI3K–AKT pathway in a PTEN-independent manner. This scenario is easily understood, as the *PTEN* mutation may not be required to activate the PI3K–AKT pathway if an HER-2/PI3K–AKT axis exists. Another interesting finding was the tendency for HER-2 expression to be associated with intact MLH1 expression ($P=0.0746$). We previously showed that low MLH1 expression is mainly caused by

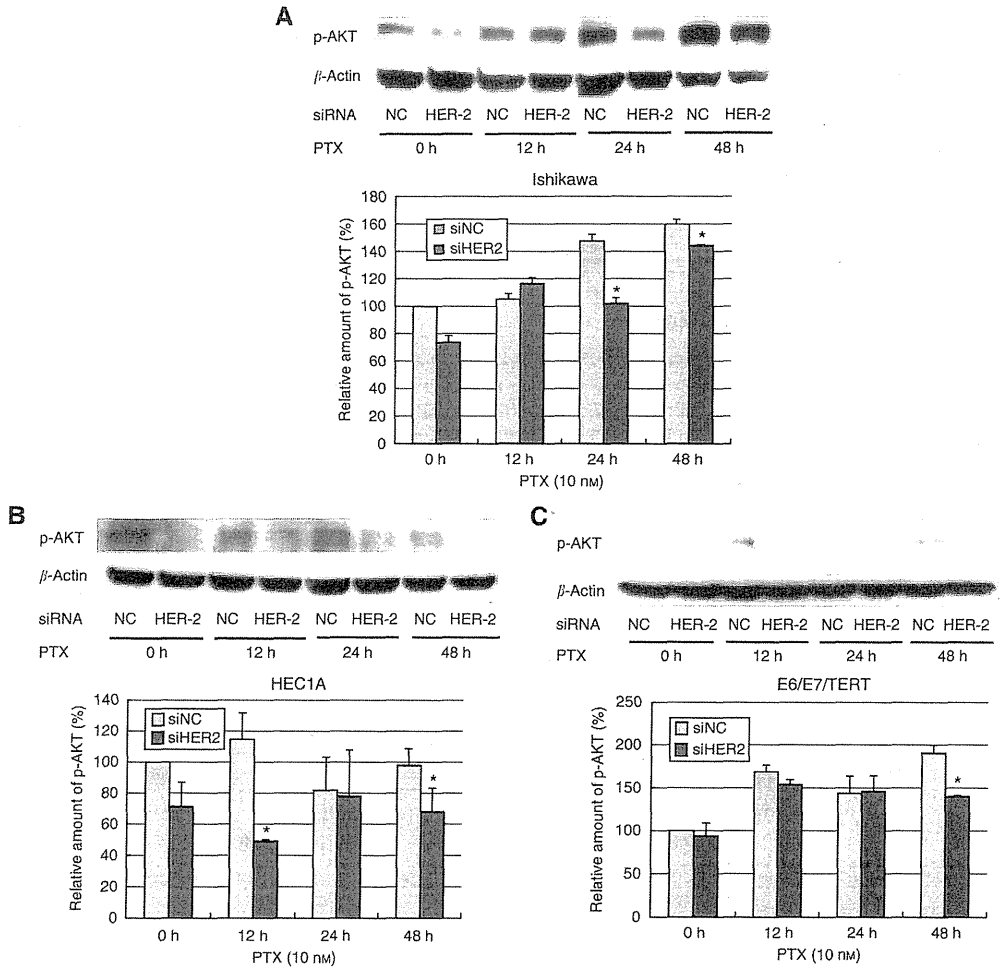

Figure 6 Induction of p-AKT expression on paclitaxel treatment is attenuated by *HER-2* knockdown. Endometrial cancer Ishikawa (**A**) and HEC1A cells (**B**) or immortalised EM-E6/E7/TERT cells (**C**) were incubated with siRNA against *HER-2* (siHER-2) or scrambled siRNA (siNC) for 48 h. Cells were then treated with 10 nM of paclitaxel for an additional 48 h. Cell lysates were prepared and western blot analyses were performed with antibodies against p-AKT. NC: negative control sample treated with scrambled siRNA. A representative image of the bands is shown. Bband intensity was measured using NIH image and is represented as the relative amount of p-AKT after normalisation against β-actin expression. Each experiment was performed in triplicate in three independent experiments. Columns, mean; bars, ± s.d. $^*P<0.05$.

its promoter inactivation through DNA hypermethylation, which triggers an accumulation of mutations of several genes, including *PTEN* (Kanaya *et al*, 2005). Thus, the possible association between HER-2 and intact MLH1 may also account for HER-2 signalling being able to directly activate the AKT pathway, thereby not requiring mismatch repair deficiency.

Although the *in vitro* knockdown study of HER-2 showed clear association with p-AKT (Figure 3), HER-2 expression *in vivo* was not significantly associated with the expression of p-AKT on the basis of immunohistochemistry ($P=0.9411$, data not shown). However, this *in vivo* result is not anomalous because, in clinically advanced cancers, there are undoubtedly several factors or signalling pathways that regulate p-AKT expression other than the PI3K–AKT pathway. Therefore, the possible linkage between two factors observed in a knockdown study is not always proven by immunohistochemical comparison with clinical cancer samples.

Among the EFGR family, only HER-2 expression is an independent prognostic factor in endometrial cancer. Morrison *et al* (2006) studied larger numbers of patients with a broad spectrum of histological type, grade and stage of endometrial carcinoma, and showed that in univariate analyses, both HER-2 expression and amplification correlated with disease-specific and progression-free survival. In multivariate analyses, HER-2 expression with amplification correlated with overall survival, but not expression without amplification. Overall survival was significantly shorter in patients who overexpressed and/or showed amplification of HER-2 compared with those who did not (Morrison *et al*, 2006). Our data support the findings of Morrison *et al* (2006) and clearly show that it is HER-2 among the EGFR family that significantly affects patient survival.

Growth factor receptor-mediated signal transduction and PI3K–AKT activation have been implicated in conferring resistance to conventional chemotherapy in breast cancer cells (Yu *et al*, 1996; Knuefermann *et al*, 2003). In uterine cancer cells (Gagnon *et al*, 2004, 2008) and ovarian cancer cells (Cao *et al*, 2008; Weng *et al*, 2009), the AKT pathway is known to have critical roles in chemoresistance. The linkage of HER-2 and p-AKT expression by our siRNA knockdown experiment indicates that HER-2 may be involved in the chemosensitivity of endometrial cancer cells. As expected, HER-2 inhibition by siRNA efficiently showed increased sensitivity to paclitaxel, concomitant with p-AKT inhibition. This increased sensitivity seemed to be at least partly p-AKT dependent, because the increased sensitivity to paclitaxel by *HER-2* knock-

down was cancelled in cells with inactive AKT (Figure 5B). Thus, it seems that HER-2 regulates the sensitivity to paclitaxel by modulating AKT activity in endometrial cancer cells. Knuefermann *et al* (2003) reported that, in MCF7 breast cancer cells, overexpression of HER-2 caused a PI3K-dependent activation of AKT, and was associated with an increased resistance of cells to multiple chemotherapeutic agents. They also found that selective inhibition of PI3K or AKT activity sensitised MCF7 breast cancer cells to the induction of apoptosis by chemotherapeutic agents. Our results are consistent with their study and show that HER-2 is one of the key regulators of paclitaxel sensitivity, as an upstream factor of AKT, in endometrial cancer.

In summary, we have shown that, among the EGFR family members, only HER-2 is an independent prognostic factor of endometrioid-type endometrial cancer, which may link to the PI3K–AKT pathway, independent of *PTEN* mutation or mismatch repair deficiency. Our *in vitro* study underscores the importance of the HER-2/PI3K–AKT signalling pathway for regulating the efficacy of paclitaxel in endometrial cancer cells. Therefore, we propose the potential clinical benefit of an appropriate combination of conventional chemotherapeutic drugs with a new generation of signal-transduction inhibitors that target the HER-2/PI3K–AKT pathway for the treatment of endometrial cancer.

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Involvement of LKB1 in epithelial–mesenchymal transition (EMT) of human lung cancer cells

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ABSTRACT

Epithelial–mesenchymal transition (EMT) is a critical phenotypic alteration of cancer cells that triggers invasion and metastasis. Lung cancer cells often show mesenchymal phenotypes; however, a causative genetic alteration for the induction of EMT in lung cancer cells remains unknown. Recent studies have shown that the LKB1 gene is mutated in up to one-third of lung adenocarcinomas. Therefore, to pursue the possible involvement of LKB1 inactivation in the induction of EMT in lung carcinogenesis, we generated immortalized lung epithelial cells and lung adenocarcinoma cells with stable or transient LKB1 knockdown. LKB1 knockdown increased cell motility and invasiveness, and induced the expression of several mesenchymal marker proteins accompanied by the expression of ZEB1, a transcriptional repressor for E-cadherin and an EMT inducer. In agreement with the recent findings, expression of miR-200a/c was inversely correlated with that of ZEB1 in LKB1 knockdown clones with mesenchymal phenotype. Furthermore, transient knockdown of LKB1 induced ZEB1 mRNA and increased cell motility, and this motility was suppressed by ZEB1 repression. These results strongly indicate that LKB1 inactivation triggers EMT in lung cancer cells through the induction of ZEB1.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. In particular, adenocarcinoma (AdC) is the most common histological type, and its highly invasive and metastatic phenotypes are the major reasons for poor prognosis of patients with lung AdC. The cellular and molecular mechanisms of lung AdC metastasis remain to be elucidated. Recently, epithelial–mesenchymal transition (EMT) has gained attention as a critical phenotypic alteration of cancer cells to acquire invasive and metastatic ability [2]. EMT is mediated through several transcription repressors, such as Snail, Slug, Twist and ZEB1, and these EMT inducers typically suppress the transcription of the E-cadherin gene, an epithelial cell marker and a potent suppressor of tumor cell invasion and metastasis [3,4]. Indeed, a considerable fraction of lung cancer cells shows mesenchymal phenotypes with loss of E-cadherin expression [5], and ZEB1 expression was shown to correlate with E-cadherin loss in lung cancer cell lines [6]. Expression of Slug was also shown to be significantly associated with shortened survival of patients with lung AdC [7].

Multiple genetic alterations are accumulated in lung AdC cells during their progression. Inactivation of the p16 and p53 genes is common and frequent, and activating mutations of the EGFR and KRAS genes occur in a mutually exclusive manner [8,9]. However, invasiveness and metastatic phenotypes of lung AdC cells cannot be explained by these alterations. Therefore, causative genetic alterations for the acquisition of invasive and metastatic phenotypes in lung AdC cells are still unclear. Recently, we showed that the LKB1 gene is inactivated preferentially in poorly differentiated AdCs in male smokers, and LKB1 inactivation often co-exists with KRAS mutation [10,11]. Involvement of the LKB1 gene in the control of initiation, differentiation and metastasis in pulmonary tumorigenesis was also shown recently [12]. LKB1 is known to play important roles in multiple biological processes, including apoptosis, cell cycle arrest, cell polarity, chromatin remodeling, and energy metabolism [13–21]. Therefore, it is possible that inactivation of the LKB1 gene is involved in the acquisition of invasiveness and metastatic ability as well as the induction of EMT in lung AdC cells. Here, we investigated the pathogenic and biological significance of LKB1

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inactivation in lung AdC progression by manipulating immortalized lung epithelial cells as well as a lung AdC cell line with epithelial phenotypes. Knockdown of LKB1 in these cells induced EMT through the enhanced ZEB1 expression. Thus, it was strongly indicated that LKB1 inactivation is a genetic alteration for the EMT induction in lung AdC cells.

2. Materials and methods

2.1. Expression vectors and antibodies

Two shRNA mediated silencing vectors for LKB1, pTER-LKB1-sh#1 and pTER-LKB1-sh#4, were used in this study [18]. Targeting sequences for LKB1 of these vectors are pTER-LKB1-sh#1; 5′-GATCCTCAAGAAGAAGAAG-3′ and pTER-LKB1-sh#4; 5′-CGAAGAGAAGCAGAAAATG-3′. A human telomerase reverse transcriptase (hTERT) retroviral expression vector, pMSCVpuro-hTERT, was constructed as described previously [22,23]. Primary antibodies, LKB1 (sc-32245), ZEB1 (sc-25388) and Twist (sc-15393), were purchased from Santa Cruz Biotechnology, Santa Cruz, CA; β-actin (clone AC-74) from Sigma, St Louis, MO; Fibronectin (cat# 610071), N-cadherin (cat# 610920), α-catenin (cat# 610193) and γ-catenin (cat# 610253) from Transduction Laboratories, Lexington, CA; E-cadherin (HECD-1) from Takara, Kyoto, Japan; and Vimentin (RB-9063-P1) from Lab Vision Corp., Fremont, CA.

2.2. Cell culture and immortalization of hSAEC

Human small airway epithelial cells (hSAECs) were purchased from Cambrex (Walkersville, USA) [24] and cultured in Defined-KFC medium (Invitrogen) containing a growth supplement. Production and infection of the hTERT recombinant retroviruses were described previously [25], and the infected cells were selected with puromycin (0.5 μg/ml). After cultivation for approximately 120 population doublings (PD), cells were cloned and subjected to further analyses. H358 was purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum.

2.3. Mutation analysis of the p53 and LKB1 genes

Genomic PCR, reverse transcription-PCR (RT-PCR) and direct sequencing were performed as described previously [10].

2.4. Karyotype analysis

Karyotype analysis was done using a standard G-banding method as described previously [23].

2.5. RT-PCR

Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were synthesized using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) with oligo-dT primers. Semiquantitative RT-PCR analysis was performed as described previously [24]. Quantitative RT-PCR analysis was performed in duplicate using the ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) with the TaqMan primer and probe sets from Applied Biosystems. Relative expression levels of each gene were normalized to GAPDH (cat# 4326317E, Applied Biosystems), and digitized by the mean of two values. In some experiments, relative mRNA amounts were shown as mean ± SD of four values in two independent PCR experiments in duplicate in order to evaluate the significance of differences in expression levels. miRNA and U6-specific cDNA were generated from total RNA, isolated with a miRVana miRNA isolation kit (Ambion), using a TaqMan MicroRNA Reverse Transcription Kit with an RNA-specific RT primer from the TaqMan MicroRNA Assay (Applied Biosystems). miRNA levels were measured by real-time PCR using the miRNA-specific TaqMan probe provided in the MicroRNA Assays. miRNA levels were normalized to U6 small nuclear RNA [26].

2.6. Transfection and small interfering RNA treatment

The pTER-LKB1-sh#1 and -sh#4 plasmids were transfected into hSAEC-T1 cells with Microporator (Digital Bio Technology, Kyonggi, Korea) and cultured in medium containing Zeocin (100 μg/ml) for 14 days. Resistant cells were cloned and checked for LKB1 expression by Western blot analysis. A Validated Stealth RNAi DuoPak containing two different siRNAs for LKB1 (cat#12938-048, Invitrogen), a non-targeting control-siRNA (cat#12935-200, Invitrogen), and an siRNA for ZEB1 (cat#AM16704, Ambion) were used according to the manufacturer's instructions. The two siRNAs for LKB1 were designated siLKB1#1 and siLKB1#2, respectively. Specific knockdown of ZEB1 mRNA was confirmed in our previous study [27]. Transfection of siRNA into H358 cells was performed with Lipofectoamine 2000 (Invitrogen) according to the manufacturers' instructions, and that into hSAEC-T1 cells was performed by electroporation because the efficiency of siRNA introduction by lipofection method was very low.

2.7. Western blot analysis

Cells were harvested, washed with PBS, and lysed in buffer A (50 mM Tris–HCl, pH 7.5, 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 100 mM NaF, 200 μM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml chymotrypsin). After boiling of the lysate in a loading buffer, proteins were separated by SDS-PAGE, transferred to PVDF membranes (Immobilon PVDF; Millipore, Billerica, MA), and reacted with respective antibodies. The signals were enhanced with secondary antibodies against alkaline phosphatase (Sigma) or horseradish peroxidase (Amersham Biosciences), and developed with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Sigma) or enhanced chemiluminescence (ECL) (Amersham Biosciences).

2.8. Immunocytochemistry

Cells were seeded on cover slips, cultured for 2 days, fixed with 3.7% formaldehyde in PBS containing 2% BSA and 0.1% Triton X-100, and incubated with primary and secondary antibodies. The secondary antibodies used were AlexaFluor 594-conjugated goat anti-mouse and anti-rabbit IgGs (red) and AlexaFluor 488-conjugated goat anti-mouse and anti-rabbit IgGs (green) (Molecular Probes, Eugene, OR). Images were obtained with an Axiovert 200 M fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and processed by Adobe Photoshop.

2.9. Wound healing assay

hSAEC-T1 cells were grown to confluence on 6-well plastic dishes, wounded by scratching with a 200 μl pipette tip, and washed with fresh media. Wounds were continuously photographed at 30 min to 1 h intervals up to 24 h with an Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany) with a 10x objective. H358 cells were treated with siRNAs of LKB1, ZEB1 or in combination. After 40 h of transfection, cells were wounded, washed and photographed as above. A portion of the cells treated with siRNAs was taken separately for Western blot analysis.

2.10. Invasion assay

Cells (1×10^4), in 0.5 ml of medium without growth supplement, were added to each well of a 24-well/8 μm pore invasion membrane chamber coated with Matrigel (BD Discovery Labware, Bedford, MA) [28]. The lower chambers contained medium with a double concentration of growth supplement. Cells were allowed to invade over the course of 48 h, and then fixed with 100% methanol, stained with 0.5% crystal violet, rinsed with water, dried, and examined under a microscope. Values for invasion were obtained by counting the total number of migrated cells in each chamber and presented by the average of three independent experiments.

3. Results

3.1. Establishment of immortalized hSAECs by hTERT

It has been shown that both hTERT expression and inactivation of either RB or p16 are required for the immortalization of primary human keratinocytes and mammary epithelial cells [29,30]. Introduction of hTERT into hSAECs led to their immortalization with a retarded growth until PD = 35. Then, fast growing cells emerged and continued to grow up to PD = 120 (Fig. 1A). Therefore, immortalized cells were cloned and karyotyped. Among 5 clones analyzed, one clone designated as hSAEC-T1 showed minimal chromosomal changes. This clone was almost diploid with trisomies of chromosomes 5 and 9 and an isochromosome of chromosome 20 (Fig. 1B). The other 4 clones carried some additional chromosomal changes, in addition to these changes (Supplementary Table 1). Since inactivation of the p16/RB and p53 pathways is required for and enhances the process of epithelial cell immortalization, respectively, the status of the p16 and p53 genes was examined in hSAEC-T1 cells. p16 was not expressed due to promoter methylation, as treatment with a DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine restored p16 expression, confirmed by RT-PCR and Western blot analyses (Fig. 1C and D). The p53 gene was mutated in this cell line. C to T transition of nucleotide 817 in exon 8, resulting in the amino acid change from arginine to cysteine (R273C), was detected by genomic PCR and sequencing. No wild-type allele was detected, therefore, only a mutant p53 protein was expressed in this cell line. The results indicate that methylational inactivation of the p16 gene, mutational inactivation of the p53 gene and several chromosomal alterations have occurred in hSAEC-T1 cells during cultivation, and the cells carrying these alterations were selected in the process of immortalization.

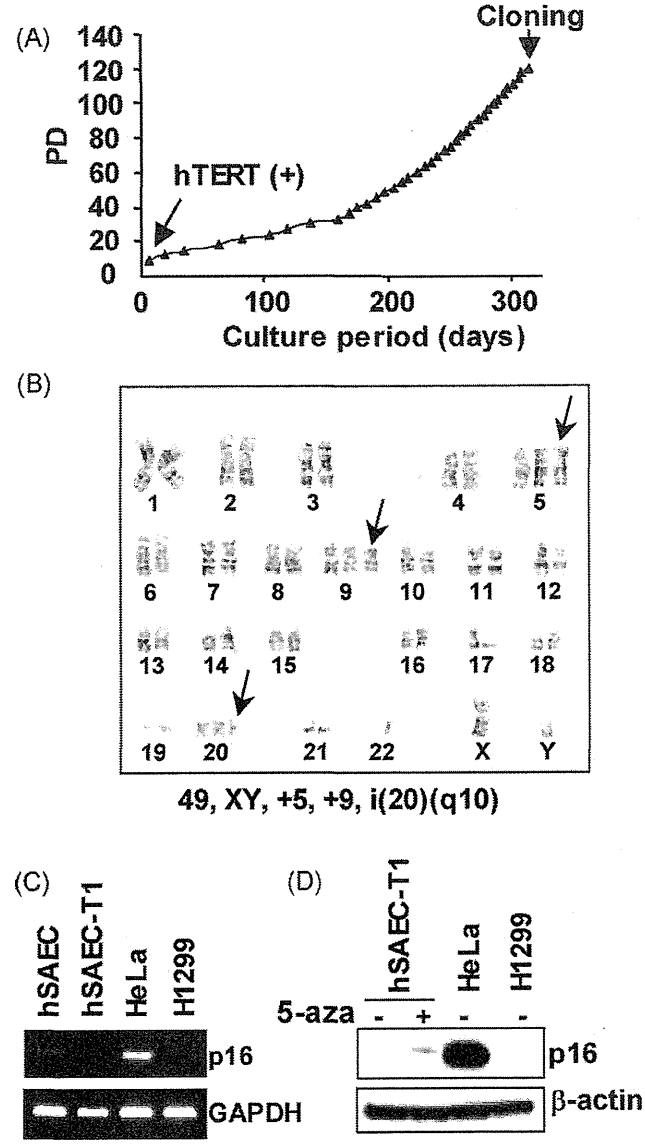

Fig. 1. Immortalization of human small airway epithelial cells (hSAEC) by hTERT. (A) Growth of hSAEC with introduction of hTERT. hSAEC was infected with the retroviral hTERT expression vector at PD = 9, cultured for up to PD = 120, and cloned. (B) Karyotype analysis of hSAEC-T1 cells. A minimal change with 49 chromosomes was observed in this clone. (C) RT-PCR analysis of p16 mRNA in hSAEC-T1 cells and primary cultured hSAEC. (D) Western blot analysis of p16 protein in hSAEC-T1 cells after 5-aza-2′-deoxycytidine (5-aza) (1 μM) treatment. β-actin was used as a loading control. HeLa and H1299 cells were used as a positive and a negative control for p16 expression in C and D, respectively.

3.2. Motility and invasiveness enhanced by LKB1 knockdown in hSAEC-T1 cells

Using the immortalized hSAEC-T1 cells, we established several stable LKB1 knockdown clones that express a small and negligible amount of LKB1 protein by introducing an shRNA mediated silencing vector, pTER-LKB1-sh#4. Suppression of LKB1 expression in individual clones was confirmed by Western blot analysis (Fig. 2A). Clone sh#4-9 expressed a little lower level of LKB1 protein than parental cells, whereas clones sh#4-8 and sh#4-12 expressed almost negligible levels of LKB1 protein. Under standard culture conditions, there were no remarkable changes in the growth of LKB1 knockdown clones (Supplementary Fig. S1A). Morphological observation of these clones revealed that LKB1 knockdown caused an increase in the population of spindle shaped cells (Fig. 2D). This finding led us to examine the motility of these clones. We used a wound healing assay to monitor the effect of LKB1 on cell motility. The velocity of LKB1 knockdown clones sh#4-8 and sh#4-12 was significantly increased (~3-fold) in comparison with that of clone sh#4-9 or parental cells (Fig. 2B; also Supplementary Fig. S1B). We then examined the invasiveness of these cells by a Matrigel invasion assay. Parental cells along with clone sh#4-9 showed a low invasiveness, whereas LKB1 knockdown clones sh#4-8 and sh#4-12 exhibited increased invasiveness (Fig. 2C). Thus, in hSAEC-T1 cells, the population of spindle shaped cells was increased and motility and invasiveness were enhanced by suppression of LKB1 expression.

3.3. Epithelial–mesenchymal transition (EMT) induced by LKB1 knockdown in hSAEC-T1 cells

EMT is often associated with spindle shaped morphology and with enhanced motility and invasiveness of cells. To assess whether

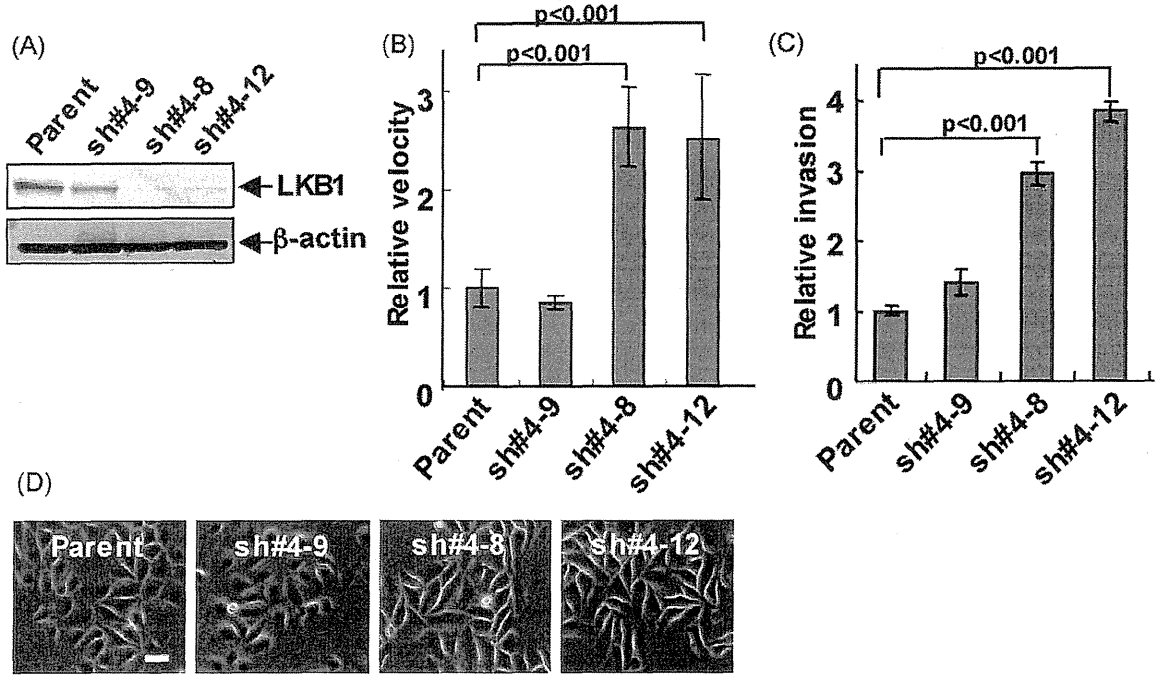

Fig. 2. Motility and invasiveness enhanced by LKB1 knockdown in hSAEC-T1 cells. (A) Western blot analysis for LKB1 expression in hSAEC-T1 cells with and without LKB1 knockdown. β-actin was used as a loading control. (B) Motility of hSAEC-T1 cells with and without LKB1 knockdown. Motility is expressed by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios to parental cells. (C) Invasiveness of hSAEC-T1 cells with and without LKB1 knockdown. Invasiveness is expressed by the number of migrated cells in each chamber. Data are presented by means ± SD of three independent measurements and expressed as ratios to parental cells. Statistical significance for the difference was determined using a Student's two-tailed *t*-test. (D) Morphology of hSAEC-T1 cells with and without LKB1 knockdown. Morphologies of cells under standard culture conditions are shown. Bar, 25 μM.

hSAEC-T1 cells had undergone an EMT by LKB1 knockdown, we examined the expression of several epithelial and mesenchymal marker proteins by Western blot analysis. Parental hSAEC-T1 cells showed an epithelial-like phenotype. Mesenchymal markers, fibronectin, N-cadherin and vimentin, were very faintly detected, whereas epithelial markers, E-cadherin, α-catenin and γ-catenin, were strongly detected in hSAEC-T1 cells. It was noted that knockdown of LKB1 resulted in a strong induction of these mesenchymal markers and a marked reduction of these epithelial markers (Fig. 3A). The Western blot results were further verified by immunostaining with antibodies against fibronectin and E-cadherin (Fig. 3B). Increased fibronectin deposition was observed in these LKB1 knockdown clones. E-cadherin staining was intense and mainly restricted to cell-cell contacts in the parental cells and clone sh#4-9, and a disappearance of E-cadherin at cell-cell contacts was observed in LKB1 knockdown clones, sh#4-8 and sh#4-12.

Several transcription factors, including Snail, Slug, Twist and ZEB1, have been shown to promote cell transitions from an epithelial to a mesenchymal phenotype [2–4]. We then examined the expression of these proteins in these LKB1 knockdown clones by Western blot analysis (Fig. 3C). Only the zinc finger and homeodomain transcriptional repressor ZEB1 was induced in LKB1 knockdown clones. Snail and Slug were not detected in these cells. Twist was detected, but the amount was not changed by LKB1 knockdown. Next, we measured the amount of mRNA for these transcription factors by quantitative RT-PCR analysis (Fig. 3D). ZEB1 was most highly induced, and the level of ZEB1 mRNA expression showed the significant inverse correlation to the level of LKB1 mRNA expression in these clones. The levels of Snail and Slug mRNA expression were also changed but did not show significant correlation to that of LKB1 mRNA expression. The level of Twist mRNA expression was not changed, as the amount of Twist protein. E-cadherin has been shown to be a target of transcriptional repression by ZEB1. Our Western blot and immunostaining results also indicated an inverse correlation of ZEB1 expression with E-cadherin expression in these cells (Fig. 3A–C). Quantitative RT-PCR results further supported this finding. E-cadherin mRNA was decreased and N-cadherin mRNA was increased in the cells with ZEB1 induction (Fig. 3D). Thus, it was concluded that LKB1 knockdown induces ZEB1 expression at both mRNA and protein levels in hSAEC-T1 cells. A similar result was found using several stable clones (sh#1-2, sh#1-5, sh#1-14 and control sh#1-7) generated by a different targeting construct pTER-LKB1-sh#1 (Supplementary Fig. S2), confirming that induction of ZEB1 as well as reduction of E-cadherin were not due to the off-target effect of siRNA but a specific effect of LKB1 knockdown. Thus, we concluded that ZEB1 mediated EMT had occurred in SAEC-T1 cells by LKB1 knockdown.

3.4. Correlation of miR-200 expression with LKB1 expression in hSAEC-T1 cells

It was recently shown that the miR-200 family and ZEB1 are reciprocally repressed in cancer cells [26,31–33]. To determine whether LKB1 expression is associated with miR-200 family expression in hSAEC-T1 cells, we quantified the expression of miR-200a and miR-200c, as representative members of the miR-200 family, by real-time PCR in parental hSAEC-T1 cells and 7 knockdown clones with various levels of LKB1 expression (Fig. 4). A significant inverse correlation ($P=0.0090$ and $P=0.0210$) was observed between ZEB1 and miR-200a/c expression in these clones (Fig. 4B and C). A similar inverse correlation ($P=0.0012$) was found for the expression of LKB1 and ZEB1 (Fig. 4A). In contrast, miR-200a/c expression was positively correlated ($P=0.0001$ and $P=0.0010$) with LKB1 expression in these clones (Fig. 4D and E). This result further supports that ZEB1 mediated EMT had occurred in SAEC-T1 cells by LKB1 knockdown.

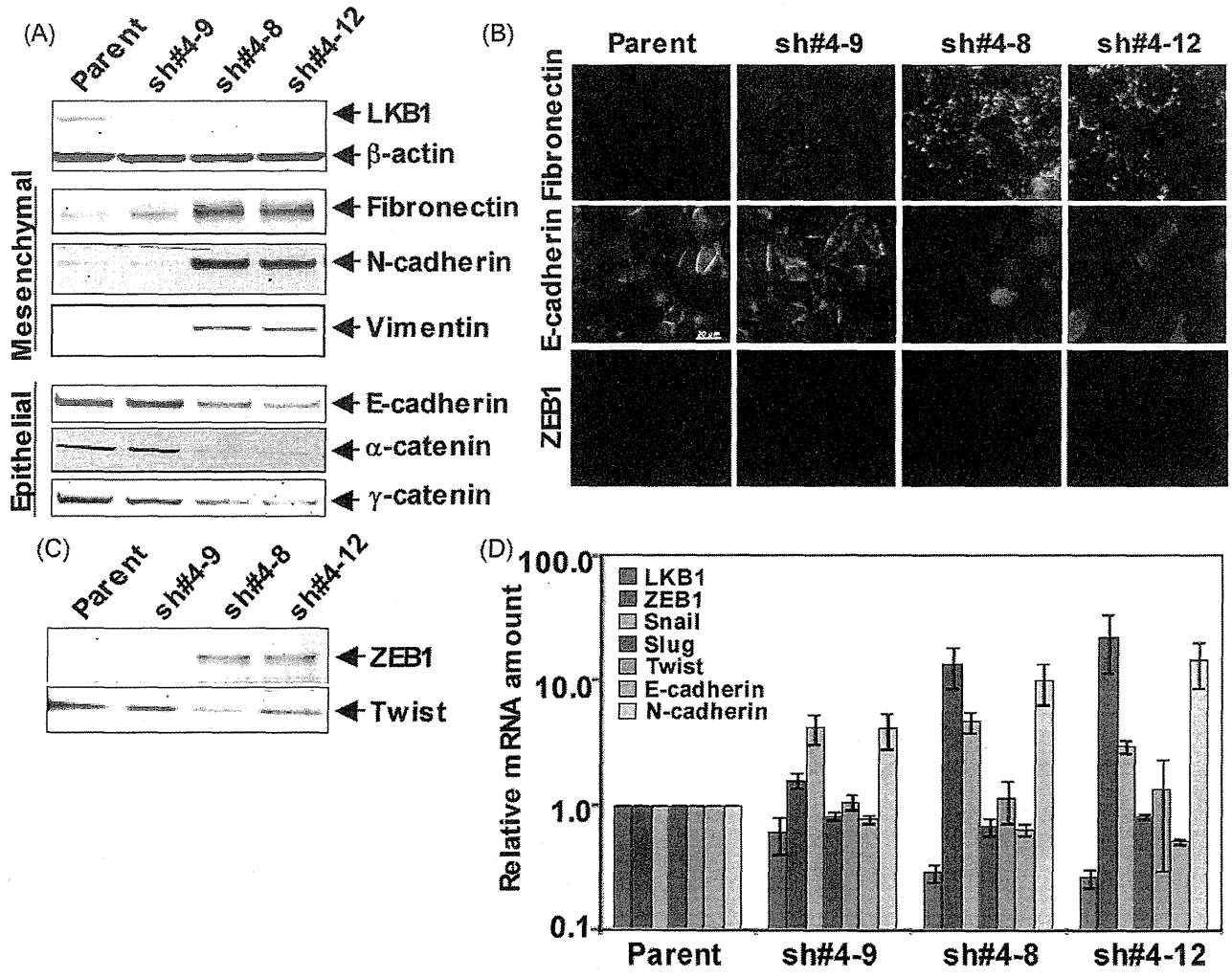

Fig. 3. EMT induced by LKB1 knockdown in hSAEC-T1 cells. (A) Western blot analysis for mesenchymal markers, fibronectin, N-cadherin and vimentin, and epithelial markers, E-cadherin, α-catenin and γ-catenin in hSAEC-T1 cells with and without LKB1 knockdown. β-actin was used as a loading control. (B) Immunohistochemistry for fibronectin, E-cadherin and ZEB1 in hSAEC-T1 cells with and without LKB1 knockdown. (C) Western blot analysis for ZEB1 and Twist in hSAEC-T1 cells with and without LKB1 knockdown. (D) Quantitative RT-PCR analysis for LKB1, ZEB1, Snail, Slug, Twist, E-cadherin and N-cadherin mRNAs in hSAEC-T1 cells with and without LKB1 knockdown. The mRNA amounts in LKB1 knockdown clones are shown as a ratio relative to those in parental cells.

3.5. Epithelial–mesenchymal transition induced by LKB1 knockdown in H358 cells

To elucidate whether LKB1 inactivation commonly induces EMT in lung adenocarcinoma cells, we next examined a lung adenocarcinoma cell line H358 carrying the wild-type LKB1 gene [12] and with retention of epithelial phenotypes. As in the case of hSAEC-T1 cells, mesenchymal markers, fibronectin, N-cadherin and vimentin, were faintly or not detected, whereas epithelial markers, E-cadherin, α-catenin and γ-catenin, were intensely detected in H358 cells [6]. Several stable LKB1 knockdown clones derived from H358 cells were generated using two different targeting vectors, pTER-LKB1-sh#1 (sh#1-1 and sh#1-5) and pTER-LKB1-sh#4 (sh#4-7, sh#4-9). Induction of mesenchymal markers such as N-cadherin and vimentin, and reduction of an epithelial marker protein E-cadherin were observed in these clones by Western blot analysis, although induction/reduction was not so evident as in hSAEC-T1 cells (Fig. 5A and data not shown). ZEB1 expression was also induced in these clones. Quantitative RT-PCR analysis for EMT-inducing transcription factors further confirmed ZEB1 to be the most highly induced one among them, and the analysis for E-cadherin and N-cadherin further supported the decrease of E-cadherin mRNA and the increase of N-cadherin mRNA in the cells with LKB1 knockdown (Fig. 5B). Increased cell motility and invasiveness were also observed in these clones (Fig. 5C and D), as observed in hSAEC-T1 cells. Thus, it was concluded that the phenotype of H358 lung AdC cells had changed from an epithelial one to a mesenchymal one accompanied by the induction of ZEB1 expression by knocking down the expression of the LKB1 gene.

3.6. Transient knockdown of LKB1 enhances ZEB1 expression in hSAEC-T1 and H358 cells

Consistent results for the induction of EMT were obtained using several clones with stable LKB1 knockdown derived from both hSAEC-T1 cells and H358 cells. To further elucidate whether such an induction can occur by transient LKB1 knockdown in the cells or not, we transiently suppressed LKB1 expression by introducing siRNAs into both hSAEC-T1 and H358 cells. To exclude the off-target effect of siRNA, siLKB1#1 and siLKB1#2 with different target sequences were used for knocking down the expression of LKB1 in the cells. Then, expression of LKB1, ZEB1, N-cadherin and

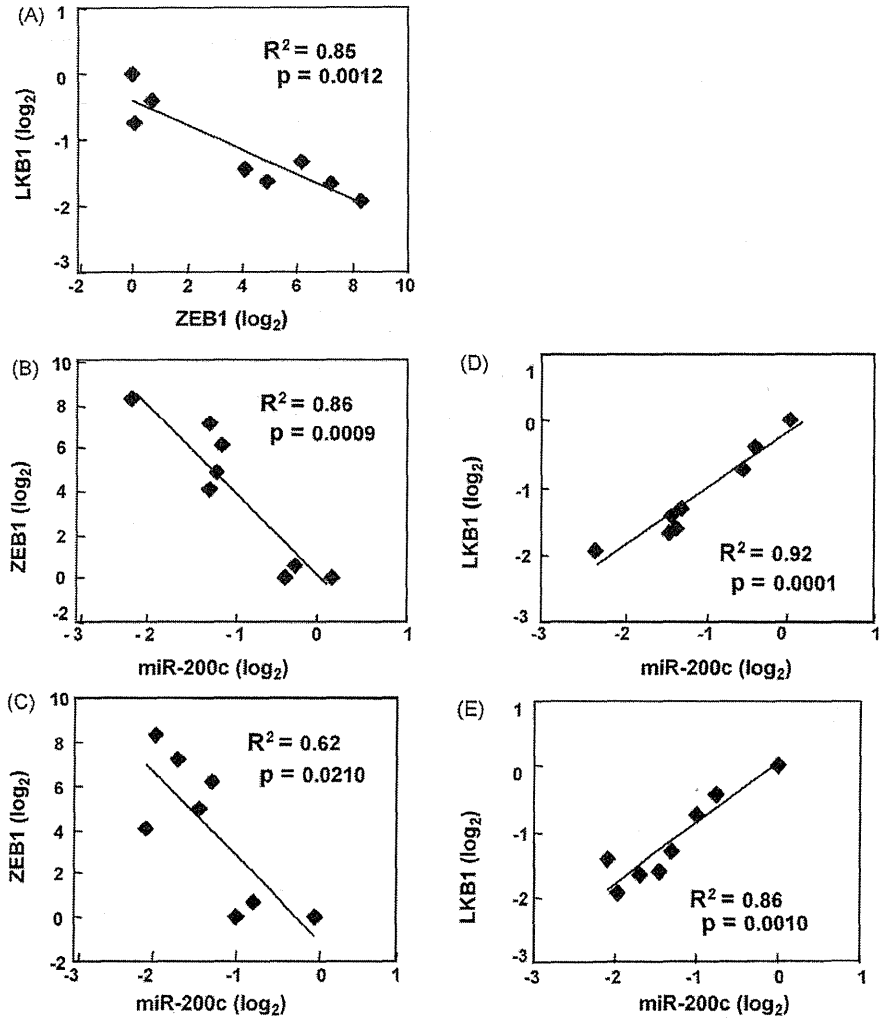

Fig. 4. Association of miRNA-200a/c expression with LKB1 and ZEB1 expression in hSAEC-T1 cells. Levels of mRNA expression were measured by real-time PCR for parental and 7 knockdown clones, and indicated by $\log_2$ ratios. Associations of two variables are shown by Pearson correlation coefficients (R^2) and *P*-values.

E-cadherin were assessed by real-time RT-PCR analysis (Fig. 6). The amount of LKB1 mRNA was markedly reduced by 4 h after siRNA transfection and was persistently suppressed over 24 h in both siRNAs. ZEB1 expression was increased at around 8–16 h of post-transfection, and, at a later period, N-cadherin expression was increased and E-cadherin expression was reduced in hSAEC-T1 cells (Fig. 6A). Similar results were obtained in H358 cells; enhanced ZEB1 expression occurred at around 8–16 h of post-transfection, followed by the increase in N-cadherin expression, although reduction of E-cadherin expression was not so evident as hSAEC-T1 cells (Fig. 6B). The induction of ZEB1 mRNA by transient LKB1 knockdown in these cells was also confirmed in another experiment (Supplementary Fig. S3).

We then measured the expression level of miR-200a/c after transient LKB1 knockdown by real-time PCR analysis. Contrary to the mRNA level of ZEB1, the expression level of miR-200a/c was inconsistent to the results of stable LKB1 knockdown clones in several experiments. A representative result was shown in Fig. 6C. Although miR-200c was not significantly reduced at any time points, miR-200a expression was slightly decreased at 16 h after siRNA transfection. Therefore, it was suggested that ZEB1 rather than miR-200 is a primary target of LKB1 for the induction of EMT in these cells, and that members of the miR-200 family are EMT regulators where ZEB1 is an EMT inducer.

3.7. Occurrence of EMT by LKB1 knockdown is dependent on ZEB1 expression in H358 cells

The above results indicate that LKB1 dependent EMT occurs through the induction of ZEB1 expression. Therefore, we next investigated the effect of LKB1 and ZEB1 double-knockdown on the motility of H358 cells. Transient knockdown of LKB1 expression significantly increased the wound healing capacity of H358 cells. The mean velocity of transient LKB1 knockdown cells was significantly higher than that of control cells, as in stable LKB1 knockdown cells (Fig. 5C). Therefore, we next examined whether or not this increase of cell motility was due to ZEB1 induction or not by introducing ZEB1 siRNA into H358 cells. Introduction of ZEB1 siRNA effectively reduced ZEB1 protein expression in H358 cells (Fig. 7A) and resulted in a drastic suppression of cell motility (Fig. 7B and C). Cell morphology also changed significantly; knockdown of LKB1 increased the population of mesenchymal-like spindle shaped cells, whereas knockdown of ZEB1 increased the population of epithelial-like polygonal shaped cells with tight cell–cell adhesions (Fig. 7B). In particular, in LKB1 knockdown cells, further repression of ZEB1 also showed a suppression of cell motility (Fig. 7C). It was confirmed that increased cell motility by LKB1 knockdown was suppressed by ZEB1 repression using another ZEB1 siRNA (Hs_ZEB1_0526 Mission siRNA, Sigma) (data not shown). These results imply that increased

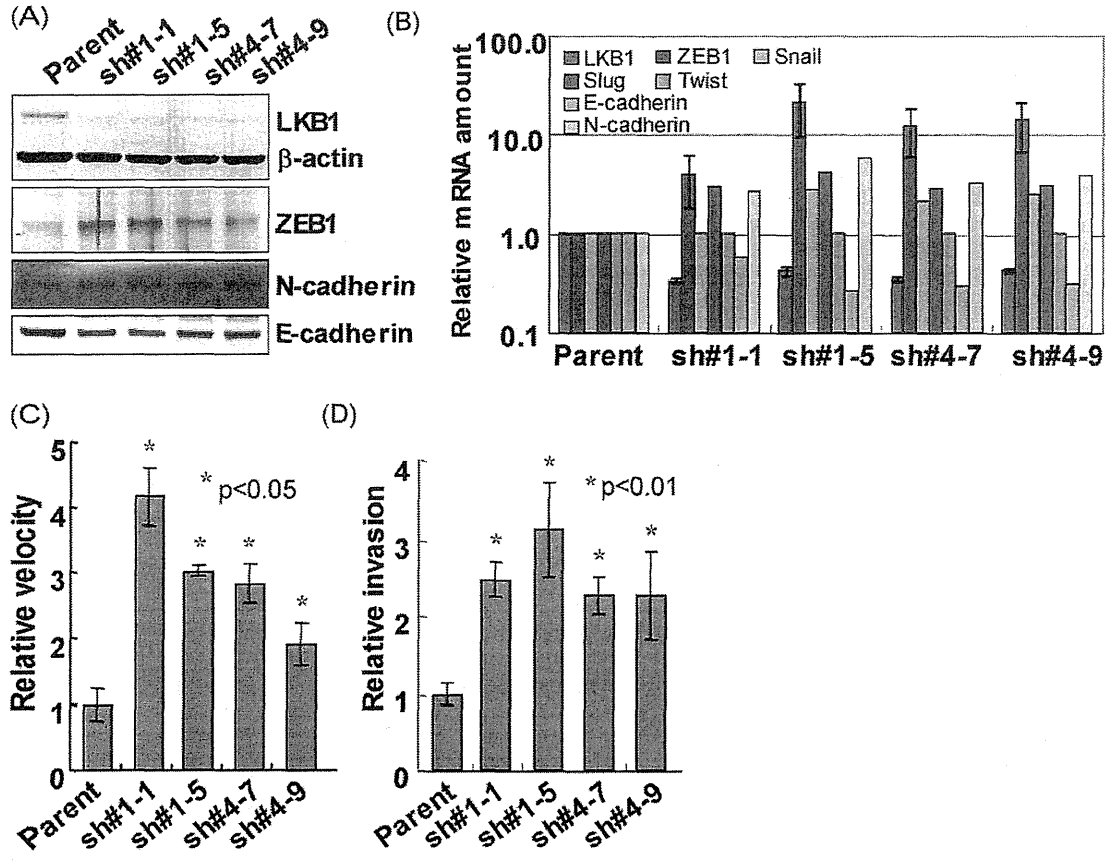

Fig. 5. Epithelial–mesenchymal transition induced by LKB1 knockdown in H358 cells. (A) Western blot analysis for the expression of ZEB1, N-cadherin and E-cadherin, in H358 derived LKB1 knockdown clones. β-actin was used as a loading control. Clones designated sh#1 and sh#4 were generated by targeting vectors pTER-LKB1-sh#1 and pTER-LKB1-sh#4, respectively (see Section 2). (B) Quantitative RT-PCR analysis of mRNAs in H358 cells with and without LKB1 knockdown. Relative mRNA amounts were determined by normalization with the amount of GAPDH mRNA. The mRNA amounts of LKB1 and ZEB1 were obtained from two independent PCR experiments in duplicate and shown as mean ± SD. (C) Motility of H358 cells with and without LKB1 knockdown. Motility is expressed by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios relative to parental cells. (D) Invasiveness of H358 cells with and without LKB1 knockdown. Invasiveness is expressed by the number of migrated cells in each chamber. Data are presented by means ± SD of three independent measurements and expressed as ratios relative to parental cells. Statistical significance for the difference was determined using a Student's two-tailed *t*-test.

cell motility exhibited by LKB1 knockdown was mediated by the induction of ZEB1 expression. Thus, it was concluded that EMT was induced by LKB1 knockdown through the augmentation of ZEB1 expression in H358 cells.

4. Discussion

Inactivation of the p53 and p16 genes is common in lung AdCs, and the majority of AdC cells carry mutations of the p53 gene and either methylation or homozygous deletion of the p16 gene [8]. The LKB1 gene is inactivated in lung cancer generally by either homozygous deletion or hemizygous deletion plus point mutation that results in the absence/truncation of LKB1 protein products, and in most cases, LKB1 inactivation is accompanied by inactivation of both the p53 and p16 genes [10,11]. In this study, to assess the specific contribution of LKB1 inactivation to multistage carcinogenic processes of lung AdC, we first investigated the biological significance of LKB1 inactivation by using a primary hSAEC derived cell line, hSAEC-T1. Introduction of hTERT into primary hSAECs allowed us to obtain the immortalized hSAEC-T1 cell line with inactivation of the p53 and p16 genes and retention of the wild-type LKB1 gene. As a model of LKB1 inactivation in human lung carcinogenesis, LKB1 gene expression was constitutively knocked down in hSAEC-T1 cells. Transition from an epithelial to a mesenchymal phenotype, i.e. EMT, was observed by LKB1 knockdown in hSAEC-T1 cells. Similar phenotypic alterations were also observed by LKB1 knockdown in the H358 lung AdC cell line, in which p53 is homozygously deleted and p16 is methylated [34,35]. The appearance of mesenchymal phenotypes was well correlated with the level of reduced LKB1 expression in two different cell lines, SAEC-T1 and H358, using two different knockdown vectors. Furthermore, such phenotypes were also induced by transient LKB1 knockdown experiments using these two cell lines. Thus, it was strongly indicated that LKB1 inactivation would be one of causative events for the induction of EMT in lung AdC cells.

Several recent studies have implicated the involvement of LKB1 gene inactivation in differentiation, invasion and metastasis of lung cancer cells [10–12,28]. Therefore, the present results are consistent with those findings and further support the involvement of LKB1 inactivation in malignant progression of lung AdC. Previous studies on LKB1 functions using several lung cancer cell lines also showed an increase in invasiveness of the cells in response to LKB1-downregulation. In those studies, invasiveness was indicated to be enhanced through the up-regulation of the PEA3 transcriptional factor or the NEDD9 adaptor protein [12,28]. Therefore, we evaluated the expression levels of PEA3 and NEDD9 in stable and transient LKB1 knockdown hSAEC-T1 and H358 cells. However, significant changes in the amounts of NEDD9 mRNA and those of PEA3 protein were not observed in the LKB1 knockdown cells (data not shown). At present, it is unclear why the mediators of

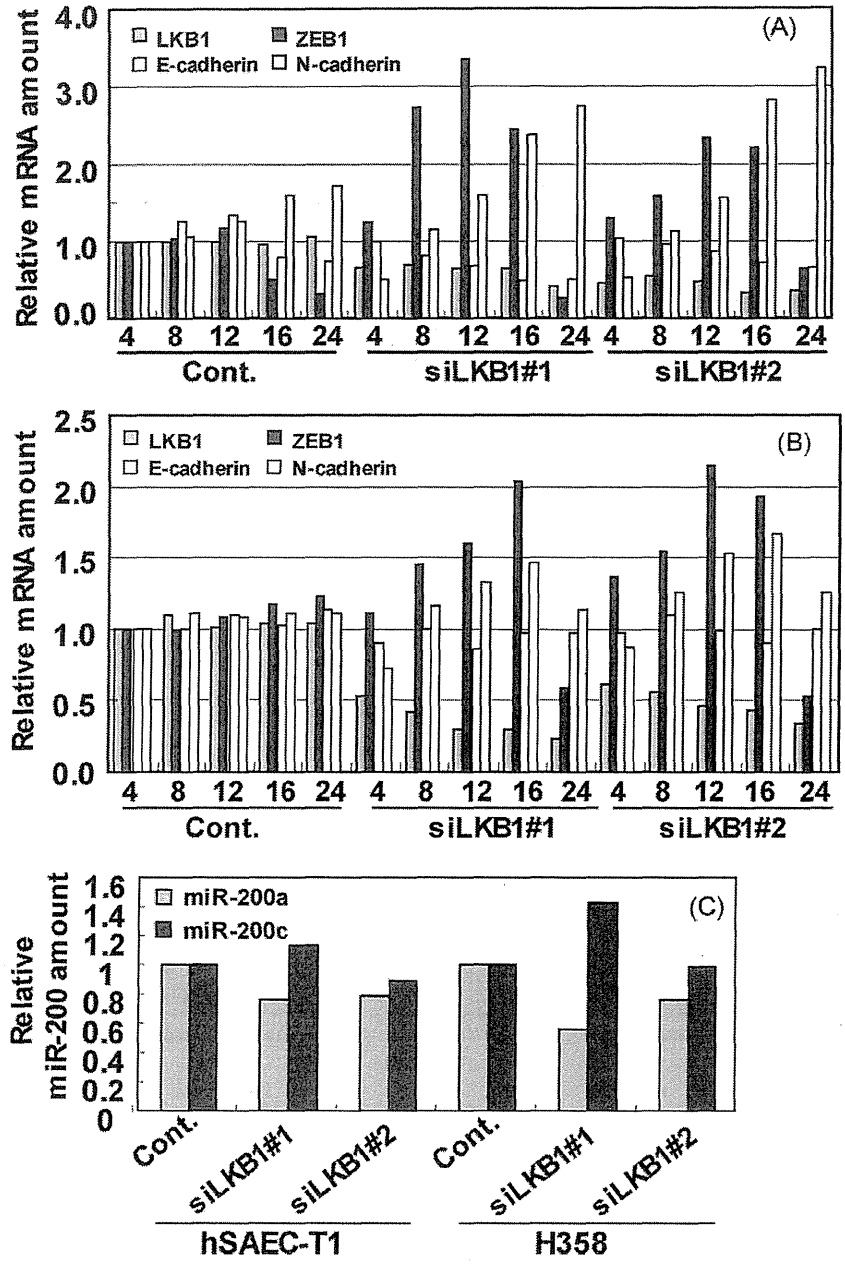

Fig. 6. Enhanced ZEB1 expression by transient LKB1 knockdown in hSAEC-T1 and H358 cells. Quantitative RT-PCR analysis was performed for LKB1, ZEB1, E-cadherin and N-cadherin mRNAs in hSAEC-T1 (A) and H358 cells (B) after LKB1 siRNA transfection. Cells were transfected with a control-siRNA or with two different siRNAs for LKB1, siLKB1#1 and siLKB1#2, respectively. Relative mRNA amounts were determined by normalization with the amount of GAPDH mRNA, and expressed as ratios relative to control samples (4 h after control-siRNA treatment). (C) Relative expression of miR-200a and miR-200c were measured by quantitative RT-PCR at 16 h of post-transfection of siRNAs and expressed as ratios to control samples (16 h after control-siRNA treatment).

LKB1 knockdown induced invasiveness are different among three studies, including the present study. Because LKB1 is not a direct transcriptional factor but a signaling mediator, it is possible that LKB1inactivation could induce mesenchymal phenotypes through multiple pathways in the cells.

EMT is known to be mediated by several transcriptional factors, as described above. Among them, ZEB1 was identified as a major transcription factor induced by stable knockdown of LKB1 expression in both hSAEC-T1 and H358 cells (Figs. 3 and 5). In addition, miR200a and miR200c, which are known to repress ZEB1 expression, were downregulated by LKB1 knockdown in both cells. Thus, acquisition of mesenchymal phenotypes in LKB1 knockdown stable clones might be due to feedforward loop between ZEB1 and miRNAs. Transient LKB1 knockdown studies indicate that the induction of ZEB1 seems to be an earlier event than miR-200a/c repression in the process of EMT induction (Fig. 6), and ZEB1 induction might remain throughout the completion of EMT and ultimately affect the expression of miR-200a/c. This phenomenon seems convincing as transient knockdown of LKB1 in hSAEC-T1 and H358 cells enhanced ZEB1 expression and slightly reduced miR-200a expression, whereas in LKB1 knockdown stable clones, derived from both hSAEC-T1 (Figs. 3 and 4 and Supplementary Fig. S2) and H358 cells (Fig. 5), expression of ZEB1 was increased and that of miR-200a/c was decreased significantly. The same is true for the expression of N-cadherin and E-cadherin; transient knockdown of LKB1 leads to an increase in N-cadherin expression and

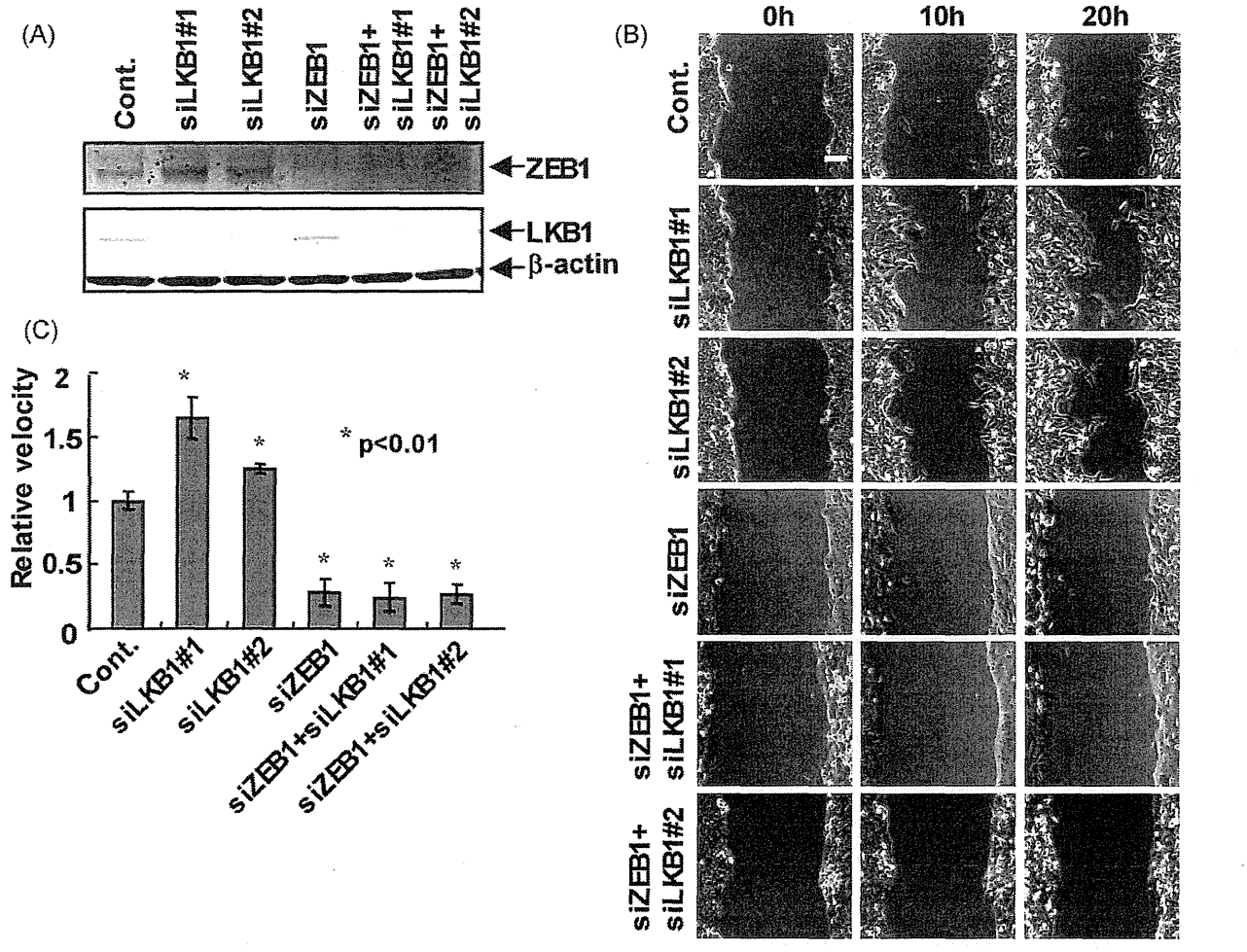

Fig. 7. EMT is dependent on ZEB1 expression in H358 cells.
(A) Western blot analysis for ZEB1 and LKB1 in H358 cells after 72 h of LKB1 and/or ZEB1 siRNA transfection. Two siRNAs for LKB1, siLKB1#1 and siLKB1#2, were used for LKB1 knockdown. β-actin was used as a loading control. (B) Wound healing assay of H358 cells treated with LKB1 and/or ZEB1 siRNA. Representative cells at the wounded edges after 0, 10 and 20 h of wounding are photographed. (C) Motility shown by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios to control (control-siRNA treated). Bar, 100 μM.

a slight decrease in E-cadherin expression, whereas in stable LKB1 knockdown clones, expression of N-cadherin and E-cadherin significantly increased and decreased, respectively (Figs. 3 and 5 and Supplementary Fig. S2). Thus, the induction of ZEB1 could be a primary effect and reduction of miR-200a/c could be a secondary effect of LKB1 inactivation. Recent studies have demonstrated that EMT is associated with breast cancer stem cell function and that downregulation of miRNA-200c is important for tumorigenicity of human breast cancer stem cells [36,37]. It will be interesting to see if LKB1 depletion induces the stem cell-like properties in lung cancer cells.

On the other hand, stable expression of LKB1 did not induce a phenotypic alteration resembling a reversion of EMT, i.e. MET, in lung AdC cell lines with LKB1 inactivation, A549 and H23 (unpublished results). This result is consistent with earlier gene expression studies using exogenous LKB1-expressed cells [12,21]. Moreover, transient knockdown of LKB1 in LKB1 wild-type lung cancer cell lines, such as PC9, H820 and H2009, induced ZEB1 expression, but the magnitude of ZEB1 induction varied among the cell lines. The reason for such a difference is uncertain at present, but could be due to the difference in accumulated genetic alterations in the cells. Indeed, there was no significant correlation between LKB1 status and E-cadherin expression in 33 lung AdC cell lines (Supplementary Fig. S4), although the mean expression level of E-cadherin in LKB1 wild-type cell lines was slightly higher than that in LKB1 mutated cell lines. Thus, various degrees of mesenchymal phenotypes are acquired among lung cancer cell lines, and the acquisition of mesenchymal phenotypes cannot be explained only by LKB1 inactivation. Several other genetic alterations could also induce EMT in lung AdC cells. Therefore, further studies using immortalized lung epithelial cells would be a strong strategy of elucidating molecular pathways of EMT in lung AdC cells. As described above, LKB1 is not a transcriptional factor but a signaling mediator; therefore, loss of LKB1 might induce ZEB1 expression through several other mediators. Therefore, it seems possible that overexpression of LKB1 could not completely reproduce the reverse course of loss-of-function phenotype and that uncertain genetic alterations in the downstream of LKB1 might also affect the EMT induction in lung AdC cells. Recent studies indicated that LKB1 is a regulator of molecules in several signaling pathways, including those of AMPK-related kinases and Cdc42-PAK signals, in cancer cells [38–40]. One hypothesis is that one of the AMPK-related kinases or a Cdc42-PAK pathway protein induces the transcription of ZEB1 by LKB1 depletion. Further studies to elucidate how LKB1 intermediates and regulates ZEB1 expression will provide deep understanding of EMT induction in lung AdC cells.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.02.004.

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