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Overexpression of MAGEA2 has a prognostic significance and is a potential therapeutic target for patients with lung cancer

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Abstract. Melanoma-associated antigens (MAGE) are expressed in different type of cancers including lung cancer and have been shown to be functionally related to p53 tumor suppressor gene. Little is known about the relationship between MAGE genes and p53 aberrant expression in lung cancer. The aims of this study were to observe the expression of MAGEA2, examine the role of MAGEA2 in lung cancer survival, investigate its correlation between MAGEA2 and p53, and explore its clinicopathologic significance as a prognostic marker. Quantitative reverse transcription-polymerase chain reaction was performed to detect the expression of MAGEA2 using 36 primary tumors and 31 metastatic lymph nodes from patients with lung cancer. The role of MAGEA2 in cancer cell growth and in the regulation of p53 downstream genes were examined using small interfering RNA. The expression of MAGEA2 and p53 were analyzed immunohistochemically using tissue microarray from 353 resected lung specimens. High-level expression of MAGEA2 (High-MAGEA2) was confirmed in lung tumors with high frequency. Inhibiting MAGEA2 expression effectively suppressed cancer cell growth and decreased the expression of p53 downstream target genes *in vitro*. In adenocarcinoma, High-MAGEA2 was strongly

associated with aberrant p53 expression ($P < 0.001$) and was associated with worse clinical outcomes (5-year OS, 87.1% in low vs. 74.1% in high, $P = 0.014$). Aberrant p53 expression was also significant worse prognostic factor ($P = 0.029$). Among the adenocarcinoma patients with wild-type p53, High-MAGEA2 had poorer prognosis than low-level MAGEA2 groups (5-year OS, 90.1% vs. 72.1%, $P = 0.037$), whereas had no difference in p53 aberrant tumors. On multivariate analysis, MAGEA2 was independently associated with survival (hazard ratio; 2.12, $P = 0.030$). In conclusion, suppression of MAGEA2 in lung cancer cells significantly reduced the growth/survival of cancer cells. High-MAGEA2 was identified as an independent prognostic factor in lung adenocarcinoma. Specific inhibition of MAGEA2 may be a promising therapeutic strategy for patients with lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). According to the results from the National Lung Screening Trial, an increase in the detection and treatment of early stage lung cancer is expected (2). However, despite curative-intent surgical resection, tumor recurrence and metastasis remain the primary causes of cancer-related death even among patients with early stage lung cancer (3). Furthermore, lung cancer is still usually not detected until it is at an advanced stage, which makes it more challenging to treat due to high frequency of metastasis. The 5-year survival for patients with regional lymph node (LN) metastasis shows poor prognosis (1,4). Inadequacy of major improvements in the survival rate for lung cancer in spite of advances in surgery, chemotherapy, and radiotherapy has driven a search for new strategies aimed at improving lung cancer management and treatment, which requires a better understanding of lung cancer biology. In an effort to identify relevant molecular targets for diagnosis and/or treatment of lung cancer (5,6). We analyzed expression profiles of our previously performed microarray using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) samples (7) and various types of database (8). Confirmatory quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed against 122 possible candidate genes using tumor samples

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Abbreviations: ADC, adenocarcinoma; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; H&E, hematoxylin and eosin; HR, hazard ratio; IHC, immunohistochemical; LCC, large cell carcinomas; LN, lymph node; MAGE, melanoma-associated antigens; NSCLC, non-small cell lung cancer; OS, overall survival; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SCC, small cell lung cancers; siRNA, small interfering RNA; SqCC, squamous cell carcinomas; TMA, tissue microarrays

Key words: biomarker, EBUS-TBNA, lung cancer, MAGEA2, p53, therapeutic target

achieved by EBUS-TBNA (8). These genes are i) overexpressed in the majority of EBUS-TBNA samples taken from advanced lung cancer cases, ii) overexpressed in at least one lung cancer cell line for RNAi screening, and iii) expressed most in the testis and less expressed in other human vital organs, which provides further evidence supporting these genes as promising molecular targets.

Throughout these screenings, we selected melanoma-associated antigens (MAGE) family member A2 (MAGEA2) as a possible candidate therapeutic gene of lung cancer. MAGE genes were initially discovered as cancer-associated antigens in melanoma patients (9) and are now known to comprise of a super-family of more than 60 genes in humans (10,11). This family of genes has been shown to be overexpressed in a variety of cancers including non-small cell lung cancer (NSCLC) and rarely expressed in normal human organs, therefore, they are potential biomarkers for early diagnosis and screening of lung cancer (12-16). MAGE family member A (MAGEA) has also been used as immunogenic target gene, and for adjuvant immunotherapy using vaccination with MAGEA3 fusion protein in resected MAGEA3 positive lung cancer, which is currently ongoing Phase III clinical trials (17,18).

Compelling evidence has been reported that MAGEA2 blocks the association between the potent tumor suppressor p53 and its cognate sites on chromatin, silencing the downstream p53-dependent transactivation during tumor development (19). MAGEA2 also binds histone deacetylase 3, suppresses p53-dependent apoptosis in response to chemotherapeutic drugs, decreases cellular senescence and increases cell proliferation (20,21). However, genetic p53 mutations are found in approximately 50% of NSCLC and 70% of small cell carcinoma (SCC) (22). Although it is recognized that the functional loss of p53 has an established role in the lung carcinogenesis, other multiple mechanisms of activation and inactivation are thought to also contribute to the progression of lung cancer (23-25). Currently, little is known about the relationship between MAGEA genes and p53 aberrant expression in lung cancer.

In the present study, we examined the expression of MAGEA2 in human lung cancer, and the role of MAGEA2 in cancer cell growth and/or survival using lung cancer cell lines with p53 mutation. We then examined its clinicopathologic significance and evaluated MAGEA2 expression as a prognostic biomarker according to histology and p53 expression. Our results demonstrate the functional role of MAGEA2 in p53 aberrant lung tumors and the therapeutic and prognostic potential of targeting MAGEA2 for lung cancer.

Materials and methods

Lung cancer clinical EBUS samples and tissue samples. Thirty-one samples obtained via EBUS-TBNA from metastatic LNs in patients with advanced lung cancers, including 13 lung adenocarcinomas (ADCs), eight lung squamous cell carcinomas (SqCCs), five lung large cell carcinomas (LCCs), and five small cell lung cancers (SCCs) were used (Toronto General Hospital, Toronto, ON, Canada, study number: 11-0109-CE). Thirty-six of the primary lung cancer samples, including 23 lung ADCs and 13 lung SqCCs, paired with normal lung tissue were also used in this study, and a total

RNA of 21 normal human tissues (Human Total RNA Master Panel II) were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA) to detect MAGEA2 expression in normal tissue distribution. A total of 353 lung cancer and adjacent normal lung tissue samples to be used for immunohistochemical (IHC) staining on tissue microarrays (TMA) and additional statistical analysis were obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals with informed consent (26-28). These were consecutive patients with lung cancer who underwent surgical lobectomy or pneumonectomy. Detailed clinical and pathological information was collected retrospectively for all patients.

Histopathological examination of resected tumors revealed that ADC was the dominant histological group (241 cases), followed by 89 cases of SqCC, 19 cases of LCC, and 4 cases of SCC. Postoperative staging evaluation identified stage IA disease in 120 cases, stage IB disease in 108 cases, stage IIA disease in 38 cases, stage IIB disease in 27 cases, stage IIIA disease in 57 cases, and stage IIIB disease in 3 cases. Follow-up lasted through October 30, 2009, with a median follow-up period of 60.5 months for living patients (range, 48.1-165.8 months). The primary end point was overall survival as measured from the date of surgery to the time of death of the patients. All specimens were fixed in formalin and embedded in paraffin wax. Representative blocks were selected (based primarily on greatest dimensions of each tumor), and serial 4- μ m-thick sections were examined by immunohistochemistry. Histological diagnoses of tumors were based on the 2015 World Health Organization Classification (29). All tumors were staged according to the pathological tumor/node/metastasis (pTNM) classification (7th edition) of the American Joint Committee on Cancer (30). All available hematoxylin and eosin (H&E) stained tumor slides were histologically reviewed by a pathologist.

Lung cancer cell lines. The human lung cancer cell lines used in this study were as follows: lung ADC NCI-H1437; lung LCC H661; and lung SCC SBC-5. All cancer cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% CO₂.

Specimen handling and EBUS-TBNA sample preparation. EBUS-TBNA was performed as previously described (31). Briefly, a dedicated 22-gauge needle equipped with an internal stylet was used (NA-201SX-4022; Olympus, Japan). Multiple passes were performed from the target LNs. After confirmation of adequate sampling for cytological evaluation, an additional pass was performed for the preservation of RNA. The aspirate was mixed with Allprotect Tissue Reagent® (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at -80°C. The samples were equilibrated to room temperature, and the stabilizing solution was completely removed. QIAzol Lysis Reagent (Qiagen) and one 5-mm stainless steel bead (Qiagen) were added before homogenizing with a TissueLyser Adapter Set (Qiagen) for 2 min at 20 Hz. Total RNA was then purified using a miRNeasy mini kit (Qiagen). The amount and purity were measured using a spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA).

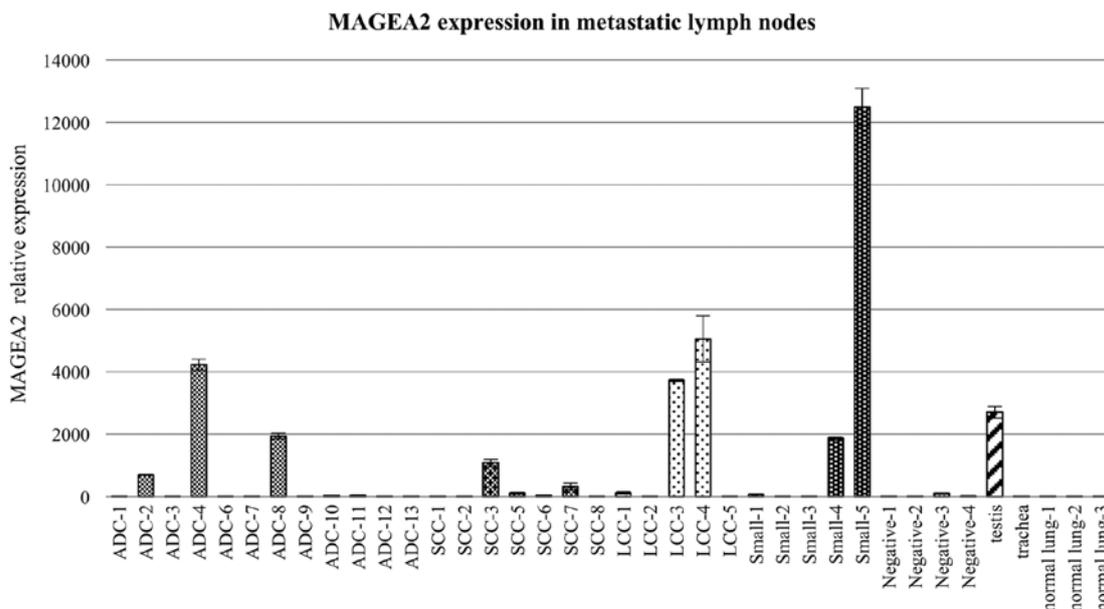


Figure 1. Expression of MAGEA2 in metastatic lymph nodes samples. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis in metastatic lymph node samples from advanced lung cancer. The relative expression levels were normalized to the ACTB level in each sample and calculated as the threshold cycle value in each sample divided by the average threshold cycle values in normal lung. Error bars represent the standard error of the mean (SEM) of duplicate. ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, small cell carcinoma; Negative; no malignancy lymph node samples.

Quantitative RT-PCR analysis. The cDNA was synthesized from 2 μ g total RNA using QuantiTect[®] Reverse Transcription kit (Qiagen). The primers were designed as follows: for MAGEA2, A2B, forward primer, 5'-gggacaggctgacaagtagg-3', and reverse primer 5'-ttgcagtgtgactcctctg-3'; for p21, forward primer, 5'-gcagaccagcatgacagattt-3' and reverse primer 5'-ggattagggtctctcttggg-3'; for MDM2, forward primer, 5'-ggatttcggacggctctcgc-3' and reverse primer 5'-cgcgcagcgttcacactactg-3'; for actin, β (ACTB), forward primer, 5'-gaaatcgtgcgtgacattaa-3', and reverse primer, 5'-aaggaaggctggaagagtg-3'. qRT-PCR analysis was performed using LightCycler 480[®] SYBR Green I Master Ready-to-use hot start reaction mix and LightCycler 480 system (Roche, South San Francisco, CA, USA). The thermal cycler conditions were as follows: 5 min at 95°C for denaturation, 45 cycles at 95°C for 10 sec, 56°C for 10 sec, and 72°C for 10 sec for PCR amplification, and 1 min at 65°C for melting. The threshold cycle value was defined as the value obtained in the PCR cycle when the fluorescence signal increased above the background threshold. PCR reactions were carried out in duplicates.

RNA interference and cell viability assay. All siRNA oligonucleotide sequences for this study were purchased from Qiagen. Negative Control siRNA and AllStar[®] Negative Control siRNA (Qiagen) were used as the negative control (NC-siRNAs-#1, -#2). siRNAs with a final concentration of 5-10 nM were incubated with HiPerFect[®] Transfection Reagent (Qiagen) according to the manufacturer's instructions. The CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used for the evaluation of the number of viable cells according to the manufacturer's instructions, and measured using a microplate spectrophotometer (μ Quant; Bio-Tek Inc., Winooski, VT, USA). Each experiment was performed in triplicates.

Tissue microarray construction and immunohistochemistry. Tissue areas for sampling were selected based on visual alignment with the corresponding H&E stained sections on slides. A core (diameter, 2 mm; height, 3-5 mm) taken from each donor-tumor block was placed into a recipient block using a tissue microprocessor (Azumaya Medical Instruments, Tokyo, Japan). MAGEA2 [anti-MAGEA2 antibody (ab64894), Abcam Japan, Tokyo, Japan] and p53 immunostaining [DO-7 anti-human p53 mouse monoclonal antibody (Dako Japan, Tokyo, Japan)] were performed using an automated IHC platform (Autostainer Plus, Dako, Carpinteria, CA, USA). Antigen retrieval was performed in pH 9.0 for 20 min. EnVision[™]+Dual Link (K4063, Dako) was used for detection, with post-primary incubation for 60 min at room temperature. A polymer-based detection system (EnVision[™]+Dual Link #K4063, Dako) was used with 3', 3-diaminobenzidine (DAB) as the chromogen. The positive control included a sample of testis, and normal lung samples were used as negative controls. Slides were dehydrated and placed on coverslips.

Evaluation of immunohistochemical staining. Digital images of IHC-stained TMA slides were obtained at x20 magnification using a whole slide scanner (ScanScope CS, Aperio ePathology; Leica Microsystems Inc., ON, Canada). Images were saved in SVS format (Aperio) and annotation of tumor regions on whole slides was performed blinded to clinical follow-up data using Aperio annotation software (ImageScope Viewing Software, Positive Pixel Count version 9.1, Aperio) (32). MAGEA2 was quantified by IHC scoring, which summated the percentage of area stained at each intensity level multiplied by the weighted intensity (0, 1, 2, or 3) reported in other studies (33-36). Initially, the weighted intensity of staining was graded as follows; grade 0 (negative), 1+ [weak positive: intensity Threshold Weak (upper limit)=240, (lower limit)=220],

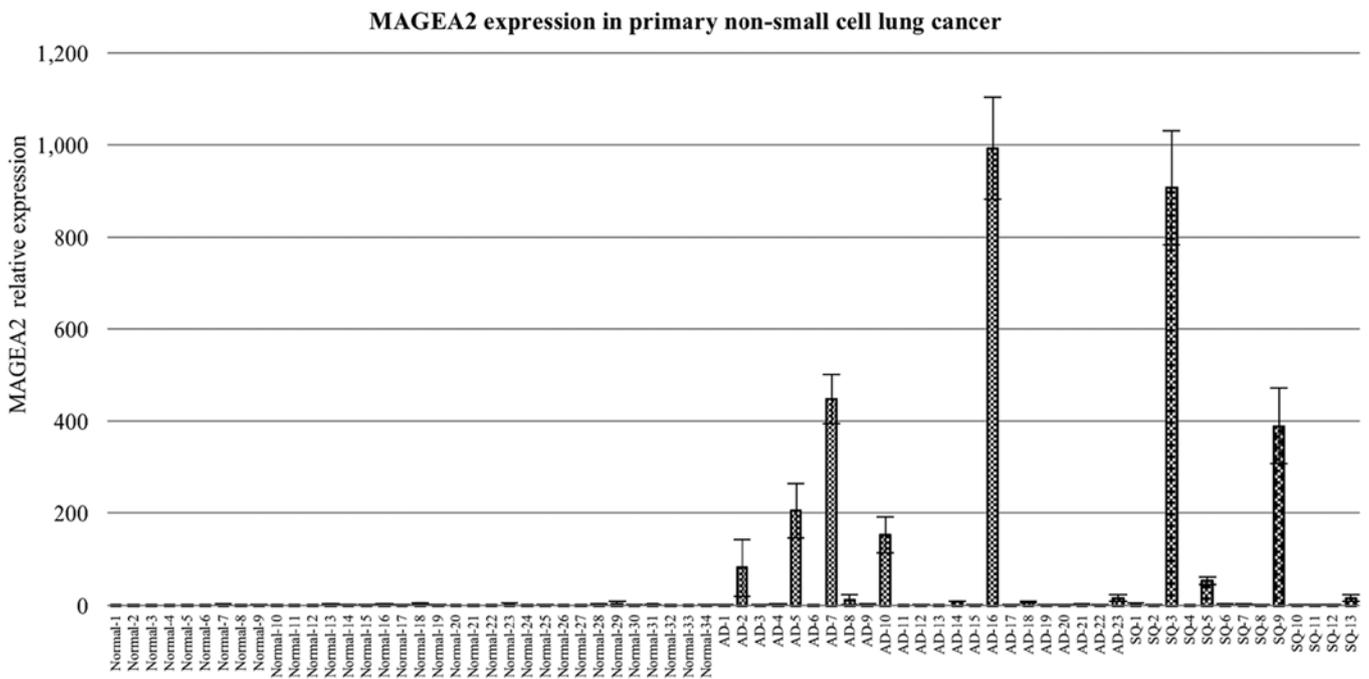


Figure 2. Expression of MAGEA2 in primary non-small cell lung cancer (NSCLC). Mean messenger RNA expression as measured by means of qRT-PCR in primary tissue from paired 34 lung cancer and normal tissue were used for this analysis. Normal, normal lung; AD, adenocarcinoma; SQ, squamous cell carcinoma.

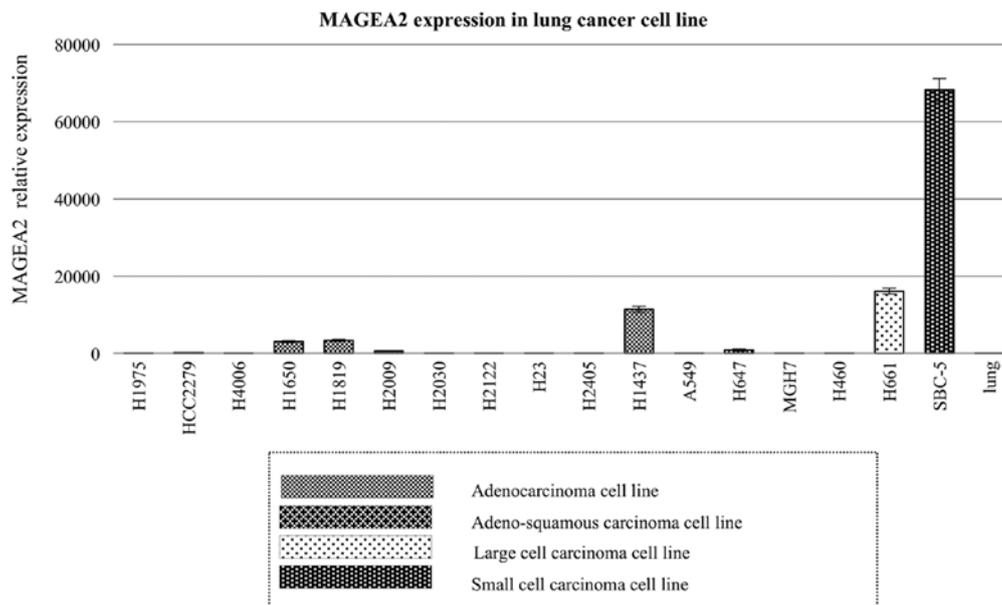


Figure 3. MAGEA2 expression in lung cancer cell lines. Mean messenger RNA expression as measured by means of qRT-PCR in primary tissue from 17 lung cancer cell lines and normal lung were used for this analysis.

2+ [moderate positive: Medium (upper)=220, (lower)=180], and 3+ (strong positive: Strong (upper)=180, (lower)=0) according to Aperio annotation software). According to the total amount of IHC scores, MAGEA2 was dichotomized into high versus low using optimal cut-points, which were found using a maximally selected log-rank statistics: low-level MAGEA2 expression (Low-MAGEA2, with an IHC score <0.65) and high-level MAGEA2 expression (High-MAGEA2, with an IHC score ≥0.65). For p53 evaluation, each core was scored semi-quantitatively for the degree of positive nuclear expression in tumor

cell under a high-power field (magnification, x200). Percentage of positive nuclear expression was calculated. p53 IHC was defined as ‘aberrant expression’ if tumor cells showed either nuclear expression in >50% or complete absence of staining and as ‘wild-type expression’ if tumor cells showed no aberrant expression (1-50% staining) (36-39).

Statistical analysis. We attempted to correlate clinicopathological variables such as age, gender, pathological TNM stage, pleural invasion, histological classification, and smoking

Table I. Patient characteristics according to MAGEA2 and p53 expression levels for all patients.

Patient characteristics	MAGEA2 expression					p53 expression				
	Low		High		P-value	Wild-type		Aberrant		P-value
	N	%	N	%		N	%	N	%	
All patients	132	37	221	63		176	50	177	50	
Age (years)					0.908					1.000
<60	46	38	76	62		61	50	61	50	
≥60	86	37	145	63		115	50	116	50	
Gender					0.818					0.436
Female	47	38	76	62		65	53	58	47	
Male	85	37	145	63		111	48	119	52	
Smoking history					0.642					0.056
Never-smoker	42	35	77	65		68	57	51	43	
Smoker	90	38	144	62		108	46	126	54	
Histological classification					0.556					0.341
ADC	95	39	146	61		127	53	114	47	
SqCC	30	34	59	66		41	46	48	54	
LCC	5	26	14	74		7	37	12	63	
SCC	2	50	2	50		1	25	3	75	
pT factor					0.309					0.029
pT1	46	34	90	66		78	57	58	43	
pT2-4	86	40	131	60		98	45	119	55	
pN factor					0.374					0.176
pN0	103	39	162	61		138	52	127	48	
pN1-2	29	33	59	67		38	43	50	57	
Pathologic stage					0.054					0.041
Stage I	85	37	143	63		120	53	108	47	
Stage II	31	48	34	52		35	54	30	46	
Stage III	16	27	44	73		21	35	39	65	
Pleural invasion					0.109					0.100
Absent	75	34	144	66		117	53	102	47	
Present	57	43	77	57		59	44	75	56	
p53 expression					0.001					
Wild-type	96	55	80	45		-		-		
Aberrant	36	20	141	80		-		-		

ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; SCC, Small cell carcinoma; CI, confidence interval. Significant P-values (<0.05) are shown in bold. P-value was analyzed using the Pearson's χ^2 test.

history with expression levels of MAGEA2 protein and aberrant p53 as determined by TMA analysis. MAGEA2 and aberrant p53 immunoreactivities were assessed for association with clinicopathologic variables using the χ^2 test for variables. Overall survival (OS) was estimated using the Kaplan-Meier method starting at the time of surgery. Patients who survived during study follow-up were censored at the last time they were known to be alive. Differences in OS between patient subgroups were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model to estimate the effect of markers of interest on OS, with adjustments for the clinicopathologic factors

that were found to be significantly associated with OS in univariate analyses. All significance tests were two-sided and used a 5% level of significance. *In vitro* experiments, tumor treated with MAGEA2 vs. no transfection were analyzed by paired Student's t-test. Statistical analyses were conducted using the 'survival' and 'maxstat' packages in R (version 3.0.1; R Development Core Team).

Results

Transcriptional expression of MAGEA2 in lung tumors and normal tissues. Expression of MAGEA2 was significantly

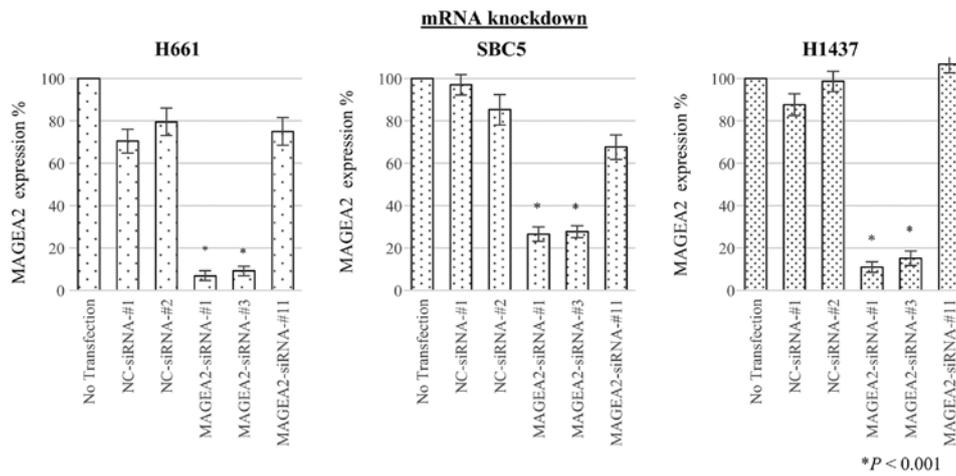


Figure 4. Effects of MAGEA2 short interfering RNA (siRNA) on lung cancer cell proliferation *in vitro*. Effects of siRNA on mRNA expression levels. qRT-PCR analysis of gene expression in each lung cancer cell lines with p53 mutation (H661, SBC5 and H1437) treated with negative control siRNAs (NC-siRNA-#1, #2) and different gene-specific siRNAs. mRNA levels of each cancer cell line transfected with independent siRNAs targeting MAGEA2 gene was significantly decreased in comparison with cells transfected with control siRNAs 48 h after transfection. Results shown are mean ± SEM (bars) (*P<0.001, paired Student's t-test).

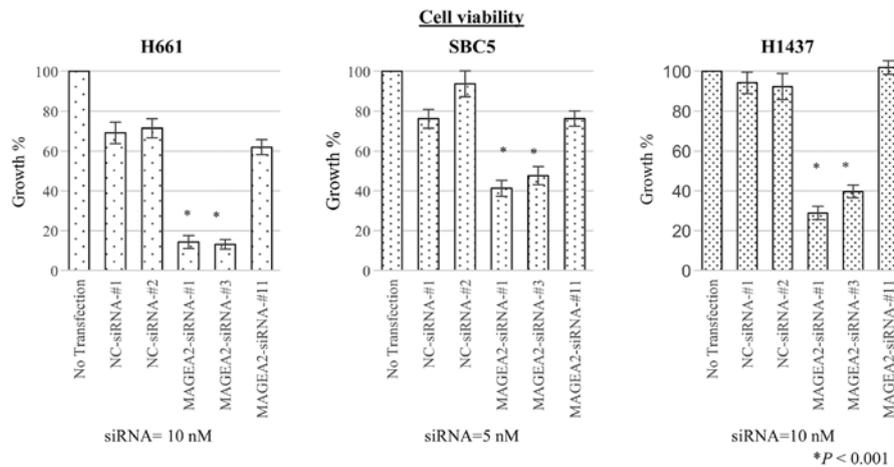


Figure 5. Effect of each siRNA on lung cancer cell proliferation *in vitro*: Cells were treated with siRNAs for 96 h, and cell viability was determined using cell proliferation assay. After siRNA treatments, the proliferation in MAGEA2 gene cells was evaluated compared with control groups at 4 days after transfection, resulting in the identification of potential MAGEA2 therapeutic candidate genes. Gene knockdown in lung cancer cell lines identified cell viability inhibition following knockdown of MAGEA2 gene. Results shown are mean ± SEM (bars) of three experiments (*P<0.001, paired Student's t-test).

higher in metastatic lymph node samples obtained from patients with advanced lung cancer with EBUS-TBNA compared to those taken from healthy individuals and non-malignant (negative) LN tissues (Fig. 1). MAGEA2 expression was also higher in primary tissue from 34 lung cancer samples taken, from patients who underwent surgery, of which 23 had lung ADCs and 13 had lung SqCCs, compared to paired normal lung tissue (Fig. 2). qRT-PCR analysis using cDNA panel containing normal human tissues also identified MAGEA2 as being expressed only in the testis with almost no expression in the other vital organs (data not shown). We also confirmed high expression levels of MAGEA2 using 17 lung cancer cell lines (Fig. 3). This step also allowed identification of relevant cell lines for RNAi experiments.

Growth inhibition of lung cancer cells by specific siRNA against candidate genes. To assess whether MAGEA2 is essential for growth of lung-cancer cells, we transfected 3 different types

of target-specific siRNAs against MAGEA2 (MAGEA2-siRNAs-#1, #3, #11) as well as two different negative control siRNAs (NC-siRNAs-#1, -#2) into H661, SBC5, and H1437 lung cancer cell lines. qRT-PCR showed that the mRNA levels of each cancer cell transfected with two of the three siRNAs targeting MAGEA2 gene (MAGEA2-siRNAs-#1 and -#3) were significantly decreased in comparison with cells transfected with control siRNAs 48 h after transfection (Fig. 4). Next, to evaluate the effect of MAGEA2 gene knockdown on cell proliferation, we conducted a cell viability assay. Proliferation of lung cancer cells transfected with these two siRNAs was significantly suppressed compared to control groups at 4 days after transfection, suggesting that upregulation of MAGEA2 is associated with survival of lung cancer cells (Fig. 5).

MAGEA2 regulates p53 targets (BAX, CDKN1A, and MDM2) in lung cancer cell lines. To determine the effects of regulating MAGEA2 on the expression level of 3 main p53

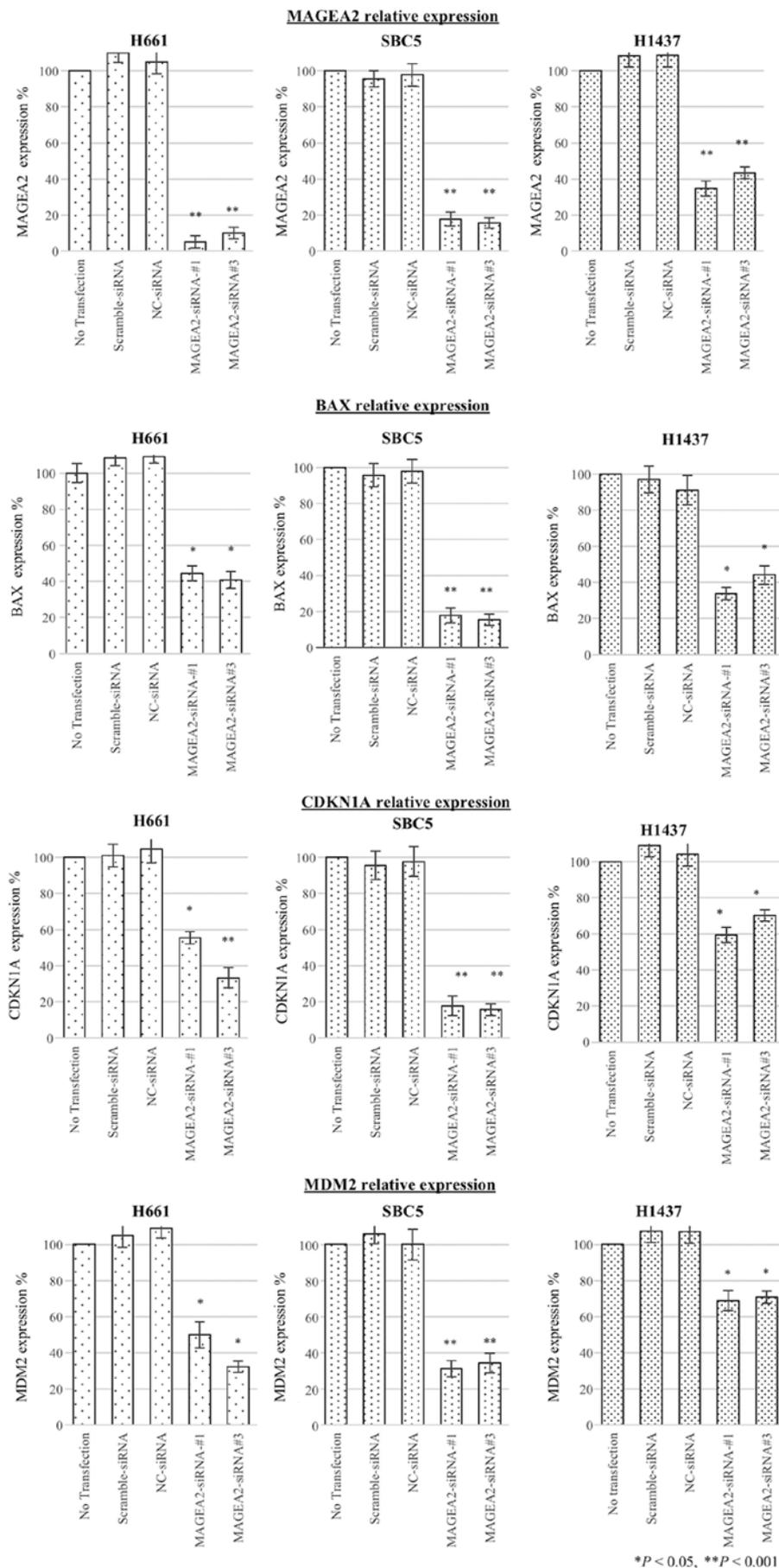


Figure 6. Expression of p53 downstream targets after suppression of MAGEA2 in lung cancer cells. MAGEA2, BAX, CDKN1A, and MDM2 expression adjusted to % of no transfection. Each gene were significantly downregulated in each lung cancer cell line (H661, SBC5 and H1437). Results shown are mean \pm SEM (bars) (*P<0.05, **P<0.001, paired Student's t-test).

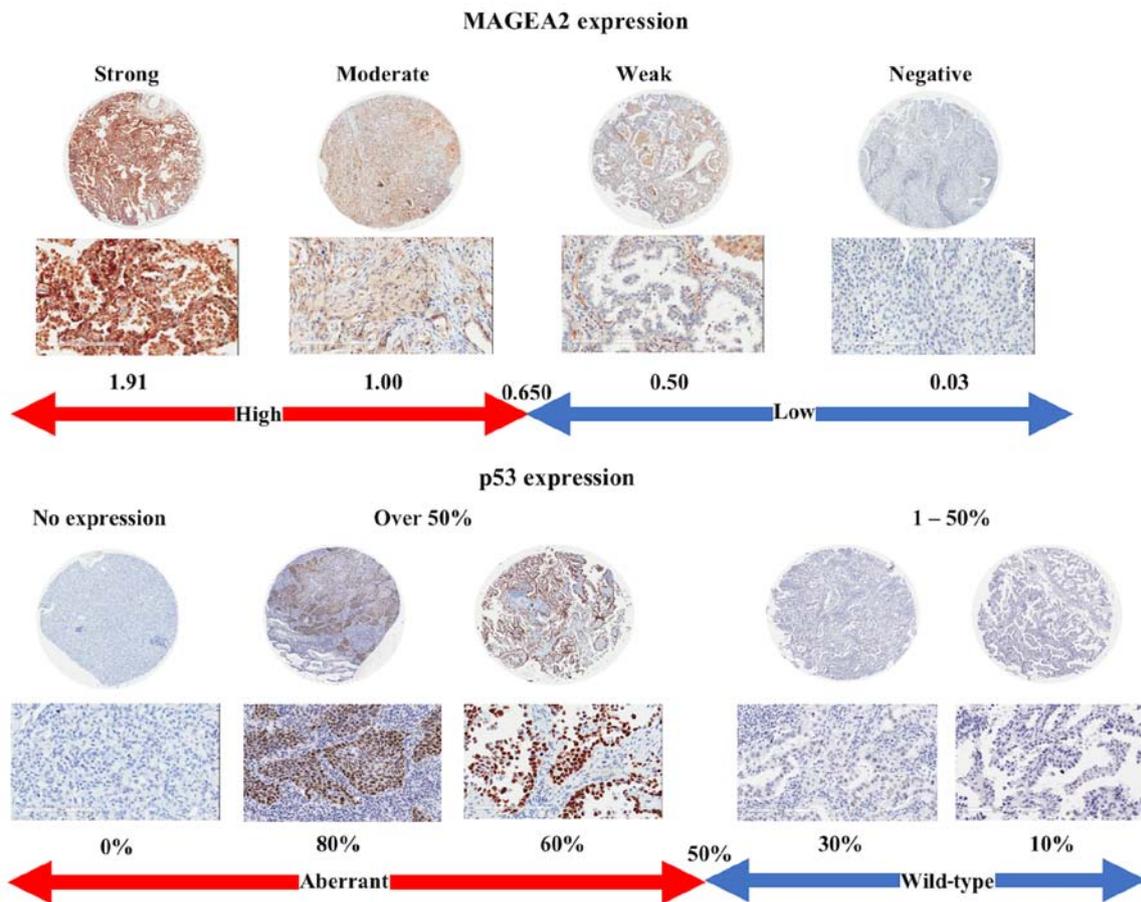


Figure 7. Representative examples of MAGEA2 protein expression in lung adenocarcinoma (ADC). Intensity and proportion scores were multiplied together to obtain the immunohistochemical (IHC) score. MAGEA2 expression was defined as ‘High expression’ if IHC score is greater than 0.65 and as ‘Low expression’ if IHC score is <0.65. Expression of aberrant p53 in lung cancer nucleus. Percentage of positive nuclear expression was calculated. p53 IHC was defined as ‘aberrant expression’ if tumor cells showed either nuclear expression in >50% or complete absence of staining and as ‘wild-type expression’ if tumor cells showed no aberrant expression (1-50% staining).

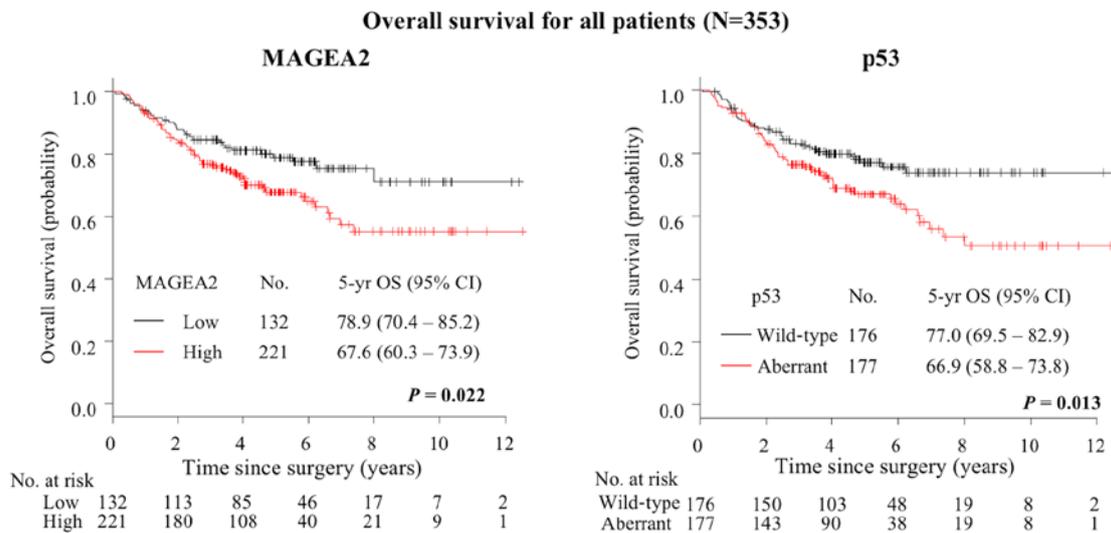


Figure 8. Expression of MAGEA2 and p53 protein and prognostic impact on lung cancers. Kaplan-Meier analysis of overall survival in all patients according to MAGEA2 and aberrant p53 expression level and combination of MAGEA2 and aberrant p53. The 5-year overall survival (OS) rate was 78.9% for patients with low expression of MAGEA2 (n=132), whereas 67.6% for patients with high expression of MAGEA2 (n=221) (P=0.022). The 5-year OS rate was 77.0% for patients with wild-type p53 expression (n=176), and 66.9% for patients with aberrant p53 expression (n=177) (P=0.013).

downstream target genes, BAX, CDKN1A, and MDM2, we performed qRT-PCR after transient transfection of siRNA

against MAGEA2 in lung cancer cell lines with p53 mutation (H661, SBC5, H1437). Subsequently, inhibition of MAGEA2

Table II. Patient characteristics according to MAGEA2 and p53 expression levels for adenocarcinoma.

Patient characteristics	MAGEA2 expression				P-value	p53 expression				P-value
	Low		High			Wild-type		Aberrant		
	N	%	N	%		N	%	N	%	
All patients	95	39	146	61		127	53	114	47	
Age (years)					0.687					0.513
<60	36	38	60	63		48	50	48	50	
≥60	59	41	86	59		79	54	66	46	
Gender					1.000					0.158
Female	44	40	67	60		64	58	47	42	
Male	51	39	79	61		63	49	67	52	
Smoking history					0.425					0.056
Never-smoker	38	36	67	64		63	60	42	40	
Smoker	57	42	79	58		64	47	72	53	
pT factor					1.000					0.027
pT1	41	40	62	60		63	61	40	39	
pT2-4	54	39	84	61		64	46	74	54	
pN factor					0.874					0.161
pN0	75	40	113	60		104	55	84	45	
pN1-2	20	38	33	62		23	43	30	57	
Pathologic stage					0.315					0.065
Stage I	68	40	102	60		94	55	76	45	
Stage II	16	47	18	53		20	59	14	41	
Stage III	11	30	26	70		13	35	24	65	
Pleural invasion					0.412					0.284
Absent	57	37	96	63		85	56	68	44	
Present	38	43	50	57		42	48	46	52	
p53 expression					<0.001					
Wild-type	78	61	49	39		-	-	-	-	
Aberrant	17	15	97	85		-	-	-	-	

Significant P-values (<0.05) are shown in bold. P-value was analyzed using the Pearson's χ^2 test.

induced a decrease in messenger RNA expression of these p53 downstream target genes, suggesting MAGEA2 was able to stimulate p53 transactivation function (Fig. 6).

Pattern of MAGEA2 and p53 expression and its clinicopathological correlation. To determine the clinical relevance of the MAGEA2, we assessed MAGEA2 protein and aberrant p53 expression using TMA analysis, MAGEA2 and p53 expression were categorized as previously described (34-36). The representative staining and its IHC score of lung ADC cases are shown in Fig. 7. Positive staining of MAGEA2 in tumor cells generally showed a cytoplasmic pattern in cancer tissue. Of the 353 lung cancer cases examined, High-MAGEA2 was observed in 221 cases (63%). Of those, 146 ADCs (61%), 59 SqCCs (66%), 14 LCCs (74%), and 2 SCCs (50%) showed High-MAGEA2. We then proceeded to correlate MAGEA2 expression and aberrant p53 combinations with various clinicopathological

parameters in all patients (Table I). High-MAGEA2 levels were strongly correlated with aberrant p53 expression (P=0.001). Aberrant expression of p53 was also correlated with pT factor (P=0.029) and pathological stage (P=0.041). We then examined any correlations between MAGEA2 and p53 expression using subset analyses according to histology. In ADC patients (Table II), High-MAGEA2 was strongly associated with aberrant p53 expression (P<0.001) and this change in p53 levels was correlated with pT factor in patients with ADC (P=0.027). In SqCC patients (Table III), High-MAGEA2 was strongly associated with pT factor (P=0.046). Association between MAGEA2 expression and p53 expression was not observed in SqCC patients (P=0.656).

Prognostic significance of MAGEA2 and p53 expression. At the end of the study period, 100 patients had died. The 5-year OS for all patients was 72.0% (95% CI, 66.0-76.7%) (Table IV).

Table III. Patient characteristics according to MAGEA2 and p53 expression levels for squamous cell carcinoma.

Patient characteristics	MAGEA2 expression				P-value	p53 expression				P-value
	Low		High			Wild-type		Aberrant		
	N	%	N	%		N	%	N	%	
All patients	30	34	59	66		176	50	177	50	
Age (years)					0.255					0.595
<60	8	47	9	53		9	53	8	47	
≥60	22	31	50	69		32	44	40	56	
Gender					1.000					0.118
Female	2	29	5	71		1	14	6	86	
Male	28	34	54	66		40	49	42	51	
Smoking history					0.479					0.498
Never-smoker	4	44	5	56		3	33	6	67	
Smoker	26	32	54	68		38	48	42	52	
pT factor					0.046					0.479
pT1	4	17	20	83		13	54	11	46	
pT2-4	26	40	39	60		28	43	37	57	
pN factor					0.630					0.819
pN0	22	36	39	64		29	48	32	52	
pN1-2	8	29	20	71		12	43	16	57	
Pathologic stage					0.070					0.590
Stage I	13	29	32	71		23	51	22	49	
Stage II	13	52	12	48		11	44	14	56	
Stage III	4	21	15	79		7	37	12	63	
Pleural invasion					0.649					0.197
Absent	17	32	37	68		28	52	26	48	
Present	13	37	22	63		13	37	22	63	
p53 expression					0.656					
Wild-type	15	37	26	63		-	-	-	-	
Aberrant	15	31	33	69		-	-	-	-	

Significant P-values (<0.05) are shown in bold. P-value was analyzed using the Pearson's χ^2 test.

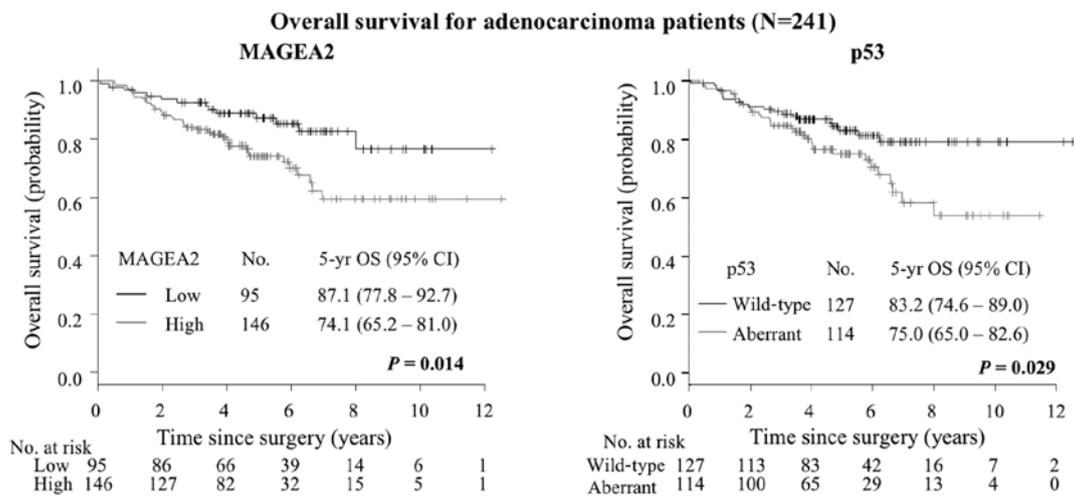


Figure 9. Expression of MAGEA2 and p53 protein and prognostic impact on lung cancers. Kaplan-Meier analysis of overall survival in patients with lung ADC according to MAGEA2, aberrant p53 expression level, and combination of MAGEA2 and p53 status. The 5-year overall survival (OS) rate was 87.1% for patients with low-level MAGEA2 expression (Low-MAGEA2, n=95), whereas 74.1% for patients with high-level MAGEA2 expression (High-MAGEA2, n=146) (P=0.014). The 5-year OS was 83.2% for patients with wild-type p53 expression (n=127), whereas 75.0% for patients with aberrant p53 expression (n=114) (P=0.029).

Table IV. Patient characteristics and univariate analyses of survival for all patients.

Factors	Patients		5-yr OS (%)	95% CI	P-value
	No.	%			
All patients	353	100	72.0	66.0-76.7	
Age (years)					0.296
<60	122	35	74.7	65.3-81.9	
≥60	231	65	70.8	64.0-76.5	
Gender					0.001
Female	123	35	81.0	72.2-87.3	
Male	230	65	67.0	60.0-73.1	
Smoking history					0.023
Never-smoker	119	34	80.0	71.1-86.4	
Smoker	234	66	67.8	60.8-73.8	
Histological classification					<0.001
ADC	241	68	79.4	73.2-84.3	
SqCC	89	25	54.2	42.4-64.6	
LCC	19	5	73.7	47.9-88.1	
SCC	4	1	NA	NA	
pT factor					<0.001
pT1	136	39	84.4	76.5-89.8	
pT2-4	217	61	64.3	57.0-70.7	
pN factor					<0.001
pN0	265	75	81.2	75.4-85.7	
pN1-2	88	25	43.9	32.4-54.8	
Pathologic stage					<0.001
Stage I	228	65	85.5	79.6-89.7	
Stage II	65	18	61.2	47.6-72.2	
Stage III	60	17	31.3	18.7-44.7	
Pleural invasion					0.014
Absent	219	62	76.1	69.3-81.6	
Present	134	38	65.2	55.9-73.1	
MAGEA2 expression					0.022
Low	132	37	78.9	70.4-85.2	
High	221	63	67.6	60.3-73.9	
p53 expression					0.013
Wild-type	176	50	77.0	69.5-82.9	
Aberrant	177	50	66.9	58.8-73.8	
MAGEA2/p53 combination					0.008
Low/Wild-type	96	27	85.5	76.2-91.4	
Low/Aberrant	36	10	61.4	42.5-75.7	
High/Wild-type	80	23	66.2	53.4-76.3	
High/Aberrant	141	40	68.4	59.2-75.9	
MAGEA2 expression in wild-type p53					0.012
Low	96	27	85.5	76.2-91.4	
High	80	23	66.2	53.4-76.3	
MAGEA2 expression in aberrant type p53					0.662
Low	36	10	61.4	42.5-75.7	
High	141	40	68.4	59.2-75.9	

ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; SCC, Small cell carcinoma; CI, confidence interval. Significant P-values (<0.05) are shown in bold.

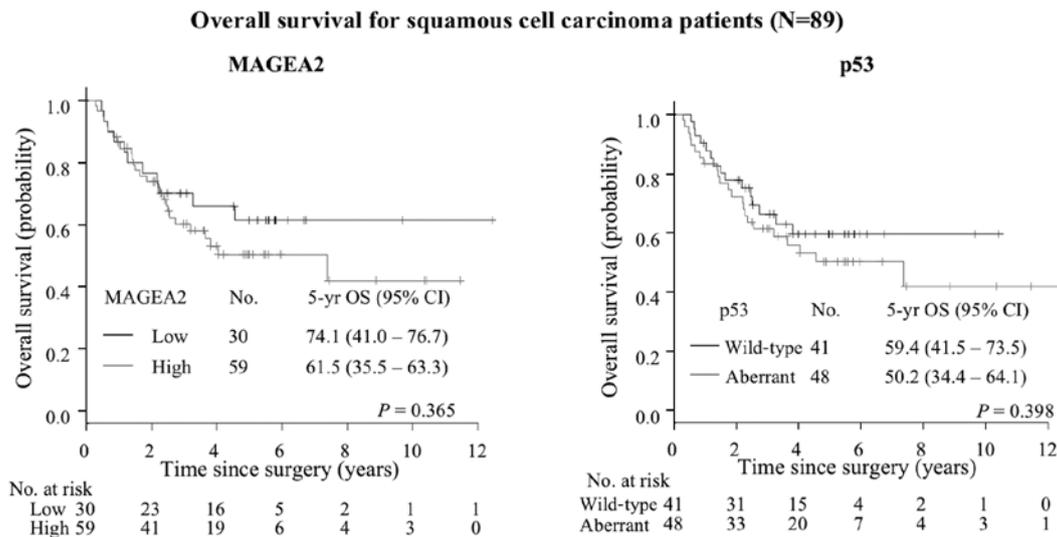


Figure 10. Expression of MAGEA2 and p53 protein and prognostic impact on lung cancers. Kaplan-Meier analysis of overall survival (OS) in squamous cell carcinoma patients according to MAGEA2 and aberrant p53 expression level and combination of MAGEA2 and aberrant p53. There were no significant differences between low expression of MAGEA2 and high expression of MAGEA2 (*P*=0.365). There were no significant differences between wild-type p53 expression and aberrant p53 expression (*P*=0.398).

Kaplan-Meier method indicated that High-MAGEA2 was associated with increased risk of death (5-year OS, 78.9% for low level vs. 67.6% for high level; *P*=0.022, Fig. 8). Aberrant p53 was associated with increased risk of death (5-year OS, 77.0% for wild-type expression vs. 66.9% for aberrant expression; *P*=0.013, Fig. 8). On univariate analysis for other clinicopathological factors, male gender (*P*=0.001), smoking history (*P*=0.023), patient with SqCC (*P*<0.001), pT factor (*P*<0.001), pN factor (*P*<0.001), pathological stage (*P*<0.001), pleural invasion (*P*=0.014) were correlated with worse OS. Multivariate analyses were performed on the MAGEA2 expression and p53 status and these models were adjusted for the prognostic clinicopathologic factors, including gender, smoking status, pT stage (T2-4 vs. T1) and pN stage (N1, 2 vs. N0) and pleural invasion (Present vs. Absent). The final multivariate model confirmed pT stage (hazard ratio [HR] 1.79, 95% CI 1.01-3.17; *P*=0.047) and pN stage (HR 2.78, 95% CI 1.83-4.21; *P*<0.001) remained independently associated with survival, but this was not observed with MAGEA2 expression and p53 status (Table V).

In patients with ADC, the 5-year OS was 79.4% (95% CI, 73.2-84.3%) (Table VI). Noteworthy, ADC patients with High-MAGEA2 revealed significantly shorter overall survival than those with Low-MAGEA2 (5-year OS, 87.1% for low level vs. 74.1% for high level; *P*=0.014) (Fig. 9), while SqCC patients did not show any difference although there was a tendency towards poorer prognosis with High-MAGEA2 (*P*=0.365) (Table VII, Fig. 10). We also performed univariate analysis to evaluate associations between prognosis and other factors in patients with ADC (Table VI). Among those parameters, advanced pT status (*P*=0.002), advanced pN status (*P*<0.001) and advanced pathological stage (*P*<0.001) were significantly associated with poor prognosis in ADC patients. Aberrant p53 expression was also significantly worse prognostic factor (5-year OS, 83.2% for wild-type expression vs. 75.0% for aberrant expression; *P*=0.029) (Fig. 9). Next, we investigated MAGEA2 and aberrant p53 combinations. Among patients

Table V. Multivariate analysis of survival for all patients.

Variable	HR	95% CI	P-value
Gender			
Male vs. Female	1.68	0.96-2.94	0.068
Smoking			
Smoker vs. Never-smoker	1.04	0.61-1.79	0.881
T stage			
T2-4 vs. T1	1.79	1.01-3.17	0.047
N stage			
N1,2 vs. N0	2.78	1.83-4.21	<0.001
Pleural invasion			
Present vs. Absent	1.04	0.65-1.65	0.883
MAGEA2 expression			
High vs. Low	1.55	0.97-2.49	0.070
p53 expression			
Aberrant vs. Wild-type	1.15	0.74-1.80	0.531

HR, Hazard ratio; CI, confidence interval. Significant *P*-values (<0.05) are shown in bold.

with wild-type p53 expression, High-MAGEA2 had worse prognosis than low MAGEA2 groups (5 year OS, 90.1% vs. 72.1%, *P*=0.037, Fig. 11). On the other hand, among patients with aberrant type p53 expression, there were no significant differences in the High- and Low-MAGEA2 expression (5-year OS, 75.3% for low level vs. 75.1% for high level; *P*=0.756) (Fig. 11). Multivariate analyses were performed for the MAGEA2 expression and p53 status and these models were adjusted for the prognostic clinicopathologic factors from univariate analysis, including gender, pT stage (T2-4 vs. T1) and pN stage (N1, 2 vs. N0). MAGEA2

Table VI. Patient characteristics and univariate analyses of survival for adenocarcinoma.

Factors	Patients		5-yr OS (%)	95% CI	P-value
	No.	%			
All patients (ADC)	241	100	79.4	73.2-84.3	
Age (years)					0.749
<60	96	40	80.0	69.6-87.2	
≥60	145	60	79.1	70.7-85.4	
Gender					0.050
Female	111	46	82.9	73.7-89.1	
Male	130	54	76.3	67.1-83.3	
Smoking history					0.115
Never-smoker	105	44	84.1	74.8-90.1	
Smoker	136	56	75.7	66.6-82.6	
pT factor					0.002
pT1	103	43	87.9	79.1-93.2	
pT2-4	138	57	73.0	63.9-80.2	
pN factor					<0.001
pN0	188	78	86.4	80.1-91.0	
pN1-2	53	22	53.8	40.5-69.6	
Pathologic stage					<0.001
Stage I	170	71	89.2	82.6-93.4	
Stage II	34	14	70.5	52.0-82.9	
Stage III	37	15	42.4	24.6-59.2	
Pleural invasion					0.291
Absent	153	63	80.7	72.7-86.6	
Present	88	37	77.0	65.6-85.0	
MAGEA2 expression					0.014
Low	95	39	87.1	77.8-92.7	
High	146	61	74.1	65.2-81.0	
p53 expression					0.029
Wild-type	127	53	83.2	74.6-89.0	
Aberrant	114	47	75.0	65.0-82.6	
MAGEA2/p53 combination					0.043
Low/Wild-type	78	33	90.1	80.1-95.2	
Low/Aberrant	17	7	75.3	46.8-89.9	
High/Wild-type	49	20	72.1	55.2-83.5	
High/Aberrant	97	40	75.1	63.9-83.2	
MAGEA2 expression in wild-type p53					0.037
Low	78	33	90.1	80.1-95.2	
High	49	20	72.1	55.2-83.5	
MAGEA2 expression in aberrant type p53					0.756
Low	17	7	75.3	46.8-89.9	
High	97	40	75.1	63.9-83.2	

ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; SCC, Small cell carcinoma; CI, confidence interval. Significant P-values (<0.05) are shown in bold.

Table VII. Patient characteristics and univariate analyses of survival for squamous cell carcinoma.

Factors	Patients		5-yr OS (%)	95% CI	P-value
	No.	%			
All patients (SqCC)	89	100	54.2	42.4-64.6	
Age (years)					0.891
<60	17	20	49.3	23.0-71.2	
≥60	72	80	55.6	42.3-66.9	
Gender					0.545
Female	7	8	66.7	19.5-90.4	
Male	82	92	53.3	41.0-64.1	
Smoking history					0.559
Never-smoker	9	10	44.4	13.6-71.9	
Smoker	80	90	55.4	42.7-66.4	
pT factor					0.034
pT1	24	27	75.9	51.2-89.2	
pT2-4	65	73	47.2	33.9-59.4	
pN factor					<0.001
pN0	61	69	69.9	55.8-80.3	
pN1-2	28	31	22.0	8.3-39.9	
Pathologic stage					<0.001
Stage I	45	51	78.8	63.2-88.4	
Stage II	25	28	48.5	27.0-67.1	
Stage III	19	21	6.9	0.5-26.4	
Pleural invasion					0.008
Absent	54	61	64.3	48.5-76.3	
Present	35	39	39.4	22.4-55.9	
MAGEA2 expression					0.365
Low	30	34	74.1	41.0-76.7	
High	59	66	61.5	35.5-63.3	
p53 expression					0.398
Wild-type	41	46	59.4	41.5-73.5	
Aberrant	48	54	50.2	34.4-64.1	
MAGEA2/ p53 combination					0.455
Low/Wild-type	15	17	59.3	30.7-79.3	
Low/Aberrant	15	17	64.2	33.3-83.6	
High/Wild-type	26	29	59.2	35.1-76.9	
High/Aberrant	33	37	44.1	25.9-60.9	
MAGEA2 expression in wild-type p53					0.821
Low	15	17	59.3	30.7-79.3	
High	26	29	59.2	35.1-76.9	
MAGEA2 expression in aberrant type p53					0.206
Low	15	17	64.2	33.3-83.6	
High	33	37	44.1	25.9-60.9	

ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; SCC, Small cell carcinoma; CI, confidence interval. Significant P-values (<0.05) are shown in bold.

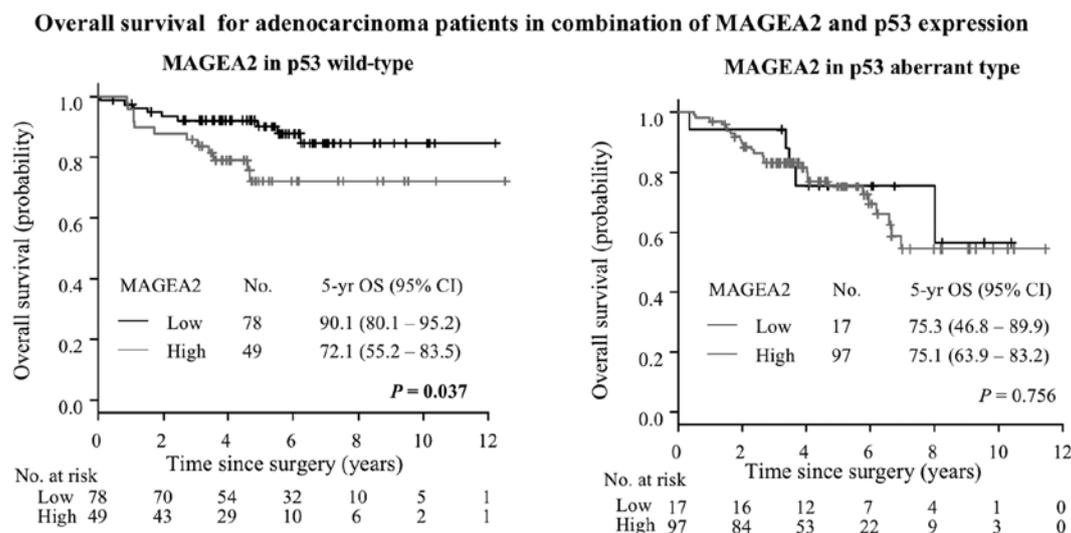


Figure 11. Combination of MAGEA2 and p53 protein expression and prognostic impact on LAC. Among the wild-type p53 patients, the 5-year OS was 90.1% for patients with Low-MAGEA2 (n=78), whereas 72.1% for patients with High-MAGEA2 (n=49) (P=0.037). Among the aberrant type p53 patients, the 5-year OS was 75.3% for patients with Low-MAGEA2 (n=17), whereas 75.1% for patients with High-MAGEA2 (n=97) (P=0.756).

Table VIII. Multivariate analysis of survival for adenocarcinoma.

Variable	HR	95% CI	P-value
Gender (Male vs. Female)	1.50	0.85-2.65	0.160
T stage (T2-4 vs. T1)	1.73	0.88-3.38	0.110
N stage (T1,2 vs. N0)	3.68	2.06-6.57	<0.001
MAGEA2 expression (High vs. Low)	2.12	1.08-4.18	0.030
p53 expression (Aberrant vs. Wild-type)	1.02	0.55-1.91	0.945

HR, Hazard ratio; CI, confidence interval. Significant P-values (<0.05) are shown in bold.

expression was identified as an independent prognostic factor of lung ADC (HR 2.12, 95% CI 1.08-4.18; P=0.030) by multivariate analysis, as was pN status (HR 3.68, 95% CI 2.06-6.57; P<0.001, Table VIII).

Discussion

MAGEA comprises of an 11-member subfamily of the broader family of MAGE proteins which are characterized by the presence of a MAGE-homology domain (11). The search for these genes as tumor-specific antigen has been active for many decades, however, the normal physiologic role of MAGEA proteins remains poorly understood and their contribution to cancer development is yet to be fully demonstrated (11). Only in recent years members of the MAGEA family have been shown

to be implicated in various tumorigenic or tumor suppressive pathways. On one hand, MAGEA4, for example, promotes apoptosis and sensitization to chemotherapeutic agents, therefore functions as a tumor suppressor protein (40). MAGEA4 is also processed by a proteasome to generate a C-terminal fragment with pro-apoptotic activities, which increases the p53 protein level, and subsequent apoptosis (41). MAGEA genes have been shown to inhibit p53-dependent apoptosis in cancer cells, and contribute to tumor aggressiveness (19,42). MAGEA protein blocked the association of p53 with its DNA binding surface of the p53 core domain, suppressing apoptosis in p53-dependent manner (19).

Herein, we showed for the first time that a high MAGEA2 expression level (High-MAGEA2) in patients with lung cancer, especially in ADC, is strongly associated with poor survival. In particular, patients with High-MAGEA2 have significantly poorer overall survival rate than those with Low-MAGEA2 in wild-type p53 tumors. In addition, High-MAGEA2 is an independent poor prognostic factor among lung ADC. In lung SqCC, however, there is no relation to p53 status and no prognostic difference between tumors with High- and Low-MAGEA2, although MAGEA2 expression is increased with tumor progression (pT factor). This prognostic significance indicates that p53/MAGEA2 interaction in cancer progression depends on histology and p53 status and suggests that therapies targeting MAGEA2 may improve patient survival for patients with ADC with p53 wild-type.

The p53 tumor-suppressor is a key transcription factor that controls cell proliferation, inducing growth arrest or apoptosis in response to different cellular stresses (43). p53 mutation is associated with invasiveness in ADC, suggesting this is a relatively late event during tumor development and plays an important role in the progression of the peripheral pulmonary ADC (24). However, little is known about the functional role of MAGE-A genes in tumorigenesis for p53 aberrant-type tumors. We found MAGEA2 expression to be significantly increased with aberrant p53 expression in ADC. In addition, downregulation of MAGEA2 in lung cancer cell

with p53 mutation showed significant growth suppressive effect *in vitro*, although, unexpectedly, there is no difference in terms of prognosis between tumor with High-MAGEA2 and Low-MAGEA2 in patients with aberrant-type p53. We also examined the effect of MAGEA2 expression on the p53 downstream targets using lung cancer cell lines with p53 mutation. In p53 wild-type cancer cells, elimination of Mage-A expression in tumor-derived cells that retain functional p53 leads to increased recruitment of p53 to p53-responsive promoters and increases in p53-dependent transcription, cell cycle arrest, and cell death (19). To our surprise, we found that there was a significant decrease in expression of p53 downstream genes after suppression of MAGEA2. Taken together, these results indicate that MAGEA2 may contribute to the stability of p53 transcriptional activity in p53-mutated lung cancer cells, and the expression of MAGEA2 itself does not affect prognostic impact in p53 aberrant-type tumor, even though MAGEA2 is associated with tumor growth/survival. There is room for discussion about different functional roles of MAGEA2 in integrations between MAGEA2 and mutated-p53. Given our results in addition to the expression pattern of MAGEA2 reported in other studies, MAGEA2 is a promising molecular and immunogenic target even in lung cancer with p53 mutation and our finding helps understanding of the mechanisms that explains how MAGEA2 interacts with p53 activity according to p53 status, which also can be used to help develop new therapeutic strategy against lung cancer.

A limitation of this study is the efficacy of TMAs in reflecting gene expression in heterogeneity of lung cancer. TMA analysis is a promising technique in the evaluation of immunohistochemical markers in tumors and may be used as an alternative for whole sections. However, TMAs represent only a small portion of tissue collected and could be subject to sampling error. This should be considered a potential limitation of this finding and warrants further investigation with larger validate cohorts and using multiple TMA cores.

There have been no previous studies addressing the functional and prognostic role of MAGEA2 in lung cancer with regards to patients with resectable lung cancer. In the present study, we demonstrated that suppression of MAGEA2 in lung cancer cells significantly reduced the growth/survival of cells. Furthermore, MAGEA2 overexpression could be a useful index for patients with a high risk of poor prognosis in ADC patients. Although it still remains unclear as to how MAGEA2 is associated in tumorigenesis and p53 status, our study has shed some light on the biological function role of MAGEA2 in promoting lung cancer cell progression. Based on these results, specific inhibition of MAGEA2 can be a promising therapeutic agent for patients with lung cancer.

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