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Characterization of an Opa interacting protein 5 involved in lung and esophageal carcinogenesis

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To identify potential molecular targets for diagnosis, treatment and/or prevention of lung and esophageal carcinomas, we screened for genes that were overexpressed in tumors through gene expression analyses of 120 lung cancers and 19 esophageal squamous-cell carcinomas using a cDNA microarray consisting of 27 648 cDNA or expressed sequence tags. In this process, we identified a gene, Opa interacting protein 5 (*OIP5*), to be highly transactivated in the majority of lung and esophageal cancers. Immunohistochemical staining using 336 archived non-small cell lung cancers and 305 esophageal squamous-cell carcinomas specimens demonstrated that OIP5 expression was significantly associated with poor prognosis of lung and esophageal cancer patients ($P = 0.0053$ and 0.0168, respectively), and multivariate analysis confirmed its independent prognostic value for non-small cell lung cancers ($P = 0.0112$). Suppression of OIP5 expression with siRNA effectively suppressed the growth of cancer cells, whereas the exogenous expression of OIP5 enhanced the growth of cancer cells. In addition, OIP5 protein is likely to be stabilized through its interaction with Raf1. OIP5 is a promising target for developing new prognostic biomarkers and anti-cancer drugs. (*Cancer Sci* 2012; 103: 577–586)

Lung cancer is the leading cause of cancer deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases.[1] Esophageal squamous-cell carcinoma (ESCC) is one of the most lethal malignancies of the digestive tract, and at the time of diagnosis most patients are at advanced stages.[2] These two major thoracic tumors could be categorized as aerodigestive tract cancers (including lung, esophagus, oral cavity, pharynx and larynx carcinomas). Aerodigestive tract cancer accounts for one-third of all cancer deaths in the USA and is the most common cancer in some areas of the world.[3] Despite the use of current surgical techniques combined with various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of ESCC remains at 40–60%,[4] and that of lung cancer is only 15%.[1] To isolate potential molecular targets for diagnosis, treatment and/or prevention of lung and esophageal carcinomas, we performed genome-wide expression profile analyses of cancer cells from 120 lung cancer and 19 ESCC patients by means of a cDNA microarray consisting of 27 648 genes or expressed sequence tags (EST).[5–11] To verify the biological and clinicopathological significance of the respective gene products, we performed high-throughput screening of loss-of-function effects by means of the RNAi technique, as well as tumor-tissue microarray analysis of clinical lung and esophageal cancer materials.[12–44] This systematic approach revealed that Opa interacting protein 5 (*OIP5*) was overexpressed in the majority of primary lung and esophageal cancers.

Opa interacting protein 5 encodes a 25-kDa protein with a coiled-coil domain, and was found by yeast two-hybrid analysis as a protein interacting with Opa proteins.[45] Previous studies have demonstrated the elevated expression of *OIP5* mRNA in human gastric and colorectal carcinomas, but no report has functionally clarified the significance of transactivation of OIP5 in human carcinogenesis and indicated its potential as a therapeutic target.[46,47]

In the present study, we report that overexpression of OIP5 could contribute to the malignant nature of lung and esophageal cancer cells. We suggest that targeting the OIP5 molecule might be promising for the development of a new diagnostic and therapeutic strategy in the clinical management of lung and esophageal cancers.

Materials and Methods

Cell lines and tissue samples. The 15 human lung cancer cell lines used in this study included five adenocarcinoma (ADC) cell lines (A549, LC319, PC-14, NCI-H1373 and NCI-H1781), five squamous cell carcinoma (SCC) cell lines (SK-MES-1, LU61, NCI-H520, NCI-H1703 and NCI-H2170), one large cell carcinoma (LCC) cell line (LX1) and four small cell lung cancer (SCLC) cell lines (DMS114, DMS273, SBC-3 and SBC-5). The human esophageal carcinoma cell lines used in the present study were: nine SCC cell lines (TE1, TE2, TE3, TE4, TE5, TE6, TE8, TE9 and TE10) and one ADC cell line (TE7) (Table S1). All cells were grown in monolayer in appropriate media supplemented with 10% FCS and were maintained at 37°C in humidified air with 5% CO_2. Human small airway epithelial cells (SAEC) used as a normal control were grown in optimized medium (SAGM) from Cambrex Bio Science (East Rutherford, NJ, USA). Primary lung cancer and ESCC samples for cDNA microarray and/or semiquantitative RT-PCR experiments had been obtained earlier with informed consent.[6,10,11] In addition to these tumor samples, a total of 336 formalin-fixed NSCLC (201 ADC, 101 SCC, 23 LCC, 11 adenosquamous carcinoma [ASC]; 103 female and 233 male patients; median age of 66 years with a range of 29–85 years) and normal lung tissue samples for immunostaining on tissue microarray were also obtained from Saitama Cancer Center (Saitama, Japan). These patients received resection of their primary cancers and among them only patients with positive lymph node metastasis were treated with platinum-based adjuvant chemotherapies after their surgery. Formalin-fixed primary 305 ESCC (36 female and 269 male patients; median age of 62 years with a range of 38–84 years) and adjacent normal esophageal tissue samples were obtained from patients undergoing surgery at Keiyukai Sapporo Hospital and Hokkaido University and its affiliated hospitals (Sapporo, Japan) (Table S2). The clinical stage of these tumor

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samples was judged according to the Union for International Cancer Control TNM classification. This study and the use of all clinical materials were approved by individual institutional ethical committees.

Semiquantitative RT-PCR. Semiquantitative RT-PCR experiments were carried out as described previously,[12] with the following *OIP5*-specific primers or with *ACTB*-specific primers as an internal control: *OIP5*, 5′-CTTCAAGAATGGAGGGGAAA-3′ and 5′-GTATTCATAACAACTGCTCCATGC-3′; and *ACTB*, 5′-GAGGTGATAGCATTGCTTTCG-3′ and 5′-CAAGTCAGTGTACAGGTAAGC-3′.

Northern blot analysis. Human multiple-tissue blots (BD Biosciences Clontech, San Jose, CA, USA) were hybridized with a ^{32}P-labeled PCR product of *OIP5*. The cDNA probes of *OIP5* were prepared by RT-PCR using the following primers: 5′-CCAGTGACAAAATGGTGTGC-3′ and 5′-GTATTCATAACAACTGCTCCATGC-3′. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed at −80°C for 1 week with intensifying screens.

Western blotting. Western blotting was performed, as previously described.[13] A commercially available rabbit polyclonal antibody to human OIP5 (Catalog No. 12142-1-AP, Proteintech group) was confirmed to be specific to endogenous OIP5 protein by western blot analysis using lysates of lung and esophageal cancer cell lines as well as normal airway epithelial cells.

Immunocytochemistry. Immunocytochemistry was carried out as described previously,[13] with a rabbit polyclonal anti-OIP5 antibody (Catalog No. 12142-1-AP, Proteintech Group, Chicago, IL, USA) as a primary antibody and a goat anti-rabbit secondary antibody conjugated to Alexa 488 (Invitrogen, Grand Island, NY, USA).

Immunohistochemistry and tissue-microarray analysis. Tumor tissue microarrays were constructed, as described previously, using formalin-fixed NSCLC and ESCC.[13] To investigate the significance of OIP5 overexpression in clinical NSCLC, we stained tissue sections using ENVISION+ kit/HRP (DakoCytomation, Carpinteria, CA, USA) and OIP5 antibody (Catalog No. 12142-1-AP, Proteintech Group), as described previously.[13] Because the intensity of staining within each tumor tissue core was mostly homogeneous, positivity for OIP5 was assessed semiquantitatively by three independent investigators without prior knowledge of the clinicopathologic data, each of whom recorded staining intensity positive or negative. Cases were accepted as positive if two or more investigators independently defined them as such.

Statistical analysis. We used contingency tables to analyze the relationship between OIP5 expression and clinicopathologic variables in NSCLC and ESCC patients. Survival curves were calculated from the date of surgery to the time of death related to NSCLC and ESCC, or to the last follow-up observation. Kaplan–Meier curves were calculated for each relevant variable and for OIP5 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were carried out with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality. Multivariate logistic regression analysis was applied to determine clinicopathologic factors that were independently associated with the OIP5 expression.

RNA interference assay. Small interfering RNA (siRNA) duplexes (Dharmacon, Yokohama, Japan) (100 nM) were transfected into an NSCLC cell line LC319, an SCLC cell line SBC-5 and an esophageal cancer cell line TE2, using 30 μL of Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The transfected cells were cultured for 7 days, the number of colonies was counted by Giemsa staining; and the viability of cells was evaluated by MTT assay (cell counting kit-8 solution; Dojindo Laboratories, Rockville, MD, USA). To confirm suppression of *OIP5* mRNA expression, semiquantitative RT-PCR experiments were carried out with synthesized primers specific to *OIP5*, as described above. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control 1 (Luciferase/LUC: *Photinus pyralis* luciferase gene), 5′-NNCGUACGCGGAAUACUUCGA-3′; control 2 (On-Target plus/CNT); 5′-UGGUUUACAUGUCGACUAA-3′; siRNA-OIP5-1, 5′-CGGCAUCGCUCACGUUGUGUU-3′; siRNA-OIP5-2, 5′-GUGACAAAAUGGUGUGCUAUU-3′ siRNA-Raf1-1, 5′-GCAAAGAACAUCAUCCAUA-3′; and siRNA-Raf1-2, 5′-GACAUGAAAUCCAACAAUA-3′. Each assay was performed in tripricate.

Cell growth assay. SBC-5, TE9 and COS-7 cells were plated at densities of 1×10^6 cells/100 mm dish, transfected with plasmids designed to express OIP5 (pcAGGSn3FC-OIP5-Flag) or mock plasmids. Cells were selected in medium containing 0.4 mg/mL of geneticin (Invitrogen) for 7 days, and cell viability was assessed by MTT assay (cell counting kit-8; Dojindo Laboratories).

Immunoprecipitation assay. Cell extracts from SBC-5 transfected with Flag-tagged OIP5-expression vector or those transfected with mock vector were precleared by incubation at 4°C for 1 h with 100 μL of protein G-agarose beads in a final volume of 1 mL of immunoprecipitation buffer (0.5% NP-40, 50 mM Tris–HCl, 150 mM NaCl) in the presence of proteinase inhibitor. After centrifugation at 70g for 1 min at 4°C, the supernatant was incubated at 4°C with anti-Flag M2 agarose beads for 2 h. The beads were then collected by centrifugation at 1800g for 1 min and washed six times with 1 mL of each immunoprecipitation buffer. The washed beads were resuspended in 20 μL of Laemmli sample buffer and boiled for 5 min, and the proteins were separated in 12% SDS PAGE gels (Bio Rad, Hercules, CA, USA). After electrophoresis, western blotting analysis was carried out using a commercially available rabbit polyclonal antibody to human Raf1 (Catalog No. 9422; Cell Signaling, Danvers, MA, USA).

Results

Opa interacting protein 5 expression in lung and esophageal cancers and normal tissues. To identify molecular targets for the development of novel therapeutic agents and/or biomarkers for lung and esophageal cancers, we performed genome-wide gene expression profile analysis of these cancers using a cDNA microarray.[5–11] Among 27 648 genes or EST for which 120 lung cancers and 19 ESCC had been screened, we identified elevated expression (threefold or higher) of *OIP5* transcript in the majority of the lung and esophageal cancers examined. We validated its overexpression by means of semi-quantitative RT-PCR experiments in nine of 15 lung cancer tissues that had been served for cDNA microarray experiments, in all 15 lung cancer cell lines (Fig. 1A,B), in six of the 10 ESCC tissues that were used for cDNA microarray analysis, and in nine of 10 ESCC cell lines (Fig. 1C,D). However, its expression was barely detectable in their adjacent normal lung and esophagus tissues and SAEC cells derived from normal airway epithelial cells. We also evaluated the expression of *OIP5* in independent sets of paired lung and esophageal cancer tissues and their adjacent normal tissues that were not used for cDNA microarray analysis, and confirmed its overexpression in seven of nine NSCLC and three of five ESCC (Fig. S1A,B). We further confirmed a high level of OIP5 expression in lung and esophageal cancer cell lines by western blot analyses using anti-OIP5 antibody (Fig. 1E). OIP5 protein was detected as double bands by western blotting, indicating a possible modification of the OIP5 protein. Because there are several predicted phosphorylation sites on OIP5 protein, we first incubated extracts from SBC-5 cells that overexpressed endogenous OIP5 as well as COS-7 cells transfected with

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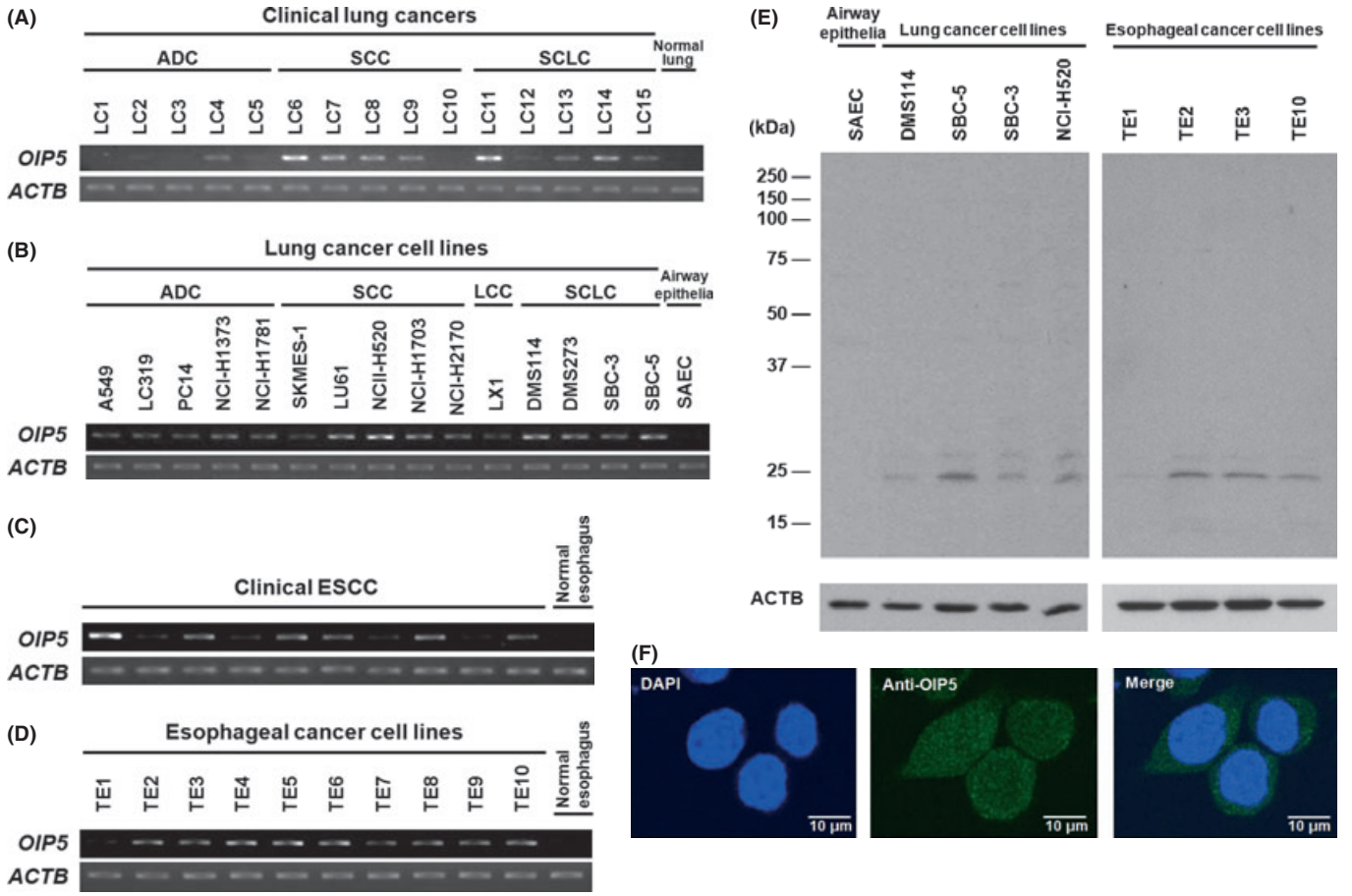

Fig. 1. Opa interacting protein 5 (*OIP5*) expression in lung and esophageal cancers and normal tissues. (A,B) Expression of *OIP5* in a normal lung tissue and 15 clinical lung cancer samples and 15 lung cancer cell lines detected by semiquantitative RT-PCR analysis. (C,D) Expression of *OIP5* in a normal esophagus and 10 clinical esophageal squamous-cell carcinoma (ESCC) tissue samples and 10 ESCC cell lines detected by semiquantitative RT-PCR analysis. (E) Expression of OIP5 in lung cancer cell lines, examined by western blot analyses. Expression of ACTB was served as a quantity control. (F) Subcellular localization of endogenous OIP5 protein in lung cancer SBC-5 cells. ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, small cell carcinoma.

OIP5-expressing plasmids in the presence or absence of protein phosphatase, and analyzed the molecular size of OIP5 protein by western blot analysis. The higher molecular weight bands of both endogenous and exogenous OIP5 protein were barely detectable in the extracts treated with phosphatase (Fig. S1C), indicating that OIP5 was possibly phosphorylated in these cells. We performed immunofluorescence analysis to examine the subcellular localization of endogenous OIP5 in a lung cancer SBC-5 cell line and found that OIP5 was located in the nucleus and cytoplasm (Fig. 1F). Northern blot analysis using *OIP5* cDNA as a probe identified a strong signal corresponding to a 1.5-kb transcript only in the testis among 23 tissues examined (Fig. 2A). Furthermore, we examined by immunohistochemical analysis using anti-OIP5 polyclonal antibodies OIP5 protein expression in six normal tissues (liver heart, kidney, lung, esophagus and testis) and in lung and esophageal cancer tissues. OIP5 was detected abundantly in the nucleus and cytoplasm of testicular cells, and lung and esophageal cancer cells, but its expression was barely detectable in the remaining five normal tissues (Fig. 2B).

Association of opa interacting protein 5 expression with poor prognosis for non-small cell lung cancer and esophageal squamous-cell carcinoma patients. To verify the clinicopathological significance of OIP5, we additionally examined the expression of OIP5 protein by means of tissue microarrays containing lung cancer tissues from 336 NSCLC and 305 ESCC patients who underwent surgical resection. OIP5 positive staining with the anti-OIP5 polyclonal antibody was observed in the nucleus and cytoplasm of lung and esophageal cancer cells, but staining was negative in any of their adjacent normal epithelial cells or stromal cells surrounding tumor cells (Fig. 2C). We found positive staining in 131 of 201 ADC tumors (65.2%), 87 of 101 SCC (86.1%), nine of 11 ASC (81.8%) and 22 of 23 LCC (95.6%) using immunohistochemical positivity criteria as described in the Materials and Methods. We then examined the correlation of OIP5 expression (positive versus negative) with various clinicopathological parameters and found a significant correlation with histology (higher in non-ADC; $P < 0.0001$ by Fisher's exact test), with pT factor (higher in pT2–T3; $P = 0.0318$ by Fisher's exact test) and with smoking (higher in a smoker; $P = 0.0187$ by Fisher's exact test) (Fig. 2D; Table 1). Multivariate logistic regression analysis for these three significant clinicopathologic variables determined that non-adenocarcinoma histology was an independent feature associated with OIP5 expression ($P < 0.0001$; Table S3). The Kaplan–Meier method indicated significant association between OIP5 positivity and shorter survival periods of NSCLC patients ($P = 0.0053$ by the log-rank test; Fig. 2E). Subgroup analysis clearly showed that the survival time of stage I or stage II–IIIA patients with OIP5-positive NSCLC tended to be shorter than that of patients with OIP5-negative tumors, although due to the smaller sample size, these results are not statistically significant (Fig. S2A,B). Using univariate analysis, non-adenocarcinoma histology, advanced pT stage (pT2–3), presence of lymph node metastasis

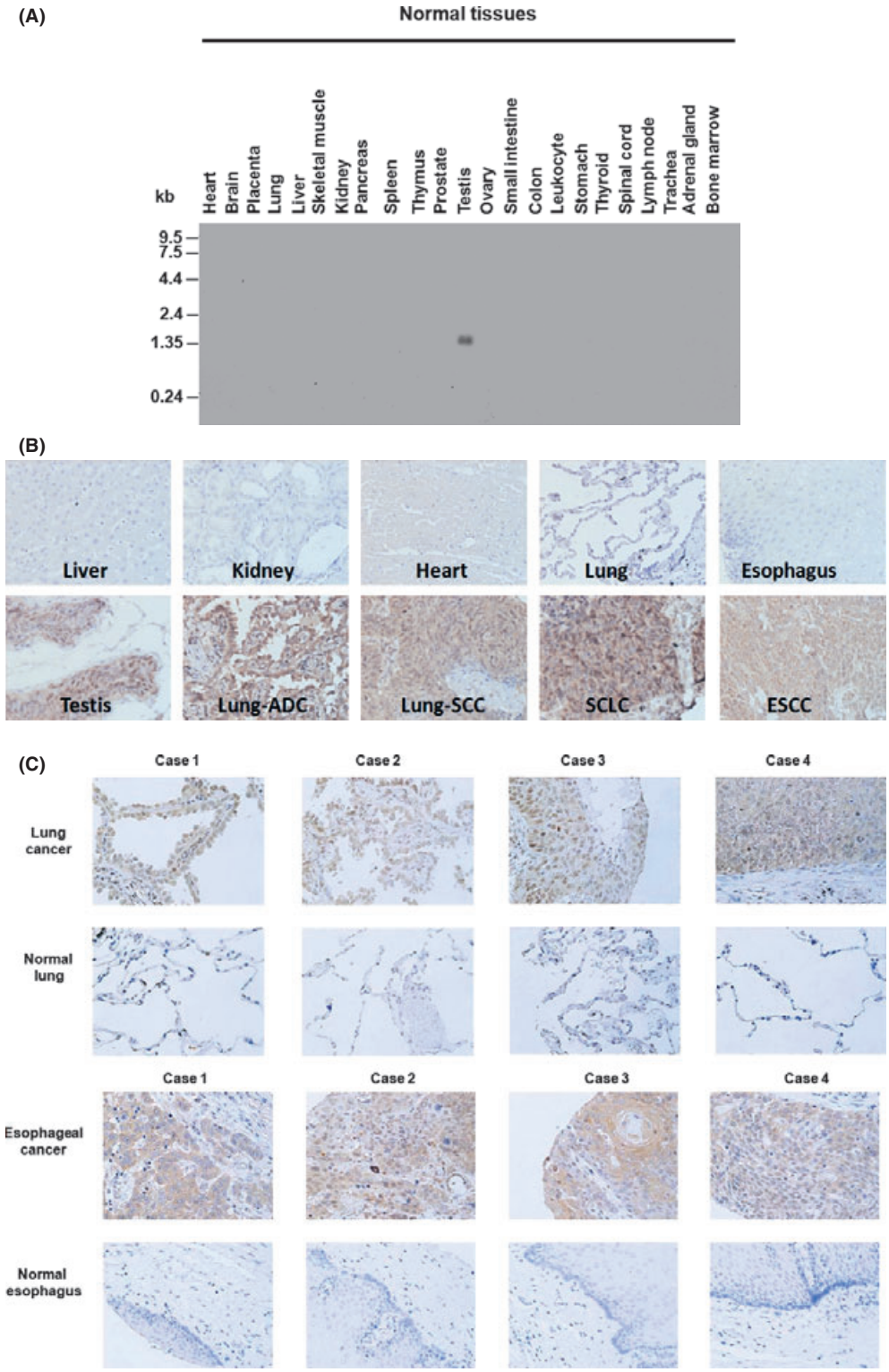

Fig. 2. Opa interacting protein 5 (*OIP5*) expression in normal tissues and lung and esophageal cancers, and association of OIP5 expression with poorer clinical outcomes for non-small cell lung cancer (NSCLC) and esophageal squamous-cell carcinoma (ESCC) patients. (A) Northern blot analysis of the *OIP5* transcript in 23 normal human tissues. (B) Expression of OIP5 in six normal human tissues as well as various histologic types of lung cancer and ESCC, detected by immunohistochemical staining (magnification ×100). (C) Immunohistochemical staining of OIP5 protein using anti-OIP5 antibody in eight representative paired lung and esophageal tumors and adjacent normal lung tissues (×200). (D) Representative example of OIP5 expression in lung cancer (squamous cell carcinomas) and normal lung (top, ×100; bottom ×200). (E) Kaplan–Meier analysis of survival in NSCLC patients according to OIP5 expression level (P = 0.0053; log-rank test). (F) Representative example of OIP5 expression in ESCC and normal esophagus (top, ×100; bottom ×200). (G) Kaplan–Meier analysis of survival in ESCC patients according to OIP5 expression level (P = 0.0168; log-rank test). ADC, adenocarcinoma; SCC, small cell carcinoma; SCLC, small cell lung cancer.

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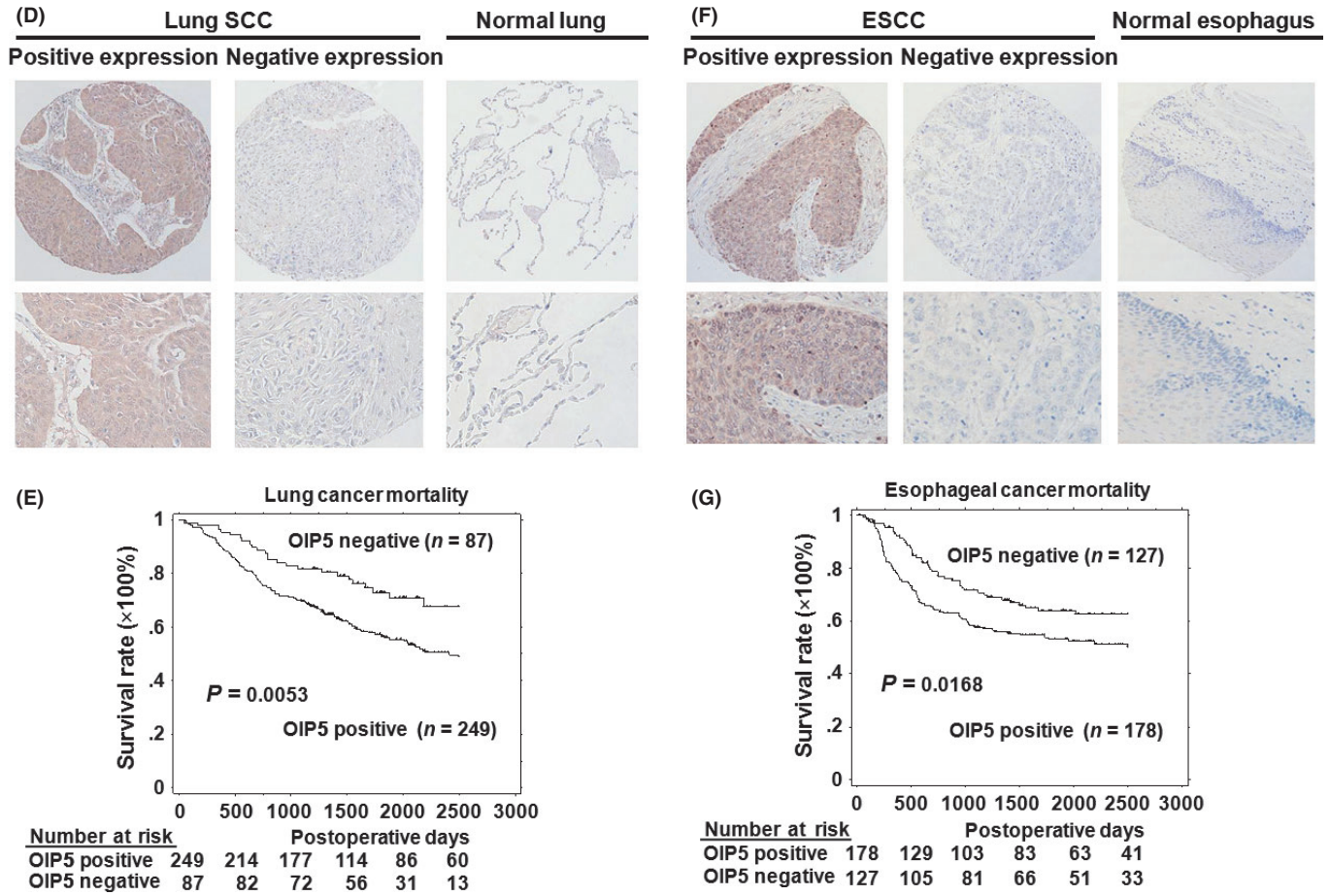

Fig. 2. (Continued)

Table 1. Cox's proportional hazards model analysis of prognostic factors in patients with non-small cell lung cancer

Variables	Hazards ratio	95% CI	Unfavorable/favorable	*P*-value
Univariate analysis				
OIP5	2	1.193–2.882	Positive/negative	0.0061*
Age (years)	2	1.103–2.199	≥65/65>	0.0119*
Gender	2	1.094–2.396	Male/female	0.0159*
Histological type	1	1.021–1.998	Non-ADC/ADC	0.0374*
pT factor	2	1.610–3.513	T2 + T3/T1	<0.0001*
pN factor	2	1.565–3.063	N1 + N2/N0	<0.0001*
Smoking	1	0.849–1.844	Smoker/non-smoker	0.2567
Multivariate analysis				
OIP5	2	1.144–2.869	Positive/negative	0.0112*
Age (years)	2	1.228–2.499	≥65/65>	0.002*
Gender	1	0.878–2.095	Male/female	0.1691
Histological type	1	0.586–1.271	Non-ADC/ADC	0.4554
pT factor	2	1.250–2.796	T2 + T3/T1	0.0023*
pN factor	2	1.491–3.001	N1 + N2/N0	<0.0001*

ADC, adenocarcinoma; non-ADC, squamous–cell carcinoma plus large-cell carcinoma and adenosquamous-cell carcinoma; OIP5, Opa interacting protein 5. *$P < 0.05$.

(pN1–2), being elderly (≥65 years), being male (female versus male) and OIP5 positivity were significantly related to poor survival of NSCLC patients (Table 2). Furthermore, multivariate analysis using the Cox proportional hazard model indicated that pT stage, pN stage, age and positive OIP5 staining were independent prognostic factors for NSCLC patients (Table 2).

Positive staining of OIP5 scored according to immunohistochemical positivity criteria, as described in the Materials and Methods, was observed in 178 of 305 (58.4%) surgically resected esophageal cancers, whereas no staining was observed in any of the adjacent normal esophageal tissues. We then examined a correlation of OIP5 expression (positive versus negative) with various clinicopathological parameters and found a significant correlation with pT factor (higher in pT2–T3; $P = 0.0003$ by Fisher's exact test) and with lymph node metastasis (higher in pN1–N2; $P = 0.0037$ by Fisher's exact test) (Fig. 2F; Table 3). The Kaplan–Meier analysis indicated significant association between OIP5 positivity and shorter survival periods of ESCC patients ($P = 0.0168$ by the log-rank test; Fig. 2G). Using univariate analysis, advanced pT stage (pT2–T3), lymph node metastasis (pN1–N2), male gender and OIP5 positivity were found to be significantly related to poor survival of ESCC patients (Table 4). Using multivariate analysis, OIP5 status did not reach a statistically significant level as an independent prognostic factor for surgically treated ESCC patients enrolled in this study ($P = 0.1310$), whereas pT and pN stages and gender did, suggesting the relevance of OIP5 expression to these clinicopathological factors in esophageal cancer (Table 4).

Table 2. Cox's proportional hazards model analysis of prognostic factors in patients with non-small cell lung cancer

Variables	Hazards ratio	95% CI	Unfavorable/favorable	*P*-value
Univariate analysis				
OIP5	1.854	1.193–2.882	Positive/negative	0.0061*
Age (years)	1.557	1.103–2.199	≥65/65>	0.0119*
Gender	1.619	1.094–2.396	Male/female	0.0159*
Histological type	1.428	1.021–1.998	Non-ADC/ADC	0.0374*
pT factor	2.4	1.610–3.513	T2 + T3/T1	<0.0001*
pN factor	2.189	1.565–3.063	N1 + N2/N0	<0.0001*
Smoking	1.252	0.849–1.844	Smoker/non-smoker	0.2567
Multivariate analysis				
OIP5	1.812	1.144–2.869	Positive/negative	0.0112*
Age (years)	1.752	1.228–2.499	≥65/65>	0.002*
Gender	1.357	0.878–2.095	Male/female	0.1691
Histological type	0.863	0.586–1.271	Non-ADC/ADC	0.4554
pT factor	1.87	1.250–2.796	T2 + T3/T1	0.0023*
pN factor	2.115	1.491–3.001	N1 + N2/N0	<0.0001*

ADC, adenocarcinoma; non-ADC, squamous–cell carcinoma plus large–cell carcinoma and adenosquamous-cell carcinoma; OIP5, Opa interacting protein 5. *$P < 0.05$.

Table 3. Association between OIP5 positivity in esophageal cancer tissues and patients' characteristics ($n = 305$)

	Total	OIP5 positive	OIP5 negative	*P*-value: Positive versus negative
	$n = 305$	$n = 178$	$n = 127$	
Age (years)				
<65	196	114	82	>0.9999
≥65	109	64	45	
Gender				
Female	36	23	13	0.5898
Male	269	155	114	
pT factor				
T1	117	53	64	0.0003*
T2 + T3	188	125	63	
pN factor				
N0	138	68	70	0.0037*
N1 + N2	167	110	57	

OIP5, Opa interacting protein 5. *$P < 0.05$ (Fisher's exact test).

Table 4. Cox's proportional hazards model analysis of prognostic factors in patients with esophageal cancers

Variables	Hazards ratio	95% CI	Unfavorable/favorable	*P*-value
Univariate analysis				
OIP5	1.56	1.080–2.254	Positive/negative	0.0177*
Age (years)	1.078	0.748–1.554	≥65/65>	0.6866
Gender	3.404	1.499–7.730	Male/female	0.0034*
pT factor	2.558	1.689–3.873	T2 + T3/T1	<0.0001*
pN factor	3.129	2.102–4.656	N positive/N negative	<0.0001*
Multivariate analysis				
OIP5	1.333	0.918–1.935	Positive/negative	0.131
Gender	3.068	1.347–6.984	Male/female	0.0076*
pT factor	1.907	1.238–2.939	T2 + T3/T1	0.0034*
pN factor	2.442	1.619–3.684	N1–4/N0	<0.0001*

OIP5, Opa interacting protein 5. *$P < 0.05$.

Effect of Opa interacting protein 5 expression on cell growth. To assess whether upregulation of OIP5 plays a role in growth or survival of cancer cells, we transfected siRNA against *OIP5* (si-1 and i-2), along with two different controls (siRNA for LUC and CNT), into two lung cancer cell lines (LC319 and SBC-5) and an esophageal cancer cell line (TE2) to suppress expression of endogenous *OIP5*. The level of *OIP5* expression in the cells transfected with si-1 and si-2 was significantly reduced, in comparison with two control siRNA (Fig. 3A). Cell viability measured by MTT and colony-formation assays were reduced significantly in the cells transfected with si-1 or si-2, compared with those transfected with control siRNA (Fig. 3B,C).

To further examine a potential role of OIP5 in tumorigenesis, we prepared plasmids designed to express OIP5 (pcAGGSn3FC-OIP5-Flag) and transfected them into a lung cancer cell line (SBC-5), an esophageal cancer cell line (TE9) and an OIP5-negative mammalian cell line (COS-7) (Fig. S3A). After confirmation of exogenous OIP5 expression by western blot analysis (Fig. 3D and Fig. S3B), we found that growth of the OIP5-overexpressed cells was promoted at a significant degree in comparison to those transfected with the mock vector as measured by MTT and colony-formation assays (Fig. 3E,F and Fig. S3C,D).

Stabilization of Opa interacting protein 5 protein through its interaction with Raf1. To elucidate the biological significance of OIP5 expression in carcinogenesis, we attempted to identify proteins that would interact with OIP5 in cancer cells. A previous report about an exhaustive yeast two-hybrid screening using N-terminal regulatory domain of human Raf1 as ''bait'' indicated that OIP5 was one of 20 candidate Raf1-interacting proteins,[48] although their physiological interaction and function in mammalian cells were not clarified. Raf1 is activated in a wide range of tumor types, which triggers a cascade of cell signaling responses, resulting in cell proliferation and survival. Therefore, we examined whether OIP5 could interact with Raf1 in lung cancer cells. Immunoprecipitation of OIP5 in SBC-5 cells transfected with Flag-tagged OIP5 expressing plasmids using anti-Flag antibody followed by immunoblotting with anti-Raf1 antibodies indicated the interaction of exogenous OIP5 with endogenous Raf1 (Fig. 4A). We then examined OIP5 expression in human lung cancer cell lines by western blotting, and found co-expression of OIP5 and Raf1 in most of lung cancer cells examined (Fig. 4B), suggesting the possibility of the importance of the complex formation of the two proteins in cancer cells.

To further assess whether expression of Raf1 plays a role in the regulation of OIP5 function in cancer cells, we examined the biological significance of the Raf1 using siRNA against Raf1. We measured the level of endogenous OIP5 protein after transfection of siRNA for Raf1 to SBC-5 cells. Interestingly, the level of OIP5 protein was decreased in cells treated with si-Raf1, whereas the expression level of *OIP5* mRNA was not changed (Fig. 4C). Concordantly, overexpression of Raf1 resulted in the increase of the level of OIP5 protein, whereas the expression level of *OIP5* was unchanged (Fig. 4D), indicating a possibility that OIP5 protein stability is regulated by its interaction with Raf1.

Discussion

Prognosis of lung cancer and ESCC are known to be poor among malignant tumors, in spite of improvement in surgical techniques and adjuvant chemoradiotherapy. Several molecular-targeting drugs have been developed and their efficacy has been proven in cancer therapy; however, the proportion of patients benefitting from these drugs is still limited and new types of adverse reactions have been reported.[5] Therefore, it is important to develop new anti-cancer agents that will be highly specific to malignant cells, with minimal or no risk of adverse

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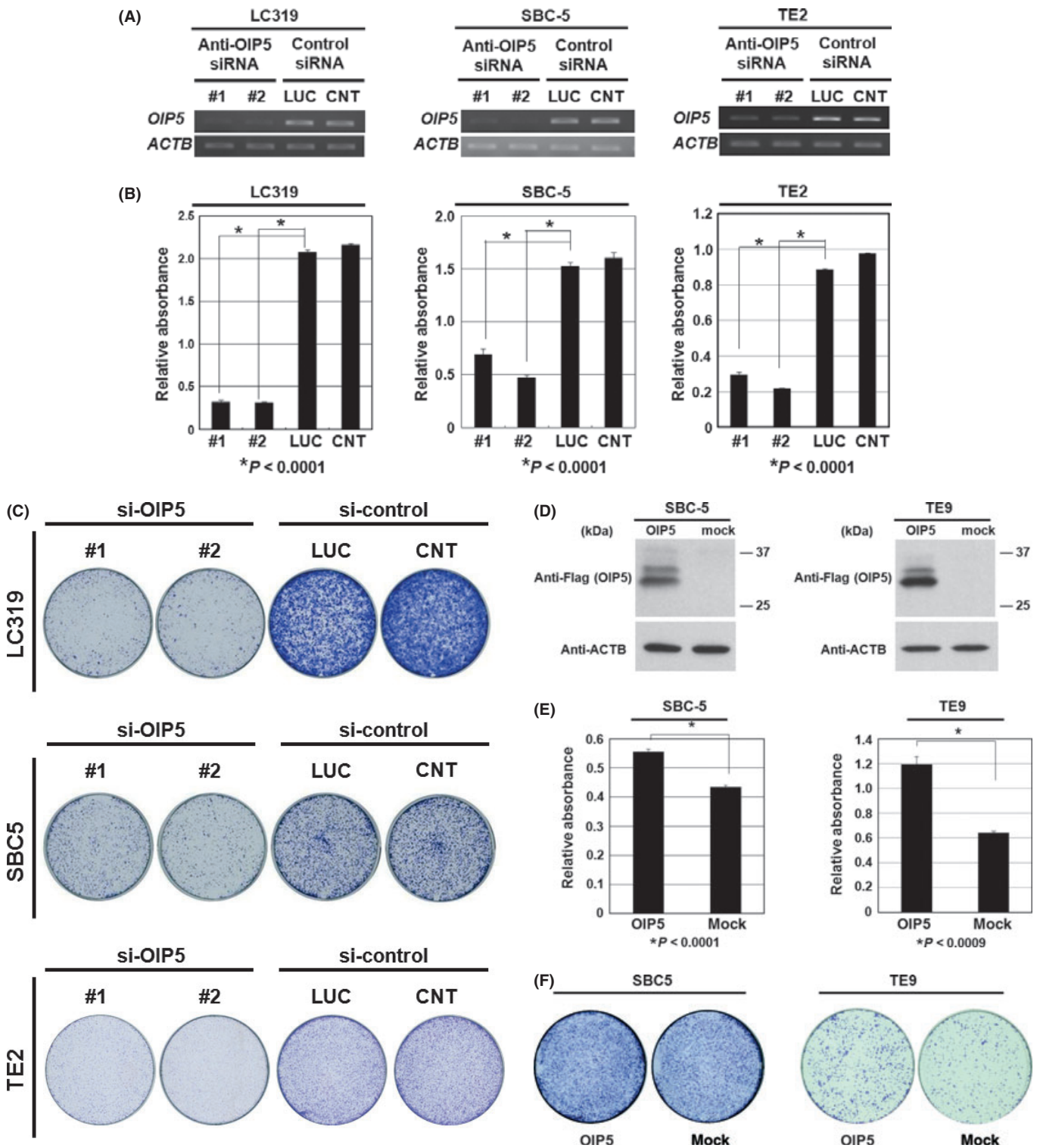

Fig. 3. Effect of Opa interacting protein 5 (*OIP5*) on cell growth. (A) Expression of *OIP5* in response to si-OIP5 (si-1 and -2) or control siRNA (LUC and On-Target plus/CNT) in LC319, SBC-5 and TE2 cells, analyzed by semiquantitative RT-PCR. (B) Viability of LC319, SBC-5 and TE2 cells evaluated by MTT assay in response to si-1, si-2, si-LUC, or si-CNT. (C) Colony-formation assays of LC319, SBC-5 and TE2 cells transfected with specific siRNA for OIP5 or control siRNA. (D) Expression of OIP5 in SBC-5 and TE9 cells examined by western blot analysis. (E,F) The cells transfected with pCAGGSn3Fc-OIP5 or mock vector were each cultured in triplicate, and the cell viability was evaluated by the MTT assay (E) and colony-formation assay (F).

reaction. We performed a genome-wide expression profile analysis of 120 lung cancers and 19 ESCC cells after enrichment of cancer cells by laser microdissection, using a cDNA microarray containing 27 648 genes.[5–11] We combined screening of candidate molecules by genome-wide expression profile analysis with high-throughput screening of loss-of-function effects, using the RNAi technique, and undertook systematic analysis of protein expression among hundreds of clinical samples on

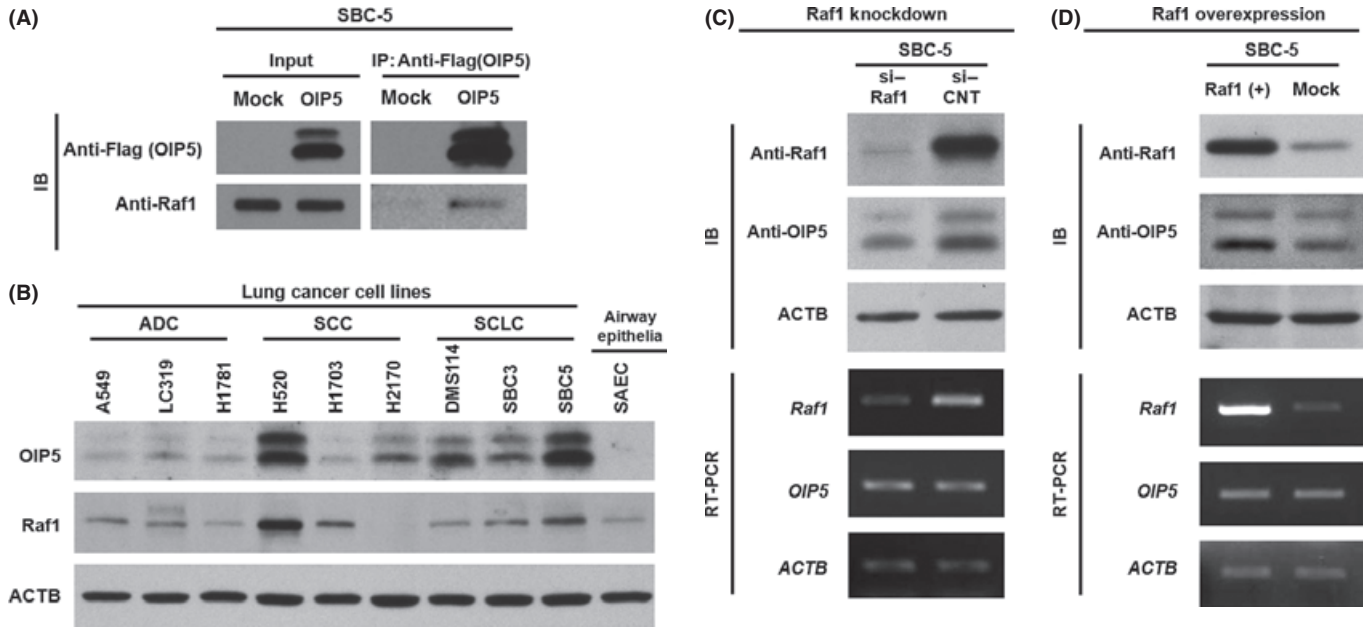

Fig. 4. Stabilization of Opa interacting protein 5 (*OIP5*) through its interaction with Raf1 protein. (A) Interaction of exogenous OIP5 with endogenous Raf1 protein in lung cancer SBC-5 cells. IB, immunoblotting; IP, immunoprecipitation. (B) Expression of OIP5 and Raf1 proteins in lung cancer cell lines. (C) Effect of Raf1 knockdown on the levels of OIP5 protein, detected by semiquantitative RT-PCR analysis and western blot analysis in SBC-5 cells. (D) Effect of Raf1 overexpression on the levels of OIP5 protein, detected by semiquantitative RT-PCR analysis and western blot analysis in SBC-5 cells. ADC, adenocarcinoma; IB, immunoblotting; IP, immunoprecipitation.SCC, small cell carcinoma; SCLC, small cell lung cancer.

tissue microarrays.[12–44] In this process, we identified OIP5 to be frequently overexpressed in lung and esophageal cancers (clinical samples and cell lines), and determined that the gene product is indispensable for growth of cancer cells.

Using yeast two-hybrid analysis and *in vitro* binding assays, OIP5 has been suggested to interact with Lamina-associated polypeptide (LAP2a), which interacts with A-type lamins and retinoblastoma protein, and regulates cell cycle progression via the E2F-Rb pathway.[49] OIP5 also forms the complex with C21orf45 and M18BP1, and is accumulated specifically at the telophase-G1 centromere.[50] Despite the availability of a considerable amount of *in vitro* data, the precise function of OIP5 in human cancer remains unclear.

Clinicopathological evidence obtained through our tissue-microarray experiments indicated that NSCLC and ESCC patients with OIP5-positive tumors had shorter survival periods than those with OIP5-negative tumors. We demonstrated that knockdown of OIP5 expression by siRNA in lung cancer cells resulted in suppression of cell growth. Importantly, Raf1 could interact with and stabilize OIP5 protein in cancer cells. The results obtained by *in vitro* and *in vivo* assays strongly suggested that OIP5 is likely to be an important growth factor and to be associated with a more malignant phenotype of lung cancer cells. Because these results indicate that OIP5 is likely to be one of the components of the Raf1 pathway, selective targeting of functional interaction between Raf1 and OIP5 is a promising therapeutic strategy, although further investigation of OIP5 pathway is necessary for a better understanding of the mechanisms of OIP5 oncogene activation. Because OIP5 should be classified as one of the typical cancer testis antigens, selective inhibition of OIP5 activity by molecular targeted agents is a promising therapeutic strategy that is expected to have powerful biological activity against cancer with minimal risk of adverse events.

In summary, OIP5 might play an important role in the growth of lung and esophageal cancers by interacting with Raf1. OIP5 overexpression in resected specimens might be a useful index for application of adjuvant therapy to the patients who are likely to have poor prognosis. In addition, the data strongly imply the possibility of designing new anti-cancer drugs and cancer vaccines to specifically target the OIP5 for human cancer treatment.

Disclosure Statement

YD and YN are scientific advisers of OncoTherapy Science.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (A,B) Expression of OIP5 in lung and esophageal cancer tissues, comparing with each adjacent normal tissues. (C) Dephosphorylation of exogenous OIP5 protein in COS-7 cells transfected with OIP5-expressing plasmid and endogenous OIP5 protein in SBC-5 cells by treatment with λ-phosphatase.

Fig. S2. (A) Kaplan–Meier analysis of survival of stage I NSCLC patients according to OIP5 expression (P = 0.0848; log-rank test). (B) Kaplan–Meier analysis of survival of stage II–IIIA NSCLC patients according to OIP5 expression (P = 0.0770; log-rank test).

Fig. S3. (A) *OIP5* expression in lung and esophageal cancer cell lines and mammalian COS-7 cells. (B) Expression of OIP5 in COS-7 cells examined by western blot analysis. (C, D) The cells transfected with pCAGGSn3Fc-OIP5 or mock vector were each cultured in triplicate, and the cell viability was evaluated by the MTT assay (C) and colony-formation assay (D).

Table S1. Resource and histological type of lung cancer and esophageal cell lines.

Table S2. Summary of 336 NSCLC and 305 ESCC patients.

Table S3. Multivariate logistic regression analysis of the clinicopathologic factors that were associated with the OIP5 expression in NSCLC patients.

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