EGFR Mutation Up-regulates EGR1 Expression through the ERK Pathway

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Abstract. *Background: DelE746_A750-type EGFR is a constitutively active type of mutation that enhances EGFR signaling. However, the changes in gene expression that occur in mutant EGFR-harboring cells has not been fully studied. Materials and Methods: A gene expression analysis of HEK293 cells transfected with wild-type or mutant EGFR was performed focusing on the significant gene. Results: Early growth response 1 (EGR1), a transcription factor, was the most strongly up-regulated gene in mutant EGFR-transfected cells among the genes examined. An increase in EGR1 expression in the mutant EGFR cells was confirmed using RT-PCR or immunoblotting. The expression was up-regulated by EGF stimulation and down-regulated by EGFR-tyrosine kinase inhibitor. In addition, the MEK inhibitor U0126 inhibited EGR1 expression, while the phosphatidylinositol 3-kinase inhibitor LY294002 did not. Conclusion: Mutant EGFR constitutively up-regulates EGR1 through the ERK pathway, and its expression is correlated with EGFR signal activation. Findings provide an insight into a target gene of mutant EGFR and further improve the understanding of the oncogenic properties of EGFR.*

Epidermal growth factor receptor (EGFR) is frequently overexpressed in various solid tumors (1, 2) and is regarded as a definitive oncogene. Accumulating data on EGFR and its signal pathway in cancer cells suggests that EGFR is a promising therapeutic target molecule; indeed, benefits from treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) and anti-EGFR antibody have been confirmed in clinical settings (3, 4). Common *EGFR* mutations of DelE746_A750 and L858R, characterized by 15-base in-frame deletions or substitutions clustered around the ATP-binding site in exons 19 and 21 of *EGFR*, have been identified in patients with non-small cell lung cancer (NSCLC); these mutations are major determinants of sensitivity to EGFR-TKIs (5-8). Such mutations confer a constitutively active EGFR signal pathway to cancer cells (9).

The activated EGFR signal pathway has been intensively investigated, including studies on alterations in downstream signaling, the underlying mechanism responsible for sensitivity to EGFR-TKIs, the involvement in carcinogenesis, oncogene addiction, and clinico-pathological analyses. It has been previously reported that a lung cancer cell line, PC-9, with a deletional mutant of *EGFR* (delE746_A750) was hypersensitive to EGFR-TKIs and that this mutant *EGFR* was constitutively active and activated the ERK and AKT pathways (10-13). However, the changes in gene expression that occur in mutant *EGFR*-harboring cells have not been fully studied.

To identify changes in the gene expressions of downstream molecules that arise as a result of *EGFR* mutation and activated EGFR signaling, a microarray analysis of cells, in which the DelE746_A750-type of *EGFR* mutation had been stably introduced, was performed.

Materials and Methods

Reagents. The purified recombinant human EGF was purchased from R&D systems (Minneapolis, MN, USA). LY294002 2-(4-Morpholinyl)-8-phenyl-4H-benzopyran- 4-one was purchased from Calbiochem (San Diego, CA, USA), U0126 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadine was purchased from Cell Signaling Technology (Beverly, MA, USA).

Expression constructs and viral production. Full-length cDNA of wild-type *EGFR* was amplified by RT-PCR from a human embryonal kidney cell line (HEK293), and mutant *EGFR* (delE746_A750) was amplified from an NSCLC cell line (PC-9) (10, 14). Wild-type and mutant *EGFR* cDNA in a pcDNA3.1 vector (Clontech, Palo Alt, CA, USA) was cut out and introduced into a pQCLIN retroviral vector (BD Biosciences Clontech, San Diego, CA, USA) together with EGFP, followed by the internal ribosome entry sequence (IRES) to monitor the expression of the inserts indirectly. A pVSV-G vector (Clontech,

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Palo Alt, CA, USA) for the constitution of the viral envelope and pQCXIX constructs were co-transfected into the GP2-293 cells using FuGENE6 transfection reagent. Briefly, 80% confluent cells cultured on a 10-cm dish were transfected with 2 μg of pVSV-G plus 6 μg of pQCXIX vectors. Forty-eight hours after transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15,000 g for 3 h at 4˚C. The viral pellet was then resuspended in fresh RPMI1640 medium. The titer of the viral vector was calculated by counting the EGFP-positive cells that were infected by serial dilutions of virus-containing medium, and the multiplicity of infection (MOI) was then determined.

Cell culture and transfection. The HEK293 cell line was cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37˚C. The HEK293 cells were retrovirally transfected with the mock, wild-type and mutant *EGFR*, and the stable established cell lines were designated as HEK293-Mock, HEK293-Wild and HEK293-Del.

Real-time RT-PCR. One microgram of total RNA from a cultured cell line was converted to cDNA using a GeneAmp® RNA-PCR kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using the Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems) under the following conditions: 95˚C for 6 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses. To amplify the target genes, the following primers were purchased from TaKaRa (Yotsukaichi, Japan): EGR1-FW, GTA CAG TGT CTG TGC CAT GGA TTT C; EGR1-RW, GAG GAT CAC CAT TGG TTT GCT TG; GAPD-FW, GCA CCG TCA AGG CTG AGA AC; and GAPD-RW, ATG GTG GTG AAG ACG CCA GT. The results of three independent experiments were analyzed.

In vitro growth-inhibition assay. The growth-inhibitory effects of AG1478 (Biomol International, Plymouth Meeting, PA, USA) on the HEK293-Mock, -Wild and -Del cells were examined using an MTT assay. A 180-μL volume of an exponentially growing cell suspension (2×10^3 cells/well) was seeded into 96-well microtiter plates and 20 μL of various drug concentrations were added. After incubation for 72 h at 37˚C, 20 μL of MTT solution (5 mg/mL in PBS) were added to each well and the plates were incubated for an additional 3 h at 37˚C. After centrifuging the plates at 400 g for 5 min, the medium was aspirated from each well and 200 μL of DMSO was added to each well to dissolve the formazan. The optical density was measured at 570 nm. The results of three independent experiments were analyzed.

Immunoblotting. The antibodies used for immunoblotting were as follows: anti-EGFR (Upstate Biotechnology), anti-phospho-EGFR (Tyr1068), anti-p44/42 MAP kinase, anti-phospho-p44/42 MAP kinase, anti-Akt (Cell Signaling), anti-EGR1, anti-βactin (Santa Cruz), and anti-phospho-Akt (Ser473) (BD Bioscience, SanJose, CA, USA). Sub-confluent cells were washed with cold PBS and harvested with Lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.0), 5 mM EDTA, 50 mM sodium chloride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor mix, complete™ (Roche Diagnostics). Whole-cell lysates and the culture medium were separated using a 2-15% gradient SDS-PAGE and blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a TBS buffer (pH 8.0) with 0.1% Tween-20, the membrane was probed with primary antibody. After rinsing twice with TBS buffer, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling) and washed, followed by visualization using an ECL detection system (Amersham) and LAS-3000 (Fujifilm, Tokyo, Japan). The immunoblotting was performed in two independent experiments.

Microarray analysis. The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA, USA). In brief, the total RNA extracted from the cell lines was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) as a quality check, and cRNA was synthesized using the GeneChip® 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNAs were then purified and used to construct the probes. Hybridization was performed using the Affymetrix GeneChip HG-U133 Plus2.0 array for 16 h at 45˚C. The signal intensities were measured using a GeneChip®Scanner3000 (Affymetrix) and converted to numerical data using the GeneChip Operating Software, Ver.1 (Affymetrix).

Statistical analysis. The microarray analysis was performed using the BRB Array Tools software ver. 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html), developed by Dr. Richard Simon and Dr. Amy Peng. The microarray analysis was performed as described previously (15). Additional statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) to calculate the standard deviation (SD) and statistically significant differences between each sample using the Student *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

Early growth response 1 (EGR1) expression in mutant EGFR. The DelE746_A750-type *EGFR* mutation mediates a constitutively active EGFR signal and induces cellular hypersensitivity to EGFR-TKIs (11, 13). Mock, wild and mutant *EGFR* was introduced and stable cell lines was established as HEK293-Mock, -Wild and -Del cells. HEK293-Del cells showed increased phosphorylation levels of EGFR and ERK1/2 and were significantly hypersensitive to the EGFR-tyrosine kinase inhibitor AG1478, compared with the other cell lines (Figure 1A and 1B). To identify which gene expressions were changed by the *EGFR* mutation, a microarray analysis was performed for these stable cell lines. Twenty-three genes were identified as differentially expressed genes, the expressions of which differed by more than three-fold between HEK293-Wild and HEK293-Del cells (Table I). These genes included several cancer-related genes such as *EGR1, GALNT3, TACSTD1* (EpCAM), *MAFF, NLK, FOXN4, RUNX3* and *CD70*. Among them, *EGR1* was the most up-regulated gene in the HEK293-Del cells (>10-fold higher than in HEK293-Mock and -Wild cells, Figure 1C, 1D). The ratios of the signal intensity relative to that in the HEK293-Mock cells were 3.0-fold in the HEK293-Wild cells and 34.5-fold in the HEK293-Del

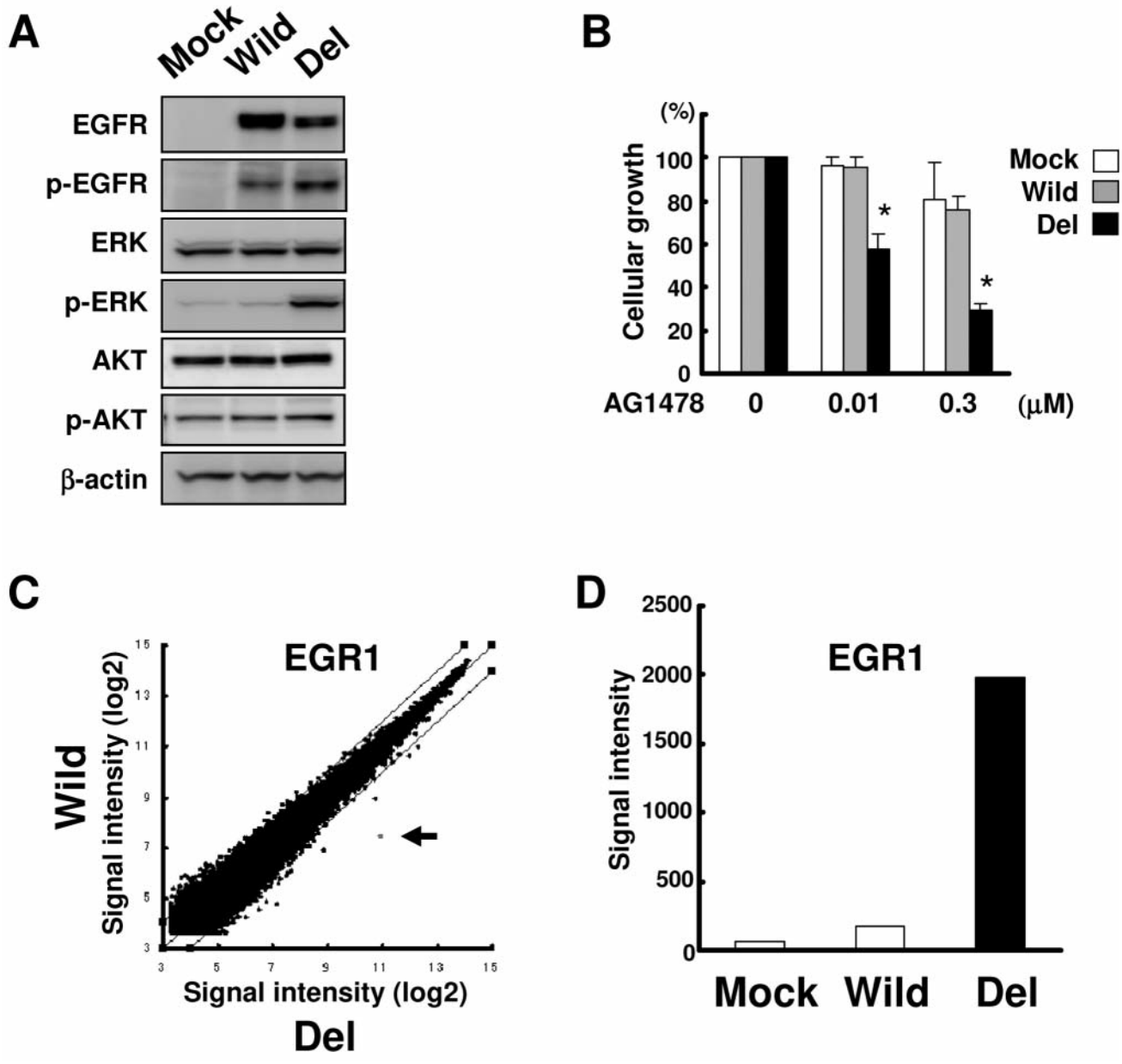

Figure 1. *Microarray analysis showing that mutant EGFR up-regulates EGR1 expression. (A) Immunoblotting for HEK293-Mock, -Wild and -Del cells cultured under normal conditions. The phosphorylation of EGFR and ERK1/2 was increased in HEK293-Del cells. (B) Growth inhibitory effect of an EGFR tyrosine kinase inhibitor. HEK293-Del cells were highly sensitive to AG1478. (C) Results of microarray analysis for genes with differential expressions between HEK293-Wild and -Del cells. The arrow indicates the EGR1 gene. (D) Signal intensity of microarray data for EGR1. EGR1 expression was up-regulated by more than 10-fold, compared with in HEK293-Wild cells. The error bars represent the SDs of three independent experiments. *: p<0.05.*

cells. Thus, the role of the *EGR1* transcription factor in EGFR signal activation was the focus of subsequent studies.

EGF stimulates EGR1 expression. The mRNA and protein levels of EGR1 up-regulation were confirmed using real-time RT-PCR and western blotting for these stable cell lines. Real-time RT-PCR revealed that *EGR1* mRNA expression in the HEK293-Wild cells was slightly (~3-fold) higher than that in the HEK293-Mock cells. On the other hand, *EGR1* mRNA expression was remarkably increased in the HEK293-Del cells (133-fold, compared with the HEK293-Mock cells). Similar results were obtained for the protein levels (Figure 2A, 2B). These results indicate that *EGR1* expression was constitutively up-regulated in the *EGFR* mutation-harboring cells.

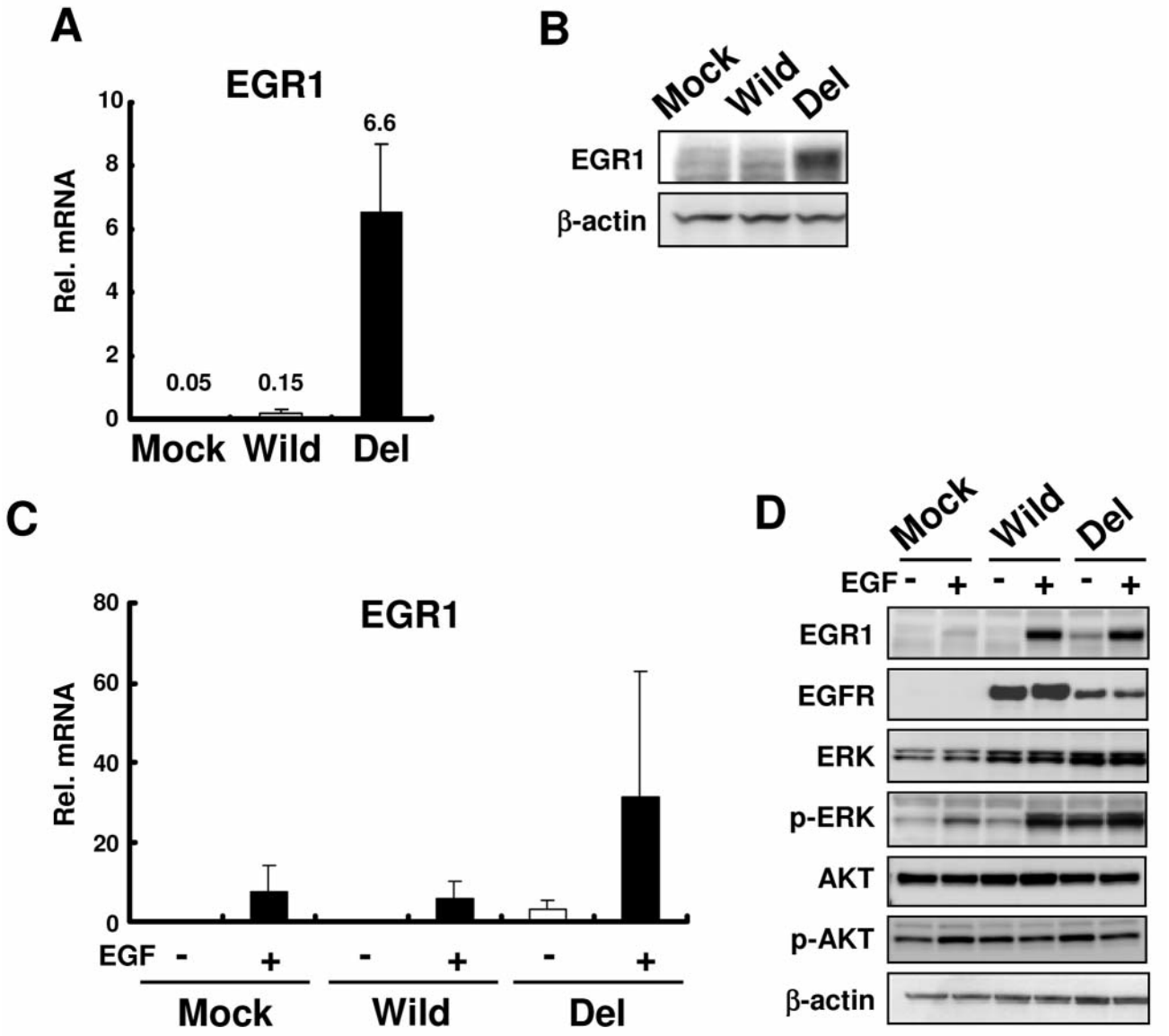

Figure 2. *Mutant EGFR up-regulates EGR1, and EGR1 expression is regulated by EGF stimulation. (A) Real-time RT-PCR shows that mutant EGFR up-regulates EGR1 by more than 40-fold, compared with in HEK293-Mock and -Wild cells cultured under normal conditions. (B) Immunoblotting shows the up-regulation of EGR1 in HEK293-Del cells. (C, D) EGF stimulation and EGR1 expression detected by real-time RT-PCR and immunoblotting. EGR1 expression was up-regulated by EGF, and the expression was correlated with the phospho-ERK1/2 levels. The error bars represent the SDs of three independent experiments. Immunoblotting was performed in two independent experiments. Rel. mRNA indicates the ratio of mRNA expression of EGR1/GAPD × 10^{-6}.*

To examine whether the up-regulation of *EGR1* expression is regulated by EGFR signaling, the change in expression induced by EGF stimulation was evaluated. EGF increased *EGR1* mRNA expression in HEK293-Mock, -Wild and -Del cells (Figure. 2C). EGR1 up-regulation by EGF was also confirmed by immunoblotting (Figure 2D). HEK293-Wild cells stimulated with EGF expressed EGR1 to the same extent as in HEK293-Del cells, possibly reflecting the constitutively active function of EGFR in the HEK293-Del cells. In addition, EGR1 expression was closely correlated with the phospho-ERK1/2 expression levels. These findings suggest that EGR1 expression is involved in the ERK1/2 pathway.

EGFR-TKI down-regulates EGR1 expression. To elucidate the further relationship between *EGR1* up-regulation and EGFR signaling activity, the three cell lines were treated with EGFR-TKI. An EGFR-TKI, AG1478, inhibited the

Table I. *The results of microarray analysis for differentially expressed genes between HEK293-Wild and HEK293-Del cells. Twenty-three genes were identified as differentially expressed genes, the expressions of which differed by more than three-fold.*

Gene	Description	Probe set	Wild	Del	Fold change
EGR1	Early growth response 1	227404_s_at	172	1979	11.5
APOBEC3B	Apolipoprotein B mRNA editing enzyme	206632_s_at	27	147	5.4
GALNT3	UDP-N-acetyl-alpha-D-galactosamine	203397_s_at	118	477	4.1
TACSTD1	Tumor-associated calcium signal transducer 1	201839_s_at	23	91	3.9
MAFF	Transcription factor MAFF	36711_at	29	114	3.9
LOC646903	Hypothetical LOC646903	237116_at	19	67	3.6
UCHL1	Ubiquitin carboxyl-terminal esterase L1	201387_s_at	483	1733	3.6
RABL3	RAB, member of RAS oncogene family-like 3	226090_x_at	12	39	3.2
ZNF330	Zinc finger protein 330	213760_s_at	47	149	3.2
ERGIC2	ERGIC and golgi 2	226422_at	66	208	3.2
PHLDA2	Pleckstrin homology-like domain, family A, member 2	209803_s_at	59	183	3.1
TRIM5	Tripartite motif-containing 5	210705_s_at	13	40	3.1
KLHL23	Kelch-like 23 (Drosophila)	213610_s_at	62	192	3.1
C18orf37	Chromosome 18 open reading frame 37	1559716_at	56	13	0.23
NLK	Nemo-like kinase	238624_at	59	15	0.25
	CDNA FLJ34034 fis	238515_at	41	11	0.27
FOXN4	Forkhead box N4	241009_at	57	15	0.27
RUNX3	Runt-related transcription factor 3	204198_s_at	44	13	0.28
	transcribed locus	230746_s_at	70	21	0.31
CD70	CD70 molecule	206508_at	80	25	0.31
VDAC1	Voltage-dependent anion channel 1	217139_at	52	16	0.31
NBEA	Neurobeachin	226439_s_at	33	10	0.32
NID2	Nidogen 2 (osteonidogen)	204114_at	55	18	0.32

expression of both *EGR1* mRNA (Figure 3A) and protein (Figure 3B). EGR1 expression was also correlated with the phospho-ERK1/2 expression levels detected by immunoblotting. These results support the concept that EGR1 up-regulation by mutant EGFR is regulated by EGFR signaling.

EGR1 expression is regulated through the ERK1/2 pathway. EGR1 is thought to be a downstream molecule in the ERK1/2 pathway (16). To elucidate whether EGR1 up-regulation in mutant EGFR cells is regulated *via* the ERK1/2 pathway, ERK1/2 and AKT, two major downstream pathways of EGFR was evaluated. LY294002, a phosphatidylinositol 3-kinase inhibitor, inhibited the phosphorylation levels of AKT but did not modify the expression of EGR1 (Figure 4A). However, the MEK inhibitor U0126 clearly down-regulated EGR1 expression in HEK293-Del cells (Figure 4B). EGR1 expression was consistent with the phospho-ERK1/2 expression levels. These results strongly suggest that EGR1 up-regulation by mutant *EGFR* is regulated through the ERK pathway. Based on these findings, a model was propossed to explain the up-regulation of EGR1 by mutant *EGFR* (Figure 4C). In this model, mutant *EGFR* activates the ERK pathway and induces *EGR1* transcription.

Discussion

EGR1 transcription factor is induced by various stimuli, including growth factors, hypoxia, UV and cytokines, and mediates multiple cellular responses such as mitogenesis, differentiation, cellular survival, anti-apoptosis, angiogenesis and apoptosis (17). In cancer biology, *EGR1* is basically regarded as a tumor suppressor gene because it directly regulates p53, PTEN and TGFβ1. Deletion of the *EGR1*-containing 5q31 region has been associated with a certain type of lymphoma and small cell lung carcinoma. Low EGR1 expression in tumor tissue is frequently observed in breast cancer, glioblastoma and other solid tumors (18). In contrast, the oncogenic property of *EGR1* is observed in prostate cancer (19).

An increased expression of *EGR1* was observed in mutant *EGFR* cells. Previous reports have demonstrated that mutant *EGFR* is oncogenic in non-small cell lung cancer (20). However, Ferraro *et al*. have demonstrated that *EGR1* expression is strongly correlated with PTEN expression and that patients with high levels of EGR1 had better overall and disease-free survival periods than patients with low levels of EGR1 in patients with NSCLC (21). It was speculated that the overexpression of EGR1 in mutant *EGFR* cells may play some role in the biological behaviors of mutant *EGFR* in cancer.

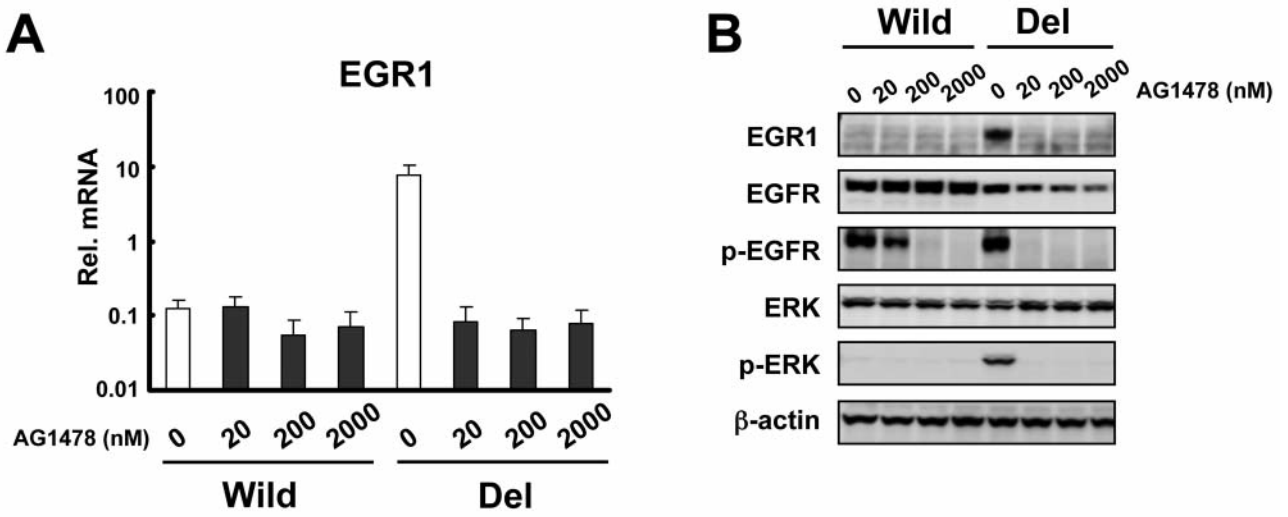

Figure 3. *EGFR tyrosine kinase inhibitor down-regulates EGR1 expression. (A) Real-time RT-PCR and (B) immunoblotting were performed for cells cultured under normal conditions and treated with four concentrations of AG1478 for 5 h. EGFR tyrosine kinase inhibitor clearly down-regulated EGR1 expression. The phosphorylation levels of EGFR decreased at a lower concentration (20 nM) in HEK293-Del cells than in HEK293-Wild cells. Note that the Y-axis is a log-scale. Error bars represent the SDs of three independent experiments. Immunoblotting was performed in two independent experiments. Rel. mRNA indicates the ratio of mRNA expression of EGR1/GAPD × 10^{-6}.*

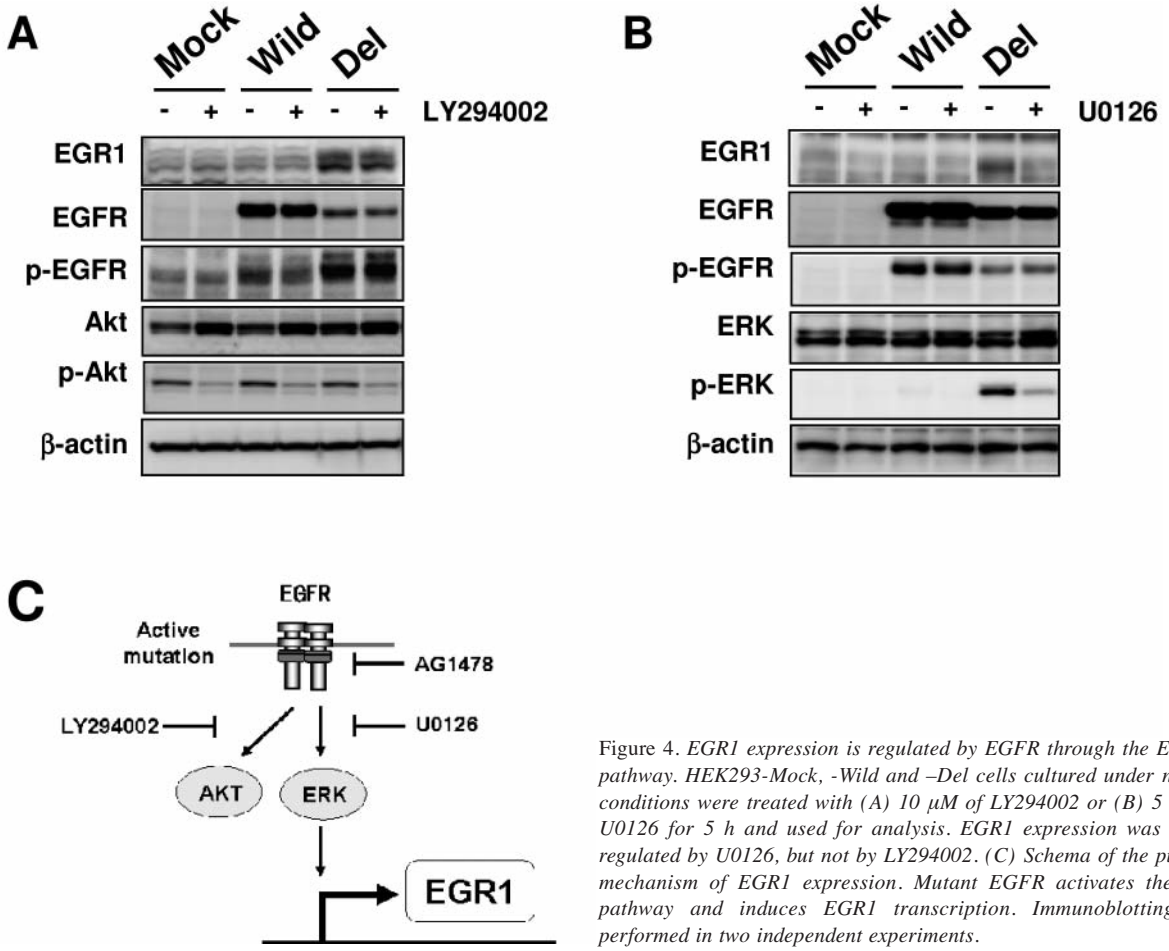

Figure 4. *EGR1 expression is regulated by EGFR through the ERK1/2 pathway. HEK293-Mock, -Wild and –Del cells cultured under normal conditions were treated with (A) 10 μM of LY294002 or (B) 5 μM of U0126 for 5 h and used for analysis. EGR1 expression was down-regulated by U0126, but not by LY294002. (C) Schema of the putative mechanism of EGR1 expression. Mutant EGFR activates the ERK pathway and induces EGR1 transcription. Immunoblotting was performed in two independent experiments.*

In general, ERK and JNK kinases phosphorylate ternary complex factors (TCF), which cooperate with serum response factor (SRF) to induce *EGR1* transcription in vascular biology (22). *EGR1* can displace Sp1 and other transcription factors, and EGR1 transactivation leads to the transcription of many EGR1-target genes. To date, several putative EGR1-target genes related to cancer have been identified, including cyclin D, EGFR, FGF, IGF-I, thymidine kinase, PDGF-A, Bcl2, CD44, p53, PTEN, TNF-α and VEGF. Further investigation of the biological role of EGR1 overexpression in mutant *EGFR* may lead to a better understanding of the roles of mutant *EGFR* in cancer cells.

In conclusion, it was found that mutant *EGFR* induced EGR1 overexpression and that this overexpression was correlated with EGFR signal activation through ERK1/2. These results provide a novel insight into the oncogenic properties of EGFR in cancer cells.

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