Erlotinib Resistance in Lung Cancer Cells Mediated by Integrin β1/Src/Akt-Driven Bypass Signaling

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Abstract
EGF receptor (EGFR) kinase inhibitors, including gefitinib and erlotinib, exert potent therapeutic efficacy in non–small cell lung cancers harboring EGFR-activating mutations. However, most patients ultimately develop resistance to these drugs. Here, we report a novel mechanism of acquired resistance to EGFR tyrosine kinase inhibitors and the reversal of which could improve clinical outcomes. In erlotinib-resistant lung cancer cells harboring activating EGFR mutations that we established, there was increased expression of Src, integrin β1, α2, and α5 along with enhanced cell adhesion activity. Interestingly, RNAi-mediated silencing of integrin β1 restored erlotinib sensitivity and reduced activation of Src and Akt after erlotinib treatment. Furthermore, Src silencing inhibited Akt phosphorylation and cell growth, with this inhibitory effect further augmented by erlotinib treatment. Increased expression of integrin β1, α5, and/or α2 was also observed in refractory tumor samples from patients with lung cancer treated with erlotinib and/or gefitinib. Together, our findings identify the integrin β1/Src/Akt signaling pathway as a key mediator of acquired resistance to EGFR-targeted anticancer drugs. Cancer Res; 73(20): 6243–53. ©2013 AACR.

Introduction
Patients with non–small cell lung cancer (NSCLC) harboring activating somatic mutations in the EGF receptor (EGFR) gene show dramatic clinical responses. Of the somatic mutations, in-frame deletions in exon 19 (del E746-A750) and the L858R- point mutation are most commonly observed in NSCLC (1). These EGFR mutations are closely associated with sensitivity to EGFR tyrosine kinase inhibitors (TKI), erlotinib and gefitinib. Both gefitinib and erlotinib have shown to improve progression-free survival as compared with chemotherapy alone when given as first-line treatment for patients with NSCLC harboring activating EGFR mutations (2–6).

However, one of the serious problems encountered during clinical treatment with EGFR-TKI is the appearance of drug-resistant tumors (7). Well-characterized mechanisms for the acquired resistance to EGFR-TKIs include the T790M mutation in exon 20 of the EGFR-TKI domain (8, 9) and Met amplification (10). Furthermore, the loss of PTEN and increased expression of mitogen-activated protein kinase (MAPK), ABCG2, insulin growth factor 1 receptor (IGF-IR), and TGF-β have also been reported (7, 11, 12). In addition to these well-characterized mechanisms, further elucidation of novel mechanisms for acquired drug resistance is essential for the development of personalized therapeutics and strategies to circumvent drug resistance.

In our laboratory, we have established various drug-resistant cell lines from human lung cancer cell lines harboring activating EGFR mutations by selecting for resistance to EGFR-TKIs (13). Previously, we have reported the loss of PTEN expression with the loss of nuclear translocation of EGR-1, a transcription factor responsible for PTEN gene expression, in gefitinib-resistant clones (14–16). We also reported that either the complete or partial loss of the activated EGFR gene allele could also result in the acquisition of erlotinib resistance (17). In this present study, we further established erlotinib-resistant clones by step-wise selection following exposure to erlotinib and observed enhanced expression and activation of integrin β1 and Src. Herein, we present a novel bypass mechanism through which the integrin β1/Src/Akt signaling may play a pivotal role in the acquisition of erlotinib resistance in lung cancer cells.

Materials and Methods
Cell culture
PC9 cells were kindly provided by Dr. Y Ichinose, National Hospital Organization, Kyushu Cancer Center (Fukuoka, Japan) and 11–18 cells were provided by Dr. K Nakagawa,
Kinki University (Osaka, Japan). These cells and the resistant clones were cultured in RPMI medium supplemented with 10% FBS in an atmosphere of 5% CO₂ (14, 18). Cells were routinely confirmed to be free of mycoplasma contamination using mycosensor QPCR Assay Kits (Agilent Technologies). Cells were transiently transfected with cDNA using Lipofectamine LTX, PLUS reagent, and Opti-MEM medium (Invitrogen) according to the manufacturers’ recommendations. Cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen) according to the manufacturers’ recommendations. Reagents are described in Supplementary Material and Methods.

**Cytotoxicity assays**

Exponentially growing cell suspensions were seeded into each well and the following day the indicated concentration of drugs was added. After incubation for 72 hours, cytotoxicity was determined as described previously (17, 18). Each value represents the average from triplicate wells ± SD.

For colony formation assay, 5 × 10² cells were plated in 35 mm dishes and the following day were transfected with integrin β1 siRNA. At 48 hours, various concentrations of erlotinib were added, followed by incubation for 7 days at 37°C. Colonies of more than 10 cells were counted after Giemsa staining as described previously (19). Each value represents the average of duplicate dishes.

**Western blot analysis**

Cells were rinsed with ice-cold PBS and lysed in Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 50 mmol/L NaF, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate) and cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore Corp.). For detection of active integrin β1, cells were plated on fibronectin- or collagen-coated dishes and incubated with antiactive integrin β1 antibody (HUTS-4) for 30 minutes at 37°C. Cells were rinsed with PBS, lysed in SDS sample buffer, and bound antibody was detected by Western blotting (20). Immunoprecipitation was described in Supplementary Material and Methods.

**Cell adhesion assay**

For cell adhesion assay, 96-well plates were coated with collagen (10 μg/well, Trevigen) or fibronectin (5 μg/well, Trevigen) and cancer cells were seeded onto the extracellular matrix components, allowed to adhere for 30 minutes at 37°C. Each value represents the average from triplicate trials ± SD.

**Quantitative real-time PCR**

RNA was reverse transcribed from random hexamers using avian myeloblastosis virus reverse transcriptase (Promega). In brief, 20 μL of PCR amplification reaction mixtures contained cDNA, primer pairs (Applied Biosystems), a dual-labeled fluorogenic probe (Applied Biosystems), and TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 minutes and 95°C for 10 minutes and then 40 cycles alternating between 95°C for 15 seconds and 60°C for 1 minute. The relative gene expression for each sample was determined using the formula 2−(ΔΔCt) = 2(Ct(GAPDH) − Ct(target)), which reflected the target gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Each value represents the average from triplicate trials ± SD.

**Flow cytometry (FACS)**

Cell suspension (10⁴ cells/100 μL) in incubation buffer [0.5% bovine serum albumin (BSA) in PBS] was stained with anti-integrin β1 mAb or purified PE-Mouse IgE2a Control (Becton-Dickinson) for 1 hour at 4°C. Flow cytometry was conducted using the FACSCalibur System (Becton-Dickinson).

**Tumor samples**

Informed consent for the use of tumor tissue specimens was obtained from patients according to the legal guidance (21, 22). Tumor samples, case 4 and case 10, are collected from University Hospital, University of Occupational and Environmental Health (Kitakyushu, Japan). Tumor samples, case RB4 and case RB11, are from EURTAC Study in Spain. The EURTAC was approved by the Institutional Review Board of each participating center and written informed consent was obtained from all patients. Mutation status of EGFR (delE746-A750 and L858R) and T790M was examined by direct sequencing analysis. K-Ras mutations were investigated by PCR analysis (21–23).

**Immunohistochemical analysis**

Expression of integrins and Src was examined by immunohistochemical (IHC) staining as previously described (23). Anti-integrin β1 mAb was purchased from Abcam Inc., anti-integrin α2 mAb was from Epitomics, anti-integrin α5 mAb was from Santa Cruz Biotechnology, and anti-Src was from Cell Signaling Technology. The intensity of staining was scored using the following scale: no staining, 0; weak staining, +1; moderate staining, +2; and strong staining, +3, in more than 10% of cancer cells.

**Results**

**Establishment of the erlotinib-resistant subclones from PC9 cells and 11-18 cells**

To isolate erlotinib-resistant subclones from PC9 cells harboring the activating EGFR delE746-A750 mutation and 11-18 cells harboring activating EGFR L858R mutation, we cultured PC9 cells and 11-18 cells in increasing, step-wise doses of erlotinib up to 15 μmol/L for approximately 6 months as described previously (14, 17). We isolated drug-resistant cell lines, PC9/ER2 and 11-18/ER2, and further established drug-resistant subclones, PC9/ER2-2 and PC9/ER2-3 from PC9/ER2 and 11-18/ER2-1 from 11-18/ER2 (Fig. 1A; Table 1). Growth rates between each parental cell line and resistant subclones are similar (Table 1). Resistant subclones also manifested increased resistance to gefitinib as compared with the parental cell lines (Table 1). In contrast, these 3 resistant subclones exhibited similar sensitivities to picropodophyllin (an inhibitor of IGF-IR), SU11274 (an inhibitor of Met), and cisplatin as compared with the parental cell lines (Table 1).
Figure 1. Expression and activation of receptor tyrosine kinases and downstream signaling molecules in PC9, PC9/ER2-2, PC9/ER2-3, 11-18, and 11-18/ER2-1 cells. A, the sensitivity to erlotinib was assessed by cell proliferation assays for 3 days. B, Western blots showing the expression levels of proteins and the loading control α-Tubulin. C, Western blots showing the effect of erlotinib on expression of proteins. Each cell line was exposed to various doses of erlotinib for 5 hours.
Integrin family proteins 2 and 5 were coimmunoprecipitated with integrin 1 in PC9/ER2-2 and 11-18/ER2-1 as compared with parental cells (data not shown). However, L858R EGFR expression was less in 11–18/ER2-1 cells because of the partial loss of mutated (T790M, T854A, L747S, and D761Y) nor hot spot activating mutation of PIK3CA was detected in resistant subclones (data not shown). As compared with parental cell lines, all resistant subclones expressed relatively lower levels of pHER2(Fig. 2B). In addition, 2 resistant subclones from PC9 expressed lower levels of HER2, HER3, and pHER3. In contrast, the expression of phospho-Akt (pAkt) was moderately higher in the resistant subclones, although that of PTEN, e-Met, IGF1R, Axl, and FGFR1–4 was also similar among cell lines (Fig. 1B, data not shown). DelE746-A750 EGFR was similarly expressed among both of the resistant subclones and the PC9 cell line (data not shown). However, L858R EGFR expression was less in 11–18/ER2-1 cells because of the partial loss of mutated EGFR gene allele, as described previously (17). The phosphorylation of EGFR (pEGFR) and Erk1/2 was similarly inhibited by erlotinib when integrin 1 was silenced (Fig. 3D). Furthermore, the adhesion of cells to collagen- or fibronectin-coated plates in the resistant subclones was significantly higher than that observed in parental cell lines (Fig. 2E).

Increased expression of β1, α2, and α5 integrins in the erlotinib-resistant subclones

We further examined the expression levels of integrins in the resistant subclones by Western blot analysis. Surprisingly, all resistant subclones manifested higher levels of β1, α2, and α5 integrins than the parental cell lines. However, there were similar expression levels of αv and β4 integrins between parental cell lines and the resistant subclones (Fig. 2A). PC9/ER2-2 and PC9/ER2-3 also exhibited higher expression levels of integrin β1 mRNA than the PC9 cells, but 11–18/ER2-1 exhibited similar expression levels of integrin β1 mRNA (Fig. 2B). Increased expression of β1 in PC9/ER2-2 and 11–18/ER2-1 as compared with parental cell lines (Fig. 2C). Integrin β1 is known to be dimerized with α integrin family proteins α2 and α5 (24), and immunoprecipitation Western blot analysis revealed that α2 or α5 integrins were coimmunoprecipitated with integrin β1 in PC9/ER2-2 and PC9/ER2-3 cells (Supplementary Fig. S1A).

Integrins induce the activation of downstream regulatory signaling pathways by adopting a high-affinity state to interact with extracellular matrices (25). Ligands of integrin α2β1 and integrin α5β1 are collagen and fibronectin, respectively. As shown in Fig. 2D, increased activation of integrin β1 in PC9/ER2-2 and 11–18/ER2-1 cells as compared with parental cell lines was observed in the presence of fibronectin or collagen when a specific antibody (HUTS-4) recognizing ligand-occupied integrin β1 was used. Furthermore, the adhesion of cells to collagen- or fibronectin-coated plates in the resistant subclones was significantly higher than that observed in parental cell lines (Fig. 2E).

Integrin β1 knockdown overcomes erlotinib resistance in resistant subclones

We next examined whether integrin β1 expression was correlated with erlotinib resistance or constitutive Akt phosphorylation. Treatment with integrin β1 siRNA almost completely suppressed the expression of integrin β1 protein and mRNA (Fig. 3A and B) and there were no apparent changes in the growth rates between each parental cell line and their resistant subclones upon exposure to integrin β1 siRNA for 5 days (data not shown). In resistant subclones, integrin β1 siRNA treatment restored sensitivity to erlotinib (Fig. 3C) and erlotinib treatment almost completely blocked the constitutive phosphorylation of Akt when integrin β1 was silenced (Fig. 3D). We further examined whether integrins α2 and α5 were also involved in the constitutive Akt phosphorylation in PC9/ER2-2 cells (Supplementary Fig. S1B). The phosphorylation of Akt was moderately inhibited by erlotinib when integrin α2 or integrin α5 was silenced in PC9/ER2-2 cells. Integrin β1 knockdown was also accompanied by the downregulation of integrin α5, suggesting a close regulatory link between integrin β1 and integrin α5. These results suggest that integrin β1, in concert with integrin α2 and/or integrin α5, seems to play a key role in the acquisition of erlotinib resistance.

We further established the stable cell lines PC9/TG2 and PC9/TG11 by transfecting PC9 cells with integrin β1 cDNA (Supplementary Fig. S2A). There was no concomitant enhancement of the expression of integrin α2 or integrin α5 in either cell line and no apparent changes in the growth rates of PC9/Mock, PC9/TG2, and PC9/TG11 cells over the 5-day culture...
EGFR or integrin subclones phosphorylation and cell survival in the resistant erlotinib resistance. Thus, we next examined whether integrin induce the activation of the Src/Akt pathway and whether this overexpression of integrin corresponding integrin similarly suppressed by erlotinib in the PC9/Mock cells and the cells (Supplementary Fig. S2C). The pEGFR and Erk1/2 was pressed by erlotinib in either the PC9/ITG2 or PC9/ITG11 cell lines while Akt phosphorylation was suppressed in PC9/Mock S2B). Furthermore, the phosphorylation of Akt was not suppressed by erlotinib in either the PC9/ITG2 or PC9/ITG11 cell lines while Akt phosphorylation was suppressed in PC9/Mock cells (Supplementary Fig. S2C). The pEGFR and Erk1/2 was similarly suppressed by erlotinib in the PC9/Mock cells and the corresponding integrin β1-overexpressing cell lines. Thus, the overexpression of integrin β1 with integrin α2 and integrin α5 seems to be one of the mechanisms underlying acquired erlotinib resistance. Src knockdown and dasatinib treatment suppress Akt phosphorylation and cell survival in the resistant subclones Src protein, which interacts with PI3K, is often activated by EGFR or integrin β1 in association with extracellular matrices (26). Thus, we next examined whether integrin β1 could directly induce the activation of the Src/Akt pathway and whether this pathway could be responsible for erlotinib resistance. Relatively higher expression levels of Src and pSrc (Y416) were observed in PC9/ER2-2 and 11-18/ER2-1 cells as compared with each parental cell line (Fig. 4A). In each resistant subclone from PC9 or 11-18 cells, the phosphorylation of Src was blocked by integrin β1 knockdown and this reduction was augmented by erlotinib (Fig. 4B), suggesting a close correlation between integrin β1 and EGFR with the activation of the Src/Akt pathway. We next examined how Src activation could be involved in the integrin β1-driven activation of Akt and acquired erlotinib resistance using Src siRNA and dasatinib—a Src kinase inhibitor. Treatment with Src siRNA almost completely suppressed the expression of Src (Fig. 4C). Src siRNA treatment suppressed expression of Src and markedly suppressed Akt phosphorylation (Fig. 4D) and cell growth (Fig. 4E) in PC9/ER2-2 and 11-18/ER2-1 cells. This suppression of Akt phosphorylation was augmented by erlotinib. We further examined whether Akt phosphorylation was inhibited by erlotinib in PC9/ER2-2 cells treated with an inhibitor of Src, dasatinib. Dasatinib treatment
at 0.1 μmol/L suppressed Akt phosphorylation and this effect was augmented by erlotinib in PC9/ER2-2 cells (Supplementary Fig. S3A). Moreover, PC9/ER2-2 cells were more sensitive to dasatinib than PC9 cells (Supplementary Fig. S3B), suggesting that Src activation in the resistant subclone may sensitize those cells to the cytotoxic effects of dasatinib.
Figure 4. Effect of Src knockdown on Akt phosphorylation and the restoration of erlotinib sensitivity by Src knockdown. A, Western blots showing the expression of p-Src (Y416) and Src. B, the effect of integrin β1 knockdown on Src phosphorylation with or without erlotinib. Cells were treated for 2 days with integrin β1 siRNA, followed by further exposure to erlotinib for 5 hours. C, Western blots show the expression of Src protein after treatment with Src siRNA for the indicated number of days. Because Src and α-tubulin were detected on the same membrane, α-tubulin is pointed to with arrowheads. D, the cells were treated with or without Src siRNA for 2 days and exposed to erlotinib for another 5 hours. The quantitative analysis of the Western blots is shown and the values are normalized to pAkt levels in the absence of erlotinib and Src siRNA. E, the effect of Src knockdown on proliferation of cells. Cells were treated for 2 days with Src siRNA, followed by treatment with erlotinib. The cell number in the absence of the drug and/or siRNA was set as 100%.

Integrin β1 Contributes to EGFR-TKI Resistance in NSCLC
The expression of integrins in cancer cells from refractory lung tumors

We finally examined whether integrin expression could be observed in gefitinib- or erlotinib-refractory tumors. Four clinical samples were prepared from patients with lung adenocarcinoma treated with gefitinib or and erlotinib, but subsequently became refractory to these EGFR-TKIs (Supplementary Table S1; ref. 21). As shown in Supplementary Table S1, 2 patients harbored L858R and 2 patients harbored delE746-A750. Figure 5(A–D) shows typical IHC images from the patients. Especially expression of integrin β1 was increased in the post-erlotinib and post-gefitinib tumor samples with enhanced expression of integrin α2 or α5. Src expression was also enhanced after treatment, but only post-erlotinib sample of case 10 shows lower expression of Src than pretreatment sample. One sample, case RB4 harboring
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T790M secondary mutation of EGFR, shows higher expression of integrin β1, α2, and Src in post-gefitinib treatment. In this case, integrin overexpression might not be directly correlated with acquired resistance (Fig. 5D). Expression of integrins and Src in 4 clinical samples was summarized in Supplementary Table S1.

Discussion

In our present study, we established erlotinib-resistant subclones from PC9 cells harboring the activating del E746-A750 EGFR mutation and 11-18 cells harboring the activating L858R EGFR mutation. We observed the following characteristics in these resistant subclones: (i) Akt was constitutively activated and the phosphorylation of Akt was not suppressed by erlotinib in resistant subclones; (ii) the expression of β1, α2, and α5 integrins was upregulated along with Src activation; (iii) the knockdown of integrin β1 restored cellular sensitivity to erlotinib and reduced Akt phosphorylation; (iv) Src knockdown or dasatinib treatment effectively inhibited cell survival and reduced Akt phosphorylation in the presence or absence of erlotinib; and (v) enhanced expression of integrin β1, α2, and/or α5 was also observed in refractory tumor samples from patients with lung cancer treated with gefitinib and/or erlotinib. Together, these novel findings indicate that the acquisition of erlotinib resistance is mediated by the activation of the integrin β1/Src/Akt signaling pathway (Fig. 6).

Three major mechanisms including the alteration of oncopgenic targets, the activation of downstream regulatory molecules, and the activation of bypass effectors, are known to contribute to acquired resistance to molecularly targeted drugs in cancer cells (7). This study suggests that the acquisition of erlotinib resistance is attributable to the activation of an integrin β1-driven bypass signaling pathway. Furthermore, the study by Ju and colleagues (27) previously reported that transfection with integrin β1 cDNA protected PC9 cells from apoptosis in response to gefitinib treatment. However, this study did not evaluate how integrin β1 overexpression renders lung cancer cells resistant to EGFR-TKIs. Our study showed that transfection with integrin β1 slightly induced erlotinib resistance in PC9 cells (Supplementary Fig. S2) and in these integrin β1-overexpressing cell lines, there was no enhanced expression of integrin α2 or α5, suggesting that the coactivation of β1/α2 and/or β1/α5 integrins is required for the acquisition of increased erlotinib resistance via the integrin β1 signaling pathway.

The interactions between cancer cells and the extracellular matrices often reduce the sensitivity to anticancer agents, including gefitinib (28–31) and the expression of integrin family proteins plays an important role in the malignant progression of cancers (32–34). The cell proliferation rates were similar between parental cell lines and the resistant subclones, but the adhesion to collagen or fibronectin was much higher in the resistant subclones. Integrin β1 thus seems to be responsible for not only the acquisition of erlotinib resistance but also for cell adhesion activity.

In this study, we observed constitutive phosphorylation of Akt even in the presence of erlotinib in resistant subclones. This phosphorylation was markedly suppressed by erlotinib upon integrin β1 knockdown and cell sensitivity to erlotinib was restored by integrin β1 knockdown. These data strongly suggest an essential role for integrin β1/α2 and/or integrin β1/α5 in the constitutive activation of Akt and in the drug resistance in the resistant subclones. Our study also sought to understand how integrin β1 promotes Akt phosphorylation and the acquisition of drug resistance. Of the potentially relevant molecules, Src is known to be a downstream regulator of EGFR and/or integrins and it plays an essential role in the survival of lung cancer cells (35, 36). Src is also responsible for the acquisition of resistance to EGFR-targeted drugs (37, 38). Consistent with these studies, we observed marked activation of Src in PC9/ER2-2 and 11-18/ER2-1 cells as compared with the parental counterparts. The blockade of Src signaling by Src siRNA resulted in the marked suppression of Akt phosphorylation, suggesting a close link between Src and Akt activation.
as shown in a previous study (26). Furthermore, Src knockdown also dramatically suppressed cell growth in PC9/ER2-2 and 11-18/ER2-1 cells, even in the absence of erlotinib. Furthermore, treatment with Src kinase inhibitor dasatinib also suppressed Akt phosphorylation. Taken together, these data suggest that the activation of the integrin β1/Src/Akt signaling pathway may undergo compensatory activation during the acquisition of drug resistance (Fig. 6). On the other hand, clinical trials of Src inhibitor dasatinib in combination with erlotinib, was studied in lung cancer (39). We suggest that Src is one of the effective targets for lung cancer therapeutics.

We also examined whether integrin β1, together with integrin α2 or integrin α5, was expressed in lung cancer refractory to gefitinib and/or erlotinib. These results showed increased expression of β1, α2, and/or α5 integrins in 4 refractory tumors following treatment with gefitinib and/or erlotinib (Fig. 5 and Supplementary Fig. S1). Enhanced expression of integrin β1 and α2 were also observed in case RB4-harbored T790M secondary EGFR mutation (Fig. 5D). It remains to be further studied whether the enhanced expression of integrin β1, α2, and Src is closely correlated with T790M mutation. Recently, integrin family targeted drugs are focused on cancer therapeutics. For example, anti-integrin α5β1 antibody (volociximab) is conducted on phase II clinical trials for several cancers including NSCLC (40, 41). Further study should be also required whether these integrin-targeted drugs could be useful to overcome drug resistance in patients with NSCLC.

In conclusion, the acquisition of erlotinib resistance in our erlotinib-resistant subclones was attributable to the overexpression of integrin β1/α2/α5, which resulted in the activation of an integrin/Src/Akt pathway. This relationship between integrin expression and Src activity may be useful for the development of both personalized therapeutics involving EGFR-TKIs and for circumventing drug resistance in patients with NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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