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Original Article

EGFR over-expression and mutations lead to a change in biological characteristics of human lung adenocarcinoma cells

Jia Feng[1], Min Peng[1], Viverk Verma[2], Jianping Bi[3], Qibin Song[1], Guang Han[1]

[1]Department of Oncology, Renmin Hospital of Wuhan University, Wuhan 430060, China; [2]Department of Radiation Oncology, University of Nebraska Medical Center, Omaha, USA; [3]Department of Radiation Oncology, Hubei Cancer Hospital, Tongji Medical Colloge, Huazhong University of Science and Technology, Wuhan 430079, China

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Abstract: Brain metastasis (BM) is a frequent occurrence from lung cancer, and is associated with an overall poor prognosis. Accumulating evidence has demonstrated a high incidence of BM in lung adenocarcinoma patients with the epidermal growth factor receptor (EGFR) mutation. To investigate the potential molecular mechanisms, we constructed EGFR over-expression and three EGFR-mutant (EGFR-L858R, EGFR-E746-A750del and EGFR-T790M) human lung adenocarcinoma cell sublines by using lentiviral transfection of NCI-H1563 cells. The effects of EGFR over-expression and mutations on proliferation, migration, and invasion *in vitro* were investigated. Herein, we found that EGFR over-expression and the EGFR-E746-A750del (19 exon deletion) mutation significantly enhanced cellular proliferation, migration, and invasion. Moreover, the expression of BM-associated genes, such as matrix metalloproteinase-9 (MMP-9), vascular epithelial growth factor (VEGF), and S100β, was significantly increased in both EGFR over-expression and EGFR-E746-A750del mutated cell sublines. Therefore, our study demonstrates that EGFR over-expression and the 19 exon deletion mutation could enhance cellular invasion and even promote the occurrence of BM, possibly by up-regulating the expression of MMP-9, VEGF, and S100β.

Keywords: EGFR, mutations, biological characteristics, lung adenocarcinoma

Introduction

Lung cancer is the most common cause of cancer-related death, with 158,080 deaths estimated for 2016 in the United States [1]. Representing 80% of lung cancer cases, non-small cell lung carcinoma (NSCLC) has several histological subtypes, the most common of which is adenocarcinoma. Occurring in more than half of NSCLC patients, adenocarcinoma is more aggressive than other NSCLC subtypes and is often associated with rapid disease progression and early distant metastasis [2, 3]. An example of such, brain metastasis (BM), associated with an overall poor prognosis, may occur more frequently in adenocarcinoma [4, 5]. Approximately 60% of patients with lung adenocarcinoma develop BM at some point in their disease course, which is clearly higher than a < 20% incidence for squamous cell carcinoma [6]. Notably, the precise mechanisms underlying this high BM rate in lung adenocarcinoma remain unclear.

The epidermal growth factor receptor (EGFR) is a tyrosine kinase (TK) receptor of the ErbB family that encodes a 1186 amino acid (170-kD) membrane-bound protein [7]. Studies have reported that EGFR signaling pathways may play important roles in oncogenic progression and metastasis via at least three major mechanisms: over-expression of EGFR ligands, amplification of EGFR, and EGFR mutations [8-10]. In addition, mutations in the EGFR gene exert a dominant oncogenic effect, resulting from the increased and enhanced effect of the receptor itself [11, 12]. Several types of EGFR mutations, which are predominantly found in adenocarcinomas [13], have been confirmed: G719X (exon 18), E746-A750 (exon 19), T790M or D770-N771 (exon 20), and L858R or L861Q (exon 21). Of these, the most common muta-

tions occur at exon 19 (E746-A750 deletion) and exon 21 (L858R substitution), accounting for roughly 85% to 90% [14] of mutations. These may warrant treatment with EGFR tyrosine kinase inhibitors (TKIs) for selected patients. On the contrary, mutations in exon 20 (T790M) correlate with resistance to EGFR-TKIs [15].

A clinical retrospective study [16], including our previous study [17], have shown a significant association between EGFR mutations and proclivity for BM in patients with lung adenocarcinoma, and indicate that EGFR mutations may play an important role in promoting BM. However, research regarding precise molecular mechanisms between EGFR mutations and brain metastases are currently lacking.

Molecular mechanisms underlying BM are very complex and incompletely understood. In order to successfully metastasize to the brain, tumor cells must cross the blood brain barrier (BBB) and form colonies thereafter. Several genes are thought to promote cancer cells' infiltration into the brain, not limited to plasmin, heparinase, E-cadherin, integrins, matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF). Of these, MMP-9 and VEGF will be briefly highlighted. MMP-9 plays a critical function in tumor progression and metastasis by stimulating cell migration, tumor invasion, and angiogenesis [18]. VEGF is an important positive regulator of angiogenic cytokines, which can promote cellular migration by regulating blood vessel (BV) formation and growth [19]. Thus, it has been posited that VEGF and MMP-9 play a synergistic role in the development and progression of BM [20]. Also understudied with regard to BM, S100 proteins are a family of low-molecular-weight proteins found in vertebrates and characterized by two calcium-binding sites that have helix-loop-helix (EF-hand type) conformation. The variegated recombination of S100 genes in human cells can lead to various malignancies, especially central nervous system (CNS) metastases [21]. It has been reported that S100β contributes to tumorigenesis by inhibiting the function of the tumor suppressor protein p53, along with regulating cell proliferation and differentiation by stimulating the activity of the mitogenic kinase Akt [22, 23]. S100β has thus been utilized in a sense as a "predictor" of brain metastases in lung carcinoma [24].

Because molecular mechanisms characterizing the relationship between EGFR and BM remain underdefined, we used the H1563 human lung adenocarcinoma cell line to construct EGFR over-expression and various EGFR-mutant cell sublines. We then compared the biological characteristics and expression of VEGF, MMP-9, EGFR, and S100β in these cell lines.

Materials and methods

Cell culture and transfection

A human lung adenocarcinoma cell line, NCI-H1563 (EGFR-wild type) (blank control, BC), purchased from American Type Culture Collection (ATCC), was grown in RPMI 1640 with fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml), at 37°C with 5% CO_2. Regarding EGFR transfection, the retroviral vector (GV358) containing either over-expressed EGFR (OE), EGFR-L858R (LR), EGFR-E746-A750del (DEL), EGFR-T790M (790M), or an empty vector (negative control, NC) were transfected into a competent cell line (DH5α cells) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h, viral supernatant was harvested, it was filtered (0.45 μm pore size) and used to infect H1563 cells at a density of 5×10^5 cells/dish. For transfection, 5×10^5 NCI-H1563 cells were seeded into each well of 24-well plates, cultured in complete medium for 48 h, and subsequently transfection efficiency was analyzed via a fluorescence-activated cell sorter (FACS) assay.

Proliferation assays

Cells were seeded at a density of 1×10^4 cells/well, in triplicate, in a 96-well dish and counted daily for 5 days with the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. For the MTT assay, MTT was diluted 1:5 with dilution buffer, and 50 μl added to each well. The dish was then incubated for 4 hours at 37°C with 5% CO_2, and stopped with the addition of 150 μl DMSO. Absorbance was read at 570 nm.

Migration and invasion assays

Migration was assessed using the wound healing assay. For migration, 5×10^4 cells suspended in 2 ml RPMI-1640 medium were planted in

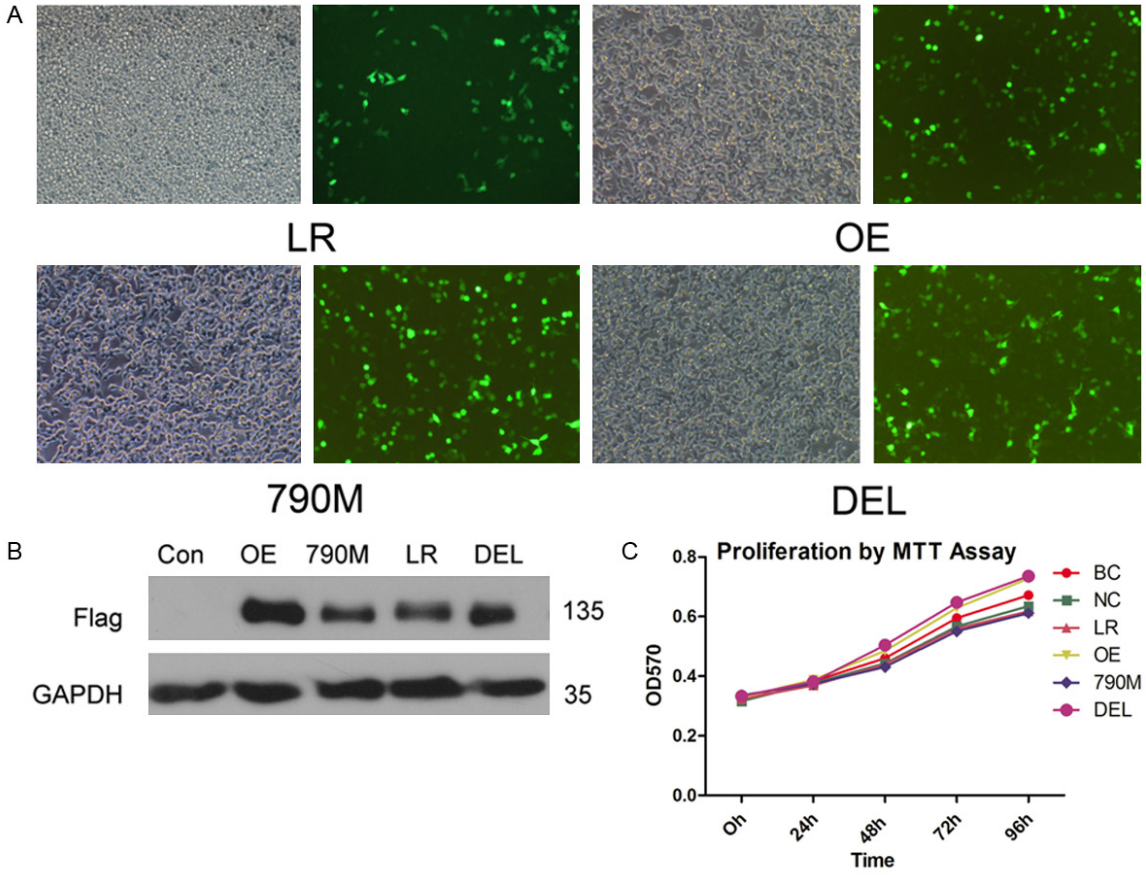

Figure 1. Construction of EGFR over-expression and mutated NCI-H1563 cells, and proliferation assays. A. Obvious fluorescence were observed in cells, indicating the target plasmids were successfully transfected into cells (×100); B. The expression of the EGFR fusion gene was assessed by Western blot analysis using anti-flag antibody in flag-tagged EGFR mutation plasmids. C. Cell viability was measured by MTT and expressed as the absorbance at 570 nm. EGFR over-expression and the EGFR-E746-A750-del mutation enhance proliferation. [NC: negative control (empty vector), BC: blank control (EGFR-wild type), LR: EGFR-L858R, OE: EGFR over-expression, 790M: EGFR-T790M, LR: EGFR-L858R, DEL: EGFR-E746-A750del].

a 6-well plate. Cells were then scratched with a 20 μl micropipette tip when the cells reached >90% confluence. The migrated distances were measured by phase microscopy at 0, 24, and 48 h after wounding.

Invasion assay was performed in Transwell chambers. Briefly, 5×10^4 cells/well in serum-free media containing BSA were added to the upper microporous membrane of a Transwell chamber and were subsequently triggered by addition of complete media containing 10% FBS. After 24 h, cells were fixed with 95% ethanol and stained with toluidine blue for 15 min and 10 min, respectively. Invaded cells were counted from five fields with phase contrast microscopy.

RT-PCR and Western blot assays

Total RNA was first extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used as a template to synthesize cDNA. The expression of mRNA was quantified by RT-PCR (ABI Prism 7900). In this study, the following primers were used for amplification: MMP9, forward (5'-TCTATGGTCCTCGCCCTGAA-3') and reverse (5'-CATCGTCCACCGGACTCAAA-3'); VEGF, forward (5'-CTGTCTAATGCCCTGGAGCC-3') and reverse (5'-ACGCGAGTCTGT-GTTTTTGC-3'); S100β, forward (5'-ATTCTGGAAGGGAGGGAGACA-3') and reverse (5'-TGGAAGTCACATTCGCCGTC-3'); EGFR, forward (CTAAGATCCC-GTCCATCGCC) and reverse (GGAGCCCAGCACTTTGATCT); and GAPDH (neg-

ative control), forward (5'-CAATGACCCTTCATTGACC-3') and reverse (5'-G-ACAAGCTTCCCGTTCTCAG-3').

Proteins were lysed in the modified radio immunoprecipitation assay (RIPA) buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. After blocking with 5% skim milk diluted in PBS-Tween (0.1%), membranes were incubated with antibodies against MMP-9 (1:400), VEGF (1:800), S100β (1:400), EGFR (1:400), or GAPDH (1:800) overnight at 4°C. Blots were subsequently probed with appropriate secondary antibodies conjugated to goat radish peroxidase (goat anti-rabbit or -mouse) and subsequently analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

GraphPad Prism5 was used for cell migration and invasion assays as well as statistical analysis of RT-PCR. Comparisons among multiple groups were analyzed with one-way ANOVA by Dunnett method. Results were deemed statistically significant if $P < 0.05$ (*$P < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

Construction of EGFR over-expression and mutated NCI-H1563 cells

We first constructed EGFR over-expression and three EGFR mutated (LR, DEL, and 790M) human lung adenocarcinoma sublines by using lentivirus to infect NCI-H1563 cells. Results from the FACS assay showed that obvious fluorescence was observed in transfected cells, indicating that the target plasmids were successfully transfected into the cells, and all four lentiviral constructs expressed EGFR well *in vitro* (**Figure 1A**). At the same time, results from the Western blot assay also demonstrated that four sublines expressed the EGFR fusion gene (**Figure 1B**). We observed a characteristic band around 135 kD, which was consistent with the target gene fusion protein.

EGFR over-expression and EGFR-E746-A750del mutation enhances proliferation

After transfection for 48 hours, the proliferation of the various cell lines was measured by the MTT assay. The data (**Figure 1C**) showed that NCI-H1563 cells transfected with overexpressed EGFR and EGFR-E746-A750del proliferated more effectively [OE (1.475±0.021 fold/48 h, 1.898±0.010 fold/72 h, 2.196±0.019 fold/96 h), DEL (1.509±0.014 fold/48 h, 1.926±0.008 fold/72 h, 2.185±0.016 fold/96 h), respectively) as compared to control [NC (1.400±0.021 fold/48 h, 1.776±0.017 fold/72 h, 1.989±0.019 fold/96 h, $p < 0.001$, BC (1.400±0.011 fold/48 h, 1.796±0.016 fold/72 h, 2.027±0.023 fold/96 h, $p < 0.001$), respectively].

EGFR over-expression and EGFR-E746-A750del mutation increases migration and invasion

The migrated ability of the various cell lines were measured by phase microscopy at 0, 24, and 48 h after wounding (**Figure 2A**). The wound healing results after 48 h showed that EGFR over-expression and EGFR-E746-A750del mutation substantially increased cellular migration (**Figure 2B**, $p < 0.05$, respectively). Neither EGFR-L858R nor EGFR-T790M affected migration of the NCI-H1563 cells compared to control cell lines with empty vector or wild-type EGFR (**Figure 2B**, $p>0.05$). Additionally, invaded cells were counted with phase contrast microscopy (**Figure 2C**). Coincidentally, overexpressed EGFR and the EGFR-E746-A750-del mutation significantly promoted invasion (**Figure 2D**, $p < 0.001$). The EGFR-L858R mutation restricted invasion as compared to negative control ($p < 0.01$). However, there was no difference between EGFR-T790M and control ($p>0.05$, negative control; $p>0.05$, blank control).

EGFR over-expression and EGFR-E746-A750del mutation regulate expression of MMP-9, VEGF, and S100β

Expression of the MMP-9, VEGF, and S100β proteins detected in EGFR over-expression cells (MMP9, 2.822±0.067 fold; VEGF, 1.667±0.029 fold; S100β, 1.6676±0.009 fold) and EGFR-E746-A750del mutated cells (MMP9, 3.276±0.041 fold; VEGF, 1.662±0.015 fold; S100β, 1.671±0.006 fold) were much higher than that in negative or blank control cells ($p < 0.001$) (**Figure 3A** and **3B**).

mRNA expression of MMP-9, VEGF, and S100β in cell lines

To further confirm the above results, MMP-9, VEGF, and S100β were amplified by RT-PCR,

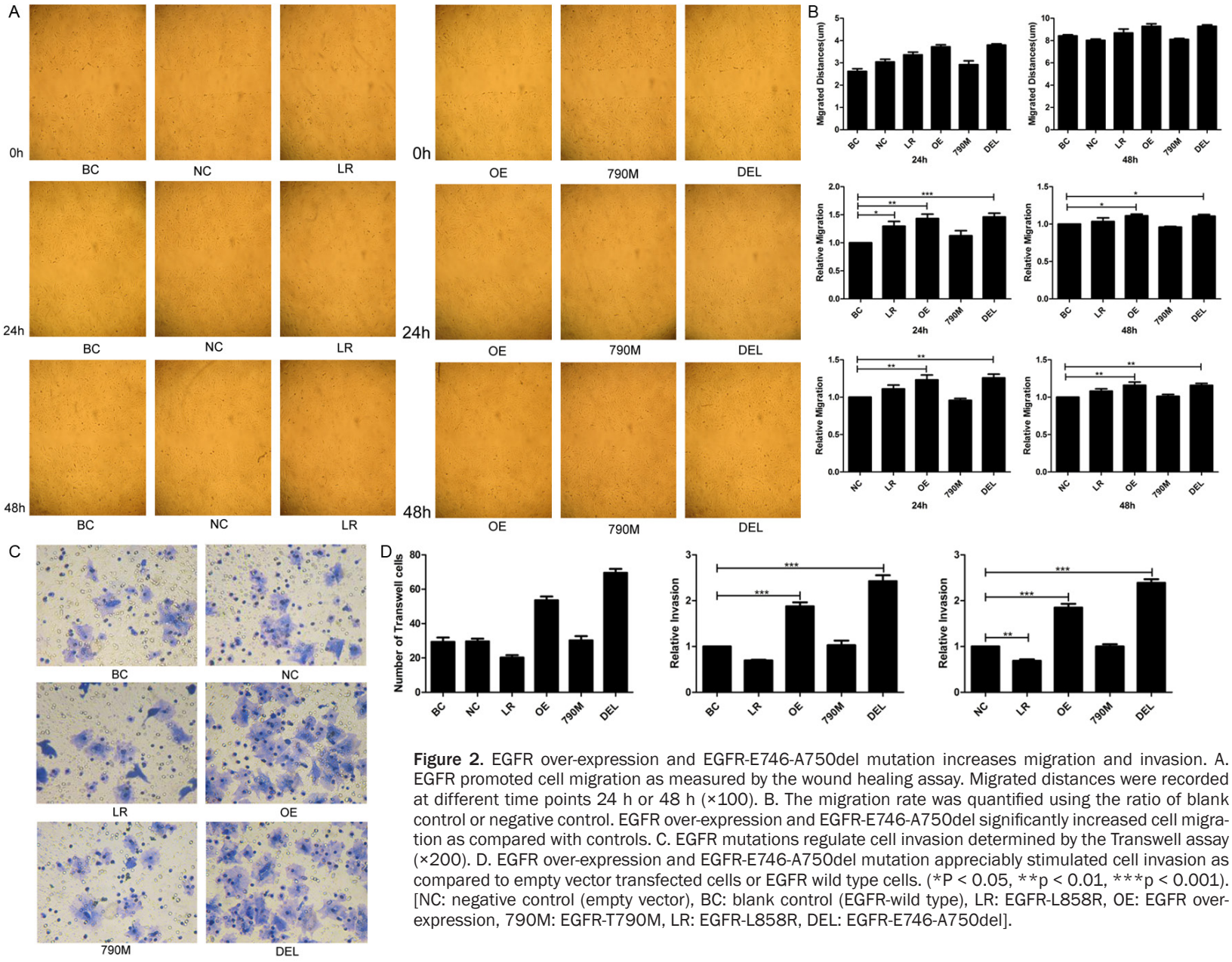

Figure 2. EGFR over-expression and EGFR-E746-A750del mutation increases migration and invasion. A. EGFR promoted cell migration as measured by the wound healing assay. Migrated distances were recorded at different time points 24 h or 48 h (×100). B. The migration rate was quantified using the ratio of blank control or negative control. EGFR over-expression and EGFR-E746-A750del significantly increased cell migration as compared with controls. C. EGFR mutations regulate cell invasion determined by the Transwell assay (×200). D. EGFR over-expression and EGFR-E746-A750del mutation appreciably stimulated cell invasion as compared to empty vector transfected cells or EGFR wild type cells. (*P < 0.05, **p < 0.01, ***p < 0.001). [NC: negative control (empty vector), BC: blank control (EGFR-wild type), LR: EGFR-L858R, OE: EGFR over-expression, 790M: EGFR-T790M, LR: EGFR-L858R, DEL: EGFR-E746-A750del].

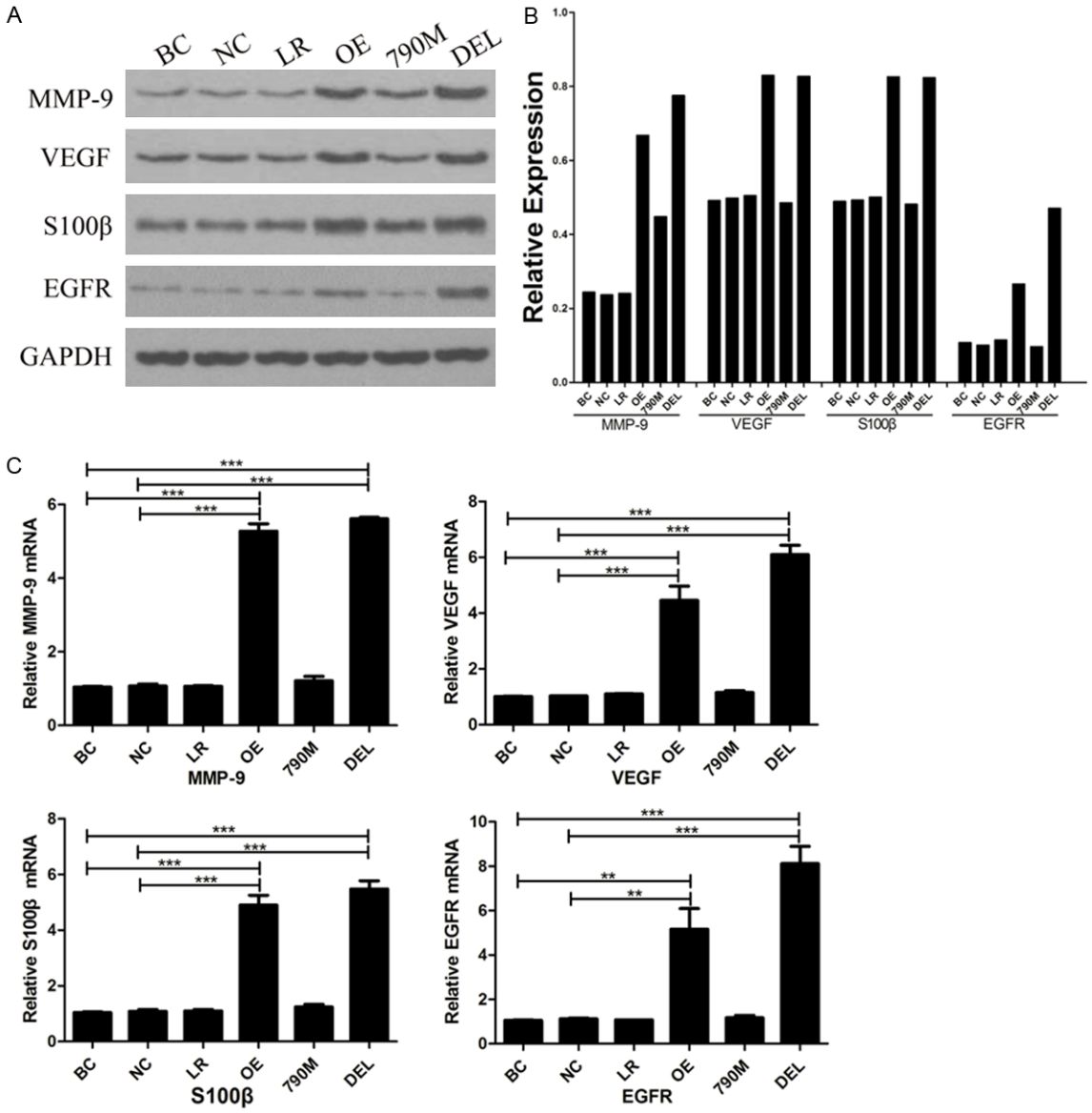

Figure 3. EGFR over-expression and EGFR-E746-A750del mutation regulate expression of MMP-9, VEGF, and S100β. A, B. The protein expression of MMP-9, VEGF, and S100β were analyzed by Western blot in different cells. The results showed up-regulation of MMP-9, VEGF, and S100β proteins in NCI-H1563 cells transfected with overexpressed EGFR or EGFR-E746-A750del as compared with other cells. GAPDH was used as a control; C. mRNA levels of MMP-9, VEGF, and S100β in different cells were analyzed by real-time PCR, with GAPDH as a control. (*P < 0.05, **p < 0.01, ***p < 0.001). [NC: negative control (empty vector), BC: blank control (EGFR-wild type), LR: EGFR-L858R, OE: EGFR over-expression, 790M: EGFR-T790M, LR: EGFR-L858R, DEL: EGFR-E746-A750del].

with GAPDH utilized as an internal control. As shown in **Figure 3C**, NCI-H1563 cells with over-expressed EGFR and EGFR-E746-A750del mutation significantly up-regulated the mRNA levels of MMP-9, VEGF, and S100β ($p < 0.001$).

Discussion

BM is a significant cause of morbidity and mortality in lung cancer patients. The median survival is just 1-2 months in untreated patients [25], and estimated as 4-5 months with whole brain radiation therapy (WBRT) [26], although survival of 12-24 months has been demonstrated in certain subsets [27]. Nevertheless, the overall poor prognosis necessitates further investigation into metastatic mechanisms, in hopes of subsequently identifying genes to create targeted agents for clinical therapy.

Mounting data have suggested involvement of EGFR mutations in BM [16]. However, the underlying mechanisms remain unclear. At the same time, Li et al. found that there were differential incidences of BM among NSCLC patients with specific EGFR mutations [28]. Sekine et al. found that patients with exon 19 deletions have smaller sized BMs (albeit more in quantity) than patients with wild-type EGFR or EGFR exon 21 L858R mutation [29]. These results suggest that EGFR mutations at different sites might have differential effects in terms of BM. In this study, we constructed three different EGFR-mutant cell sublines, and found that only exon 19 deletion could result in a change of biological characteristics, significantly enhanced cellular proliferation, migration, and invasion. Furthermore, to explore the possible relationship between the changes in activity and BM, we investigated the mRNA and protein expression of several genes related to BM. We observed that still only the exon 19 deletion could significantly up-regulate the expression of BM-associated genes. Indirectly, our study showed that EGFR exon 19 deletion mutation correlate with cerebral metastatic capacities. The results of our study could also be used as a potential explanation for the results presented by Li and colleagues, wherein patients with EGFR mutations at exon 19 had the highest incidence of BM among patients with EGFR mutations [28].

Similarly regarding the role of EGFR mutations in BM, a stepwise increasing frequency of EGFR amplification has been reported in NSCLC with the development of BM [30, 31]. Nie et al. also found that EGFR over-expression can promote BM in patients with breast cancer [32]. In this study, we constructed EGFR over-expression cell subline and found EGFR over-expression could also enhanced cellular proliferation, migration, and invasion and significantly up-regulate the expression of MMP-9, VEGF, and S100β. Our study also indirectly showed that EGFR over-expression associated with cerebral metastatic capacities.

Based on the above results, we speculated that EGFR over-expression and exon 19 deletion mutation (EGFR-E746-A750del) could be taken as predictive factor for BM in patients with NSCLC (although more work is needed to validate this notion). Moreover, in the future, it may be necessary to guide early intervention in patients with certain EGFR over-expression or mutations, such as over-expression with cetuximab and exon 19 deletions with TKIs.

Though prophylactic cranial irradiation (PCI) is a standard treatment for small cell lung cancer (SCLC), it reduces the cumulative incidence of BMs without OS improvement [33]. This is in part due to differences in tumor biology and genetics across various pathological subtypes of NSCLC; it is perceived that only patients with higher risks of BM may benefit from PCI. Based on our findings, we hypothesize that PCI could provide benefits for NSCLC patients with EGFR over-expression or exon 19 deletions, who cannot receive cetuximab or EGFR-TKIs for various reasons. Well-designed prospective randomized clinical trials are warranted to validate our presupposition.

There were several limitations in our study. First, the conclusions were summarized in only one cell line, which must be confirmed in more cell lines. Second, some signaling pathways, such as CXCL12/CXCR4 or Wnt/β-catenin, also thought to be associated with BM, were not included into this study. Further analysis of these pathways in conjunction with EGFR over-expression and mutations should be carried out in the future. Finally, only some changes in biological characteristics and expression of several BM-related genes were observed in this *in vitro* study; however, these results were not confirmed *in vivo*. In order to explore the association between EGFR over-expression or mutations and BM, further work should construct BM animal models by using EGFR over-expression and different EGFR-mutant cell sublines.

In conclusion, it is plausible that EGFR over-expression and E746-A750 deletion mutations could change the biological characteristics of tumor cells and even might promote the progress of BM, possibly by up-regulating the expression of MMP-9, VEGF, and S100β.

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Disclosure of conflict of interest

None.

Address correspondence to: Qibin Song and Guang Han, Department of Oncology, Renmin Hospital of Wuhan University, 99 Zhangzhidong Street, Wuhan 430060, Hubei, China. Tel: 0086-13517281931; E-mail: qibinsong@163.com (QBS); Tel: 0086-13886048178; E-mail: hg7913@163.com (GH)

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