Erlotinib inhibits osteolytic bone invasion of human non-small-cell lung cancer cell line NCI-H292

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Abstract Previous preclinical and clinical findings have suggested a potential role of epidermal growth factor receptor (EGFR) in osteoclast differentiation and the pathogenesis of bone metastasis in cancer. In this study, we investigated the effect of erlotinib, an orally active EGFR tyrosine kinase inhibitor (TKI), on the bone invasion of human non-small-cell lung cancer (NSCLC) cell line NCI-H292. First, we established a novel osteolytic bone invasion model of NCI-H292 cells which was made by inoculating cancer cells into the tibia of scid mice. In this model, NCI-H292 cells markedly activated osteoclasts in tibia, which resulted in osteolytic bone destruction. Erlotinib treatment suppressed osteoclast activation to the basal level through suppressing receptor activator of NF-κB ligand (RANKL) expression in osteoblast/stromal cell at the bone metastatic sites, which leads to inhibition of osteolytic bone destruction caused by NCI-H292 cells. Erlotinib inhibited the proliferation of NCI-H292 cells in in vitro. Erlotinib suppressed the production of osteolytic factors, such as parathyroid hormone-related protein (PTHrP), IL-8, IL-11 and vascular endothelial growth factor (VEGF) in NCI-H292 cells. Furthermore, erlotinib also inhibited osteoblast/stromal cell proliferation in vitro and the development of osteoclasts induced by RANKL in vitro. In conclusion, erlotinib inhibits tumor-induced osteolytic invasion in bone metastasis by suppressing osteoclast activation through inhibiting tumor growth at the bone metastatic sites, osteolytic factor production in tumor cells, osteoblast/stromal cell proliferation and osteoclast differentiation from mouse bone marrow cells.

Keywords Epidermal growth factor receptor · Tyrosine kinase inhibitor · Osteolytic bone invasion model · Osteoclast

Abbreviation EGFR Epidermal growth factor receptor NSCLC Non-small-cell lung cancer PTHrP Parathyroid hormone-related protein VEGF Vascular endothelial growth factor SRE Skeletal-related event TKI Tyrosine kinase inhibitor MEM Minimum essential medium HE Hematoxylin and eosin TRAP Tartrate-resistant acid phosphatase ALP Alkaline phosphatase OPG Osteoprotegerin RANKL Receptor activator of NF-κB ligand

Introduction

Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of lung cancers. Bone metastases occur in 30–40% of NSCLC patients, and are often associated with significant morbidity [1]. Common bone metastatic complications, which are also called skeletal-related events (SREs), include bone pain, pathological fractures, malignant hypercalcemia and spinal cord compression. These SREs are clinically meaningful...
sequelae that are associated with health care cost and decreased quality of life. Zoledronic acid is one of the widely used bisphosphonates that has been demonstrated to significantly prevent SREs in lung cancer patients with bone metastases. However, there are no significant differences in time of bone-lesion progression and median survival between zoledronic acid and a placebo [2]. Thus, it is necessary to develop an effective therapy that could inhibit the tumor growth and extend survival in NSCLC patients with bone metastases.

Erlotinib is an oral, small-molecule epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that reversibly binds to the intracellular tyrosine kinase domain of EGFR and is used as a standard treatment for previously-treated advanced NSCLC. The expression of EGFR and its ligand has been found in the majority of human carcinoma. Erlotinib blocks autophosphorylation of EGFR with subsequent inhibition of the downstream signaling cascades involving RAS/RAF/MAPK and PI3K/AKT. The EGFR signaling network, one of important signaling cascades involving RAS/RAF/MAPK and PI3K/AKT. The EGFR signaling network, one of important processes involved in tumor progression affecting cell proliferation, inhibition of apoptosis, metastasis and angiogenesis, is often dysregulated in cancer cells [3]. Erlotinib monotherapy has been favorable to the presence of EGFR mutations and both EGFR and KRAS wild-types in NSCLC [4].

In clinical studies, treatment with EGFR-TKI, erlotinib or gefitinib, for NSCLC patients with bone metastases was reported to significantly decrease bone pain, to improve pathologic fractures, to prolong disease control of bone metastatic site and also to significantly extend overall survival [5–9]. Although, these clinical observations indicate the possibility of EGFR signal involvement in the progression of bone metastases of NSCLC, there are no prospective, large size clinical studies.

In preclinical studies, various activities of EGFR-TKI on the pathogenesis and progression of bone metastases of prostate cancer and renal cancer have been described in previous reports [10–12]. Normanno et al. [13] described that gefitinib has a potential in bone metastases by inhibiting the ability of human bone marrow stromal cells to induce osteoclast differentiation. Moreover, a number of different mechanisms have been suggested to be involved in these effects [14]. However there has been no report to show an activity of EGFR-TKI on bone metastases of NSCLC cells.

In this study, for evaluating whether or not erlotinib would be effective for bone metastases of NSCLC, we tried to establish a novel osteolytic bone invasion model which resembles the clinical characteristics of NSCLC. Then we examined whether erlotinib could be a useful agent for bone metastases of NSCLC using our novel osteolytic bone invasion model.

Materials and methods

Chemicals

Erlotinib was provided by F. Hoffman-La Roche Ltd. (Basel, Switzerland). Erlotinib was dissolved in DMSO for the in vitro assay and in 6% Captisol® (CyDex Pharmaceuticals, Inc., Lenexa, KS) solution for the in vivo experiment. Captisol was dissolved in distillated water.

Cell lines

Human NSCLC cell line NCI-H292, human osteoblast cell line MG-63 and human stromal cell line HS-5 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI1640 (Sigma-Aldrich Co., Ltd., St. Louis, MO) supplemented with 10% FBS (Japan Bio Serum Co., Ltd., Fukuyama, Japan). Mouse osteoblastic cell line MC3T3-E1 and mouse stromal cell line ST2 were purchased from RIKEN Bio-Resource Center (Tsukuba, Japan) and maintained in αMEM (Invitrogen, Carlsbad, CA) and in RPMI1640 supplemented with 10% FBS, respectively. Mouse primary osteoblasts were purchased from Primary Cell Co., Ltd. (Sapporo, Japan) and maintained in αMEM supplemented with 10% FBS. All cells were cultured at 37°C in 5% CO₂.

Animals

Male 5-week-old ddY mice, BALB-nu/nu mice and scid mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), Charles River Japan, Inc. (Yokohama, Japan) and Clea Japan, Inc. (Osaka, Japan), respectively. Animals were housed in a pathogen-free environment under controlled conditions (temperature: 20–26°C, humidity: 40–70%, light–dark cycle: 12–12 h). Chlorinated water and irradiated food were provided ad libitum. The animals were allowed to acclimatize and recover from shipping-related stress for more than 7 days prior to the study. The health of the mice was monitored daily. All animal protocols were reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd and all animal experiments were performed in accordance with the ‘Guidelines for the Accommodation and Care of Laboratory Animals’ of Chugai Pharmaceutical Co., Ltd.

Intratibial osteolytic bone invasion model of NCI-H292 cells

Tibial implantation of NCI-H292 cells was performed as described [15]. Briefly, 2 × 10⁵ NCI-H292 cells in 20 μl of culture medium/matrigel (BD Biosciences, San Jose, CA)
In vitro cell proliferation assay

Cells were seeded $1 \times 10^3$ cells/well in 96-well plates. From the following day, the cells were treated with erlotinib for 4 days and then MTT assays were done using WST-8 solution (Dojindo, Tokyo, Japan) according to the manufacturer’s instructions. The viability of cells was calculated as: $\frac{\text{mean absorbance of erlotinib-treated wells} - \text{mean absorbance of cell-free wells}}{\text{mean absorbance of vehicle wells} - \text{mean absorbance of cell-free wells}} \times 100$.

Coculture of NCI-H292 cells and mouse primary osteoblasts or stromal cells

To evaluate interaction between these cells, three coculture conditions were performed as described previously [18]. Briefly, $1 \times 10^6$ mouse primary osteoblasts or stromal cell line ST2 were cocultured with NCI-H292 confluent cell monolayer with or without 3% paraformaldehyde fixation. Or $1 \times 10^4$ mouse primary osteoblasts/ST2 cells were cocultured with $1 \times 10^6$ NCI-H292 cells in suspension with or without a 8.0 μm pore size cell culture insert (Corning, Inc., Lowell, MA) to separate from each cell. After 8 h coculture, cDNA of primary osteoblasts and ST2 were synthesized using the Cells-to-cDNA kit (Ambion, Inc., Austin, TX), and the real-time RT-PCR was performed to examine the mouse RANKL, osteoprotegerin (OPG) and actin mRNA expression level using LightCycler system (Roche Diagnostics, Ltd., Basel, Switzerland). Applied Biosystems Assay-on-Demand primer probe sets for the detection of mouse RANKL, OPG and actin were used with Mm00441908m1, Mm0046190m1 and Mm00435452m1, respectively (Life Technologies Co., Ltd., Carlsbad, CA). Relative gene expression compared to non-treated cells was quantified using the comparative threshold cycle (C_\text{T}) method by subtracting the internal control of actin C_\text{T} value from the RANKL or OPG C_\text{T} value. These C_\text{T} values were calculated by the 2nd derivative maximum method in LightCycler system.

In vitro production of osteolytic factors in NCI-H292 cells

The $2 \times 10^6$ NCI-H292 cells were seeded in 6-well plates. On the following day, cells were treated with erlotinib for 1 day and then protein levels of osteolytic factors (parathyroid hormone-related protein (PTHrP), IL-1β, 6, 8, 10, 11, GM-CSF, TNF-α and vascular endothelial growth factor (VEGF)) [19–21] in the culture media and the cellular DNA contents were determined by immunostaining and a double-strand DNA Quantification Kit (Invitrogen). PTHrP and IL-11 were measured by IRMA in Mitsubishi...
Chemical Medience Corporation (Tokyo, Japan) and ELISA kit (R&D systems), respectively. The others were measured by Bio-plex human cytokine assays (Bio-Rad Laboratories, Inc., Hercules, CA). The protein level of osteolytic factors normalized by the DNA contents were calculated as: 
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\frac{\text{([concentration of erlotinib-treated wells} - \text{mean concentration of cell-free wells})/\text{DNA contents of erlotinib-treated wells])}}{\text{([concentration of vehicle-treated wells} - \text{mean concentration of cell-free wells})/\text{DNA contents of vehicle-treated wells})} \times 100.
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The data were shown as mean + SD of duplicates.

In vitro osteoclast differentiation from mouse bone marrow cells

The protocol was performed as described [22]. Briefly, mouse bone marrow cells were isolated from 6 to 10-week-old male ddY mice and then cultured in MEM supplemented with 10% FBS, 30 ng/ml mouse M-CSF (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 30 ng/ml mouse RANKL (Wako) with or without erlotinib. After osteoclast differentiation, cells were fixed and stained with TRAP. The number of osteoclasts was determined by counting the multinucleated (>3 nuclei) TRAP-positive cells under a microscope. The data were shown as mean + SD of quadruplicates.

Statistical analysis

The Wilcoxon test was used to detect the statistical differences in tumor volume. Probability values <0.05 were considered to be significant. The t test was used to detect the statistical differences in number of osteoclasts. Probability values <0.05 were considered to be significant. Statistical analyses were performed using an SAS preclinical package (version 8.2; SAS Institute, Inc., Cary, NC).

Results

Establishment of mouse osteolytic bone invasion model of NCI-H292 cells

To examine the effects of erlotinib on bone metastasis of NSCLC, we established a novel in vivo intratibial osteolytic bone invasion model of the human NSCLC cell line NCI-H292. In mice injected with NCI-H292 cells into tibia, osteolytic bone lesions appeared from day 14 and expanded in a time-dependent manner (Fig. 1a, b). The tumor take rate in this model was 90% (osteolytic bone lesions were observed only in eighteen of twenty legs inoculated on day 28). ALP/TRAP double staining demonstrated that many TRAP-positive activated osteoclasts were present within the ALP-positive osteoblast/stromal cell layer covering the bone surfaces close to the metastatic tumor (Fig. 1c). In contrast, activated osteoclasts could hardly be seen in normal mouse (Fig. 1c). These findings indicate that direct contact between NCI-H292 tumor cells and osteoblasts/stromal cells induced the activation of osteoclastic precursors in the metastatic lesions.

NCI-H292 cell-induced RANKL expression in mouse osteoblasts/stromal cells ST2

Osteoclasts are developed by cellular interaction between osteoclastic precursors and osteoblasts or stromal cells thorough RANK-RANKL signal transduction [23]. To examine the mechanism of NCI-H292 cell-induced osteoclast activation in this model, we investigated the effects of NCI-H292 cells on RANKL expression in mouse primary osteoblasts and mouse stromal cells ST2. The expression of RANKL mRNA in osteoblasts/ST2 cells was drastically increased when cultured with live adherent NCI-H292 cells, but were strongly abolished when cultured with fixed tumor cells (Fig. 1d). Furthermore, RANKL mRNA expression in osteoblasts/stromal cells was also drastically increased when cultured with NCI-H292 cells in suspension, but were strongly abolished when cultured apart from tumor cells (Fig. 1d). In contrast, there was almost no change in OPG expression in osteoblasts/stromal cells when these were cocultured with adhesion and suspension of NCI-H292 cells (data not shown). These findings indicate that not only cell-to-cell direct contact but also any soluble factors from tumor cells are indispensable for NCI-H292 cell-induced RANKL expression in osteoblasts/stromal cells.

Inhibitory effects of erlotinib on osteolytic bone invasion model of NCI-H292 cells

Administration of erlotinib significantly decreased the area and incidences of NCI-H292 cell-induced osteolytic lesions on days 21 and 28 (Fig. 2a, b, c). Administration of erlotinib starting from days 7 or 14 exerted almost the same inhibitory effects of osteolytic lesions on day 28 as starting from day 1 (data not shown). A difference in body weight loss was not observed in the erlotinib treatment group compared with the vehicle group on days 21 and 28 (data not shown). Furthermore, erlotinib completely suppressed the tumor induced-RANKL expression in osteoblasts/stromal cells (Fig. 2d). Erlotinib markedly suppressed the osteoclast activation to the basal level of non-tumor bearing mouse (P = 0.07) (Fig. 2e, f). These findings indicate that erlotinib inhibited osteolytic bone destruction of NCI-H292 cells in the tibia by inhibiting the accumulation of activated osteoclasts.
Inhibitory effects of erlotinib on cell proliferation of NCI-H292 cells in vivo and in vitro

Administration of erlotinib tended to inhibit the metastatic tumor growth in this osteolytic bone invasion model ($P = 0.24$) (Fig. 3a, b). The reduced cell proliferative activity of NCI-H292 cells was observed by immunostaining of proliferative markers Ki-67 ($P = 0.24$) (Fig. 3c). Furthermore, erlotinib showed a marked inhibition of NCI-H292 cell proliferation in vitro in a dose-dependent manner, and the IC$_{50}$ value was 0.11 $\mu$M (Fig. 3d). An addition of 1 $\mu$M of erlotinib markedly blocked not only EGFR phosphorylation but also ERK and Akt phosphorylation which are the downstream of EGFR signaling cascades in NCI-H292 cells (Fig. 3e). Meanwhile, phosphorylated EGFR expression in tumor cells at the metastatic sites was detected in not only the vehicle treatment but also the erlotinib treatment, a difference of the level of EGFR phosphorylation was not observed with or without erlotinib treatment (Fig. 3f).

Inhibitory effects of erlotinib on production of osteolytic factor in NCI-H292 cells

To elucidate the soluble factor from NCI-H292 cells which is related to RANKL expression in osteoblasts/ST2 cells, we investigated the production of major osteolytic factors including PTHrP, IL-6 and IL-8 etc. As a result, NCI-H292 cells produced a sufficient amount of PTHrP, IL-6, IL-8, IL-11 and VEGF but the production of IL-1$\beta$, IL-10, GM-CSF and TNF-$\alpha$ was lower than the detection limit (data not shown). Erlotinib blocked the production of PTHrP, IL-8, IL-11 and VEGF in NCI-H292 cells in a dose-dependent manner (Fig. 4). Meanwhile, IL-6 production was not changed by erlotinib treatment, indicating that IL-6 expression is independent of the EGFR signal in NCI-H292 cells (Fig. 4).
Inhibitory effects of erlotinib on cell proliferation of osteoblasts/stromal cells and osteoclast differentiation from mouse bone marrow cells

Recently, the role of EGFR in osteoblast and osteoclast has been focused on [5, 24]. Therefore, we examined the effects of erlotinib on these cells. The mouse primary osteoblasts, mouse osteoblastic cell line MC3T3-E1, mouse stromal cell line ST2, human osteoblastic cell line MG-63 and human stromal cell line HS-5 expressed EGFR (Fig. 5a). Erlotinib inhibited the proliferation of these cell lines in vitro (Fig. 5b, c). In contrast, the reduced cell proliferative activity of osteoblasts/stromal cells in vivo by erlotinib treatment could not be estimated, because there was no detectable Ki-67 immunostaining in osteoblasts/stromal cells with or without erlotinib treatment (data not shown). In addition, erlotinib completely suppressed the formation of mononuclear and multinuclear TRAP-positive cells in a dose-dependent manner (Fig. 5d, e), although erlotinib did not exhibit any cytotoxicity against bone marrow cells in this condition (data not shown).

Discussion

Bone is one of the commonest target organs of distant metastasis in lung cancer [25]. Many steps are involved in the metastasis from primary site to bone; that is, new vessel formation, invasion, arrest in secondary capillary beds, extravasation, adaptation to bone microenvironment and proliferation of cancer cells in bone [26, 27]. The main mechanism responsible for bone destruction is considered to be bone resorption, caused not directly by tumor cells but by tumor cell-activated osteoclasts through interaction with osteoblasts/stromal cells, which altogether form a “vicious circle” [28]. In the present study, we investigated
the role of EGFR in the vicious circle of NSCLC bone metastasis by examining whether EGFR-TKI has the potential to inhibit bone metastases of NSCLC cells.

Although the effects of EGFR-TKI on bone metastases in NSCLC patients have not been investigated in a large clinical study, occasional reports have suggested a potential activity of this drug on the progression of bone disease [5–9]. Recently, Okano et al. [6] have reported cases of NSCLC in which gefitinib, an EGFR-TKI, showed a dramatic response to pathological fractures due to bone metastases and eventually the fractures were almost completely healed. Moreover, gefitinib showed a significant improvement of metastatic bone pain in breast cancer [29]. However, the impact of EGFR-TKI on bone metastases seems to be different in each patient.

To evaluate the potential of erlotinib on bone metastases in preclinical study, we tried to establish a novel bone invasion model using NSCLC cell line NCI-H292. Because the experimental bone metastasis models of NSCLC cells were very limited. NCI-H292 cells carry wild-types of both EGFR and KRAS, which are the most major characteristics (60–76%) in all NSCLC patients [30, 31]. This is the first osteolytic bone invasion model using a NSCLC cell line with both EGFR and KRAS wild-types. In this model, our histological studies showed that osteolytic bone destruction induced by NCI-H292 cells is caused by activated...
osteoclast accumulation in the metastatic lesions through the interaction between NCI-H292 tumor cells and osteoblasts/stromal cells (Fig. 1). Furthermore, RANKL expression in osteoblasts/stromal cells caused by NCI-H292 cells was indispensable not only for cell-to-cell contact but also some soluble factors, such as osteolytic factors, from NCI-H292 tumor cells (Fig. 1). RANKL is a critical osteoclast differentiation factor highly expressed on the membranes of the osteoblasts/stromal cells in the bone marrow environment [23]. RANKL combines with RANK, a receptor of RANKL expressed in osteoclastic precursors, and delivers intracellular signals involved in osteoclast differentiation [19]. At the site of bone metastases, RANKL is also considered as a direct mediator of tumor-associated osteolytic lesions [32]. These findings indicate that a vicious circle is formed in NCI-H292 osteolytic bone invasion model.

The molecules involved in cell-to-cell recognition between NCI-H292 cells and osteoblasts/stromal cells are unclear. Integrins are reported to be candidates. Previous reports have shown that osteoblasts as well as tumor cells express various integrins, such as $\beta_1$ integrin and $\alpha_v\beta_3$ integrin, and integrin signals regulate osteoclast formation [33, 34]. However, we do not investigate whether or not integrins are involved in interaction between NCI-H292 cells and osteoblasts/stromal cells, further studies are needed to identify the molecules.

In the present study, erlotinib treatment suppressed osteoclast activation, which led to inhibit osteolytic bone destruction caused by NCI-H292 cells (Fig. 2). To clarify the bone metastasis inhibitory mechanism of erlotinib would be a useful approach to clinically select NSCLC patients with bone metastases who could benefit from erlotinib treatment. We demonstrated that erlotinib affects the vicious circle of NCI-H292 osteolytic bone invasion which results in inhibition of osteoclast activation. The effects of erlotinib appear to be related to four different mechanisms: the suppression of (1) tumor growth at the bone metastatic sites, (2) osteolytic factor production, such as PTHrP, IL-6, IL-8, IL-11 and VEGF in tumor cells, (3) osteoblasts/stromal cell proliferation and (4) osteoclast differentiation from mouse bone marrow cells (Fig. 6).

First, erlotinib strongly inhibited the proliferation of NCI-H292 cells in vitro by blocking EGFR phosphorylation with subsequent inhibition of the downstream signaling cascades involving RAS/RAF/MAPK and PI3K/AKT.
Fig. 6 A schematic diagram of the mechanism of erlotinib in a NCI-H292 osteolytic bone invasion model. Erlotinib suppressed NCI-H292 cell-induced osteoclast activation by suppressing metastatic tumor growth, osteolytic factor production in tumor cells, osteoblasts/stromal cell proliferation and osteoclast differentiation from mouse bone marrow cells. Abbreviations: OB/ST osteoblast/stromal, OC osteoclast
found that RANKL expression levels in primary osteoblasts decreased about 50% with erlotinib and there was no change in RANKL expression levels of ST2 cells. In contrast, OPG expression levels of either type of cell were not changed (data not shown).

Fourth, erlotinib completely suppressed the RANKL-induced osteoclast differentiation from mouse bone marrow cells without exhibiting cytotoxicity. Our results are consistent with the previous reports. Wang et al. [24] have shown that EGFR is expressed in osteoclasts and osteoclast recruitment is impaired in EGFR deficient mice. In addition, Yi et al. [40] have demonstrated that EGFR plays a crucial role in RANKL-induced osteoclast differentiation from mouse bone marrow cells by regulating RANKL-activated signaling pathways through cross-talking with RANK. These findings indicate that erlotinib may inhibit the activation of osteoclasts by affecting on non-cancer cells including osteoblasts/stromal cells and osteoclasts, independently by affecting on NCI-H292 tumor cells. Therefore, it is important to highlight the activity of anti-EGFR agents on non-cancer cells including osteoblasts/stromal cells and osteoclasts in the bone metastatic microenvironment. Meanwhile, in clinical, abnormal bone metabolism such as hypercalcemia and bone fracture has not been reported as a side effects of erlotinib, therefore the effects of erlotinib against osteoblasts/stromal cells and osteoclasts may be specific in bone metastatic sites.

Meanwhile, only one cell line, NCI-H292, was used in this study. We have tried to construct another bone metastasis model for generalization of our NCI-H292 model using HCC827 EGFR-mutated NSCLC cell line. Unfortunately, we could not establish HCC827 bone metastasis model. Therefore, we used only the NCI-H292 osteolytic bone invasion model in the present study. Further studies are needed to clarify whether or not erlotinib could inhibit bone metastases in the other models. Additionally, in this NCI-H292 intra-tibial tumor inoculated osteolytic bone invasion model, it could not be evaluated whether erlotinib have a potential to inhibit forming bone metastasis from primary site through the bloodstream. However, anti-EGFR agents were reported to reduce invasive capacity and angiogenic activity through the inhibition of (1) molecules associated with tissue invasion such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-9, (2) the proliferation and survival of endothelial cells and (3) the production of VEGF in bone marrow stromal cells (14). Therefore, erlotinib would inhibit the progression of bone metastasis from the primary site through the bloodstream.

Altogether, it is plausible that the inhibition of NCI-H292 tumor growth and cell proliferation of osteoblasts/stromal cells by erlotinib treatment would decrease the chance of interaction between tumor cells and osteoblasts/stromal cells, resulting in the suppression of the activation of RANKL expression in osteoblasts/stromal cells.

In conclusion, we demonstrated that erlotinib inhibits tumor-induced osteolytic invasion in bone metastasis by suppressing metastatic tumor growth, osteolytic factor production in tumor cells, osteoblasts/stromal cell proliferation and osteoclast differentiation from mouse bone marrow cells. These findings suggest that erlotinib has potential therapeutic benefits against bone metastases of NSCLC and may be a useful therapeutic agent for the treatment of NSCLC patients with bone metastases. Finally, the evidence of our preclinical study would support prospective clinical trials which will clarify the role of erlotinib in the management of NSCLC patients with bone metastases.

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