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Investigation of Biomarkers and Handling Strategy of Erlotinib-Induced Skin Rash in Rats

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Skin rash is a common adverse event associated with erlotinib therapy. In severe conditions, the rash could affect patients’ QOL. If the rash occurrence can be predicted, erlotinib treatment failures can be prevented. We designed an in vivo study that applied erlotinib regimens resembling its clinical application to evaluate possible erlotinib-induced skin rash biomarkers for humans and simultaneously observe the effects of erlotinib discontinuation, followed with or without dose reduction, on rash development. Rats were divided into four groups: placebo, constant (erlotinib 35 mg/kg on d1–d21), intermittent (erlotinib 70 mg/kg on d1–d7 and d15–d21), and mimic (erlotinib 70 mg/kg on d1–d7 and erlotinib 35 mg/kg on d15–d21). Blood sampling was performed on d1, d8, d15, and d22. The samples were used to measure erlotinib concentrations, the level of hepatic and renal function markers, immune cell percentages, and immune cells’ CD45 expression levels. Erlotinib 70 mg/kg generated high mean circulating erlotinib concentrations (>1800 ng/mL) that led to severe rashes. Erlotinib dose reduction following rash occurrence reduced circulating erlotinib concentration and rash severity. After the treatment, the escalation of neutrophil percentages and reduction of neutrophils’ CD45 expression levels were observed, which were significantly correlated with the rash occurrence. This study is the first to show that erlotinib-induced skin rash may be affected by the reduction of neutrophils’ CD45 expression levels, and this is a valuable finding to elucidate the erlotinib-induced skin rash formation mechanism.

Key words erlotinib; skin rash; biomarker; protein tyrosine phosphatase; neutrophil; in vivo study

INTRODUCTION

Erlotinib is a medicine for non-small cell lung cancer (NSCLC) approved to be used in Japan on October 19th, 2007.1,2 It reversibly blocks the downstream signaling pathway of a surface protein called epithelial growth factor receptor (EGFR), resulting in the reduction of cancer cell development and enhancement of cancer cell apoptosis.3 Erlotinib is predominantly metabolized by CYP3A4 and excreted by the biliary system.4 However, EGFR is not only expressed by cancer cells but also keratinocytes in skin tissue.5 Erlotinib action at the skin site corresponds to the rash emergence in 75% of patients.6 In severe conditions, the rash could affect erlotinib therapy continuation and patients’ QOL.7 If the rash occurrence can be predicted, erlotinib treatment failures occurring at a certain rate can be prevented.

Biomarker utilization is a way to predict the rash occurrence. The potential biomarkers can be obtained one of which by understanding the rash formation mechanism. Several studies have been conducted at skin and blood sites to understand the erlotinib-induced rash formation mechanism.8,9 Their findings suggest an important role of the immune system in rash development. Paul et al. stated EGFR signaling blockade by erlotinib increased CCL2 and CCL5 and reduced CXCL8 expressions in a study on human keratinocyte cultures.7 Furthermore, Lichtenberger et al. stated, apart from CCL2 and CCL5 elevations, erlotinib also elevated CCL27 expression in a study on patients’ skin samples.8 Considering CCR10, expressed by T-helper 22 CD4+ T cells,9 is CCL27 receptor,10 we initially hypothesized Th22 CD4+ T cells escalation in the blood as the response to the CCL27 escalation after erlotinib treatment.12 In this preliminary study, circulating neutrophils, monocytes, and CD8+ T cells were also involved. However, circulating CCR10 expression and Th22 CD4+ T cells’ presence did not change in the skin rash model by erlotinib treatment. It is possibly due to erlotinib inhibitory effects on T cell proliferation and activation.13 On the other hand, neutrophil percentage escalation and unexpectedly, neutrophils’ CD45 reduction were observed. Neutrophils are part of innate immunity that ingest not only microbes but also cell debris.14 In healthy humans, it is primarily distributed in the circulating system.15 Any trigger on innate immunity, such as skin cell apoptosis, will change circulating neutrophil levels.

Neutrophils and other immune cell subsets exclusively express CD45 on their surfaces.16 CD45 is a transmembrane protein tyrosine phosphatase which the extracellular domain exists in multiple isoforms as a result of three exons (4–6) alternative splicing.17 The extracellular domain involvement in antigen receptor signaling is dispensable, but its presence reflects the cell type as well as the cell’s developmental stage and activation state.17 The cytoplasmic domain consists of enzymatic properties involved in Src family kinase activation, which are important for the immune responses of T and B cells18 and migration of immune cells to the inflamed tissues.19

In this study, we investigated the correlation of neutrophil percentages and neutrophils’ CD45 expression levels to erlotinib-induced skin rash occurrence by designing an in vivo model that applied erlotinib regimens resembling its clinical application. Simultaneously, erlotinib discontinuation followed with readministration with or without dose reduction effects.
on rash development were observed.

MATERIALS AND METHODS

Animals and Ethics  Wild-type Sprague–Dawley male rats aged 8 weeks were purchased from Japan SLC (Shizuoka, Japan) and were maintained for up to 7 d for adaptation. The rats were kept in a room with 12 h light exposure daily. Food and water were provided ad libitum. The guidelines of the Ministry of Education, Culture, Sports, Science and Technology of Japan for the care and use of animals were followed. All procedures performed in this study have followed the ethical standards of the Mie University Animal Ethics Committee.

Study Design  Figure 1 shows a chart of the treatment design used to evaluate erlotinib-induced skin rash biomarkers in rats. Rats were divided into four groups: constant (C), intermittent (I), mimic (M), and placebo (P). Group C was treated with erlotinib 35 mg/kg daily from d1 to d21, group I with erlotinib 70 mg/kg daily from d1 to d7 and from d15 to d21, and group M with erlotinib 70 mg/kg daily from d1 to d7 and erlotinib 35 mg/kg daily from d15 to d21. Groups I and M had an erlotinib-free period from d8 to d14. As the control group, group P received solvent solution (as indicated in Test Solution Preparation) from d1 to d21. The doses used in this study were established by referring to erlotinib concentration levels from clinical settings (after erlotinib 150 mg multiple oral doses in humans, the minimum trough concentration level on d28 was 1200 ng/mL). Group C aimed 1) to observe the effect of reduced starting dose and continuous dose of erlotinib on skin rash development; and 2) to confirm the group M results after 22 d of treatment whether having similar results or not with group C because group C and M received, in total, the same erlotinib amount during 22 d of treatment. Groups I and M aimed to observe erlotinib discontinuation effects followed with readministration with or without dose reduction on rash occurrence and progression, respectively.

Photographs of the area around the mouth and blood samples were collected four times from each rat. The photograph was used as a reference to determine rash severity. The first, second, and third collection was performed before test solution administration on d1, d8, and d15, respectively. The fourth collection was performed 24 h following the last test solution administration (on d22).

Skin Rash Classification  Skin rash classification was evaluated by referring to criteria of the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 as follows: (a) grade ≤1: pinkish skin (no rash); (b) grade 2: clustered red skin (moderate rash); (c) grade 3: scattered blackish-red skin (severe rash), but not immediately life-threatening; (d) grade 4: blackish-red skin, life-threatening consequences; and (e) grade 5: death related to the adverse event.

Test Solution Preparation  Each test solution was prepared daily as a single dose by dissolving erlotinib hydrochloride (LC Laboratories, U.S.A.) in solvent solution (2 mL). The solvent solution was a mixture comprising sodium chloride 0.9% (m/v), polysorbate 80 0.1% (v/v), and sodium carboxymethylcellulose 0.3% (m/v) in distilled water.

Blood Collection and Processing  Blood (1 mL) was collected from the rat orbital sinus. The blood was stored in a heparinized 1.5 mL tube at 4 °C for further treatment. The blood sample (100 µL) was separated for flow cytometric analysis. The residue was treated to obtain plasma for the measurement of circulating erlotinib concentration and markers of hepatic and renal functions. Plasma was collected following centrifugation of the blood sample at 10 °C, 1700 × g for 10 min.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)  Plasma (100 µL) was mixed with 5 µL of 1 µg/mL erlotinib-d6 (the internal standard, Cosmo Bio Co. Ltd., Tokyo, Japan). Then, the sample was mixed with methanol (695 µL), vortexed, and centrifuged at 4 °C, 10 000 × g for 10 min. The supernatant was filtered with a 0.45-micron filter for chromatographic analysis.

An Agilent 1260 Infinity (Agilent Technologies, Tokyo, Japan) LC system equipped with a Zorbax Eclipse plus C18 2.1 × 100 mm 1.8-micron (Agilent Technologies) was used and eluted using a gradient. The mobile phase comprised 5 mM ammonium acetate in distilled water and acetonitrile. Following sample injection (5 µL), elution was performed by generating a gradient from 10 to 50% of acetonitrile in the initial 3 min, followed by a gradient from 50 to 90% of acetonitrile from min3 to 6. The total run time was 10 min per run and the flow rate was maintained at 0.35 mL/min at 40 °C. The reten-

Fig. 1. Treatment Design of Erlotinib-Induced Skin Rash Observation in Rats
P: placebo; C: constant; I: intermittent; and M: mimic. Dash line: blood sampling point.
tion time was 4.6 min for both erlotinib and erlotinib-d6.

The LC system was coupled with triple quadrupole MS/MS (Agilent 6490, Agilent Technologies). The MS/MS was equipped with an electrospray ionization source, operated in the positive ion mode, and quantification was performed in the multiple reaction monitoring mode with mass-to-charge (m/z) transition at 394.4 > 278.1 for erlotinib and 400.2 > 278.1 for erlotinib-d6. Nitrogen gas was used for nebulization, desolvation, and collision. The instrument conditions were set as follows: collision energy 28 V for erlotinib and 32 V for erlotinib-d6, capillary voltage 4.0 kV, dwell time 200 ms, sheath gas flow 12 L/min at 300°C, desolvation gas flow 14 L/min at 250°C, and 50 psi nebulization gas pressure.

Samples used for calibration were prepared by mixing erlotinib stock solution with L-Consena (a drug-free solution, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to obtain a 7-points standard curve (1000, 500, 100, 50, 10, 5, and 1 ng/mL). The detection lower limit was 10 ng/mL.

**Hepatic and Renal Functions** Six circulating markers were used to determine rat hepatic and renal function conditions before, during, and after the treatment, as follows: blood urea nitrogen, creatinine (Cre), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and total bilirubin (T-Bil). The markers were measured by FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Flow Cytometry** The blood sample (100 μL) was divided equally into two tubes. The first tube was used to detect neutrophils, and the second was used to detect monocytes and lymphocytes. For neutrophil detection, the blood sample was stained with phycoerythrin-cyanine7 (PE-Cy7) anti-rat CD45 antibody (BioLegend, U.S.A.), phycoerythrin (PE) anti-rat CD11b antibody (BD Pharmingen, U.S.A.), and fluorescein isothiocyanate (FITC) anti-rat CD16 antibody (Novus Biologicals, U.S.A.). For monocyte and lymphocyte detection, the blood sample was stained with PE-Cy7 anti-rat CD45 antibody (BioLegend, U.S.A.), peridinin chlorophyll protein (PerCP) anti-rat CD3 antibody (Invitrogen, U.S.A.), PE anti-rat CD4 antibody (Invitrogen), and allophycocyanin (APC) anti-rat CD45RA antibody (BioLegend).

Samples for flow cytometric analysis were prepared by the following procedure: (1) each antibody mentioned above (1 μL) was added into the designated blood sample-containing tubes, (2) the stained blood samples were incubated in the dark at 20–25°C for 30 min, (3) red blood cell lysing solution (1 mL) (Beckman Coulter, France) was added into each tube, (4) the mixtures were centrifuged at 20°C, 500 × g for 5 min, and the supernatants were discharged; (5) the remaining residues were resuspended with phosphate-buffered saline (PBS) solution (1 mL) followed by centrifugation as mentioned in step 4; and (6) the supernatants were discharged and the residues were resuspended with PBS solution (1 mL).

The mixtures in step 6 were the end-products of flow cytometric sample preparation. The processed samples were analyzed using FACS Canto II (BD Bioscience, U.S.A.). The gating strategy for neutrophil detection followed the steps performed by Fujimoto et al., whereas the gating strategy for monocyte and lymphocyte detection was performed as shown in Fig. 2.

**Data and Statistical Analyses** All data were collected from 3 to 6 individual experiments and are displayed as the mean ± standard deviation. The n value in each table and figure describes the number of rats, except in Figs. 6, S2B–D, and S3A–C. The n value in Figs. 6 and S3A–C gathers the number of observations from d1 to d22 in all groups, whereas Figs. S2B–D gathers the number of observations from weight-loss erlotinib-treated rats. LC-MS/MS data were acquired and analyzed using MassHunter Workstation (Agilent Technologies). Erlotinib to erlotinib-d6 peak area ratio was used to calculate the erlotinib concentration in blood samples. Flow cytometric data were analyzed using FACS Diva (BD Bioscience) to measure immune cell population percentages and marker expressions. Statistical data were analyzed using GraphPad Prism v5 (San Diego, U.S.A.). The t-test was performed to compare three groups by comparing erlotinib-treated groups to group P, except in Figs. 6, S1, and S3C. The one-way ANOVA was performed to compare three or more groups, while the Pearson r correlation to quantify the association between two variables. Significances were determined with * (p < 0.05), ** (p < 0.01), and *** (p < 0.001).

**RESULTS**

**Circulating Erlotinib Concentrations** Table 1 shows circulating erlotinib concentrations after erlotinib treatment in rats. There was a high difference in mean circulating erlotinib concentrations in rats treated with erlotinib 35 mg/kg and 70 mg/kg as shown in group C compared to groups I and M on d8. Erlotinib 70 mg/kg generated high mean circulating erlotinib concentrations (>4000 ng/mL) followed by high standard deviation on d8 as shown in groups I and M. Mean circulating erlotinib concentrations after erlotinib readministration for 7d following erlotinib-free period was lower than that before the discontinuation (group I on d22 compared to d8). The reduced mean concentrations were also followed by a relatively low standard deviation.

**Hepatic and Renal Functions** Table 2 shows hepatic and renal function marker levels after erlotinib treatment in rats. Among six circulating markers, three marker levels increased...
Table 1. Circulating Erlotinib Concentrations

| Day | Placebo (n = 6) (ng/mL) | Constant (n = 6) (ng/mL) | Intermittent (n = 6) (ng/mL) | Mimic (n = 3) (ng/mL) |
|-----|------------------------|--------------------------|----------------------------|----------------------|
| 1   | ND                     | ND                       | ND                         | ND                   |
| 8   | ND                     | 235.42 ± 187.50          | 4007.15 ± 4772.38          | 7578.63 ± 6325.32   |
| 15  | ND                     | 85.38 ± 43.44            | ND                         | ND                   |
| 22  | ND                     | 161.89 ± 164.69          | 1898.98 ± 980.49           | 229.60 ± 172.05     |

Table 2. Hepatic and Renal Function Marker Concentrations

| Day | Placebo (n = 6) | Constant (n = 6) | Intermittent (n = 6) | Mimic (n = 3) |
|-----|----------------|-----------------|---------------------|--------------|
|     | BUN (mg/dL)    |                 |                     |              |
| 1   | 17.85 ± 3.80   | 18.95 ± 3.29    | 20.75 ± 2.33        | 18.53 ± 4.07 |
| 8   | 18.20 ± 2.46   | 18.42 ± 1.74    | 20.87 ± 4.71        | 16.20 ± 7.11  |
| 15  | 19.25 ± 1.46   | 19.78 ± 1.26    | 21.22 ± 2.20        | 19.10 ± 1.08  |
| 22  | 19.18 ± 0.90   | 19.98 ± 2.33    | 18.78 ± 2.35        | 18.63 ± 0.90  |

|     | Cre (mg/dL)  |                 |                     |              |
| 1   | 0.23 ± 0.2    | 0.25 ± 0.03     | 0.23 ± 0.02         | 0.26 ± 0.2   |
| 8   | 0.24 ± 0.03   | 0.27 ± 0.04     | 0.34 ± 0.05***      | 0.33 ± 0.03** |
| 15  | 0.24 ± 0.02   | 0.28 ± 0.03*    | 0.22 ± 0.02         | 0.25 ± 0.01  |
| 22  | 0.25 ± 0.03   | 0.29 ± 0.04     | 0.29 ± 0.01         | 0.28 ± 0.02  |

|     | AST (IU/L)  |                 |                     |              |
| 1   | 68.17 ± 9.02 | 61.17 ± 7.22    | 75.67 ± 19.62       | 67.00 ± 9.54 |
| 8   | 68.50 ± 9.54 | 66.67 ± 3.08    | 68.00 ± 12.92       | 53.00 ± 11.00 |
| 15  | 68.00 ± 8.25 | 68.33 ± 4.89    | 78.17 ± 17.87       | 73.67 ± 4.73  |
| 22  | 67.33 ± 8.41 | 76.17 ± 12.88   | 73.33 ± 12.60       | 78.00 ± 8.19  |

|     | ALT (IU/L)  |                 |                     |              |
| 1   | 45.17 ± 12.59 | 43.17 ± 12.22    | 54.83 ± 11.34       | 56.33 ± 8.02  |
| 8   | 45.00 ± 9.57 | 53.50 ± 12.52   | 44.67 ± 6.77        | 35.67 ± 1.53  |
| 15  | 46.33 ± 7.74 | 51.17 ± 9.28    | 65.83 ± 13.47**     | 72.00 ± 13.00** |
| 22  | 42.50 ± 7.15 | 52.00 ± 13.10   | 51.67 ± 10.84       | 60.67 ± 4.04  |

|     | ALP (IU/L)  |                 |                     |              |
| 1   | 978 ± 212   | 1014 ± 192      | 1354 ± 484          | 952 ± 47     |
| 8   | 902 ± 177   | 618 ± 135**     | 432 ± 152**         | 382 ± 88**   |
| 15  | 898 ± 128   | 628 ± 168***    | 1090 ± 297          | 838 ± 261    |
| 22  | 826 ± 122   | 678 ± 168       | 557 ± 152**         | 713 ± 401    |

|     | T-Bil (µg/dL) |                 |                     |              |
| 1   | 33.33 ± 16.33 | 30.00 ± 8.94    | 25.00 ± 10.49       | 33.33 ± 11.55 |
| 8   | 30.00 ± 8.94 | 83.33 ± 65.32*  | 3065 ± 1874**       | 2333 ± 596.00*** |
| 15  | 38.33 ± 14.72 | 25.00 ± 12.25   | 31.67 ± 9.83        | 30.00 ± 10.00 |
| 22  | 41.67 ± 13.29 | 58.33 ± 30.61   | 948.3 ± 513.1***    | 43.33 ± 15.28 |

BUN, blood urea nitrogen; Cre, creatinine; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; T-Bil, total bilirubin.

after erlotinib treatment, namely Cre, ALT, and T-Bil, whereas ALP levels decreased. Increased Cre levels occurred in group C on d15 and in groups I and M on d8. Increased ALT levels occurred in groups I and M on d15, while increased T-Bil levels occurred in group C on d8, in group I on d8 and d22, and group M on d8. Decreased ALP levels occurred in group C on d8 and d15, in group I on d8 and d22, and group M on d8.

Skin Rash Figures 3A–C show rash appearance comparison among grades in erlotinib-treated rats. Each erlotinib-treated rat experienced a skin rash, which was also characterized by hair loss. The grade 2 rash emerged after erlotinib initial administration for 7d in approximately 80% of erlotinib 70mg/kg-treated rats (Fig. 3D, groups I and M combined on d8), whereas only approximately 20% of erlotinib 35mg/kg-treated rats (Fig. 3D, group C on d8). Erlotinib discontinuation for 7d restored the skin condition in approximately 60% of rash-observed rats (groups I and M combined on d15 compared to d8). After erlotinib readministration for 7d, the rash on half of erlotinib 70mg/kg-treated rats progressed to grade 3 (group I on d22). Erlotinib dose reduction restored the skin condition in half of the rats within 7d (group M on d22 compared to d15). In group M, no rats experienced grade 3 rash on d22.

Circulating Immune Cell Percentages The term percentage generated from flow cytometric analysis refers to the number of designated circulating immune cell subsets per total number of immune cells. Figure 4 shows the percentage dynamics of the designated circulating immune cell subset after erlotinib treatment in rats. The percentage of several circulating immune cell subsets was affected by erlotinib treatment.

Percentage escalation was observed on neutrophils and monocytes. The neutrophil percentage escalated in each erlotinib-treated group (Fig. 4A), whereas monocyte only in erlotinib 70mg/kg-treated groups (Fig. 4B, group I on d8 and d22 and group M on d8). Percentage reduction was observed on CD8+ T cells and B cells. CD8+ T cell percentages reduced on d15 in group I (Fig. 4D), and B cell on d8 in groups I and
CD45 Expression on Circulating Immune Cells

CD45 expression was evaluated based on the median fluorescence intensity (MFI) of fluorochromes bound to CD45 molecules. Figure 5 shows CD45 expression dynamics on the designated circulating immune cell subset after erlotinib treatment in rats. Neutrophils’ CD45 expression levels, but not monocytes, CD4+ T cells, CD8+ T cells, and B cells, were reduced by erlotinib and it could be seen in all erlotinib-treated groups (Fig. 5A). Erlotinib discontinuation for 7 d did not fully restore the CD45 expression levels (Fig. 5A, groups I and M on d15).

Correlation between Expected Immune Biomarkers and Skin Rash

Figure 6 shows the correlation between rash occurrence in rats with circulating neutrophil percentages and neutrophils’ CD45 expression levels. The rash occurrence was significantly correlated with the escalation of circulating neutrophil percentages and reduction of neutrophils’ CD45 expression levels.

DISCUSSION

We designed an in vivo study that applied erlotinib regimens resembling its clinical application to identify possible erlotinib-induced skin rash biomarkers for humans. Simultaneously, erlotinib discontinuation and readministration with dose reduction effects on rash development were observed. We comprehensively investigated erlotinib-induced skin rash biomarkers among circulating immune cells and found that the rash emergence in erlotinib-treated rats was significantly correlated with the escalation of circulating neutrophil percentages and reduction of neutrophils’ CD45 expression levels. Particularly, the reduction of neutrophils’ CD45 expression levels was the first phenomenon to be observed related to studies involving erlotinib. Based on this study, the rash onset in rats was an average of 14 and 7 d following erlotinib 35 and 70 mg/kg administration, respectively, whereas neutrophil percentage escalation and neutrophils’ CD45 expression reduction have occurred beforehand. In group C, only 1 out of 6 rats generated the rash on d8 (Fig. 3D), while neutrophil percentages and neutrophils’ CD45 expression have already changed (Figs. 4A, 5A). It was followed by rash incident escalations on d15 when 4 out of 6 rats experienced the rash. Thus, we believe neutrophil percentages and neutrophils’ CD45 reduction could be the predictive biomarker candidates of erlotinib-induced skin rash.

Our study revealed circulating neutrophil and monocyte percentage escalations after erlotinib treatment. Circulating neutrophil escalation was probably related to high neutrophilic infiltration in the skin as the erlotinib-induced skin rash characteristic.25) The rash was also followed by hair losses. Keratinocyte apoptosis followed by neutrophil accumulation and activation in the hair follicle sites are likely responsible for the rash and hair loss. Like neutrophils, monocytes also act as a part of innate immunity. However, monocytes have a longer life span than its counterpart, particularly when it has been differentiated to macrophages.26,27) This could be the rationale that monocyte percentage escalation was weaker than neutrophils and observed only in erlotinib 70 mg/kg-treated rats. We showed that erlotinib treatment reduced neutrophils’ CD45 expression levels, but not monocytes, CD4+ T cells, CD8+ T cells, and B cells. This selectivity is likely related to hematopoietic stem cell differentiation tendency towards neutrophils as compared to the other subsets under erlotinib-induced inflammatory conditions. CD45 has been expressed by im-
Immune cells since the hematopoietic stem cell stage. During cell differentiation, CD45 expression on hematopoietic stem cells increased. Erlotinib may interrupt CD45 synthesis at this stage, resulting in neutrophils’ CD45 reduction compared to normal neutrophil differentiation. CD45 itself is a receptor-type protein tyrosine phosphatase (PTP) distributed in all nucleated hematopoietic cells. It is a transmembrane molecule with enzymatic properties in the cytoplasmic region. CD45, through Src family kinases (SFKs) dephosphorylation, is involved in one of which in chemoattractant signaling. During chemoattractant signaling, CD45 is activated after E-selectin and CXCL1 stimulation which eventually results in immune cell rolling velocity reduction and adherence enhancement to the endothelial cell of vasculatures, respectively. CD45 reduction leads to function declines mentioned above.28

Figure 7 shows the proposed erlotinib induced-skin rash mechanism. Erlotinib is distributed into peripheral organs such as bone marrow and skin. Then, erlotinib binds to the EGFR on EGFR-expressing cells, such as keratinocytes of the skin, hence promotes keratinocyte apoptosis. The apoptosis initiates inflammation that contributes to neutrophil production elevation in the bone marrow. However, the produced neutrophils possess erlotinib-induced CD45 deficiency. This deficiency reduces neutrophils’ ability to migrate into the inflamed tissues. Yet, neutrophil migration from the circulating system is likely to increase in narrower vasculatures, such as the capillary blood vessel in hair follicle sites due to the concentrated neutrophils in those regions. A previous report supports this idea by showing that the skin histopathology analysis of erlotinib-treated patients revealed that neutrophil infiltration mostly occurred in the hair follicle sites and did not occur in the dermis as compared to lapatinib, cetuximab, and panitumumab. Inadequate responses by neutrophils and

Fig. 4. Percentage Dynamics of the Designated Circulating Immune Cell Subset per Total Immune Cells after Erlotinib Treatment in Rats
P: placebo; C: constant; I: intermittent; and M: mimic.
continuous keratinocyte apoptosis elevate skin inflammation which eventually promotes skin rash appearance and stimulates more CD45-deficient neutrophil production in the bone marrow. Furthermore, more monocytes are also produced by the hematopoietic stem cells to cope with the severe inflammation in the skin.

A high difference in mean circulating erlotinib concentrations was observed between erlotinib 35 mg/kg- and 70 mg/kg-treated rats on d8 as shown in group C, compared to groups I and M (Table 1) as the results of high mean circulating erlotinib concentrations in erlotinib 70 mg/kg-treated rats (>4000 ng/mL). This suggests erlotinib metabolic enzyme in-

![Fig. 5. CD45 Expression Dynamics on the Designated Circulating Immune Cell Subset after Erlotinib Treatment in Rats](image)

Fig. 5. CD45 Expression Dynamics on the Designated Circulating Immune Cell Subset after Erlotinib Treatment in Rats

MFI: median fluorescence intensity; P: placebo; C: constant; I: intermittent; and M: mimic.

![Fig. 6. Erlotinib-Induced Skin Rash Correlation to (A) Neutrophil Percentages and (B) Neutrophils’ CD45 Expression Levels](image)

Fig. 6. Erlotinib-Induced Skin Rash Correlation to (A) Neutrophil Percentages and (B) Neutrophils’ CD45 Expression Levels

The n value = number of observations from d1 to d22 in all groups. MFI: median fluorescence intensity.
Inhibition occurred in erlotinib 70 mg/kg-treated rats. However, there were conflicting reports regarding the erlotinib effect on its metabolic enzyme. Dong et al. stated erlotinib inhibits CYP3A activity in vitro, while Svedberg et al. stated erlotinib induced CYP3A activity after a 2-month treatment in a clinical study. To confirm the erlotinib effect on CYP3A activity in our study, specifically in group I, a CYP3A activity analysis in vivo was performed. In this experiment, CYP3A activity was analyzed through the quinine/3-hydroxyquinine concentration ratio with the ratio on d1 as the negative control. Quinine hydrochloric dihydrate was administered to group I 15 h before blood sampling on d1, d8, d15, and d22. Quinine and 3-hydroxyquinine concentrations were measured by LC-MS/MS with Quinine-d3 as the internal standard. The (−)-(3S)-3-hydroxyquinine for stock solutions was purchased from Novus Biologicals as the stock standard. The ALP level reduction has been reported in a clinical case report. At that time, the authors suggested the ALP level reduction was derived specifically the bile duct inflammation (cholangitis) rather than the liver. Cholangiocyes, the bile duct epithelial cells, express a considerable amount of EGFR as many as keratinocytes, hence potentially inflamed by erlotinib. Bile duct cavity contractions due to the inflammation reduce bile secretions to the duodenum and conversely increases circulating T-Bil levels. Fewer bile secretions reduce fat and fat-soluble vitamin absorption. Thus, the rats will use fat deposits as an energy source that contribute to their weight losses. Indeed, cholangitis in small animals is characterized by weight losses. Figure S2A shows the weight losses in erlotinib-treated groups. There were strong correlations between rat weight losses with circulating erlotinib concentration and T-Bil level escalations that likely prove the causal relationship among them (Figs. S2B, C). On the other hand, erlotinib has an inhibitory effect on uridine diphosphate glucuronosyltransferase1A1 (UGT1A1), a phase II conjugative enzyme involved in endogenous bilirubin elimination, which could also be responsible for the T-Bil level escalation. The C-reactive protein (CRP) test, performed using Rat CRP ELISA (ICL Lab., U.S.A.), shows a moderate negative correlation between weight losses and CRP levels (Fig. S2D). It is understood that apart from the liver, adipose tissue is a rich source of CRP. Thus, weight loss may contribute to CRP level reduction. The Cre and ALT level recoveries in group I on d22 were likely related to erlotinib concentration reductions (Table 1). High circulating erlotinib concentrations potentially increase Cre, ALT, and T-Bil plasma concentrations, reflecting a decline in hepatic and renal functions and leading to a further erlotinib concentration escalation. Therefore, it is important to monitor circulating erlotinib concentration at certain times in clinical settings, especially in patients with reduced hepatic or renal functions. It was also reported that B cell promotes hepatic inflammation in autoimmune cholangitis. In fact, B cell percentage reduction in erlotinib 70 mg/kg-treated groups (Fig. 4E, groups I and M on d8) is possibly related to the hepatic damage observed on d8 (Table 2).

ALP levels were reduced by erlotinib. The ALP level reduction has been reported in a clinical case report. At that time, the authors suggested the ALP level reduction was derived...
from multiple bone metastases. However, in our study, the ALP level reduction was observed in healthy wild-type erlotinib-treated rats (Table 2). The ALP levels were moderately correlated with neutrophil percentages (negative correlation) and neutrophils’ CD45 expression levels (positive correlation) (Figs. S3A, B). Along with neutrophile percentage escalation and neutrophils’ CD45 reduction, the ALP level reduction was also significantly correlated with the rash occurrence (Fig. S3C). Hence, the ALP levels can be the complement biomarker of erlotinib-induced skin rash, in addition to neutrophil percentages and neutrophils’ CD45 expression levels, to its vast use in clinical applications. Because by far of our knowledge, no information reveals the correlation of erlotinib-induced inflammation with the ALP level reduction, further investigations are needed to elucidate the erlotinib-induced ALP level reduction mechanism.

In conclusion, we comprehensively investigated erlotinib-induced skin rash biomarkers among circulating immune cells and revealed novel relations of neutrophil percentages and neutrophils’ CD45 expression levels on the rash occurrence. The reduction of neutrophils’ CD45 expression levels may be a trigger to the erlotinib-induced skin rash development and severity escalation. Erlotinib readministration at a reduced dose following skin rash is a reasonable strategy for preventing high circulating erlotinib concentration and skin damage escalation. Our findings will be informative for researchers and clinicians to understand and manage erlotinib-induced skin rash.

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Conflict of Interest I.J. performed this study as a graduate student of the Department of Clinical Pharmaceutics, Graduate School of Medicine, Mie University. T.I. declares no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Cohen MH, Johnson JR, Chen YF, Sridhara R, Pazdur R. FDA drug approval summary: erlotinib (Tarceva®) tablets. Oncologist, 10, 461–466 (2005).
2) Takeuchi K, Ito F. Receptor tyrosine kinases and targeted cancer therapeutics. Biol. Pharm. Bull., 34, 1734–1780 (2011).
3) Sridhara SS, Seymour L, Shepherd FA. Inhibitors of epidermal-growth-factor receptors: a review of clinical research with a focus on non-small-cell lung cancer. Lancet Oncol., 4, 379–406 (2003).
4) Ling J, Johnson KA, Miao Z, Rakbit A, Pantez MP, Hamilton M, Luth BL, Prakash C. Metabolism and excretion of erlotinib, a small molecule inhibitor of epidermal growth factor receptor tyrosine kinase, in healthy male volunteers. Drug Metab. Dispos., 34, 420–426 (2006).
5) Mascia F, Mariani V, Girolomoni G, Pastore S. Blockade of EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. Am. J. Pathol., 163, 303–312 (2003).
6) Joshi SS, Ortiz S, Witherspoon JN, Rademaker A, West DP, Anderson R, Rosenbaum SE, Lacouture ME. Effects of epidermal growth factor receptor inhibitor-induced dermatologic toxicities on quality of life. Cancer, 116, 3916–3923 (2010).
7) Paul F, Schumann C, Rüdiger S, Boeck S, Heinemann V, Küchele V, Steffens M, Scholl C, Hichert V, Seufferlein T, Stingl JC. Cyto-
kine regulation by epidermal growth factor receptor inhibitors and epidermal growth factor receptor inhibitor-associated skin toxicity in cancer patients. Eur. J. Cancer, 50, 1855–1863 (2014).
8) Lichtenberger BM, Gerber PA, Holmman M, Buhren BA, Amberg N, Smolle V, Schumph B, Boecke E, Ansari P, Mackenzie C, Wol-
lenberg A, Kislat A, Fischer JW, Kock K, Harder J, Schröder JM, Homey B, Sibilia M. Epidermal EGFR controls cutaneous host defense and prevents inflammation. Sci. Transl. Med., 5, 199ra111 (2013).
9) Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorioso T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodel-
ing. J. Clin. Invest., 119, 3573–3585 (2009).
10) Eyerich K, Eyerich S. Th22 cells in allergic disease. Allergy. Int. J., 24, 1–7 (2015).
11) Homey B, Alenius H, Müller A, Soto H, Bowman EP, Yuan W, McEvoy L, Laemmerer M, Assmann G, Bunemann E, Lehlo M, Wolf B, Rey M, Marschhausen H, Ho W, Stegwerk J, Ruzicka T, Lehmann P, Zlotnik A. CCL27–CCR10 interactions regulate T cell-mediated skin inflammation. Nat. Med., 8, 157–165 (2002).
12) Huang V, Lonsdorf AS, Fang L, Kakinuma T, Lee VC, Cha E, Zhang H, Nagao K, Zaleska M, Olszewski WL, Hwang ST, Cutting Edge: rapid accumulation of epidermal CCL27 in skin-draining lymph nodes following topical application of a contact sensitizer recruits CCR10-expressing T cells. J. Immunol., 180, 6462–6466 (2008).
13) Luo Q, Gu Y, Zheng W, Xu R, Gong F, Gu L, Sun Y, Xu Q. Erlo-
tinib inhibits T-cell-mediated immune response via down-regulation of the c-Raf/ERK cascade and Akt signaling pathway. Toxicol. Appl. Pharmacol., 251, 130–136 (2011).
14) Lee WL, Harrison RE; Grinstein S. Phagocytosis by neutrophils. Microbes Infect., 5, 1299–1306 (2003).
15) Kobayashi SD, DeLeo FR. Role of neutrophils in innate immunity: a systems biology-level approach. Wiley Interdiscip Rev. Syst. Biol. Med., 1, 309–333 (2009).
16) Trowbridge IS, Thomas ML. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and develop-
ment. Annu. Rev. Immunol., 12, 85–116 (1994).
17) Hermiston ML, Xu Z, Weiss A. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol., 21, 107–137 (2003).
18) Yu CL, Yu HS, Sun KH, Hsieh SC, Tsai CY. Anti-CD45 isoforms enhance phagocytosis and expression of gene activation of IL-8 and TNF-α in human neutrophils by differential suppression on protein tyrosine phosphorylation and p56lck tyrosine kinase. Clin. Exp. Immunol., 129, 78–85 (2002).
19) Germena G, Volmering S, Sohlbach C, Zarbock A. Mutation in the mutation CCR10-expressing T cells. J. Immunol., 163, 6462–6466 (2015).
20) Barnett-Vanes A, Sharrock A, Birrell MA, Rankin S. A single
defense and prevents inflammation. PLoS ONE, 11, e0142520 (2016).
21) Hidalgo M, Suo LL, Nemunaitis J, Rizzo J, Hammond LA, Taki-
moto C, Eckhardt SG, Tolcher A, Britten CD, Denis L, Ferrante K, Von Hoff DD, Silberman S, Rowinsky EK. Phase I and pharmaco-
logic study of OSI-774, an epidermal growth factor receptor tyro-
sine kinase inhibitor, in patients with advanced solid malignancies. J. Clin. Oncol., 19, 3267–3279 (2001).
22) Smith NF, Baker SD, Gonzalez FJ, Harris JW, Figg WD, Spar-
reboon A. Modulation of erlotinib pharmacokinetics in mice by a
novel cytochrome P450 3A4 inhibitor, BAS 100. Br. J. Cancer, 98, 1630–1632 (2008).

23) Thermo Fisher Scientific. “Red blood cell lysis protocols using eBio-science Lysis Buffers.” https://www.thermofisher.com/jp/en/home/references/protocols/cell-and-tissue-analysis/protocols/red-blood-cell-lysis.html, accessed 27 March, 2021.

24) Fujimoto H, Sakata T, Hamaguchi Y, Shiga S, Tohyama K, Ichiyama S, Wang F, Houwen B. Flow cytometric method for enumeration and classification of reactive immature granulocyte populations. Cytometry, 42, 371–378 (2000).

25) Nardone B, Nicholson K, Newman M, Guitart J, Gerami P, Talarico N, Yang XJ, Rademaker A, West DP, Lacouture ME. Histopathologic and immunohistochemical characterization of rash to human epidermal growth factor receptor 1 (HER1) and HER1/2 inhibitors in cancer patients. Clin. Cancer Res., 16, 4452–4460 (2010).

26) Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, Bigley V, Flavell RA, Asquith B, Macallan D, Yona S. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J. Exp. Med., 214, 1913–1923 (2017).

27) Tetley TD. Macrophages and the pathogenesis of COPD. Chest, 121 (Suppl), 156S–159S (2002).

28) Zhu JW, Dean K, Park J, Chau AH, Zhang H, Lowell CA, Weiss A, Dean K, Park J, Chau AH, Zhang H, Lowell CA, Weiss A, Dean K, Park J, Chau AH, Zhang H, Lowell CA, Weiss A, Dean K, Park J, Chau AH, Zhang H, Lowell CA, Weiss A. Receptor-like tyrosine phosphatases CD45 and CD148 have distinct functions in chemokine-activated neutrophil migration and response to S. aureus. Immunity, 35, 757–769 (2011).

29) Dong P, Fang Z, Zhang Y, Ge G, Mao Y, Zhu L, Qu Y, Li W, Wang L, Liu C, Yang L. Substrate-dependent modulation of the catalytic activity of CYP3A by erlotinib. Acta Pharmacol. Sin., 32, 399–407 (2011).

30) Svedberg A, Vikingsson S, Vikström A, Hornstra N, Kentson M, Branden E, Koyi H, Bergman B, Greén H. Erlotinib treatment induces cytochrome P450 3A activity in non-small lung cancer patients. Br. J. Clin. Pharmacol., 85, 1704–1709 (2019).

31) FDA. “Tarceva® (erlotinib) tablets package insert.” https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021743s010lbl.pdf, accessed 20 April, 2021.

32) Smith DA, Beaumont K, Maurer TS, Di L. Volume of distribution in drug design. J. Med. Chem., 58, S691–S698 (2015).

33) Pullen R. A clinical review of primary biliary cholangitis. Gastroenterol. Nurs., 43, E48–E55 (2020).

34) The Human Protein Atlas. "Cell Expression of EGFR.” https://www.proteinatlas.org/ensg00000146648-egfr/celltype, accessed 13 April, 2021.

35) Zekarias B, Stockhöfe-Zurwieden N, Post J, Balk F, van Reenen C, Gruys E, Rebel MJ. The pathogenesis of and susceptibility to malabsorption syndrome in broilers is associated with heterophil influx into the intestinal mucosa and epithelial apoptosis. Avian Pathol., 34, 402–407 (2005).

36) Cheng X, Lu X, Qu H, Li D, Hu M, Guo W, Ge G, Dong R. Comparison of the inhibition potentials of icotinib and erlotinib against human UDP-glucuronosyltransferase 1A1. Acta Pharm. Sin. B, 7, 657–664 (2017).

37) Sproston NR, Ashworth JJ. Role of C-reactive protein at sites of inflammation and infection. Front. Immunol., 9, 754 (2018).

38) Lau DCW, Dhillon B, Yan H, Szmitko PE, Yerma S. Adipokines: molecular links between obesity and atherosclerosis. Am. J. Physiol. Heart Circ. Physiol., 288, H2031–H2041 (2005).

39) Moritoki Y, Tsuda M, Tsucayama K, Zhang W, Yoshida K, Lian ZX, Yang GX, Ridgway WM, Wisker LS, Ansari AA, Gershwin ME. B cells promote hepatic inflammation, biliary cyst formation, and salivary gland inflammation in the NOD.C3c4 model of autoimmune cholangitis. Cell. Immunol., 268, 16–23 (2011).

40) Kunimasa K, Yoshioka H, Iwasaku M, Nishiyama A, Korogi Y, Masuda G, Takaiwa T, Ishida T. Successful treatment of non-small cell lung cancer with gefitinib after severe erlotinib-related hepatoxicity. Intern. Med., 51, 431–434 (2012).