Mutational activation of the epidermal growth factor receptor down-regulates major histocompatibility complex class I expression via the extracellular signal-regulated kinase in non–small cell lung cancer

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The efficacy of programmed cell death–1 (PD-1) blockade in patients with non–small cell lung cancer (NSCLC) positive for epidermal growth factor receptor (EGFR) gene mutations has been found to be limited, but the underlying mechanisms for this poor response have remained obscure. Given that the recognition by T cells of tumor antigens presented by major histocompatibility complex class I (MHC-I) molecules is essential for an antitumor immune response, we examined the effects of EGFR tyrosine kinase inhibitors (TKIs) on MHC-I expression in NSCLC cell lines. Appropriate EGFR-TKIs increased MHC-I expression at the mRNA and cell surface protein levels in NSCLC cells positive for EGFR mutations including those with the T790M secondary mutation. Trametinib, an inhibitor of the extracellular signal–regulated kinase (ERK) kinase MEK, also increased MHC-I expression, whereas the phosphatidylinositol 3-kinase (PI3K) inhibitor buparlisib did not, suggesting that the MEK-ERK pathway mediates the down-regulation of MHC-I expression in response to EGFR activation. Immunohistochemical analysis of EGFR-mutated NSCLC specimens obtained before and after EGFR-TKI treatment also revealed down-regulation of phosphorylated forms of EGFR and ERK in association with up-regulation of MHC-I, an increased number of infiltrating CD8+ T cells, and increased PD-1 ligand 1 expression after such treatment. Our results thus suggest that mutational activation of EGFR inhibits MHC-I expression through the MEK-ERK pathway in NSCLC and thereby contributes to the poor response of such tumors to immunotherapy. Further studies are warranted to evaluate the relation between EGFR-MEK-ERK signaling in and the immune response to EGFR-mutated NSCLC.

Keywords: epidermal growth factor receptor, major histocompatibility complex class I, non–small cell lung cancer, phosphatidylinositol 3-kinase, T790M mutation

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IFN, interferon; MHC, major histocompatibility complex; NSCLC, non–small cell lung cancer; PCR, polymerase chain reaction; PD-1, programmed cell death–1; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; TKI, tyrosine kinase inhibitor.
Lung cancer is the most common cause of cancer deaths worldwide.\(^1\) Mutations of the epidermal growth factor receptor (EGFR) gene are common drivers of non–small cell lung cancer (NSCLC),\(^2\) and EGFR tyrosine kinase inhibitors (TKIs) are considered the best choice for first-line treatment of advanced or recurrent NSCLC harboring activating mutations of EGFR. All patients treated with EGFR-TKIs eventually develop resistance to these drugs, however, with a secondary, gatekeeper mutation (T790M) of EGFR being responsible for the resistance in almost half of such patients. Osimertinib is a third-generation EGFR-TKI that has been found to confer a survival benefit compared with cytotoxic chemotherapy in T790M-positive patients,\(^3\) but no specific agents or treatment strategies have been developed to overcome resistance mechanisms other than that mediated by T790M. New treatment options are thus needed for patients whose tumors have developed resistance to EGFR-TKIs by such mechanisms.

Immune-checkpoint inhibitors are emerging as an important new mode of treatment for NSCLC. Programmed cell death-1 (PD-1) is a cell surface receptor of the immunoglobulin superfamily that is expressed on T cells and pro-B cells and which binds the ligands PD-L1 and PD-L2. PD-1 and its ligands play an important role as a checkpoint in regulation of the immune system by inhibiting T cell activation, thereby suppressing autoimmunity and promoting autoimmune tolerance.\(^4\) Many types of tumor cells express PD-L1, and inhibition of the binding of PD-L1 to PD-1 has been found to enhance T cell responses in vitro as well as to have an antitumor effect in preclinical studies.\(^5\) Immune-checkpoint inhibitors that interfere with the interaction between PD-1 and PD-L1 have recently been found to be superior to docetaxel in terms of both overall survival and tolerability for the treatment of NSCLC, although a durable benefit is observed in only ~20% of patients and some individuals may experience unprecedented immune-related adverse events.\(^6-9\)

Subgroup analysis has suggested that inhibition of the PD-1–PD-L1 axis is less effective in NSCLC patients positive for EGFR mutations than in those wild type for EGFR.\(^7,10\) Low levels of both PD-L1 expression and CD8\(^+\) tumor-infiltrating lymphocytes (TILs) within the tumor microenvironment have been suggested to underlie such an unfavorable clinical response to immune-checkpoint inhibitors in patients with NSCLC positive for activating EGFR mutations.\(^11\) However, the mechanisms responsible for the low efficacy of immune therapy in such patients have remained obscure.

Major histocompatibility complex class I (MHC-I) molecules expressed on the cell surface present peptide fragments from foreign or native intracellular proteins. The induction of a CD8\(^+\) TIL response for tumor eradication requires the recognition by these cells of tumor antigens presented by MHC-I molecules on tumor cells, with limited presentation of such antigens by MHC-I being a key obstacle to effective immunotherapy.\(^12\) We have now examined whether EGFR signaling might inhibit surface MHC-I expression, resulting in loss of immunogenicity, in EGFR mutation–positive NSCLC. We found that inhibition of a specific EGFR signaling pathway by targeted agents was able to increase MHC-I expression in such NSCLC cells.

## MATERIAL AND METHODS

### 2.1 Human NSCLC cell lines and reagents

The PC9 cell line was kindly provided by Dr. Hayata (Tokyo Medical University). The PC9GR cell line was previously described.\(^13\) The cell lines H1944, HCC827, and H1975 were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were maintained under a humidified atmosphere of 5% CO\(_2\) at 37°C in RPMI 1640 medium digested with 10% fetal bovine serum. The cells were routinely tested and found to be negative for mycoplasma contamination with the use of a MycoAlert system (LT07, Lonza, Basel, Switzerland). Erlotinib, osimertinib, trametinib, and buparlisib were obtained from Chemietek (Indianapolis, IN, USA). Recombinant human interferon (IFN) γ was obtained from PeproTech (Rocky Hill, NJ, USA).

### 2.2 RNA isolation, RT, and real-time PCR analysis

Total RNA was extracted from cells with the use of a RNasey Mini Kit (74106, Qiagen, Valencia, CA, USA) and was subjected to RT with a High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems, Carlsbad, CA, USA). The resulting cDNA was subjected to reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis with PowerUp SYBR Green Master Mix (A25741, Thermo Fisher Scientific, Waltham, MA, USA) and a StepOnePlus Real-Time PCR system (Applied Biosystems), and the final results were calculated with the \(\Delta\Delta^Ct\) method and normalized by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. The primer sequences (forward and reverse, respectively) were 5′-GGATCCGGAGCCACAGGTC-3′ and 5′-CTTATAGAGGATTTCC-3′ for GAPDH. The primers were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3 Flow cytometry

Cells were dissociated and collected with the use of Accutase cell-deterachment solution (561527, BD Biosciences, San Jose, CA, USA), washed three times with 0.5% bovine serum albumin in phosphate-buffered saline, and incubated for 30 minutes at room temperature with phycoerythrin-conjugated antibodies to HLA-A, -B, and -C (557349, BD Biosciences) or a similarly conjugated isotype control antibody (556640, BD Biosciences). The cells were then washed three times with Stain Buffer containing fetal bovine serum (554656, BD Biosciences) before suspension in Stain Buffer for analysis with a FACS Canto II instrument (BD Biosciences). Viable and dead cells were discriminated with the use of 7-aminoactinomycin D (559925, BD Biosciences).

### 2.4 Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline and then lysed with 1× Cell Lysis Buffer (Cell Signaling Technology,
Danvers, MA, USA). The protein concentration of the lysates was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% gel (Bio-Rad, Hercules, CA, USA). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated for 20 minutes at room temperature with Blocking One or (for phosphorylated proteins) Blocking One-P solution (both from Nakalai Tesque, Kyoto, Japan) before incubation overnight at 4°C with primary antibodies including those to EGFR (#4405), to phosphorylated EGFR (Tyr\textsuperscript{1068}, #2234), to AKT (#9272), to phosphorylated AKT (Ser\textsuperscript{473}, #9271), or to extracellular signal–regulated kinase (ERK) (#9102) from Cell Signaling Technology; with those to phosphorylated ERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}, sc-16982) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); or with those to \(\beta\)-actin (#10021) from Sigma-Aldrich. The membrane was washed with phosphate-buffered saline containing 0.05% Tween 20 before incubation for 2 hours at room temperature with horseradish peroxidase-conjugated secondary antibodies (NA934, GE Healthcare, Indianapolis, IL, USA). Immune complexes were detected with enhanced chemiluminescence reagents (RPN3244, GE Healthcare).

2.5 | Immunohistochemistry

Immunohistochemical staining was performed as previously described.\textsuperscript{14} The primary antibodies included those to HLA class I (ab70328, Abcam, Cambridge, MA, USA), to CD8 (GA623, Agilent Technologies, Palo Alto, CA, USA), to PD-L1 (ab205921, Abcam), to phosphorylated EGFR (Tyr\textsuperscript{1068}, #3777, Cell Signaling Technology), and to phosphorylated ERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}, #4370, Cell Signaling Technology).

2.6 | Patients and tumor specimens

The use of human tissue was in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kindai University. Tumor samples were obtained from NSCLC patients with EGFR mutations both before initiation of EGFR-TKI therapy as well as after disease progression during the treatment.

2.7 | Statistical analysis

Quantitative data are presented as means ± SE and were compared with the two-sided unpaired Student’s t test. The analysis was performed with the use of GraphPad Prism v.7 software (GraphPad Inc., La Jolla, CA, USA). A P value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | EGFR mutated NSCLC cells tended to show lower expression level of HLA-B compared to EGFR wild type NSCLC cells

Epidermal growth factor receptor is a transmembrane protein that is activated by binding of its ligands including epidermal growth factor and transforming growth factor–\(\alpha\). Mutations in the kinase domain of EGFR have been identified in a subset of NSCLC patients, the growth of whose tumors is dependent on the associated activation of EGFR signaling. In vitro studies have shown that EGFR signaling suppresses the expression of immunoregulatory genes including those for MHC-I molecules.\textsuperscript{15} We therefore hypothesized that mutational activation of EGFR in NSCLC might reduce MHC-I expression and thereby lower the efficacy of PD-1–targeted therapy. To examine if mutational activation of EGFR inhibits MHC-I expression, we compared basal expression level of MHC-I gene in the presence of IFN-\(\gamma\) in EGFR mutation positive and negative cells by RT-PCR (Figure 1). As expected, HLA-B expression level of EGFR mutation positive cells tended to be lower than those of EGFR wild type positive cells.

3.2 | EGFR inhibition increases MHC-I expression in the absence or presence of IFN-\(\gamma\)

We next examined the effects of inhibition of EGFR activity with the EGFR-TKIs erlotinib and osimertinib on HLA-B gene expression in various NSCLC cell lines (Figure 2A). RT-PCR analysis revealed that the first-generation, reversible EGFR-TKI erlotinib induced only a small increase in the amount of HLA-B mRNA in the H1944 cell line, which is wild type for EGFR. In the EGFR-mutated cell lines HCC827, erlotinib significantly increased HLA-B mRNA abundance. In another EGFR-mutated cell line, PC9, the increase of HLA-B expression...
by erlotinib was small, but was statistically significant. On the other hand, in the T790M-positive cell line H1975, whereas erlotinib had no significant effect, the third-generation, T790M-selective EGFR-TKI osimertinib significantly increased the amount of HLA-B mRNA. Neither erlotinib nor osimertinib affected the HLA-B mRNA level in the T790M-positive cell line PC9GR.

Given that IFN-γ up-regulates cell surface expression of MHC-I in vivo, we next exposed NSCLC cells to EGFR-TKIs in the presence of IFN-γ (Figure 2B). Under this condition, erlotinib did not affect HLA-B mRNA abundance in H1944 cells but significantly increased it in HCC827 and PC9 cells. Osimertinib significantly increased the HLA-B mRNA level in both H1975 and PC9GR cells, whereas erlotinib had no such effect. Treatment of osimertinib resulted in no increase of HLA-B in H1944 cells, and only small increase compared to erlotinib in HCC827 and PC9 cells (Figure S1). The effect of EGFR-TKIs on MHC-I gene expression was also seen in HLA-A and HLA-C, which was determined by RT-PCR (Figure S2). In consistent with HLA-B expression, appropriate EGFR-TKI increased HLA-A or C expression in EGFR-mutated cells, whereas, there was no effect of EGFR-TKIs on EGFR wild type H1944 cells.

To determine whether these effects of EGFR-TKIs on MHC-I gene (HLA-A, B, and C) expression were accompanied by up-regulation of MHC-I protein at the cell surface, we treated PC9 and PC9GR cells with these drugs in the absence or presence of IFN-γ and then subjected them to flow cytometric analysis with antibodies to HLA-A, -B, and -C (Figure 3). In EGFR-mutated PC9 cells, erlotinib increased the expression of MHC-I in the absence or presence of IFN-γ, although these effects were not statistically significant. In T790M-positive PC9GR cells, erlotinib and osimertinib each increased the expression of HLA-B in the absence or presence of IFN-γ, with the effect of osimertinib in the presence of IFN-γ being the most pronounced and achieving statistical significance. Overall, these findings were thus similar to those obtained by RT and real-time PCR analysis of HLA-B mRNA and suggested that EGFR inhibition by appropriate EGFR-TKIs increases both MHC-I gene expression and cell surface protein abundance.

3.3 Role of the MEK-ERK pathway in induction of MHC-I expression by EGFR inhibition

The phosphatidylinositol 3-kinase (PI3K)-AKT and ERK signaling pathways are the major mediators of EGFR signal transduction. Immunoblot analysis revealed that, in T790M-positive PC9GR cells, osimertinib (which increased MHC-I expression) inhibited the phosphorylation of AKT and ERK, whereas erlotinib (which did not significantly induce MHC-I expression) had minimal effects on AKT and ERK phosphorylation (Figure 4A), suggesting that inhibition of either PI3K-AKT or ERK pathways may play a role in up-regulation of MHC-I expression. To investigate further the role of these two pathways, we examined the effects of trametinib, which inhibits the upstream ERK kinase MEK, and of the pan-PI3K inhibitor buparlisib on MHC-I expression in PC9GR cells. We first confirmed that trametinib and buparlisib inhibited the activation of ERK and AKT, respectively, in PC9GR cells (Figure 4B). Flow cytometric analysis then revealed that trametinib significantly increased MHC-I expression in PC9GR cells.
in the absence or presence of IFN-γ, whereas buparlisib had no such effect (Figure 3C,D). These results thus suggested that the inhibition of MHC-I expression by EGFR activity is mediated by MEK-ERK signaling.

3.4 | Down-regulation of phospho-EGFR and phospho-ERK associated with up-regulation of MHC-I in tumor tissue

To investigate the possible role of EGFR-MEK-ERK signaling in MHC-I expression in vivo, we examined the abundance of MHC-I and phosphorylated forms of EGFR and ERK as well as of CD8 and PD-L1 in specimens of an EGFR-mutated NSCLC tumor obtained before and after EGFR-TKI treatment (Figure 5). Immunohistochemical analysis revealed that the amounts of phosphorylated EGFR and ERK were markedly decreased after the onset of EGFR-TKI treatment, possibly as a result of the inhibition of EGFR activation. In contrast, expression of MHC-I at the cell surface and in the cytoplasm was substantially increased after treatment onset. Furthermore, the number of CD8+ TILs and the expression of PD-L1 were also increased after EGFR-TKI exposure. These results are thus consistent with the notion that increased...
signaling by the EGFR-MEK-ERK pathway in EGFR-mutated NSCLC results in down-regulation of MHC-I expression, and that inhibition of EGFR activity with EGFR-TKIs attenuates MEK-ERK signaling and thereby increases MHC-I expression, possibly leading to activation of CD8$^+$ TILs.

4 | DISCUSSION

Epidermal growth factor receptor is a receptor tyrosine kinase that is commonly activated or overexpressed in numerous types of cancer. Up-regulation of EGFR activity is mediated by various mechanisms including kinase domain mutations that confer ligand-independent signaling. MEK-ERK and PI3K-AKT signaling pathways mediate the biological effects of EGFR activation and are well-characterized targets of cancer therapy. With the use of EGFR-mutated NSCLC cell lines, we have now shown that pharmacological inhibition of the MEK-ERK pathway, but not that of the PI3K-AKT pathway, restored the expression of MHC-I molecules at the cell surface that had been attenuated as a result of EGFR activation. Our results thus support previous findings that inhibition of EGFR or MEK increases the expression of MHC-I in primary and malignant human keratinocytes or NSCLC cell lines.15,18

Although the development of PD-1-PD-L1 inhibitors has improved treatment outcome for NSCLC patients, the presence of EGFR mutations, which have been identified in ~40% to 60% of lung adenocarcinomas in East Asia,19 has been associated with a low response rate to such drugs.10,11 Antibodies to PD-1 or to PD-L1 relieve effector T cell suppression, with CD8$^+$ TILs being key determinants of the efficacy of these agents. Tumor infiltration by CD8$^+$ T cells and their distribution at tumor invasive margins that precede PD-1 blockade appear to predict the efficacy of PD-1-PD-L1 inhibitors in advanced melanoma.20,21 Expression of MHC-I and antigen presentation at the
but treated individuals eventually develop resistance to these drugs, most often as a result of the emergence of a secondary T790M mutation of EGFR. Osimertinib was designed to overcome such resistance conferred by the T790M mutation and has been approved and now widely adopted for treatment of NSCLC positive for this mutation.\textsuperscript{23} On the other hand, we previously found that, among EGFR-TKI-resistant patients, those negative for T790M tended to show a more favorable response to the PD-1-targeted antibody nivolumab compared with those positive for T790M.\textsuperscript{24} The results of the present and previous studies suggest that EGFR pathway activation is maintained in T790M-positive tumors that generally show a durable response to osimertinib and may lead to suppression of MHC-I expression.\textsuperscript{18} We found that osimertinib overcomes such suppression of MHC-I in T790M-positive PC9GR and H1975 cells. Therefore, even in NSCLC patients with EGFR-TKI resistance due to T790M, appropriate EGFR-TKI therapy may increase MHC-I expression through inhibition of EGFR signaling and thereby improve the efficacy of immune-checkpoint inhibitors.

Our present histopathologic analysis of an EGFR-TKI and immunotherapy combination of the MEK inhibitor in combination with immunotherapy in patients with NSCLC patients,\textsuperscript{34-37} although the data have been disappointing as a result of high toxicity as manifested by adverse events such as interstitial lung disease and elevation of liver enzymes.\textsuperscript{35,36} Further studies will thus be required to identify optimal treatment strategies based on concurrent or sequential administration of an EGFR-TKI or MEK inhibitor in combination with immunotherapy in patients with NSCLC positive for EGFR mutations.

In summary, our results have suggested that mutational activation of EGFR signaling inhibits MHC-I expression through the
MEK-ERK pathway in NSCLC cells. An appropriate EGFR-TKI or MEK inhibitor can inhibit EGFR-MEK-ERK signaling and thereby increase MHC-I expression and possibly activate an immune response to the tumor (Figure 6). Down-regulation of MHC-I expression may be responsible, at least in part, for the low response rate of immune-checkpoint blockade in patients with EGFR-mutated NSCLC. Further study is thus warranted to evaluate the relation between EGFR-MEK-ERK signaling in EGFR-mutated NSCLC and the antitumor immune response.

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CONFLICT OF INTERESTS

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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