CD73 Is Regulated by the EGFR-ERK Signaling Pathway in Non-small Cell Lung Cancer

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Abstract. *Background/Aim:* Successful therapy of EGFR-mutant NSCLC remains a challenging task despite initial benefits with the usage of EGFR tyrosine kinase inhibitors. Cancer immunotherapy has shown promising results in certain tumors, but response rate in EGFR-mutant NSCLC is low, because these tumors are thought to have weak immunogenicity. *Materials and Methods:* We used data from in vivo NSCLC databases as well as from in vitro cell culture experiments to investigate the regulation of CD73 by EGFR. *Results:* EGFR expression was correlated with CD73 expression in patients' datasets, with EGFR-mutant tumors showing higher expression than their EGFR wildtype counterparts. Treatment of EGFR-mutant NSCLC cell lines with EGFR TKI reduced expression of CD73 at both mRNA and protein level. Among EGFR downstream signaling pathways, the Ras-Raf-ERK pathway was involved in the regulation of CD73 expression directly via ERK1/2 without the engagement of RSKs or MSKs. *Conclusion:* The results of this study may provide novel therapeutic strategies for the treatment of oncogene-driven NSCLC.

With more than 1.5 million deaths worldwide, lung cancer remains the leading cause of cancer-related deaths, despite major progresses in lung cancer research and therapy. Tumors with activating mutations in the epidermal growth factor receptor (EGFR) gene are frequently observed in non-small lung cancer (NSCLC) patients in East Asia (1). These mutations lead to autophosphorylation of EGFR and subsequent activation of down-stream signaling pathways,

including the MAPK cascade (Ras-Raf-ERK), the PI3K-AKT signaling pathway and the JAK/STAT signaling pathway, all of which contribute to cell growth and -survival (2). EGFR-tyrosine kinase inhibitor (EGFR TKI) therapy is effective in treating patients with activating EGFR mutation, but despite initial response, the median progression-free survival (PFS) is only 9-13 months and acquired resistance is inevitable (3-7). Thus, the development of novel therapy approaches is as important as ever. In recent years, cancer immunotherapy has gained wide attention, with successful application in the treatment of melanoma and renal cancer (8, 9). In contrast, EGFR-mutant NSCLC do not respond well to checkpoint inhibitor therapy, because they are thought to possess weak immunogenicity and immunological ignorance (10-13). However, the underlying mechanisms remain unclear and poorly understood.

CD73, a GPI-anchored membrane protein widely expressed on tumor cells, is an important enzyme in the conversion of adenosine monophosphate (AMP) to adenosine and organic phosphate. Adenosine activates A(2A) receptors and creates an immunosuppressive niche by inhibiting the activation and function of macrophages, neutrophils, dendritic cells and T-cells (14, 15). Accumulating evidence suggests that non-immunomodulating functions of CD73 play an equally important role for cancer cell survival, progression and migration (16-21). Elevated expression of CD73 is frequently observed in solid tumors and high CD73 expression was reported to be an indicator of poor prognosis in NSCLC and other cancers (22, 23). Targeting CD73 as a potential cancer treatment has so far focused on the inhibition of its immunomodulatory functions in the context of rendering immunologically "cold" tumors sensitive to treatment with checkpoint inhibitors (24). However, the prognostic role of CD73 and its role of predicting response to immune checkpoint inhibitor monotherapy is still controversial.

Expression of CD73 in cancer has been shown to be increased under hypoxic conditions by HIF-1 α (25), as well as by the activation of the NF κ B- and Wnt/ β -catenin

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Key Words: CD73, EGFR, ERK1/2, oncogene-driven NSCLC.

Table I. *Drugs used in this study.*

Name	Purchased from	Working concentration
ZD1839 (gefitinib)	Adooq Bioscience, Irvine, CA, USA	20 nM
BIBW 2992 (afatinib)	Adooq Bioscience, Irvine, CA, USA	20 nM
AZD9291 (osimertinib)	Adooq Bioscience, Irvine, CA, USA	20 nM
AZD6244 (selumetinib)	Adooq Bioscience, Irvine, CA, USA	1 μ M
MK-2206	Adooq Bioscience, Irvine, CA, USA	1 μ M
INCB018424 (ruxolitinib)	Adooq Bioscience, Irvine, CA, USA	1 μ M
U0126	Adooq Bioscience, Irvine, CA, USA	10 μ M
BVD-523 (ulixertinib)	Adooq Bioscience, Irvine, CA, USA	1 μ M
LJH685	Adooq Bioscience, Irvine, CA, USA	10 μ M
SB747641A	Adooq Bioscience, Irvine, CA, USA	10 μ M
GDC-0994 (ravoxertinib)	Adooq Bioscience, Irvine, CA, USA	1 μ M

signaling pathways (26, 27). However, the regulation of basal CD73 expression in NSCLC and the relationship with EGFR and its downstream signaling pathways remains vague. In the present study, we show that expression of CD73 is directly regulated by the Ras-Raf-ERK pathway on transcriptional level, and that this regulation is mediated by ERK1/2 in both EGFR-mutant as well as KRAS-mutant cell lines. These findings may provide novel therapeutic strategies for the treatment of NSCLC with driver oncogene.

Materials and Methods

Cell culture. The HCC827 and NCI-H441 lung adenocarcinoma cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were grown in Roswell Park Memorial Institute (RPMI-1640; Cytiva, Marlborough, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-Glutamine and 1x Antibiotic-Antimycotic Solution (all Corning, New York, NY, USA), and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Medium was replaced with fresh medium every two days and cells were sub-cultured when reaching about 90% confluency to ensure optimal conditions for experiments. EGFR mutation status was confirmed by Sanger sequencing.

Drug treatment and establishment of afatinib-resistant PC-9 cell line. Origin and working concentrations of all drugs used in this study can be found in Table I. For drug treatment of cells, 2×10⁵ cells were seeded into 6 cm dishes and grown for 48 h before medium was replaced with fresh medium containing the drug of interest. After 48 h incubation time, cells were washed once with ice-cold Phosphate Buffered Saline (PBS; Cytiva) and subsequently lysed with 200 μ l Radioimmunoprecipitation Assay Buffer (RIPA; Cell Signaling, Danvers, MA, USA), supplemented with protease and phosphatase inhibitors (Thermo Fischer Scientific, Waltham, MA, USA), or 350 μ l TRI Reagent (Zymo Research, Irvine, CA, USA) for isolation of protein and RNA, respectively. Afatinib-resistant PC-9 cells (PC-9-AR) were established by growing parental PC-9 cells in escalating concentrations of afatinib (starting from 0.1 nM) for six months. The occurrence of T790M mutation as a resistance mechanism was confirmed by Sanger sequencing.

Western blot analysis. Protein concentration was measured using a Bradford Protein Assay Kit (Sigma, St. Louis, MO, USA). A total of 20 μ g of protein were then subjected to SDS-PAGE on a 10% polyacrylamide gel and subsequently blotted on a Hybond Nitrocellulose Membrane (Cytiva, Marlborough, MA, USA). After air-dry for 1 h, membranes were blocked in 5% milk in Tris-buffered Saline + 0.1% Tween (TBST; Omics Bio, New Taipei City, Taiwan) for 1 h at room temperature and subsequently probed with the indicated primary (at 4°C overnight) and secondary antibodies (at room temperature, 1 h). Following washes with TBST, membranes were developed using Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate (Millipore, Billerica, MA, USA). All antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). RNA was extracted from cells using an RNA Isolation Kit (Zymo Research) and subsequently quantified using NanoDrop (Thermo Fischer Scientific). 500 ng RNA were reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan). For qRT-PCR analysis, 10 ng cDNA were mixed with 5 μ l KAPA SBYR Green (Roche, Basel, Switzerland) and 500 nM of gene-specific primers and amplification was performed on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Expression of each gene was normalized to 18S and GAPDH expression levels, and calculated using the comparative Ct method. Primers were designed to span exon-exon junctions, when possible, to avoid background amplification from genomic DNA. Primer Sequences used were: GAPDH-F: 5'-TGGTATCGTGGGAAGGACTCATGAC-3', GAPDH-R: 5'-ATGCCA GTGAGCTTCCCGTTCAGC-3', 18S-F: 5'-AATCAGGGTTCGAT TCCGGA-3', 18S-R: 5'-CCAAGATCCAACACTACGAGCT-3', CD73-F: 5'-CAAAGAGGCCAAATTTCCAA-3', CD73-R: 5'-TTCCCA CAACTTCATCACCA-3'.

Cell viability assay. 1×10³ cells were seeded into 96-well plates, treated with various concentration of afatinib (ranging from 0.1 nM to 1 μ M) and incubated for 96 h before cytotoxicity was determined using Sulforhodamine B (SRB) assay according to standard procedures. Cell viability was determined by dividing the absorbance values of treated cells to that of untreated cells. IC₅₀ calculated from the dose response curve was defined as the concentration of afatinib which resulted in 50% growth inhibition.

Figure 1. CD73 expression is correlated with expression of EGFR in NSCLC cells and patient samples. (A) Correlation analysis of CD73- and EGFR expression in three data sets. (B) Comparison of CD73 expression between normal lung, EGFR-wildtype and EGFR-mutant tumors in two NSCLC patient data sets. Expression is plotted as the log-normalized and Z-score standardized value of raw data (r, correlation coefficient).

Statistics and data analyses. The Cancer Genome Atlas (TCGA) data was downloaded from the Genomic Data Commons Data Portal (GDC portal, <https://portal.gdc.cancer.gov/>), whereas The Cancer Cell Line Encyclopedia (CCLE) data was downloaded from the CCLE database (<https://portals.broadinstitute.org/>). Raw data was log transformed, followed by normalization using z-scores. All statistical analyses were done using Prism 5.0 (GraphPad Software Inc., La Jolla, San Jose, CA, USA), R or Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical differences were then determined using two-tailed Student's *t*-test. Statistical significance was considered at a minimum value of $p < 0.05$. All results presented represent the mean $\pm$ SD of triplicate determinations of three independent experiments performed under the same conditions.

Results

Expression of CD73 correlates with EGFR expression in NSCLC patients. In order to investigate the relationship between the EGFR pathway and CD73 expression, we first analyzed their correlation in three data sets: the The Cancer Genome Atlas

(TCGA) data set (<https://www.cancer.gov/tcga>), comprising data from 488 NSCLC patients, a dataset published by Selamat *et al.* with data from 54 NSCLC patients (28), as well as the data set of the Cancer Cell Line Encyclopedia (CCLE), including data from 138 NSCLC cell lines (29). In all three data sets, expression of CD73 mRNA was significantly correlated with expression of EGFR mRNA (Figure 1A). Expression of CD73 mRNA was strongly elevated in tumor samples compared to normal lung tissue and expression was significantly higher in the EGFR-mutant subset compared to EGFR wildtype tumors (Figure 1B). Taken together, analysis of clinical data sets showed that CD73 expression correlated with expression of EGFR and is increased in NSCLC compared to normal lung with a higher expression in the EGFR-mutant group.

Treatment of EGFR-mutant NSCLC cell lines with EGFR TKIs decreased mRNA- and protein expression of CD73. We next sought to investigate whether treatment of EGFR-mutant

NSCLC cell lines with various EGFR TKIs would affect expression of CD73. Thus, we treated HCC827 and PC-9 cells, both harboring deletions in exon 19 of EGFR, with first-generation TKI gefitinib (30), second-generation TKI afatinib (31) and third-generation TKI osimertinib (32) and analyzed expression levels of CD73 mRNA by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). In both cell lines, all three TKIs significantly reduced expression of CD73 mRNA (Figure 2A). Western blot analysis showed that reduction of CD73 expression was also observed on the protein level as all three TKIs strongly decreased protein expression of CD73 (Figure 2B). To rule out any off-target effects of the EGFR TKIs, we established an afatinib-resistant PC-9 cell line (PC-9-AR) by dose escalation protocol. PC-9-AR cells displayed a high resistance to afatinib, as shown by a cell viability assay (Figure 2C) with an IC_{50} of 132 nM, compared to 0.3 nM of the parental PC-9 cell line. Sanger sequencing revealed that PC-9-AR cells acquired T790M mutation as a resistance mechanism (Figure 2D). Interestingly, PC-9-AR cells showed an increased expression of CD73 compared to PC-9 parental cells (Figure 2E), indicating an importance of CD73 in the survival of afatinib-resistant PC-9 cells. Increased expression of CD73 after EGFR TKI treatment has also been recently observed by Isomoto *et al.* (33). Treatment of PC-9-AR cells with EGFR TKIs showed that gefitinib treatment failed to decrease protein expression of CD73 (Figure 2F), whereas expression of CD73 was partially rescued in PC-9-AR cells compared to parental PC-9 cells under afatinib treatment. In summary, we showed that EGFR TKIs could decrease mRNA and protein expression of CD73 in a specific manner, further strengthening the hypothesis that CD73 is regulated by the EGFR pathway.

Inhibition of the Ras-Raf-ERK signaling pathway, but not of the PI3K-AKT- or JAK/STAT- pathway decreased expression of CD73. The EGFR pathway branches into three major downstream signaling pathways: the Ras-Raf-ERK-, the PI3K-AKT-, and the JAK/STAT- signaling pathway, all of which fulfill distinct roles in the growth and survival of EGFR-mutant tumors (2, 34). Hence, we next investigated which of these EGFR downstream pathways regulates the expression of CD73. To this end, we treated HCC827-, PC-9 and PC-9-AR cells with selumetinib (35), MK-2206 (36) and ruxolitinib (37), inhibitors of the Ras-Raf-ERK-, PI3K-AKT- and JAK-STAT signaling pathway, respectively (Figure 3A). qRT-PCR analysis revealed that CD73 mRNA expression was significantly decreased in all cell lines treated with selumetinib (Figure 3B). In contrast, MK-2206 treatment increased mRNA expression of CD73, whereas ruxolitinib treatment had no effect on CD73 expression. The decrease of CD73 mRNA under selumetinib treatment was not due to altered half-life of CD73 mRNA since selumetinib did not affect degradation rate of CD73 mRNA in HCC827 cells, indicating regulation of CD73 mRNA on

transcriptional level (data not shown). Protein expression of CD73 was also strongly decreased in selumetinib treated cells, similar to the decrease seen under osimertinib treatment (Figures 3C). On the contrary, MK-2206 treatment increased protein expression of CD73 in all cell lines, indicating distinct roles of the Ras-Raf-ERK pathway and the PI3K-AKT pathway in the regulation of CD73 expression in EGFR-mutant NSCLC. Efficiency of selumetinib, MK-2206 and ruxolitinib was confirmed by the inhibition of phosphorylation of ERK1/2 (T202/T204), AKT (S473) and STAT1 (Y701), respectively. In order to confirm the involvement of the Ras-Raf-ERK pathway in the positive regulation of CD73, we treated HCC827 and PC-9 cells with another commonly used inhibitor of the Ras-Raf-ERK pathway, U0126, and observed similar decrease in expression of CD73 protein (Figure 3D). Moreover, treatment of KRAS-mutant H441 cells with selumetinib, but not with MK-2206 or ruxolitinib, reduced CD73 protein levels (Figure 3E), indicating that the regulation of CD73 by the Ras-Raf-ERK pathway is also relevant in KRAS-mutant tumors. KRAS-mutant cells were shown to have elevated expression of CD73 (38, 39) and KRAS mutation was suggested to be associated with high expression of CD73 in cancer patients (40, 41). Taken together, these data show that expression of CD73 is positively regulated by the Ras-Raf-ERK pathway in EGFR- and KRAS- mutant lung cancers.

ERK1/2, but not MSKs or RSKs, regulate the expression of CD73. Signaling by the Ras-Raf-ERK pathway is moderated by three crucial mediators: the 90 kDa ribosomal S6 kinases (RSKs), the mitogen- and stress-activated protein kinases (MSKs), both of which are directly phosphorylated and activated by ERK1/2, and ERK1/2 itself (42). Thus, we next sought to evaluate which ERK downstream mediator is involved in the regulation of CD73 expression. By that means, we treated HCC827-, PC-9 and PC-9-AR cells with ulixertinib (43), LJH685 (44) and SB747651A (45), inhibitors of ERK1/2, RSKs and MSKs, respectively (Figure 4A). Ulixertinib, but not LJH685 or SB747651A treatment significantly reduced expression of CD73 mRNA in all cell lines (Figure 4B). Expression of CD73 protein was similarly reduced in cell lines treated with ulixertinib (Figures 4C), indicating that regulation of CD73 is mediated by ERK1/2 itself and not *via* activation of RSKs or MSKs. Efficiency of LJH685 and SB747651A was evaluated by inhibition of phosphorylation of RSK2 (S227) and CREB (S133), respectively. Interestingly, phosphorylation of ERK was increased rather than decreased in cell lines treated with ulixertinib (Figure 4C). It was reported previously that treatment with ulixertinib inhibited ERK kinase activity, but resulted in marked increase in MEK-mediated phosphorylation of ERK driven by the release of negative feedback signaling (43). We observed similar results by treatment of GDC-0994, another commonly used ERK inhibitor (46): GDC-0994 treatment decreased expression of CD73 in HCC827- and PC-

Figure 2. Treatment of EGFR-mutant NSCLC cell lines with EGFR TKIs decreases expression of CD73. (A) qRT-PCR analysis of CD73 mRNA expression in HCC827- and PC-9 cells after treatment with gefitinib (GEF), afatinib (AFA) and osimertinib (OSI). GAPDH and 18S served as internal controls. (B) Western blot analysis of CD73 protein expression in HCC827- and PC-9 cells after treatment with EGFR TKIs. β-Actin served as an internal control. (C) Cell viability assay of PC-9 cells and afatinib-resistant PC-9 cells (PC-9-AR) at various concentrations of afatinib. (D) Sanger sequencing of EGFR Exon 20 in PC-9-AR cells showing occurrence of T790M. (E) Western blot analysis of CD73 protein expression in PC-9 and PC-9-AR cells. β-Actin served as an internal control. (F) Western blot analysis of CD73 protein expression in PC-9-AR cells after treatment with EGFR TKIs. β-Actin served as internal control. All experiments were carried out in triplicate and data shown represent mean±SD. * $p < 0.05$; ** $p < 0.01$, two-tailed Student's *t*-test. n.s., not significant.

Figure 3. CD73 expression is regulated by the Ras-Raf-ERK pathway in EGFR-mutant NSCLC. (A) Schematic overview of EGFR downstream pathways and its inhibitors. (B) qRT-PCR analysis of CD73 mRNA expression in HCC827-, PC-9- and PC-9-AR cells after treatment with selumetinib (SEL), MK-2206 and ruxolitinib (RUX). GAPDH and 18S served as internal controls. (C) Western blot analysis of CD73 protein expression in HCC827, PC-9- and PC-9-AR cells after treatment with SEL, MK-2206 and RUX. β -Actin served as an internal control. (D) Western blot analysis of CD73 protein expression of HCC827- and PC-9 cells after treatment with U0126. β -Actin served as internal control. (E) Western blot analysis of CD73 protein expression in KRAS-mutant H441 cells after treatment with SEL, MK-2206 and RUX. β -Actin served as an internal control. All experiments were carried out in triplicate and data shown represent mean $\pm$ SD. * p <0.05; ** p <0.01, two-tailed Student's t -test. n.s., not significant.

9 cells; however, phosphorylation of ERK was markedly increased (Figure 4D). In order to confirm that ERK kinase activity is indeed inhibited by ulixertinib, we investigated expression of two prominent ERK pathway target genes, DUSP6 (47) and FOS (48) by qRT-PCR. Ulixertinib significantly decreased mRNA expression of DUSP6 and FOS, similar to treatment with osimertinib and selumetinib, indicating successful inhibition of ERK1/2 by ulixertinib (data not shown). CD73 expression was also down-regulated in KRAS-mutant

cell line H441 when treated with ulixertinib (Figure 4E), suggesting that ERK1/2-mediated regulation of CD73 expression also occurs in KRAS-mutant NSCLC cells. Collectively, we showed that regulation of CD73 expression in EGFR- and KRAS-mutant NSCLC is mediated downstream of the Ras-Raf-ERK pathway by ERK1/2 itself, rather than by engagement of RSKs and MSKs. These results could provide novel therapeutic strategies on how to inhibit CD73 expression in oncogene-driven NSCLC.

Figure 4. CD73 expression is regulated by ERK1/2 directly without involvement of RSKs or MSKs. (A) Schematic overview of ERK downstream pathways and its inhibitors. (B) qRT-PCR analysis of CD73 mRNA expression in HCC827-, PC-9- and PC-9-AR cells after treatment with ulixertinib (ULIX), LJH685 and SB747651A. GAPDH and 18S served as internal controls. (C) Western blot analysis of CD73 protein expression in HCC827-, PC-9- and PC-9-AR cells after treatment with ULIX, LJH685 and SB747651A. β -Actin served as an internal control. (D) Western blot analysis of CD73 protein expression of HCC827- and PC-9 cells after treatment with GDC-0994. β -Actin served as internal control. (E) Western blot analysis of CD73 protein expression in H441 cells after treatment with ULIX, LJH685 and SB747651A. β -Actin served as an internal control. All experiments were carried out in triplicate and data shown represent mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$, two-tailed Student's *t*-test. n.s., Not significant.

Discussion

In this study, we sought to investigate the relationship between EGFR and CD73 in NSCLC. We first found a correlation of EGFR mRNA expression with CD73 mRNA expression in clinical data sets of NSCLC patients (Figure

1A). CD73 expression was significantly elevated in tumors compared to normal lung tissue with EGFR-mutant tumors displaying higher expression than EGFR wildtype tumors (Figure 1B). Next, we observed down-regulation of CD73 mRNA- as well protein expression in EGFR-mutant NSCLC cells when treated with several EGFR TKIs (Figure 2). This

is the first study to evaluate the importance of EGFR and its downstream signaling pathways in the regulation of CD73 expression in NSCLC. We report that CD73 expression is regulated by the Ras-Raf-ERK pathway in EGFR-mutant as well as KRAS-mutant NSCLC cell lines (Figure 3). Interestingly, inhibition of the PI3K-AKT pathway increased mRNA- and protein expression of CD73, indicating that CD73 is delicately regulated in EGFR-mutant NSCLC. It is generally thought that genes regulated by the Ras-Raf-ERK pathway are involved in cell proliferation and progression, whereas those regulated by the PI3K-AKT pathway mediate cell survival and differentiation. Further studies are warranted to investigate the significance of inhibition of CD73 expression by the PI3K-AKT pathway in NSCLC. Finally, we report that signaling *via* ERK1/2 directly, without activation of RSKs or MSKs, controls expression of CD73 in both EGFR- and KRAS- mutant cells (Figure 4). The regulation of CD73 mRNA expression by the Ras-Raf-ERK pathway was not the result of altered CD73 mRNA half-life (data not shown), indicating that regulation takes place at the transcriptional level. Transcriptional regulation of CD73 has been previously studied in other cancers and cell entities (49). In cervical cancer cells, CD73 expression was shown to be induced by the Wnt/ β -Catenin pathway (26) and the TNF α /NF κ B signaling pathway was reported to regulate CD73 expression in colorectal cancer (27). However, the regulation of basal CD73 expression in NSCLC remains poorly understood. The CD73 promoter has been described as a 201 bp core region upstream of the transcriptional start site (50). Indeed, we found this region to be active in PC-9 cells (data not shown). Interestingly, longer fragments of the CD73 promoter, 969 bp- and 1964 bp- fragments, decreased promoter activity in PC-9 cells, indicating that regulation of CD73 expression in NSCLC is complex and involves both activating as well as inhibiting factors. The 201 bp CD73 core promoter contains several predicted binding sites of transcription factors which are regulated by the Ras-Raf-ERK pathway, such as CREB (51), SP1 (52) and AP-1 (26, 53). In our study, inhibition of MSKs by SB747651A resulted in reduced phosphorylation of CREB, but did not change expression of CD73, indicating that CREB is not involved in the regulation of basal CD73 expression in NSCLC (Figure 4C). To our surprise, activity of the CD73 promoter was not changed upon treatment with selumetinib, suggesting that the Ras-Raf-ERK pathway acts on CD73 expression *via* other mechanisms (data not shown). It has been reported that c-Jun/AP-1 induces CD73 in melanoma cells by binding to an intronic enhancer (54). Further studies are needed to clarify whether this intronic enhancer is employed by the Ras-Raf-ERK pathway to induce CD73 expression in NSCLC.

In recent years, cancer immunotherapy with antibodies against PD-1 or PD-L1 has been successfully applied in the

treatment of several tumors, including NSCLC (55). However, EGFR-mutant NSCLC tumors do not respond well to treatment with these checkpoint inhibitors due to weak immunogenicity (56). PD-L1 has been thought to play a key role in this phenomenon. It has been reported that PD-L1 itself is regulated by EGFR as EGF treatment could induce PD-L1 expression and inhibition of EGFR by EGFR TKI resulted in decrease of PD-L1 expression (57). Moreover, EGFR mutation status is correlated with high PD-L1 expression in NSCLC (58). In contrast, other reports suggest that EGFR-mutant tumors are generally thought to possess low expression of PD-L1, resulting in poor response to checkpoint inhibitor treatment (59, 60). Thus, the relationship between EGFR and PD-L1 remains controversial. Regulation of CD73 has been suggested as another possible explanation as to why EGFR-mutant tumors are immunologically “cold”. Indeed, herein we report that CD73 expression is regulated by the EGFR pathway in EGFR-mutant NSCLC and that EGFR-mutant tumors display higher CD73 expression than their EGFR wildtype counterparts. However, the prognostic role of high CD73 expression in EGFR-mutant NSCLC when treated with checkpoint inhibitors remains disputed. Ishii *et al.* recently reported that high CD73 expression may predict a favorable response to checkpoint inhibitors in NSCLC patients; however, the patient cohort analyzed in this study was relatively low (61). Moreover, KRAS-mutant NSCLC were shown to respond rather well to cancer immunotherapy (62, 63), despite their association with increased CD73 expression (40, 41) and CD73 being regulated by the Ras-Raf-ERK pathway as shown in this study. In contrast, other studies showed that inhibition of the adenosine pathway increased the efficiency of checkpoint inhibitor therapy in NSCLC patients (64). Future studies are needed to clarify the role of Ras-Raf-ERK-induced CD73 expression in EGFR- and KRAS-mutant NSCLC with regard to response to cancer immunotherapy.

Besides its functions in converting extracellular ATP to immunosuppressive adenosine, CD73 also possesses other important functions essential for the survival and proliferation of NSCLC, which are, in part, independent of its enzymatic activity (16, 65). It has been reported that knock-down of CD73 inhibits cell growth, cell-cycle progression and migration of KRAS-mutant A549 NSCLC cell line (19). In addition, high CD73 expression was an independent indicator of poor prognosis for overall survival in NSCLC patients (19, 22). We found increased expression of CD73 in PC-9-AR cells which acquired resistance to afatinib by the T790M mutation (Figure 2E). A recent study by Isomoto *et al.* also showed increased expression of CD73 in NSCLC patients after progression during EGFR-TKI treatment (33), indicating an important role of CD73 in the survival of PC-9-AR cells. It would be intriguing to investigate whether the increase of CD73 expression in PC-9-AR cells indeed contributes to

resistance to afatinib and thus, represents a novel therapeutic target for the treatment of afatinib-resistant NSCLCs. Currently, inhibition of CD73 by neutralizing antibodies or small-molecule inhibitors has focused on combination therapy with checkpoint inhibitors in the context of cancer immunotherapy (40, 66). Given the enzyme activity dependent and -independent functions of CD73 in cancer cell survival and progression, it would be intriguing to test whether CD73 inhibition as monotherapy or in combination with EGFR TKIs could result in a better response in patients with EGFR-mutant NSCLC. Indeed, small-molecule inhibition of CD73 activity has shown promising results in pre-clinical studies of Glioblastoma multiforme (67), warranting future studies on the effectivity and feasibility of CD73 inhibition in NSCLC and other cancers.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, S.G. and J.C.-H.Y.; investigation, S.G.; methodology, S.G.; data curation, S.G.; formal analysis, S.G.; software, S.G.; visualization, S.G.; validation, S.G.; formal analysis, S.G.; resources, J.C.-H.Y.; writing - original draft preparation, S.G.; writing - review and editing, S.G., B.-C.L. and J.C.-H.Y.; supervision, J.C.-H.Y.; project administration, J.C.-H.Y. and B.-C.L.; funding acquisition, S.G. and B.-C.L. All Authors have read and agreed to the published version of the manuscript.

Acknowledgements

The Authors thank Chia-Chi Hsu (National Taiwan University Cancer Center) for his advice on experimental design and writing the manuscript.

Funding

The present study was supported by grants from the Ministry of Science and Technology of Taiwan to B.-C.L. (grant number: 108-2314-B-002-095-MY3).

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Received January 12, 2021

Revised January 28, 2021

Accepted February 8, 2021