PD-L1 Upregulation by the mTOR Pathway in VEGFR-TKI–Resistant Metastatic Clear Cell Renal Cell Carcinoma

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Purpose Tyrosine kinase inhibitors (TKI) targeting vascular endothelial growth factor receptor (VEGFR) signaling pathways have been used for metastatic clear cell renal cell carcinoma (mCCRCC), but resistance to the drug develops in most patients. We aimed to explore the underlying mechanism of the TKI resistance with regard to programmed death-ligand 1 (PD-L1) and to investigate signaling pathway associated with the resistant mechanism.

Materials and Methods To determine the mechanism of resistance, 10 mCCRCC patients from whom tumor tissues were harvested at both the pretreatment and the TKI-resistant post-treatment period were included as the discovery cohort, and their global gene expression profiles were compared. A TKI-resistant renal cancer cell line was established by long-term treatment with sunitinib.

Results Among differentially expressed genes in the discovery cohort, increased PD-L1 expression in post-treatment tissues was noted in four patients. Pathway analysis showed that PD-L1 expression was positively correlated with the mammalian target of rapamycin (mTOR) signaling pathway. The TKI-resistant renal cancer cells showed increased expression of PD-L1 and mTOR signaling proteins and demonstrated aggressive tumoral behaviour. Treatment with mTOR inhibitors down-regulated PD-L1 expression and suppressed aggressive tumoural behaviour, which was reversed with stimulation of the mTOR pathway.

Conclusion These results showed that PD-L1 expression may be increased in a subset of VEGFR-TKI–resistant mCCRCC patients via the mTOR pathway.

Key words Renal cell carcinoma, Receptor protein-tyrosine kinases, B7-H1 antigen, TOR serine-threonine kinases
While trying to define the TKI-resistance mechanism by comparing gene expression of paired RCC tissues harvested before and after TKI treatment, we found that PD-L1 expression was increased in a proportion of patients with progression of mCCRCC after TKI treatment. The findings encouraged us to explore the underlying mechanism of PD-L1 expression in TKI-resistant mCCRCC.

Materials and Methods

1. Patients

As a discovery cohort, we retrospectively searched for mCCRCC patients treated with VEGFR-TKIs from whom tumor tissues were harvested before and after the TKI treatment period. To define differential gene expression responsible for TKI resistance, we included only those patients from whom post-TKI mCCRCC tissue was harvested within a month after the diagnosis of progressive disease after TKI treatment. Although post-TKI biopsy is not usually performed once the diagnosis of CCRCC is confirmed at pretreatment period, we were able to find 10 patients that satisfied the above inclusion criteria among 553 patients with recurrent or metastatic CCRCC who were treated with VEGFR-TKI at the Asan Medical Centre, Seoul, Republic of Korea, from 1997 to 2013. Pre-TKI tumor samples were obtained from the primary kidney tumor in six patients and from metastatic tumors of the brain, cheek, leg, and lung in the remaining four patients. In cases in which the tumor tissues were biopsied more than once before TKI treatment, the sample obtained closest to TKI treatment was chosen as the pre-TKI tumor sample for further analyses. The post-TKI tumor samples were biopsied from metastatic tumors in the ileum in two patients; the lung in two patients; and the scalp, retroperitoneum, brain, stomach, thigh, and abdominal wall (n=1 each) in the remaining six patients.

The patients’ clinical information was obtained from electronic medical records and/or hospital charts. Each patient was reviewed according to the 2016 World Health Organization (WHO) Tumor Classification, graded according to the International Society of Urological Pathology (ISUP) grading system, and staged according to the American Joint Committee on Cancer (AJCC) Staging System, 8th edition.

2. Gene expression profiling and pathway analysis

Total RNA was extracted from the 10 pairs of pre- and post-treatment formalin fixed and paraffin embedded tumor tissues of the discovery cohort. Gene expression profiling analyses were performed using Human Genome Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA) according to the manufacturer’s instruction at an outside laboratory under the supervision of two authors (H.G. and H.S.H.). The raw data were normalised by the robust multi-array average method implemented in Transcriptome Analysis Centre ver. 3.0 (Affymetrix). The probes were annotated using chip files for HTA 2.0 platform for gene set enrichment analysis (GSEA) provided by the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp). The differentially expressed genes (DEGs) in the PD-L1–expressing and –nonexpressing groups were investigated by an empirical Bayes moderated t-test using R package limma [10]. The cut off criteria for the DEGs were < 0.05 of normal p-value instead of the false discovery rate (FDR) because of the small number of patients. No annotated genes were excluded.

To determine the enriched pathways for PD-L1 expression, both the hypergeometric test and GSEA were carried out. Hallmark and canonical pathway gene sets (C2cp) of the Molecular Signatures Database (MSigDB) ver. 6.0 (Broad Institute, Cambridge, MA; http://www.broad.mit.edu/gsea/msigdb/index.jsp) were used for the pathway analysis. Gene set permutation rather than sample permutation was performed during the analysis because of the small number of patients. An FDR below 0.05 was considered significant enrichment.

3. Immunohistochemistry in the tissue microarray cores and the whole-tumor sections

All available specimens were formalin-fixed and paraffin-embedded and used for tissue microarray (TMA) construction, which consisted of three representative cores 1.5 mm in diameter. The tumor areas were randomly selected while trying to exclude necrotic and degenerative areas and maximize tumor cell content. To exclude the issue of intratumoral heterogeneity, immunohistochemistry was also performed on whole-tumor sections when residual tumor cells were available.

The discovery cohort was assessed using the anti–PD-L1 antibody (clone E1L3N, 1:100, rabbit monoclonal, catalog No. 13684, Cell Signaling Technology, Danvers, MA) on the TMA construct. In the whole-tumor sections, PD-L1 expression was assessed using the anti–PD-L1 antibody E1L3N and a US Food and Drug Administration–approved PD-L1 assay (22C3 pharmDx, code 5K006, Agilent, Santa Clara, CA).

Immunohistochemical (IHC) staining was performed using the BenchMark XT automated staining system (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s protocol. The nuclei were counterstained with hematoxylin.

The IHC staining results were assessed in a semiquantitative manner. PD-L1 expression was first evaluated according to the intensity (negative, weak, moderate, or strong) and the percentage of positive tumor cells and immune cells, includ-
ing lymphocytes and macrophages. Either tumor cells with membranous patterns or immune cells with cytoplasmic or membranous patterns showing more than weak intensity in >1% were considered positive for PD-L1 expression [11]. The investigator was blinded to the associated clinicopathological information.

4. Cell culture
The human RCC cell lines 769-P, 786-O, ACHN, Caki-1, and A704 were purchased from the American Type Culture Collection. A704 was cultured in modified Eagle medium (Gibco, Grand Island, NY), and the others were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. To develop TKI-resistant RCC cell lines, a dose-response study was first performed to assess their sensitivity to sunitinib (Sutent, Pfizer Pharmaceutical, New York, NY), which is the most frequently administered TKI in mCCRCC, by treating them with various concentrations from 0.1 to 50 μM for 72 hours; then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to estimate the half-maximal inhibitory concentration (IC₅₀) value. The cells were then treated with sunitinib for 6 months at various concentrations ranging from 0.5 to 5 μM, which were chosen to span the IC₅₀ value. The culture medium was changed every 3 days. Sunitinib-sensitive parental cell lines were used as control cell lines. Sunitinib-sensitive parental and sunitinib-resistant cell lines were designated as suS and suR, respectively.

5. MTT assay
The proliferation activity of RCC cell lines was evaluated by MTT assay (Sigma Chemical Co., St. Louis, MO). Briefly, cells (2×10⁴) at the exponential growth phase were seeded in 96-well plates and allowed to attach overnight at 37°C. MTT reagent was added to each well and incubated for 4 hours at 37°C. The supernatant was discarded; the MTT-formazan crystals formed by metabolically viable cells were solubilized with 100 μL of dimethyl sulfoxide (DMSO) on a shaker for 10 seconds; then absorbance was measured in an enzyme-linked immunoassay microreader at 540 nm. Each experiment was performed in triplicate and repeated three times for reliable comparison.

6. Mammalian target of rapamycin inhibitors and activator
A dual phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) inhibitor, dactolisib (NVP-BEZ235), and an mTOR inhibitor, everolimus (RAD001), dissolved in DMSO were purchased from KIM & FRIENDS (Seoul, Korea) and used at final concentrations of 0.5 μM and 5 nM, respectively based on earlier studies [12-14]. An mTOR activator, MHY1485 (synthesised 4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine; Sigma-Aldrich), dissolved in DMSO was used at a final concentration of 2 μM [15].

7. Western blotting
The following antibodies were used in western blotting: rabbit antibodies against PD-L1 (1:100 dilution), phospho-mTOR (Ser2448) (p-mTOR; 1:100 dilution) and phospho-p65 ribosomal protein (Ser 235/236) (p-S6RP; 1:100 dilution) from Cell Signal Technology (Beverly, MA). Whole cell extracts were prepared using radioimmunoprecipitation assay lysis buffer (89900, Thermo Scientific, Waltham, MA) and protease inhibitor cocktail (BPI-9200, Tech & Innovation Co., Chuncheon, Korea). Total protein was subjected to electrophoresis on 12% (PD-L1 and p-S6RP) and 8% (p-mTOR) of sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels for 2 hours at 80 V. β-Actin served as the internal control.

8. Clonogenic survival assay
The cells were plated onto six-well plates in triplicate at low density (0.5×10⁴ cells per well). After 2 weeks in culture, the colonies were stained with 1.0% crystal violet for 15 minutes, then the grossly visible colonies were counted manually. The clonogenic survival fraction (SF) was calculated as follows: SF=(number of colonies formed at a given dose/number of cells plated at a given dose)×(control number of cells plated/control number of colonies formed) [16].

9. Wound healing assay
The cells were plated onto six-well plates at 8×10⁴ cells in triplicate. After overspreading on the plate, the cells were wounded by drawing vertical scratches with a plastic pipette tip, then incubated in regular medium. Migration of cells was observed and photographed under an optical microscope regularly.

10. Matrigel invasion assay
A total of 100 μL of serum-free medium including Matrigel was added to each well of a 24-well plate containing inserts with 8-μm pores and incubated overnight at 37°C to form an artificial basement membrane. Then 2.5×10⁵ cells were seeded in triplicate on the upper chamber of the transwell, and 10% FBS-containing medium was added to the lower chamber. Following 48 hours of incubation at 37°C, the cells remaining on the top of the inserts in the upper membrane were removed by wiping with a cotton swab. Those cells invading the underside of the membrane were fixed in 3.7% formaldehyde, then stained with 1% crystal violet. Cells penetrating the Matrigel were photographed.
Table 1. Clinicopathologic features of 10 pairs of metastatic clear cell renal cell carcinoma patients of discovery cohort

| Variable                          | Patient No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------------------------------|-------------|---|---|---|---|---|---|---|---|---|----|
| Age (yr)                          |             | 56| 54| 42| 53| 54| 66| 40| 53| 45| 55 |
| Sex                               |             | Male | Male | Male | Female | Male | Female | Male | Male | Male | Male |
| Pre-TKI treatment (regimen)       |             | None | Interferon | None | None | None | None | None | Radiation | Interferon | IL-2 |
| TKI treatment                     |             | From initial diagnosis to TKI initiation (mo) | 1 | 61 | 71 | 2 | 4 | 1 | 194 | 3 | 2 | 132 |
| Pre-TKI biopsy                    |             | Regimen | Sunitinib | Sunitinib | Sunitinib | Sunitinib | Sunitinib | Pazopanib | Sunitinib | Pazopanib | Sunitinib |
| Organ                             |             | Procedures | RN | M | M | RN | RN | NB | NB | PN | RN | NB |
| WHO/ISUP grade                   |             | 3 | 1 | 3 | 4 | 4 | 2 | 2 | 4 | 4 | 2 |
| Sarcomatoid area (%)             |             | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 50 | 80 | 0 |
| Necrosis (%)                     |             | 0 | 0 | 0 | 30 | 15 | 0 | 0 | 5 | 30 | 0 |
| Post-TKI biopsy                   |             | Organ | Ileum | Scalp | Lung | Retropertitoneum | Brain | Stomach | Thigh | Abdominal wall | Ileum | Lung |
| Procedures                       |             | Resection | Resection | Resection | Resection | Resection | Resection | Resection | Resection | Resection | Resection |
| WHO/ISUP grade                   |             | 4 | 1 | 3 | 4 | 4 | 4 | 3 | 4 | 4 | 4 |
| Sarcomatoid area (%)             |             | 5 | 0 | 0 | 40 | 60 | 60 | 0 | 80 | 95 | 0 |
| Necrosis (%)                     |             | 0 | 0 | 0 | 80 | 0 | 0 | 5 | 0 | 5 | 0 |
| Follow-up                         |             | Progression-free survival (mo) | 9 | 70 | 46 | 4 | 12 | 46 | 15 | 20 | 1 | 6 |
| Time to death from TKI initiation (mo) | 12 | 89 | 96 | 8 | 39 | 55 | 26 | 23 | 5 | 10 |

IL-2, interleukin-2; M, metastatectomy; NB, needle biopsy; PN, partial nephrectomy, RN, radical nephrectomy; TKI, tyrosine kinase inhibitor; WHO/ISUP, World Health Organization/International Society of Urological Pathology.
Results

1. Characteristics of patients in the discovery cohort

The clinicopathological features of the 10 patients in the discovery cohort are summarised in Table 1. The median age at initial diagnosis was 51.8 years (range, 40 to 66 years), with a 4:1 male/female ratio. At initial diagnosis, three patients presented with metastatic disease, but all patients eventually developed distant metastases (median, 2 months; range, 0 to 169 months). Four patients had received treatment prior to TKI therapy, including treatment with interferon (two patients), interleukin-2 (one patient), and radiation (one patient). As the TKI regimen, seven patients received sunitinib and three patients received pazopanib.

The pre-TKI tumors were of high grade (WHO/ISUP grades 3 or 4) in six patients, with accompanying sarcomatoid features observed in three patients. Compared with pre-TKI tumors, post-TKI tumors demonstrated aggressive pathological features, with more patients with high-grade tumors (nine patients) and tumors with sarcomatoid features (six patients).

The patients developed tumor progression at a median of 13.5 months after initiation of TKI (range, 1 to 70 months), and all died of the disease at a median of 24.5 months after progression (range, 5 to 96 months).

2. Expression of PD-L1 was increased in a subset of TKI-resistant CCRCC patients in the discovery cohort

Global gene expression was compared by microarray experiments on paired tumor tissues harvested at pre-TKI treatment and those harvested at progression of the disease after TKI treatment. Among DEGs, increase of PD-L1 expression in post-treatment tissues was identified in three patients (patients 3, 4, and 10) (Fig. 1A). To verify the microarray results at the protein level, PD-L1 IHC staining was performed on the 10 paired patients. In TMA, PD-L1 expression of tumor cells was noted in the post-treatment tissues of three patients. Patient 3’s tissue yielded a membranous pattern and the other patient samples yielded mixtures of cytoplasmic and membranous patterns (Fig. 1B and C). In consideration of tumor heterogeneity, the PD-L1 IHC staining was performed using whole-tumor sections. In addition to the three patients who were positive in terms of TMA, patient 6 was confirmed to be positive in terms of tumor cells (S1 Table). Therefore, an increase in PD-L1 expression in post-treatment tissues was noted in four patients. Both anti–PD-L1 antibodies, E1L3N and 22C3, showed similar expression patterns in the whole-tumor sections.

PD-L1 expression of immune cells was noted in the post-treatment tissues of two patients. In the TMA construct, PD-L1 expression was noted in only one patient (patient 5).
Fig. 2. Differential gene expression associated with programmed death-ligand 1 (PD-L1) upregulation in tyrosine kinase inhibitor–resistant post-treatment clear cell renal cell carcinoma of the discovery cohort. (A) Heat map overview of differential gene expression between three patients showing PD-L1 upregulation and the remaining seven patients. The levels of mammalian target of rapamycin complex 1 (mTORC1)–associated genes, including PLOD2, SYTL2, NAMPT, SLC7A5, HSPA9, BHLHE40, ATP2A2, LDHA, ACLY, EIF2S2, CALR, CDKN1A, HSPA5, and SLC2A, were increased in patients with increased PD-L1 expression (individual gene names do not appear). Shades of red represent elevated levels and green and blue represent reduced levels of gene expression (see colour scale). (B) Enrichment result for the “hallmark mTORC1 signaling” gene set from the gene set enrichment analysis of microarray data from the above two groups with PD-L1 upregulation noted or not. The enrichment score is calculated by walking down a list of genes ranked by their correlation with up-regulated PD-L1 expression, increasing a running-sum statistic when a gene in that gene set is encountered (each black vertical line underneath the enrichment plot) and decreasing it when a gene that is not in the gene set is encountered. The enrichment score is the maximum deviation from zero encountered in the walk. FDR, false discovery rate.
in both pretreatment and post-treatment tissues. Another patient (patient 6) showed PD-L1 expression in the immune cells of the whole-tumor section. However, PD-L1 expression status could not be assessed in the pretreatment needle biopsy tissue because the tumor tissue was not available.

3. DEGs associated with PD-L1 upregulation

To define DEGs associated with PD-L1 upregulation, the global gene expression of post-treatment CCRCC tissues was compared between three patients whose PD-L1 increased and the remaining seven patients whose PD-L1 expression was not increased in the TKI-resistant post-treatment tissues. When the selection criterion of nominal p-value < 0.05 was applied, 434 DEGs were obtained (Fig. 2A, S2 Table).

The analysis of DEGs in the discovery cohort by the hypergeometric test and GSEA resulted in the selection of mTOR complex 1 (mTORC1) signaling, integrin signaling, hypoxia and glycolysis as the gene sets positively associated with PD-L1 upregulation (Table 2, S3-S5 Tables). Since mTOR inhibitors have been widely used to treat patients with advanced RCC, the mTORC1 signaling pathway was chosen for further study (Fig. 2B). Among the DEGs, those overlapping with the hallmark mTORC1 gene sets on GSEA were GBE1, PLOD2, HSPD9, PDAP1, HSPA5, NMT1, PSMC2, TES, ACLY, and HSPD1. The gene sets negatively associated with PD-L1 upregulation included those associated with allograft rejection, graft- versus- host disease, cytotoxic T lymphocyte antigen-4, T-cell receptor and various interleukin signals, which suggests a decreased tumor immune response (S6 Table).

4. Development of VEGFR-TKI-resistant CCRCC cell line with PD-L1 upregulation

To examine the effect of TKI resistance on the tumoral behaviour of CCRCC, TKI-resistant CCRCC cell lines were developed by long-term treatment of five RCC cell lines, 769-P, 786-O, A-704, ACHN and Caki-1, with sunitinib. Although sunitinib resistance was successfully established in these five cell lines, upregulation of PD-L1 was noted only in 769-P in their corresponding sunitinib-resistant cells (Fig. 3A). After long-term treatment with 0.5, 1.5, 2.5, and 5 μM sunitinib, the IC50 of 769-P sublines increased to 15, 15.4, 15.5, and 17.7 μM, respectively, compared with 10 μM for 769-P/suS (Fig. 3B). Therefore, 769-P cells treated with 5 μM sunitinib were chosen for further experiments.

5. Sunitinib-resistant 769-P with aggressive behavior and upregulation of mTOR signaling

In addition to its high proliferative capacity (Fig. 3B), 769-P/suR showed higher clonogenic survival with greater numbers and larger sizes of colonies in the colony-forming assay compared with 769-P/suS (Fig. 3C). Furthermore, 769-P/suS showed more cells invading the Matrigel membrane in the Matrigel invasion assay and migrating rapidly into the scratched area in the scratch test assay, suggesting its higher invasion and migration capacities (Fig. 3D and E). In addition to PD-L1 upregulation, the expression of p-mTOR and p-S6RP, which has been used as a surrogate marker for mTORC1 activity [17], was also increased in 769-P/suR (Fig. 3F).

### Table 2. Signalling pathways positively associated with the PD-L1-up-regulated group according to the hypergeometric test in the discovery cohort

| Pathway name                                             | Length | Overlap | p-value     | FDR     |
|----------------------------------------------------------|--------|---------|-------------|---------|
| Glycolysis                                               | 200    | 8       | 0.000092    | 0.016   |
| Hypoxia                                                  | 200    | 9       | 0.000013    | 0.006   |
| mTORC1 signaling                                         | 200    | 10      | 0.000001    | 0.001   |
| Starch and sucrose metabolism                           | 52     | 5       | 0.000032    | 0.010   |
| Integrin 3 pathway                                       | 43     | 4       | 0.000238    | 0.027   |
| UPA Upar pathway                                         | 42     | 4       | 0.000217    | 0.027   |
| Calnexin calreticulin cycle                              | 11     | 3       | 0.000054    | 0.012   |
| Glucuronidation                                          | 18     | 3       | 0.000260    | 0.027   |
| Integrin alpha IIb/beta 3 signaling                     | 27     | 4       | 0.000037    | 0.010   |
| Integrin cell surface interactions                      | 79     | 7       | 0.000001    | 0.001   |
| N glycan trimming in the ER and calnexin calreticulin cycle | 13     | 3       | 0.000993    | 0.016   |
| Platelet aggregation plug formation                     | 36     | 4       | 0.000118    | 0.016   |
| Insulin receptor pathway in cardiac myocytes            | 51     | 4       | 0.000462    | 0.045   |
| PIP3 signalling in cardiac myocytes                      | 67     | 5       | 0.000112    | 0.016   |

ER, endoplasmic reticulum; FDR, false discovery rate; mTORC1, phospho-mammalian target of rapamycin complex 1; PD-L1, programmed death-ligand 1; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; UPA, urokinase-type plasminogen activator; Upar, urokinase plasminogen activator receptor.
Fig. 3. Development of 769-P/suR cells with programmed death-ligand 1 (PD-L1) expression and increased aggressive tumoral behavior. (A) Expression of PD-L1 at the protein level in each of the five sunitinib-sensitive and sunitinib-resistant cell lines. 769-P successfully established sunitinib resistance with increased PD-L1 expression. (B) 769-P/suS cells were treated with sunitinib at the indicated concentrations, and relative cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. 769-P cells treated with 5 μM sunitinib were chosen for further studies. (C) The proliferative capacities of 769-P/suS and 769-P/suR cells were assessed by colony formation assay. Representative photographs of crystal-violet-stained cell colonies showed that colony numbers and sizes were increased more in 769-P/suR cells than in 769-P/suS cells (survival fraction, 35). The invasion (D) and migration (E) abilities of the 769-P/suR cells were determined by the Matrigel invasion assay and the scratch test assay, respectively. The numbers of 769-P/suR cells that invaded the underside of the Matrigel membrane and migrated into the wound area were greater than the number of 769-P/suS cells. (F) Expression of PD-L1, phospho-mammalian target of rapamycin (p-mTOR) and phospho-S6 ribosomal protein (p-S6RP) was greater in 769-P/suR cells than in 769-P/suS cells on Western blot analysis.
Fig. 4. Down-regulated programmed death-ligand 1 (PD-L1) expression and decreased aggressive tumoral behavior in 769-P/suR cells treated with dactolisib and everolimus. (A) Inhibition of the mammalian target of rapamycin (mTOR) signaling pathway decreases PD-L1 expression by 769-P/suR cells in a time-dependent manner. (B) Dactolisib and everolimus, which inhibit mTOR signaling pathway-related proteins, reduced PD-L1 expression by 769-P/suR cells after 72 hours. (C) 769-P/suR cells treated with dactolisib and everolimus showed decreased colony numbers and sizes compared with 769-P/suR cells treated with dimethyl sulfoxide (DMSO) in the colony formation assay (survival fraction, 0.01 and 0.4, respectively). (D) The migration ability of 769-P/suR cells treated with dactolisib and everolimus was determined by the scratch test assay. The number of 769-P/suR cells treated with dactolisib and everolimus that migrated into the wound area was less than the number of 769-P/suR cells treated with DMSO. (Continued to the next page)
6. Inhibition of the mTOR pathway down-regulates PD-L1 and suppresses aggressive behavior of VEGFR-TKI-resistant CCRCC

To evaluate whether PD-L1 expression was dependent on activation of the mTOR signaling pathway, 769-P/suR was treated with two pharmacologic inhibitors, dactolisib and everolimus. They inhibited PD-L1 expression in a time-dependent manner (Fig. 4A). In addition, they inhibited the expression of p-mTOR and p-S6RP (Fig. 4B). Treatment of 769-P/suR with dactolisib and everolimus resulted in smaller numbers and sizes of colonies in the colony-forming assay (Fig. 4C) and inhibited migration and invasion in the scratch test assay and the Matrigel invasion assay, respectively (Fig. 4D and E).

7. Stimulation of the mTOR pathway up-regulates PD-L1 expression and represses aggressive behavior of VEGFR-TKI-resistant CCRCC

Stimulation of the mTOR pathway with MHY1485 increased the expression not only of p-mTOR and p-S6RP, but also of PD-L1, in a time-dependent manner in 769-P/suS cells (Fig. 5A). MHY1485 stimulation increased the number and size of colonies in the colony-forming assay (Fig. 5B) and promoted migration and invasion in the scratch test assay and the Matrigel invasion assay, respectively (Fig. 5C and D).

Discussion

We demonstrated that PD-L1 expression was increased in a subset of TKI-resistant CCRCC patients. An in vitro study using a TKI-resistant CCRCC cell line revealed that mTOR signaling increased PD-L1 expression and was responsible for aggressive tumoral behavior. These results may provide an explanation for the clinical therapeutic effects of the use of combination therapy with TKIs and mTOR inhibitors to prevent PD-L1 expression and the aggressiveness of TKI-resistant CCRCC.

VEGF has been isolated as an endothelial cell-specific mitogen that mainly functions through binding to VEGF receptors on endothelial cells [18]. The therapeutic effect of VEGF-targeted therapy has been largely explained by its antiangiogenic effect [19]. In addition, VEGF-mediated autocrine signaling has been reported in breast carcinoma, squamous carcinoma, and glioma to promote the growth, survival, migration, and invasion of tumor cells [20-22]. The previous reports are in accordance with our results, in that an endothelium-independent mechanism explains, at least in part, resistance to VEGFR-TKI in CCRCC.

VEGF is also known to play an important role in immune modulation and to establish immune privilege of tumor cells by inhibiting the infiltration of T cells and blocking dendritic cell differentiation [19]. Paradoxically, VEGFR-TKI treatment resulted in upregulation of PD-L1 expression in a subset of CCRCC patients and RCC cell lines in this present study. In a previous study, RCC patients treated with antiangiogenic therapy showed enhanced expression of PD-L1 compared with untreated patients, which was inversely correlated with patient survival [23]. These findings suggest that TKI-resistant CCRCC develops an immune evasion mechanism by upregulation of PD-L1 expression through the mTOR signaling pathway.

As a downstream effector of the PI3K/AKT pathway, mTOR exerts its function via protein synthesis and affects cell proliferation, angiogenesis, and metabolism [24]. It acts through two distinct multiprotein signaling complexes, mTORC1 and mTOR complex 2. mTORC1 activates ribosomal S6 kinase (p70S6K) and inhibits a translational repressor eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which eventually initiates translation. Through its effect on protein synthesis, mTORC1 modulates the activity of cell-cycle–regulating proteins, including hypoxia-inducible factor 1α and VEGF [25]. Our results imply that the increased mTOR signaling pathway may serve as an
alternative oncogenic mechanism in a subset of TKI-resistant CCRCC patients in spite of the inhibition of the major oncogenic VEGF signaling pathway by VEGFR-TKI. They also suggest that combination therapy with VEGFR-TKIs and mTOR inhibitors might help prevent PD-L1 expression and the aggressiveness of TKI-resistant CCRCC.

Fig. 5. Increased programmed death-ligand 1 (PD-L1) expression via activation of the mammalian target of rapamycin (mTOR) pathway in 769-P/su5 cells by MHY1485. (A) Western blot analysis was performed to detect the change in total protein level of mTOR pathway-related protein and PD-L1 in 769-P/su5 cells treated with MHY1485 as an mTOR activator for 24, 48, or 72 hours. (B) 769-P/su5 cells treated with MHY1485 showed increased colony numbers and sizes compared with 769-P/su5 cells treated with dimethyl sulfoxide (DMSO) in the colony formation assay (survival fraction, 27). The number of 769-P/su5 cells treated with MHY1485 that migrated into the wound area and invaded the underside of the Matrigel membrane was greater than the number of 769-P/su5 cells treated with DMSO in the scratch test assay (C) and the Matrigel invasion assay (D), respectively.
Upregulation of PD-L1 through the mTOR pathway has been reported in non–small cell lung cancer. In an IHC analysis of human lung cancer where p-S6RP expression was used as a marker of active mTOR signaling, 40% of lung cancer patients had active mTOR signaling and PD-L1 expression, with a statistically significant correlation between the two markers. mTOR activation and PD-L1 expression were detected in non–small cell lung cancer cell lines, where PD-L1 expression was not only decreased by inhibition of the PI3K/Akt/mTOR pathway but was also induced by epidermal growth factor and interferon γ in an mTOR-dependent manner [26].

Clinical studies on concomitant treatment with VEGFR-TKI and mTOR inhibitors have been conducted. Phase I trials of sunitinib with everolimus or temsirolimus resulted in significant acute and chronic dose-limiting toxicity, which prevented their clinical adoption. A recent phase II study of combined therapy with another TKI, lenvatinib, plus everolimus found a progression-free survival benefit, with an ORR of 43%, and the combination therapy received Food and Drug Administration approval for the treatment of advanced RCC after failure of prior TKI therapy [27].

In our study, only a subset of TKI-resistant CCRCC patients showed upregulation of PD-L1 expression in the tumor cells at the mRNA level (3 of 10 patients) and at the protein level (4 of 10 patients). Although PD-L1 expression of immune cells in post-treatment tissues was noted in two patients, neither of the patients were confirmed to have increased PD-L1 expression in the immune cells of post-treatment tissues compared with pretreatment tissues. Previous studies have reported PD-L1 expression of immune cells to be associated with worse prognosis, but the significance of such expression is uncertain in the context of TKI-resistant CCRCC [28].

In accordance with our results, clinical studies showed that PD-L1 inhibitors were effective in a proportion of patients with metastatic CCRCC who developed drug resistance after treatment with VEGFR-TKI inhibitors. The ORRs of the PD-L1 inhibitors (atezolizumab and avelumab) were 36% to 55.2% [8,29]. Nivolumab was the first PD-1 inhibitor, and its ORR was 41% to 42% in patients with advanced RCC [8,29]. As shown in the present study, the authors reported that RCC with increased PD-L1 expression was associated with worse prognoses [30,31], although drug efficacy was observed irrespective of PD-L1 expression [32].

The present study had some limitations. Although we demonstrated upregulation of PD-L1 and mTOR signaling in a subset of TKI-resistant CCRCC patients using post-treated CCRCC tissue compared with matched pretreated tissues, the number of these patients was small (10 patients only). It is mainly because once the diagnosis of CCRCC is confirmed at pretreatment, responsiveness to treatment is determined clinically by imaging studies without pathologic examination. Despite the limitation, this study provides an insight into the acquired resistance mechanism of mCCRCC. Therefore, prospective multi-institutional studies need to be performed to collect a large number of paired samples to confirm the results of the present study. In addition, future prospective studies will allow procurement of viable tumor cells and in vivo studies using animal models.

Electronic Supplementary Material
Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement
This retrospective study was approved by the Asan Medical Centre Institutional Review Board (2012-0788). The patient consent was waived due to retrospective nature of the study.

Author Contributions
Conceived and designed the analysis: Jeong SU, Lee JL, Cho YM. Collected the data: Jeong SU, Shin SJ. Contributed data or analysis tools: Jeong SU, Park JM, Yoon SY, Cho YM. Performed the analysis: Jeong SU, Hwang HS, Go H, Jeong G. Wrote the paper: Jeong SU, Cho YM.

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Conflicts of Interest
Conflict of interest relevant to this article was not reported.

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