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HLA-DR and CD74 Expression and the Immune Microenvironment in Renal Cell Carcinoma

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Abstract. *Background/Aim: Expression of human leukocyte antigen (HLA) class I and II and CD74, which functions as a chaperone of MHC class II, play essential roles in T-cell recognition. The aim of this study was to elucidate the association between the HLAs and CD74, and their correlation with the infiltrated immune cells in renal cell carcinoma (RCC). Materials and Methods: We retrospectively investigated the expression of HLA-A/B/C, HLA-DR, and CD74 in 38 patients with advanced RCC (T3/T4), and evaluated their correlations with CD4 and CD8-positive T-cell infiltration using immunohistochemistry. Results: The expression of HLA-A/B/C, HLA-DR, and CD74 on cancer cells was observed in 37, 20, and 31 patients, respectively. The density of CD8- and CD4-positive T cells showed a positive correlation with HLA-DR expression. The density of CD4-positive lymphocytes was significantly associated with CD74 expression. Conclusion: The expression of HLA-DR, rather than CD74, on cancer cells was potentially associated with the anti-cancer immune microenvironment.*

Renal cell carcinoma (RCC) is the most common malignant tumor of all primary kidney neoplasms (1). The prognosis of RCC is generally poor; 21% of patients diagnosed with RCC have metastases at the initial presentation, and 20% to 30% of patients with localized RCC who undergo radical or partial nephrectomy experience a recurrence (2, 3). According to the most recent GLOBOCAN data provided by the World Health Organization, there are 175,098 deaths due to kidney cancer every year worldwide (4). Recently, the development of immune checkpoint inhibitors has dramatically improved the survival of patients with advanced RCC (5, 6), and several studies in multiple cancer types have suggested that the tumor immune microenvironment (TIME) might impact the efficacy of immune checkpoint inhibitors as well as the prognosis (7).

The expression of human leukocyte antigen (HLA) class I (A/B/C) and II (DR/DP/DQ) molecules is widely known to be critical for T-cell recognition. It is also well-known that cancer cells with a higher HLA class I expression level are more sensitive to CD8-positive and antigen-specific cytotoxic T lymphocytes (CTLs), and the down-regulation of HLA class I expression causes the acquired resistance of cancer cells to immunotherapies (8). In contrast, HLA class II molecules are predominantly expressed on antigen-presenting cells, such as dendritic cells and macrophages, and they interact with CD4-positive T cells. Since the anti-HLA-DR antibody has long been available for immunohistochemistry (IHC) on paraffin sections, many articles described HLA-DR expression in human samples instead of HLA-class II. HLA-DR molecules are expressed on tumor cells in various types of cancers, and are associated with a favorable prognosis (9). The over-expression of HLA class I and HLA-DR in cancer cells was detected in 98% and 67% of RCC cases, respectively (10). Another study, reported the down-regulation of HLA class I in 38% of RCC cases, and was significantly associated with a poor clinical course (11); however, no study investigating the significance of HLA-DR expression in RCC has yet been reported. The loss of HLA class II in colorectal cancer was associated with

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a lower infiltration of CTLs and an increased risk of metastasis (12). The expression of HLA-DR in cancer cells was tested by IHC using paraffin-embedded sections, and the HLA-DR expression was found to predict the response to immunotherapy in patients with melanoma (13).

In addition to HLA-DR, CD74 has also been suggested to be a marker that predicts the response to immunotherapy in patients with melanoma (13). CD74 is a transmembrane glycoprotein that is expressed on antigen-presenting cells, such as macrophages, dendritic cells, and B-cells, and has multiple functions (14). CD74 expression was found on leukemic cells and epithelial cancer cells, including breast cancer (15, 16). CD74 expression was observed in RCC, and was involved in cancer cell survival and proliferation through the activation of the HIF-1α pathway (17). CD74 is also referred to as MHC class II invariant chain, and functions as a specialized chaperone of MHC class II (18).

In the present study, we tested whether there is a significant association between HLA molecules and CD74, and their correlation with the cancer immune microenvironment in patients with advanced RCC. It is well known that the composition of TIME in RCC differs between tumor stages (19). Therefore, we selected patients with advanced stage RCC and tumor thrombosis but without distant metastases, who are known to have a poor prognosis, to have an appropriate number of death events to perform the survival analysis (20).

Materials and Methods

Patients and tissue arrays. Tissue microarrays were produced from the preserved formalin-fixed paraffin-embedded tissue samples in our previous work (20). All tumor cores were identified. Four to six areas without necrosis and hemorrhage were grossly selected by 2 experienced pathologists and a 3-mm core was removed from each selected area using a needle punch. These were subsequently embedded in previously arranged recipient paraffin blocks through a precisely spaced 24-hole array pattern. Core positions in the recipient paraffin block were noted on a tissue microarray map. Tumor cores cut from intra-renal tumors were evaluated in the present study.

Immunohistochemistry. Specimens were cut into 3-μm sections. Anti-HLA-A/B/C (clone EMR8-5, Hokudo, Hokkaido, Japan), anti-HLA-DR (clone TAL1B5, Santa Cruz Biotech, Dallas, TX, USA), anti-CD74 (clone LN2, Santa Cruz Biotech), anti-CD3 (clone SP7, Nichirei Biosciences, Tokyo, Japan), anti-CD8 (clone C8/144B, Nichirei Biosciences), and anti-CD163 (clone 10D6, Leica Biosystems, Nussloch, Germany) were used as primary antibodies. Samples were incubated with horseradish peroxidase-labeled goat anti-mouse or rabbit secondary antibodies (anti-mouse, #424131, and anti-rabbit, #424141, Histofine, Nichirei Biosciences). We visualized immunoreactions using a diaminobenzidine substrate kit (425011, Nichirei Biosciences). HistoGreen substrate (green color; #AYS-E109, Eurobio Scientific, Les Ulis, France) was used for peroxidase-based immunostaining. HIGHDIF Red solution (red color; #Enzo Life Sciences, Farmingdale, NY, USA) was used for alkaline phosphatase-based immunostaining. For double-immunofluorescence staining, anti-CD74 (rabbit polyclonal, Abcam, Cambridge, UK) antibody and anti-HLA-DR antibody (TAL1B5) were used, following incubation with Alexa 488-labeled anti-mouse antibody and Alexa 546-labeled anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) as secondary antibodies. HLA and CD74 positivity on cancer cells was classified into three groups depending on the area of positive staining: score 0: 0% positive; score 1: <30% positive; and score 2: ≥30% positive. It is well-known that CD4 is expressed not only on Th2 lymphocytes, but also on macrophages (21). Therefore, anti-CD3 and -CD8 antibodies were used for detecting T lymphocytes in the present study, and CD3-positive/CD8-negative lymphocytes were considered CD4-positive T lymphocytes. For the cell counting of lymphocytes and macrophages, eight non-overlapping high-power fields (400× magnification) were randomly selected in tumor areas without necrosis and hemorrhage, and the cell counting was performed using KEYENCE BZ-X800 software (KEYENCE Itasca, IL, USA).

Statistics. Statistical analyses of the pathological findings were carried out using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Spearman's correlation test and the Kruskal–Wallis test were used to perform comparisons between groups. Statistical analyses of survival were performed with JMP version 14.2 (SAS, Cary, NC, USA). Cancer-specific survival (CSS) was calculated from the date of surgery to the date of death from cancer-related causes. Data from the survivors at the end of the study period were censored at the date of the last follow-up examination. Survival analyses were performed according to the Kaplan–Meier method and the log-rank test. For all analyses, differences were considered to be statistically significant at $p<0.05$.

Results

Patients and tumor characteristics. The patient characteristics are shown in Table I. There were 27 males (71.1%) and 11 females (28.9%). The median age was 66.5 years (range=27-80 years). All patients, except for one patient with pT4 disease, had pT3 disease (pT3b in 18 patients and pT3c in 19 patients). No patient had clinical node-positive disease; however, four patients had pathological node-positive disease. No patient had distant metastasis. Histologically, 34 patients (89.5%) had clear cell RCC, and four (10.5%) had papillary RCC.

The expression of HLA-A/B/C, HLA-DR, and CD74 in RCC. The IHC results revealed strong HLA-A/B/C expression on cancer cells in 37 of the 38 cases (Figure 1A and B), HLA-DR expression in 20 cases (Figure 1A and B), and CD74 expression in 31 cases (Figure 1A and B). HLA-DR and CD74 were detected on the cell membrane and double-positive cancer cells were detected in HLA-DR-positive cases (Figure 1B). Weakly HLA-A/B/C and CD74 expression and no HLA-DR expression was detected in normal tubules (Figure 1C). Regarding the expression of HLA-A/B/C, the 37 positive cases were all classified as score 2 (Figure 1D). Among the HLA-DR-positive cases, 11 and 9 cases were

Table I. *Patient characteristics (n=38).*

Median age (range) at time of surgery	66.5 (27-80)
Gender (%)	
Male	27 (71.1)
Female	11 (28.9)
pT stage (%)	
pT3b	18 (47.4)
pT3c	19 (50.0)
pT4	1 (2.6)
pN stage (%)	
pN0	15(39.5)
pN1	4 (10.5)
Unknown	19 (50.0)
Histology (%)	
Clear cell	34 (89.5)
Papillary	4 (10.5)
Nuclear grade* (%)	
Grade 1, 2	6 (15.8)
Grade 3, 4	32 (84.2)

*Fuhrman nuclear grade.

classified as score 1 and score 2, respectively (Figure 1D). Among the CD74-positive cases, 11 and 20 cases were classified as score 1 and score 2, respectively (Figure 1D). There was a significant correlation between HLA-DR expression and CD74 expression (Figure 1E). We evaluated the relationship between CSS and the expression of HLA-A/B/C, HLA-DR, and CD74, and no significant differences were observed (unpublished data). The median follow-up duration was 49.8 months (range=1.3-108.4 months).

The correlation between HLA-DR expression and T-cell infiltration. Since T cells were simply divided into CD8-positive lymphocytes with affinity for HLA class I and CD4-positive lymphocytes with affinity for HLA class II, we next tried to evaluate the CD8- and CD4-positive lymphocytes that infiltrate cancer tissues. The results of the double-IHC (CD8, brown color; CD3, green color) showed that the number of CD8-positive cells was positively associated with CD4-positive lymphocytes (Figure 2A and B). The density of CD8-positive lymphocytes and CD4-positive lymphocytes correlated well with HLA-DR expression (Figure 2C). The density of CD8-positive lymphocytes showed a tendency to be correlated with CD74 expression, but the results were not statistically significant (Figure 2D). The density of CD4-positive lymphocytes was significantly associated with CD74 expression (Figure 2D). Representative triple-IHC showed increased number of CD8-positive lymphocytes, and CD4-positive lymphocytes were observed in HLA-DR-positive cancer areas (Figure 2E).

The correlation between T cells and macrophages. Macrophages infiltrating cancer tissues have been known to have protumor and immunosuppressive functions, and CD163 is one of the markers of protumor macrophages (22). High macrophage infiltration was linked to worse prognosis, whereas high lymphocyte infiltration was associated with better prognosis in RCC (19, 23). Therefore, we next tested whether there was a significant correlation between T cells and macrophages. Results of IHC and cell counting showed that there was no association between the density of macrophages and the HLA-DR score (Figure 3A and B). The density of CD163-positive macrophages showed a tendency to be correlated with CD4- or CD8-postive lymphocytes, but the results were not statistically significant (Figure 3C).

Discussion

In the present study, we evaluated the expression of HLA-A/B/C, HLA-DR, and CD74 on cancer cells. However, these molecules were also detected in non-cancer stromal cells, which were mainly infiltrating macrophages and lymphocytes (Figure 1A). Since it was difficult to set a scoring method for the expression of these molecules on lymphocytes and macrophages, the expression of HLA-A/B/C, HLA-DR, and CD74 on stromal cells was not evaluated in the present study.

The down-regulation of HLA-A/B/C was only seen in one case among the 38 enrolled cases, and this case showed a significantly shorter overall survival; this frequency of HLA class I antigen was consistent with that reported in a previous study (11). The down-regulation of HLA class I molecules is one of the mechanisms related to immune evasion. Atkins *et al*. previously reported the detection of HLA class I deficiency in 5% of cases with clear cell RCC and 20% of cases with papillary/chromophobe RCC; they also demonstrated that defects of antigen-processing machinery (APM) components were more frequent in papillary/chromophobe RCC (24). Matsushita *et al*. demonstrated that cases with a low number of neoantigens/epitopes and low expression of HLA-A had a significantly worse prognosis and low tumor-infiltrating lymphocyte infiltration into tumor tissues (25). These data suggest that not only HLA/APM expression, but also the presence of neoantigens is involved in anti-cancer immune responses.

In the present study, HLD-DR expression was observed in 52% of RCC cases. Although the significance of HLA class II expression on cancer cells has never been clarified, Johnson *et al*. have recently shown by means of murine lung cancer models that MHC class II expression on cancer cells was associated with an increased number of infiltrating CD4- and CD8-positive lymphocytes and an effective response to anti-PD-1 therapy (26). We previously reported a metastatic RCC case that showed a complete response to anti-PD-1 therapy, and this case was HLA-DR-positive (27). CD4-

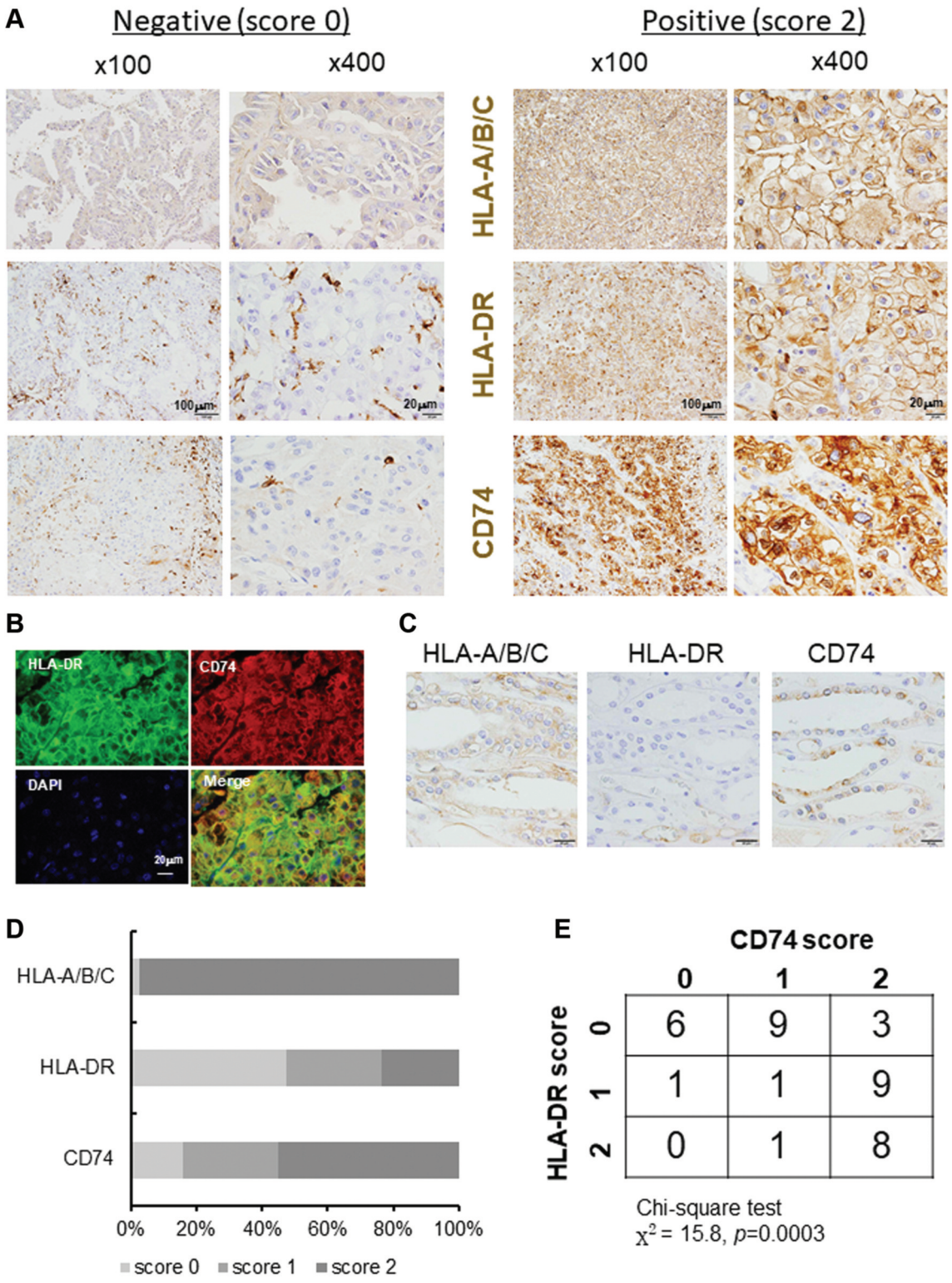

Figure 1. *Immunohistochemistry (IHC) of HLA-A/B/C, HLA-DR, and CD74. (A) Representative IHC results of positive and negative cases are presented. (B) Double immunofluorescence staining of CD74(red) and HLA-DR (green) was performed. (C) Representative IHC images of HLA-A/B/C, HLA-DR, and CD74 in non-cancer kidney areas. Scale bar; 20 mm. (D) Positive staining was scored as described in the Materials and Methods section, and the percentages of HLA-A/B/C, HLA-DR, and CD74 expression are shown. (E) The correlation between HLA-DR and CD74 expression on cancer cells was analyzed by a cumulative Chi-square test.*

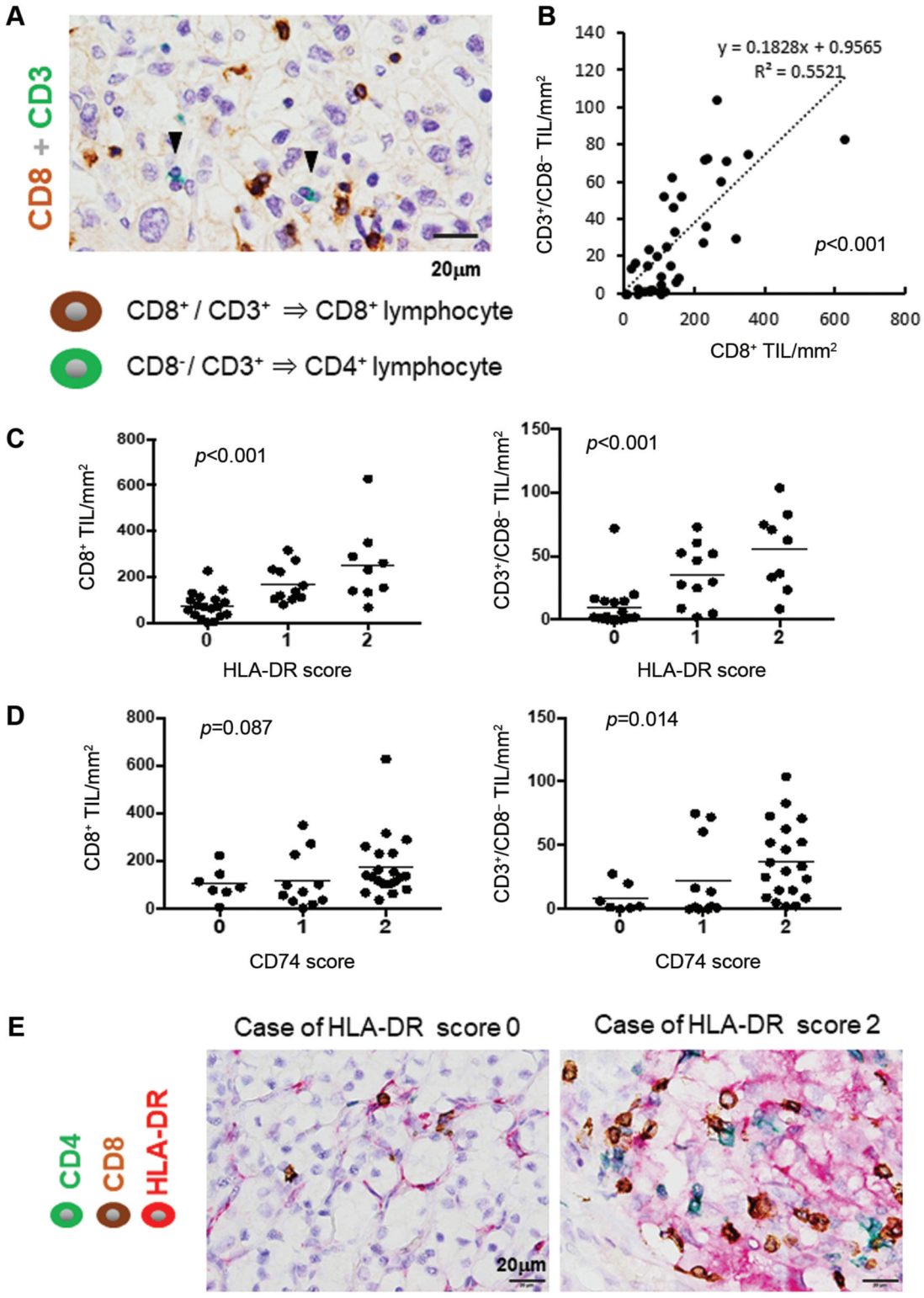

Figure 2. *Immunohistochemistry (IHC) of infiltrated lymphocytes. (A) Double-IHC of CD3 (green) and CD8 (brown) was performed. CD8-positive lymphocytes were positive for both CD3 and CD8, and were detected as a dense brown color. CD4-positive lymphocytes were positive for CD3 and negative for CD8, and were detected as a green color (arrowhead). (B) Correlation between the densities of CD8-positive and CD4-positive ($CD3^+/CD8^-$) lymphocytes. Spearman's correlation test was performed. The correlations between the densities of lymphocytes and the expression of HLA-DR (C) and CD74 (D) are shown. The Kruskal-Wallis test was performed.*

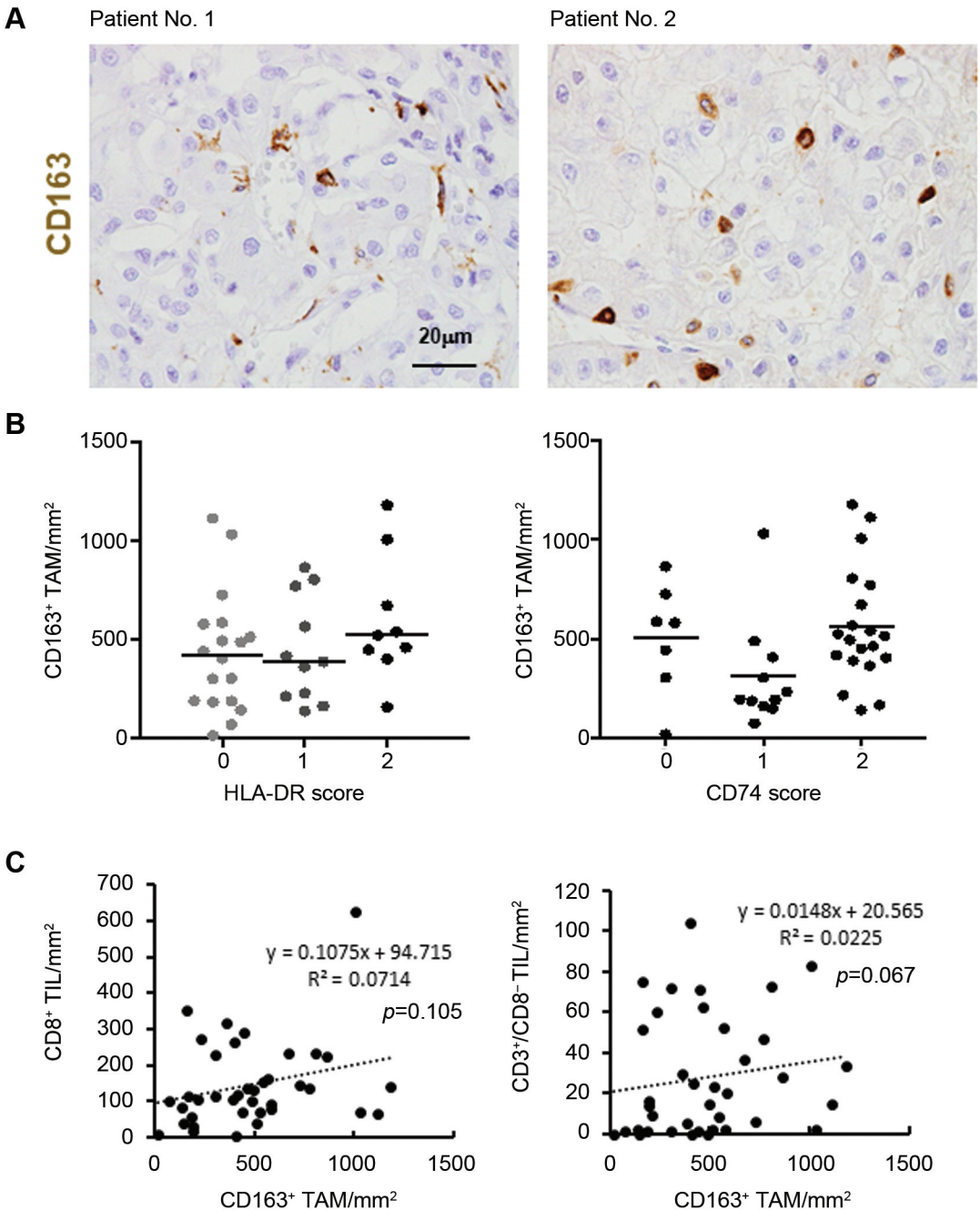

Figure 3. *Immunohistochemistry (IHC) of tumor-associated macrophages (TAMs). (A) IHC of CD163 was performed to detect M2-like TAMs. (B) The correlations between the densities of TAMs, HLA-DR and CD74 expression are shown. The Kruskal-Wallis test was performed. (C) Correlation between the densities of lymphocytes and TAMs. Spearman's correlation test was performed.*

positive T-cell responses to neoantigens were more prevalent and potentially more effective for antitumor immunity than CD8-positive T-cell responses in a murine model (28). Neoepitope-specific CD4-positive T cells have also been detected in human cases (29). MHC class II-restricted neoantigens and the activation of CD4-positive helper T cells were necessary for the induction of anti-tumor responses by CD8-positive T cells (30).

Previous research reported high CD74 expression in 78% of cases, which was associated with high nuclear grade in RCC

(17). The CD74 expression in the present study was 81%, in consistent with a previous study, however, there was significant correlation between CD74 and nuclear grade in this study. In the present cohort, 84% of cases showed high nuclear grade, and this bias obstructed the statistical analysis on nuclear grade. Regarding the functions of CD74, down-regulation of CD74 inhibited growth and invasion *via* suppressing the HIF-1alpha pathway (17) in RCC. Macrophage migration inhibitory factor (MIF), a ligand of CD74, was shown to activate CD74 and MAPK activation, which induce HIF-1alpha activation in breast cancer cells (31). Furthermore, in a previous study, 98% of RCC cases were shown to express MIF, and MIF/CD74 binding stimulated cell growth *via* regulating the Src/p27 pathway (32). High expression of MIF was significantly related to a worse clinical course in several cancers (33). These studies indicate that CD74 might be a promising target for anti-cancer therapy, however, the function of the MHC chaperon should not be abrogated, since MHC expression is required for the immune cell reactions.

In conclusion, the expression levels of HLA-DR (or MHC class II), rather than CD74, on cancer cells were associated with T-lymphocyte infiltration. Further investigations on the relationship between these findings and the response of anti-RCC immune therapy is warranted.

Conflicts of Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. All Authors have no financial competing interests to declare.

Authors' Contributions

Y.M., T.A., and Y.K. carried out the experiments. Y.M. and Y.K. wrote the manuscript with support from T.K. Furthermore, J.Y., T.M., S.O., K.Y., K.K., and S.U. contributed to sample preparation.

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