Annals of Diagnostic Pathology 47 (2020) 151537

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

journal homepage: www.elsevier.com/locate/anndiagpath

Original Contribution

Abundant CD8+ tumor infiltrating lymphocytes and beta-2-microglobulin are associated with better outcome and response to interleukin-2 therapy in advanced stage clear cell renal cell carcinoma

Dale Davis[a,1], Maria S. Tretiakova[b,1], Chris Kizzar[a], Randy Woltjer[a], Victoria Krajbich[a], Scott S. Tykodi[c,d], Christian Lanciault[a], Nicole K. Andeen[a,*]

[a] Oregon Health & Science University, Department of Pathology, Portland, OR 97239, United States of America
[b] University of Washington, Department of Pathology, Seattle, WA 98195, United States of America
[c] University of Washington, Department of Medicine, Division of Medical Oncology, Seattle, WA 98109, United States of America
[d] Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, United States of America

ARTICLE INFO

Keywords:
CD8
Tumor infiltrating lymphocyte
TIL
Immunoscore
Clear cell renal cell carcinoma
Beta-2-microglobulin
Tumor microenvironment
Outcome
Interleukin 2

ABSTRACT

Studies assessing tumor-infiltrating lymphocytes (TILs) in clear cell renal cell carcinoma (ccRCC) and clinical outcomes have mixed results. Given fundamental interaction of MHC class I with CD8+ T-cells, we hypothesized that expression of MHC class I associated protein, beta-2-microglobulin (B2M), may be an important immunologic marker in RCC. We sought to understand potential implications of CD8 + TILs and tumor B2M expression on overall survival and response to high-dose interleukin-2 (IL-2) therapy, in a cohort of patients with high-stage (clinical stage III and IV) ccRCC. Four tumor regions from 56 patients with ccRCC were retrospectively assessed immunohistochemically. At a median follow-up time of 33 months, 22 (39%) patients had died of disease, 23 (41%) were alive disease, and 11 (20%) had no evidence of disease. Tumors with high CD8 + TILs had a significantly lower death rate [hazard ratio (HR): 0.33, $p = 0.02$]. CD8 + TILs correlated with B2M expression ($p = 0.007$). On multivariable analyses, patients with both high B2M and CD8 + TILs had lower death rate (HR: 0.27, $p = 0.03$). Within the subgroup treated with IL-2 ($n = 27$, 48%), tumors with high CD8 + TILs were more likely to respond to IL-2 therapy [coefficient (coef): 1.6, $p = 0.05$]. On multivariable analyses, tumors with a combination of both high B2M expression and high CD8 + TILs also showed trend to responding to IL-2 therapy (coef: 2.5, $p = 0.06$). In conclusion, abundant CD8+ TILs and high tumor expression of beta-2-microglobulin were good prognostic indicators associated with longer survival in patients with high-stage ccRCC. Abundant CD8+ TILs may predict response to IL-2 therapy.

1. Introduction

There are multiple investigations of the tumor microenvironment in renal cell carcinoma (RCC) [1], and conflicting results regarding prognostic impact of tumor infiltrating lymphocytes (TILs) [2,3], although it has been generally concluded that immune response and outcome are closely linked [4]. Although studies of other cancers have revealed favorable correlation between increased numbers of TILs and survival [5-7], some studies of RCC including those examining CD8+ TILs have corroborated a similar phenomenon while others have not [3,4,8-12]. Beta-2-microglobulin (B2M) is found on the surface of all nucleated cells and acts to stabilize the major histocompatibility complex (MHC) class I trimer by non-covalently bonding to the alpha-3 subunit [13]. Extrinsic anchoring of B2M is required for cells, including tumor cells, to express MHC class I and participate in tumor-antigen processing via CD8+ T-cells [4,13-15].

Before the advent of tyrosine kinase and checkpoint inhibitors, the mainstay of treatment for advanced RCC was the cytokines interleukin-2 (IL-2) and interferon-alpha [16]. High-dose IL-2 therapy is effective against advanced stage clear cell RCC (ccRCC) [16,17], but use is limited by toxicity and lack of distinct biomarkers predicting response. Since cytotoxic T-cell TILs have been associated with a better prognosis in some studies, we hypothesized that 1) increased tumor B2M expression would be associated with higher CD8+ T-cell response and potentially better prognosis, and 2) tumor B2M expression and TILs could potentially predict response to IL-2 therapy, in patients with high-

* Corresponding author at: 3181 SW Sam Jackson Park Road, Mail code: L113, Portland, OR 97239, United States of America.
E-mail address: andeen@ohsu.edu (N.K. Andeen).
[1] These authors contributed equally.

https://doi.org/10.1016/j.anndiagpath.2020.151537

stage RCC.

2. Materials and methods

This study was approved by the Oregon Health & Science University and University of Washington Institutional Review Boards. 56 patients with high-stage ccRCC were retrospectively identified; high-stage was defined as pathologic stage T3 (AJCC 8th edition) and/or nodal or metastatic disease at presentation, which corresponded with clinical stage III or IV disease. Nephrectomy specimens ($n = 47$) or metastases ($n = 9$) were reviewed, and four tumor regions were assessed: highest grade (HG), highest density of TILs (HD), infiltrative margin (IM), and central tumor (CT), as previously described [5]. These were examined by (TMA) ($n = 25$) or whole slide ($n = 31$) (Olympus BX43, Tokyo, Japan).

Given previously described pilot immunohistochemical findings identified with PD-L1 (Cell Signaling, Danvers MA, USA; clone E1L3N, 1:200), CD4 (Cell Marque, Rocklin CA, USA; clone SP35, 1:25), CD8 (Leica/Novocastra, Buffalo Grove IL, USA; clone 4B11, 1:200), CD25 (IL-2 receptor; Cell Marque, Rocklin CA, USA; clone 4C9, 1:100), FoxP3 (Abcam, Cambridge UK; clone 236A/E7, 1:100), Gata3 (Biocare, Pacheco CA, USA; clone L50-823, ready-to-use), Tia1 (Immunotech, Marseille France; clone 2G9A10FS, 1:500) and summarized below [18], the cohort of 56 patients was evaluated by H&E and CD8 and B2M immunohistochemistry (Abnova, Taipei, Taiwan; clone 3F9-2C2, 1:5000). CD8+ cells infiltrating the tumor and B2M+ tumor cells were both manually counted per high-powered (400×) microscopic field. CCRCC was considered CD8 + TIL high if both 4-point case median exceeded the overall group median (61/400×) and CD8 + TILs in HD area exceeded HD median (138/400×). For B2M, positive tumor cells showed membranous staining, and the percentage of positive tumor cells in each region was evaluated; the case was considered B2M high if 4-point case median exceeded overall group median (7.5%). Clinical follow-up was available in all patients. Primary outcome was death. Statistical analyses were performed in Stata 13 (College Station, TX, USA). Findings were correlated with other parameters and outcome data using Fischer's exact tests, logistic regression, and Cox proportional hazard analyses. A p-value less than or equal to 0.05 was considered statistically significant.

3. Results

Detailed pathologic findings from 56 patients (40 men, 16 women; median age 60 years) with high-stage ccRCC are summarized in Fig. 1; most patients had WHO/ISUP nucleolar grade 3 tumors and presented at AJCC stage pT3NXM0. Eleven patients (20%) presented with metastatic disease (7 lung, 2 adrenal, 1 bone, 1 lymph node). Forty-seven (85%) had stage pT3 tumors including 29 (52%) pT3a, 14 (25%) pT3b, 2 (4%) pT3c, and 2 (4%) pT3 not sub-staged. Eighteen (33%) had grade 4 tumors including 5 with sarcomatoid and 2 with rhabdoid differentiation. Twenty-seven patients (48%) were treated with high dose IL-2, and 30 (54%) received other therapies including a multitargeted tyrosine kinase and/or VEGF inhibitor ($n = 26$), PD-1 inhibitor ($n = 3$), and/or mTOR inhibitor ($n = 8$). At a median follow-up time of 33 months, 22 (39%) patients had died of disease, and 34 (61%) were alive: 23 (41%) of whom had known disease and 11 of whom (20%) had no evidence of disease. Thirty-two (57%) of patients had experienced subsequent metastasis. Among established prognostic variables, lymph node metastases at presentation was associated with death [hazard ratio (HR): 2.93, 95% confidence interval (CI): 1.02–8.39, $p = 0.04$]; grade 4 tumors showed a non-significant trend toward higher mortality when compared with grade 2 and 3 tumors (HR: 2.19, 95%CI: 0.94–5.1, $p = 0.07$), and clinical stage IV patients ($n = 17$, 30%) had a non-significant trend toward higher mortality (HR: 2.16, 95%CI: 0.87–5.4, $p = 0.09$) than clinical stage III patients ($n = 39$, 70%).

A pilot cohort of 22 cases was first tested with a panel of multiple immunologic immunohistochemical markers, including PD-L1, CD8, CD4, CD25 (IL-2 receptor), FoxP3, Gata3, and Tia1. Of these, only CD8+ TILs ($p = 0.01$) were associated with survival; CD4+ TILs ($p = 0.07$), FoxP3+ TILs ($p = 0.10$), PD-L1, CD25+ TILs, Gata3+ TILs, TIA1+ TILs, and CD8/CD4 ratios were not significantly associated with survival nor IL-2 response [18].

Based on pilot results, the total cohort of 56 patients was tested with CD8 and B2M. CD8 + TILs correlated with B2M expression ($p = 0.007$); 39% of cases were both CD8 + TIL high and B2M high, and 33% were low for both (Fig. 2). On univariate analysis, tumors with high CD8 + TILs had a significantly lower death rate (HR: 0.33; 95%CI: 0.13–0.87, $p = 0.02$, Fig. 3), and there was a similar but non-significant trend for B2M high tumors to have longer survival (HR: 0.38; 95%CI: 0.14–1.0, $p = 0.06$). On multivariable analyses, patients with a combination of both high B2M expression and high CD8 + TILs had lower death rate (HR: 0.27, CI: 0.08–0.89, $p = 0.03$, Fig. 3). CD8+ TILs correlated with lymphocyte count on H&E ($p = 0.05$) and the pathologist's gestalt impression of abundant TILs ($p = 0.03$), but there was no significant association between CD8+ TILs and any of the other tested baseline variables, including sex, age, stage at presentation, or WHO/ISUP nucleolar grade.

Within the total cohort, 27 (48%) patients were treated with IL-2. In this subgroup, at a median follow up time of 36 months, 5 (19%) had complete remission with no evidence of disease at last follow-up, 12 (44%) were alive with disease, and 10 (37%) had died of disease. There was a non-significant trend for patients who responded to IL-2 therapy to remain alive at the end of analysis (83% of responders alive vs. 17% dead of disease, compared with 50% of non-responders alive vs. 50% dead of disease, $p = 0.08$). Within the cohort treated with IL-2, tumors with high CD8 + TILs were more likely to respond to IL-2 therapy [coefficient (coef): 1.6, $p = 0.05$]. On multivariable analyses, tumors with a combination of both high B2M expression and high CD8 + TILs also showed trend to responding to IL-2 therapy (coef: 2.5, $p = 0.06$). The relationship between high CD8+ TILs and survival was stronger for the subgroups treated with IL-2 (HR: 0.09, 95%CI: 0.1–0.8, $p = 0.03$) and clinical stage III ccRCCs (HR: 0.24, 95%CI 0.06–0.8, $p = 0.03$) than for the overall cohort including stage IV ccRCCs (Fig. 3).

4. Discussion

In this study, we evaluated patients with high-stage clear cell RCC from two institutions, selected as they are more likely to die from disease and/or be considered for adjuvant systemic therapy. We analyzed TILs and B2M expression – a component of the MHC class I complex required for immune presentation to CD8+ T-cells – in four distinct location, as this “immunoscore”-based evaluation has shown prognostic significance in other cancers [5]. After examination of an initial panel of immunohistochemical markers, we identified abundant CD8+ TILs and high tumor cell expression of B2M as good prognostic indicators associated with longer survival. As far as we are aware, this is the first time an “immunoscore”-type approach has been utilized for ccRCC. Compared with prior studies, cytotoxic T-cell TILs have been generally associated with better oncologic outcome [19], although increased T-cells have not indicated a better prognosis in all RCCs [2]. Differences in our results may be due in part to cohort selection, methodologic differences, and limitations inherent to assessing immune system function and cellular interactions by immunohistochemistry on a single slide.

In addition to prognostic value, we demonstrate that abundant CD8+ TILs may predict response to IL-2 therapy. The findings suggest that response to IL-2 therapy may in part reflect a pre-existing immunologic milieu that has already recognized the carcinoma and generated TILs. High dose IL-2 was approved for treatment of metastatic RCC 28 years ago [20]. While IL-2 therapy has been supplanted by immune checkpoint inhibitors (ICIs), it is still used in limited settings as a standalone treatment [21-23]. Recent and ongoing studies combine

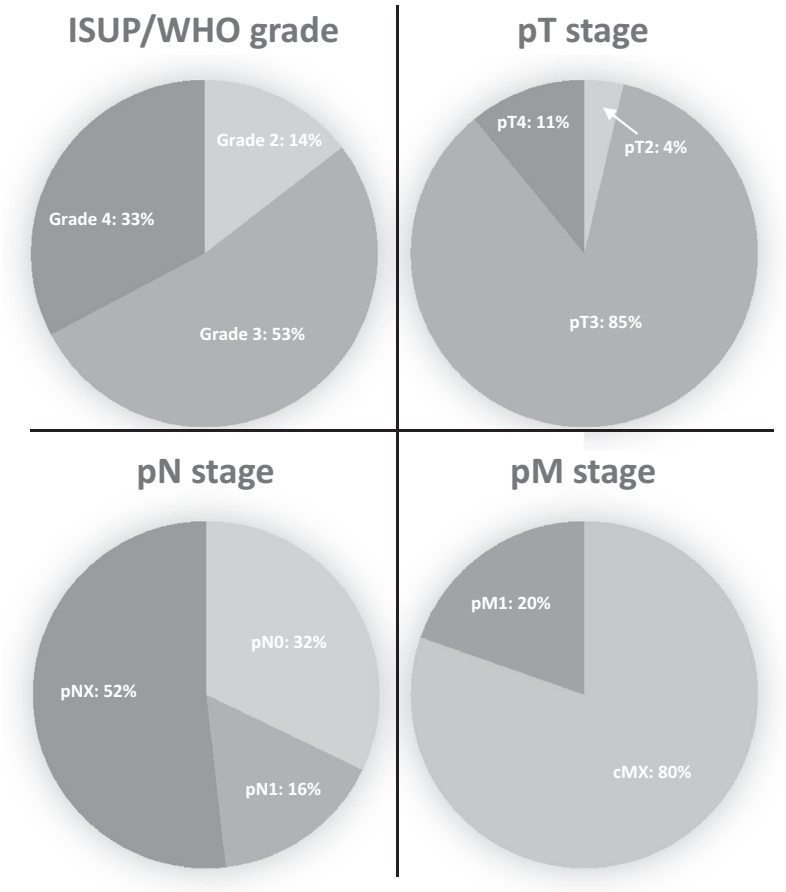

Fig. 1. Detailed pathologic findings from 56 patients with high-stage clear cell renal cell carcinoma.

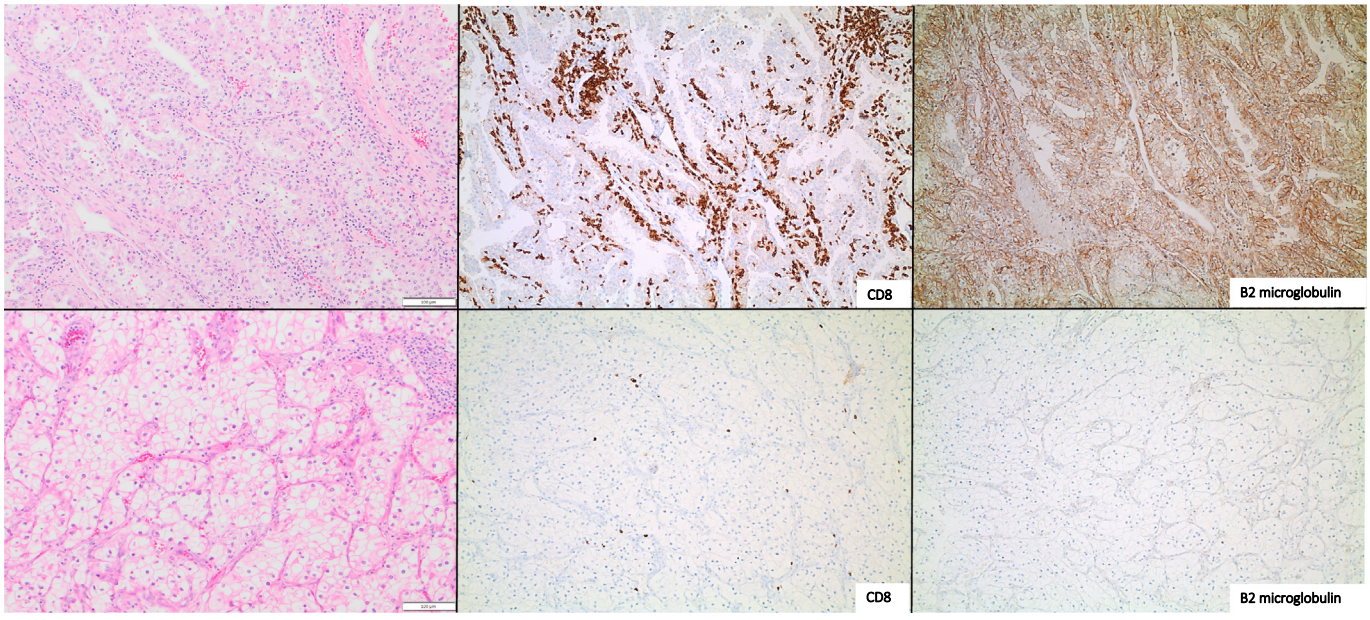

Fig. 2. Top row: in clear cell RCC, CD8+ tumor infiltrating lymphocytes (TILs) were associated with tumor beta-2-microglobulin expression, a component of the MHC class I complex required for immune presentation to CD8+ T-cells. Bottom row: ccRCCs with low numbers of CD8+ TILs had little or no tumor cell expression of beta-2-microglobulin (all images at 100×).

IL-2 with ICIs, such as the anti-PD-1 agent pembrolizumab [21,24]. One study looking at the combination of pembrolizumab and high dose IL-2 reports a preliminary response rate of 72% response rate, which was greater than either of the two therapies combined [24]. Currently there is no guidance beyond clinical status on how to choose which patients will respond to IL-2 therapy [16,21]. Previous studies have correlated a better response with clear cell histology, and one examined the combination of clear cell histology and carbonic anhydrase IX expression,

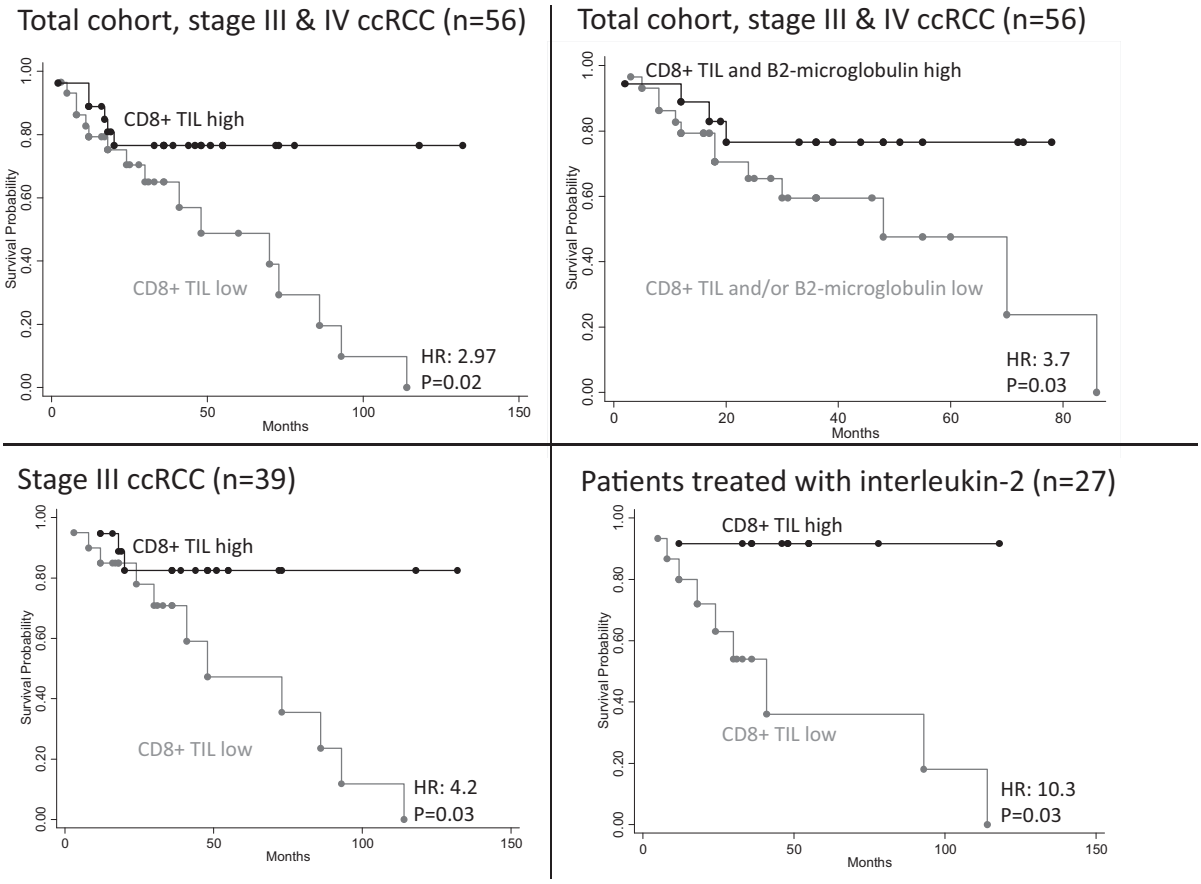

Fig. 3. Top row: In high-stage clear cell RCC, CD8 + tumor infiltrating lymphocytes and high tumor expression of beta-2-microglobulin were associated with longer survival. Bottom row: high CD8 + TILs portended a better prognosis in the subgroups of patients with stage III ccRCC, and those treated with IL-2.

but found this did not improve selection criteria [25]. Toxicity and side effect profiles, combined with inconsistent results and non-standardized treatment [26], make additional criteria for treatment beneficial. Thus the association between abundant CD8 + TILs and IL-2 response observed in our study could be used to help identify patients likely to benefit from this therapy in the future.

Finally, we identified a correlation between tumor beta-2-microglobulin expression and CD8 + TILs. Without B2M, MHC class I antigens are not expressed on the cell's surface, which leads to a lack of trained cytotoxic T-cells [13]. Loss of MHC expression and subsequent loss of antigen presentation has long been studied as a method of tumor escape [27]. Loss of B2M also has been linked to worse prognoses for patients receiving cytokine therapies [15,28,29]; further, RCCs with low B2M expression in the cancer genome atlas (TCGA) have shown modestly lower survival rates [30]. In our study, we demonstrate the association between CD8 + TILs and high tumor B2M expression, which likely increases CD8 + TIL recruitment and activity, and we demonstrate the improved prognosis in clinical stage III and IV ccRCCs with both high B2M expression and CD8 + TILs. Expression of B2M warrants additional investigation as a prognostic marker in other cancers.

Limitations of this study include its retrospective nature, and the findings should be confirmed in larger, prospective studies. Like other retrospective studies, we relied on immunohistochemical markers performed on formalin-fixed paraffin embedded tissue as a surrogate of immune system function. However, not all CD8 + T-cells are cytotoxic, as a variety of cytokines and signaling pathways regulate proliferation, migration, and effector functions of CD8 + T-cells [31]. Although selected areas for immunohistochemical study were chosen based on published methods [5] and after review of all tumor slides, intra-tumoral heterogeneity contributing to outcome differences was not specifically evaluated.

5. Conclusions

In this study of patients with high-stage ccRCC, we identified abundant CD8 + TILs and high tumor cell expression of B2M – a component of the MHC class I complex required for antigen presentation to CD8 + T-cells – as good prognostic indicators associated with longer survival. In addition, we demonstrate that abundant CD8 + TILs may predict response to IL-2 therapy. These novel observations may enhance selection of patients for IL-2 therapy as well as inform prognosis for patients with advanced-stage ccRCC.

Sources of support

University of Washington House Officer Research Grant.

Declaration of competing interest

All authors declare no conflicts of interest and nothing to disclose.

Acknowledgements

Portions of this work were presented in poster format at the US and Canadian Academy of Pathology (USCAP) 2015 and 2019 meetings.

References

[1] Banner BF, Burnham JA, Bahnson RR, Ernstoff MS, Auerbach HE. Immunophenotypic markers in renal cell carcinoma. Mod Pathol 1990;3(2):129–34.

[2] Drake CG, Stein MN. The immunobiology of kidney cancer. J Clin Oncol 2018;36(36):3547–52. JCO2018792648.
[3] Stenzel PJ, Schindeldecker M, Tagscherer KE, Foersch S, Herpel E, Hohenfellner M, et al. Prognostic and predictive value of tumor-infiltrating leukocytes and of immune checkpoint molecules PD1 and PDL1 in clear cell renal cell carcinoma. Transl Oncol 2020;13(2):336–45.
[4] Zhang S, Zhang E, Long J, Hu Z, Peng J, Liu L, et al. Immune infiltration in renal cell carcinoma. Cancer Sci 2019;110(5):1564–72.
[5] Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313(5795):1960–4.
[6] Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012;12(4):298–306.
[7] Fridman WH, Remark R, Goc J, Giraldo NA, Becht E, Hammond SA, et al. The immune microenvironment: a major player in human cancers. Int Arch Allergy Immunol 2014;164(1):13–26.
[8] Kang MJ, Kim KM, Bae JS, Park HS, Lee H, Chung MJ, et al. Tumor-infiltrating PD1-positive lymphocytes and FoxP3-positive regulatory T cells predict distant metastatic relapse and survival of clear cell renal cell carcinoma. Transl Oncol 2013;6(3):282–9.
[9] Ghatalia P, Gordetsky J, Kuo F, Dulaimi E, Cai KQ, Devarajan K, et al. Prognostic impact of immune gene expression signature and tumor infiltrating immune cells in localized clear cell renal cell carcinoma. J Immunother Cancer 2019;7(1):139.
[10] Yao J, Xi W, Zhu Y, Wang H, Hu X, Guo J. Checkpoint molecule PD-1-assisted CD8(+) T lymphocyte count in tumor microenvironment predicts overall survival of patients with metastatic renal cell carcinoma treated with tyrosine kinase inhibitors. Cancer Manag Res 2018;10:3419–31.
[11] Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res 2001;61(13):5132–6.
[12] Kondo T, Nakazawa H, Ito F, Hashimoto Y, Osaka Y, Futatsuyama K, et al. Favorable prognosis of renal cell carcinoma with increased expression of chemokines associated with a Th1-type immune response. Cancer Sci 2006;97(8):780–6.
[13] Abbas ALA, Pillai S. Basic immunology. Elsevier; 2019.
[14] Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. Annu Rev Immunol 2011;29:235–71.
[15] Restifo NP, Marincola FM, Kawakami Y, Taubenberger J, Yannelli JR, Rosenberg SA. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. J Natl Cancer Inst 1996;88(2):100–8.
[16] Rini BI, McDermott DF, Hammers H, Bro W, Bukowski RM, Faba B, et al. Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of renal cell carcinoma. J Immunother Cancer 2016;4:81.
[17] McDermott DF, Regan MM, Clark JI, Flaherty LE, Weiss GR, Logan TF, et al. Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. J Clin Oncol 2005;23(1):133–41.
[18] Andeen N, Giannakopoulos N, Doan K, Tykodi S, Tretiakova M. Laboratory investigation. 95. 75 Varick St, 9th Flr, New York, NY 10013-1917 USA: Nature Publishing Group; Feb 1, 2015. p. 204A.
[19] Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol 2006;90:1–50.
[20] Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, Louie AC. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. J Clin Oncol 1995;13(3):688–96.
[21] Fishman M, Dutcher JP, Clark JI, Alva A, Miletello GP, Curti B, et al. Overall survival by clinical risk category for high dose interleukin-2 (HD IL-2) treated patients with metastatic renal cell cancer (mRCC): data from the PROCLAIM(SM) registry. J Immunother Cancer 2019;7(1):84.
[22] Motzer RJ, Jonasch E, Michaelson MD, Nandagopal L, Gore JL, George S, et al. NCCN guidelines insights: kidney cancer, version 2.2020. J Natl Compr Cancer Netw 2019;17(11):1278–85.
[23] Ljungberg B, Albiges L, Abu-Ghanem Y, Bensalah K, Dabestani S, Fernandez-Pello S, et al. European Association of Urology guidelines on renal cell carcinoma: the 2019 update. Eur Urol 2019;75(5):799–810.
[24] Chatzkel JASJ, Ludlow S, Lombardi K, Croft C, Artigas Y, Rodriguez Y, et al. Overall responses with coordinated pembrolizumab and high dose IL-2 (5-in-a-row schedule) for therapy of metastatic clear cell renal cancer: a single center, single arm trial. Proc Am Soc Clin Oncol 2019;37(7). https://doi.org/10.1200/JCO.2019.37.7_suppl.657. 657–657.
[25] McDermott DF, Cheng SC, Signoretti S, Margolin KA, Clark JI, Sosman JA, et al. The high-dose aldesleukin "select" trial: a trial to prospectively validate predictive models of response to treatment in patients with metastatic renal cell carcinoma. Clin Cancer Res 2015;21(3):561–8.
[26] Unverzagt S, Moldenhauer I, Nothacker M, Rossmeissl D, Hadjinicolaou AV, Peinemann F, et al. Immunotherapy for metastatic renal cell carcinoma. Cochrane Database Syst Rev 2017(5):CD011673.
[27] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331(6024):1565–70.
[28] Speiser DE, Ho PC, Verdeil G. Regulatory circuits of T cell function in cancer. Nat Rev Immunol 2016;16(10):599–611.
[29] Trujillo JA, Sweis RF, Bao R, Luke JJ. T cell-inflamed versus non-T cell-inflamed tumors: a conceptual framework for Cancer immunotherapy drug development and combination therapy selection. Cancer Immunol Res 2018;6(9):990–1000.
[30] Beta 2 microglobulin in renal cancer, Human Protein Atlas. https://www.proteinatlas.org/ENSG00000166710-B2M/pathology/renal+cancer.
[31] Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. Immunity 2011;35(2):161–8.