Elevated eosinophils as predictor of immune-related adverse events after ipilimumab and nivolumab treatment of advanced and metastatic renal cell carcinoma

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Eosinophil could be biomarkers for irAE

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Abstract
Ipilimumab and nivolumab treatment against advanced and metastatic renal cell carcinoma (RCC) cause severe and lethal immune-related adverse events (irAEs). Predicting irAEs might improve clinical outcomes, however no practical biomarkers exist. This study examined whether eosinophils could be effective biomarkers for irAEs in RCC. We retrospectively analyzed 75 patients with RCC treated with ipilimumab and nivolumab between August 2018 and March 2021 in a multicenter study. The median overall and progression-free survival of patients who experienced irAEs (irAE group) were longer than those of the non-irAE group. Grade ≥2 irAEs were associated with poor mPFS. The eosinophil level two weeks after treatment was significantly elevated in the irAEs compared to non-irAE group (mean, 3.0% vs. 5.7%; P < 0.05). The receiver operating characteristic curve revealed the optimal cut-off value for eosinophil levels against ≥ grade 2 irAEs two weeks after treatment was 3.0% (area under the curve=0.699). In multivariate analyses, an eosinophil level ≥3.0% was a risk factor for ≥ grade 2 irAEs (odds ratio 4.18, 95% confidence interval 1.16–15.1). An increased eosinophil level two weeks after treatment might be an effective biomarker for ≥ grade 2 irAEs in patients with RCC treated with ipilimumab and nivolumab.

Keywords
eosinophil, ipilimumab, nivolumab, immune-related adverse event, renal cell carcinoma

Introduction
Advanced and metastatic renal cell carcinoma (RCC) was previously recognized as one of the most aggressive human malignancies, in which the five-year survival rate was around 13% ¹. However, the approval and initiation of immune checkpoint inhibitor (ICI) therapy improved overall survival in this disease ²⁻⁷. The standard therapy for RCC of
intermediate/poor risk according to the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) uses ipilimumab and nivolumab combination therapy as a first-line therapy. A four-year follow-up of the CheckMate 214 trial showed that median overall survival (mOS) was 48.1 months and the overall survival (OS) probability at four years in patients with RCC treated with ipilimumab and nivolumab was 53.4% \(^2\). However, many patients with RCC who were treated with ipilimumab and nivolumab inevitably developed immune-related adverse events (irAEs) with treatment discontinued or death due to the onset of irAE.

Immune-related adverse events occur when the immune system becomes non-specifically activated and can affect most organ systems. The CheckMate 214 trial demonstrated that 46% of patients had suffered from a grade 3–4 irAE \(^8\). In particular, comprehensive studies have shown that ipilimumab and nivolumab therapy was associated with higher mortality rates than ICI monotherapy \(^9\). Therefore, the development of an effective biomarker to predict the onset of irAE is imperative. However, to date, no such practical biomarker has been reported in RCC treated with ipilimumab and nivolumab.

In this study, our aim was to identify peripheral blood biomarkers that were associated with the onset of irAE in patients with RCC that underwent ipilimumab and nivolumab therapy in a retrospective, multicenter study.
Materials and Methods

Patients and treatment

Data were retrospectively obtained from 75 patients who had been diagnosed with RCC and had undergone ≥1 course of treatment with ipilimumab and nivolumab (1 mg/kg ipilimumab and 240 mg/body nivolumab on day 1) between Aug 2018 and April 2021 in a multicenter study. Diagnosis of RCC was determined histologically by experienced pathologists. We evaluated primary and metastasis tumor site such as bone, liver, lung or others (lymph node, brain, adrenal glands, abdominal wall, tooth ridge, iliopsoas muscle and pleura) by using Computed Tomography and Magnetic Resonance Imaging. The classification of responses was determined by experienced physicians based on radiology reports or imaging reviews using the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 such as a complete response (CR), partial response (PR), stable disease, and progressive disease (PD). The characteristics of patients are listed in Table 1.

Immune-related adverse events

An irAE was defined as an adverse effect that promotes immune system activity and requires intensive monitoring or treatment with steroids. We divided patients into two groups: irAE and non-irAE; depending on whether they had experienced an irAE or not, respectively. In addition, we analyzed the association with clinical outcome. Each irAE was divided into six disease groups, such as endocrine, gastrointestinal, skin, pulmonary, and others, and graded according to National Cancer Institute Common Terminology Criteria for Adverse Events, version 5.0. A profile of irAEs is listed in Table 2.

Data collection
In order to identify a predictive biomarker, we examined peripheral blood parameters after blood sampling immediately after the occurrence of an irAE (irAE sample), before one course of treatment with ipilimumab and nivolumab (baseline samples), two weeks after treatment with one course of ipilimumab and nivolumab (two-week samples), and before two course of treatment with ipilimumab and nivolumab (two-course samples), including hemoglobin, white blood cells, neutrophils, eosinophils, lymphocytes, monocytes, platelets, lactate dehydrogenase, calcium, C-reactive protein, and creatinine. This study was undertaken according to the World Medical Association Declaration of Helsinki and approved by an institutional review board (approval number 60-19-0196).

**Statistical analyses**

Data are presented as boxplots. A middle horizontal line was drawn inside each box that signifies the median value. The 25th and 75th percentiles were represented by the bottom and top of each box, respectively. The ends of the whisker plots signify the minimum and maximum of the data for that plot, respectively. *P*-values of statistical significance are indicated as *P* < 0.05; **P** < 0.01. Differences in the quantified data between groups were compared using one-way ANOVA, followed by a Bonferroni post hoc test, or t-test. Fisher’s exact test was used to assess differences in the characteristics of patients. The optimal cut-off points for potential peripheral blood biomarkers to predict irAE onset were determined from the analysis of receiver operating characteristic (ROC) curves. Overall survival and progression-free survival (PFS) were calculated using a Kaplan–Meier method and log-rank test. Univariate and multivariate logistic regression analyses were used to assess risk factors for irAE. All reported *P*-values were two-sided. Statistical analyses were performed using GraphPad Prism 9 software, and EZR (Saitama Medical Center, Jichi
Tasaki Y, *et al.*

Medical University, Saitama, Japan, \(^{11}\).
Results

*Patient characteristics and frequency of onset of irAEs*

Patient characteristics are shown in Table 1. Of the 75 patients, 48 (64%) had experienced an irAE (irAE group) while 27 (36%) had not experienced an irAE (non-irAE group). The age, ratio of genders, distribution of IMDC risk, Karnofsky Performance Status, histological subtype, metastasis site (bone, liver, lung and others), Nephrectomy before ipilimumab and nivolumab, number of courses, and use of steroids before ipilimumab and nivolumab did not significantly differ between the two groups. The irAE group showed a significantly better response to ipilimumab and nivolumab than the non-irAE group ($P < 0.01$).

The irAE profiles are shown in Table 2. A total of 68 irAEs were observed. The most affected systems were endocrine ($n=17; 25.0\%$), followed by gastrointestinal ($n=14; 20.6\%$), skin ($n=11; 16.2\%$), and pulmonary ($n=9; 13.2\%$), as well as others ($n=17; 25.0\%$). Of the irAE group, 58.8% of patients had experienced an irAE of grade $\geq 2$.

*IrAE of grade $\geq 2$ is related to poor clinical outcome*

The mOS and median (m)PFS of the irAE group was significantly longer than that of the non-irAE group (mOS, $P < 0.05$; mPFS, $P < 0.05$, respectively; Fig. 1a and b). The mOS of patients in the irAE group who experienced an irAE of grade $\geq 2$ was no different to that of patients in the irAE group who experienced an irAE of grade $1$ ($P = 0.61$; Fig. 1c). Notably, the mPFS of patients in the irAE group who tolerated an irAE of grade $\geq 2$ was significantly shorter than of patients in the irAE group who tolerated an irAE of grade $1$ ($P < 0.05$; Figure 1d), indicating that irAE onset of grade $\geq 2$ was associated with a poor clinical outcome.
**Blood sampling immediately after occurrence of grade $\geq 2$ irAE**

Differential outcomes in PFS between irAE of grade $\geq 2$ and grade 1, inspired us to examine the effective biomarkers for predicting occurrence of grade $\geq 2$ irAE. To identify effective biomarkers associated with the onset to grade $\geq 2$ irAE, we examined blood samples from patients of the irAE group collected immediately after the occurrence of irAE (irAE samples) with respect to: hemoglobin, white blood cells, neutrophils, lymphocytes, eosinophils, monocytes, platelets, lactate dehydrogenase, calcium, C-reactive protein, and creatinine. The eosinophil count in irAE samples derived from patients who had experienced grade $\geq 2$ irAE, was significantly upregulated compared to that before one course of treatment with ipilimumab and nivolumab (baseline sample; $P < 0.05$, Fig. 2a), whereas other factors were not apparently different between baseline and irAE samples derived from patients who had experienced grade $\geq 2$ irAE (Supplementary Fig. S1). The factors in irAE samples derived from patients who had experienced grade 1 irAE did not change in baseline samples (Supplementary Fig. S2)

**Upregulation of eosinophil count two weeks after treatment predicts occurrence of grade $\geq 2$ irAE**

To elucidate whether the eosinophil level predicts the onset of grade $\geq 2$ irAEs, we examined this at an early phase of treatment with ipilimumab and nivolumab. The eosinophil count in before two course of treatment with ipilimumab and nivolumab (two-course sample), was upregulated in irAE group derived from patients who had experienced grade $\geq 2$ irAEs compared to that in non-irAE group (Supplementary Fig. S3a); however, this was not observed for baseline samples (Fig. 2b). To find out if it is predictable before two course of treatment, we next examined the eosinophil count specifically in two weeks after treatment.
with one course of ipilimumab and nivolumab (two-week sample). Notably, the eosinophil count of the irAE group derived from patients who had experienced grade $\geq 2$ irAE, was found to be significantly higher than that of the non-irAE group and irAE group derived from patients who had experienced grade 1 irAE in two-week sample (Fig. 2c).

**Cut-off value of eosinophil count in two-week samples for predicting occurrence of grade $\geq 2$ irAE**

The optimal cut-off value for the eosinophil count in two-week samples to differentiate the occurrence of grade $\geq 2$ irAE was 3.0% as determined by ROC curve analysis (area under the curve = 0.699, 95% confidence interval = 0.54–0.84, sensitivity = 0.75, specificity = 0.64; Fig. 3a). Univariate and multivariate logistic regression analyses showed that an eosinophil count of $\geq 3.0\%$ in two-week samples was found to be an independent risk factor for the development of grade $\geq 2$ irAEs (Table 3). Consistently, an eosinophil count of $<3.0\%$ in two-week samples correlated with a poor mOS and mPFS (mOS, $P < 0.05$; mPFS, $P < 0.05$, respectively; Fig. 3b and c).
Discussion

In this study, we demonstrated that upregulation of the eosinophil count $\geq 3.0\%$ two weeks after treatment might be a predictive biomarker for the onset of irAEs of grade $\geq 2$ in patients with RCC treated with ipilimumab and nivolumab. Recent comprehensive studies on onset to irAE induced by ipilimumab and nivolumab have shown that the median-time onset of all-grade irAEs ranged from 2.4 to 13.9 weeks $^{12}$, indicating the need to predict irAEs before two course of treatment with ipilimumab and nivolumab. Although several reports have suggested an association of blood cell count parameters with the prediction of irAEs in patients under ICI therapy $^{13}$, no reports exist on effective biomarkers, especially in RCC treated with ipilimumab and nivolumab. Eosinophils in two weeks after treatment might be one of the key determinants of irAEs onset of grade $\geq 2$ in patients with RCC and who underwent ipilimumab and nivolumab therapy, as reported for other, different types of cancers. Our approach using eosinophils as an effective prediction biomarker might help to diagnose and treat irAEs.

Eosinophils have a homeostatic role in the immune response. They combat several parasitic, bacterial, and viral infections by interacting with B cells, T cells, macrophages, and neutrophils. Eosinophils are further involved in the regulation of several diseases, including allergic asthma, esophagitis, myopathies, and autoimmune disorders due to directly or indirectly promoting tissue damage or altering the local immune status $^{14-16}$. A recent study showed that upregulation of the eosinophil count in the peripheral blood is linked with a better prognosis and response to ICI therapy. In patients with recurrent or metastatic head and neck squamous cell carcinoma treated with nivolumab, a higher eosinophil count was associated with better survival $^{17-19}$. The eosinophil count positively correlated with overall survival in patients with melanoma treated with ipilimumab $^{20}$. Indeed, eosinophils induced
by ICI play an important role in the elimination of tumors. Mechanistically, functional experiments showed that anti-cytotoxic T-lymphocyte–associated protein 4 increased eosinophil infiltration into tumors. Activated tumor-infiltrating eosinophils produced chemokines and recruited CD4+ T cells and CD8+ T cells, which resulted in tumor elimination \(^{21-23}\). Furthermore, eosinophils associate with the onset of irAE induced by ICI. A recent case report described how specific tissue-infiltrating eosinophils occurred as an adverse effect, such as in eosinophilic fasciitis and eosinophilic pneumonia triggered by ICI \(^{24,25}\). Therefore, several studies have focused on the eosinophil count as an effective biomarker of the onset of irAEs \(^{26-28}\). Our data showed that the upregulation of eosinophils is associated with the clinical outcome and onset of irAEs, as reported in different types of cancers. These results collectively indicated that the upregulation of eosinophils in peripheral blood reflects the efficacy and toxicity of ICI by tumor and tissue-infiltrating eosinophils.

Previous studies on eosinophils as a biomarker to predict irAEs have focused on levels before treatment or one month after treatment. For example, a high eosinophil count before or one month after nivolumab or pembrolizumab monotherapy increased the risk of occurrence of irAEs by 1.3 times in solid tumors \(^{26}\). Additionally, upregulation of eosinophils before or one month after nivolumab or pembrolizumab monotherapy may be an effective biomarker to predict endocrine irAEs in patients with melanoma \(^{27}\). Interestingly, our data revealed that patients in the irAE group who experienced endocrine, gastrointestinal, and skin disorders had a significantly higher eosinophil count in two-week samples than patients in the non-irAE group \( (P < 0.05 \text{ for all; Supplementary Fig. S3b–d}) \). Patients in the irAE group who experienced pulmonary disease tended to show a higher eosinophil count than those in the non-irAE group \( (P = 0.06; \text{Supplementary Fig. S3e}) \), but not for other disorders.
Furthermore, the eosinophil count in two-week samples of patients in the irAE group was significantly upregulated at the onset of irAE, not only during early courses (one or two courses) but also during late courses (three or four courses) compared to that of patients in the non-irAE group ($P < 0.05$; Supplementary Fig. S3g and h), indicating that the eosinophil level two weeks after treatment was upregulated by the onset of any type of irAE, excluding other diseases, regardless of when the adverse event occurred. These results suggest the eosinophil count is regulated by different ICIs and types of cancers. Our data also suggest the necessity of examining the eosinophil count during one course of ipilimumab and nivolumab to predict the onset of irAEs.

In the past, most studies had shown ICI-mediated irAEs might be associated with an improved response to therapy and survival outcome in several types of cancers. However, especially in RCC, no consensus of evidence exists in the association between the onset of irAEs and clinical outcome. The CheckMate 214 trial showed that OS between patients with and without irAE did not reveal a significant difference in RCC after ipilimumab and nivolumab therapy. In contrast, Ikeda et al. revealed that the onset of irAEs was significantly associated with an improvement of clinical outcome in RCC treated with ipilimumab and nivolumab. Our data revealed the significantly longer mOS of patients showing irAEs, along with the upregulation of eosinophils in peripheral blood, than that in patients without irAEs, as reported in different types of cancers. These controversial results might be reflected in different patient characteristics.

Interestingly, we found that having an irAE of grade $\geq 2$ was associated with a poor clinical outcome, compared with having an irAE of grade 1 (mPFS; 16.7 months and 29.0 months, respectively; Figure 1d). Consistently, in a study of 42-month results of the CheckMate 214 Trial, treatment-free survival of patients who experienced an irAE of grade
≧3 tended to be shorter than that of patients who did not experience an irAE of grade ≧3 (0.6 months and 6.1 months, respectively; 33). Several studies have shown that the discontinuation of treatment by the onset of irAEs was associated with a poor clinical outcome 34,35. Whereas mPFS of grade ≧2 irAE was shorter than that of Grade 1 irAE, mOS was not apparently different between Grade 1 and grade ≧2 irAEs. These results indicated that the follow-up period of OS may not be sufficient. Although further investigation is required, treatment discontinuation due to the onset of grade ≧2 irAEs might be related to a poor clinical outcome. Therefore, we need to predict and suppress the onset of irAEs, particularly irAEs of grade ≧2, to improve clinical outcomes. Our observations indicated that the prediction of grade ≧2 irAEs using the eosinophil level as an effective biomarker could further improve clinical outcome and prognosis due to treatment continuation.

The present study had several limitations. Only a small number of participants were involved in the study. In addition, because of the retrospective nature of the study, we could not control biases in the selection of patients. An interventional prospective study will be required to confirm our data.

In conclusion, our data provides a novel rationale for measuring the blood eosinophil level, with an elevated eosinophil count likely to be an effective biomarker for predicting grade ≧2 irAE onset in patients with RCC under ipilimumab and nivolumab therapy.

References


Statements & Declarations

Authors’ Contributions

Y.T., S.H., T. Yasui, and K.K. designed and directed the project. Y.T. analyzed most of the data with assistance from S.H., N.T., T. Naiki, T.E., K.T., S.I., N.M., Y.S., H.K., Y.N., Y. Hashimoto, T. Sakakura, M.A., Y.I., Y.K., Y.M., S.N., T.T., S.K., Y.M., K.O., A.O., and N.K. who carried out the acquisition of data. Y.S. conducted statistical analyses for the study. S.H. and Y.T. both wrote the manuscript. All authors discussed the results and made comments on the manuscript.

Competing interests

The authors have declared that no conflict of interest exists.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Ethics approval
This study was approved by the ethical review board at Nagoya City University Graduate School of Medical Sciences (approval number: 60-19-0196).

Consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For retrospective study, the need for consent to participate was deemed unnecessary according to national regulations, “Ethical Guidelines for Medical and Health Research Involving Human Subjects.” The protocol summary was described on the hospital website, and the subjects were provided with the opportunity to opt-out.

Consent to publish
All authors consented the last version of this manuscript.

List of abbreviations
CI: confidence interval
CR: complete response
ICI: immune checkpoint inhibitor
IMDC: International Metastatic Renal Cell Carcinoma Database Consortium
irAEs: immune-related adverse events
mOS: median overall survival
mPFS: median progression-free survival
OR: odds ratio
PD: progressive disease
PR: partial response
RCC: renal cell carcinoma
ROC curve: receiver operating characteristic curve
SD: stable disease

Figure Legends

Fig. 1 Occurrence of irAE associated with clinical outcome of treatment with ipilimumab and nivolumab

a–d, Kaplan–Meier survival curves for: (a) overall survival (non-irAE group; n=26, and irAE group; n=48); (b) progression-free survival (non-irAE group; n=20, and irAE group; n=44); (c) overall survival rate (irAE group [Grade 1]; n=13, and irAE group [Grade ≥2]; n=35); and (d) progression-free survival (irAE group [Grade 1]; n=11, and irAE group [Grade ≥2]; n=33) in patients. (a–d) log-rank test. CI, confidence interval; irAE, immune-related adverse events; mOS, median overall survival; mPFS, median progression-free survival; NA, not applicable.

Fig. 2 Upregulation of eosinophil count reflects onset of grade ≥2 irAE

a, Boxplots showing values for eosinophils (baseline samples, n=34, and irAE samples [Grade ≥2], n=24) in the blood samples of patients who had experienced irAE. b, Boxplot showing eosinophil counts in baseline samples; non–irAE (n=26) and irAE ([Grade 1]; n=13, [Grade ≥2]; n=34) groups. c, Boxplot showing eosinophil counts in two-week samples; non-irAE (n=19) and irAE ([Grade 1]; n=6, [Grade ≥2]; n=24) groups. The median value is
represented by the middle horizontal line in each box. The bottom and top of each box indicate, respectively. The 25th and 75th percentiles are represented by the ends of the whiskers that indicate the minimum and maximum of all data, respectively. *P < 0.05. (a) Unpaired t-test. irAE, (b–c) one-way ANOVA with Bonferroni post hoc tests, immune-related adverse events; n.s., not significant.

Fig. 3 Optimal cut-off value of eosinophil count two weeks after treatment with ipilimumab and nivolumab

a, Receiver operating characteristic curve analysis of the eosinophil count for the occurrence of grade ≥2 irAE. b–c, Kaplan–Meier survival curve for (b) overall survival rate (eosinophils <3.0%; n=22, and eosinophils ≥3.0%; n=27) and (c) progression-free survival (eosinophils <3.0%; n=19, and eosinophils ≥3.0%; n=24) in patients. (b–c) log-rank test. CI, confidence interval; irAE, immune-related adverse events; mOS, median overall survival; mPFS, median progression-free survival; NA, not applicable.
Figure 1.

a) Non-irAE group
   mOS; 13.3 months (95%CI; 4.3-NA)
   irAE group
   mOS; 29.3 months (95%CI; NA-NA)

Overall survival  P<0.05

Number at risk
Non-irAE group 26 14 9 3 2 1 0
irAE group 48 36 26 14 8 2 0

b) Non-irAE group
   mPFS; 13.2 months (95%CI; 2.0-NA)
   irAE group
   mPFS; 29.0 months (95%CI; 13.5-NA)

Progression-free survival  P<0.05

Number at risk
Non-irAE group 20 7 7 2 1 1 0
irAE group 44 28 19 10 6 2 0

c) irAE group (Grade 1)
   mOS; Not reached (95%CI; NA-NA)
   irAE group (Grade ≥2)
   mOS; 29.3 months (95%CI; 10.8-NA)

Overall survival  P=0.61

Number at risk
irAE group (Grade 1) 13 11 9 5 3 1 0
irAE group (Grade ≥2) 35 25 17 9 5 1 0

d) irAE group (Grade 1)
   mOS; 29.3 months (95%CI; 10.8-NA)
   irAE group (Grade ≥2)
   mPFS 16.7 months (95%CI; 10.9-NA)

Progression-free survival  P<0.05

Number at risk
irAE group (Grade 1) 11 9 7 5 3 1 0
irAE group (Grade ≥2) 33 19 12 5 3 1 0
Figure 2.

(a) Eosinophils (%) in baseline samples compared to irAE samples (Grade ≥2).

(b) Eosinophils (%) in non-irAE group and irAE group (Grade 1).

(c) Eosinophils (%) in baseline samples and irAE samples (Grade ≥2).

**Non-irAE group**

**irAE group**

**Baseline sample**

**2-weeks sample**

* indicates statistical significance; n.s. indicates no significant difference.
Figure 3.

- **a**
  - Eosinophils (%)
  - Sensitivity vs. Specificity
  - PFS: Probability
  - Number at risk
  - Eosinophils ≥3.0%
  - mPFS: Not reached (95%CI; 10.9-NA)
  - Eosinophils <3.0%
  - mOS; Not reached (95%CI; 13.7-NA)

- **b**
  - Eosinophils ≤3.0%
  - mOS; 5.4 months (95%CI; 2.9-NA)
  - Eosinophils >3.0%
  - mOS; Not reached (95%CI; 13.7-NA)

- **c**
  - Eosinophils ≤3.0%
  - mPFS; 5.25 months (95%CI; 2.0-NA)
  - Eosinophils >3.0%
  - mPFS; Not reached (95%CI; 10.9-NA)

- **Overall survival**
P<0.05

- **Progression-free survival**
P<0.05

**Number at risk**

- Eosinophils ≤3.0%
  - 22 9 6 2 1 1 0
  - 27 22 12 7 4 0 0

- Eosinophils >3.0%
  - 19 7 4 1 1 1 0
  - 24 14 9 5 2 0 0
Table 1. Clinical features of patients

<table>
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irAE: immune-related adverse event
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CI: confidence interval; IMDC: International Metastatic Renal Cell Carcinoma Database Consortium; irAE: immune-related adverse event; OR: odds ratio
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 4supplementaryfigure.pdf