Am J Cancer Res 2025;15(9):3919-3932
www.ajcr.us /ISSN:2156-6976/ajcr0166300

Original Article

Extracellular vesicle lncRNA AGAP2-AS1 as a non-invasive prognostic biomarker in patients with advanced renal cell carcinoma

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Received May 23, 2025; Accepted August 6, 2025; Epub September 15, 2025; Published September 30, 2025

Abstract: Immune checkpoint inhibitor (ICI)-based combination therapies, such as dual ICI therapy or ICI plus vascular endothelial growth factor (VEGF) inhibitors, are recommended as first-line treatment for advanced renal cell carcinoma (aRCC). ICI-based combination therapy has improved the prognosis of patients with aRCC compared with the era of VEGF inhibitor monotherapy. Long noncoding RNAs (lncRNAs) are involved in the prognosis and metastasis of several cancers, and extracellular vesicle (EV)-derived lncRNAs play important roles in tumor progression and metastasis, serving as prognostic biomarkers. The lncRNA AGAP2-AS1 is highly expressed in RCC and is associated with poorer prognosis in patients with elevated expression levels. This study aimed to investigate the function of lncRNA AGAP2-AS1 in RCC and explore whether serum-derived EV lncRNA AGAP2-AS1 serves as a prognostic biomarker in patients with aRCC. Firstly, we examined lncRNA AGAP2-AS1 expression in RCC and normal kidney tissues with pathologically confirmed RCC at our institution. We also performed a functional analysis of lncRNA AGAP2-AS1 in RCC cell lines. Additionally, we analyzed the relationship between the EV lncRNA AGAP2-AS1 expression and the prognosis of 47 patients with aRCC treated with ICI-based combined therapy. We observed higher lncRNA AGAP2-AS1 expression in RCC tissues than in normal tissues. Furthermore, AGAP2-AS1 knockdown in RCC cells using small interfering RNA significantly decreased cell viability, invasion, and migration. Patients with progressive disease (PD) receiving ICI-based combination therapy exhibited significantly higher expression of the EV lncRNA AGAP2-AS1 than patients without PD. We then classified 47 patients into two groups by median lncRNA AGAP2-AS1 expression. Notably, the high-expression group exhibited significantly worse progression-free survival and overall survival than the low-expression group (log-rank $P = 0.0193$ and log-rank $P = 0.0256$, respectively). In multivariate analysis, high EV lncRNA AGAP2-AS1 expression was an independent risk factor for disease progression (hazard ratio = 3.6, $P = 0.0287$). Overall, high EV lncRNA AGAP2-AS1 expression was associated with poorer prognosis in patients with aRCC. Therefore, serum-derived EV lncRNA AGAP2-AS1 may be an effective non-invasive prognostic biomarker in patients with aRCC treated with ICI-based combination therapy.

Keywords: Advance renal cell carcinoma, long non-coding RNA AGAP2-AS1, extracellular vesicles, non-invasive biomarker, ICI-based combination therapy

Introduction

Renal cell carcinoma (RCC) is the 10th most commonly diagnosed cancer and is one of the most common urological cancers of the kidney, accounting for approximately 90% of all renal malignancies [1, 2]. Clear cell RCC and papillary RCC are the most common and second most common subtypes of RCC, representing approximately 80% and 13-20% of RCC cases, respectively [3]. Approximately 25% of patients with RCC present with metastatic disease at initial diagnosis [4]. Although localized RCC can be treated with partial or radical nephrectomy, approximately 40% of patients with localized RCC experience local or distant recurrence following curative surgical resection [5]. For patients with advanced RCC (aRCC) who have metastatic disease or recurrence postoperatively, systemic therapy - such as combination therapy with a vascular endothelial growth factor (VEGF) inhibitor and an immune checkpoint inhibitor (ICI) or dual ICI therapy-is recommended as first-line treatment [6, 7]. ICI and VEGF

https://doi.org/10.62347/YCEB1273

inhibitor combination therapy or dual ICI therapy has been shown to improve the prognosis of patients with advanced RCC compared with VEGF inhibitor monotherapy [8-12].

Long non-coding RNAs (lncRNAs) are non-coding RNAs longer than 200 nucleotides that regulate gene expression at epigenetic, transcriptional, and post-transcriptional levels [13]. lncRNAs also regulate microRNA (miRNA) expression and play important roles in early cancer development and progression, making them promising biomarkers [14]. Furthermore, lncRNAs are important regulators of adaptive and innate immunity and serve as biomarkers for predicting the efficacy of ICI therapy [15-18].

Extracellular vesicles (EVs) are small lipid bilayer-enclosed particles released by all cell types [19, 20]. Previously considered cellular waste products, EVs contain DNA, RNA, and proteins and play an important role in intercellular communication [19-21]. EVs remain stable and resistant to degradation in body fluids because of their lipid bilayer structure, making them attractive candidates for liquid biopsy [22]. EVs are classified into three major categories based on their size and biosynthetic pathway: exosomes, microvesicles, and apoptotic bodies [19, 20, 23]. The term "EVs" includes all subtypes according to the guidelines of the International Society for Extracellular Vesicles [24]. EVs are also involved in organ-specific metastases. Specifically, tumor cell-secreted EVs express distinct integrins that target organ-specific cells and promote metastatic organotropism [25]. Additionally, EV-derived lncRNAs are associated with tumor progression, metastasis, and resistance to anticancer drug therapy in several urological cancers [26-32].

lncRNA AGAP2 antisense RNA 1 (AGAP2-AS1) is a newly identified cancer-associated antisense lncRNA that is aberrantly expressed in several carcinomas and linked to prognosis [33, 34]. It has also been reported as a potential prognostic biomarker in RCC [35, 36]. In particular, lncRNA AGAP2-AS1 competitively binds to miR-9-5p, activates the PI3K/AKT signaling pathway, induces macrophage M2 polarity, and promotes RCC development [37]. However, reports on the EV lncRNA AGAP2-AS1 as a predictive biomarker for treatment efficacy or prognosis in patients with aRCC are lacking. Although there are no established biomarkers for predicting efficacy or prognosis, ICI-based combination therapy improves the prognosis of patients with aRCC. The identification of predictive biomarkers is crucial to further improve the prognosis of patients with aRCC. This study aimed to investigate the function of EV lncRNA AGAP2-AS1 in RCC and explore whether it serves as a useful non-invasive prognostic biomarker in patients with aRCC treated with ICI combination therapy. We also performed a functional analysis of lncRNA AGAP2-AS1 in RCC using RCC cell lines.

Methods

Clinical samples

In total, 54 patients with aRCC who received ICI-based combination therapy at our institution between January 2019 and November 2024 were initially included. Blood samples were collected from these patients, and EVs were extracted from their serum. Seven patients were excluded due to unavailable serum samples. Finally, 47 patients were included and were analyzed retrospectively. This study was approved by the Institutional Ethics Committee of the Graduate School of Medicine, Yamaguchi University, and written informed consent was obtained from all patients enrolled in the study.

RCC and normal kidney cell lines

Primary RCC cell lines (786-O; ATCC number: CRL-1932 and A-498; ATCC number: HTB-44 and ACHN; ATCC number: CRL-1611) were used. 786-O cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). A-498 and ACHN cells were cultured in Eagle's Minimum Essential Medium (Thermo Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich). Human renal proximal tubule cells (RPTEC; catalog #: CC-2553) were used as the normal kidney cell line. RPTEC were cultured in REGM™ Renal Epithelial Cell Growth Medium BulletKit™ (catalog #: CC-3190). All cell lines were maintained in humidified incubators at 5% CO_2 and 37°C. All the cells were passaged upon reaching approximately 80% confluence.

Total RNA extraction from tissues and cell lines

Total RNA was extracted from frozen human RCC tissues and adjacent normal kidney tissues using a PureLink™ RNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions. RNA was extracted from RCC cell lines and RPTEC using the same method with the PureLink™ RNA Mini Kit (Thermo Fisher Scientific).

Cell transfection

Small interfering RNA (siRNA) for the lncRNA negative control or lncRNA APAG2-AS1 (Thermo Fisher Scientific) was transiently transfected into ACHN and A-498 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific) following the manufacturer's instructions. Briefly, these cells were seeded into six-well plates at a density of 0.25-1 × 10^6 cells/well and individually transfected with si-lncRNA AGAP2-AS1 and si-lncRNA negative control at a concentration of 50 pmol/well. The cells were then incubated at 37°C for 48 h until further experiments.

Quantitative real-time RT-PCR

The extracted RNA was reverse-transcribed using a PrimeScript RT Reagent Kit (Takara, Shiga, Japan) following the manufacturer's instructions. Quantitative real-time RT-PCR was performed in triplicate with Applied Biosystems StepOnePlus using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The TaqMan probes and primers were purchased from Thermo Fisher. GAPDH (assay ID: Hs02786624_g1) was used as an endogenous control. lncRNA AGAP2-AS1 expression (assay ID: Hs01096080_s1) was determined using StepOnePlus software (Applied Biosystems). Expression levels were calculated using the $2^{-\Delta\Delta ct}$ method [38].

Cell viability, cell invasion, and wound healing assay

The viability of RCC cells was assessed using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. Approximately 5000 cells were seeded into 96-well plates and maintained for 24 h. Cell viability was measured at 24, 48, and 72 h after cell plating by assessing optical density (OD) at 490 nm. Cell invasion assays were performed using a CytoSelect 24-well cell invasion assay kit (Cell BioLab, San Diego, CA, USA). Transfected cells were placed in the upper chamber with FBS-free medium in triplicate. After 48 h of incubation at 37°C and 5% CO_2, cells migrated through the membrane were stained, extracted, and quantified by measuring OD at 560 nm following the manufacturer's instructions. A wound healing assay was performed to evaluate the migratory ability of RCC cells. Transfected RCC cells were seeded into six-well plates. Once cells reached approximately 80% confluence, a wound was created by scratching with a 200-μL pipette tip. The cells were then washed twice with the medium to remove debris and floating cells, observed at 0 and 24 h, and photographed using a BZ-X800 fluorescence microscope (KEYENCE, Osaka, Japan). All experiments were performed in triplicate. Data are presented as the mean ± standard deviation from three independent experiments.

Isolation of EVs from serum

EVs were extracted from the serum of patients with aRCC receiving ICI-based combination therapy using a Total Exosome Isolation Kit (Thermo Fisher Scientific), as previously reported [39]. Briefly, the serum was centrifuged at 2000 × *g* for 30 min to remove cellular components and debris. The supernatant containing the clear serum was transferred to new centrifuge tubes and mixed with the total exosome isolation reagent. After incubation at 4°C for 30 min, the sample was then centrifuged at 10,000 × *g* for 10 min, and EVs were collected from the resulting pellet.

EV lncRNA isolation

EV lncRNAs were isolated from the extracted EVs using a Total Exosome RNA and Protein Isolation Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Digital PCR

EV lncRNA expression was measured using digital PCR (QuantStudio™ 3D Digital PCR System; Thermo Fisher Scientific). EV-derived lncRNAs were reverse-transcribed using the PrimeScript RT Reagent Kit (Takara) with digital PCR master mix (Thermo Fisher Scientific) following the manufacturer's instructions. GAPDH (assay ID: Hs02786624_g1) was used as the endogenous control. The expression of EV lncRNA AGAP2-AS1 (assay ID: Hs01096080_

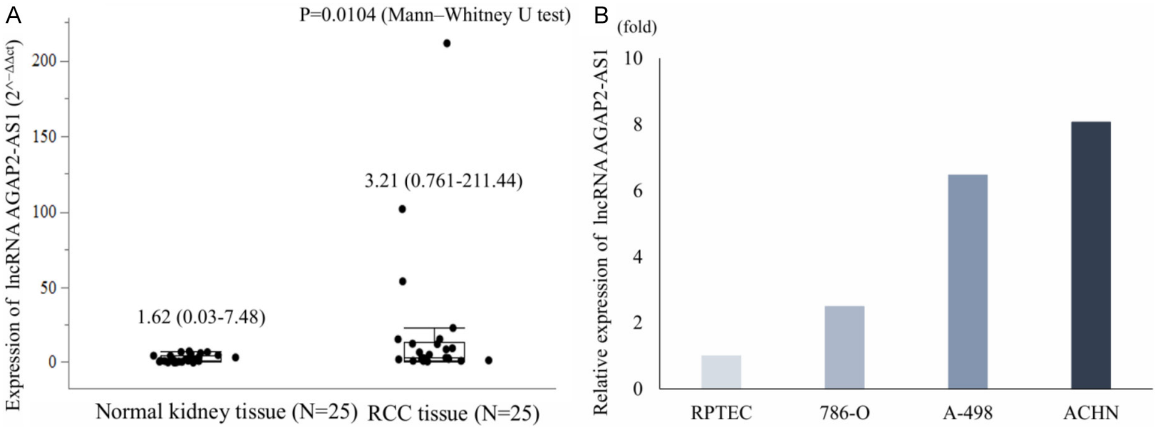

Figure 1. lncRNA AGAP2-AS1 expression in RCC tissues, matched kidney tissues, and RCC cell lines. A. lncRNA AGAP2-AS1 expression in RCC tissues (n = 25) and matched normal kidney tissues (n = 25). lncRNA AGAP2-AS1 expression was significantly elevated in RCC tissues compared to that in normal matched kidney tissues (P = 0.0261). B. lncRNA AGAP2-AS1 expression in RCC cell lines and human renal proximal tubule cells was determined using quantitative real-time RT-PCR. ACHN and A-498 cells exhibited higher lncRNA AGAP2-AS1 expression than RPTECs and 786-O cells. Subsequent functional analysis using ACHN and A-498 cells revealed high lncRNA AGAP2-AS1 expression.

s1) in absolute numbers (copies/μL) was determined using QuantStudio™ 3D AnalysisSuite™ (Thermo Fisher Scientific).

Statistical analysis

Categorical variables were compared using the chi-square test, and continuous variables were compared using the Mann-Whitney U test. Progression-free survival (PFS) was defined as the time from the initiation of ICI-based combination therapy to disease progression (PD) or death. Overall survival (OS) was defined as the time from the initiation of ICI-based combination therapy to death from any cause. Survival analysis was performed using the Kaplan-Meier method and compared using the log-rank test. For multivariate analysis, the Cox proportional hazards model was used to identify the risk factors for PD and OS. All data are presented as the mean ± standard deviation from three independent experiments. Statistical analyses were performed using the JMP software (Pro.18; SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$.

Results

lncRNA AGAP2-AS1 expression in RCC tissues and cell lines

We analyzed lncRNA AGAP2-AS1 expression in RCC tissues and adjacent normal kidney tissues from 25 patients who underwent radical or partial nephrectomy at our institution. Quantitative real-time RT-PCR revealed significantly upregulated lncRNA AGAP2-AS1 expression in RCC tissues (n = 25) compared to the adjacent normal kidney tissues (n = 25) (**Figure 1A**). We also examined lncRNA AGAP2-AS1 expression in RCC cell lines (786-O, ACHN, and A-498) and RPTECs. Notably, lncRNA AGAP2-AS1 expression was upregulated in RCC cell lines compared to that in RPTECs (**Figure 1B**). ACHN and A-498, which had relatively high LncRNA AGAP2-AS1 expression, were chosen for subsequent functional analysis.

We confirmed AGAP2-AS1 expression in the GDC TCGA database using UCSC Xena (https://xenabrowser.net/). lncRNA AGAP2-AS1 expression was significantly higher in RCC tissues than in normal kidney tissues (Supplementary Figure 1A). Furthermore, patients in the high-expression group had a significantly shorter OS than those in the low-expression group (Supplementary Figure 1B).

Function of lncRNA AGAP2-AS1 in RCC cell lines (ACHN, and A-498)

LncRNA AGAP2-AS1 expression in ACHN and A-498 cells was downregulated following siRNA-mediated knockdown of lncRNA AGAP2-AS1 (**Figure 2A**). Furthermore, cell viability and invasiveness significantly decreased in A-498 and

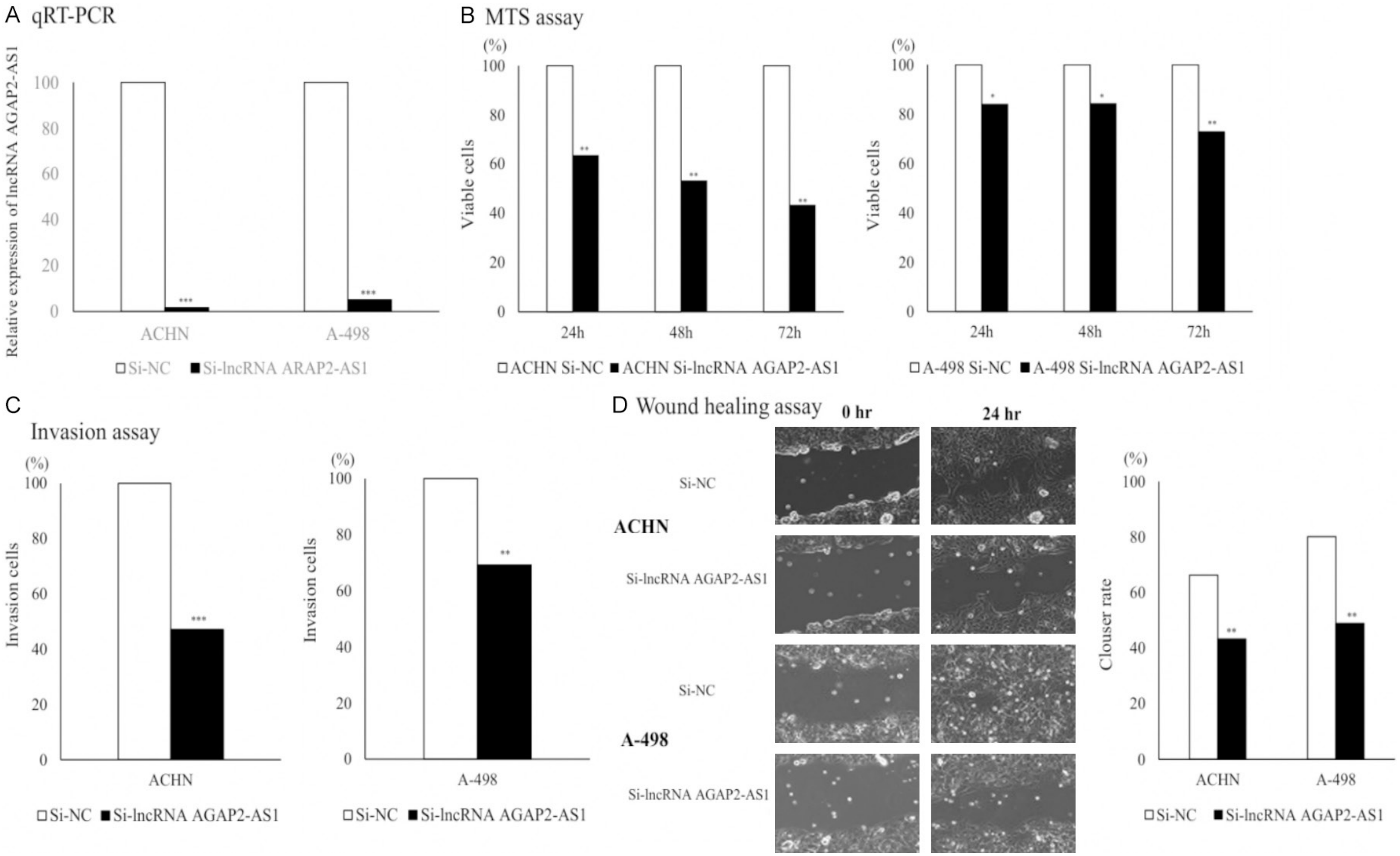

Figure 2. Function of lncRNA AGAP2-AS1 in RCC cell lines (ACHN and A-498). A. qRT-PCR revealed significantly decreased lncRNA AGAP2-AS1 expression in ACHN and A-498 cells transfected with siRNA lncRNA AGAP2-AS1 than in those transfected with siRNA-NC. B. MTS assay revealed significantly decreased cell viability at 24, 48, and 72 h in cells transfected with the siRNA lncRNA AGAP2-AS1 than in control cells. C. Invasion assay revealed significantly decreased invasiveness of ACHN and A-498 cells transfected with siRNA AGAP2-AS1 compared to that of control cells. D. Wound-healing assay revealed significantly reduced migration ability of ACHN and A-498 cells transfected with siRNA AGAP2-AS1 compared to that of control cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 1. Characteristics of 47 patients with aRCC at baseline

Age (years, IQR)	69 (61-72)
Gender	
Male	32 (68.1%)
Female	15 (31.9%)
ECOG PS	
0-1	45 (95.7%)
≥ 2	2 (4.3%)
Subtypes	
Clear	32 (68.1%)
Non-clear	7 (14.9%)
Unknown	8 (17.0%)
Metastasis sites	
Lung	21 (44.7%)
Bone	8 (17.0%)
Lymph	13 (27.7%)
Pancrease	4 (8.5%)
Adrenal glands	4 (8.5%)
Others	11 (23.4%)
IMDC risk score	
Favorable	7 (14.9%)
Intermediate	28 (59.6%)
Poor	6 (12.8%)
Unresectable	6 (12.8%)

IQR; interquartile range, ECOG PS; eastern cooperative oncology group performance status, IMDC; international metastatic RCC database consortium.

ACHN cells transfected with siRNA lncRNA AGAP2-AS1 compared to those in siRNA negative control cells (**Figure 2B**, **2C**). Additionally, cell migration ability was significantly decreased in cells transfected with siRNA lncRNA AGAP2-AS1 compared to that in the control cells (**Figure 2D**).

Correlation between lncRNA AGAP2-AS1 expression and prognosis in patients with aRCC

We investigated the correlation between the EV lncRNA AGAP2-AS1 expression and the prognosis in 47 patients with aRCC. **Table 1** presents the patient characteristics. Of the 47 patients with aRCC, 41 had metastatic disease and 6 patients had unresectable RCC. Twelve patients received dual ICI therapy (25.5%), while 35 patients received ICI plus VEFGR inhibitors therapy (74.5%). Supplementary Figure 2 shows the percentage of patients receiving ICI-based combination therapy. The median lncRNA AGAP2-AS1 expression was 1.90 copies/μL (range: 0.15-39.66). No significant difference was observed in lncRNA AGAP2-AS1 expression between patients with complete response (CR) or partial response (PR) and those with stable disease (SD) or PD (**Figure 3A**), whereas its expression was significantly higher in patients with PD than in those without PD (**Figure 3B**). We then classified 47 patients into two groups by the median AGAP2-AS1 expression. Patient backgrounds for these groups, divided by the median EV lncRNA AGAP2-AS1 expression, are shown in Supplementary Table 1. No significant differences were observed in baseline patient characteristics between the two groups.

PFS and OS were significantly shorter in the high-expression group than in the low-expression group (log-rank P = 0.0193 and 0.0256, respectively; **Figure 4A**, **4B**). We then performed univariate and Cox proportional analyses to evaluate the risk factors associated with PD and OS in the 47 patients with aRCC (**Table 2**). High lncRNA AGAP2-AS1 expression was an independent risk factor for PD in multivariate analysis (hazard ratio [HR] = 3.67, P = 0.0287). No other significant factors related to PD, such as therapy or metastasis sites, were found. In contrast, AGAP2-AS1 expression was not significantly associated with OS in multivariate analysis. The correlation between lncRNA AGAP2-AS1 expression and neutrophil-lymphocyte ratio (NLR), assessed using Spearman's rank correlation coefficient, revealed a positive relationship (rs = 0.3309, P = 0.0264; **Figure 5**). Patients with higher expression levels of lncRNA AGAP2-AS1 also tended to have a higher NLR.

Discussion

In this study, we investigated whether the EV lncRNA AGAP2-AS1 could serve as a useful non-invasive prognostic biomarker in patients with aRCC treated with ICI-based combination therapy. Patients with high serum-derived EV lncRNA AGAP2-AS1 expression exhibited worse PFS and OS than those with low expression. Moreover, high expression was an independent risk factor for PD. In RCC, lncRNA AGAP2-AS1 expression has also been previously associated with prognosis [35, 36]. Xu et al. reported the oncogenic function of lncRNA AGAP2-AS1 in RCC and its association with RCC develop-

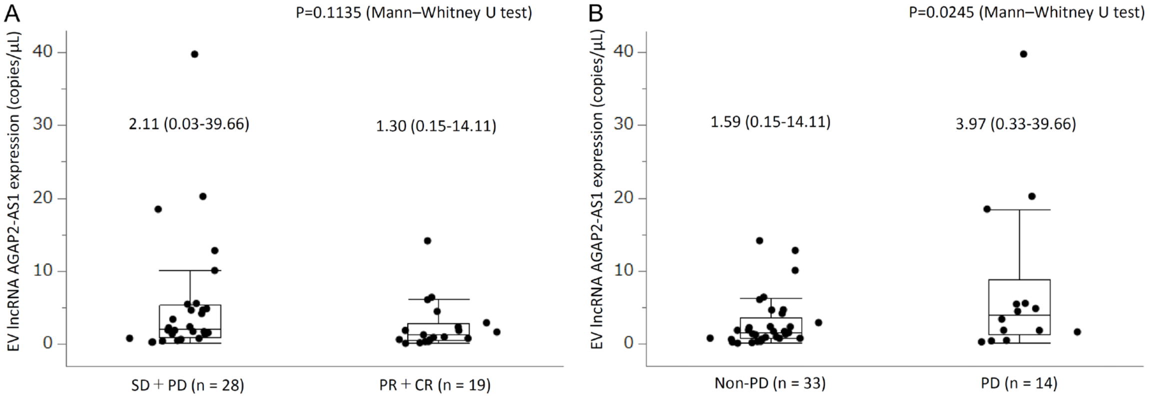

Figure 3. Correlation between EV lncRNA AGAP2-AS1 expression and the efficacy of ICI-based combination therapy. No significant difference was observed in the EV lncRNA AGAP2-AS1 expression between patients who showed PR or CR with ICI-based combination therapy and those who did not respond to ICI-based combination therapy (SD or PD). However, EV lncRNA AGAP2-AS1 expression was significantly higher in patients who eventually progressed to PD after ICI-based combination therapy than in those who did not (P = 0.0219).

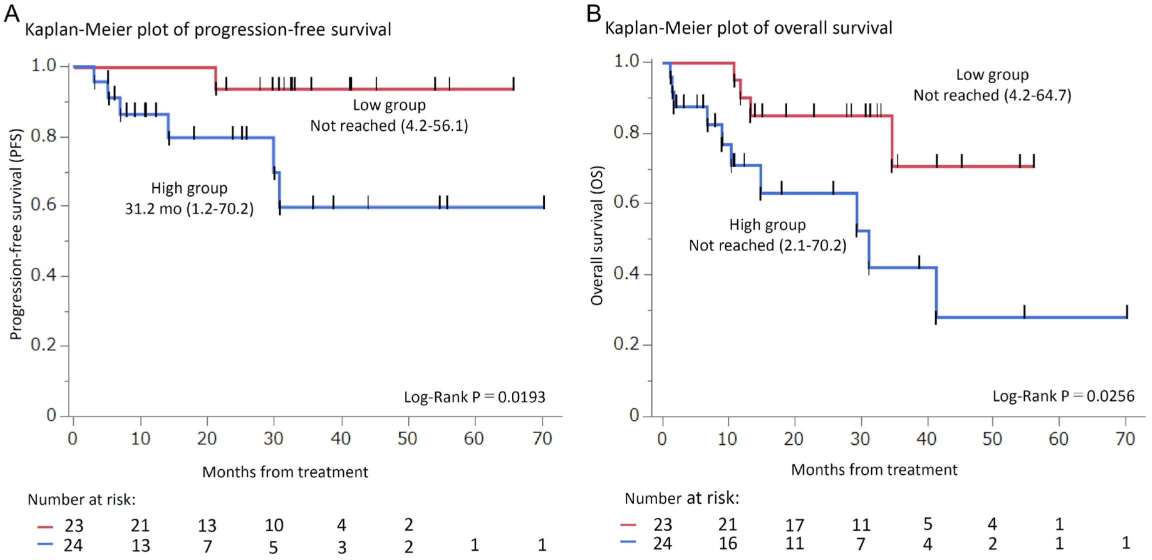

Figure 4. Correlation between EV lncRNA AGAP2-AS1 expression and prognosis. Forty-seven patients were classified into two groups by the median lncRNA AGAP2-AS1 expression. A. Kaplan-Meier survival plot for progression-free survival (PFS). The high-expression group had a significantly shorter PFS than the low-expression group (log-rank P = 0.0193). B. Kaplan-Meier survival plot for overall survival (OS). The high-expression group had a significantly shorter OS than the low-expression group (log-rank P = 0.0256).

ment through the PI3K/AKT signaling pathway [37]. Using RCC cell lines, we confirmed that lncRNA AGAP2-AS1 promotes malignant features, supporting its oncogenic function.

Several studies have reported EV miRNAs and lncRNAs as biomarkers associated with resistance to systemic therapy in aRCC [29-31, 40-43]. Soleimani et al. reported that EV-derived miR-155-3p is associated with resistance to first-line ICI-based combination therapy [43]. They revealed higher miR-155-5P levels in patients with aRCC (n = 40) than in healthy volunteers (n = 30) and significantly lower EV-derived miR-155-5P levels in patients who responded to ICI-based combination therapy (n = 27) than in those who did not respond to treatment (n = 13). In our study, patients with PD receiving ICI-based combination therapy exhibited significantly higher lncRNA AGAP2-AS1 expression than patients without PD. Although EV lncRNAs have been reported to be

Table 2. Univariate and cox hazard proportional hazards model analysis of factors related progression and OS

Variables	Progression				Overall survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95%CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age								
≤ 69/> 69	1.45 (0.48-4.36)	0.5108			2.18 (0.42-11.25)	0.3532		
Gender								
Male/Female	1.28 (0.35-4.69)	0.7134			2.51 (0.30-21.11)	0.3972		
ECOG PS								
< 2/≥ 2	0.61 (0.08-4.78)	0.6410			0.82 (0.53-1.27)	0.1750		
Subtype								
Clear/Non-clear	0.61 (0.16-2.25)	0.4544			0.75 (0.09-6.22)	0.7887		
T stage								
≤ 2/> 3	0.74 (0.24-2.29)	0.6055			0.78 (0.25-2.42)	0.6634		
Metastasis sites								
Lung	1.49 (0.50-4.44)	0.4767			2.78 (0.54-14.40)	0.2236		
Bone	2.77 (0.57-13.56)	0.2081			1.46 (0.17-12.08)	0.6933		
Lymph	1.27 (0.34-4.72)	0.7168			8.77 (1.69-45.24)	0.0097	8.42 (1.62-43.90)	0.0114
CRP								
< ULN/≥ ULN	1.20 (0.39-3.72)	0.7439			0.61 (0.13-2.78)	0.5257		
Anemia								
Present/Absent	2.36 (0.70-7.91)	0.1609			1.47 (0.32-6.74)	0.6180		
PLT counts								
< ULN/≥ ULN	0.68 (0.25-1.83)	0.3353			0.58 (0.07-4.86)	0.6171		
Neutrophils counts								
< ULN/≥ ULN	0.86 (0.66-1.12)	0.2002			1.09 (0.99-1.20)	0.3674		
Therapy								
ICI-ICI/ICI-TKI	0.50 (0.16-1.51)	0.2173			0.72 (0.35-1.49)	0.2465		
LncRNA AGAP2-AS1								
High/Low	3.67 (1.14-11.78)	0.0287	3.67 (1.14-11.78)	0.0287	0.51 (0.32-0.81)	0.0212	0.13 (0.02-0.62)	0.0648

HR; hazard ratio, CI; confidence interval, ULN; upper limit of normal, TKI; tyrosine kinase inhibitor.

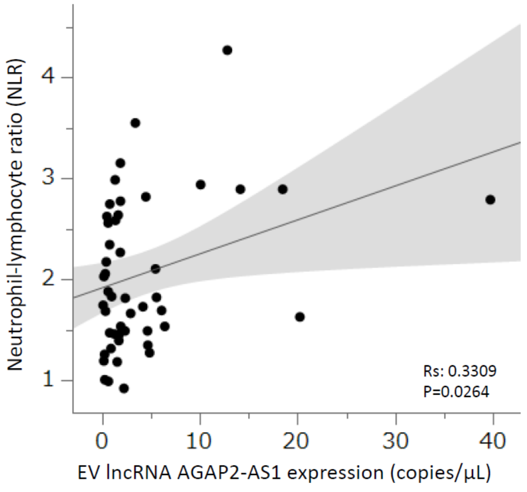

Figure 5. Correlation between EV lncRNA AGAP2-AS1 expression and neutrophil-lymphocyte ratio (NLR). Spearman's rank test demonstrated a positive correlation between AGAP2-AS1 expression and NLR (rs = 0.3309, P = 0.0264). Patients with higher lncRNA AGAP2-AS1 expression also tended to have higher NLR.

associated with resistance to systemic VEGF inhibitor monotherapy [30, 31], reports of resistance to ICI-based systemic therapy remain lacking [44]. To the best of our knowledge, this is the first report describing the association between EV lncRNAs and resistance to ICI-based systemic therapy.

In recent years, EV-based liquid biopsy has emerged as a potential diagnostic and prognostic marker for RCC [44, 45]. EV-based liquid biopsies mainly target miRNAs, lncRNAs and proteins. Several reports have demonstrated the high sensitivity and specificity of EV-based liquid biopsies for diagnosis and prognosis [44, 45]. However, to further improve the accuracy of EV-based liquid biopsies, it will be necessary to evaluate combinations of miRNAs and lncRNAs with rather than miRNAs and lncRNAs alone. Cuproptosis is a form of cell death induced by free intracellular copper. It has been reported that RCC is susceptible to cuproptosis in various cancers [46]. Liu H. reported that the cuproptosis gene signature was superior to conventional ICI therapy biomarkers such as microsatellite instability (MSI) score and tumor mutation burden (TMB), in predicting the prognosis in patients with RCC treated with ICI therapy [47]. Combining the cuproptosis-related genes with lncRNA AGAP2-AS1 may improve the accuracy of prognostic biomarkers for patients with aRCC treated with ICI therapy. In the future, combining various genes will be necessary to improve the accuracy and specificity of EV-based liquid biopsies for clinical application.

In this study, we focused on lncRNA AGAP2-AS1. LncRNAs act as regulators of gene expression and are involve in diverse physiological and pathological processes [13, 48]. In cancers, they can act as oncogenes or tumor suppressors. Oncogenic lncRNAs regulate cancer cell proliferation, invasion, and metastasis, as well as metabolic pathways, promoting energy metabolism and cancer progression through post-translational modifications of metabolism-related proteins [49]. LncRNAs play a central role in regulating immune responses within the tumor microenvironment (TME) by establishing an immunosuppressive environment that influences immune responses and contributes to tumorigenic growth [50]. In particular, they have been reported to play a pivotal role in the polarization of macrophages, the infiltration of neutrophils, T cell interactions and B cell function within the TME [51-54]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been reported to regulate the expression of genes associated with chemokine production and the mobilisation of neutrophils and T cells within the TME. Wei et al. reported that knockdown of MALAT1 in lung cancer promoted immune cell infiltration and increased tumor cell recognition by immune cells, improving the response to ICI therapy [55]. Previous reports have suggested that lncRNAs with oncogenic functions can regulate immune cells and immune responses in the TME. This may promote cancer growth and progression, and possibly contribute to resistance to ICI therapy [51-55]. In addition, tumor-derived EVs have also been found to play an important role in the TME remodelling, and are considered to influence the regulation of macrophage polarization [56]. Previous reports have shown that tumor-derived EV-lncRNA polarises the M2-like phenotype macrophages [57, 58]. M2-like phenotype macrophages are tumor-associated macrophages (TAMs), and TAMs derived EVs have been reported to be involved in tumor growth, chemoresistance, and immune escape [59-62]. Although this study could not investigate the detailed mechanism by which lncRNA AGAP2-AS1 confers resistance to ICI-based combination therapy, it is possible that lncRNA AGAP2-AS1 and EVs may act in combination to affect the TME, and confer resistance to ICI-based combination therapy. In the field of chimeric antigen receptor (CAR) T cell therapy, the next generation of immunotherapy, various studies are underway to address metabolic changes in the TME with cancer [63]. Real-time profiling of the TME and associated biomarkers is being considered as a potential way to monitor anti-tumor activity in real time. Choi et al using longitudinal liquid biopsies to examine EV RNA in a CAR-T cell trial against glioblastoma, reported that they were able to monitor the copy number of the target antigen in both blood and cerebrospinal fluid during treatment [64]. Such innovations are expected to contribute to elucidating the complex metabolic mechanisms of the TME and the mechanisms of resistance to ICI therapy, thereby improving efficacy.

A positive correlation was observed between the expression of EV lncRNA AGAP2-AS1 and NLR. A high NLR is a poor prognostic factor in various carcinomas and is driven by neutrophils producing inflammatory cytokines that promote tumorigenesis, including angiogenesis, within the TME [65, 66]. Inflammatory cytokines also promote the induction of immunosuppressive cells, such as regulatory T cells [67]. Furthermore, several reports have shown that NLR could be a biomarker for predicting the efficacy and prognosis of patients treated with ICI therapy in several cancers [68-70]. Previous research has reported a correlation between the percentage of neutrophil and lymphocyte infiltration in the TME and their levels in peripheral blood, suggesting that peripheral blood NLR may reflect immune cell infiltration in the TME [71]. Patients with high peripheral blood NLR levels are considered to have “cold tumors” with less immune cell infiltration in TME and are less likely to respond to ICI therapy [72]. Therefore, patients with high EV lncRNA AGAP2-AS1 expression may have tumor characteristics associated with resistance to ICI therapy. Blood-based biomarkers associated with ICI therapy resistance have also been reported, including LDH, white blood cell count and eosinophil count, and in malignant melanoma, soluble immune checkpoint molecules, cytokines and chemokines have also been reported to be associated with ICI therapy resistance [68]. In the future, a combination of various biomarkers may be necessary to more accurately predict the therapeutic efficacy and prognosis of ICI therapy. Identifying the most effective predictive markers will enable personalised cancer care and achieve the best possible outcomes for patients.

Currently, EVs are not only targets for liquid biopsy but also represent a promising therapeutic strategy. EVs play an important role in cell-cell communication, and tumor cell-derived EVs have been reported to transport oncogenic non-coding RNAs to receptor cells and are involved in tumor progression and metastasis [19-21]. Inhibiting the secretion of tumor-derived EVs is currently being considered a potential therapeutic strategy. Inhibiting tumor-derived EV secretion and uptake by receptor cells may lead to new EV-targeted therapies. Urabe et al. comprehensively analyzed the mechanism of EV secretion in prostate cancer cells and the potential of new therapeutic strategies to inhibit EV secretion. They identified miR-26a as a regulator of EV secretion in prostate cancer cells and reported that its target genes - SHC4, PFDN4, and CHORDC1 - inhibit EV secretion from prostate cancer cells and suppress progression [73]. Nishida et al. reported a novel therapeutic strategy using antibodies to eliminate EVs and inhibit their uptake by recipient cells. Specifically, in a mouse model of breast cancer, depletion of EVs using human-specific anti-CD9 or anti-CD63 antibodies significantly reduced lung and lymph node metastases [74]. New EV-mediated therapies, if able to inhibit the EV lncRNA AGAP2-AS1, could potentially improve the prognosis of patients with aRCC treated with ICI combination therapy.

The limitations of this study include its retrospective design and the small sample size of the single-center study. Furthermore, although EVs were targeted, the lack of standardisation in EV isolation protocols may affect the accuracy and reproducibility of the isolation process. Technical bias, and biological biases, such as cancer heterogeneity and sample purity, have been reported to affect transcriptomic data, including microarrays and RNA sequencing [75]. It is possible that these technical and biological biases may also affect EV isolation. A standardized EVs isolation protocol is needed to minimise these biases. The ability to stably extract EVs with high purity and efficiency using such a protocol would facilitate the clinical application of EV-based biomarkers. This study did not examine the role of lncRNA AGAP2-AS1 in the immune response and its effect on the TME. Further studies are needed to investigate the role of lncRNA AGAP2-AS1 in the immune response and the mechanism by which it affects the TME.

We investigated whether the EV lncRNA AGAP2-AS1 could be used as a prognostic biomarker for patients with aRCC treated with ICI-based combination therapy. Patients with high lncRNA AGAP2-AS1 expression were more likely to have PD and had significantly worse clinical outcomes with ICI combination therapy. Additionally, EV lncRNA AGAP2-AS1 expression was positively correlated with NLR, suggesting that patients may exhibit tumor characteristics associated with resistance to ICI combination therapy.

In conclusion, EV lncRNA AGAP2-AS1 may serve as a non-invasive and useful prognostic biomarker in patients with aRCC receiving ICI-based combination therapy. EV lncRNA AGAP2-AS1 could be also a potential new therapeutic target in patients with aRCC.

Acknowledgements

We would like to thank Editage (www.editage.jp) for English language editing.

Disclosure of conflict of interest

None.

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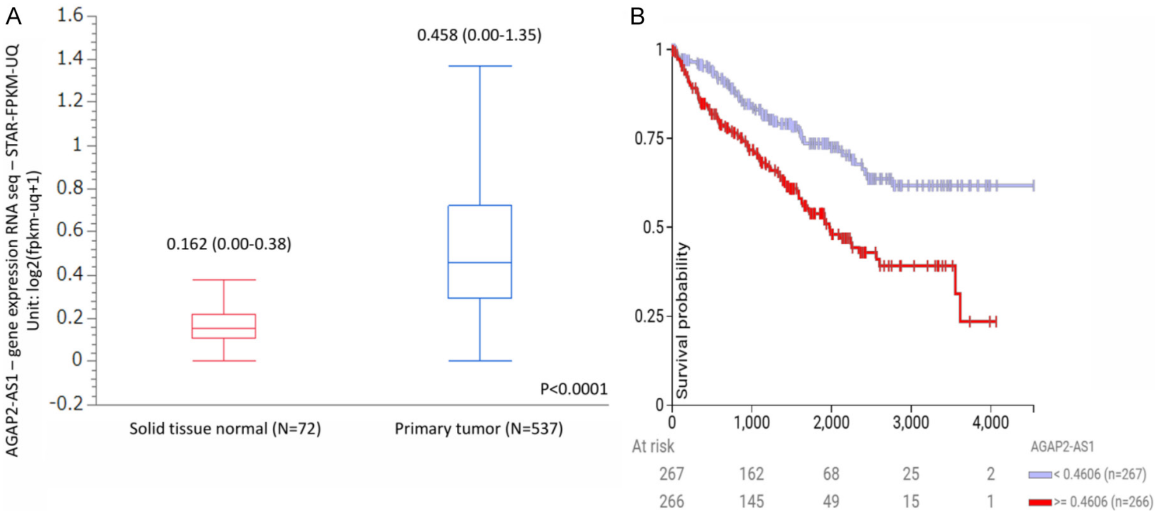
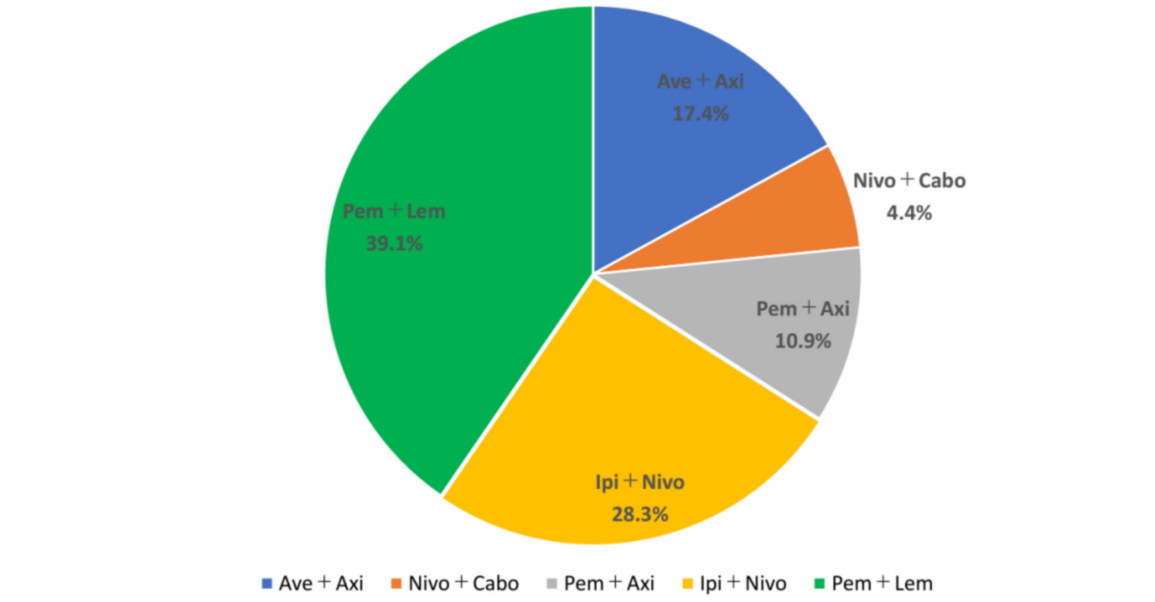


Supplementary Figure 1. Expression of lncRNA AGAP2-AS1 and its association with prognosis in the GDC TCGA database. Violin plots in the GDC TCGA database showed that lncRNA AGAP2-AS1expression was significantly higher in RCC tissue (n = 537) than in normal kidney tissue (n = 72). The Kaplan-Meier survival plot showed that the AGA2-AS1 high expression group (n = 266) had significantly shorter overall survival (OS) than the low expression group (n = 267) (log-rank P < 0.0001).



* Nivo; Nivolumab, Ipi; Ipilimumab, Pem; Pembrolizumab, Axi; Axitinib, Ave; Avelumab, CAabo; Cabozantinib, Lem; Lenvatinib

Supplementary Figure 2. Percentage of patients receiving ICI-based combination therapy. The largest group of patients received treatment with pembrolizumab plus lenvatinib, with 19 patients in this group. This was followed by ipilimumab plus nivolumab, which was given to 12 patients. Eight patients received avelumab plus axitinib, five received pembrolizumab plus axitinib, and three received nivolumab plus cabozantinib.

Supplementary Table 1. Patient background based on serum-derived EV lncRNA AGAP2-AS1 expression levels

	Low group (n = 23)	High groups (n = 24)	*P*-value
Age (years, IQR)	68 (58-75)	69 (62-71)	0.5367
Gender			
Male	15 (65.2%)	17 (68.1%)	0.6797
Female	8 (34.8%)	7 (70.8%)	
ECOG PS			
0-1	22 (95.7%)	23 (95.8%)	0.9755
≥ 2	1 (4.3%)	1 (4.2%)	
Subtypes			
Clear	17 (73.9%)	15 (62.5%)	0.6864
Non-clear	3 (13.0%)	4 (16.7%)	
Unknown	3 (13.0%)	5 (20.8%)	
Metastasis sites			
Lung	8 (34.8%)	13 (54.2%)	0.1799
Bone	4 (17.4%)	4 (17.4%)	0.9473
Lymph	5 (21.7%)	8 (33.3%)	0.3726
Pancrease	2 (9.1%)	2 (8.33%)	0.9275
Adrenal glands	2 (8.7%)	2 (8.33%)	0.9645
Others	5 (21.7%)	6 (25.0%)	0.7032
IMDC risk score			
Favorable	4 (17.4%)	3 (12.5%)	0.3520
Intermediate	15 (65.2%)	13 (54.2%)	
Poor	3 (13.0%)	3 (12.5%)	
Unresectable	1 (4.4%)	5 (20.8%)	
Therapy			
ICI-ICI	4 (17.4%)	8 (33.3%)	0.3726
ICI-TKI	19 (82.6%)	16 (66.7%)	