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Original Article

Hypermethylated SHOX2 in circulating cell-free DNA post renal cell carcinoma surgery as TNM-independent biomarker for recurrence risk

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Abstract: Introduction: Adjuvant immune checkpoint inhibitor trials in renal cell carcinoma (RCC) call for improved recurrence risk stratification. Due to limitations of circulating tumor DNA (ctDNA) use in RCC, the use of hypermethylated SHOX2 gene (mSHOX2) in circulating cell-free DNA is explored as a surrogate marker for identifying high-risk patients after RCC surgery. Methods: Liquid biopsies were collected post-surgery from 45 RCC patients (mean duration 4.3 days). Real-time polymerase chain reaction was used to analyze SHOX2 methylation in circulating cell-free DNA. Patients were categorized as mSHOX2 positive or negative by cut-off. Metastasis-free survival (MFS), cancer-specific survival (CSS), and overall survival (OS) were assessed using Cox regression and Log-rank analyses (median follow-up time: 60 months). Results: 17 patients were mSHOX2 positive, showing unfavorable OS/CSS (Log-rank P = 0.004 and 0.02) and nearly 6-fold higher recurrence risk (hazard ratio 5.89, 95% CI 1.46-23.8). Multivariable Cox analysis confirmed mSHOX2 as an independent recurrence risk factor, disregarding TNM-based stratification. Conclusions: mSHOX2 effectively identifies high-risk RCC patients post-surgery, indicating minimal residual disease. This easy to implement biomarker has potential for guiding of adjuvant therapy decisions.

Keywords: Renal cell carcinoma, recurrence, biomarker, SHOX2, adjuvant therapy

Introduction

Adjuvant immunotherapy has entered genitourinary oncology. The KEYNOTE-564 trial demonstrated a disease-free survival benefit for adjuvant therapy with the immune checkpoint inhibitor (ICI) pembrolizumab in patients with renal cell carcinoma (RCC) at high risk of recurrence [1].

While some patients treated with adjuvant ICI appear to have a survival benefit, for a yet uncharacterized proportion of patients this therapy regimen represents an overtreatment with potentially severe side effects [1-3]. This issue was emphasized in the 2022 updated European Association of Urology (EAU) guidelines [4]. The reasons may include insufficient patient selection through histopathological TNM staging alone [3]. This implies new challenges, but also, due to new molecular analysis technologies, opportunities to identify subgroups of patients who will particularly benefit from adjuvant therapy or who do not need adjuvant therapy [5].

Adjuvant therapy can only be effective in patients who harbor micrometastases after tumor surgery that are not yet radiologically detectable, a stage called minimal residual disease (MRD) [3]. The concept of identifying MRD to guide adjuvant therapy decisions in RCC was recently highlighted at *The Society for Immunotherapy of Cancer*'s annual meeting (SITC 2022). Among the possible approaches, the detection of circulating tumor DNA (ctDNA)

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following tumor surgery is an emerging and powerful therapeutic concept to identify MRD patients at highest risk of disease recurrence [6]. The ctDNA-guided adjuvant treatment for patients with stage II colon cancer reduces the use of adjuvant chemotherapy without compromising recurrence-free survival [7]. This concept can also be applied to patients with urothelial carcinoma, where only ctDNA-positive patients at high risk of recurrence had a survival benefit from adjuvant therapy with atezolizumab in the phase 3 IMvigor010 trial [8]. Imvigor011 (NCT04660344) will further explore this therapeutic concept by administering either atezolizumab or placebo to patients with ctDNA detection after cystectomy.

This promising new therapeutic concept to guide adjuvant treatment decision on the detection of MRD may also support adjuvant therapy decisions for patients with RCC.

However, detection of ctDNA is costly and not widely available, and for RCC in particular, further technical difficulties arise for this diagnostic tool: Detected levels of ctDNA in patients with RCC are low, and highly sensitive methods are required [9]. Also, the fraction of ctDNA in liquid biopsy was found to be comparatively low in RCC patients compared to other solid tumor entities [10]. In addition, clear cell RCC is the prime example of a heterogeneous tumor with substantial intrapatient heterogeneity between metastatic lesions throughout tumor evolution; thus, detection of relevant mutations for ctDNA monitoring is complicated [11]. Therefore, even for truncal mutations such as the Von Hippel-Lindau gene, DNA-based genomic profiling in peripheral blood for metastatic RCC resulted in low detection rates [12]. As an alternative approach, the measurement of tumor-specific methylation signatures seems to be a promising concept to detect ctDNA in patients with RCC [9].

Research has identified specific methylation patterns across various entities with the potential to serve as pan-cancer biomarkers [13]. Hypermethylated *short stature homeobox gene 2 (SHOX2)* within circulating cell-free DNA (ccfDNA) is a highly sensitive surrogate parameter for the presence of ctDNA in diverse entities including RCC, where it has high prognostic potential as a surrogate for tumor burden prior to nephrectomy [14]. An U.S. Food and Drug Administration (FDA)-approved test kit (*Epi proLung)* detecting hypermethylated *SHOX2* (mSHOX2) for diagnostic purposes in lung cancer would allow a straight forward clinical implementation of this promising liquid biopsy biomarker for patients with RCC as well.

Therefore, in our study, we aimed to investigate whether the presence of mSHOX2 in ccfDNA after nephrectomy represents a surrogate for MRD to identify patients at risk for disease recurrence who might particularly benefit from adjuvant immunotherapy.

Methods

Patient cohort

From a prospectively collected biobank registry, a single-center observational cohort study comprising 45 patients with RCC was assembled between 2014 and 2015. Male and female patients undergoing renal tumor surgery for radiologically suspected RCC aged 18-99 years were included. Patients with no histological evidence of malignancy after surgery were excluded retrospectively.

Assessment of SHOX2-methylation

A plasma blood sample was collected at a mean of 4.3 days (interquartile range 2.3-6.7 days) after surgery. The timing was chosen to exclude false positive data obtained by high pre-surgical levels regarding the half-life of ctDNA (approximately 2 hours) [15]. The method of measuring SHOX2-methylation in ccfDNA has been described previously in detail [13, 14, 16, 17]. In brief, blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and processed to plasma within 6 hours. Next, ccfDNA was bisulfite converted and purified [16]. Afterwards, *SHOX2*-methylation was measured using a methylation-specific quantitative real time polymerase chain reaction amplifying a 112 base pair fragment within the body of the *SHOX2* gene as described in this publicly available protocol [18]. Relative methylation levels were calculated using the ΔΔCq method [17]. Using the previously validated cutoff > 0.25%, samples were dichotomized as either SHOX2 hypermethylated (mSHOX2-positive) or not hypermethylated (mSHOX2-negative) [14].

Follow-up data and statistical analysis

The patient cohort was observed from enrollment until December 2021. Overall survival (OS), cancer-specific survival (CSS), and metastasis-free survival (MFS) after surgery were estimated using univariable Kaplan-Meier regressions and tested with log-rank tests. Starting with the day of surgery, OS was defined as the period until death of any cause, CSS as the period until death causally related to RCC and MFS as the period until tumor recurrence or metastasis. Patients with completely resected metastatic disease without radiological evidence of further metastases were included as stage *M1 with no evidence of disease* (M1 NED) as defined in KEYNOTE-564 [19]. Patients undergoing palliative cytoreductive nephrectomy in the metastatic disease stage were excluded from the MFS analyses. Uni- and multivariable Cox regression analyses were performed to compare the independent prognostic value of SHOX2 hypermethylation with baseline characteristics (age, sex, Eastern Cooperative Oncology Group (ECOG) performance status, and high risk of recurrence based on histopathological findings according to the KEYNOTE-564 trial: pT2, G4; pT3-4; pN+; pM+ [19]). Variables were only included in the multivariable Cox regression models if survival effects were significant in the univariable analyses. Statistical analyses were performed with RStudio (version 1.4.1106) using "base" and "survminer" package (version 0.4.9, https://CRAN.R-project.org/package=survminer). Categorical variables are reported as median, frequency, range, and interquartile range (IQR). The Kruskal-Wallis rank sum test and Wilcoxon-Mann-Whitney test were used for intergroup comparisons. All tests were two-sided, and *P*-values < 0.05 were considered significant.

Results

Characteristics of the study cohort

45 patients with RCC who underwent surgery between 2014 and 2015 met the inclusion criteria. 73% of patients were male, and the median age at nephrectomy was 67 years (interquartile range (IQR): 57-74 years). Only 1 patient (2.2%) had simultaneously undergone resection of a singular pancreatic distant metastasis (M1 NED), while in another 6 patients (13.3%), the surgical intention was a cytoreductive palliative nephrectomy with known distant metastases. The main histologic subtype was clear cell renal carcinoma in 30 patients (66.7%), as well as 9 patients (20%) with papillary and 5 patients (11%) with chromophobe RCC. Data are summarized in **Table 1**. The median follow-up period was 63 months (mean: 51.8; IQR: 20-73 months). 18 (40%) patients died during the follow-up period, and 9 patients (20%) experienced disease recurrence.

SHOX2 hypermethylation and survival outcom

17 patients (37.8%) were defined as mSHOX2-positive and 28 patients (62.2%) were mSHOX2-negative. There were significant differences in baseline clinical parameters (**Table 1**); mSHOX2-positive patients were of higher tumor stage and risk of recurrence according to stratification based on histopathological findings.

In univariable Cox regression, the detection of mSHOX2 was associated with shortened OS (hazard ratio (HR) = 3.65, 95% Confidence Interval [CI] 1.41-9.46, *P* = 0.004), CSS (HR = 3.59, 95% CI 1.17-11.0, *P* = 0.02), and MFS (HR = 5.89, 95% CI 1.46-23.8, *P* = 0.005). The other factor significantly associated with survival outcomes was high-risk constellation based on histopathological TNM staging, namely pT2, G4; pT3-4; pN+ or pM+ tumors (OS: HR = 2.99, 95% CI 1.18-7.64, *P* = 0.02; CSS: HR = 5.16 95% CI 1.58-16.9, *P* = 0.002; MFS: HR = 5.15, 95% CI 1.37-19.4, *P* = 0.007). Age, sex, and ECOG performance status showed no statistically significant association with survival outcomes. In the multivariable Cox analysis, mSHOX2 was found to be an independent risk factor for both reduced OS and MFS (*P* = 0.034 and 0.042, respectively), while high-risk tumors were independently associated with reduced CSS (*P* = 0.030). The Cox regression analyses are presented in **Table 2**.

The Kaplan-Meier estimators (KM) are presented in **Figure 1**. Detection of mSHOX2 was associated with significantly reduced OS (**Figure 1A**; log-rank *P* = 0.004), CSS (**Figure 1B**; log-rank *P* = 0.017), and MFS (**Figure 1C**; log-rank *P* = 0.005). Additional KM estimators, excluding patients with metastatic disease stages, are depicted in **Figure 2**.

Of 17 mSHOX2-positive patients, 6 met the low-risk criteria according to TNM staging,

Table 1. Comparison of baseline parameters between patients with detection or absence of hypermethylated short stature homeobox 2 (mSHOX2-positive vs. -negative) in circulating cell-free DNA (ccfDNA)

Characteristic	Overall, *N* = 45	mSHOX2-positive, *N* = 17	mSHOX2-negative, *N* = 28	*p*-value[1]
Age				0.223
Median, IQR	67 (56.5, 74)	70 (61.5, 75)	67 (47, 73.5)	
Range	23, 89	56, 89	23, 86	
Sex				0.760
Male	33 (73.3%)	12 (70.6%)	21 (75%)	
Female	12 (26.7%)	5 (29.4%)	7 (25%)	
ECOG				0.795
0	30 (66.7%)	11 (64.7%)	19 (67.9%)	
1	14 (31.1%)	5 (29.4%)	9 (32.1%)	
2	1 (2.2%)	1 (5.9%)	0 (0%)	
Histological Subtype				0.150
Clear cell	30 (66.7%)	14 (82.2%)	16 (57.1%)	0.072
Non clear-cell	15 (33,3%)	3 (17,8%)	13 (42,9%)	
Papillary	9 (20%)	2 (11.8%)	7 (25%)	0.262
Chromophobe	5 (11.1%)	0 (0%)	5 (17.9%)	0.099
Tubulocysytic	1 (2.2%)	1 (5.9%)	0 (0%)	0.427
AJCC Stage				0.013*
I	24 (53.3%)	4 (23.5%)	20 (71.4%)	
II	3 (6.7%)	2 (11.8%)	1 (3.6%)	
III	11 (24.4%)	6 (35.3%)	5 (17.9%)	
IV	7 (15.6%)	5 (29.4%)	2 (7.1%)	
Risk classification[2]				0.005**
High-risk	18 (40%)	11 (64.7%)	7 (25%)	
Low-risk	27 (60%)	6 (35.3%)	21 (75%)	

mSHOX2 = hypermethylated short stature homeobox 2, IQR = interquartile range, ECOG = Eastern Cooperative Oncology Group performance status, AJCC = American Joint Committee on Cancer. [1]Wilcoxon-Mann-Whitney-Test; Kruskal-Wallis rank sum test. [2]According to KEYNOTE-564 [19]: High-risk = pT2, G4; pT3-4, pN+, pM+ (M1 NED No Evidence of Disease). *P < 0.05, **P < 0.01.

Table 2. Uni- and multivariable Cox regression analysis regarding overall survival, cancer-specific survival and metastasis-free survival after surgery

Univariable	OS			CSS			MFS		
Characteristic	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Sex: Female	0.7	0.23-2.1	0.522	1.11	0.34-3.6	0.872	1.30	0.32-5.4	0.715
Age	1.04	0.99-1.1	0.086	1.04	0.99-1.1	0.107	1.01	0.97-1.1	0.462
ECOG	2.30	0.90-5.9	0.084	2.14	0.67-6.7	0.195	0.57	0.07-4.6	0.598
mSHOX2-positive	3.59	1.41-9.5	0.004**	3.59	1.17-11	0.026*	5.89	1.46-23.8	0.005**
High-risk-tumor[1]	2.99	1.18-7.6	0.022*	5.16	1.58-16.9	0.002**	5.15	1.37-19.4	0.007**
Multivariable	OS			CSS			MFS		
Characteristic	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
High-risk-tumor	2.22	0.83-5.9	0.11	3.95	1.14-13	0.030*	3.63	0.92-14	0.066
mSHOX2-positive	2.94	1.09-7.9	0.034*	2.36	0.72-7.7	0.153	4.51	1.05-19	0.042*

OS = overall survival, CSS = cancer-specific survival, MFS = metastasis-free survival, HR = hazard ratio, 95% CI = 95% confidence interval, mSHOX2 = hypermethylated short stature homeobox 2, ECOG = Eastern Cooperative Oncology Group performance status. [1]According to KEYNOTE-564 [19]: High-risk = pT2, G4; pT3-4, pN+, pM+ (M1 NED No Evidence of Disease). *P < 0.05, **P < 0.01.

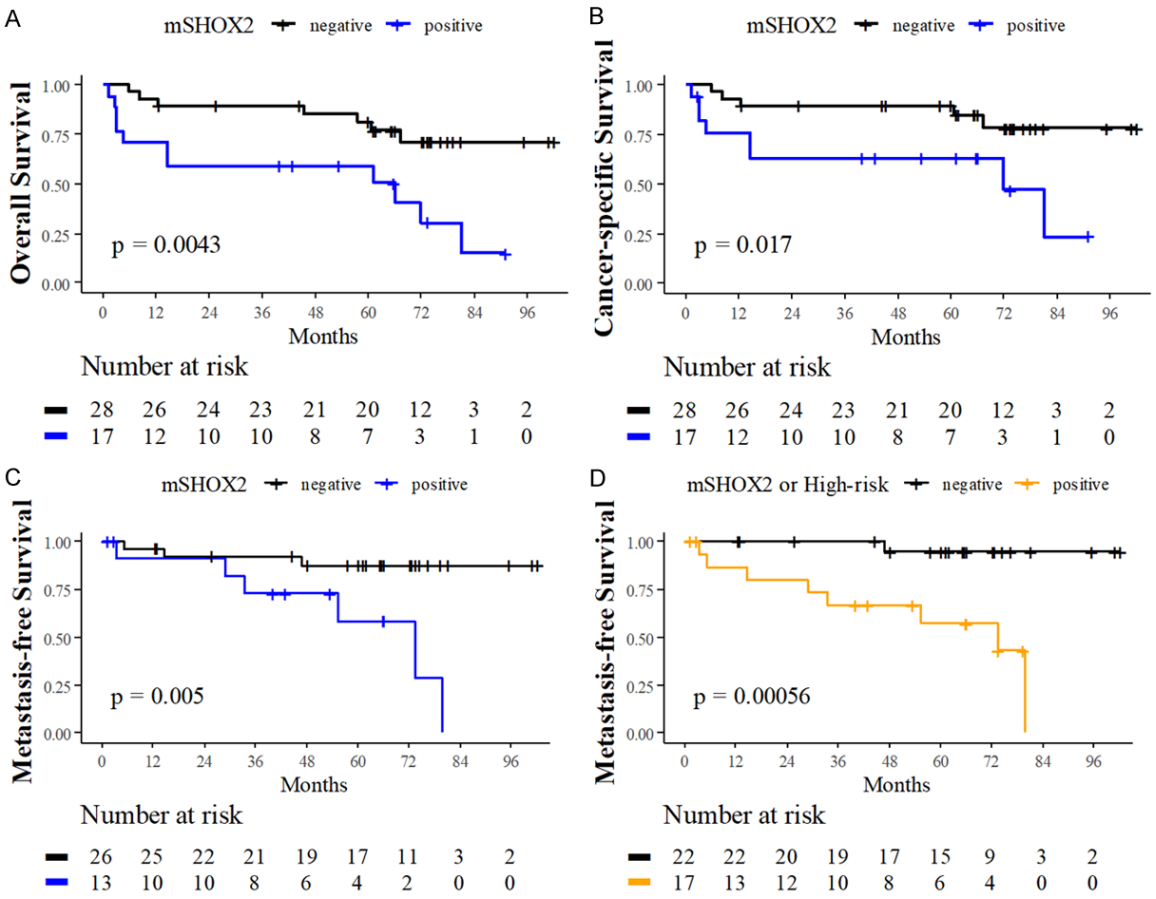

Figure 1. Kaplan-Meier estimators of survival after renal cell carcinoma surgery: All 45 patients were divided by methylation status of short stature homeobox 2 (SHOX2) gene in circulating cell-free DNA into either hypermethylated (mSHOX2-positive) or non-hypermethylated (mSHOX2-negative). Significant survival benefit was shown for mSHOX2-negative patients in: (A) Overall survival (Log rank P = 0.004), (B) Cancer-specific-survival (Log rank P = 0.017) and (C) Metastasis-free-survival (MFS, Log rank P = 0.005). For MFS analysis primary metastatic cases were excluded. (D) By composing mSHOX2 with high-risk criteria according to KEYNOTE-564 trial (pT2, G4; pT3-4; pN+, pM+ [19]) one event of recurrence is missed out (Log rank P < 0.001).

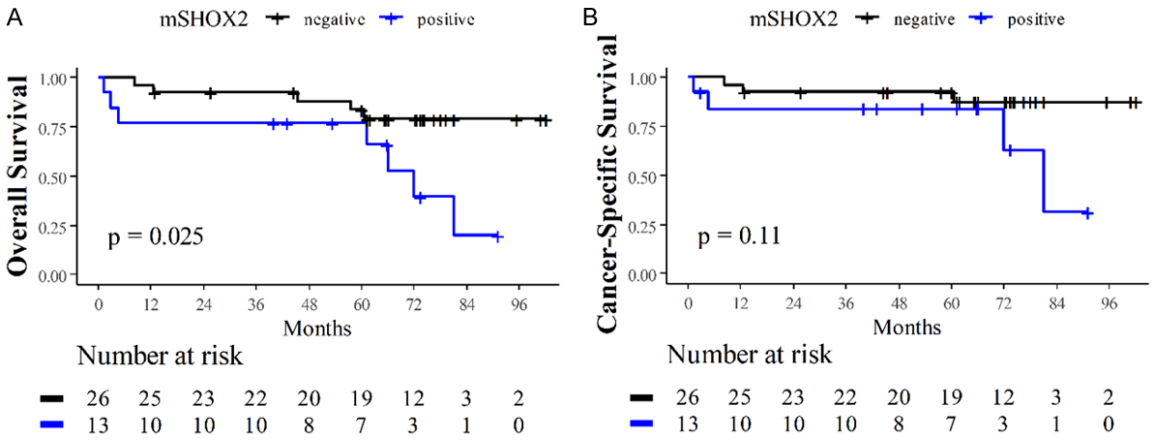

Figure 2. Kaplan-Meier estimators of survival after renal cell carcinoma surgery. After exclusion of primary metastatic cases, 39 patients are divided by methylation status of short stature homeobox 2 (SHOX2) gene in circulating cell-free DNA into either hypermethylated (mSHOX2-positive) or non-hypermethylated (mSHOX2-negative). A significant survival benefit was shown for mSHOX2-negative patients in (A) overall survival (Log rank P = 0.025) with a non-significant trend for (B) cancer-specific-survival (Log rank P = 0.11).

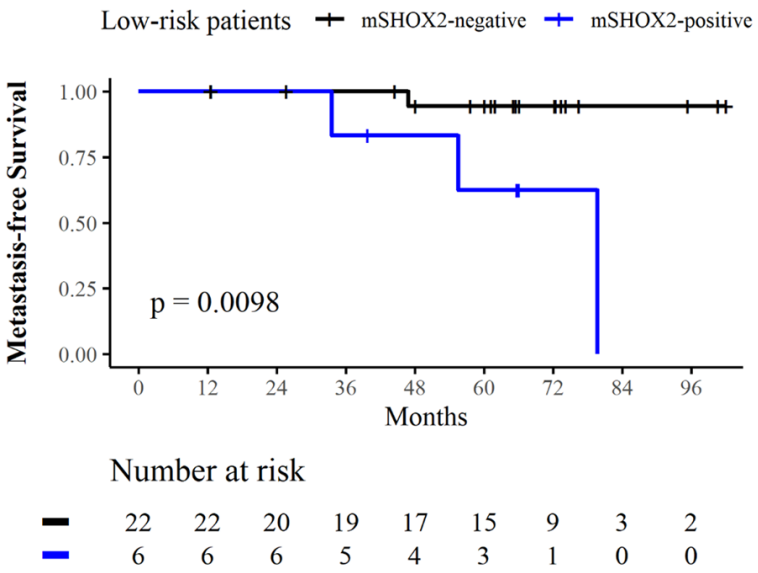

Figure 3. Kaplan-Meier estimator of metastasis-free survival in the low-risk subgroup after renal cell carcinoma surgery. Additional analysis including only patients with low risk of recurrence according to KEYNOTE-564 trial (pT1-2, G1-3, N0, M0) [19]. Hypermethylation of short stature homeobox 2 (mSHOX2) in circulating cell-free DNA was associated with impaired MFS (Log rank $P < 0.01$).

excluding them from eventual adjuvant treatment according to current approval, while out of these patients 3 (50%) suffered from disease recurrence. The MFS was significantly shortened in low-risk patients with SHOX2 hypermethylation ($P = 0.01$). In high-risk patients, no significant difference was found in survival outcomes depending on SHOX2 methylation status ($P = 0.73$). The KM plots are shown in **Figure 3**.

In an additional MFS analysis, we included all 16 patients (41.0%) with either mSHOX2 hypermethylation or a high-risk status as a composite score. By combining these two predictive indicators, only 1 event of recurrence was missed (**Figure 1D**).

Discussion

Recurrence of renal cell carcinoma is common, and to date, we have not progressed beyond predicting the risk of recurrence based on histopathology alone. Since the approval of pembrolizumab as an adjuvant treatment, accurate risk assessment has become increasingly important. As the IMmotion010 (ClinicalTrials.gov Identifier: NCT03024996) and CheckMate914 (NCT03138512) trials failed to show a benefit of adjuvant treatment with atezolizumab or nivolumab + ipilumumab, this further underscores the paramount clinical importance of accurate patient selection [20, 21]. In the era of precision oncology, molecular biomarkers that identify patients who will benefit from adjuvant ICB would be desirable to guide adjuvant treatment decisions [22].

Postoperative detection of ctDNA as a surrogate for MRD has strong potential to guide adjuvant treatment decisions. This concept has been proven in large phase 3 clinical trials, such as the DYNAMIC and IMvigor010 [7, 8]. Adjuvant atezolizumab only prolonged disease-free survival in the ctDNA-positive subgroup of the negative IMvigor010 trial [8], which now led to the initiation of the IMvigor011 trial, which tested atezolizumab versus placebo in patients with high-risk muscle-invasive bladder cancer who are ctDNA-positive following cystectomy (NCT04660344).

However, there are limitations in the determination of ctDNA, particularly in RCC, owing to difficult detection and intratumor heterogeneity as recently highlighted by Geertsen et al. [9]. Even in advanced RCC, detection of ctDNA is achieved within approximately half of the patients at most [23-26]. Therefore, despite its proven relevance throughout other solid tumor entities and indications for potential in MRD detection even in RCC, ctDNA is not gaining widespread use among RCC patients [26]. In order to avoid these technical difficulties, DNA methylation markers such as mSHOX2 might provide a promising approach.

Numerous studies have suggested the diagnostic value of methylation markers in RCC [27]. However, to date there are very limited data on the prognostic role of liquid biopsy in RCC. Nevertheless, our results demonstrating a significant survival difference according to postoperative SHOX2 hypermethylation status, as well as the identification of mSHOX2 as an

independent risk factor in addition to TNM staging, are in line with previously discovered survival signals obtained from liquid biopsy studies: Jung et al. found that SHOX2 hypermethylation in preoperative blood samples of RCC patients is a prognostic factor for overall survival and a molecular staging parameter [14]. Yamamoto et al. found that increased fragmentation of ccfDNA after surgery was negatively correlated with progression-free survival (PFS) [28]. Bacon et al. observed shorter OS and PFS with fist-line therapy in ctDNA-positive patients, but only included metastatic RCC [29].

From our results we conclude that liquid biopsy for *SHOX2* methylation assessment may have an additive value in recurrence risk stratification, a field in particular need of improvement [22]. Within days after surgery, we were able to identify patients with an approximately 6-fold increased risk of recurrence, including several "low-risk" patients with late-onset metastases. These findings indicate that mSHOX2-positive patients might derive increased benefits from adjuvant ICI therapy, especially when forming a composite score with TNM staging.

The eventual utility of these findings remains to be assessed; however, it seems likely that advising our patients regarding adjuvant ICI therapy after surgery will sometimes not be an easy and straightforward decision [5]. Given the high number needed to treat and adverse events following overtreatment without additional clinical benefit, adjuvant therapies must be used rationally. While in KEYNOTE-564, patients with stage M1 NED or tumors with sarcomatoid features had the strongest disease-free survival benefit on adjuvant pembrolizumab; for example, in non-sarcomatoid pT3a, G3, N0, and R0 tumors, additional tools may play a helpful role in evaluating the risk of recurrence. Therefore, under the objective of precision oncology, we envision mSHOX2 as a supportive biomarker.

However, a biomarker should not only be useful; it should also have potential for broad use in clinical practice. As an FDA-approved test kit already exists for mSHOX2, we believe that there is potential for simple implementation in clinical workflow. Pre-analytics should be considered, as the isolation of cell-free DNA should be completed within 6 h after blood plasma sample collection to avoid dilution of methylation patterns by background ccfDNA from disintegrating blood cells.

Our results have limitations. The study was monocentric; likewise, the cohort size was not large, accounting for 45 patients. The design deliberately focused on a single measurement point and dichotomy between mSHOX2-positive and -negative patients, refraining from quantitative analyses or longitudinal observations.

Conclusion

The findings of the study indicate that the evaluation of mSHOX2 methylation through liquid biopsy holds promise as a valuable biomarker in predicting the likelihood of recurrence and aiding in the selection of appropriate adjuvant treatments for patients with renal cell carcinoma.

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Written informed consent was obtained from all the participants.

Disclosure of conflict of interest

DD has been an employee of Epigenomics AG, Berlin, Germany. This company aims to commercialize DNA methylation biomarker SHOX2. DD is co-inventor and owns patents on methylation biomarkers and related technologies. DD receives inventor's compensation from Epigenomics AG.

Abbreviations

RCC, Renal Cell Carcinoma; ICI, Immune Checkpoint Inhibitor; MRD, Minimal Residual Disease; ctDNA, Circulating Tumor DNA; SHOX2, Short Stature Homeobox 2; mSHOX2, Hypermethylated Short Stature Homeobox 2; ccfDNA, Circulating Cell-Free DNA; NED, No Evidence of Disease.

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