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RESEARCH

Genomic analysis of small renal masses reveals mutations linked with renal cell carcinoma and fast-growing tumors

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Abstract

Purpose Small renal masses (SRMs) SRMs are a heterogeneous group of small kidney lesions. Currently, the genomic landscape of SRMs is understudied, and clinically relevant tools for malignancy detection and fast tumor growth prediction are lacking. The aim of the study was to evaluate whether mutations in SRMs are associated with increased risk of renal cell carcinoma (RCC) or aggressive tumors.

Methods In this pilot study, 52 patients with SRMs were divided based on tumor histology into RCC and benign tumors, while RCC cases were divided into fast-growing and slow-growing tumor groups. Tissue biopsy samples evaluated for mutations in 51 cancer hotspot genes using next generation sequencing and qPCR. Non-benign mutations were tested for associations with RCC and clinical features. Receiver operating curve analysis used for evaluation of mutation biomarker models prediction of RCC and fast-growing tumors.

Results 75% of SRMs harbored non-synonymous alterations in 16/51 genes. 38.5% of detected mutations were listed in ClinVar and correlated with smaller SRM volume ($p=0.023$). *KRAS*, *VHL*, *HNF1A*, *TP53*, and *ATM* mutations were predominantly detected in RCC rather than benign SRMs ($p=0.046$). SRMs with pathogenic mutations were at three times higher risk of being RCC and four times higher risk of fast growth.

Conclusion Genomic biomarkers may improve risk stratification and management of patients with SRMs, however a more extensive genomic analysis of SRMs is still needed.

Keywords Small renal mass · Renal cell carcinoma · Kidney tumor · Somatic mutations · Next generation sequencing

Introduction

Small renal masses (SRMs) are defined as renal lesions ≤ 4 cm in size. SRMs are usually incidentally detected by imaging, with up to 84% of these masses showing no symptoms (Sánchez-Martín et al. 2008). Advancements in cross-sectional imaging of SRMs over the past 20 years have increased the incidence of renal cell carcinoma (RCC) rates, more frequently detecting early stage pT1a tumors. However, around 25% of SRMs are benign cysts and tumors (40.4% for tumors less than 1 cm in diameter), thus invasive treatment poses a risk of overtreatment when considering the prevalence of older age and additional comorbidities among patients (Johnson et al. 2015). SRMs are generally regarded as slow-growing tumors with low metastatic potential, thus active surveillance (AS) with delayed treatment is generally recommended for patient management (Sebastià et al. 2020). However, there is still the risk of metastasis (detected in 2% of patients) and fast growth of aggressive tumors requiring intervention (up to 45% of cases) (Smaldone et al. 2012), thus proper biomarkers for separation of these aggressive SRMs are greatly needed.

RCC is a heterogeneous disease group arising from renal tubular epithelial cells (Hsieh et al. 2017). It is the seventh most prevalent cancer type worldwide, with the highest incidence and mortality rates in Northern Europe, specifically Lithuania (Wong et al. 2017). Histologically the main

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subtypes of RCC are clear cell (ccRCC) representing ~ 75% of RCCs, papillary (pRCC) making up ~ 15%, and chromophobe (chRCC) with ~ 5% of RCC cases (Hsieh et al. 2017). The most common benign kidney SMRs are angiomyolipoma (AML) and oncocytoma (OCT) (Smaldone et al. 2012).

Risk factors for RCC include obesity, hypertension, cigarette smoke, and diabetes mellitus (Hsieh et al. 2017). Moreover, a genetic component also contributes to RCC risk with familial disease cases accounting for 2–3% of RCC cases (Wong et al. 2017). Most notably, mutations in the *VHL* gene are closely associated with ccRCC histology tumors and present in both sporadic and familial cancers (the latter associated with von Hippel-Lindau syndrome). In *VHL* mutated tumors, stabilization of hypoxia-inducible factors (HIF1 and HIF2) activates genes that regulate cell metabolism and survival signaling. However, additional (epi) genetic events are required for ccRCC development (Hsieh et al. 2017). Other notable RCC mutations described by The Cancer Genome Atlas (TCGA) include chromatin remodeling genes (such as *PBRM1*, *SMARCB1*) in ccRCC and pRCC, and *CDKN2A* loss in chRCC (Linehan and Ricketts 2019). Although genome-wide studies such as TCGA have described the main genomic alterations of the three main RCC histologies, these observations mainly come from more advanced kidney tumors and may not include SRMs. Thus, molecular profiling of SRMs may provide valuable information not only about the tumor type but also help to inform decisions on AS protocols or selection of personalized treatments.

In this pilot study of tissue biopsies from 52 SRMs, targeted next-generation sequencing of 50 cancer-related genes frequently associated with renal tumorigenesis was applied and supplemented by qPCR-based *CHEK2* hotspot mutations detection. The potential of identified mutations to predict SRM malignancy and growth potential was evaluated for improved understanding on clinical value of genetic testing for SRMs stratification.

Methods

Patient selection and sample collection

The study included 52 kidney percutaneous needle biopsies collected at the National Cancer Institute in Vilnius, Lithuania between 2018 and 2021. The biopsies were taken during routine procedure for confirmation of diagnosis of RCC, immediately flash-frozen after the procedure, and stored until DNA extraction at -80 °C.

The patients were selected if (1) older than 18 years; (2) had a solid renal mass less than 4 cm in diameter diagnosed by imaging (CT, MRI, or ultrasonography); (3) patient's agreement for active surveillance of SMR was obtained and the informed consent form was signed. The patients that had an uninformative biopsy, a life expectancy of less than a year, or had undergone systematic therapy for malignancy were excluded. The cases with suspected hereditary cancer were also excluded. The study was approved by the regional bioethics committee (No. 158200-17-952-457).

Of the 52 biopsies, 38 were confirmed RCC (5 chRCC, 26 ccRCC, 6 pRCC, and 1 p/ccRCC case), and 14 were benign (10 OCT, 3 AML, and 1 other). Clinical data is provided in Table 1.

DNA extraction

DNA extraction from kidney needle biopsy samples was performed using standard phenol-chloroform extraction and ethanol precipitation protocols. In brief, the needle biopsy tissue samples digested for 18 h at 55 °C with Proteinase K (Thermo Scientific, Wilmington, DE, USA) and 500 µL lysis solution containing (50 mM Tris-HCl, pH 8.5; 1 mM EDTA; 0.5% Tween-20, Carl Roth, Karlsruhe, Germany). After incubation, Phenol/Chloroform/Isoamyl alcohol (25:24:1, Carl Roth, Karlsruhe, Germany) was used for DNA extraction following the chloroform (Carl Roth, Karlsruhe, Germany) step. The final DNA is precipitated using 40 µL 5 M ammonium acetate (Thermo Fisher, Kandel, Germany), 1 µL glycogen (Thermo Scientific, Vilnius, Lithuania), and 1 mL 96% ethanol. The DNA was then washed twice using 70% ethanol and dissolved in nuclease-free water. DNA quantity was measured using Qubit™ dsDNA BR Assay Kit on a Qubit™ 2.0 Fluorimeter (Invitrogen, TFS, Eugene, OR, USA). The DNA samples were stored at -80 °C until further experiments.

CHEK2 hotspot mutation qPCR analysis

All 52 biopsy samples were analyzed for predominant mutations in *CHEK2* using TaqMan SNP Genotyping assays (rs17879961 c.470T > C; rs555607708 c.1100delC; rs121908698 c.444 + 1G > A). The qPCR reactions were conducted using 2X TaqMan Universal Master Mix II (Applied Biosystems (AB), Thermo Fisher Scientific Baltics, Vilnius, Lithuania) on QuantStudio 5 Real-Time PCR System (AB, Singapore) following the manufacturer's instructions.

Targeted next-generation sequencing

A custom 50 gene panel consisting of *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAQ*, *GNAS*, *HNF1A*, *HRAS*,

Table 1 Clinical and demographic features of the study cohort. RCC- renal cell carcinoma, N/A – no data available, SD – standard deviation

Clinicopathological features	All samples	RCC	Benign	*p* =
n=	52	38	14	
Gender				
Male, n (%)	23 (44.20)	15 (39.50)	8 (57.10)	0.35
Female, n (%)	29 (55.80)	23 (60.50)	6 (42.90)	
Average age at diagnosis (±SD)	76.1 (±7.54)	76.1 (±7.77)	76.1 (±7.15)	1
Average body mass index (BMI) (±SD)	28.99 (±5.56)	29.8 (±5.67)	25.9 (±4.00)	**0.04**
Average waist circumference, cm (±SD)	101.27 (±17.83)	102.2 (±18.32)	96.3 (±15.20)	0.38
Median tumor volume, mm3 (min-max)	5155.57 (690.23–29563)	5156 (690-29104)	15796 (2028–29563)	0.68
Mean maximal tumor diameter, mm (±SD)	24.01 (±7.59)	23.7 (±6.98)	25.7 (±10.70)	0.64
Disease progression rate (fast/slow growing tumor)				
Progressive, n (%)	9 (17.31)	9 (23.68)		
Stable (non-progressive), n (%)	26 (50.00)	26 (68.42)		
N/A[1], n (%)	17 (32.69)	3 (7.90)	14 (100.00)	
ISUP:				
ISUP 1, n (%)	9 (17.31)	9 (23.68)		
ISUP 2, n (%)	19 (36.54)	19 (50.00)		
N/A[2], n (%)	24 (46.15)	10 (26.32)	14 (100.00)	
Tumor histology:				
chRCC, n (%)	5 (9.62)	5 (13.16)		
pRCC, n (%)	6 (11.54)	6 (15.79)		
ccRCC + cc/pRCC, n (%)	27 (51.92)	27 (71.05)		
OCT, n (%)	10 (19.23)		10 (71.43)	
AML, n (%)	3 (5.77)		3 (21.43)	
Other, n (%)	1 (1.92)		1 (7.14)	
Median high-density lipoprotein (HDL) mmol/L (min-max)	1.55 (0.77–6.83)	1.36 (0.77–2.39)	1.68 (1.18–6.83)	**0.04**
Median cholesterol mmol/L (min-max)	5.12 (3.04–7.47)	5.12 (3.04–7.47)	5.0 (3.32–7.38)	0.79
Mean MTL mmol/L (±SD)	3.49 (±1.11)	3.51 (±1.10)	3.4 (±1.17)	0.86
Diabetes mellitus (Yes vs. No)	13 vs. 32	11 vs. 26	2 vs. 6	1
Metabolic syndrome (Yes vs. No) (artery hypertension)	41 vs. 4	34 vs. 3	7 vs. 1	0.56

[1] N/A – no patient follow-up

[2] N/A – ISUP was not determined

IDH1, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3A*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL* was used for targeted next generation sequencing of the 52 DNA samples from kidney percutaneous needle biopsies.

The sequencing libraries were prepared using Ion AmpliSeq™ Library Kit 2.0 and custom On-Demand Panel (Life Technologies (LT), Carlsbad, CA, USA) following the manufacturer's protocols. Library quantification was conducted using Ion Library TaqMan™ Quantification Kit (AB, TFS, Vilnius, Lithuania). The final libraries were sequenced on the Ion 520™ chip using the Ion Torrent™ Ion S5™ system (LT, Singapore). The NGS data analysis was conducted on the Ion Reporter 5.18 tool (LT, Carlsbad, CA, USA).

Variant classification

Each variant was first classified using ClinVar database (Landrum et al. 2018) according to the American College of Medical Genetics and Genomics (ACMG) / the College of American Pathologist (AMP) guidelines (Richards et al. 2015) into benign/likely benign, pathogenic/likely pathogenic or uncertain significance (VUS) groups. Variants not listed or without interpretation on ClinVar were regarded as VUS. Every VUS was then evaluated using five publicly available interpretation knowledgebases: Varsome (Saphetor SA) (Kopanos et al. 2019), Franklin (Genoox) (2024), InterVar (Li and Wang 2017), Cancer Genome Interpreter (CGI) (Tamborero et al. 2018), and CancerVar (Li et al. 2022) for mutation pathogenicity interpretation. The VarSome, Franklin, CancerVar, and InterVar databases provide predictions for ACMG/AMP classification using unique data aggregation algorithms and use the available data to automatically classify each variant according to ACMG/AMP criteria. Franklin database separately classify each variant according to both ACMG (classifying each variant into benign/likely benign, VUS, low or moderate oncogenic support, and pathogenic/likely pathogenic variants) and AMP classification (using tier system: Tier 1 – strong

clinical significance, Tier 2 – potential clinical significance, Tier 3 – unknown clinical significance, Tier 4 – benign/likely benign) (Li et al. 2017). CancerVar OPAI algorithm takes CancerVar interpretation and combines the 23 in silico scores for semi-supervised deep learning oncogenicity prediction (Li et al. 2022). CGI base provides a rule based OncodriveMUT algorithm to designate variants as passenger or driver mutations (Tamborero et al. 2018). All knowledgebases accessed in January 2024.

Statistical analysis

For statistical analysis, the Shapiro-Wilk's W test was used for testing the normal distribution. Mann-Whitney U test or Welch two sample t test was used for testing associations between two independent samples as appropriate, while Fisher's exact test was used for categorical variable associations. Univariable and multivariable odds ratios calculated using logistic regression models. The data was analyzed using R x64 (version 4.3.1, R Foundation for statistical computing, Vienna, Austria) on the RStudio (version 2023.06.0, Posit, PBC, Boston, MA, USA) software. ComplexHeatmap package (version 2.16.0) was used for oncoprint visualization. Receiver operating characteristic (ROC) analysis was performed using pROC package (version 1.18.5). Statistical significance was considered when p-value was <0.050.

Results

Genetic alterations in kidney biopsies

Out of 52 kidney needle biopsies 75.0% (39/52) had non-synonymous alterations, of them 38.5% (15/39) had pathogenic mutations (*via* ClinVar) and the rest were regarded as VUS (Fig. 1). In all, 61 alterations were detected in 39 biopsies from 52 patients with SRMs and out of 51 genes included in analysis 16 were identified with genetic alterations.

Genetic alterations were identified in 73.7% (28/38) of the cases with RCC, and 34.2% (13/38) had pathogenic mutations (listed in ClinVar). Analyzing patients by different

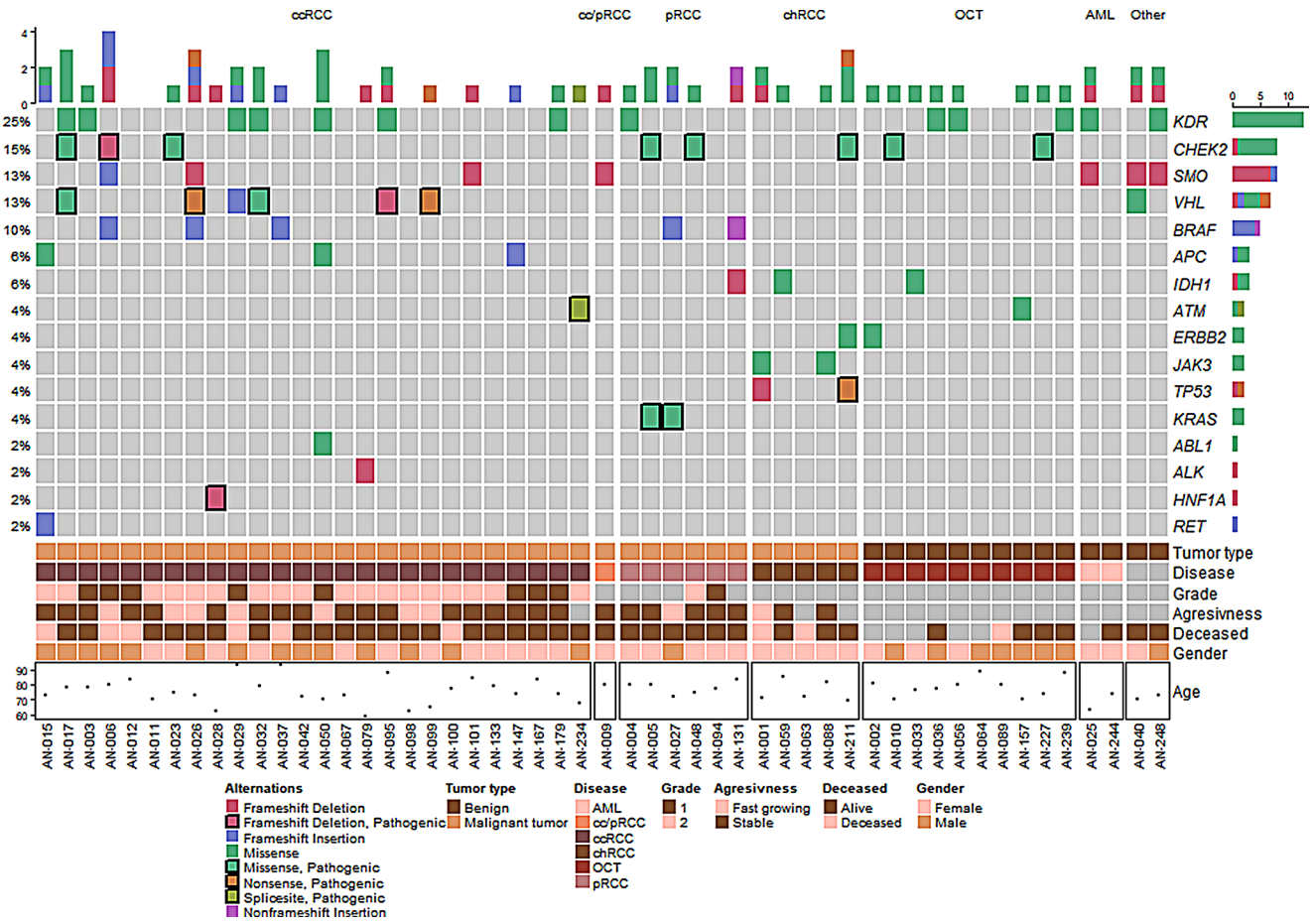


Fig. 1 Oncoprint depicting mutation analysis of 52 kidney needle biopsy samples. Black boxes indicate clinically pathogenic mutations (identified *via* ClinVar). Abbreviations: AML – angiomyolipoma, ccRCC – clear cell renal cell carcinoma, chRCC – chromophobe RCC, OCT – oncocytoma, pRCC – papillary RCC

histological groups, 80% (4/5) chRCC, 83.3% (5/6) pRCC, and 70.4% (19/27) ccRCC (including one p/ccRCC case) had alterations, of them pathogenic mutations were found in 20% (1/5) chRCC, 50% (3/6) pRCC, 33.3% (9/27) ccRCC cases. The chRCC group showed unique *TP53* c.595G>T mutation, *TP53* and *JAK3* VUS alterations. *KRAS* gene mutations were only found in pRCC cases, while 22.2% (6/27) of ccRCC cases had *VHL* alterations not found in other RCC cases. Clear cell histology RCC was unique for *HNF1A*, *ABL1*, *ALK*, *APC*, and *RET* gene alterations.

Alterations detected 78.6% (11/14) of benign cases, 18.2% of them (2/11) were pathogenic *CHEK2* c.470T>C mutations. 80% (8/10) of OCT cases had alterations. Most distinctively *ERBB2* c.1466 C>T gene alteration was only found in OCT and chRCC cases, while *ATM* alterations were only observed in OCT and ccRCC cases. Two of the three AML cases had *SMO* c.73delG alteration, alongside *KDR* and *VHL* gene alterations. Changes in the *SMO* gene were also found in ccRCC and other benign (non-AML or OCT) cases, while *KDR* c.889G>A mutation was found in 25.0% (13/52) of all cases, both p/ccRCC or benign. On average patients with RCC had 1.27 alterations (0–4 per patient), while patients with benign conditions on average had only 1.07 alterations per patient (0–3 per patient).

Of the 18 detected mutations listed in ClinVar, eight (44.4%) were *CHEK2* identified *via* qPCR (seven c.470T>C and one c.1100delC). *CHEK2* mutations were found in 15.8% (6/38) RCC cases in every type of histology group, as well as 20.0% (2/10) OCT cases. The only *CHEK2* deletion was found in a ccRCC patient alongside *BRAF* and *SMO* alterations.

Overall, 11 unique pathogenic mutations (Table 2) and 22 unique VUS were detected (Table S1). Of them, 5 pathogenic and 10 VUS were missense, 3 pathogenic and 11 VUS frameshift indel alterations, one pathogenic splice-site alteration, two pathogenic nonsense mutations, and one VUS non-frameshift insertion were detected.

Prediction of variant with uncertain significance pathogenicity using online databases

Variants not listed or listed as uncertain significance in the ClinVar database were regarded as uncertain significance variants (VUS). For these, 7 algorithms from 5 mutation knowledgebases were used for predicting pathogenicity. Franklin, VarSome, CancerVar, and InterVar provide automated variant interpretations according to ACMG/AMP rules, and CGI classifies variants into driver and passenger mutations. CancerVar (OPAI) and VarSome used *in* silico algorithms to predict variant pathogenicity. Only VarSome and Franklin provided interpretation for all VUS, others not providing predictions for most deletions and splice site alteration.

None of the VUS alterations that had classification by all five knowledge bases had a complete concordance of predicted pathogenicity. However, *TP53* c.466delC, *VHL* c.479_480insA, and *APC* c.3634_3635insA predicted pathogenic by all three algorithms reporting on the variants. All 11 frameshift indel alterations were predicted as either pathogenic or VUS, while 40.0% (4/10) of missense alterations were predicted VUS or benign by all prediction algorithms. Comprehensive variant interpretation is provided in Table S1. Overall, 81.8% (18/22) VUS were predicted pathogenic by at least one of the knowledgebases, and 54.5% (12/22) were predicted by at least two algorithms. 51.9% (27/52) of all cases had either pathogenic mutations (via ClinVar) or VUS, predicted pathogenic by at least two algorithms, these mutations are regarded as predicted pathogenic mutations in further analyses.

Table 2 Pathogenic mutations (classified by ClinVar) detected in the study

No.	Sample(s)	Gene	Coding sequence	Amino Acid Change	Variant Type	dbSNP
1	AN-005; AN-010; AN-017; AN-023; AN-048; AN-211; AN-227	*CHEK2*	c.470T>C	p.Ile157Thr	Missense	rs17879961
2	AN-006	*CHEK2*	c.1100delC	p.Thr367fs	Frameshift Deletion	rs555607708
3	AN-017	*VHL*	c.340G>C	p.Gly114Arg	Missense	rs869025636
4	AN-032	*VHL*	c.194 C>G	p.Ser65Trp	Missense	rs5030826
5	AN-095; AN-026	*VHL*	c.575delC	p.Pro192GlnfsTer10	Frameshift Deletion	-
6	AN-099	*VHL*	c.394 C>T	p.Gln132Ter	Nonsense	rs5030813
7	AN-005	*KRAS*	c.35G>T	p.Gly12Val	Missense	rs121913529
8	AN-027	*KRAS*	c.35G>A	p.Gly12Asp	Missense	rs121913529
9	AN-028	*HNF1A*	c.872delC	p.Pro291GlnfsTer51	Frameshift Deletion	rs587776825
10	AN-211	*TP53*	c.595G>T	p.Gly199Ter	Nonsense	rs1567551821
11	AN-234	*ATM*	c.3994-1G>T	p.?	Splice site	rs1057516238

Variant association with clinical data

When analyzing the pathogenic mutation association with clinical features, we found that patients with pathogenic mutations had significantly smaller tumors in volume compared to the non-mutated group (38 cases with tumor size data, median volume 2526 mm^3 in mutated ($n=14$) vs. 8907 mm^3 in non-mutated ($n=24$), $p=0.02$) (Fig. 2A), while maximum tumor radius only marginally correlated with pathogenic mutation status (median tumor size 18 mm in mutated ($n=15$) vs. 26 mm in non-mutated ($n=28$), $p=0.05$) (Fig. 2B).

Patients who had pathogenic alterations or predicted pathogenic mutations had an increased body mass index (mean BMI in mutated 30.80 (SD=4.54, $n=23$) vs. 27.1 (SD=5.96, $n=22$) in non-mutated, $p=0.02$) (Fig. 2C), as well as bigger waist circumference (mean waist circumference in mutated 106.71 (SD=13.25, $n=24$) vs. 94.75 (SD=20.61, $n=20$) in non-mutated, $p=0.03$, data not shown). Moreover, patients with predicted pathogenic mutations were of younger age (mean age in 74 years (SD=7.72, $n=25$) vs. 78.3 years (SD=6.82, $n=27$) in non-mutated, $p=0.040$) (Fig. 2D). No other significant associations with cholesterol (HDL, MTL), metabolic syndrome, diabetes status, ISUP grade, gender, or disease progression were detected (data not shown).

Prediction of RCC and fast-growing tumors

Patients with pathogenic mutations had three times higher risk of RCC compared to non-mutated cases (OR=3.12, 95% CI: 0.71–22.02, $p=0.17$, $n=52$), and four times the risk of fast-growing tumors (OR=4.17, 95% CI: 0.85–22.28, $p=0.08$, $n=35$). Adjusted for gender and HDL, the risk of

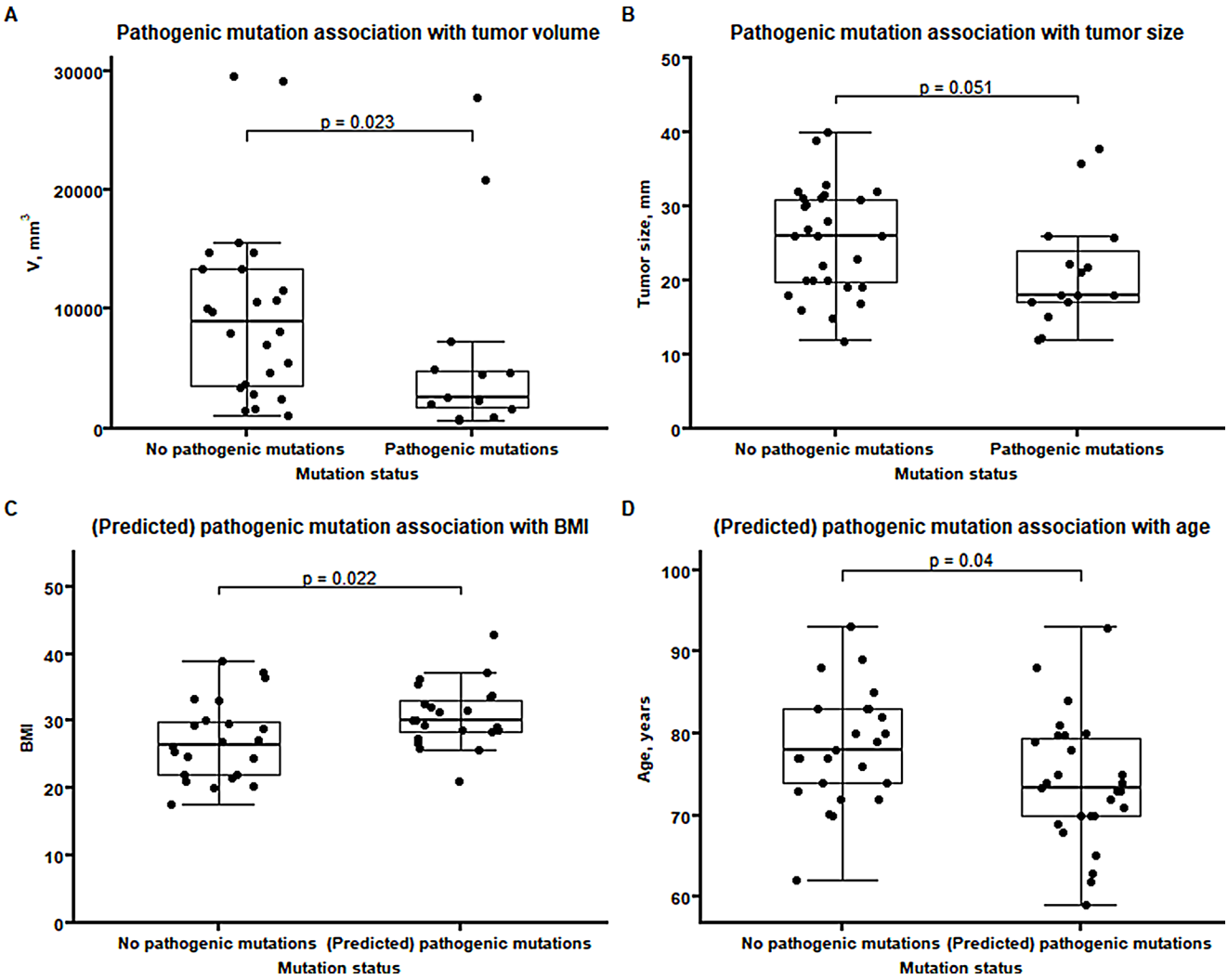


Fig. 2 Mutation association with clinical features **A** Pathogenic mutation association with tumor volume, mm^3 **B** Pathogenic mutation association with tumor size, mm. **C** Predicted pathogenic mutation association with BMI **D** Predicted pathogenic mutation association with patient age at diagnosis

RCC was almost 2.5 times higher than in non-mutated cases (OR = 2.44, 95% CI: 0.30–52.50, $p = 0.46$, $n = 45$), and 9 times higher for fast-growing tumors (OR = 9.00, 95% CI: 1.33–131.09, $p = 0.04$, $n = 35$) (Figure S1).

KRAS, *VHL*, *HNF1A*, *TP53*, and *ATM* pathogenic mutations were more likely to be detected in RCC than in benign cases ($p = 0.046$). However, it did not accurately distinguish RCC from benign cases (AUC = 0.63, sensitivity 0.26, specificity = 1) or fast-growing tumors from the slower-growth counterparts (AUC = 0.57, sensitivity 0.33, specificity = 0.81). Combining these mutations with HDL and gender improved the prediction of RCC (AUC = 0.83, sensitivity = 1, specificity = 0.62) as well as fast-growing tumors (AUC = 0.77, sensitivity = 0.89, specificity = 0.61) (Fig. 3).

KRAS, *VHL*, *HNF1A*, *TP53*, and *ATM* pathogenic mutations showed highest performance predicting RCC, while prediction sensitivity of fast-growing tumors were further improved by the addition of predicted pathogenic mutations (AUC = 0.68, sensitivity = 0.78, specificity = 0.58), and even further improved by the addition of gender and HDL biomarkers (AUC = 0.84, sensitivity = 0.89, specificity = 0.77) (Fig. 3B and D).

Discussion

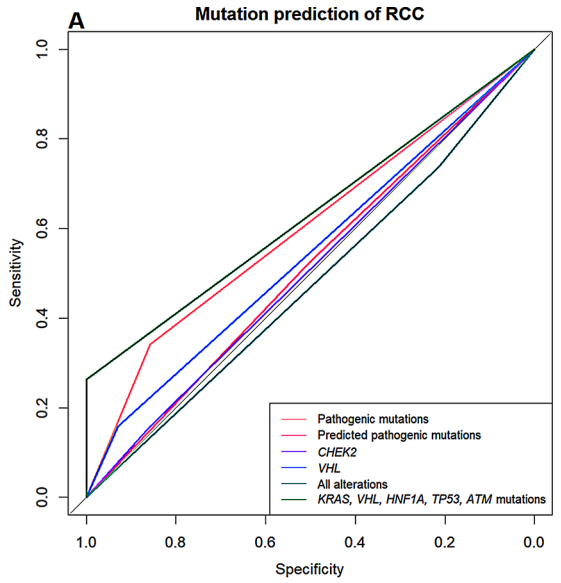
Small renal masses are asymptomatic lesions that make up more than 65% of all renal tumors (Conti et al. 2015). SRMs are a highly heterogenous entity, with up to 40% benign lesions (Johnson et al. 2015) and 45% aggressive tumors (Smaldone et al. 2012). Very little is known about SRM-specific genetic makeup, most of the information coming from large RCC studies. In this study of 52 kidney needle biopsies, 73% of SRMs were identified with non-familial RCC. Genetic alterations of proved pathogenicity were predominantly detected in RCC (13/15), while only two pathogenic variants were identified in OCT cases. The *KRAS*, *VHL*, *HNF1A*, *TP53*, and *ATM* pathogenic mutations were significantly associated with RCC cases and in combination with a risk factor of RCC (HDL) and gender were predictive of SRMs malignancy and fast growth.

Several studies have demonstrated that somatic mutations, which can be detected through kidney biopsies, are associated with pathological and clinical outcomes of kidney tumors. A few attempts of kidney tumor risk stratification using genetic biomarkers have already been made. A recent study found that ccRCC mutation status in combination with clinical features increased the prediction of metastasis free probability when compared to only clinical feature model (Mano et al. 2021). *TP53*, *BAP1* and *PBRM1* mutations have shown both independent prognostic value and improved an established risk model in patients with first-line tyrosine kinase inhibitor treated metastatic renal-cell carcinoma (Voss et al. 2018). In our study, a 5 gene pathogenic mutation signature has shown stratification of both RCC tumors from benign SRMs and fast-growing from slow-growing tumors, which was improved by the addition of features of gender and HDL which are typically associated with artery hypertension, one of the main comorbidities of kidney tumors (Hsieh et al. 2017). Metabolic syndrome or artery hypertension, of which low level of serum triglyceride HDL is an indication, is more common in males (Alipour et al. 2024). A combination of clinical and genetic predictors was able to separate RCC cases from benign SRMs with a diagnostic accuracy of 93%, which is concordant with overall morphological diagnostic accuracy achieved by kidney biopsies of > 90% (Ficarra et al. 2011).

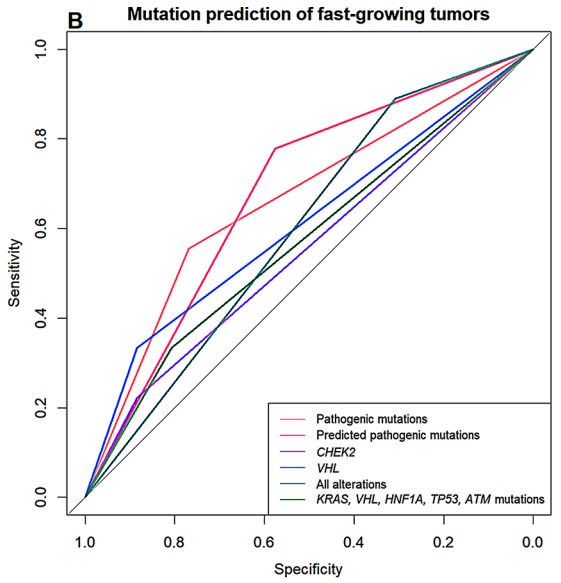
The highest prediction of fast-growing tumors (AUC = 0.84) was achieved when combining clinical predictors with mutations, predicted pathogenic with public-access knowledgebases, showing clinical value in reclassification of VUS. A recent report showed that 52% of patients with kidney disease have VUS, and 63% of patients have upgraded diagnosis and change in clinical management after reclassification (Lim et al. 2024). However, clinical variant interpretation remains challenging due discrepancies between knowledgebases and their prediction algorithms (Wagner et al. 2020), as well as labor-intensive process of functionality testing though experimental approaches. Nevertheless, efforts in VUS pathogenicity interpretations are important for improving cancer risk assessment and choosing personalized treatment plans.

An interesting association was found between pathogenic mutations and smaller initial tumor size, despite similar associations with singular gene mutation status were not observed. Although observations between somatic mutations and tumor size are rare, a pooled genomic cohort study which included 50% of pT1 ccRCC tumors also found a smaller median size of tumors with *VHL* and *TP53* mutations, while *BAP1* and *PTEN* mutations were significantly associated with larger tumors (Manley et al. 2017).

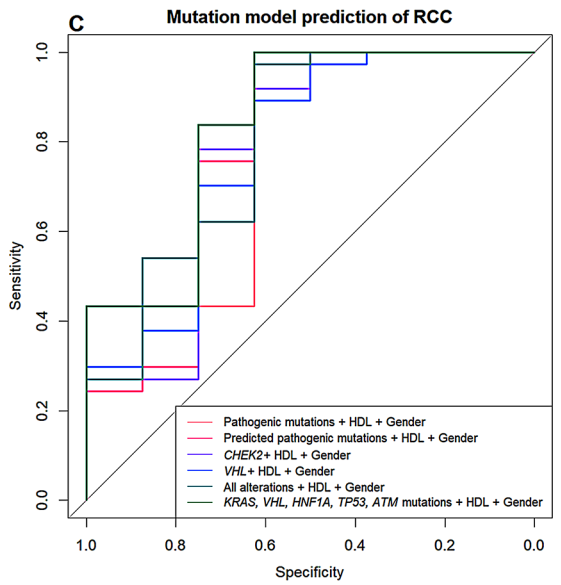
Our SRM study found a striking abundance of the DNA damage repair pathway gene *CHEK2* mutations c.470T > C (13.2% of RCC cases, and 14.3% of all benign SRMs), as well as a case of *CHEK2* deletion c.1100delC. *CHEK2* mutation c.1100delC, despite being more common than the missense variant in the United States of America, was greatly outnumbered by the c.470T > C variant. The missense variant is likely the dominant variant in the Northern Europe region, as a recent study found that 9.3% of polish RCC patients and 5% of controls has this variant (Zlowocka-Perlowska et al. 2019). Notably, 3.5% of patients with advanced RCCunselected for suspicion of hereditary syndromes harbor *CHEK2* mutations and LOH in tumor



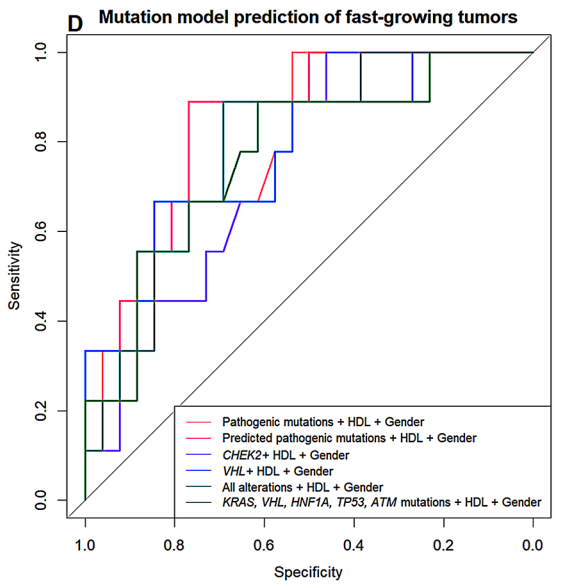
Model	AUC	Accuracy	Sensitivity	Specificity	Precision	NPV
Pathogenic mutations	0.60	0.48	0.34	0.86	0.87	0.32
Predicted pathogenic mutations	0.51	0.52	0.53	0.50	0.74	0.28
CHEK2	0.51	0.35	0.16	0.86	0.75	0.27
VHL	0.54	0.37	0.16	0.93	0.86	0.29
All alterations	0.48	0.60	0.74	0.21	0.72	0.23
KRAS, VHL, HNF1A, TP53, ATM mutations	0.63	0.46	0.26	1.00	1.00	0.33



Model	AUC	Accuracy	Sensitivity	Specificity	Precision	NPV
Pathogenic mutations	0.66	0.48	0.34	0.86	0.87	0.32
Predicted pathogenic mutations	0.68	0.63	0.78	0.58	0.39	0.88
CHEK2	0.55	0.35	0.22	0.88	0.40	0.77
VHL	0.61	0.74	0.33	0.88	0.50	0.79
All alterations	0.60	0.46	0.89	0.31	0.31	0.89
KRAS, VHL, HNF1A, TP53, ATM mutations	0.57	0.69	0.33	0.81	0.38	0.78



Model	AUC	Accuracy	Sensitivity	Specificity	Precision	NPV
Pathogenic mutations + HDL + Gender	0.79	0.93	1.00	0.62	0.92	1.00
Predicted pathogenic mutations + HDL + Gender	0.77	0.76	0.76	0.75	0.93	0.40
CHEK2 + HDL + Gender	0.78	0.78	0.78	0.75	0.94	0.43
VHL + HDL + Gender	0.78	0.71	0.70	0.75	0.93	0.35
All alterations + HDL + Gender	0.80	0.91	0.97	0.62	0.92	0.83
KRAS, VHL, HNF1A, TP53, ATM mutations + HDL + Gender	0.84	0.82	0.84	0.75	0.94	0.50



Model	AUC	Accuracy	Sensitivity	Specificity	Precision	NPV
Pathogenic mutations + HDL + Gender	0.81	0.80	0.67	0.85	0.60	0.88
Predicted pathogenic mutations + HDL + Gender	0.84	0.80	0.89	0.77	0.57	0.95
CHEK2 + HDL + Gender	0.74	0.63	0.89	0.54	0.40	0.93
VHL + HDL + Gender	0.77	0.80	0.67	0.85	0.60	0.88
All alterations + HDL + Gender	0.79	0.74	0.89	0.69	0.50	0.95
KRAS, VHL, HNF1A, TP53, ATM mutations + HDL + Gender	0.77	0.69	0.89	0.62	0.44	0.94

Fig. 3 ROC analysis of pathogenic mutations, predicted pathogenic mutations, *CHEK2*, *VHL* mutations, all detected alterations, and *KRAS*, *VHL*, *HNF1A*, *TP53*, and *ATM* pathogenic mutations predicting RCC (**A**) or fast-growing tumors (**B**) and these mutation combinations with HDL and gender predicting RCC (**C**) or fast-growing tumors (**D**). AUC – area under the curve, NPV – negative predictive value, RCC – renal cell carcinoma, HDL - high-density lipoprotein

tissues. *CHEK2* mutations pose an increased risk of RCC compared to general population with OR ranging from 2.1 to 3.6 (Carlo et al. 2018), notably higher than in our comparison with benign tumors (OR = 1.12).

In the RCC cases, besides the *CHEK2*, the other most frequently mutated gene with known pathogenic mutations was the von Hippel-Lindau tumor suppressor *VHL*. Its mutations were specifically detected in 22% ccRCC cases and one benign AML case. Somatic *VHL* mutations are present in up to 90% of non-familial ccRCC cases, however, there is no known association between *VHL* mutation status and clinical ccRCC outcomes. A similar study from a geographically neighboring Poland detected *VHL* somatic mutations in 40% (8/20) of the ccRCC cases, almost double the frequency of somatic mutations observed in our study (Marek-Bukowiec et al. 2021), while another kidney tumor study from Hungary not only detected a comparable frequency (25%, 6/24) of *VHL* mutations in ccRCC patients of whom 2 had SRMs, but also detected *VHL* mutations in two AML and one OCT patient as well (Szegedi et al. 2023).

Other notable genes with mutations specific to ccRCC belonged to the WNT signaling pathway (*APC*, *ALK*, *HNF1A*) and protooncogene *RET*. Interestingly, the WNT pathway is connected to *VHL*, and its loss enables WNT signaling, thus both *VHL* and activating WNT signaling pathway mutations may derepress the same β-catenin signaling (Majid et al. 2012). Indeed, in our study, the *VHL* and WNT signaling pathway genes mutations are not detected together. As for the *RET* mutation in ccRCC, other studies have found its gene and protein expression significantly decreased in ccRCC cases when compared to healthy kidney tissue (Van Den Heuvel et al. 2019). *RET* mutations may pose significant implications on the first-line treatment with tyrosine kinase inhibitor sunitinib of which RET is one of the targets, as approximately 20% of patients with ccRCC do not respond to this treatment and an additional 30% develop resistance within a year (Van Den Heuvel et al. 2019).

In the non-clear cell RCC cases, unique mutations were found in *KRAS*, *TP53*, and *JAK3* genes. In our study, 30% of papillary RCC cases had pathogenic *KRAS* mutations not detected in other cases. Generally, about 2% of pRCC present with *KRAS* mutations (Li et al. 2021). *KRAS* mutations may provide a possible therapeutic avenue for RCC patients with such mutations as inhibitors for *KRAS* G12 codon mutation are already approved for non-small cell lung cancer and could be repurposed for other solid tumors as well (Batrash et al. 2023). In the chromophobe RCC cases the predominant mutations were detected in *TP53* and *JAK*, which is consistent with the general frequency of *TP53* and *JAK* mutations in chRCC (previously detected in 30% and 5% of cases respectively) (Mollica et al. 2021).

There were several limitations to our study design. First, the statistical power in this study was greatly limited by the small study population. Secondly, the use of needle biopsy tissue samples may lead to undersampling of the SRMs, as these tumors may be heterogeneous and only a small percentage of the SRMs are collected (Litchfield et al. 2020). Third, 66.7% of the unique variants detected in the study were of uncertain significance. Despite our best efforts to classify these alterations using mutation variant knowledgebases, variant interpretation was greatly limited by the lack of unification in knowledgebase descriptions of pathogenicity evidence levels, as well as sparse and conflicting interpretations of VUS by the selected knowledgebases. Finally, a limitation of our study was the use of a general cancer hotspot gene panel. Further analysis of a large panel of kidney cancer susceptibility-related genes as well as analysis of non-invasive lesions may improve the sensitivity and specificity of the predictive SRMs test.

Conclusion

Our study suggests that mutation analysis in SRMs could be useful for separation of RCC cases from benign lesions, risk stratification and identification of possible therapeutic targets.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Institutional review board The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Regional Bioethics Committee No. 158200-17-952-457.

Informed consent Informed consent was obtained from all subjects involved in the study.

Competing interests The authors declare no competing interests.

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