ORIGINAL ARTICLE

Identification of Somatic Mutations in Thai Patients with Renal Cell Carcinoma Using Whole Exome Sequencing Analysis

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Background: Whole exome sequencing (WES) is increasingly used to identify genetic alterations of renal cell carcinoma (RCC). However, in Thailand, there is no report that clarifies the common somatic mutations and tumor mutational burden (TMB) in RCC. Therefore, the understanding of the tumor somatic mutational landscape could improve RCC management in the authors' country.

Objective: To perform a descriptive study to identify common somatic mutated genes and TMB in clear cell renal cell carcinoma (ccRCC), papillary renal cell carcinoma (pRCC), and clear cell papillary renal cell carcinoma (ccpRCC).

Materials and Methods: The authors enrolled 13 patients, which consisted of 10 cases of ccRCC, two cases of pRCC, and one case of ccpRCC. DNA was isolated from peripheral blood mononuclear cells and tumor tissues for WES to identify tumor somatic mutations. The results were analyzed for correlations with tumor-aggressive features and compared with the public database.

Results: The authors identified common somatic mutations in VHL, SVIL, MUC16, CSMD3, CSMD1, and BAP1 in the study patients. In ccRCC cases, VHL mutation was detected in 90% of the cases corresponding to its high frequency in the TCGA's ccRCC database. In pRCC cases, KDM6A was the only common mutated gene that overlapped with the top-ten most common genes in the TCGA's pRCC database. Somatic mutations in BAP1, SETD2, and PBRM1 were significantly associated with tumor-aggressive features in the present study. The mean TMB of ccRCC, pRCC, and ccpRCC were 2.017, 2.143, and 6.61 mutations per megabase, respectively.

Conclusion: Identification of common somatic mutations and TMB in all subtypes of RCC from the present study showed the diversity of genetic alterations between Thai patients and the public database. This leads to specific therapeutic approaches for Thai patients. Moreover, the authors also detected similar associations between significant mutations reported in prior studies with the tumor-aggressive features in the present cases.

Keywords: Renal cell carcinoma; Somatic mutation; Tumor mutational burden; Whole exome sequencing

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Renal cell carcinoma (RCC) is one of the common malignancies[1] with an age-standardized incidence and mortality rates of 4.6 and 1.8 per 100,000 persons according to the GLOBOCAN 2020[2]. The diagnosis of RCC is almost always made by cross-sectional imaging, either with or without clinical manifestations[3]. The mainstay of current treatment in localized RCC and selected metastatic RCC aims to eradicate tumors by various interventions, such as nephrectomy or thermal ablation[4], while radiation and chemotherapy have limited efficacy due to inferior responsiveness[5,6]. Recent developments of new therapeutic modalities such as targeted therapy and immunotherapy provide outstanding advantages in managing metastatic RCC[7]. Furthermore, the cancer vaccine, another novel modality, has potential benefits and may become more attractive in modern treatments of RCC[8]. All these novel treatments benefit from fundamental knowledge about cancer genomics, which helps clarify the underlying genetic aberrant of cancers[9].

Nowadays, knowledge about genetic alterations

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using whole exome sequencing (WES) plays an essential part in precision medicine and is utilized for personalized RCC management in many developed countries[10]. Regarding the Cancer Genome Atlas (TCGA) database[11], somatic mutations in VHL, PBRM1, SETD2, BAP1, and MTOR were commonly reported in clear cell RCC (ccRCC). In contrast, the most common somatic mutations in papillary RCC (pRCC) were detected in MET, KMT2C, SETD2, FAT1, and BAP1. Lastly, chromophobe RCC (chRCC) frequently contains somatic mutations in TP53, PTEN, FLT4, ZNF521, and TSC1[11]. Many mutated genes exhibit correlations to overall survival, prognosis, and treatment response, especially with immune checkpoint inhibitors[12]. Consequently, utilization of WES is expected to help identify significantly mutated genes and guide therapeutic approaches for patients with RCC. Nevertheless, the data involving common somatic mutations and tumor mutational burden (TMB) of RCC in Thai patients are still lacking.

To enhance effectiveness in RCC treatment in Thailand, the present study aimed to identify common somatic mutated genes and TMB in each subtype of RCC, including ccRCC, pRCC, and clear cell papillary RCC (ccpRCC) as a primary objective. In addition, the authors attempted to compare the common somatic alterations of RCC between Thai patients and the public (TCGA) database to seek the differences that may impact the management of RCC in the authors' country.

Materials and Methods

Study design

The present study was a descriptive study that investigated the common somatic mutations and TMB of RCC in Thai patients by WES technique. The authors performed the present study according to the Strengthening in Reporting Observational Studies in Epidemiology-Molecular Epidemiology (STROBE-ME) reporting recommendations: extended from STROBE statement[13]. The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the present study, which is to be carried out in compliance with the international guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP), IRB No. 411/63 and COA No. 752/2020. All written informed consents were acquired from all enrolled patients.

Patient selection

All patients diagnosed with RCC by clinical and radiological findings who underwent nephrectomy in the King Memorial Chulalongkorn Hospital (KCMH) between June 2020 and June 2021 were enrolled in the present study without bias. According to the final pathological diagnosis, patients with other histologic subtypes than RCC were excluded. Eventually, the absolute number of patients participating in the present study was 13 cases.

Specimen collection

Blood was collected to isolate peripheral blood mononuclear cells (PBMCs) from the participants before the operation. Germline DNAs were isolated from PBMCs. All tumor tissues were gathered immediately after nephrectomy to maintain the freshness of tissues and lessened DNA breakdown. Tumor tissue sampling was randomly performed in multiple areas and masses of the tumor to gain more tissue diversities and then rapidly preserved in liquid nitrogen during transportation to the laboratory. The remaining specimens were preserved in formalin and sent to uropathologists for subtype classification according to the International Society of Urological Pathology (ISUP) classification[14].

DNA isolation

DNA was isolated from PBMCs (germline DNA) and tumor tissue (somatic DNA) using the genUp dDNA kit (Promega, Madison, WI, USA) by following the manufacturer's instructions. The quantity of extracted genomic DNA was assessed by a fluorometric method with a Qubit device.

Whole exome capture and sequencing

Two hundred nanograms of genomic DNA were used for library preparation using the Agilent SureSelectXT reagent kit (Agilent Technologies, Santa Clara, USA). The totality of enriched library was used in the hybridization and captured with the SureSelect All Exon v5 (Agilent Technologies) baits. Following hybridization, the captured libraries were purified according to the manufacturer's recommendations and amplified by polymerase chain reaction for 12 cycles. Normalized libraries were pooled, and then DNA was sequenced on an MGC sequencing device using 2×150 bp paired-end reads and multiplexed.

Data analysis

The short reads were aligned with the human

Table 1. Patient characteristics and pathological data

Sample	Sex	Age (years)	Operation	Laterality	Histologic subtype	ISUP grade	Variant feature	LVI	TNM stage	Tumor stage	Clinical progression
RCC3	Male	59	ORN	Right	ccRCC	3		No	T1bN0M0	I	NR
RCC5	Male	66	LRN	Left	ccpRCC	1		No	T1aN0M0	I	NR
RCC6	Female	52	ORN	Right	ccRCC	2		No	T1aN0M0	I	NR
RCC8	Male	69	ORN	Right	ccRCC	1		No	T1bN0M0	I	NR
RCC9	Female	53	LRN	Left	ccRCC	3		No	T3aN0M0	III	NR
RCC10	Female	76	OPN	Left	pRCC	2		No	T1bN0M0	I	NR
RCC11	Female	71	ORN	Right	ccRCC	2		No	T1bN0M0	I	NR
RCC12	Male	72	LRN	Left	pRCC	3		No	T1aN0M0	I	NR
RCC13	Male	65	ORN	Left	ccRCC	1		NA	T3aN0M0	III	NR
RCC15	Male	42	ORN	Left	ccRCC	2		Yes	T3aN0M0	III	NR
RCC17	Female	52	OPN	Left	ccRCC	4	Sarcomatoid	Yes	T1bN0M0	I	NR
RCC18	Female	63	ORN	Right	ccRCC	3		Yes	T3aN0M0	III	NR
RCC19	Male	77	ORN	Left	ccRCC	4	Sarcomatoid, Rhabdoid	Yes	T4N0M0	IV	PD

ISUP=International Society of Urological Pathology; LVI=lymphovascular invasion; ORN=open radical nephrectomy; OPN=open partial nephrectomy; LRN=laparoscopic radical nephrectomy; ccRCC=clear cell renal cell carcinoma; ccpRCC=clear cell papillary renal cell carcinoma; pRCC=papillary renal cell carcinoma; NR=no recurrence; PD=progressive disease; NA=not available

reference genome (GRCh38) by using Burrows Wheeler Aligner (BWA). After the alignment, polymerase chain reaction (PCR) duplicates were removed using Picard MarkDuplicates. The alignments were then recalibrated and filtered by the Genome Analysis Toolkit (GATK). Varscan was then applied to identify somatic mutations by comparing tumor against normal tissues. The oncoprint diagram was created using the Maftools software.

Results

Clinical and pathological characteristics of patients

Thirteen participants with RCC were enrolled in the present study after excluding some specimens with inadequate tissue quality for DNA extraction. All participants were in the fourth to the seventh decade of life with a mean age of 62.8 years. Almost all patients had undergone radical nephrectomy, except for two patients (RCC10 and 17) who had partial nephrectomy. All tumor tissues were classified into subtypes by tissue pathology. There were 10 patients with ccRCC (RCC3, 6, 8, 9, 11, 13, 15, 17, 18, and 19), two patients with pRCC (RCC 10 and 12) and one patient with ccpRCC (RCC5) (Table. 1). RCC10 and 12 were defined as type 1 and type 2 pRCC, respectively, from microscopic findings. Interestingly, RCC9 was presumably RCC with clear cell and papillary feature. This mixed feature was challenging to characterize subtypes distinctively by uropathologists using routine microscopic examination and immunohistochemistry (IHC) evaluation.

Aggressive features that affect the prognosis of RCC, including tumor grade, variant features, tumor stage, and the presence of lymphovascular invasion (LVI) had been reported in previous articles[15-17]. Among all participants, four cases, which were RCC3, 9, 12, and 18, were noted as tumor grade III. Tumor grade IV was classified in RCC17 and 19. LVI was demonstrated in RCC 15, 17, 18, and 19. Moreover, the sarcomatoid variant was discovered in two cases, which were RCC17 and 19. RCC19 was also found to have rhabdoid variant concomitantly. A majority of participants (8/13) were in clinical stage I, which were RCC3, 5, 6, 8, 10, 11, 12, and 17, whereas four patients, which were RCC9, 13, 15, and 18, were in clinical stage III. RCC19 was the only patient in clinical stage IV at the beginning of the study (Table 1).

Summary of somatic mutations

The six most common genes containing somatic mutations among all RCC subtypes in the present study were VHL, SVIL, MUC16, CSMD3, CSMD1, and BAP1, respectively (Figure 1). In ccRCC, the most common mutated genes were VHL, MAP3K4, KIF15, BAP1, and ATM (Figure 2a). MYO16, MUC16, GABRA4, RALGAPA2, and AHNAK were the mutated genes frequently exhibited in pRCC (Figure 2b). Missense mutation and single nucleotide polymorphism (SNP) were the main variant class and type, respectively, reported in both ccRCC and pRCC. The dominant single nucleotide variation (SNV) classes were C>T in ccRCC and T>A in pRCC.

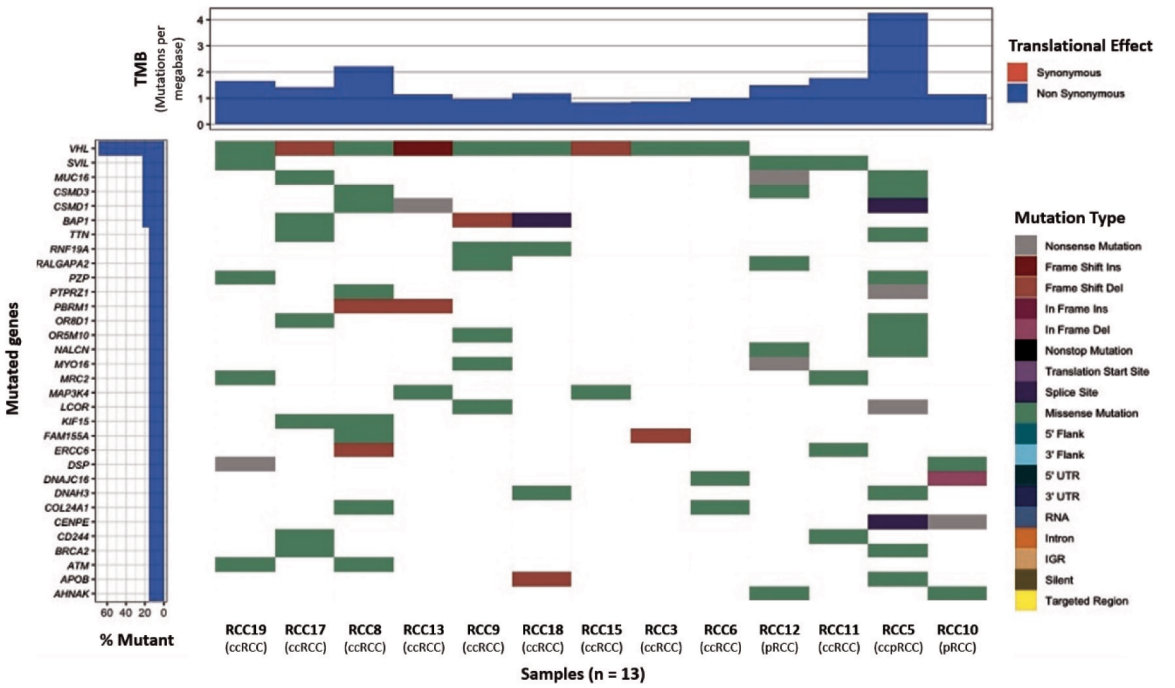

Figure 1. Diagram of common somatic mutations and tumor mutational burden (TMB) of all RCC cases.

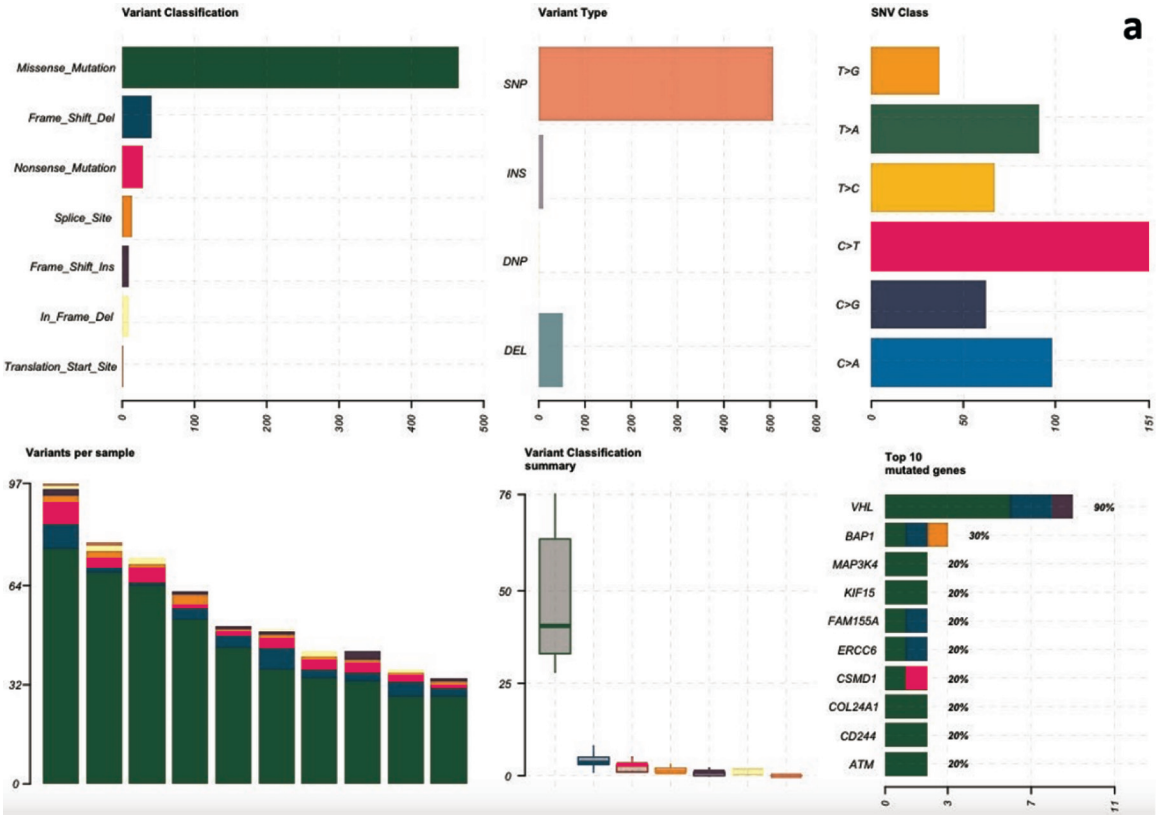

Figure 2. Bar charts of somatic mutations, (a) ccRCC, (b) pRCC.

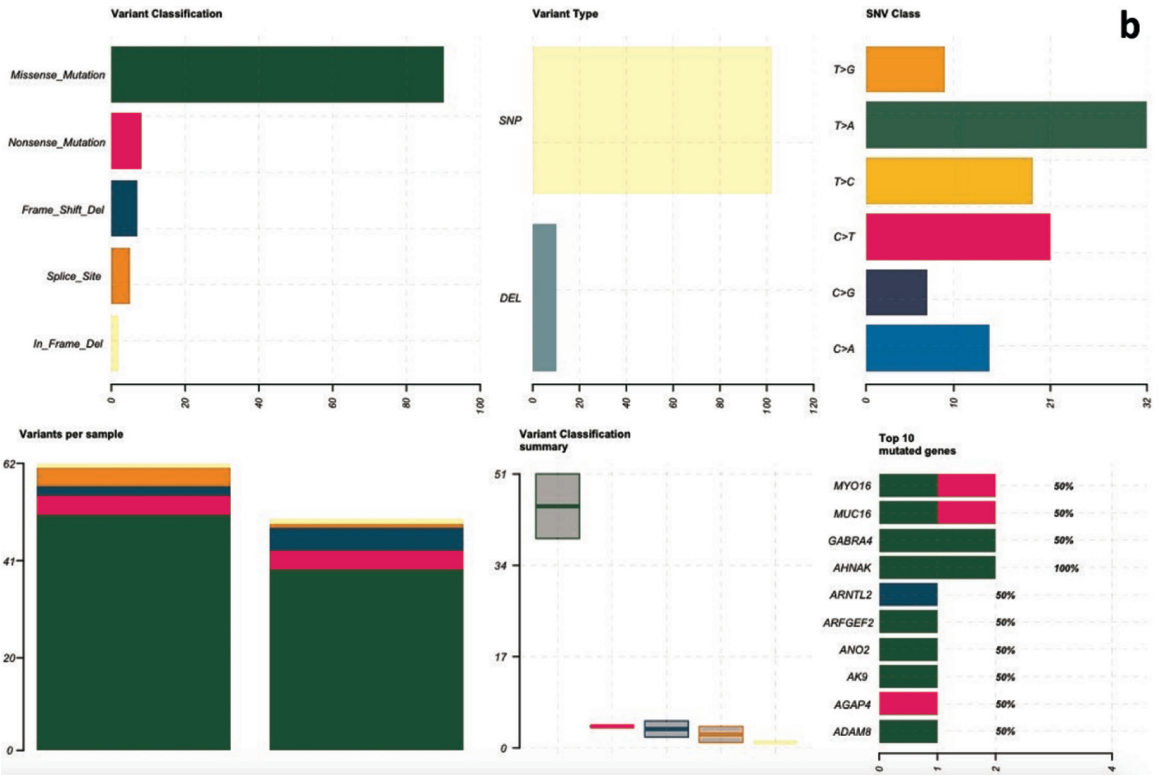

Figure 2. (continued).

Among the top-ten somatic mutated genes commonly found in the TCGA's ccRCC database, the authors identified somatic mutations in eight of these top-ten genes in the present study ccRCC cases, including VHL, PBRM1, SETD2, BAP1, MTOR, KDM5C, ATM, and ARID1A (Figure 3a). VHL is the most common mutated gene in the TCGA's ccRCC database and in the present study ccRCC cases with RCC3, 6, 8, 9, 13, 15, 17, 18, and 19. KDM6A, a gene regulating histone demethylation process, was the only somatic mutated gene discovered in the present pRCC cases, which was in the top-ten genes of the TCGA's pRCC database (Figure 3b).

Clinical stages are associated with patient survival in RCC. The higher clinical stages, the lower cancer-specific survival of the patients[18]. Additionally, numerous genetic alterations also relate to prognosis and response to therapies. One review article[19] described several mutated genes affecting the survival in RCC. BAP1 and TP53 mutations were correlated with poor survival in ccRCC[20-22], while PBRM1 and PTEN mutations were associated with worse survival in pRCC and chRCC, respectively[19,23,24]. Loss of CDKN2A was related to shortened survival in ccRCC, pRCC, and

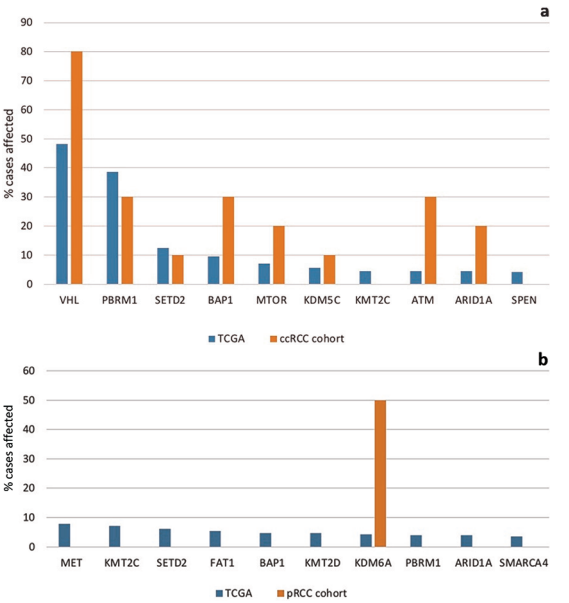

Figure 3. Bar charts of comparison of common somatic mutations between our study and the TCGA database, (a) ccRCC, (b) pRCC.

chRCC[19,25]. Another study demonstrated that SETD2 and EZH2 mutations were observed in ccRCC with

Table 2. Somatic mutations potentially related to poor prognosis in the present cases

Mutation	Sample	Type of mutation	Amino acid change
BAP1	RCC9	Frameshift deletion	p.Cys39SerfsTer29
	RCC17	Missense mutation	p.Tyr173Cys
	RCC18	Splice site mutation	
SETD2	RCC13	Frameshift deletion	p.Asp372GlufsTer111
PBRM1	RCC8	Frameshift deletion	p.Ser205ArgfsTer9
	RCC13	Frameshift deletion	p.Asp1064MetfsTer70
MTOR	RCC11	Missense mutation	p.Lys1466Glu

poorer survival and the presence of MET alteration in pRCC was associated with a better response to MET inhibitors[26,27]. MTOR mutation was also linked to poor survival in chRCC[24,28]. Those aforementioned poor prognosis-related mutations found in the present study patients were identified (Table 2) with BAP1 mutation in RCC9, 17, and 18, SETD2 mutation in RCC13, PBRM1 mutation in RCC8 and 13, and MTOR mutation in RCC11. Meanwhile, the authors did not detect TP53, PTEN, EZH2, CDKN2A, and MET alterations in the present cases. Even though VHL mutation was not associated with the prognosis of ccRCC regarding earlier articles[26,29], VHL was the most addressed mutated gene in almost all ccRCC cases, except for RCC11.

Summary of TMB

TMB is the total number of mutations per megabase detected in the DNA of cancer cells[30]. The higher TMB may indicate the higher response to certain types of immune checkpoint inhibitors such as PD-1 inhibitors[31]. In the present study, the mean TMB of ccRCC and pRCC were 2.017 and 2.143 mutations per megabase, respectively (Figure 4). Furthermore, ccpRCC showed the highest TMB at 6.61 mutations per megabase, compared to the other cases (Figure 1).

Special considerations

RCC5 (clear cell papillary RCC)

The ccpRCC is a distinctive subtype of RCC with indolent tumor behavior and favorable prognosis[32]. It is the fourth most common subtype of RCC and may arise in patients with ESRD or VHL disease[15,33]. Microscopic examination shows low grade clear epithelial cells organized in linear papillae and tubules[14], recognized in the tissue pathology of RCC5 (Figure 5a). Somatic alterations of ccpRCC were previously investigated in a few studies with small sample size. Therefore, ccpRCC was not displayed in the accessible TCGA databases. ATM

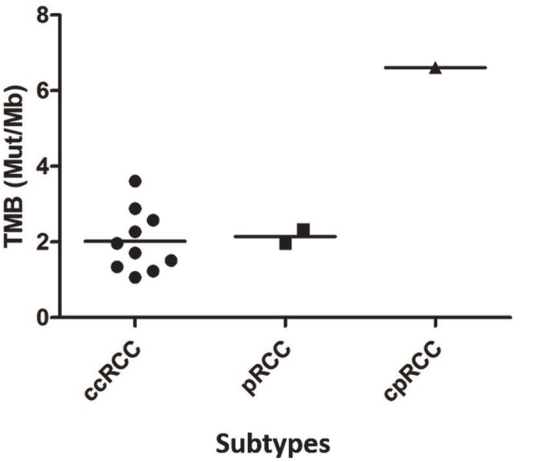

Figure 4. Box plot of distribution of TMB in each subtype.

and ASXL1 mutations were the most frequent somatic mutations reported in a prior study of ccpRCC[34], but RCC5 showed a different mutated gene pattern, including APOB, PTPRZ1, MYH13, CSMD1, and ADAM7. Resembling ccRCC and pRCC, missense mutation and SNP were mainly detected, and T greater than A was the main SNV class in this case.

RCC9 (RCC with clear cell and papillary features)

Microscopic examination of RCC9 exhibits both clear cells in acinar growth pattern interspersed by delicate fibrovascular area and prominent papillary architectures within the same tumor (Figure 5b). Further investigation by IHC reveals negative CK7, diffusely positive AMACR, and focally positive TFE3, which were still inconclusive to discriminate RCC subtypes. Although the patient had already had the radical nephrectomy, it is essential to verify the subtype of the tumor because this information may affect the treatment approaches in case of disease recurrence or progression in the future. Thus, the mutated gene pattern could be beneficial in helping the physician definitively classify RCC9 as ccRCC subtype due to the presence of VHL mutation in this case, based on the fact that VHL mutation was primarily detected in ccRCC[11].

Discussion

Common somatic mutations

In the present study, the authors explored the common somatic mutations of ccRCC, pRCC, and ccPRCC and compared the differences between the present study results and the TCGA database. VHL, PBRM1, and BAP1 were the three frequently mutated genes in the present study ccRCC cases

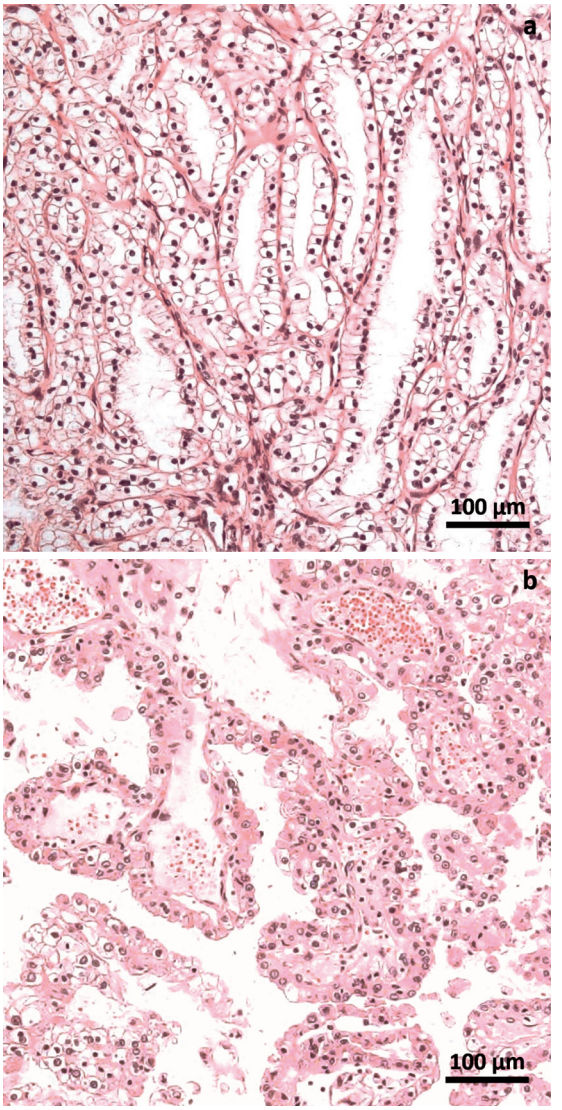

Figure 5. Tissue pathology of RCC5 (a) and RCC9 (b).

shared with the TCGA database. Remarkably, VHL was the most frequently mutated gene described in most databases[11,35,36], including the present study. On the contrary, the two cases of pRCC shared only KDM6A mutation with the TCGA database, which had no significant association with the prognosis, unlike MET and PBRM1 mutations. However, the interpretation of pRCC mutations was limited by the number of pRCC cases enrolled in the present study.

The ccpRCC (RCC5) showed a unique mutated gene pattern without ATM and ASXL1 mutations, which were repeatedly found in an earlier study[34]. Genetic alterations of ccpRCC had not yet been demonstrated to obviously play a role in prognosis[34], and the accessible TCGA database did not include ccpRCC in their analyses. Therefore, the present reports could provide additional specific information about this particular subtype.

In the case of unclear tissue pathology, many cases of RCC possibly present with more than one pathological feature, or even IHC assays are still indecisive. It is essential to differentiate ccRCC subtype from the others because the choices of preferred systemic therapy such as tyrosine kinase inhibitors and immune checkpoint inhibitors are dissimilar between each subtype. Hence, utilization of WES helps support a diagnosis of ccRCC by the presence of VHL alteration, as the authors found in RCC9.

Moreover, the four cases of ccRCC, which consisted of RCC9, 13, 15, and 18, were found as clinical stage III at the time of diagnosis, and only RCC19 was found as clinical stage IV with a clinical progression during the follow-up period. Correspondingly, the authors identified many significant genetic alterations in the present study RCC cases with high tumor aggressiveness as higher tumor grades, LVI, variant features, higher tumor stages, and poorer disease progression, which are as follows, 1) RCC9 with BAP1 mutations had tumor grade 3 and tumor stage III, 2) RCC13 with SETD2 and PBRM1 mutations had tumor stage III. 3) RCC17 with BAP1 mutation had tumor grade 4, LVI and sarcomatoid feature, and 4) RCC18 with BAP1 mutation had tumor grade 3, LVI, and tumor stage III. On the contrary, RCC19 did not manifest any remarkable mutations despite the presence of tumor grade 4, LVI, sarcomatoid with rhabdoid variants, tumor stage IV, and the poorest disease progression.

In consequence of a small number of participants, the authors were incapable of defining a solid relationship between somatic mutations and tumor-aggressive features from the present study results. However, these findings could help predicting prognosis and plan further management for the patients.

TMB

Surprisingly, ccpRCC showed the highest TMB among all subtypes, whereas TMB of ccRCC was equivalent to pRCC. For this reason, ccpRCC may be the subtype that has the outstanding response to immune checkpoint inhibitors if further investigations of ccpRCC could validate the enrichment of high TMB. Despite the results, it is too early to conclude an association between TMB with tumor subtypes or tumor aggressiveness. Nevertheless, physicians could

use TMB to plan appropriate treatment, especially anti-PD-1 therapies.

Disease recurrence and progression

Along with the entire study, the mean follow-up period was 12 months, between 6 and 20 months. Only RCC19, who had several aggressive features such as LVI, tumor grade IV, tumor stage IV, sarcomatoid, and rhabdoid variants, developed a disease progression with multiple pulmonary metastases. After receiving a course of pazopanib, he had a good response in lung nodules.

Besides RCC19, neither disease recurrence nor progression was detected in the remaining cases by surveying cross-sectional imaging at the first year of follow-up. Owing to the short follow-up period, the authors could not notice an association between previously reported somatic mutations with disease progression, recurrence, or patient survival in the present study.

Limitation

The significant limitations of the present study are a small number of participants and a short follow-up interval. Apart from ccRCC and pRCC, the authors could not gather chRCC cases, the third most common subtype of RCC. Therefore, further study including more patients and more extended follow-up period may gain more kinds of RCC subtypes and clarify some inconclusive issues encountered. In other words, the ongoing study the authors aim to perform will expectantly eliminate the limitations of the present study.

Conclusion

To summarize, WES is a helpful investigation for the modern RCC treatment. Not only ccRCC and pRCC were included in this study, but ccpRCC was also examined. The authors identified common somatic mutations in Thai RCC patients, which were distinct from the public databases. Several mutated genes such as BAP1, SETD2, and PBRM1 identified in the present study RCC cases had been reported to correlate with tumor-aggressive features. The authors recommend taking the evidence of VHL mutation from WES to facilitate subtype classification in case of uncertain diagnosis due to the high frequency of mutated VHL in ccRCC. Finally, ccpRCC exhibited the highest TMB. Therefore, ccpRCC may be one of RCC subtypes that respond well to immune checkpoint inhibitors.

Even though unclear aspects in the present study were not concluded at this time, the authors expected the commonly mutated genes reported and TMB will provide basic knowledge about RCC in Thai patients. The present study is the first step of investigating WES in Thailand.

What is already known on this topic?

According to TCGA database, there are separate common somatic mutations of ccRCC, pRCC, and chRCC, which were identified by WES. Several mutated genes exhibit correlations to overall survival, prognosis, and treatment response. However, there is no reported data in Thai patients with RCC.

What does this study add?

This study demonstrated somatic mutated genes and TMB of ccRCC, pRCC, and ccpRCC in Thai patients were different from the TCGA database. Hopefully, most practitioners can apply the present study results to guide appropriate treatment directions for their patients in current practice.

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Conflicts of interest

The authors declare no conflict of interest.

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