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The Initial Results for Identifying Distinctive Genetic Markers from the Genetic Profiling of RCC Patients

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ABSTRACT. Clear cell Renal Cell Carcinoma (ccRCC) accounts for more than 70% of cases among RCC patients and is caused by a significant number of mutations leading to health complications. However, the molecular mechanisms responsible for RCC metastasis remain poorly understood. The aim of this study was to investigate molecular genetic markers in ccRCC patients. A pilot study was conducted, including 10 patients who underwent radical nephrectomy between January 2019 and May 2021. Demographic and clinical characteristics were recorded. DNA was extracted from both tumor tissue and corresponding normal tissue samples from each patient. Exome libraries were then constructed using the Ampliseq™ Exome RDY libraries kit. Data analysis was performed using Ion Torrent Suite™ v5.12.2 and Ion Reporter™ v5.10, with variant classification and characterization of results. We identified 13 key genes: VHL, SETD2, SH3RF1, CDC27,

MUC6, LIG1, ATIC, PITPNM3, AHNAK2, ZNF717, MLXIPL, OR4C3, and PRPF4B, which were found to be mutated and potentially responsible for metastasis. Most cases (50%) showed variations in the VHL gene. In conclusion, genetic variations were identified in these genes, supporting their role in cancer progression. Further studies are needed to explore the molecular pathways associated with these genes and to identify potential therapeutic targets.

Key words: Renal Cell Carcinoma; Whole exome sequencing, Molecular genetic markers.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for about 2-3% of all cancer cases worldwide, with the highest incidence recorded in the Western countries (King SC et al., 2015). About 1.7% of all cancer mortalities are caused by RCC (Ferlay J, 2015). The disease can be diagnosed with Computer Tomography (CT) scans and is confirmed by histopathological examinations preoperatively or perioperatively (Hsieh JJ, 2008).

Additionally, the heterogeneity of RCC is a challenge as renal masses can range from benign (e.g., oncocytomas or angiomyolipoma) to clinically indolent (e.g., chromophobe RCC) to aggressive with a high potential for metastasis (e.g., high-grade clear cell RCC) (Barrisford GW et al., 2011). Therefore, RCC progresses in more than ten molecular and histopathological subtypes. The major subtypes are clear cell RCC (ccRCC), occurring at the rate of ˜75%, papillary RCC (pRCC) at ˜15% and chromophobe RCC (RCC) at ˜5%. The remaining subtypes are sporadic and account for less than ˜ 5% of the cases (Montironi R, 2020).

However, RCC could become metastasized, with about 30 % of patients diagnosed with metastatic RCC at the time of diagnosis and have a 5-year overall survival of 8% (Gupta K, 2008; Choueiri TK ,2017). While localized RCC can be successfully managed with partial or radical nephrectomy, however, metastatic RCC (mRCC) is known to be refractory to chemotherapy. Over the last decades, multiple targeted therapies have substantially improved outcomes (Vallet S, 2015). We have known treatments like inhibition of vascular endothelial growth factor (VEGF), vascular endothelial growth factor (PDGF) and mammalian target of rapamycin (mTOR), but more recently, targeted immunotherapy with PDL1- and PD1 – inhibitors are available therapeutic options for treatment of mRCC (Motzer RJ, 2006; Motzer RJ, 2015). Large scale sequencing achievements have revealed the genomic landscape of ccRCC (Sato Y, 2013; Creighton CJ, 2013). Previous research identifies multiple significantly mutated genes in ccRCC, among which *VHL, PBRM1, SETD2, KDM5C, PTEN, BAP1, MTOR*, and *TP53* were most frequently seen (Creighton CJ, 2013).

However, the patients are not cured, and the progression of the disease will, in time, lead to the death of the patient. Molecular biomarkers are still missing to predict prognosis and support for potential treatment strategies. Moreover, RCC has shown to have extremely high intratumor heterogeneity and can lead to underestimation of the tumor genomic landscape from a single tumor biopsy sample (Gerlinger M, 2013). Therefore, a better molecular understanding of RCC is needed. Here, we report the results of a pilot study using whole exome sequencing (WES) of tumor tissue and normal kidney tissue to examine the tumor genomics of clear cell RCC (ccRCC). Our aim was to investigate the genomic landscape of ccRCC in a small group of patients.

MATERIAL AND METHODS

Ethics

The study was approved by The Danish Data Protection Agency (File #: 18-000315/2017-02) and the Zealand Region Committees on Health Research Ethics (File #: SJ-608). Approval was obtained from the ethical board to review all medical records and to examine tumor tissue and healthy kidney tissue with WES.

Experimental Study

We included 10 patients who underwent radical nephrectomy in our institute between January 2019 and May 2021. All teen patients provided written informed consent. Both metastatic and non-metastatic diseases were included.

Data Collection

Demographic information, date of the surgery, histological examination, metastatic disease, lymph nodes spread, survival data and follow-up CT scans, including recurrence, were recorded retrospectively from medical records in the national Danish patient registry.

DNA extraction

Genomic DNA was extracted from Frozen dry tissue samples using the Allprep® DNA/RNA/miRNA kit (QiaGen, Inc), following the manufacturer's instructions. DNA concentration was quantified using the Qubit™ ds DNA High-Sensitive Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) on the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Library Preparation and Sequencing

All Exome libraries were prepared manually using the Ion Ampliseq™ Exome RDY libraries kit following the manufacturer's protocol (MAN0010084 E.0, Thermo Fischer Scientific, Inc) using 100 ng genomic DNA and the Ion Xpress™ Barcode Adapters 1-96 Kit (Thermo Fischer Scientific, Inc). Each library was normalized to library concentration at 100 pM using the Ion Library Equalizer™ Kit (Thermo Fischer Scientific, Inc). Template preparation and chip loading was performed on the Ion Chef™ system using the Ion 550™ kit- Chef and loaded onto an Ion 550™ Chips (Thermo Fisher Scientific, Inc) with two libraries (normal tissue and tumor tissue from the same patient) diluted to a final concentration of 50 pM. Sequencing was performed using the Ion S5XL™ Sequencer (Thermo Fisher Scientific, Inc).

Data Analysis and Variant Classification

Sequencing data from the S5XL runs were initially processed using Ion Torrent Suite™ v5.12.2 (Thermo Fisher Scientific, Inc.) and data quality-verified using CoverageAnalysis v5.12. Variant calling from the sequencing data was generated using Ion Reporter™ v5.10 with workflow AmpliSeq Exome tumor-normal pair v5.10 and hg19 as the reference genome. To eliminate erroneous base calling, a sorting filter was set with the following parameters: allele frequency

>5%; alternate allele count >10; minor allele frequency >5%; homopolymer length <5; locations in exonic, splicesite_3, splicesite_5; variant effect in missense, non-frameshift, frameshift, nonsense, stopless; and not in UCSC common SNPs. Each variant was visually examined and confirmed using the software Integrative Genomics Viewer (IGV) (Robinson JT, 2011).

RESULTS

Demographic Data and Clinical pathologies

In total 10 patients with clear cell RCC fulfilled the selection criteria for the present study. The patients undergoing radical surgical nephrectomy between January 2019 to May 2021 were involved as participants with both metastatic and non-metastatic conditions. The demographic information was collected from each patient and are presented in Table 1. One of the included patients was a

Table 1. Patient's demographic information and clinical characteristics: Pt No. Patient Number; BMI. Body Mass Index; CCI. Charlson Comorbidity Index.

Clinical Characteristics										
	Pt No.									
Characteristics	1	2	3	4	5	6	7	8	9	10
Gender	Male	Male	Male	Male	Male	Male	Female	Male	Male	Male
BMI	21	29.6	27.5	37.46	41.9	25.8	25.26	22.2	24.3	24.5
Risk factor (smoking)	Unknown	Stopped in 1986	Unknown	Former smoker	Former smoker	Smoke daily	10 pks/ year	Stopped in 1999	Former smoker	Never smoked
CCI	3	5	4	1	1	4	0	1	3	2
Histological examination	Necrosis with grade 3 (WHO proposed)	Grade 3 with no necrosis	Necrosis, Grade 3.	Grade 3 with necrosis	Grade 3, no necrosis	Grade 4, necrosis	Grade 4, necrosis	Grade 4, necrosis, sarcomatoid dedif	Grade 3, no necrosis	ccRCC with lymph node metastasis
Tumor diameter	8 cm	17 cm	7 cm	8 cm	9.2 cm	7 cm	10 cm	8.1 cm	4.5 cm	9 cm
Metastasis (lymph node spread)	Vascular invasion/ Slight progression of lungs metastasis	Growth and progression in lymph nodes	Tumor thrombus	No metastasis	Large tumor in the right kidney, no metastasis	Tumor and suspected of metastasis	No spread in lymph nodes, but CT detected Kidney metastasis	No metastasis	Benign	Small nodules and suspected lymph nodes in retroperit-noeum
Heredity	No	No	No	No	No	Unknown	No, but screened for some hereditary genes	No	No	No
Surgery, Leibovich score	Free resection, 8	Free resection, 6	Free resection margins, 6	Free resection, 6	Free resection, 5	Free resection with tumor thrombus in renal vein, 8	Free resection, 9	Free resection, 7	Free edges after surgery, 5	Metastasis after surgery, 8
CT-follow up detection	AS	AS	No recurrence and spread	No recurrence and spread	No recurrence and spread	Enlarged lymph nodes metastasis	RF ablation	No spread detected	Unchanged condition in Lungs	No recurrence and small lymph nodes detected

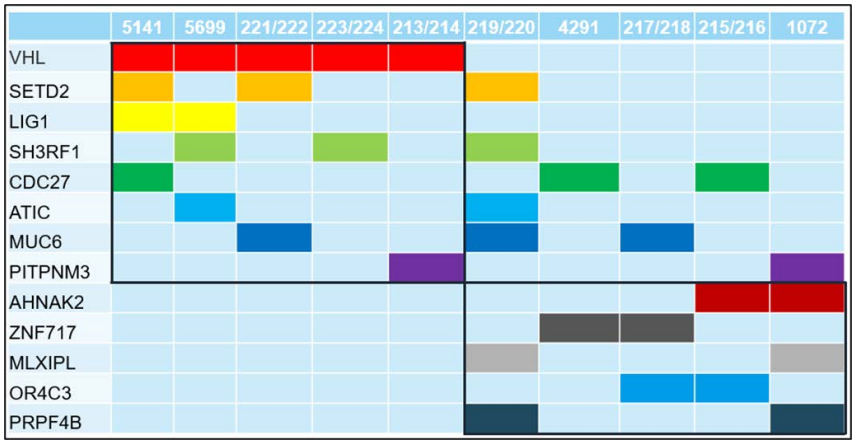

Figure 1. Represents mutations and studies variation in 13 potential genes of RCC in different samples.

female participant. The patients' average BMI was 27.9 (range: 21-41,9) at the time of diagnosis. Median age for the patients were 62,5 years (range: 49-81 years). Only one patient presented a tumor less than 7 cm (range: 7-17 cm). The data from surgical resection indicated the chances of metastasis with a Leibovich score of 8 and greater in most cases (Pichler M, 2011).

In addition, approximately two-thirds of the sample reported metastasis, with most of them reporting grade III and IV cancer stages. The patients had significant clinical indications to justify the selection and to carry out genome sequencing for profiling of RCC.

DNA Sequencing

The samples from study patients, when undergoing exome sequencing, resulted in variations of sequences with different types of variant effects and frequency of mutations. The genomic sequences from different samples were compared to sequencing results from tumor tissue and several genes were found mutated Subject 1 (39 genes), Subject 2 (74 genes), Subject 3 (66 genes), Subject 4 (43 genes), Subject 5 (39 genes), Subject 6 (107 genes), Subject 7 (13 genes), Subject 8 (39 genes), Subject 9 (57 genes), and Subject 10 (101 genes).

Summary of Mutations

Among all mutations, most of them were missense that are nonsynonymous mutations with single nucleotide alteration resulting in an amino acid change. The variation analysis was performed for 13 most top candidate genes in RCC. About 50% of the sample reported variation in the *VHL* gene, 30% reported *SETD2, SH3RF1, CDC27* and *MUC6* variations, variations in *LIG1 ATIC, PITPNM3, AHNAK2, ZNF717, MLXIPL, OR4C3,* and *PRPF4B* were reported in 20% sample. It indicates that far the most frequent variations occur in the *VHL* gene, which exceeded 20% of the mutation rate in all cases (Fig 1, Supporting Information file).

DISCUSSION

The study aimed to conduct genomic profiling of patients diagnosed with ccRCC, focusing on the molecular profiling of tissue samples (both tumor and corresponding normal tissues) through

whole exome sequencing. The results revealed significant insights into the heterogeneity of ccRCC, which aligns with existing literature. Ball et al., (2016) studied that RCC may be associated with tumor heterogeneity and that heterogeneity can be found among different lesions at the tissue necrosis site producing different effects of necrosis. The study found that ccRCC can exhibit variability in its causes and progression, with heterogeneity seen even within a single lesion, possibly due to different mutations (Ball MW, 2016).

Dizman, Philip and Pal (2020) observed that researchers are increasingly focused on understanding the role of genes involved in RCC's localization and metastasis. This focus is largely due to advances in genomic sequencing, which have revealed distinct genomic profiles in ccRCC patients.

The study demonstrated the clinical relevance of the top 13 genes responsible for causing ccRCC in study patients. The top four genes were *VHL, SETD2, SH3RF1, CDC27* and *MUC6*. Literature has also studied the *VHL* and *SETD2* as potential genes with significant alterations[18]. However, the genes such as *MUC6, CDC27*, and *SH3RF1* were not studied before. Among all cases, the most frequent alterations were seen in the *VHL* gene indicating the mutated VHL pathway as an important pathogenic pathway. In addition, our findings reported the highest percentage of mutation frequency in the *VHL* gene. The three studied mutations and variance effects were missense, frameshift deletion, and nonsense mutations. It was found that somatic *VHL* alterations are highly linked with RCC metastasis (Dizman N, 2020).

Alongside VHL, we also detected mutations within the *SETD2, SH3RF1, CDC27, MUC6, LIG1 ATIC, PITPNM3, AHNAK2, ZNF717, MLXIPL, OR4C3*, and *PRPF4B*. Farber et al., (2017) also reported the *SETD2* mutation from the molecular examination and studied variations in SETD2 pathways (Farber NJ, 2017). The *SETD2* variation was reported among the potential biomarkers of ccRCC. *SETD2* is a tumor suppressor gene accounting for variations in almost 10% of the ccRCC cases and it has been reported significantly associated to a decrease in the overall survival (Piva F, 2015). A Japanese study of mutational analysis of genes revealed that *SETD2* and *VHL* mutation is not accountable for histopathological parameters. In a Taiwanese population study, a mutation causing loss of function of both genes promoted evolution with branching of cancer cells. The impairment of these genes is associated with inherent genomic instability (Lin PH, 2021). The haploinsufficiency of *SETD2* genes interferes with the coding of the gene to histone methyltransferase affecting the DNA replication at the early phase of RCC (Lin PH, 2021). Therefore, validating the downstream roles of both *VHL* and *SETD2* is important to predict the consequences of genomic changes in these genes.

The earlier whole exome sequencing studies have also identified the genetic changes associated with *CDC27*. Our findings showed missense variations in the CDC27 gene that shown to interact with mitotic checkpoint proteins. Mendoza-Alvarez et al., (2019) proposed that somatic variations are mostly missense substitutions in the *CDC27* (Alvarez AM, 2019).

On the other hand, the literature mostly studied the genes *VHL, PBRM1, BAP1, SETD2, TP53, PTEN, KDM5C* and *TERT* for the metastasis analysis in RCC patients (Piva F, 2015; Alvarez AM, 2019). However, the present findings confirm earlier reported mutations in genes but in addition add information of variations in genes not reported before. It is of much importance that these variations must be accountable while preparing a treatment intervention for the RCC patients. For instance, treatments have been introduced for VHL-induced mutations and consequences. It was studied that the inactivation of the VHL pathway may lead to activating HIF1α and HIF2α, which

causes cell cycle progression in the ccRCC. Wallace et al., (2016) proposed an HIF2α antagonist as a potential molecular therapeutic for the treatment of such variations. The findings showed the successful inhibition of HIF2α function with the antagonist (Wallace EM, 2016). The Journal of Scientific Reports published a peer-reviewed study determining the high selectivity of candidates against the ccRCC.

According to the findings of the study, PI3Kß inhibitor termed " TGX221" selectively targeted both VHL and SETD2 altered molecular pathways. The results showed the highest selectivity with inhibition of cell mortality (Feng C, 2015). Moreover, molecular diagnostics were proposed to find personalized therapies for molecular targets, *SETD2*, *BAP1*, and *PBRM1* (Piva F, 2015). Hence, it can be suggested that genetic profiling and identification of variations in genes in ccRCC patients can facilitate possible molecular targets for drug discovery.

The study resulted in various findings associated with changes in the histological examinations in all cases. Similarly, there were differences in the BMI with some cases of overweightness. According to the literature reports, the transcriptomic signatures can be investigated in patients with ccRCC and comorbid obese conditions. In the present study, some patients have BMI over the healthy BM index (Sanchez A, 2020). The obese BMI can contribute to the pathological changes in ccRCC patients. Not only this, but the tumor in these patients reported high angiogenic scores compared to healthy weight individuals depicting that relevance of comorbid conditions is also suggested and must be studied alongside genetic markers (Sanchez A, 2020).

Our findings for the clinical characteristics also showed the presence of more than two comorbid conditions in study samples except for one or two cases. In this regard, the study of such diseases and their interference is also important since metastasis is often progressed by comorbidity. Apart from these, the study has significant strengths in its findings where it deduced mutations in the top gene candidates. Secondly, the variations and percentage of frequency of mutation indicated the intensity by which the gene can affect the molecular pathway of cancer progression. In addition, the number of different types of missenses, frameshift, nonsense, insertion and deletion mutations projected the need for studying these concepts in future research for more critical and relevant findings.

However, the study also has some limitations. Firstly, analysis of the mutation cannot be performed. Secondly, exom sequencing was performed considering only the top 13 genes for comparison. Thirdly, the downstream functions of mutated genes were not studied that can potentially be responsible for deteriorated outcomes[18]. Similarly, the expression changes in the protein profile were not studied since most of the mutations were nonsynonymous and may lead to differential protein expression. The profiling of proteins and the study of pathways is therefore important. Lastly, the primary limitation was the sample size, which may not indicate the generalizability of the findings. However, the support from evidence showed the relevance of the findings and the need for studying large samples in future studies.

CONCLUSION

The present study achieved the aim of constructing the genetic profile and genomic landscape of clear cell RCC patients. The findings revealed highly frequent mutations in several genes, with a notable similarity across samples, consistent with reported frequencies in the literature. Most cases reported mutations in the VHL gene, demonstrating the potential role of this gene. Additionally,

mutation in several other genes are reported for the first time. Future studies are needed to explore the relevance of their target role and search for the nearest molecular targets.

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CONFLICT OF INTEREST STATEMENT

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REFERENCES

Alvarez AM, Guio BG, Ortega AB. (2019). Whole-exome sequencing identifies somatic mutations associated with mortality in metastatic clear cell kidney carcinoma. *Front Genet*.

Ball MW, Gorin MA, Guner G. (2016). Circulating Tumor DNA as a Marker of Therapeutic Response in Patients With Renal Cell Carcinoma: A Pilot Study. *Clin Genitourin Cancer*. 14: e515-e520.

Barrisford GW, Singer EA, Rosner IL, Linehan WM, et al. (2011). Familial Renal Cancer: Molecular Genetics and Surgical Management. *Int J Surg Oncol*. 2011:658767.

Choueiri TK, Motzer RJ. (2017). Systemic Therapy for Metastatic Renal-Cell Carcinoma. *N Engl J Med*. 376: 354-366.

Creighton CJ, Morgan M, Gunaratne PH. (2013). Comprehensivemolecular characterization of clear cell renal cell carcinoma. *Nature*. 499: 43-49.

Dizman N, Philip EJ, Pal SK. (2020). Genomic profiling in renal cell carcinoma. *Nat Rev Nephrol*. 16: 435-451.

Farber NJ, Kim CJ, Modi PK, Hon JD, et al. (2017). Renal cell carcinoma: The search for a reliable biomarker. *Transl Cancer Res*. 6: 620-632.

Feng C, Sun Y, Ding G. (2015). PI3Kβ inhibitor TGX221 selectively inhibits renal cell carcinoma cells with both VHL and SETD2 mutations and links multiple pathways. *Sci Rep*. 5: 9465-9465.

Ferlay J, Soerjomataram I, Dikshit R. (2015). Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 136: E359-E386.

Gerlinger M, Rowan AJ, Sc B. (2016). Europe PMC Funders Group Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N Engl J Med.* 366: 883-892.

Gupta K, Miller JD, Li JZ, Russell MW, et al. (2008). Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): A literature review. *Cancer Treat Rev*. 34: 193-205.

Hsieh JJ, Thurnher M, Putz T, et al. (2008). Renal Cell Carcinoma. *Handbook of Dendritic Cells*. 3: 1117-1127.

King SC, Pollack LA, Li J, King JB, et al. (2015). Continued increase in incidence of renal cell carcinoma, especially in young patients and high grade disease: United States 2001 to 2010. J Urol. 191: 1665-1670.

Lin PH, Huang CY, Yu KJ. (2021). Genomic characterization of clear cell renal cell carcinoma using targeted gene sequencing. Oncol Lett. 21: 169.

Montironi R, Cheng L, Scarpelli M. (2020). Platinum Priority – Guidelines The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs — Part A : Renal , Penile , and Testicular Tumours. *Eur Urol*. 70: 93-105.

Motzer RJ, Escudier B, McDermott DF. (2015). Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. New England Journal of Medicine. 373: 1803-1813.

Motzer RJ, Michaelson MD, Redman BG. (2006). Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. Journal of Clinical Oncology. 24: 16-24.

Pichler M, Hutterer GC, Chromecki TF. (2011). External Validation of the Leibovich Prognosis Score for Nonmetastatic Clear Cell Renal Cell Carcinoma at a Single European Center Applying Routine Pathology. J Urol. 186: 1773-1778.

Piva F, Santoni M, Matrana MR. (2015). BAP1, PBRM1 and SETD2 in clear-cell renal cell carcinoma: molecular diagnostics and possible targets for personalized therapies. Expert Rev Mol Diagn. 15: 1201-1210.

Robinson JT, Thorvaldsdóttir H, Winckler W. (2011). Integrative genomics viewer. Nat Biotechnol. 29: 24-26.

Sanchez A, Furberg H, Kuo F. (2020). Transcriptomic signatures related to the obesity paradox in patients with clear cell renal cell carcinoma: a cohort study. Lancet Oncol. 21: 283-293.

Sato Y, Yoshizato T, Shiraishi Y. (2013). Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet. 45: 860-U191.

Vallet S, Pahernik S, Höfner T. (2015). Efficacy of Targeted Treatment Beyond Third-Line Therapy in Metastatic Kidney Cancer: Retrospective Analysis From a Large-Volume Cancer Center. Clin Genitourin Cancer. 13: e145-e152.

Wallace EM, Rizzi JP, Han G. (2016). A small-molecule antagonist of HIF2α is efficacious in preclinical models of renal cell carcinoma. Cancer Res. 76: 5491-5500.