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# Genetic alterations of clear cell renal cell carcinoma: RNA sequencing technology

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**ABSTRACT**

Clear cell renal cell carcinoma (ccRCC), the most common subtype of renal cell carcinomas, is associated with a wide range of clinical outcomes. After surgery, approximately one-third of localized ccRCC patients relapse with poor clinical outcomes. Molecular profiling provides the tumor genomic landscape, revealing novel insight into mechanism and therapeutic target for cancer. A literature review challenges the current knowledge of the molecular and genetic basis of ccRCC. Next generation sequencing indicates the most frequent ccRCC driver events including Hipple-Lindau tumor suppressor gene, histone-modifying gene, alteration in the SWI/SNF complex and the PI3K/AKT/mTOR pathway or driver somatic copy number alteration. Intratumor heterogenous landscape using the RNA sequencing technology can provide mechanisms of biological and tumor behavior in ccRCC to discover *de novo* diagnostic biomarkers in the early stage and *de novo* prognostic biomarkers in the tumor stage. More research is needed on clinical outcome to prognostic value therapeutic strategies as special subtypes for clinical decision-making. The specific molecular target can thereby be moved towards precision medicine.

**Keywords**: clear cell renal cell carcinoma (ccRCC); RNA sequencing; mutation; evolution; prognosis; therapy

## INTRODUCTION

Renal cell carcinoma (RCC) is a kidney cancer representing the 7th most frequently diagnosed cancer and the highest incidence in developed countries (Mohammadian *et al*., 2017). ccRCC, the most common histological subtype, is associated with a wide range of aggressive clinical behaviors. One-third of patients treated for localized ccRCC relapses die, and 15% die from metastatic potential (Park *et al*., 2019). Previous studies provide insights into ccRCC regarding the origin of complex genetic alterations evolution and metastatic progression (Turajlic *et al*., 2018). The Molecular classification of ccRCC indicates the inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene on the short arm of chromosome 3p. Additionally, other driver mutations on tumor suppressor genes, including *polybromo 1* called PBRM1, *BRCA1-associated 1* called BAP1, and *SET domain-containing 2* called SETD2, PI3K/AKT/mTOR pathway mutations, or driver somatic copy number alteration called SCNAs involved in pathogenesis of ccRCC. Recently, molecular profiling of intra-tumor genetic heterogeneity does not play an important role in treatment decision-making processes and clinical management. The molecular characteristics of ccRCC in terms of transcriptome sequencing and evolutionary pathways, which are required for discovery of accurate genomic markers, are still underinvestigated. Hence, this study aims to review ccRCC in terms of molecular biology with RNA sequencing technology.

### Epidemiology

Renal carcinoma (or RCC) is one of the most common malignant tumors of the urinary system, which accounts for approximately 90% of renal malignancies. The patient with RCC is diagnosed in more than 200,000 individuals worldwide each year (Campbell *et al*., 2017; Ljungberg *et al*., 2019). By utilizing the Surveillance, Epidemiology, and End Results (or SEER) database, the overall incidence rate of ccRCC patients between 1973 and 2014 in the United States was estimated to be 3.59 cases per 100,000 population. The age of 60 to 79 years (13.61 cases per 100,000 population) is the advancing age at diagnosis. Males have a significantly higher incidence rate of ccRCC than females. The primary origin of ccRCC on the left side was slightly lower than the primary origin on the right side and grade II had the highest incidence rate of ccRCC: 1.35 cases per 100,000 population (Ljungberg *et al*., 2019).

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In Thailand, the data from Ramathibodi Hospital Cancer Report 2018 showed that 81 cases of individuals were diagnosed as kidney cancers. The majority of kidney cancers are renal cell carcinomas (54 cases), 36 (66.7%) males and 18 (33.3%) females (Wilailak *et al*., 2018).

**Genetic alterations**

The most common genetic alteration related to ccRCC development is loss of the short arm of chromosome 3 (loss of 3p). This alternation is approximately 95% of ccRCC patients (Ross *et al*., (1989). Genetic factors contribute to the development of sporadic RCC (Maher *et al*., 2018; Rossi *et al*., 2018). Mutations in the *VHL* gene underlying VHL disease is characterized to be an increased risk factor of developing ccRCC (Kaelin, 2007). Several novel mutations in *PBRM1*, *SETD2*, *BAP1*, *KDM5C* and *MTOR* were identified to be involved in disease progression. Six susceptibility loci on chromosome regions 2p21, 2q22.3, 8q24.21, 11q13.3, 12p11.23, and 12q24.31 were discovered through genome-wide association studies (GWAS) (Henrion *et al*., 2013; Purdue *et al*., 2011; Wu *et al*., 2012). The locus 2p21 mapped to *EPAS1* encoding the HIFα subunit of transcription factor and the 11q13.3 locus seems to change the regulatory function of *CCND1* (encoding cyclin D1) in cell cycle regulation (Schödel *et al*., 2012). The 12p11.23 locus has a role in the regulation of *BHLHE41* (encoding basic helix-loop-helix family member e41) (Bigot *et al*., 2016). The other GWAS susceptibility loci need further identification.

**VHL deficiency**

The *VHL* tumor suppressor gene, which is located at 3p25, undergoes bi-allelic knockout (causing point mutations, insertions and deletions and 3p25 loss) and/or epigenetic modification (promoter methylation) in the majority of ccRCC (Gnarra *et al*., 1994). Over 95% of VHL haploinsufficiency occurs via arm-level loss of chromosome 3p in childhood or late adolescence. Accumulation of genetic mutations arises for decades prior to diagnosis (Mitchell *et al*., 2018) (Figure 1) Either non-synonymous mutations or epigenetic down-regulation of *VHL* gene alterations usually occur at the late onset of the disease (Cancer Genome Atlas Research, 2013; Mitchell *et al*., 2018; Sato *et al*., 2013; Scelo *et al*., 2017; Scelo et al., 2014; Turajlic *et al*., 2018). For the VHL-HIF pathway, the VHL protein (pVHL) controls levels of the HIF transcription factors in an oxygen-dependent manner (Gossage *et al*.,2015). Under normoxic conditions, HIF-α is hydroxylated on two conserved proline residues and binds the pVHL. The hydroxylation process plays a role in rapid degradation of HIF-α via the ubiquitin proteasome pathway. The reaction involves the biding of proline-hydroxylated HIF-α to VHL, which turns out to be a target for proteasomal degradation by ubiquitylation (Gossage *et al*., 2015).

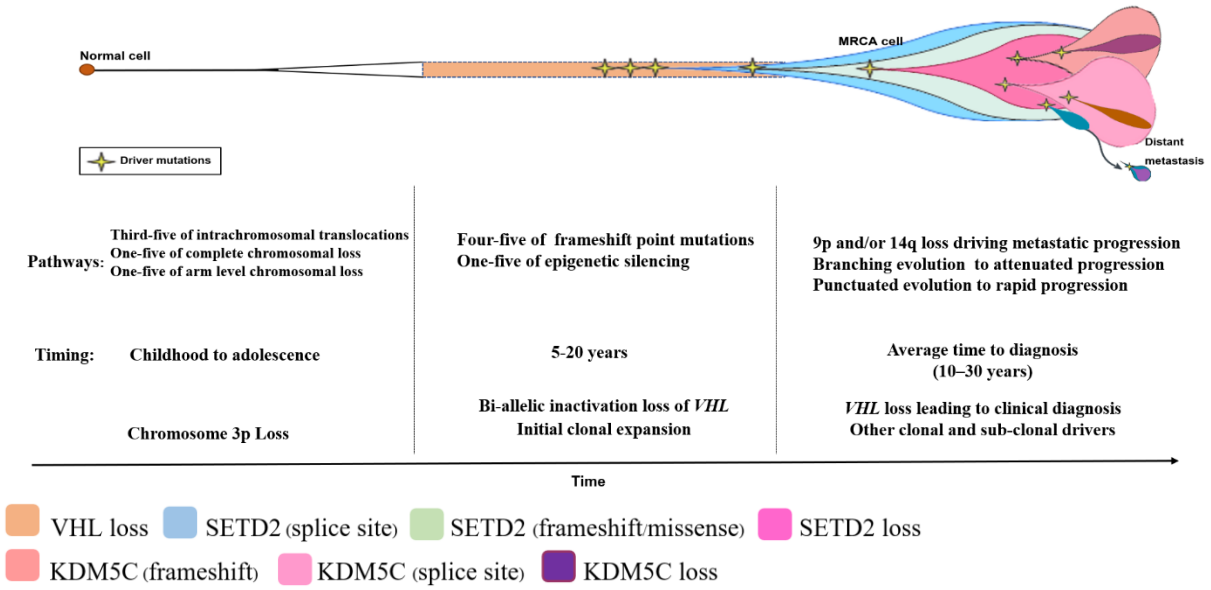


**Figure 1** Timeline for ccRCC genetic alterations [modified from Mitchell *et al*., (2018)].

In the absence of pVHL or under hypoxic circumstances in blood, HIF-α does not undergo hydroxylation at these critical proline residues. The absence of hydroxyl groups stabilizes the HIF-α complex, allowing it to promote binding to the HIF-β complex rather than pVHL to inactivate mutations in VHL also causes the HIF-α complex to stabilize, resulting in the formation of a HIF-α/β complex molecule. The evidence points to the likelihood that the transcriptional complex relocates to the nucleus in the cell, whereas, it activates gene expression of the HIF target. It is recognized that the extracellular complex proteins containing glucose transporter 1, epidermal growth factor receptor, vascular endothelial growth factor A, and others have substantial mRNA expression. This implies that the molecule complex enhances cell proliferation, angiogenesis, glycolysis, invasion, and metastasis at a lower rate of apoptosis and a significant rate of tumorigenesis compost of cell proliferation, angiogenesis, glycolysis, invasion, and metastasis (Harris *et al*., 2002) (Figure 2). The pathogenesis of ccRCC tumors, as well as prognostic or predictive biomarkers, are still unknown. VHL mutations have little effect on clinical characteristics, according to previous studies, whereas other mutations are linked to disease development and clinical results (Hakimi *et al*., 2013; Kapur *et al*., 2013; Nam *et al*., 2015).

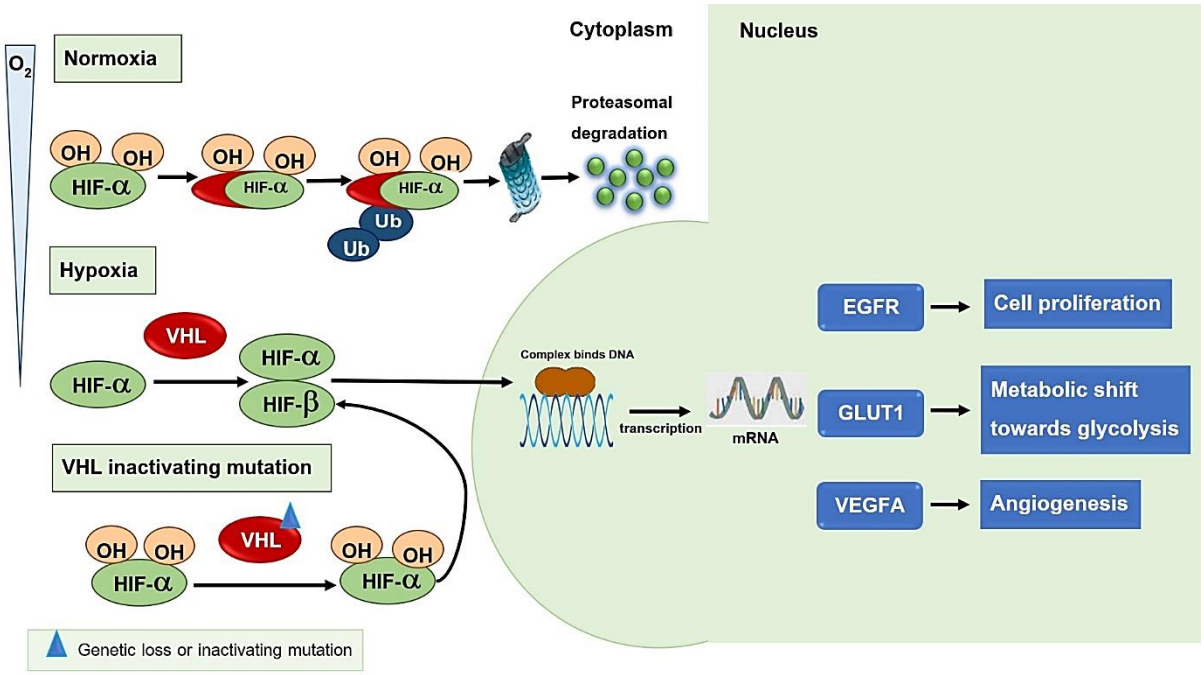


**Figure 2** The VHL-HIF pathway in ccRCC.

Under regular oxygen requirements, VHL promotes HIF-α degradation by binding to the ubiquitin-ligase complex, which ubiquitylates HIF-α and tags it for proteasomal degradation. In hypoxic conditions, the absence of hydroxylation inhibits VHL from recognizing HIF-α, allowing it to stabilize and dimerize with HIF-α. The HIF-α/β complex that was formed then leads to the activation into the nucleus, where it induces the expression of genes with hypoxia-response elements in their promoters. VHL deficiency, which is widespread in ccRCC due to genetic or epigenetic defects, results in HIF-α accumulation. Hypoxia-responsive genes are deregulated as a result, subsequently promote tumor growth and aggressive behavior.

**Malfunction of epigenetic machinery**

In ccRCC, the *VHL* gene is a common initiating mutation. Other genes reported to be involved in ccRCC include *PBRM-1* (29-41%), *BAP-1* (6%-10%), *SETD-2* (8%-12%), and *KDM5C* (4%-7%), which are involved in chromatin remodeling and histone modification and function as tumor suppressors (Hsieh *et al*., 2017). Following the loss of 3p, these haploinsufficient genes are completely inactivated by a non-synonymous mutation.

*PBRM-1* gene encoding for Protein polybromo-1, a methyltransferase, is reported to contain the second most common mutation. Protein polybromo-1, also known as BAF180, is a tumor

suppressor protein that is a subunit of the nucleosome remodeling complex. BAF180 controls gene expression by accessing the condensed part of the DNA. Mutations in the *PBRM-1* gene would result in an abnormal/malfunctioning BAF180 resulting in unchecked cell growth and subsequent tumorigenesis and correlated with tumor invasiveness (Brugarolas, 2013; Hakimi, *et al*., 2013). Moreover, data from both human cancer genomics and therapeutics support a three-driver event orchestrating the step-by-step pathogenesis of ccRCC, starting VHL loss (1st), PBRM1 loss (2nd), and MTORC1 activation (3rd) (Hsieh et al., 2017; Nargund et al., 2017).

BRCA1 associated protein-1 (or BAP-1), a histone deubiquitinase, is encoded by a tumor suppressor *BRCA-1*gene. BAP-1 plays an important role in cell proliferation and regulation of DNA repair mechanisms. It interacts with a transcription factor known as host cell factor-1 (HCF-1) that binds to a number of transcription factors. Previous research has shown that a high-risk BAP-mutant group of ccRCC has a distinct clinical profile. (Kapur *et al*., 2013; Peña-Llopis *et al*., 2012; Shankar and Santagata *et al*., 2017; Wu and Lu *et al*., 2010).

SETD2 protein is an enzyme that catalyzes histone H3 lysine 36 trimethylation, H3K36Me3. SETD2 participates in intra-tumoral heterogeneity and convergents evolution and regulates transcription elongation, RNA processing, and double-stranded DNA break repair. *SETD2* mutation is associated with worse cancer-specific survival and metastasis. It promotes tumor progression via replication stress and impaired DNA repair, which may activate the p53-mediated checkpoint in the absence of specific p53 mutations (Carvalho *et al*., 2014). *SETD2* mutations are found in 10% of ccRCC primary tumors and increases to 30% in metastatic ccRCC. In addition, *SETD2* mutations are frequently found together with *PBRM* mutation, resulting in cooperating mutations (Peña-Llopis *et al*., 2013).

*KDM5C* gene, located on Xp11.22-p11.21, embeds a histone demethylase that removes a metyl group from lysine 4 on histone H3 or H3K4Me3. *KDM5C* mutations occurred mainly in male patients (Arseneault *et al*., 2017). *KDM5C* mutations tend to co-occur with *PBRM1* mutations, whereas both *KDM5C* and *PBRM1* mutations tend to occur with *BAP1* mutations (de Cubas *et al*., 2018). H3K4Me3 is a histone label associated with genes that are actively transcribed and plays a role in transcriptional initiation (Hsieh *et al*., 2017).

*PTEN* gene encodes a dual lipid, the tyrosine phosphatase and tensin homolog. *PTEN* maps to chromosome 10q23.3 and plays a role as a dormant tumor suppressor that controls signaling through the phosphatidylinositol-3 kinase or PI3K)/Akt, cell signaling pathways involved in cell growth regulation, proliferation, apoptosis, and cell cycle. Loss of the *PTEN* gene function is related to tumor progression and adverse outcomes. In ccRCC, *PTEN* mutations may influence disease progression, prognosis, and drug selectivity. Therefore, the loss of *PTEN* expression may cause the initiation of tumorigenesis. Moreover, the *PTEN* mutation mechanism in ccRCC needs to be further analyzed by molecular experiments and additional studies involving clinical outcome to prognostic value and development of therapeutic strategies as special ccRCC subtypes. (Fan *et al*., 2019).

*mTOR* is a component of the *PI3K/Akt* signaling pathway, which is associated with the regulation of protein translation, cell growth, proliferation, and metabolism. Previous studies demonstrated highly clustered in small region of ccRCC conferring *mTOR* hyperactivation (Guo *et al*., 2015). Interestingly, *PI3K/Akt/mTOR* signaling is correlated with aggressive outcomes and poor prognosis in RCC. Overexpression of activated growth factor receptor and mutations in *PI3K/Akt* can be occurred by hyperactivity of *mTOR* signaling. *mTORC1* is a crucial regulator that works downstream of the PI3K activator and the *TSC1/TSC2* repressing signals. Patients who responded to mTOR inhibitors had more somatic mutations in mTOR pathway genes, such as *MTOR*, *TSC1*, and *TSC2*. *mTOR* has presented itself as a valid target for the treatment of cancer in RCC (Pal & Quinn, 2013). Moreover, TCGA dataset showed that the VHL/HIF and PI3K/AKT pathways have elevated rates of gene mutations or deletions of *PBRM1*, *SETD2*, *BAP1*, and *KDM5C* (Guo *et al*., 2015). The involvement of the VHL/HIF and PI3K/AKT pathways in ccRCC chromatin remodeling regulation is uncertain, and it would be a fascinating subject for research.

Tumor protein p53, also known as TP53 or p53, is encoded by *p53* gene on chromosome 17p13.1. Tumor protein p53 is involved in cell cycle checkpoints, DNA repair, cell cycle arrest, and apoptosis. ccRCC patients had low frequency (2.2%) of TP53. *p53* positive expression is associated with high grade, advanced stage, or distant metastasis in RCC patients. The International Agency for Research on Cancer (IARC) Database reported that the median

disease survival time in cases without TP53 mutations was longer than those with TP53 mutations (Li et al., 2019). However, further experiments are needed to increase the predictability of TP53 mutations and ccRCC survival and disease-free survival times.

Telomerase reverse transcriptase (or *TERT* gene), which encodes telomerase's catalytic subunit, is a ribonucleoprotein complex that ensures genomic integrity. *TERT* gene is located on chromosome 5p15.33. Activating somatic mutations in the core promoter and *5'UTR* of telomerase reverse transcriptase (*TERT*) in 6-14% ccRCCs. *TERT* promoter mutations were associated with poor disease-specific survival and increased tumor progression, and may be applied as a marker to define a small subset of tumors with aggressive behavior. *TERT* promoter mutations generate binding motifs for the E-twenty six (Ets)/ternary complex factors (or TCFs) transcription proteins. VHL inactivation was associated with accumulation of hypoxic inducible factors (or *HIFs*) leading to enhanced *Ets* expression. Significantly higher levels of *TERT* mRNA in *TERT* promoter mutation result in poorer prognosis than in those lacking the mutation in ccRCC (Hosen *et al.*, 2015; Mitchell *et al*., 2018).

**Peroxisome proliferator-activated receptors**

Peroxisome proliferator-activated receptors (or PPARs), which are ligand-activated receptors and a member of the nuclear hormone receptor super-family, are composed of the following three subtypes; PPARα, PPARγ, and PPARß/γ. The PPAR family of nuclear receptors related to energy homeostasis and metabolic function. Activated PPARs are also involved in transcriptional repression through DNA-independent protein-protein interactions with other transcription factors. Some studies report that *PPARα* and *PPARγ* are associated with ccRCC, providing a potential diagnostic and prognostic biomarker (Luo *et al*., 2019).

*PPARα* gene is located on chromosome 22q12. 2-13.1 and plays a crucial role in lipid metabolism. *PPARα* has prognostic significance in ccRCC. The expression of *PPARα* in ccRCC was related to stages, gender, tumor size, dimension, grade and clinical stage. The *PPARα* gene is linked to the *mTOR*, *AKT*, and *Wnt* signaling pathways. Down-regulation of *PPARα* expression correlates with poorer survival and progression. Although *PPARα* may promote ccRCC progression, further investigation is needed to elucidate the understanding of molecular mechanisms and VHL correlation with ccRCC prognosis.

*PPAR-γ* gene, located on chromosome 3p25 nearby the *VHL* gene in 3p25-26, plays an essential role in carcinogenesis as a tumor suppressor by promoting cell differentiation and activating increased cell apoptosis. Previous studies showed that *PPAR-γ* can modulate *PTEN* gene transcription, collaborate with *PTEN* to induce cell cycle arrest and become an attractive anticancer mechanism. *PPAR-γ* and *PTEN* may be prognostic biomarkers for RCC. The evidence suggests that high expression of *PPAR-γ* in RCC may induce *PTEN* transcription and provide a new targeted therapy for RCC. This data in human ccRCC cell lines suggests that *PPAR-γ* ligands induce apoptosis and cell cycle arrest, and decrease the production of potent angiogenic factors such as VEGF and FGF. However, the role of *PPAR-γ* and *PTEN* transcription in ccRCC is still unknown. Understanding of *PPAR-γ* and *PTEN* may be a promising new potential target for ccRCC treatment (Collet *et al*., 2011; Luo *et al*., 2019).

**Association between programed cell death-1 and somatic mutations**

Immune checkpoint blockade targeting programmed cell death-1 (or PD-1) receptor is a T-cell receptor and its ligands (PD-L1and PD-L2) are expressed on the surface of activated T cells, B cells, natural killer T cells, dendritic cells and tumor cells. PD-1 regulates effector T cell activity and plays a role in immune resistance in the tumor environment. RCC patients with PD-1 and PD-L1 expression show tumor infiltrating lymphocytes (TIL) leading to developing larger tumors, higher grade tumors, advanced stage, and sarcomatoid (McDermott *et al*., 2013). It appears that involvement with PD-1 on T-cells by its ligand causes downregulation of antigen driven immune responses. Previous studies in ccRCC show PD-1 and PD-L1 expression are significantly associated with clinical features. PD-L1 expression was found in two-thirds of ccRCC, and those with high expression had a lower cancer-specific survival rate. Furthermore, positive PD-L1 is related to aggressive clinicopathological features in patients with sporadic and VHL-associated hereditary ccRCC. It is reasonable that PD-L1 is a promising biomarker for predicting PD-1/PD-L1 checkpoint inhibitor in immunotherapy ccRCC (Hong *et al*., 2019; Ueda *et al*., 2018). PD-1 inhibitor immunotherapeutic drugs are recommended in several reports, such as Lenvatinib and Pembrolizumab in metastatic ccRCC (Taylor *et al*., 2020). However, VHL-associated hereditary ccRCC and prognostic clinical correlation require further exploratory study.

**PD-L1 association with TFE3 and HHLA2**

***PDL1 and TFE3 relation***

TFEB, TFE3, TFEC, and MITF are members of the MiT-TFE family of simple helix-loop-helix leucine-zipper transcription factors. They play critical roles as lysosome biogenesis regulators, controls cellular energy homeostasis and immune responses, and were thus initially classified as oncogenes (Raben *et al*., 2016). TFE3 and TFEB, which are transcription factors, have increased expression and activity in a variety of human cancers, and this has been linked to increased cancer cell proliferation and motility. Consequently, chromosomal translocation events that result in TFEB or TFE3 fusion and overexpression are linked to a poor prognosis in a subset of RCC patients with high recurrence and metastasis rates (Kauffman *et al*., 2014). TFE3 has intrinsic effects on cell proliferation and survival in ccRCC, but not TFEB. This corresponded to patient prognosis. The discovery of TFE3-regulated genes in ccRCC cells may lead to a better understanding of TFE3 functions in the regulation of ccRCC tumorigenesis and the interaction of ccRCC cells with the immune microenvironment. TFEB has been shown to mediate immune evasion in RCC by positively regulating PD-L1 expression (Zhang *et al*., 2019). According to a recent study, TFE3, like TFEB, is a potent tumor promoter due to its significant proliferative effect. Importantly, it promotes PD-L1 expression in ccRCC (Guo *et al*., 2020).

***PD-L1 and HHLA2 relation***

Human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2), also known as B7-H7, is a recently discovered B7 family member that is related to PD-L1, PD-L2, and B7-H3 (Zhao *et al*., 2013). HHLA2 induces CD4+ and CD8+ T angiogenesis and cytokine production by binding to its putative receptors in a wide range of immune cells (Zhao *et al*., 2013, Cheng *et al*., 2018). To date, the only HHLA2 receptor that has been identified is the transmembrane and immunoglobulin domain containing protein 2 (TMIGD2) (Janakiram *et al*., 2015). Because TMIGD is primarily expressed on navie T cells but not mature T cells, it is possible that HHLA2's immunosuppressive role is mediated by other unidentified molecules (Zhu *et al*., 2013). TMIGD2 was discovered in endothelial cells as well, implying that HHLA2 may play a role in tumor angiogenesis (Rahimi *et al*., 2012). HHLA2 has also been found to be overexpressed in RCC when compared to normal renal tissue, and its expression has been linked to a poor prognosis of RCC. (Chen *et al*.,2019). According to a recent study, co-expression of HHLA2/PD-L1 has a significant prognostic value for ccRCC patients' progression-free and overall survival (Zhou *et al*., 2020).

**Intratrumoral heterogeneity**

High levels of intra- and inter-tumoral heterogeneity in ccRCC were caused by clonal expansion and parallel evolution triggered by initiating genetic events. With multiregional genetic sequencing, at least seven distinct evolutionary patterns of ccRCC tumorigenesis are associated with clinical phenotypes and patient outcomes. The variety of cell populations inside the tumor leads to a wide range of therapeutic sensitivity responses depending on intra-individual genetic heterogeneity. Through exome sequencing technology, ccRCC has been shown to have intra-tumoral heterogeneity. On multiple spatially separated samples from primary renal carcinoma and associated metastatic sites, chromosome aberration analysis and ploidy profiling were performed. Multiple genes of intra-tumoral mutational heterogeneity are demonstrated on *PBRM1*, *BAP1*, *SETD2*, *PTEN*, *PIK3CA*, and *KDM5C*. These genes have undergone multiple independent mutations inactivating distinct clonal populations within a single tumor. Several low frequency single-nucleotide variants, including two separate clonal expansions with distinct VHL mutations, were discovered using next generation sequencing. These techniques increase the likelihood of several VHL mutations in a single tumor. The classification of VHL missense mutations is critical in driver and passenger mutations. These events can be used to forecast the effect on protein structure and function in order to assess therapeutic response. The missense mutations are divided into three groups: (i) pVHL is severely disrupted, (ii) interactions with HIF-α, elongin B, and elongin C have no destabilizing effects on pVHL, and (iii) the roles of pVHL are comparable to those of the wild type (Okumura *et al*., 2016; Rechsteiner *et al*., 2011).

**Trajectories of evolution**

A variety of clonal and subclonal driver cases describe evolutionarily distinct subtypes. With analysis of multiregion sequencing, patterns of evolution can classify tumor subtypes by altering chromosomal complexity and genetic diversity shown in table 1. SCNAs affect a small portion of the genome and are used to assess chromosomal complexity and are pronounced as weighted genome instability index (or WGII). Genetic variation or intratumor heterogeneity

(or ITH) is evaluated as the proportion of clonal to subclonal drivers which is expressed as an ITH score differing metastatic abilities.

Primary tumors with low chromosomal complexity and low genetic diversity have a low overall metastatic potential and evolve in a linear pattern with a VHL monodriver event. Primary tumors with high ITH and high genomic instability, follow an attenuated progression to solitary metastases, resulting in a branced evolutionary pattern. Early *PBRM1* mutations are frequently found in this mode of evolution, followed by subclonal SCNAs, mutational activation of the PI3K-AKT-mTOR pathway, or *SETD2* mutation.

Primary tumors with multiple driver mutations (defined as two *BAP1*, *SETD2*, *PTEN*, or *PBRM1* clonal mutations) had high levels of wGII with low ITH, resulting in a punctuated evolutionary behavior.This pattern is connected with early metastasis, rapid disemination, and poor overall survival, resulting in aggressive growth dynamics.Clonal selection of subclonal primary alterations is found to be rich in all matastatic sites with enrichment for both chromosome 9p21.3 loss and 14q loss in a late event in tumor evolution.These occurrences have the potential to hasten the progression of metatstic disease and increase overall mortality (Mitchell *et al*., 2018; Turajlic *et al*., 2018)

**Table 1** Trajectories of evolution in ccRCC [modified from Tippu *et al*., (2016); Samra *et al*., (2018); Christopher *et al*., (2018).

| Evolutionary subtype | Primary tumor | Genomic characteristics | Metastatic potential |
|---|---|---|---|
| Punctuated (more aggressive) | Multiple Clonal Drivers (VHL and in two or more of genes, BAP1, SETD2, PTEN or PBRM1) | high: wGII low: ITH | - rapid progression to metastasis in multiple tissues - poor overall survival |
| | BAP1-driven | | |
| | VHL wildtype | high: wGII | |
| Branched (less aggressive) | PBRM1-PI3K- driven | high: wGII high: ITH | - attenuated progression to solitary metastasis - improved overall survival |
| | PBRM1-SETD2-driven | | |
| | PBRM1-SCNA-driven | | |
| Linear (less aggressive) | VHL mono-driver | low : wGII low: ITH | - non-metastatic |

wGII: weighted genome integrity index, and ITH: intratumor heterogeneity

*VHL* mutations are almost universally preceded by mutations in *PBRM1*, *SETD2*, *PI3K*, *SCNA* and *BAP1*. The subtype of VHL mono-driver exhibits linear evolution, which is characterized by low wGII and low ITH. Branched evolution occurs as a result of a series of highly ordered events in which *PBRM1* mutation occurs before *SETD2* or mutations in *PI3K* or acquisition of SCNA. These pathways of evolution result in a slower disease progression with oligometastatic metastasis patterns. Punctuated evolution is triggered by the clonal acquisition of multiple driver mutations (which is involved with VHL and in two or more of genes *BAP1*, *SETD2*, *PTEN*, or *PBRM1*), or by the addition of a *BAP1* mutation. VHL wildtype tumors evolve in a similar manner. With early development, this mode of evolution produces an aggressive phenotype. SCNA is an abbreviation for somatic copy number alteration, wGII for weighted genome integrity index, and ITH for intratumor heterogeneity.

## METHODS

### RNA sequencing

The simple workflow of RNA sequencing can be performed by using one or many sites of a tumor sample (Figure 3). The transcriptome heterogeneous gene expression profile, loss of essential genes and checkpoint blockage immunotherapy can be correlated. It is possible to discover new oncogenes or novel versions of previously described cancer genes. Furthermore, RNA sequencing can track the sequential mutations or other biomarkers between individuals. These results may deconvolution of the diverse clonal relationships encompassed by a bulk tumor. It allows the identification of cell populations that have persisted over time and can narrow down the

list of mutations, potentially conferring growth advantage or treatment resistance on specific subclones. With RNA sequencing data, bioinformatic tools including SCITE, OncoNEM, SiFit, SiCloneFit and PhISCS algorithms can infer tumor phylogeny, whereas Declust, MuSiC, BSEQ-sc, MOMF, quanTIseq and DENDRO can improve the accuracy of subclone detection (Bolck *et al*., 2019; Finotello *et al*., 2019; Wang *et al*., 2019). Applications of RNA sequencing technology have a great impact on cancer research, including (1) Intratumor heterogeneity must be resolved, (2) clonal evolution in primary tumors is being investigated, (3) examining invasion of tumors in their early stages, (4) tracing the spread of metastatic disease, (5) circulating tumor cell genomic profiling, (6) investigating mutator genetic traits and mutation frequencies, (7) addressing the issues of the progression of therapy resistance, (8) cell hierarchies and cancer stem cells, (9) studying epithelial-mesenchymal transformation and cell plasticity, and (10) assignment of neoantigens to each tumor subclone (Ellsworth *et al*., 2017; Navin, 2015; Navin, 2019). Recently, profiling of intratumor genetic heterogeneity has not been correlated with therapeutic decision making and clinical management. As a result, RNA sequencing may enable the deconvolution of a bulk tumor's complex clonal relationships. A new dimension of complexity to the molecular mechanism of ccRCC could be an attractive approach to understand tumor biology and explore new therapeutic regiment options. (Li and Hou, 2018).

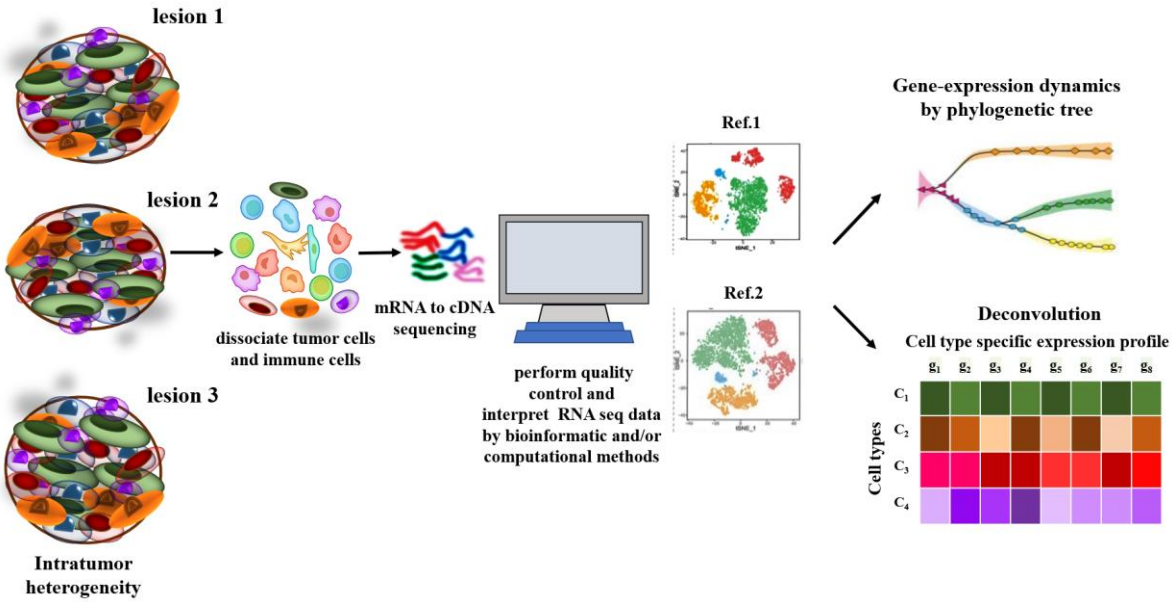


**Figure 3** The simple workflow scenario depicts RNA sequencing RNA-seq) experiments.

RNA-seq workflow following steps: (i) isolation from intratumor heterogeneous lesions consisting of different tumor and immune cell types. (ii) dissociate tumor cells and immune cell types (iii) reverse transcription of primed RNA into cDNA and cDNA application (iv) use bioinformatic tools to perform quality and variability and use of specialized tools to analyst and interpret the RNA-seq data. (v) The RNA-seq profile depicts the expression of the constituent cell types. The gene-expression gradients data can be projected onto the phylogenetic tree and the complete deconvolution outputs the estimation of both cell-type-specific expression profiles and cell-type proportions for each sample.

## CONCLUSIONS

Scientific literature demonstrates more detailed information about the genomic and transcriptomic landscapes of ccRCC. Recently, the most common altered mutations including VHL, PBRM1, BAP1, SETD2, KDM5C, PTEN, mTOR, TP53, TERT genes are frequently reported. The transcriptomic landscapes of ccRCC are well characterized through RNA sequencing and expression profile signature via microarray technology. Through complex genetic, epigenetic, and protein alterations, intra-tumoral heterogeneity is essential in driving phenotypic selection based on environmental stresses. RNA sequencing technology provides clinicians with a

more comprehensive picture of cancer cells' genetic and epigenetic heterogeneity. Through this perception, the tumor heterogeneity and characteristics evolve and provide critical insight of more remarkable treatment regimens based on prognosticated drug response in the clinical realm remains in its infancy but is rapidly advancing. Emerging evidence suggests that future personalized treatments may include a routine strategy to reveal intratumor heterogeneity, providing opportunities to improve classification schemes and laying the groundwork for RCC rare subtype response to newer target therapeutic approaches.

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