Succinate dehydrogenase A (SDHA) gene mutation in renal cell carcinoma: A new subset of hereditary renal cancer

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Abstract

Background: Succinate dehydrogenase (SDH)-deficient renal cell carcinoma (RCC) is mainly associated with SDHB gene mutation and sometimes with SDHC or SDHD mutation. However, only three case of SDHA-deficient RCC have been reported, and the relation of SDHA mutation to RCC has not been clarified. Methods: We investigated SDHA/B/C/D gene mutations in 72 human RCCs by targeted next-generation sequencing, and also assessed SDHA and nuclear factor erythroid 2–related factor 2 (Nrf2) protein expression in 30 tumors. Results: NGS revealed SDHA gene mutations associated with amino acid sequence variations in 12 tumors, while no tumors had SDHB/C/D mutations. The SDHA gene mutations were identical in somatic and germline DNA of all 12 patients. Tumors with SDHA mutations showed a decrease of SDHA protein ($p = 0.0177$) and an increase of Nrf2 protein ($p = 0.0120$), with an inverse correlation between SDHA and Nrf2 protein expression ($p = 0.0321$). Tumor cells with SDHA mutations characteristically had eosinophilic cytoplasm and showed various patterns of proliferation. Conclusions: Our observations suggested that germline SDHA gene mutations might be linked to hereditary RCC associated with mitochondrial dysfunction.

1. Introduction

There is increasing evidence that germline mutations of mitochondrial DNA are associated with various hereditary and sporadic tumors [1,2]. To date, five such genes have been identified, which are fumarate hydratase (FH; 1q43 region) and genes encoding the four subunits of succinate dehydrogenase (SDHA; 5p15 region, SDHB; 1p36.1 region, SDHC; 1q21 region, and SDHD; 11q23 region). SDH is a mitochondrial enzyme complex consisting of four protein subunits (SDHA, SDHB, SDHC, and SDHD) that is involved in the tricarboxylic acid (TCA) cycle (Krebs cycle) and the electron transport chain, and is required for intracellular energy metabolism. The catalytic subunits SDHA and SDHB, which are immobilized on SDHC and SDHD, convert succinic acid to fumaric acid and deliver it to FH, the next enzyme in the cycle [1]. Mutations of genes encoding subunits of SDH are predominantly linked to phaeochromocytoma and paraganglioma [3,4], whereas mutations of FH are associated with leiomyoma, leiomyosarcoma, and renal cell carcinoma (HLRCC) [5-7]. These mitochondrial genes have been reported to act as tumor suppressors and loss-of-function mutations have been detected so far [2]. Intrinsic loss of the normal TCA cycle presumably promotes tumorigenesis due to metabolic alterations with enforced dependence on glycolysis for energy production, mimicking the pattern of metabolic reprogramming known as the Warburg effect that is well-known to be associated with malignancy [8-10]. The Warburg effect involves an increase of glycolysis that generates adenosine triphosphate (ATP) for cell proliferation and also enhances fatty acid synthesis by dephosphorylation of acetyl CoA carboxylase, which is involved in the rate limiting step of fatty acid synthesis. Conversion of glucose metabolism from oxidation to glycolysis is one of the representative strategies for ATP generation by cancer cells [11].

Renal cell carcinoma (RCC) generally displays the Warburg effect [12]. HLRCC-associated renal cancer cells do not possess a complete TCA cycle due to loss of *FH*

enzyme activity and are effectively unable to perform oxidative phosphorylation, indicating that these cancers exist in a state of enforced dependence on glycolysis and are a notable example of the Warburg effect [12]. Thus, FH-deficient HLRCC might be a useful model for studying deregulation of energy metabolism, as well as for developing new therapies to target cancers with TCA cycle enzyme deficiencies [13].

SDH-deficient RCC was accepted as a provisional entity in the 2013 International Society of Urological Pathology (ISUP) Vancouver Classification, and as a unique subtype of RCC by the WHO in 2016 [14]. Similar to FH-deficient HLRCC-associated RCC, SDH-deficient RCC is characterized by impairment of oxidative phosphorylation and a metabolic shift to aerobic glycolysis [18]. So far, most genomic alterations in patients with SDH-deficient RCC have been found to affect SDHB, with the associated renal tumors being negative for immunohistochemical expression of SDHB and having distinctive morphologic features [15-17], while involvement of SDHC or SDHD is less common. Thus, information about SDH-deficient RCC has largely been obtained by studying tumors with SDHB/C/D deficiency and the role of SDHA in RCC is not fully understood because only three cases of genetically confirmed SDHA-deficient RCC have been reported [19-21]. In the present study, we utilized a combination of genetic and biological techniques to investigate the role of SDHA in human RCC. The findings could be useful for understanding the diverse roles of SDHA/B/C/D in cancer progression and for developing individualized strategies to treat SDH-deficient RCC.

2. Results

Next-generation sequencing

Targeted next-generation sequencing (NGS) of coding exons revealed numerous single nucleotide variants (SNVs), de novo mutations, and germline/somatic mutations. NGS of

resected primary tumor tissue samples detected SNVs of the SDHA gene accompanied by amino acid sequence variants in 12 out of 72 patients, while there were no mutations of the SDHB, SDHC, SDHD, or FH genes. We found three SNVs of the SDHA gene (chr5:224487, exon 3, c.163T>C, p.Tyr55His, variant effect: missense; chr5:226091, exon 5, c.550G>A, p.Gly184Arg, variant effect: missense; and chr5:256509, exon 15, c.1969G>A, p.Val657Ile, variant effect: missense) (Table 1). These three SDHA gene mutations have not been identified in previous studies of SDHA-deficient RCC [19-21].

Interestingly, the missense mutation of SDHA was identical between somatic and germline DNA in all 12 patients. The mutation was also consistent within each family. That is, the germline and somatic mutations of SDHA were completely identical in a father (case 5-1) and his son (case 5-2), while family members from another pedigree had the same germline mutation of SDHA and the two RCC patients in this family (father and daughter: cases 6-1 and 6-2, respectively) showed identical somatic and germline mutations of SDHA.

Clinical and pathological characteristics

The clinicopathological characteristics of the patients whose tumors showed SDHA gene mutation are summarized in Table 1. Most of the patients who had smaller tumors without lymph node and/or distant metastasis (< 4cm in diameter; pT1aN0M0) showed a better prognosis, while some patients had less differentiated tumors (Fuhrman grade 3/4) with local invasion (>pT3), regional lymph node involvement (>pN1), or metastasis and an unfavorable prognosis.

RCC with SDHA gene mutations displayed diverse histological features, including tumor cells with pale eosinophilic cytoplasm, vascular stroma, and various patterns of proliferation from tubular structures to solid alveolar nests. Both clear cells and strongly eosinophilic cells existed at different sites in the tumors (Figure 1).

These infiltrative tumors were composed of polygonal cells with abundant pale eosinophilic cytoplasm arranged in tubular, acinar, and papillary structures. The tumor cells contained large pleomorphic nuclei with prominent nucleoli (ISUP nucleolar grade 3). Some tumor cells had eosinophilic inclusions in the cytoplasm (Figure 2A), and some tumor cells had enlarged, eosinophilic nucleoli surrounded by a noticeable clear space or halo within normally stained nuclei (Figure 2B). These microscopic findings were similar to those of FH-deficient HLRCC-associated RCC [22].

Immunohistochemistry

SDHA gene mutations associated with amino acid sequence variants (missense mutations) were generally related to decreased expression of SDHA protein ($p = 0.0177$) (Table 1, Table 2, Figure 3).

Missense mutations of SDHA do not necessarily lead to reduced production or deletion of SDHA protein, and even when tumors had the same missense mutation, some showed a weak immunohistochemical reaction for SDHA and others showed a strong reaction (Table 1).

The tumors with SDHA gene mutations all showed increased expression of Nrf2 protein ($p = 0.0120$), and there was an inverse correlation between SDHA and Nrf2 protein levels ($p = 0.0321$) (Table 2).

3. Discussion

Mutations of TCA cycle genes have been implicated in several aspects of carcinogenesis and tumor progression [23]. Regarding the link between mitochondrial dysfunction and tumorigenesis, there has been significant progress in identifying genetic and molecular factors contributing to the malignant phenotype of FH-deficient HLRCC-associated RCC. On the other hand, there has been limited investigation of the genetic and genomic profile of SDH-deficient RCC, with few studies of mitochondrial gene/protein expression in

SDH-deficient RCC, particularly SDHA-associated RCC.

There have only been three previous reports about SDHA-deficient RCC [19-21]. It has been shown that the SDHA gene is located at p15.33 and this gene comprises 15 exons found at the genomic coordinates chr5:218356 to 256815. Previously reported SDHA gene deletions were located at chr5:218437 through chr5:235454, at splice site 622-2_622-2delA, and at c.91C>T (p.Arg31*) with a somatic missense variant c.1765C > T (p.Arg589Trp). Also, deletion of both SDHA and SDHB has been observed in SDHA-deficient RCC by immunohistochemistry [19-21]. It was reported that the entire mitochondrial complex 2 becomes unstable after biallelic inactivation of any of its components leads to degradation of the SDHB subunit, so biallelic inactivation of SDHA results in loss of both SDHA and SDHB [4,24]. In contrast, tumors with SDHB, SDHC, or SDHD mutations were reported to exhibit loss of staining for SDHB but retain staining for SDHA, although it is not clear why SDHA protein remains stable in the presence of SDHB, SDHC, or SDHD mutations.

In the present study, we used NGS to find three new SNVs of the SDHA gene in human RCC [chr5:224487 (exon 3), chr5:226091 (exon 5), and chr5:256509 (exon 15)], but we did not detect any germline or somatic mutations of the SDHB, SDHC, and SDHD genes. NGS was performed by targeting the coding exons, and revealed numerous SNVs, de novo mutations, and germline/somatic mutations in the coding regions. A nonsynonymous substitution is a nucleotide mutation that alters the amino acid sequence of a protein. Missense mutations are nonsynonymous substitutions that arise from point mutations, which are mutations of a single nucleotide that result in substitution of a different amino acid in the protein encoded by the gene. We found that missense mutations of SDHA did not necessarily lead to reduced expression or deletion of SDHA protein, and even when tumors had the same missense mutation, some tumors showed a weak immunohistochemical reaction for SDHA and others showed a strong reaction. SDHA acts as a tumor suppressor gene in relation to

paraganglioma and pheochromocytoma [25]. There was complete deletion of SDHA in the previously reported RCCs with SDHA mutation [19-21], but our tumors did not show SDHA deletion. Thus, it is unclear whether or not SDHA had a tumor suppressor role in our RCC patients. However, our findings suggested that even without complete deletion, reduction of SDH expression related to gene mutation might play a role in tumorigenesis. Interestingly, the SDHA gene mutation was identical between somatic and germline DNA in all 12 patients. In brief, the germline and somatic mutations of SDHA were completely identical in cases 5-1 and 5-2. Similarly, members of the case 6 family had the same germline mutation of SDHA and the two RCC patients (cases 6-1 and 6-2) displayed an identical somatic mutation, suggesting that SDHA gene mutation-associated hereditary RCC might be a new tumor entity.

Fumarate hydratase (fumarase, *FH*) is the enzyme that follows SDH in the mitochondrial TCA cycle. Accordingly, loss of SDH function and consequent TCA cycle impairment may result in a similar metabolic shift to aerobic glycolysis (Warburg effect) in FH-deficient HLRCC-associated RCC and SDH-deficient RCC, suggesting that these cancers could share common features and be similarly aggressive [18,26,27].

In the present study, some tumor cells with SDHA mutation demonstrated histopathological features associated with HLRCC, i.e., enlarged heavily stained (eosinophilic) nucleoli surrounded by a clear space or halo within normally stained nuclei [22], even though the number of such cells was small and there were variations between the tumors.

Although the mechanism underlying the association of SDH gene mutations with RCC has yet to be elucidated, two biochemical mechanisms have been suggested to explain how mutations of mitochondrial tumor suppressor genes contribute to tumorigenesis. These mechanisms are redox stress resulting from increased mitochondrial production of reactive

oxygen species (ROS), or metabolic signalling with TCA cycle metabolites as intracellular messengers [1,2]. Some SDH mutations lead to generation of ROS [1,2]. There is growing evidence that the transcription factor nuclear factor E2-related factor 2 (Nrf2) is the major regulator of antioxidant and detoxification pathways for ROS with a pivotal role in tumor proliferation, invasion, and chemoresistance, and elevated tumor expression of Nrf2 protein has been linked to a poor prognosis [28,29] It was reported that activation of Nrf2-dependent antioxidant pathways is a key step in the development of FH-deficient papillary renal cell carcinoma type 2 (pRCC2). In HLRCC, FH deficiency leads to succination of Kelch-like ECH-associated protein 1 (Keap1), stabilization of Nrf2, and induction of stress-response genes to promote the survival of FH-deficient cells [30,31]. Our previous study showed an increase of Nrf2 expression and aerobic glycolysis in tumor cells from FH-mutated HLRCC [32]. Thus, among the various cancers with TCA cycle enzyme deficiencies, FH-deficient HLRCC-associated RCC is characterized by an anti-oxidant response phenotype [26,27].

We found that RCC with SDHA gene mutation showed decreased expression of SDHA protein and increased expression of Nrf2 protein, with the two proteins demonstrating an inverse relationship. On the other hand, some tumors showed low expression of SDHA protein regardless of the presence or absence of SDHA gene mutation. Furthermore, most of the SDHA-deficient RCCs had eosinophilic cytoplasm and seemed to be oncocytic tumors. The kidney is one of the most common sites for oncocytic tumors and such tumors are characterized by abundant mitochondria [1]. While it is unclear whether the molecular mechanism is Nrf2-dependent or -independent, our findings suggest that progression of SDHA-mutated or SDHA-deficient RCC might have some relation to Nrf2 signaling. It is also unclear how SDHA gene mutation is related to SDHA protein expression and to the development and progression of human RCC. It is possible that not only the gene mutation itself, but also an unknown interaction between SDHA and other signaling pathways, leads to

loss or substantial reduction of SDHA protein function. Accordingly, identification of loss- or gain-of-function mutations affecting other key metabolic enzymes may shed more light on the alterations of metabolism in SDHA-associated tumorigenesis.

FH gene alterations reported in the literature, including missense, frameshift, and nonsense mutations, as well as whole gene deletion, cause loss of FH protein activity. However, some tumors with decreased FH activity did not have an identifiable FH mutation, and no genotype-phenotype correlations have been observed [6,7,34]. The high mutation rate in HLRCC families has led to screening of at-risk individuals for early detection of RCC and initiation of therapy while the tumor is still small [6,7,34]. Our observations suggest that family members with germline SDHA mutation should also be under active surveillance for early detection of RCC.

Superoxide is the ROS generated in the electron transport chain by transfer of a single electron to molecular oxygen. Generation of ROS in SDH-deficient cells is attributed to SDH acting as an electron carrier in the electron transport chain (succinate–ubiquinone oxidoreductase) and not to its role in the TCA cycle (succinate dehydrogenase) [1,2]. As both SDH and FH are involved in the TCA cycle, catalyzing sequential steps (oxidation of succinate by SDH to form fumarate, followed by hydration of fumarate by FH to form malate), it is tempting to speculate that these roles in the TCA cycle are relevant to their tumor suppressor function [1,2]. However, it remains unclear how mutant forms of SDH catalyze ROS production under normoxic conditions. Changes of metabolic signalling were recently proposed to be an alternative, but not mutually exclusive, mechanism to ROS for induction of pseudo hypoxia, since succinate levels are increased in SDH-deficient tumors and succinate can also inhibit prolyl hydroxylase, leading to induction of HIF. Generation of ROS by some SDH mutations leads to inhibition of hypoxia-inducible factor (HIF) prolyl hydroxylase, an enzyme that degrades HIF under normoxic conditions, indicating that

increased ROS production can mediate pseudo hypoxia in tumors with SDH mutation.

The present study had several limitations, including its retrospective design, a relatively small number of subjects, and a follow-up period that was too short to draw definite conclusions regarding the possible influence of SDHA gene mutation on the prognosis of RCC. Therefore, our findings need to be confirmed by further investigations, preferably large-scale prospective controlled clinical trials. An SDHB gene mutation was recently reported in SDHB-deficient cancer that was associated with impairment of iron sulfur cluster delivery, resulting in accumulation of succinate and metabolic remodeling [35]. We did not perform immunostaining for FH in the present study. However, as described above, FH-deficient HLRCC-associated RCC and SDH-deficient RCC may have morphological and biological similarities, suggesting that we should examine the functions and mutations of both FH and SDH in more detail to evaluate the mechanistic basis and support the development of new treatment options for these mitochondrial-associated hereditary RCCs.

4. Materials and Methods

Patients

This retrospective study investigated 72 patients (45 men and 27 women) with a histopathological diagnosis of RCC who underwent nephrectomy at our hospital between 2011 and 2018. Nephrectomy was performed before any other treatment. Preoperative imaging with CT and/or MRI was done for tumor staging in all 72 patients. The postoperative follow-up period ranged from 5 to 93 months (median: 29 months). All patients had no past or family history of paraganglioma, pheochromocytoma, or gastrointestinal stromal tumors.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics review board of Dokkyo Medical University Hospital (approval no. 24017.R-6-5.). Each patient signed an informed consent form that had been

approved by our institutional Committee on Human Rights in Research. All samples were anonymized before analysis to guarantee protection of patient privacy.

Next-generation sequencing

Next-generation sequencing (NGS) was performed for detection of single nucleotide variants (SNVs), as well as short insertions and deletions (indels). We investigated mutations of SDH subunit genes (SDHA, SDHB, SDHC, and SDHD) and the von Hippel-Lindau (VHL), polybromo-1 (PBRM1), rearranged during transfection (RET), Akt and FH genes by sequencing the coding exons and intron flanking regions using both blood samples and tumor specimens, as described previously [33]. The custom primers for these regions were designed using Ampliseq Designer (Life Technologies). Library construction and sequencing were carried out with an Ion AmpliSeq Library Kit 2.0, Ion PGM IC 200 kit, and Ion PGM (Life Technologies) according to the manufacturer's instructions.

Data analysis

After each sequencing reaction, the raw data were analyzed using Torrent Suite version 4.2.1, which performed processing of the signals, base calling, quality score assignment, adapter trimming, mapping to GRCH37/hg19 reference, assessment of mapping quality, and variant calling. After completion of primary data analysis, a list of the sequence variants [SNVs and indels] detected was compiled in a variant call file format and presented via the web-based user interface. The results of mapping and variant calling were visualized using Integrative Genome viewer (Broad Institute).

Immunohistochemistry

Immunohistochemical staining for SDHA was performed using tumor tissue specimens from 30 RCC patients (12 tumors with SDHA gene mutations and 18 tumors without such mutations). Staining was done using 4-μm thick formalin-fixed, paraffin-embedded whole tissue sections with a polymer-based detection system and a Dako EnVision FLEX Mini Kit,

High pH K8024 (Dako, Carpinteria, CA, USA). A mouse monoclonal antibody for SDHA was employed (Abcam, # ab-2E3GC12FB2AE2; dilution 1:1000, Cambridge, UK). The results were interpreted as negative when cytoplasmic staining was absent in tumor cells and as positive when cytoplasmic staining was present, according to a previous report [19]. The tumors were divided into two groups: a low SDHA expression group in which many tumor cells were negative or weakly positive for anti-SDHA antibody (< 30% of all tumor cells positive) and a high SDHA expression group in which many tumor cells showed moderate to strong positivity for anti-SDHA antibody (> 30% of all tumor cells positive).

Tumor specimens from 30 patients were also subjected to immunohistochemical analysis using an anti-Nrf2 monoclonal antibody (Abcam, # ab-62352, Cambridge, UK), as described previously [32]. Then the tumors were divided into two groups, which were a low Nrf2 expression group (< 30% of all tumor cells positive) and a high Nrf2 expression group (> 30% of all tumor cells positive).

Statistical analysis

Associations among SDHA gene mutation, SDHA protein expression, and Nrf2 protein expression were analyzed by Pearson's χ^2 test for contingency tables. Analyses were performed with commercially available software, and $P < 0.05$ was considered to indicate significance.

5. Conclusions

Our observations suggested that germline SDHA gene mutations might be linked to mitochondrial-associated hereditary RCC. Future studies may be warranted to investigate novel functions and mechanisms of mitochondrial enzymes beyond those related to energy production, which could eventually lead to new treatments for RCC and other cancers.

Authors contributions: Conception and design. T.K.; Development of methodology. T.K., S.M., H.K., T.J.; Acquisition of data. T.K., H.A.; Analysis and interpretation of data. T.K., S.M., H.K.; Writing, review, and/or revision of the manuscript. T.K.; Administrative, technical, or material support. K.A.; Study supervision. K.Y.

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Figure Legends

Figure 1: Diverse histological patterns.

Tumor cells have diverse histological patterns; small alveolar structure of cells with eosinophilic granular cytoplasm in case 1 (A), acinar architecture composed of eosinophilic granular cells with large nuclei exhibiting prominent nucleoli in case 4 (B), sheet arrangement with mixed clear and eosinophilic cytoplasm in case 9 (C), and a single layer of cuboidal cells cover a fibrovascular stalks and form papillary structures in case 10 (D).

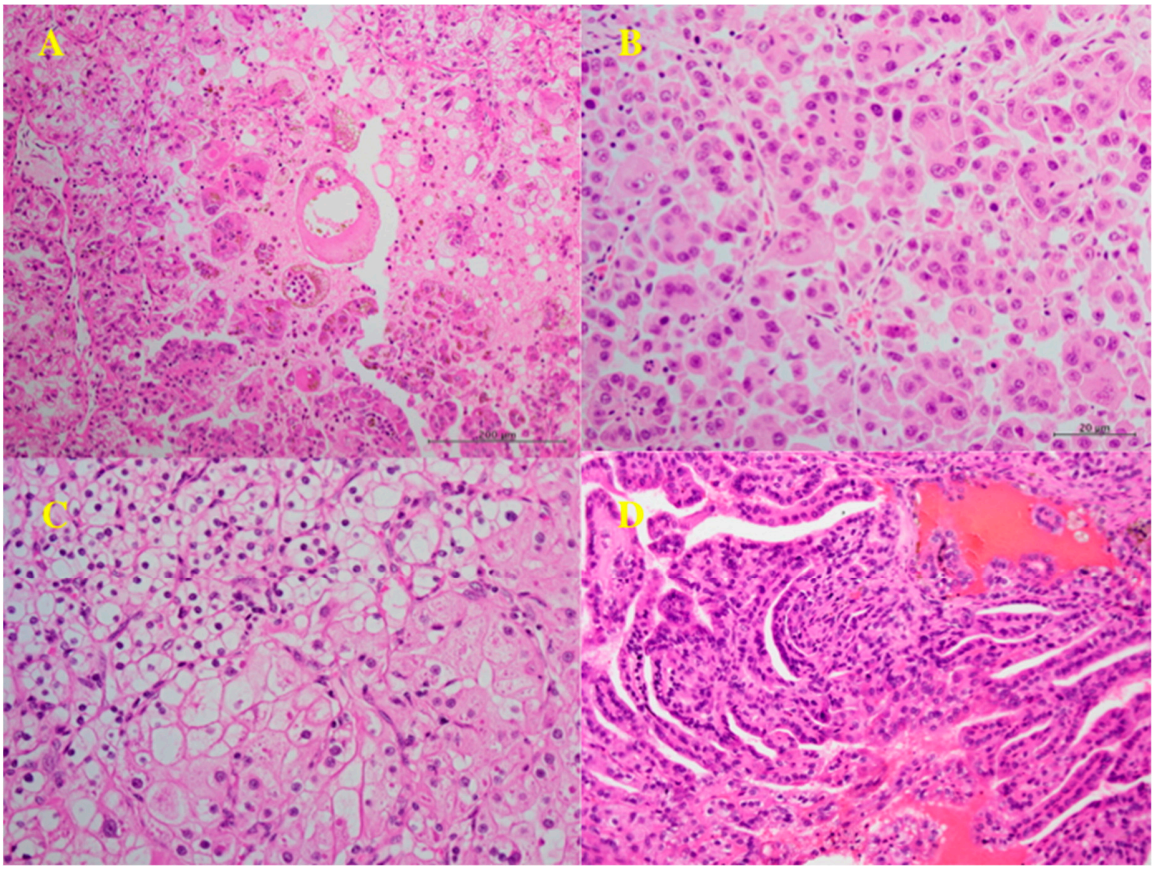

Figure 2: Prominent pale eosinophilic cytoplasmic inclusions within the tumor cells.

Some neoplastic cells contained cytoplasmic eosinophilic inclusions. The tumor cells are pleomorphic appearance with cytoplasmic eosinophilic inclusions (yellow arrows) in case 2 (A) or enlarged, heavily stained (eosinophilic) nucleoli surrounded by a noticeable unstained space or halo within normally stained nuclei (yellow arrows) in case 9 (B).

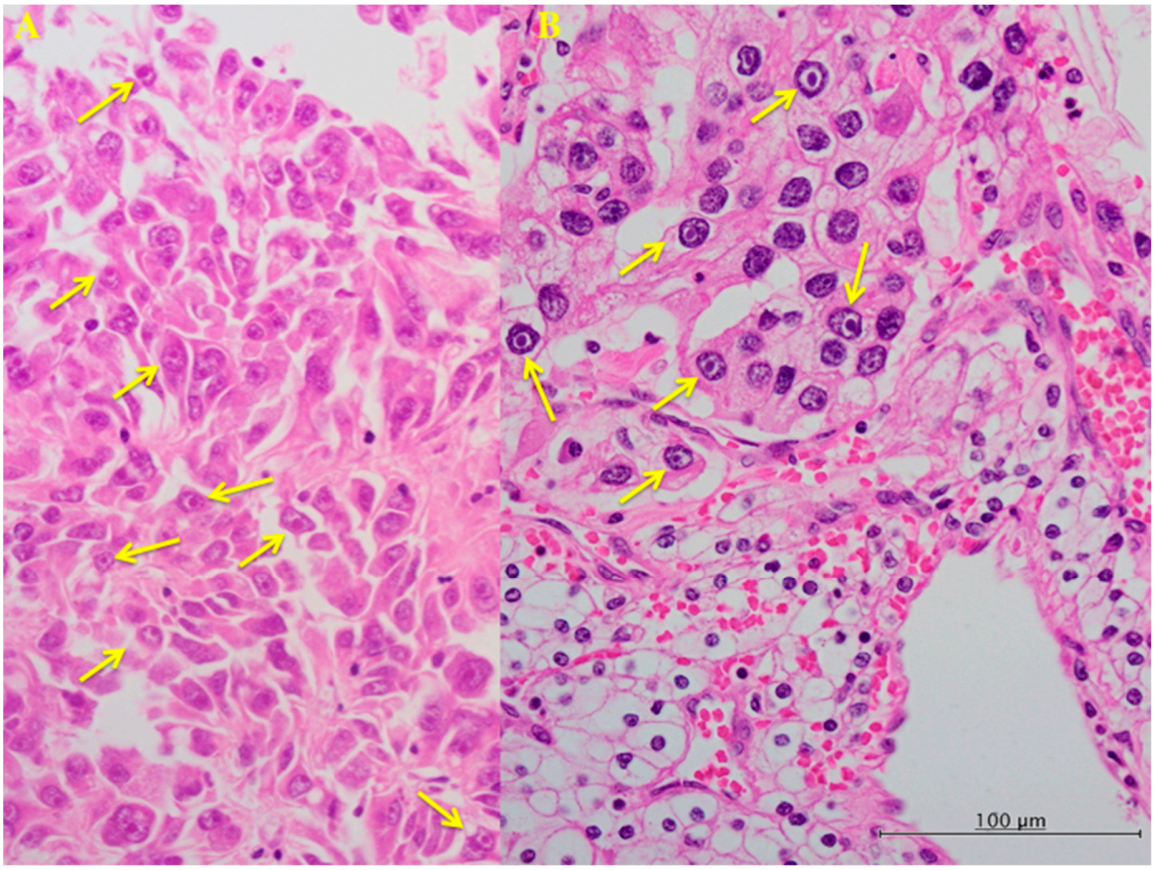

Figure 3: Immunohistochemistry for SDHA and Nrf2.

Clear cell RCC with SDHA gene mutation in case 5-1 (A-1 to A-3). Hematoxylin and eosin (A-1). The tumor cells show lower reaction for an anti-SDHA antibody (A-2), and higher staining for an anti-Nrf2 antibody (A-3). Papillary RCC type 2 with SDHA gene mutation in case 8 (B-1 to B-3). Hematoxylin and eosin (B-1). Immunohistochemical staining demonstrates strong staining for an anti-SDHA antibody in tumor cells (E), and positive staining for an anti-Nrf2 antibody (B-3). Clear cell RCC with non-SDHA gene mutation (C-1 to C-3). Hematoxylin and eosin (C-1). Positive reaction for an anti-SDHA antibody in tumor cells (C-2), while negative reaction for an anti-Nrf2 antibody (C-3).

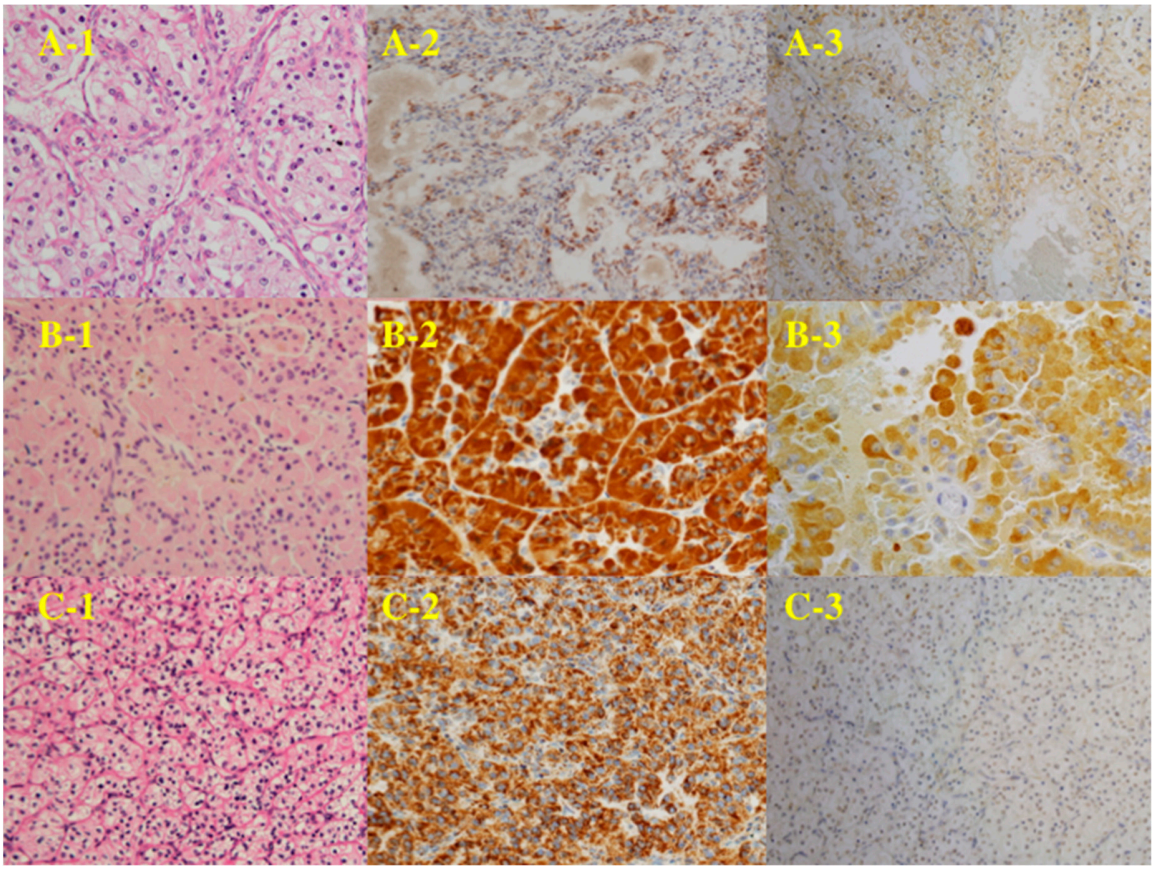

Table 1. Patients and tumor characteristics.

Case	Age/Gender	Tumor Size (cm)	Fuhrman Grade	TNM Stage	Histology	Outcome	SDHA		VHL	Others	RCC Family Histoly	Uterine Leiomyomatosis
							Germline/Tumor	Immuno-histochemistry	Tumor	Tumor		
1	48 / F	9	3	pT3aN1M1 (Pul, OSS)	Eosinophilic variant of ccRCC	DOD, 7 months	c.163T>C p.Tyr55His	Low	none	RET: c.3275A>G, p.Asn1092Ser	None	None
2	63 / M	11	4	pT3bN0M1 (PUL)	pRCC2	DOD, 13 months	c.1969G>A p.Val657Ile	High	none	PBRM1: c.3215_3216insT, p.Ala1073fs, RET: c.2071G>A, p.Gly691Ser	None	
3	67 / M	11	3	pT3aN0M1 (PUL)	ccRCC	AWD, 95 months	c.163T>C p.Tyr55His	High	c.449delA, p.Asn150fs		None	
4	68 / F	8	3	pT1bN1M0	pRCC2	DOD, 17 months	c.163T>C p.Tyr55His	High	none	Akt1: c.726delG, p.Glu242fs	None	None
5-1	66 / M	6	3	pT1bN0M1 (PUL)	ccRCC	AWD, 97 months	c.1969G>A p.Val657Ile	Low	c.292T＞A, p.Tyr98Asn		RCCs in son (case 5.2) and paternal cousin	
5-2	46 / M	9	3	pT3aN0M1 (PUL)	ccRCC	AWD, 38 months	c.1969G>A p.Val657Ile	Low	c.563T＞C, p.Leu188Pro		RCC in father (case 5.1)	
6-1	34 / F	8	2	pT3aN0M0	pRCC2	NED, 90 months	c.550G>A p.Gly184Arg	High	none	RET: c.2166G>A, p.Val706Met	RCCs in father (case 6.2)	None
6-2	66 / M	13	3	pT3aN2M1 (PUL, OSS)	pRCC2	AWD, 19 months	c.550G>A p.Gly184Arg	High	none	Akt1: c.726delG, p.Glu242fs RET: c.2166G>A, p.Val706Met	RCC in daughter (case 6.1)	
7	32 / M	3	2	pT1aN0M0	pRCC2	NED, 27 months	c.1969G>A p.Val657Ile	Low	none		None	
8	69 / M	9	3	pT2aN0M0	pRCC2	NED, 27 months	c.1969G>A p.Val657Ile	High	none	PBRM1: c.4337_4338insG, p.Gly1447fs	RCC in father	

9	49 / F	12	3	pT3aN0 M0	Eosinophilic variant of ccRCC	NED, 7 months	c.550G>A p.Gly184Arg	Low	c.346_357delCTTTGGCT CTTCT>C, p.Leu186_Phe119del	PBRM1: c.2567G>C, p.Arg856Pro, PBRM1: c.4337_4338insG, pGly1447fs, RET: c.1465G>A, p.Asp489Asn	None	None
10	71 / F	2	3	pT1aN0 M0	pRCC2	NED, 7 months	c.163T>C p.Tyr55His	Low	none		None	hysterectomy for uterine leiomyomatosis

Table 2. Relationship between SDHA gene mutation and proteins (n = 30).

		SDHA Protein		
		High (n = 20)	Low (n = 10)	*p* value
SDHA gene	mutation (-) (n = 18)	15	3	0.0177
	mutation (+) (n = 12)	5	7	
		Nrf2 protein		
		High (n = 19)	Low (n = 11)	*p* value
SDHA gene	mutation (-) (n = 18)	8	10	0.0120
	mutation (+) (n = 12)	11	1	
		Nrf2 protein		
		High (n = 19)	Low (n = 11)	*p* value
SDHA protein	High (n = 20)	10	10	0.0321
	Low (n = 10)	9	1	