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REVIEW
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Approaches to Gene Mutation Analysis Using Formalin-Fixed Paraffin-Embedded Adrenal Tumor Tissue From Patients With Primary Aldosteronism

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Aldosterone production is physiologically under the control of circulating potassium and angiotensin II as well as adrenocorticotropic hormone and other secretagogues such as serotonin. The adrenal's capacity to produce aldosterone relies heavily on the expression of a single enzyme, aldosterone synthase (CYP11B2). This enzyme carries out the final reactions in the synthesis of aldosterone and is expressed almost solely in the adrenal zona glomerulosa. From a disease standpoint, primary aldosteronism (PA) is the most common of all adrenal disorders. PA results from renin-independent adrenal expression of CYP11B2 and production of aldosterone. The major causes of PA are adrenal aldosterone-producing adenomas (APA) and adrenal idiopathic hyperaldosteronism. Our understanding of the genetic causes of APA has significantly improved through comprehensive genetic profiling with next-generation sequencing. Whole-exome sequencing has led to the discovery of mutations in six genes that cause renin-independent aldosterone production and thus PA. To facilitate broad-based prospective and retrospective studies of APA, recent technologic advancements have allowed the determination of tumor mutation status using formalin-fixed paraffin-embedded (FFPE) tissue sections. This approach has the advantages of providing ready access to archival samples and allowing CYP11B2 immunohistochemistry-guided capture of the exact tissue responsible for inappropriate aldosterone synthesis. Herein we review the methods and approaches that facilitate the use of adrenal FFPE material for DNA capture, sequencing, and mutation determination.

Keywords: primary aldosteronism, CYP11B2, somatic mutation, immunohistochemistry, next-generation sequencing

INTRODUCTION

Technologic advances in genetic analysis have provided us a better understanding of the molecular pathogenesis of endocrine-related tumors. Aldosterone-producing adenoma (APA) is one of the major subtypes of primary aldosteronism (PA), the most common cause of endocrine-related hypertension. The application of next-generation sequencing (NGS) has resulted in the identification of disease-causing mutations in APA and familial PA. Aldosterone-driver mutations can occur in genes encoding membrane ion channels or pumps (1–9). Thus far, APA have been found with mutations in *KCNJ5* (1), *ATP1A1* (2, 4), *ATP2B3* (2), *CACNA1D* (3, 4), *CACNA1H* (9), and *CLCN2* (8) (aldosterone-driver mutations). These mutations cause excess aldosterone production by raising intracellular calcium levels which leads to enhanced CYP11B2 (aldosterone synthase) expression and renin-independent aldosterone production (10). Like other adrenocortical tumors, activating mutations in *CTNNB1* gene (encoding β-catenin) have also been documented in a subset of APA (11–14) but the mechanism of *CTNNB1* mutation activation of aldosterone production remains to be clearly defined. So far more than 90 APA somatic mutations have been reported (**Table 1**). Of note, only part of the previously reported somatic mutations has been functionally tested so far. To assess the pathologic role of these mutations, it would be ideal to perform cell-based studies for each mutation. In addition to tumor somatic mutations, PA aldosterone production may be regulated by hormones that include adrenocorticotropic hormone, serotonin, or luteinizing hormone (37–44).

Since the development of specific antibodies against human CYP11B2, which is required for aldosterone biosynthesis, CYP11B2 immunohistochemistry (IHC) has played an important role in defining the histopathologic characteristics of adrenals from patients with PA (45, 46). CYP11B2 IHC has revealed diversities in the histopathology of adrenals from patients with PA, including APA (CYP11B2-expressing adrenocortical adenoma) and adrenals with small CYP11B2-expressing cell nests, called aldosterone-producing cell clusters (APCCs) (45) or aldosterone-producing micronodules (APMs) (47). Advanced sequencing methods combined with CYP11B2 IHC have significantly improved the detection rate of somatic mutations in APA (14, 48, 49). CYP11B2 IHC-guided targeted NGS has also allowed the detection of aldosterone-driver mutations in APCCs (APMs) using small amounts of DNA (50–52). Herein, we provide an overview of recent advances in the genetic analysis of APA and introduce a streamlined sequencing approach using formalin-fixed paraffin-embedded (FFPE) tumor tissue material.

IMPORTANCE OF CYP11B2 IHC AND TARGETED DNA CAPTURE

Development of specific antibodies against human CYP11B2 has allowed detection of the source of pathologic aldosterone production in the resected adrenal tissue (45, 46). Unique characteristics of adrenals from patient with PA have been documented by CYP11B2 IHC. Importantly, adrenal tumors detected by cross-sectional imaging study are not always the cause of aldosterone excess even when adrenal vein sampling lateralizes autonomous aldosterone production to the tumor side (53). In such cases, APA can be below the detection limit of imaging studies and/or imaging-detected tumors can be non-functioning adrenocortical adenomas (CYP11B2-negative tumors by IHC). Cases with multiple APAs within one adrenal have also been documented (14, 48, 49, 54).

Traditionally, DNA and RNA have been isolated from snap frozen tumor pieces obtained during pathologic gross dissection at the time of adrenalectomy. Mutational analysis has subsequently been performed without consideration of CYP11B2 expression prior to sequencing. In the largest mutation prevalence study using this conventional approach, aldosterone-driver somatic mutations were detected in 54% of 474 adrenal tumors from PA patients (55). Considering the aforementioned diversities in the histology of PA, this approach could negatively affect the accuracy of mutational analysis. As such, we recently developed an advanced molecular profiling method using selective DNA isolation from FFPE sections based on CYP11B2 IHC, followed by NGS (14, 56). The step-by-step sequencing method using the CYP11B2 IHC-guided approach is shown in **Figure 1**. Many laboratories, including ours, use a mouse monoclonal antibody specific for human CYP11B2 that was produced and characterized by Dr. Celso Gomez-Sanchez (46). This antibody is commercially available from Millipore Sigma (MABS1251, RRID: AB_2783793), making it useful for both research and pathologic diagnosis purposes. As is needed for most antibodies, laboratory testing for individual in-house protocols should be done to optimize specificity and sensitivity for CYP11B2 detection. Initial protocol testing is particularly important due to the variable CYP11B2 expression seen between APAs. The scanned slide images of adrenal tumor tissue from a PA patient are shown in **Figure 2**. The adrenal contains two distinct adrenocortical tumors (an APA and a CYP11B2-negative tumor) which exist close to each other. This example highlights the importance of targeted DNA capture method for accurate mutation analysis. Importantly, past studies demonstrated that no aldosterone-driver mutation was detected in CYP11B2-negative adrenocortical tumors from PA patients (49, 57).

Using this CYP11B2 IHC-guided approach, aldosterone-driver mutations have been identified in 88-96% of APAs (14, 48, 49). A recent study demonstrated a better mutation detection rate using CYP11B2-IHC guided sequencing (94%) as compared to the authors' previous use of conventional tumor tissue approaches (71%) (58).

For the laboratories using traditional material, i.e., DNA/RNA from macro-dissected snap frozen tumor pieces, confirmation of *CYP11B2* mRNA expression by quantitative reverse transcription polymerase chain reaction (RT-qPCR) prior to sequencing could also improve the mutation detection

TABLE 1 | Previously reported somatic mutations in aldosterone-producing adenomas.

Gene	Somatic Mutations	
KCNJ5	c.343C>T (p.R115W) (15)	c.445_446insTGG (p.T149delinsMA) (49)
	c.376T>C (p.W126R) (16)	c.447_448insATT (p.T149delinsTI) (14)
	c.414_425dupGCTTTCCTGTTC (p.A139_F142dup) (17)	c.450_451insATG (p.I150_G151insM) (13)
	c.420C>G (p.F140L) (14)[a]	c.451G>A (p.G151R) (1)
	c.433_434insCCATTG (p.I144_E145insAI) (13)	c.451G>C (p.G151R) (24)
	c.433G>C (p.E145Q) (18)	(p.G151_Y152del)* (25)
	c.433G>A (p.E145K) (4)	c.457_492dupG_G (p.G153_G164dup) (20)
	c.432_439delTGAGACCGinsCA (p.E145_E147delinsK) (19)	c.461T>G (p.F154C) (13)
	c.439G>C and c.448_449insCAACAACCA (p.E147Q_T149_I150insTTT) (20)	c.467_469delTCA (p.I157del) (26)
	c.443C>T (p.T148I) (21)[b]	c.470_471delinsAA (p.I157K) (13)
	c.445_446insGAA (p.T148_T149insR) (22)	(p.I157_E159del)* (25)
	c.446insAAC (p.T149_I150insT) (23)	c.472A>G (p.T158A) (27)
	c.445A>T (p.T149S) (21)	c.503T>G (p.L168R) (1)
		(p.G184E)* (25)
		c.737A>G (p.E246G) (15)
ATP1A1	c.295G>A (p.G99R) (16)	c.2874_2882delCTTTGAAGA (p.F959_E961del) (29)
	c.299_313delTCTCAATGTTACTGT (p.F100_L104del) (2)	c.2877_2882delTGAAGA (p.F959_E961delinsL) (28)
	c.304_309delATGTTA (p.M102_L103del) (28)	c.2878_2895delGAAGAGACAGCCCTGGCTinsGCCCTGGTT (p.E960_A965delinsALV) (48)
	c.306_317delGTTACTGTGGAT (p.M102_I106delinsW) (28)	c.2877_2888delTGAAGAGACAGC (p.E960_A963del) (29)
	c.308_313delTACTGT (p.L103_L104del) (28)	c.2878_2887delGAAGAGACAGinsT (p.E960_A963delinsS) (4)
	c.311T>G (p.L104R) (2)	c.2879_2890delAAGAGACAGCCC (p.E960_L964delinsV) (28)
	c.995T>G (p.V332G) (2)	c.2878_2892delGAAGAGACAGCCCTGinsGCCGTG (p.E960_L964delinsAV) (14)
	c.2864_2878delTATTTGGCCTCTTTG (p.I955_E960delinsK) (49)	
	c. 2867_2882delTTGGCCTCTTTGAAGAinsG (p.F956_E961delinsW) (28)	
ATP2B3	c.367G>C (p.G123R) (30)	c.1273_1278delCTGGTC (p.L425_V426del) (2)
	c.1228T>G (p.Y410D) (31)	c.1277_1282delTCGTGG (p.V426_V427del) (2)
	c.1264_1278delGTCACTGTGCTGGTCinsAGCACACTC (p.V422_V426delinsSTL) (22)	c.1276_1287delGTCGTGGCTGTC (p.V426_V429del) (28)
	c.1264_1275delGTCACTGTGCTGinsATCACT (p.V422_L425delinsIT) (14)	c.1276_1298insGACA_delTCGTGGCTGTCCCAGAGGGCCT (p.V426G_V427Q_A428_L433del) (13)
	c.1269_1274delTGTGCT (p.V424_L425del) (32)	c.1279_1284delGTGGCT (p.V427_A428del) (33)
	c.1270_1275delGTGCTG (p.V424_L425del) (55)	c.1281_1286delGGCTGT (p.A428_V429del) (34)
	c.1272_1277delGCTGGT (p.L425_V426del) (2)	
CACNA1D	c.776T>A (p.V259D) (4)	c.2906C>T (p.S969L) (48)
	c.776T>G (p.V259G) (14)	c.2936T>A (p.V979D) (55)
	c.926T>C (p.V309A) (49)	c.2943G>C (p.V981N) (55)
	c.1201C>G (p.V401L) (28)	c.2968C>G (p.R990G) (49)
	c.1207G>C [p.G403R (exon8A)] (3, 4)	c.2969G>A (p.R990H) (4)
	c.1207G>C [p.G403R (exon8B)] (3)**	c.2978G>C (p.R993T) (49)
	c.1229C>T (p.S410L) (30)	c.2978G>T (p.R993M) (29)
	c.1856G>C (p.R619P) (49)	c.2992_2993GC>AT (p.A998I) (55)
	c.1955C>T (p.S652L) (55)	c.2993C>T (p.A998V) (55)
	c.1964T>C (p.L655P) (55)	c.3019T>C (p.C1007R) (49)
	c.2182G>A (p.V728I) (20)	c.3044T>G (p.I1015S) (49)
	c.2222A>G (p.Y741C) (55)	c.3044T>C (p.I1015T) (58)
	c.2239T>G (p.F747V) (3)	c.3451G>T (p.V1151F) (55)
	c.2239T>C (p.F747L) (4)	c.3452T>C (p.V1151A) (29)
	c.2241C>G (p.F747L) (4)	c.3455T>A (p.I1152N) (55)
	c.2240T>G (p.F747C) (56)	c.3458T>G (p.V1153G) (35)
	c.2240T>C (p.F747S) (29)[c]	c.4007C>G (p.P1336R) (4)
	c.2250C>G (p.I750M) (3, 4)	c.4012G>A (p.V1338M) (3)
	c.2248A>T (p.I750F) (55)	c.4062G>A (p.M1354I) (4)
	c.2261A>G (p.N754S) (29)	
CACNA1H	c.4289T>C (p.I1430T) (9)	
CLCN2	c.71G>A (p.G24D) (8)	
	c.64-2_74del (36)	

*[a-c]Associated with another somatic mutation ([a]KCNJ5 p.G151R; [b]KCNJ5 p.T149S; [c]CACNA1D p.N754S). * Base change information was not provided in the original article. For the CACNA1D mutations, amino acid substitutions are described based on the reference sequence NM_001128839 otherwise noted (**NM_000720 for the mutation in exon 8B). Mutations that are covered by the primer sets in **Table 2** are highlighted in blue.*

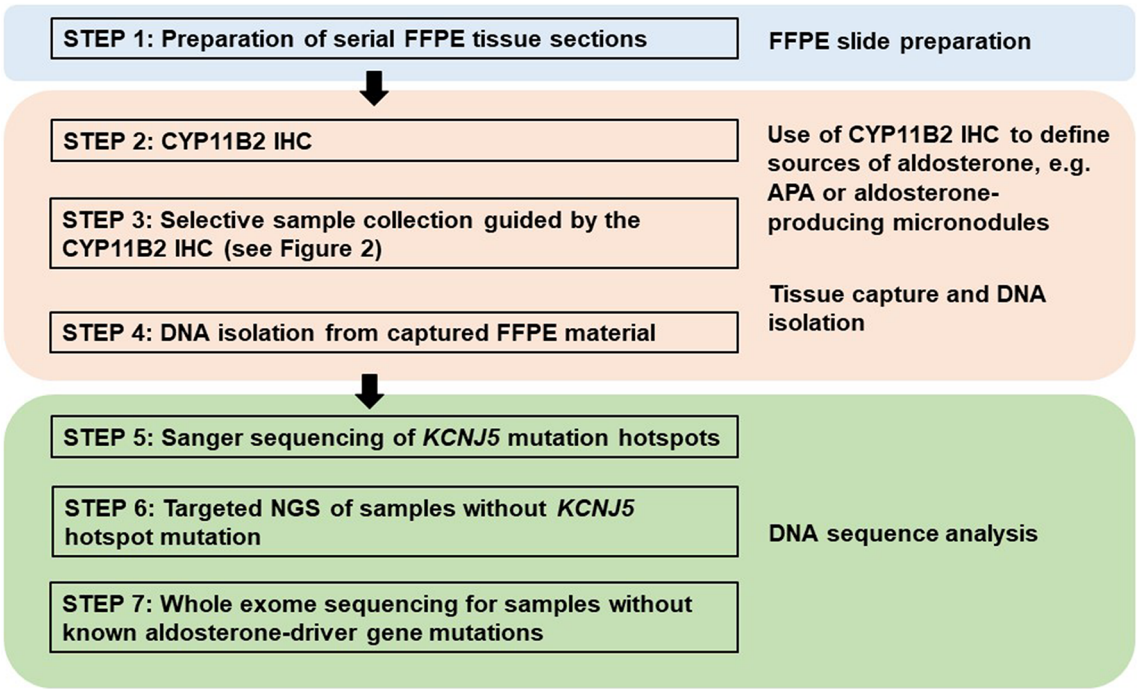

FIGURE 1 | Proposed method for DNA mutation analysis using excised adrenal tissue sections from patients with primary aldosteronism. This approach uses CYP11B2 immunohistochemistry (IHC) to define the source of aldosterone for DNA capture in FFPE tissue sections. Captured DNA is then used for Sanger or gene-targeted deep sequencing to detect known and/or novel drivers of aldosterone production.

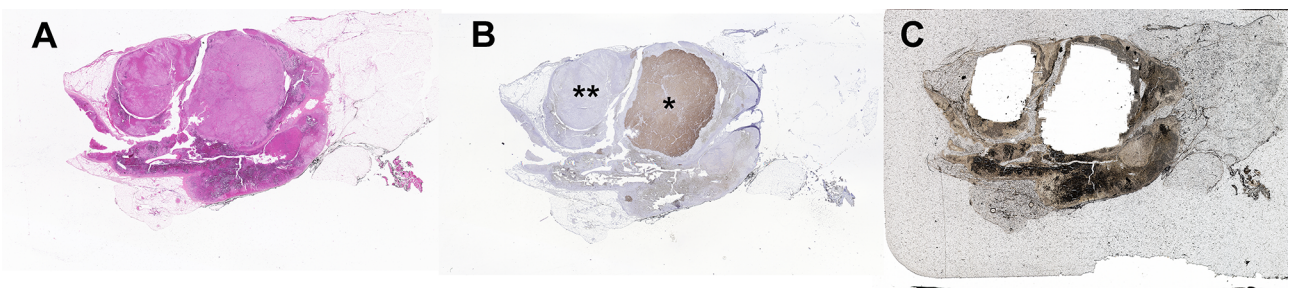

FIGURE 2 | Example of a multinodular adrenal sample from a patient with primary aldosteronism that illustrates the benefit of CYP11B2 IHC-guided DNA capture. **(A)** Hematoxylin and eosin staining, **(B)** CYP11B2 immunohistochemistry; *, aldosterone-producing adenoma (APA); **, CYP11B2-negative tumor, **(C)** Post-captured unstained FFPE adrenal tissue section. For DNA isolation, an APA and a CYP11B2-negative tumor were selectively scraped based on the results of CYP11B2 immunohistochemistry.

rate. A proposed method for mutational analysis using banked snap frozen material is shown in **Figure 3**.

DEFINING SOMATIC MUTATIONS IN ALDOSTERONE-PRODUCING LESIONS

Sanger Sequencing

Traditional direct Sanger sequencing has been widely used for the mutational analysis of APA. As new APA-related genes have continuously been identified, it is challenging to perform Sanger sequencing for the screening of multiple genes – particularly for genes like *CACNA1D*, which have a large coding region with dispersed mutation hotspot areas. Targeted NGS is rapidly becoming the preferred method due to its high sensitivity and ability to utilize small amounts of DNA; however, Sanger sequencing is still an attractive method considering the high per sample cost of NGS. As the prevalence of *KCNJ5* hotspot mutations in APA is relatively high, one option to decrease sample throughput is screening for *KCNJ5* mutation hotspots

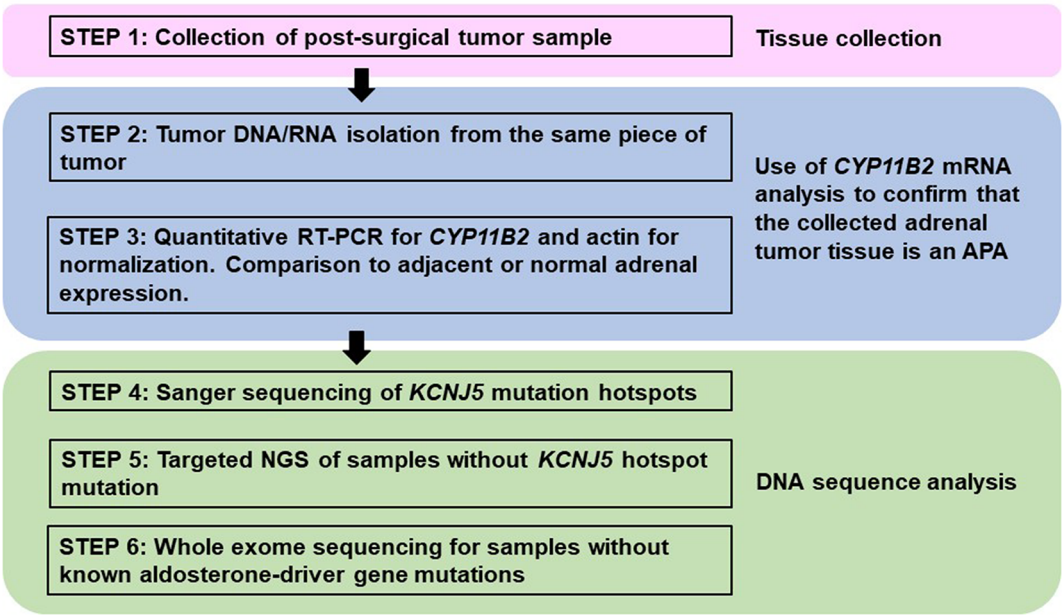

FIGURE 3 | Proposed method for processing fresh or frozen adrenal tumor tissue from patients with primary aldosteronism for mutational analysis. This approach varies from FFPE processing (Figure 1) by the method of tissue collection and the need to use *CYP11B2* mRNA detection for confirmation of APA status. However, mutational analysis is similar using Sanger and/or next-generation sequencing to detect known and/or novel drivers of aldosterone production.

using Sanger sequencing, followed by targeted NGS of *KCNJ5* mutation-negative samples (29, 48) (**Figure 1**). This approach significantly reduces cost and can also be applied to material isolated from traditional snap frozen tissue (**Figure 3**). For researchers without available NGS to screen entire coding regions, targeted Sanger sequencing that covers the majority of known aldosterone-driver mutations can be done in a systematic manner. Based on the APA mutation prevalence from our previous study (14), the use of five primer pairs (one for the *KCNJ5*, one for *ATP1A1*, one for *ATP2B3*, and two for *CACNA1D*, **Table 2**) appear to be able to identify over 70% of mutations by direct Sanger sequencing in a Caucasian American cohort. Special consideration is required for primer design when using genomic DNA (gDNA) from FFPE as a template, since FFPE-extracted DNA can be heavily degraded and fragmented. The authors recommend designing primer sets that target the amplicon size below 250 base pairs (bp) if possible.

Next-Generation Sequencing

NGS has rapidly become the standard approach for comprehensive molecular profiling of human tumors due to its ability to generate sequence-level genetic data simultaneously for tens, hundreds, or even thousands of genes. Although a variety of NGS methods and platforms exist, there are two broad approaches: amplicon-based and hybridization capture-based (**Table 3**). Amplicon-based approaches utilize multiplex PCR reactions to amplify genomic regions of interest, while hybridization capture-based methods utilize biotinylated oligonucleotide baits to pull down target regions from pools of sheared gDNA. In general, amplicon-based methods are

TABLE 2 | PCR primer sets for aldosterone-driver mutation hotspots.

Gene	Exon		Primer Sequences	Amplicon Size (bp)	Reference
KCNJ5	2	Forward	GGACCATGTTGGCGACCAAGAGTG	211	(21)
		Reverse	GACAAACATGCACCCCACCATGAAG		
ATP1A1	4	Forward	ATTAACATCTGCTCGTGCAGCTGAG	227	
		Reverse	CCATATGCTGAATTACAGAACTCAC		
ATP2B3	8	Forward	TGTCTGCCATCACCGTCATCATC	255	(14)
		Reverse	CCCAGTTTCCGAGTCTGTAAACAG		
CACNA1D	8A	Forward	CCCACTCCTATGAGACCATC	190	
		Reverse	TCTTGGCAACTGTCCTCAGG		
	16	Forward	GGTGTGTGGCGTTGCCATTG	253	(29)
		Reverse	AACTGTTGCAGGGCTCCCA		

TABLE 3 | Comparison of NGS approaches for molecular profiling of aldosterone-producing adrenal cortical lesions.

	Amplicon-based	Hybridization Capture-based
Enrichment method	Multiplex PCR	Biotinylated oligonucleotide baits
Input DNA	Less	More
# of genomic targets	Fewer	More
Experimental time	Less	More
Cost per sample	Lower*	Higher*
Application(s)	Targeted sequencing	Targeted sequencing or WES

**Depends on depth of sequencing and # of genomic targets.*
WES, whole-exome sequencing.

preferred for targeted sequencing of small numbers of genomic regions or when available input DNA for NGS library preparation is very low – particularly for FFPE samples – while hybridization capture-based approaches are favored for analyzing a large number of genomic regions [e.g., whole-exome sequencing (WES)] when ample input DNA is available. These and other differences between the NGS approaches inform how they may be best utilized for molecular profiling of aldosterone-producing lesions using FFPE tissue (**Figure 1**).

Given the relatively limited number of established aldosterone-driver mutations – coupled with the fact that most of these mutations occur at specific hotspot regions within the affected genes – targeted amplicon-based NGS is ideal for characterizing FFPE APA samples. As mentioned earlier, recent studies utilizing this approach have identified somatic aldosterone-driver mutations in the vast majority of APA. In addition to the ability to interrogate multiple genomic regions simultaneously, one of the important advantages of NGS over Sanger sequencing is improved sensitivity for detecting genetic variants. This is particularly important for detecting somatic mutations in microscopic lesions (i.e., APCC/APM), for which the expected allelic variant fraction may be less than 20% (depending on the purity of the isolated tissue for sequencing). Application of targeted amplicon-based NGS to APCC in normal adrenal glands and from patients with adrenal idiopathic hyperaldosteronism has identified somatic aldosterone-driver mutations in 34-58% of these lesions (50–52). For aldosterone-producing lesions that are mutation-negative by targeted amplicon-based NGS, hybridization capture-based WES of CYP11B2 IHC-guided FFPE tissue may identify novel aldosterone-driver mutations (9, 36). Finally, despite several clear advantages of NGS-based molecular profiling, application of these approaches to FFPE tissue is potentially limited by FFPE-associated DNA degradation (e.g., increased genomic fragmentation, artifactual nucleotide deamination) and technical issues (e.g., PCR amplification bias, sequencing error). Emerging NGS methods, including the use of unique molecular identifiers (UMI; as known as "molecular barcodes"), and novel NGS technologies may begin to address some of these limitations and will continue to revolutionize genomic characterization of human tumors, including aldosterone-producing lesions.

CONCLUSIONS

Recent advances in sequencing technology have significantly accelerated PA research to elucidate its molecular pathogenesis. Unique histologic characteristics of adrenals from patients with PA require special attention to tumor CYP11B2 expression for accurate somatic mutation identification. The streamlined approach using CYP11B2 IHC-guided DNA capture combined with NGS appears to be a preferred method for mutational analysis of adrenals from patients with PA. The use of this CYP11B2 IHC-guided sequencing approach in a large prospective cohort will allow us to accurately determine APA mutation prevalence as well as genotype-phenotype correlations.

AUTHOR CONTRIBUTIONS

KN and WR conceived the idea of this review article. KN and AU drafted the manuscript. WR reviewed the manuscript and made edits on the contents. All authors contributed to the article and approved the submitted version.

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