SHORT COMMUNICATION

Next-Generation Sequencing to Detect Deletion of *RB1* and *ERBB4* Genes in Chromophobe Renal Cell Carcinoma

A Potential Role in Distinguishing Chromophobe Renal Cell Carcinoma from Renal Oncocytoma

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Overlapping morphologic, immunohistochemical, and ultrastructural features make it difficult to diagnose chromophobe renal cell carcinoma (ChRCC) and renal oncocytoma (RO). Because ChRCC is a malignant tumor, whereas RO is a tumor with benign behavior, it is important to distinguish these two entities. We aimed to identify genetic markers that distinguish ChRCC from RO by using next-generation sequencing (NGS). NGS for hotspot mutations or gene copy number changes was performed on 12 renal neoplasms, including seven ChRCC and five RO cases. Matched normal tissues from the same patients were used to exclude germline variants. Rare hotspot mutations were found in cancer-critical genes (*TP53* and *PIK3CA*) in ChRCC but not RO. The NGS gene copy number analysis revealed multiple abnormalities. The two most common deletions were tumor-suppressor genes *RB1* and *ERBB4* in ChRCC but not RO. Fluorescence *in situ* hybridization was performed on 65 cases (ChRCC, $n = 33$; RO, $n = 32$) to verify hemizygous deletion of *RB1* (17/33, 52%) or *ERBB4* (11/33, 33%) in ChRCC, but not in RO (0/32, 0%). In total, ChRCCs (23/33, 70%) carry either a hemizygous deletion of *RB1* or *ERBB4*. The combined use of *RB1* and *ERBB4* fluorescence *in situ* hybridization to detect deletion of these genes may offer a highly sensitive and specific assay to distinguish ChRCC from RO. (*Am J Pathol* 2018; 188: 846–852; <https://doi.org/10.1016/j.ajpath.2017.12.003>)

Both chromophobe renal cell carcinoma (ChRCC) and renal oncocytoma (RO) are tumors that originate from the intercalated cells of the collecting system. Microscopically, ChRCC contains polygonal cells with cytoplasm ranging from pale to eosinophilic and displays nested, alveolar, or sheet-like growth patterns. RO shares many histologic features with ChRCC. Histologically, RO is composed of uniform round/polygonal tumor cells with abundant eosinophilic, granular cytoplasm, and small round nuclei, arranged in nests, solid, and/or tubular patterns.^{1,2} Atypical features have been reported in RO, despite the benign behavior associated with this tumor, and include chromophobe

histology, significant nuclear atypia (bizarre cells with high nuclear/cytoplasm ratio and hyperchromatic nuclei), invasion into the renal sinus and perirenal fat and vessels, as well as rare mitoses and focal areas of necrosis.¹ These morphologic features may generate diagnostic dilemmas. In routine clinical practice, special and immunohistochemical stains are commonly used to distinguish ChRCC from RO, and include

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Hale's colloidal iron and cytokeratin 7.³ These markers are usually diffusely and are strongly positive in ChRCC, whereas they are typically negative or show scattered positivity in RO. However, subsets of ChRCC are reported to be negative for these markers, whereas a variable degree of positive staining has also been documented in RO. Therefore, the results of immunohistochemical studies are not always conclusive.^{3–6}

It is well established that ChRCC and RO have different patterns of chromosomal anomalies, including copy number gains and losses at distinct chromosomal loci.^{7–10} Specifically, ChRCC are frequently associated with gains in chromosomes 4, 7, 11, 12, 14q, and 18q, and losses in chromosomes Y, 1, 2, 6, 10, 13, 17, and 21. Among the latter, losses of chromosomes 2, 6, 10, and 17 seem to be specific for ChRCC.^{9,11} RO occasionally exhibits losses of chromosomes 1 and Y and/or balanced translocation of the 11q13 breakpoint region.^{12,13} Common losses of chromosomes in both ChRCC and RO suggest that there is a close relationship between ChRCC and RO.^{14–16} The chromosomal anomalies are usually identified by conventional cytogenetic analysis, together with other approaches, such as comparative genomic hybridization and restriction fragment length polymorphism analysis.^{17–19} Herein, next-generation sequencing (NGS) was used to distinguish these two entities from each other and fluorescence *in situ* hybridization (FISH) was used to confirm these findings.

Materials and Methods

Sixty-five cases (ChRCC, $n = 33$; RO, $n = 32$) were retrieved from the surgical pathology files of the Departments of Pathology at the University of Massachusetts Medical School (Worcester, MA) and at the Indiana University School of Medicine (Indianapolis, IN). Relevant clinical records were reviewed for clinical presentation, tumor size, stage, treatment, and clinical outcome. Representative hematoxylin and eosin–stained sections were reviewed to confirm the diagnoses by two genitourinary pathologists (Q.L. and Z.J.). At the University of Massachusetts Medical School, ChRCC cases ($n = 11$) were selected between 1995 and 2010 and were composed of three female and eight male patients, with ages ranging from 28 to 75 years (mean age, 50.8 years) with all localized tumors (T1, $n = 1$; T2, $n = 5$; T3, $n = 5$); RO cases ($n = 10$) were selected between 1999 and 2009 and included two female and eight male patients, with ages ranging between 41 and 84 years (mean age, 64.7 years). At the Indiana University School of Medicine, ChRCC cases ($n = 22$) were selected between 2015 and 2017 and were composed of 12 female and 10 male patients, with ages ranging from 31 to 81 years (mean age, 58.3 years), with all localized tumors (T1, $n = 12$; T3, $n = 10$); RO cases ($n = 22$) were selected between 2012 and 2017 and included six female and 16 male patients, with ages ranging between 41 and 87 years (mean age, 66.1 years).

Targeted Mutational Sequencing and Copy Number Variation Analysis by NGS

Tumor and matched normal tissue from the same patient was identified on a hematoxylin and eosin–stained slide, then macrodissected from sequential sections, typically three to five slides of formalin-fixed, paraffin-embedded tissue (7 μ m thick). DNA was purified using the QIAamp DNA FFPE Tissue kit (Qiagen, Valencia, CA), then quantified by spectrometry (NanoDrop 1000 DNA/RNA Calculator; Thermo Scientific, Wilmington, DE) and fluorometry (Qubit DNA High-Sensitivity Assay Kit; Thermo Scientific). Pooling with short known nucleotides known as barcodes could be adapted to the amplicons with the IonXpress Barcode Kit (Life Technologies, Carlsbad, CA) if multiplexing of samples was desired.

Next-generation mutation sequencing and gene copy number analysis were performed as previously described.²⁰ Briefly, AmpliSeq Cancer Hotspot Panel v2 libraries were generated from genomic DNA (10 ng) using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific) and quantified using the Ion Library Quantification KIT (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Genes in this panel are *AKT1*, *ALK*, *APC*, *ATM*, *ABL1*, *BRAF*, *FGFR1*, *GNAS*, *IDH1*, *FGFR2*, *KRAS*, *NRAS*, *PIK3CA*, *MET*, *RET*, *EGFR*, *JAK2*, *MPL*, *PDGFRA*, *PTEN*, *TP53*, *FGFR3*, *FLT3*, *KIT*, *ERBB2*, *EZH2*, *IDH2*, *GNA11*, *GNAQ*, *HNFI1A*, *HRAS*, *RB1*, *CDH1*, *SMAD4*, *STK11*, *SRC*, *SMARCB1*, *VHL*, *MLH1*, *CTNNB1*, *KDR*, *FBXW7*, *CSF1R*, *NPM1*, *SMO*, *ERBB4*, *CDKN2A*, *NOTCH1*, *JAK3*, and *PTPN11*. Libraries were diluted and combined together, and emulsion PCR was performed on the One Touch 2 System (Thermo Scientific) using the OT200 template kit to amplify library DNA onto IonSphere particles. Enrichment of IonSphere particles with clonally amplified sequencing template relied on the OneTouch ES System (Thermo Scientific). Sequencing was performed on an Ion Torrent PGM (IC200 Sequencing Kit, Ion 318 chip; Thermo Fischer), which generates a FASTQ from the raw data file.

FASTQ files were analyzed using three pipelines to identify somatic mutations: Variant Caller version 4 (Thermo Scientific), Ion Reporter version 5 (Thermo Scientific), and NextGENe version 2.4 (Softgenetics, State College, PA). Initial criteria used to call sequence variants relied on detection by multiple pipelines with amplicon coverage of $>500\times$, a variant frequency $>3\%$ with at least 20 reads in a wild-type background, and absence of the same variant in matched normal tissue. After review, variants were confirmed as somatic mutations in the Catalogue of Somatic Mutations in Cancer database²¹ or ruled out as a known germline single-nucleotide polymorphisms with the Single Nucleotide Polymorphism Database.²²

Copy number variants (CNVs) were identified using Ion Reporter and NextGENe. Specimens were excluded from copy number analysis for the following reasons: i) low

sequencing coverage ($n = 4$, $<500\times/\text{amplicon}$), ii) poor-quality DNA, producing DNA sequencing artifacts ($n = 2$, >50 low-level variants with $<2\%$ allele frequency), or iii) an estimated tumor percentage of $<50\%$ ($n = 2$). CNV detection was achieved by comparison of tumor amplicon coverage with matched normal tissue (paired-sample workflow) or comparison to a group average of 10 normal control tissues (multiple control workflow). The analysis incorporated the tumor percentage estimated by the pathologist to determine copy number. Any gene with a single amplicon probe was excluded from the analysis. Results from multiple probes were combined to generate a single gene gain/deletion score. Copy numbers >3.3 were defined as a gain, and copy numbers <1.2 were defined as a loss. CNV calls made by both software packages were tabulated and reported both by gene and chromosome region. CNV changes present in $>60\%$ of ChRCC specimens were then confirmed by FISH.

For NextGENe, a coverage ratio (sample divided by sample plus control) is the basis for CNV detection. Coverage is converted to a normalized read count value to account for differences in coverage from sample to sample. A β -binomial model is fit to the coverage ratio to model the amount of noise (dispersion). The likelihood a copy number variant is present is calculated from the coverage ratio and dispersion value and can factor estimated tumor purity and expected CNV percentage. A hidden Markov model makes the CNV classification of duplication, normal, or deletion with accompanying Phred-scaled probability scores.

The Ion Reporter predicts the copy number or ploidy state (0, 1, 2, 3, etc) of the tumor by comparing normalized read coverage (after correction for GC bias) to a baseline constructed from control sample(s) with known ploidy. A statistical model adjusts for regions with low read coverage and significant variation, then calculates the likelihood a gene belongs to an alternate ploidy state. A hidden Markov model framework determines the optimal path through the ploidy state to maximize the total ploidy state likelihoods when state transition penalties are considered. A medium setting using medium transition penalties was used to enable robust CNV detection with few false-positive calls. Confidence (log ratio of called ploidy versus expected ploidy state likelihoods) and precision (log ratio of assigned ploidy versus next-closest ploidy state likelihoods) scores accompany the copy number predictions.

Fluorescence *in Situ* Hybridization

FISH analysis was performed as described previously.^{23–28} Briefly, multiple sections (4 μm thick) were obtained from formalin-fixed, paraffin-embedded tissue blocks containing neoplastic tissue. A hematoxylin and eosin–stained slide from each block was examined to identify areas of neoplastic tissue for FISH analysis. The slides were deparaffinized with two washes of xylene, 15 minutes each, and subsequently washed twice with absolute ethanol,

10 minutes each, and then air dried in a fume hood. Next, the slides were treated with 0.1 mmol/L citric acid (pH 6.0) (Zymed, South San Francisco, CA) at 95°C for 10 minutes, rinsed in distilled water for 3 minutes, followed by a wash of $2\times$ standard saline citrate for 5 minutes. Digestion of nuclear proteins was performed by applying 0.4 mL of pepsin (5 mg/mL in 0.01 N HCl/0.9% NaCl; Sigma, St. Louis, MO) at 37°C for 40 minutes. The slides were rinsed with distilled water for 3 minutes, washed with $2\times$ standard saline citrate for 5 minutes, and air dried.

FISH used a cocktail of dual-color DNA probes from Empire Genomics (Buffalo, NY) that targeted *RB1*-Orange (MAC clone RP11-893E5) and 13q11-Green (RP11-408E5). The second cocktail of dual-color probes consisted of *ERBB4*-Green (Empire Genomics) and CEP2-Orange (Abbott Molecular, Lake Bluff, IL). Probes were diluted at 1:25 with tDenHyb 2 (Insitus, Albuquerque, NM). Diluted probe (5 μL) was applied to each slide in reduced light conditions. The slides were then covered with a $22 \times 22\text{-mm}$ coverslip and sealed with rubber cement. Denaturation was achieved by incubating the slides at 83°C for 12 minutes in a humidified box, followed by hybridization at 37°C overnight.

The coverslips were removed, and the slides were washed twice with $0.1 \times$ standard saline citrate/1.5 mmol/L urea at 45°C (20 minutes for each), followed by a wash with $2 \times$ standard saline citrate for 20 minutes, and with $2 \times$ standard saline citrate/0.1% NP-40 for 10 minutes at 45°C . The slides were further washed with room temperature $2 \times$ standard saline citrate for 5 minutes. The slides were air dried and counterstained with 10 mL DAPI (Insitus), covered with coverslips, and sealed with nail polish.

FISH Evaluation

The slides were examined using a Zeiss Axioplan 2 microscope (ZEISS, Göttingen, Germany) with the following filters (Chroma, Brattleboro, VT): SP-100 for DAPI, fluorescein isothiocyanate MF-101 for Spectrum Green, and Gold 31003 for Spectrum Orange. The hybridized slides were analyzed and documented using a MetaSystem system (Belmont, MA) under a $100\times$ oil objective. Four sequential focus stacks with 0.3- μm intervals were acquired and integrated into a single image to reduce thickness-related artifacts. Signals from each color channel (probe) were counted under pseudocolor, with computerized translation of each color channel into blue, green, and red. A minimum of 100 nonoverlapping cancer cells were evaluated for each case. Slides were counted only if $>90\%$ of cells demonstrated satisfactory signal quality.^{23–28}

The deletion threshold for *RB1* and *ERBB4* copy number was based on previous literature, including studies of chromosome 1p/19q deletions in oligodendrogliomas.^{29–32} Homozygous deletions of *RB1* or *ERBB4* were defined as the absence of both gene-specific signals with intact control signals in $\geq 50\%$ of cells. Hemizygous deletions were

Table 1 Next-Generation Sequencing Data for RO and ChRCC

Chromosome	Gene	RO		ChRCC												% with loss	% with gain
		% with loss	% with gain	1	2	3	4	5	6	7	8	9	10	11	12		
1	<i>NRAS</i>	0	0						(-)							13	0
2	<i>ALK</i>	0	20						(-)		(-)			(-)		38	0
2	<i>ERBB4</i>	0	0						(-)		(-)		(-)	(-)		63	0
3	<i>VHL</i>	0	0													0	0
3	<i>PIK3CA</i>	0	0							(+)						13	13
4	<i>FGFR3</i>	0	20					(+)		(-)			(+)	(+)		13	38
4	<i>PDGFRA</i>	0	0													0	0
4	<i>KIT</i>	0	0													0	0
4	<i>KDR</i>	0	0													0	0
4	<i>FBXW7</i>	0	0								(-)					13	0
5	<i>APC</i>	0	0													13	0
5	<i>CSF1R</i>	0	0													0	13
7	<i>EGFR</i>	0	0													0	0
7	<i>MET</i>	0	0													0	0
7	<i>SMO</i>	0	0										(+)	(+)		0	38
7	<i>BRAF</i>	0	0										(-)	(-)		38	0
8	<i>FGFR1</i>	0	0													0	0
9	<i>CDKN2A</i>	40	20	(-)	(-)			(+)		(-)		(-)				25	0
9	<i>ABL1</i>	0	0											(+)		0	25
9	<i>NOTCH1</i>	40	20	(-)	(-)			(+)		(-)			(+)	(+)		13	38
10	<i>RET</i>	20	0		(-)				(-)	(-)						25	0
10	<i>PTEN</i>	0	0						(-)					(-)		38	0
10	<i>FGFR2</i>	0	0						(-)							13	0
11	<i>HRAS</i>	0	20					(+)	(-)				(+)	(+)		13	38
11	<i>ATM</i>	0	0						(-)							13	0
12	<i>KRAS</i>	0	0													13	0
12	<i>PTPN11</i>	0	0											(-)		25	0
12	<i>HNF1A</i>	0	0							(-)			(+)	(+)		13	38
13	<i>FLT3</i>	0	0								(-)			(-)		25	0
13	<i>RB1</i>	0	20					(+)			(-)	(-)	(-)	(-)	(-)	75	0
14	<i>AKT1</i>	20	0		(-)					(-)			(+)	(+)		13	38
16	<i>CDH1</i>	20	0			(-)										0	13
17	<i>TP53</i>	0	0						(-)	(-)	(-)	(-)	(+)			50	13
17	<i>ERBB2</i>	0	0						(-)	(-)	(-)		(+)			38	13
18	<i>SMAD4</i>	0	0													0	0
19	<i>STK11</i>	0	0							(-)			(+)	(+)		13	38
19	<i>JAK3</i>	0	0										(+)	(+)		0	38
20	<i>GNAS</i>	0	0											(+)		0	13
22	<i>SMARCB1</i>	20	0		(-)											0	13

Two or more cases carrying loss or gain ($\geq 29\%$) of total cases tested are in bold.

Loss (-), <1.2 gene copies; Gain (+), >3.3 gene copies; %, loss or gain, percentage of gains or losses.

ChRCC, chromophobe renal cell carcinoma; RO, renal oncocytoma.

defined by the presence of only one of the gene-specific signals in $\geq 50\%$ of nuclei.^{31,32}

Results

Rare Hotspot Mutations in 50 Oncogenes and Tumor-Suppressor Genes Identified in ChRCC but Not RO

It was first examined whether ChRCC ($n = 7$) or RO ($n = 5$) carried any hotspot somatic mutations in 50 oncogenes and tumor-suppressor genes using NGS. The 50

oncogenes and tumor-suppressor genes are those most commonly involved in human cancers ([Materials and Methods](#)). Approximately 2800 Catalogue of Somatic Mutations in Cancer mutational loci in 50 genes are targeted and sequenced. These regions are described as hotspot because they are frequently mutated in human cancers. Single *TP53* or *PIK3CA* mutations were detected in two ChRCCs at allele frequencies of 9% and 21%, respectively. The tumor percentage of the *TP53* mutant ChRCC was estimated to be 50%, indicating the mutation is present only in a subset of tumor cells.

Table 2 Hemizygous Deletions of *RB1* and *ERBB4* Genes and Their Frequency of Occurrence in RO and ChRCC by FISH Study

Diagnosis	Cases with hemizygous <i>RB1</i> deletion	Cases with hemizygous <i>ERBB4</i> deletion	Cases with either <i>RB1</i> or <i>ERBB4</i> deletions	Total cases
ChRCC	17/33 (52)	11/33 (33)	23/33 (70)	33
RO	0/32 (0)	0/32 (0)	0/32 (0)	32

Data are given as *n* or *n*/total (%).
ChRCC, chromophobe renal cell carcinoma; FISH, fluorescence *in situ* hybridization; RO, renal oncocytoma.

Copy Number Analysis Reveals Hemizygous Deletions of Tumor-Suppressor Genes *RB1* and *ERBB4* in ChRCC, Which Were Verified by FISH

It was further examined if there were any copy number variations in these 50 cancer-critical genes in ChRCC and RO genes that showed copy number gain or loss in at least 35% of ChRCC or RO (ie, not disomic). In the ChRCC subgroup, seven genes showed copy number loss and eight genes showed copy number gain, whereas the RO subgroup showed only two genes with copy number loss (Table 1). Both *RB1* and *ERBB4* showed loss of copy numbers in >60% of ChRCC, but were not deleted in RO (Table 1). The deletions of *RB1* and *ERBB4* genes were subsequently verified by FISH, with chromosomal probes directed against *RB1* and *ERBB4*. It was confirmed that there is a good correlation between the NGS and the FISH results in each specific case (Pearson correlation coefficient = 1, *P* < 0.01) (Table 1 and Supplemental Table S1). FISH studies demonstrated a hemizygous deletion of the *RB1* gene in 17 of 33 ChRCC cases (52%), but not in any of 32 RO cases (0%) (Table 2, Figure 1, and Supplemental Table S1). Consistent with the NGS data, a hemizygous deletion of the *ERBB4* gene was detected in 11 of 33 ChRCC cases by FISH (33%),

whereas none of the 32 RO cases showed *ERBB4* deletion (0%) (Table 2, Figure 1, and Supplemental Table S1). Furthermore, there was no significant correlation between ChRCC tumor stages and *RB1* gene deletion (T1, 7/13, 54%; T2, 2/5, 40%; T3, 8/15, 53%; *P* = 0.31, χ^2 test). Similarly, ChRCC tumor stage did not correlate with *ERBB4* gene deletion (T1, 2/13, 23%; T2, 2/5, 40%; and T3, 6/15, 40%; *P* = 0.32, χ^2 test). Taken together, 23 of 33 ChRCC cases carried either an *RB1* and/or *ERBB4* gene deletion (70%) (Table 2). In contrast, none of the RO cases showed deletion of these two genes (0%) (Table 2).

Discussion

In this study, NGS was used to detect mutations and copy number variation in ChRCC and RO. This approach showed deletion of tumor-suppressor genes *RB1* and *ERBB4* in most ChRCC cases, but not in RO cases. On the basis of these NGS results, a simple FISH assay was adopted to confirm these findings. *RB1* and/or *ERBB4* deletions occurred in 70% of ChRCC cases but not in RO cases. Consequently, the combined use of *RB1* and *ERBB4* deletions by FISH yielded a highly specific and sensitive diagnostic assay to differentiate these tumors. Like other FISH assays, the *RB1* and *ERBB4* probes can be applied to surgical biopsy specimens or cell blocks derived from fine-needle aspiration of renal tumors. If applied to clinical practice, the presence of *RB1* and *ERBB4* deletion may help to exclude an RO and rule in a ChRCC. Nevertheless, the results of this study are based on a few cases. The diagnostic significance of the combined use of *RB1* and *ERBB4* gene deletions as a diagnostic marker should be further examined in larger numbers of patients with ChRCC and RO. The molecular mechanism of ChRCC tumorigenesis remains largely unclear. ChRCCs are frequently associated with large multiallelic copy number variations that include gains in chromosomes 4, 7, 11, 12, 14q, and 18q and losses in

Figure 1 Fluorescence *in situ* hybridization analysis of *RB1* and *ERBB4* genes in chromophobe renal cell carcinoma (ChRCC) and renal oncocytoma (RO). **A:** Normal tissue, with green signals indicating chromosomal centromere regions and red signals showing the presence of disomic *RB1* gene. **B and C:** Disomic *RB1* genes (red; **B**) and disomic *ERBB4* genes (green; **C**) in RO cells. **D and E:** A hemizygous deletion of *RB1* gene (loss of one red signal; **D**) and *ERBB4* gene (loss of one green signal; **E**) in ChRCC. Original magnification, $\times 400$ (**A–E**).

chromosome Y, 1, 2, 6, 10, 13, 17, and 21.³³ It has been reported that the loss of chromosomes Y, 1, 2, 6, 10, 13, 17, and 21 is nonrandom and might lead to tumor suppressor gene inactivation, promoting tumorigenesis.^{33,34} In the FISH assay, accompanying losses of centromere signals were observed in ChrCC cases carrying *RB1* or *ERBB4* deletions. These data suggest either a whole chromosomal deletion or a large partial chromosomal deletion that is composed of *RB1* or *ERBB4* and the corresponding centromere region. The NGS data favor the latter because genes present in different areas of chromosomes 2 or 13 are identified in the same ChrCC cases with centromere loss (Table 1). Deletions that span a centromere will not affect chromosomal DNA replication because an origin of replication is found every 250 kilobase of human DNA. However, the resulting acentric chromosome will most likely be lost during cell division, unless there is a selective pressure that helps to maintain the acentric chromosome in the genome. Centromere deletion has been described in naturally occurring precancer and cancer genomes, including myelodysplastic syndrome and acute myeloid leukemia.³⁵

Previous reports indicate RO occasionally exhibits losses of chromosome 1.^{12,13} In this study, gene amplicons targeting chromosome 1 did not reveal obvious alterations in the RO cohort. Copy number changes in *CDKN2A* and *NOTCH* on chromosome 9 were observed in RO, but these abnormalities were also found in ChrCC.

In contrast to the numerous chromosomal anomalies found in ChrCC, this tumor type is reported to have a low rate of somatic mutation.³⁶ Similarly, only two somatic mutations were observed in 10 ChrCCs tested. Davis et al³⁷ used mitochondrial DNA and whole-genome sequencing to show *TP53* mutations and *PTEN* mutations in 21 and six ChrCC cases (32% and 9%), respectively. In this study, a *TP53* mutation was identified in one ChrCC (10%), but *PTEN* mutations were not observed. In agreement with previous studies, no *KIT* mutations were detected in either ChrCC or RO, despite overexpression of C-kit in these two tumors.^{38–40}

In summary, NGS demonstrated multiple gene copy abnormalities, the two most common being *RB1* and *ERBB4* in ChrCC, but not in RO. FISH was used to confirm these gene deletions in ChrCC. The FISH assay combining *RB1* and *ERBB4* probes for formalin-fixed, paraffin-embedded tissue sections offers a highly sensitive and specific genetic marker in distinguishing ChrCC from benign RO.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.003>.

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