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Differing von Hippel Lindau genotype in paired primary and metastatic tumors in patients with clear cell renal cell carcinoma

Susan A. J. Vaziri†, Emmanuel J. Tavares†, Ali R. Golshayan, Brian I. Rini, Hakan Aydin, Ming Zhou, Linda Sercia, Laura Wood, Mahrukh K. Ganapathi, Ronald M. Bukowski and Ram Ganapathi*

Taussig Cancer Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Edited by:
Charles Swanton, Cancer Research UK London Research Institute, UK

Reviewed by:
Rosalie Fisher, Royal Marsden Hospital, UK
Nicolai Juul Birkbak, Dana-Farber Cancer Institute, USA
Trevor A. Graham, University of California San Francisco, USA
Stuart David Horswell, Cancer Research UK, UK

***Correspondence:**
Ram Ganapathi, Levine Cancer Institute, Cannon Research Center, Carolinas HealthCare System, 1000 Blythe Boulevard, Charlotte, NC 28203, USA.
e-mail: ram.ganapathi@carolinashealthcare.org

†Susan A. J. Vaziri and Emmanuel J. Tavares have contributed equally to this work.

In sporadic clear cell renal cell carcinoma (CCRCC), the von Hippel Lindau (VHL) gene is inactivated by mutation or methylation in the majority of primary (P) tumors. Due to differing effects of wild-type (WT) and mutant (MT) *VHL* gene on downstream signaling pathways regulating angiogenesis, *VHL* gene status could impact clinical outcome. In CCRCC, comparative genomic hybridization analysis studies have reported genetic differences between paired P and metastatic (M) tumors. We thus sequenced the *VHL* gene in paired tumor specimens from 10 patients to determine a possible clonal relationship between the P tumor and M lesion(s) in patients with CCRCC. Using paraffin-embedded specimens, genomic DNA from microdissected samples (>80% tumor) of paired P tumor and M lesions from all 10 patients, as well as in normal tissue from 6 of these cases, was analyzed. The DNA was used for PCR-based amplification of each of the 3 exons of the *VHL* gene. Sequences derived from amplified samples were compared to the wild-type *VHL* gene sequence (GenBank Accession No. AF010238). Methylation status of the *VHL* gene was determined using *VHL* methylation-specific PCR primers after DNA bisulfite modification. In 4/10 (40%) patients the *VHL* gene status differed between the P tumor and the M lesion. As expected, when the *VHL* gene was mutated in both the P tumor and M lesion, the mutation was identical. Further, while the *VHL* genotype differed between the primary tumor in different kidneys or multiple metastatic lesions in the same patient, the *VHL* germline genotype in the normal adjacent tissue was always wild-type irrespective of the *VHL* gene status in the P tumor. These results demonstrate for the first time that the *VHL* gene status can be different between paired primary and metastatic tissue in patients with CCRCC.

Keywords: VHL genotype, renal cancer, genetic heterogeneity

INTRODUCTION

The major types of epithelial renal tumors include clear cell (75%), papillary (15%), chromophobe (5%), and oncocytoma (5%). Mutations in the von Hippel Lindau (*VHL*) tumor suppressor gene are associated with hereditary and sporadic forms of clear cell renal carcinoma only. The product of the *VHL* gene forms a heterodimeric complex with elongin C, elongin B, Cul-2, and RBX1 and targets the hypoxic inducible factors (HIF1α and HIF1β) for ubiquitin-mediated degradation. Mutation of the *VHL* gene in clear cell kidney cancer prevents the VHL complex from targeting HIFs for degradation, resulting in their accumulation. Increased levels of HIF result in increased transcription of downstream targets including VEGF and angiogenic pathways (Linehan et al., 2010).

Over the last few years, anti-VEGF therapies have made a major impact in the standard of care for patients with advanced clear cell renal cell carcinoma (CCRCC; Patard et al., 2011). These include bevacizumab (Yang et al., 2003), sunitinib (Motzer et al., 2006), axitinib (Rixe et al., 2007), pazopanib (Sternberg et al., 2010), and sorafenib (Motzer et al., 2007).

We had previously reported the possible impact of *VHL* gene mutation and promoter hypermethylation on the outcome to VEGF-targeted agents in patients with advanced CCRCC (Choueiri et al., 2008). While the overall response rate (ORR) to VEGF-targeted therapy in patients with metastatic RCC was not correlated with *VHL* inactivation, subset analysis suggested that loss of function VHL mutations may identify patients with increased ORR to VEGF-targeted agents.

Extensive genetic differences between matched primary and metastatic tumors in 6 out of 19 CCRCC cases following comparative genomic hybridization (CGH) analysis (Bissig et al., 1999) and intra-tumoral heterogeneity of VHL gene deletions (Moch et al., 1998) have been reported. Based on the availability of paired primary and metastatic tumors from 10 patients, the goal of this exploratory study was to determine whether the *VHL* genotype can indeed differ in these paired tissue samples.

MATERIALS AND METHODS

DNA SEQUENCE ANALYSIS

Genomic DNA was isolated from formalin fixed paraffin-embedded (FFPE) tumor biopsies as described in Choueiri et al.

(2008) after manual micro dissection (Blaveri et al., 2005). Samples that were at least 80% tumor, as determined by a pathologist (HA and MZ), were included in the study. A total of seven primer sets were used to amplify overlapping fragments of the coding region of the *VHL* gene (**Figure 1**). Due to inherent problems with quality of DNA extracted from FFPE specimens, three primer sets, two primer sets, and two primer sets were used to amplify exons 1, 2, and 3, respectively. The PCR reactions consisted of 100 ng of genomic DNA, 67 mM Tris-HCL, pH 8.8, 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 10 mM 2-mercaptoethanol, 12.5 mM dNTP's, and 10% DMSO, 0.5 μM primers, and 0.25 units of Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA). The primer sequences used were as follows: Exon 1A–F: 5′-CGAAGACTACGGAGGTCGAC-3′; 1A–R: 5′-TCTTCAGGGCCGTACTCTTCG-3′; 1B–F: 5′-AGGC CGAGGTAGGCGCGGA-3′; 1B–R: 5′-GCAGAAGATGACCTGG GAGGGC-3′; 1C–F: 5′-CTGCGCTCGGTGAACTCG-3′; 1C–R: 5′-CTATCGTCCCTGCTGGGTCG-3′; 2A–F′: 5′-GGATTACAG GTGTGGGCCAC-3′; 2A–R′: 5′-GGCTGTCCGTCAACATTGAG-3′; 2B–F: 5′-ACGATGGGCTTCTGGTTAAC-3′; 2B–R: 5′-CCTGTACTTACCACAACAACC-3′; 3A–F: 5′-TCCTTGTACTGA GACCCTAG-3′; 3A–R: 5′-TTTGGGTGGTCTTCCAGATC-3′; 3B–F: 5′-ATTACAGGAGACTGGACATC-3′; 3B–R: 5′-CCAGTCC TGTATCTAGATC-3′.

The amplicons underwent Sanger sequencing using the ABI377 automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Genomic's Core Facility, Lerner Research Institute, Cleveland Clinic. Sequences derived from the amplified samples were compared to the wild-type *VHL* sequence (GenBank Accession No. AF010238) using LaserGene software (DNAStar, Perkin Elmer, Foster City, CA, USA) to identify mutations. A subset of samples was also sent to Transgenomics (Omaha, NE, USA) for independent analysis of the *VHL* genotype. Sequence variations were identified on PCR-amplified samples that were screened using the mismatch cleavage enzyme, Surveyor, and DHPLC/WAVE® analyses followed by sequencing at Transgenomic (Nickerson et al., 2008).

METHYLATION ASSAY

Methylation status was determined using *VHL* methylation-specific PCR primers after DNA bisulfite modification. Genomic DNA was modified using the EZ DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA). The product then underwent PCR-based amplification using methylation-specific primers described previously (Herman et al., 1996). Methylation status was determined by gel electrophoresis of the PCR products as described in Herman et al. (1996).

RESULTS AND DISCUSSION

Paired primary and metastatic tumors were obtained from 10 patients. VHL genotype analysis in this 10 patient cohort included single biopsies from a single primary and metastatic lesion (5 patients), multiple primary and/or metastatic tumors (5 patients) and adjacent normal tissue (6 patients). Sequencing data was obtained from a total of 42 samples (18 primary tumors, 14 metastatic tumors, and 10 adjacent normal tissues). Due to the intrinsic difficulty in identifying true somatic mutations in tissues with genetic heterogeneity, a subset of 16 samples comprised of 8 primary and 8 metastatic samples were also analyzed by Transgenomic for independent assessment of *VHL* gene mutation status (**Table 1**). *VHL* genotype assessment of adjacent normal tissue, as well as evaluation of multiple samples where possible contributed to the validation of our data. Patient cohort included six men and four women. While the primary and metastatic tumors were obtained in most patients during a single surgery, in some patients subsequent surgery involved additional metastatic lesions or primary tumor in the contra lateral kidney (**Table 1**, patients 6 and 10).

A total of seven patients harbored mutations in the *VHL* gene in the primary and/or metastatic lesions, 1 patient exhibited *VHL* gene methylation, and two patients had the wild-type *VHL* genotype (**Table 1**; **Figure 1**). Sequencing traces identifying VHL genotype for data in **Table 1** are included as **Figure A1** in Appendix. Mutations identified in this study were not observed in our

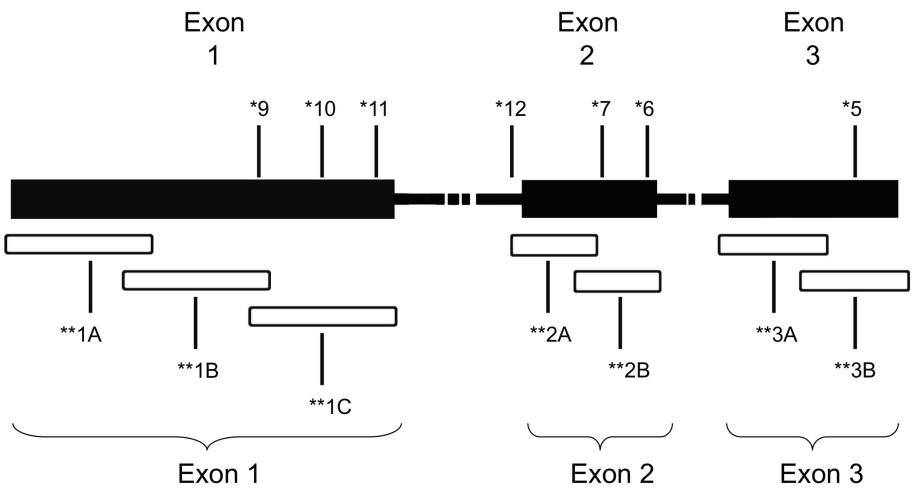

FIGURE 1 | Schematic of *VHL* gene mutations identified in the patient cohort. *Patient ID number. **Overlapping regions amplified by PCR.

Table 1 | von Hippel Lindau genotype and Fuhrman grade in primary and metastatic CCRCC tumors.

Pat. ID	Sample ID	Surgery date	Primary (P)/metastatic (M)/normal	VHL genotype	Fuhrman grade	
					Gr. 1/2	Gr. 3/4
CATEGORY 1: PATIENTS WITH IDENTICAL PRIMARY AND METASTATIC VHL GENOTYPE IN TUMORS						
4	1	12/2004	Primary, left kidney (P)	WT	Not evaluated	
4	2	12/2004	Metastatic, lymph node (M)	WT	Not evaluated	
5	3	8/2002	Primary, left kidney (P)	478delG, ex3	0	100
5	4	8/2002	Metastatic, lymph node (M)	478delG,ex3	0	100
5	5	8/2002	Adjacent normal	WT		
8	6	2/2005	Primary, right kidney (P)	Methylated Promoter	20	80
8	7	2/2005	Metastatic, adrenal (M)	Methylated Promoter	0	100
9*	8	8/2003	Primary, left kidney (P) Renal tumor	232delA,ex1	10	90
9	9	8/2003	Primary, left kidney (P) Tumor close to capsule	232delA,ex1	89	11
9*	10	8/2003	Metastatic, lymph node (M)	232delA, ex1	95	5
11	11	2/2004	Primary, left kidney (P)	349delT,ex1	10	90
11	12	2/2004	Metastatic, small bowel (M)	349delT,ex1	50	50
11	13	2/2004	Adjacent normal	WT		
13	14	3/2004	Primary, right kidney (P)	WT	100	0
13	15	3/2004	Metastatic, left adrenal (M)	WT	99	1
13	16	3/2004	Adjacent normal	WT		
CATEGORY 2: PATIENTS WITH DIFFERENT PRIMARY AND METASTATIC VHL GENOTYPE IN TUMORS						
6*	17	5/2003	Primary, left kidney (P)	407insATATATAT, ex2	100	0
6*	18	5/2004	Metastatic, fallopian tube (M)	WT	100	0
6*	19	5/2004	Metastatic, fallopian tube (M)	WT	100	0
6*	20	5/2004	Metastatic fallopian tube (M)	407insATATATAT, ex2	100	0
6	21	5/2003	Adjacent normal	WT		
7*	22	8/2004	Primary, left kidney (P)	WT	100	0
7*	23	8/2004	Metastatic, colon (M)	G463C, ex2	0	100
10*	24	9/2002	Primary, right kidney (P) Renal vein margin with tumor	WT	90	10
10*	25	9/2002	Primary, right kidney (P) Spatially separated representative section of primary tumor	C333G, ex1	10	90
10*	26	12/2003	Primary, left kidney (P) Representative section of tumor in relation to capsular margin	C333G, ex1	45	55
10*	27	12/2003	Primary, left kidney (P) Representative section of tumor in relation to parenchymal margin	C333G, ex1	66	34
10*	28	12/2003	Metastatic, lymph node (M)	C333G, ex1	100	0
10	29	9/2002	Adjacent normal	WT		
10	30	12/2003	Adjacent normal	WT		
10	31	12/2003	Adjacent normal	WT		
12	32	3/2004	Primary, right kidney (P) Representative section of closest parenchymal margin including area with renal sinus bulge of tumor	del31 bp, intron 1, 9nt before ex2	95	5

(Continued)

Table 1 | Continued

Pat. ID	Sample ID	Surgery date	Primary (P)/metastatic (M)/normal	VHL genotype	Fuhrman grade	
					Gr. 1/2	Gr. 3/4
12	33	3/2004	Primary, right kidney (P) Representative section of closest parenchymal margin including area with renal sinus bulge of tumor	del31 bp in intron 1, 9nt before ex2	95	5
12	34	3/2004	Primary, right kidney (P) Representative section of same primary	del31 bp in intron 1, 9nt before ex2	95	5
12*	35	4/2004	Primary, left kidney (P) Sections of tumor with capsule	WT	90	10
12	36	4/2004	Primary, left kidney (P) Same primary	WT	50	50
12*	37	3/2004	Lung metastasis Parenchymal line of resection	WT	10	90
12*	38	3/2004	Lung metastatsis Pleural margin of tumor	WT	10	90
12	39	3/2004	Lung metastasis Remaining metastatic tissue	WT	10	90
12	40	3/2004	Adjacent normal	WT		
12	41	3/2004	Adjacent normal	WT		
12	42	3/2004	Adjacent normal	WT		

All nucleotide positions are numbered with the adenosine of the AUG start site as position number 1. This corresponds to nt position 214 in the mRNA sequence GenBank accession no. NM_000551. DNA sequencing traces are included in Appendix.

**Samples that were also sent to Transgenomic.*

previous larger 123 patient cohort of primary tumors (Choueiri et al., 2008) suggesting that mutations were unique to the patient in which it was first described. Further, no two patients had identical *VHL* mutations and methylation in this and our previous study (Choueiri et al., 2008) was observed only where the *VHL* genotype was wild-type. The *VHL* gene was methylated in 12 out 123 patients (10%) in the Choueiri study (Choueiri et al., 2008). Notably in 4 out of 10 patients the *VHL* genotype differed between the primary and matched metastatic lesion. Also in 2 of these patients, while the primary tumor was wild-type a mutant *VHL* genotype was identified in the paired metastatic lesion (patient #7 and #10, **Table 1**) likely due to intra-tumor heterogeneity. Inter- and intra-tumor heterogeneity in *VHL* genotype is exemplified in patients 10 and 12 (**Table 1**) between the primary tumor in different kidneys as well as metastasis removed at surgery. Additional data supporting heterogeneity in *VHL* genotype is based on identification of wild-type and mutant *VHL* in the primary tumor (patient #10 and #12) and in metastasis (patient #6) when different micro dissected tumor areas were analyzed. *VHL* mutations, when present, were identical between primary and metastatic sites and the *VHL* genotype in adjacent normal tissue in all 6/10 patients analyzed was wild-type, irrespective of *VHL* gene status of tumor tissue. VHL mutation status was not found to correlate with tumor grade (**Table 1**).

Polyclonality in colorectal adenomas (Thirwell et al., 2010), genetic heterogeneity in tumors with mutations in a single gene (Dalgliesh et al., 2010) and genetic diversity based on single cell sequencing (Navin et al., 2011) suggests that the focus on specific genetic lesions for personalized targeted therapy may be overly simplistic. More recently (while this manuscript was under review), intra-tumor heterogeneity in RNA expression or inactivating mutations in renal carcinoma has been reported (Gerlinger et al., 2012). The present study and previous reports (Moch et al., 1998; Bissig et al., 1999) emphasize that multiple genetically different clones are possibly present in clear cell renal carcinoma and this could contribute to the observed differences in *VHL* genotype between the primary and metastatic tumor in the same patient. Since the patients in this study had sporadic CCRCC, it is likely that clonal heterogeneity and mutations in the *VHL* gene may occur either during tumor development or subsequent tumor progression. This possibility is supported by the heterogeneity in *VHL* genotype within a single primary (Patient 6) or metastasis (Patient 12) when different spatially separated tumor sites was analyzed for *VHL* genotype. It is also becoming increasingly apparent that several targets for current cancer therapies can also display discordance in expression or mutation status between primary and metastatic sites. Previous studies in breast (Torres et al., 2007) and melanoma (Katona et al., 2007) cases have reported extensive genetic heterogeneity between primary and metastatic tumors. Detailed assessments of PIK3CA mutations between primary and matched metastatic breast tumors report not only discordance in mutations but also microheterogeneity in mutational status of the primary tumor (Dupont Jensen et al., 2011). Similarly, discordance in HER2 expression between primary and

paired metastatic breast cancer tumors is reported to occur at a significant rate (Fabi et al., 2011; Houssami et al., 2011). Even in a CCRCC study of unmatched primary tumors and metastatic lesions, significant differences between primary and metastatic renal tumors in the expression levels of several proteins involved in the mTOR pathway including phos-AKT, phos-S6, 4EBP1, and c-myc were reported (Schultz et al., 2011). In summary, these studies suggest marked molecular heterogeneity between primary and metastatic solid tumors.

The *VHL* gene is unique in that somatic mutations are observed only with kidney tumors and the aberrant signaling due to *VHL* mutations has a direct effect on the angiogenic pathway. Although *VHL* is classified as a tumor suppressor, nearly 20% of sporadic CCRCC harbor the wild-type *VHL* genotype. While the observed differences in *VHL* genotype between the primary and metastatic tumor could be ascribed to technical issues, results from the present study were independently validated in 13/16 samples by Transgenomic (**Table A1** in Appendix) using previously reported methodology (Nickerson et al., 2008) and further, the adjacent normal tissue in all cases independent of the tumor *VHL* genotype was wild-type *VHL* since the patients had sporadic CCRCC. In summary, in CCRCC, since primary tumor nephrectomy can precede a subsequent primary in the contralateral kidney and/or metastatic disease, the present results on intra- and inter-tumor heterogeneity in wild-type or mutant VHL gene suggest that reliance on *VHL* genotype of the primary tumor for treatment strategy may not be completely informative.

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APPENDIX

Table A1 | Comparison of *VHL* genotype calls – Cleveland Clinic and Transgenomic ($n = 16$ samples).

Patient ID	Sample ID	DNA sequenced at Cleveland Clinic	DNA sequenced and reported by Transgenomic
6P[a]		407insATATATAT, ex2	412inATATATAT, ex2
6M[b]	18	wt	wt
6M	19	wt[c]	412insATATATAT, ex2
6M	20	407insATATATAT, ex2	412insATATATAT, ex2
7P		wt[d]	G463C
7M		G463C	G463C
9P		232delA	232 delA
9M		232delA[e]	P25S, 10% RSI[f]
10P	24	wt	wt
10P	25	C333G	C333G
10P	26	C333G	C333G
10P	27	C333G	C333G
10M		C333G	C333G
12P		wt	wt
12M	37	wt	wt
12M	38	wt	wt

[a] *Primary.*

[b] *Metastatic.*

[c,d,e] *Calls based on sequence traces presented in* ***Figure A1****.*

[f] *Relative signal intensity (RSI).*

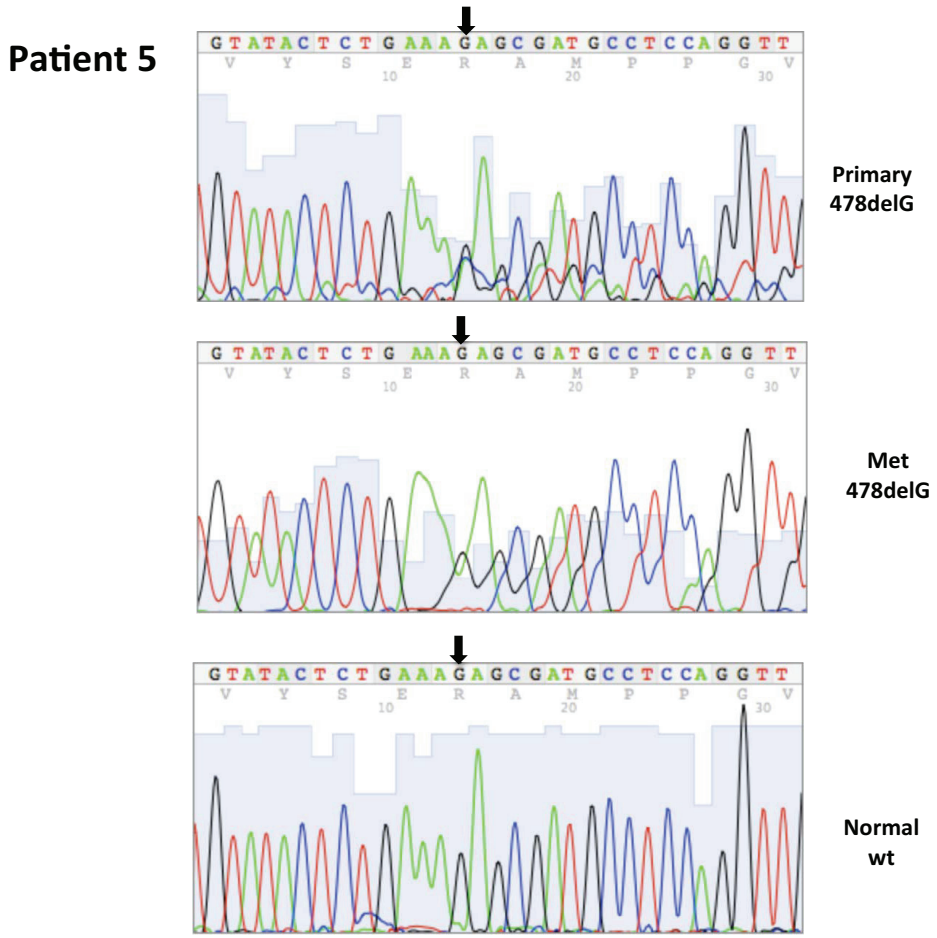

FIGURE A1 | Continued

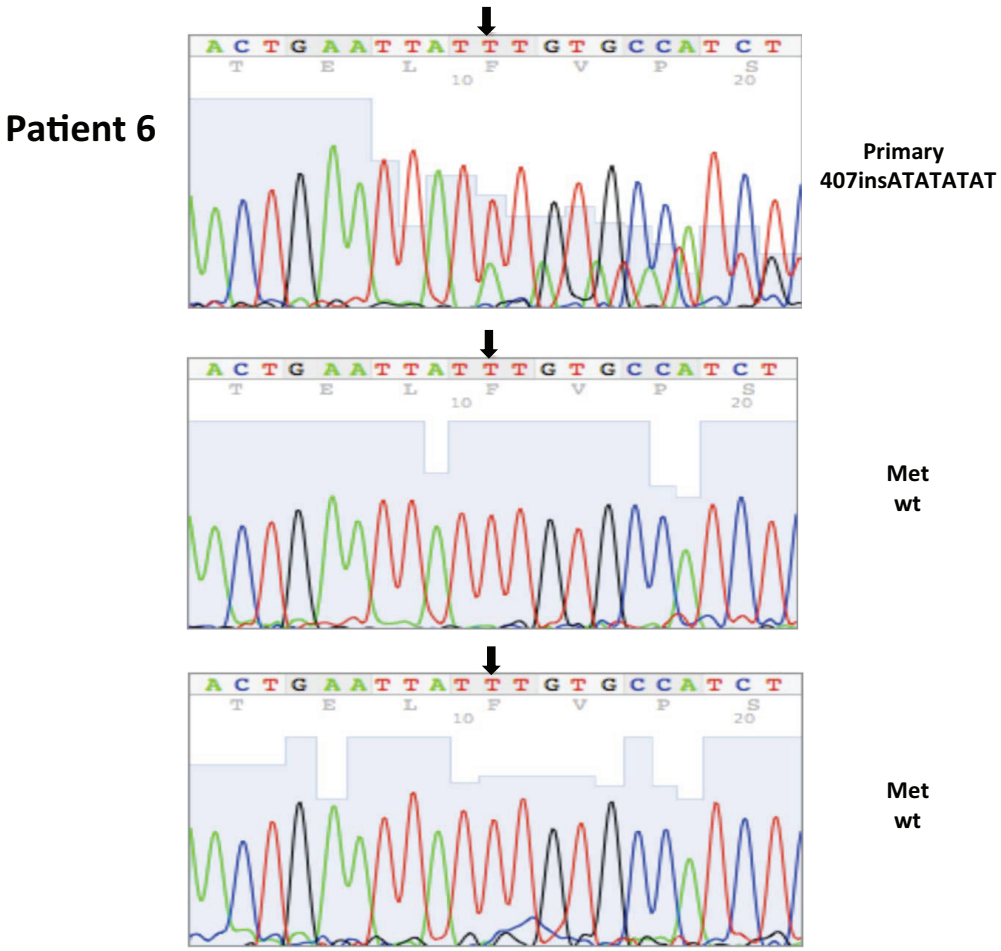

FIGURE A1 | Continued

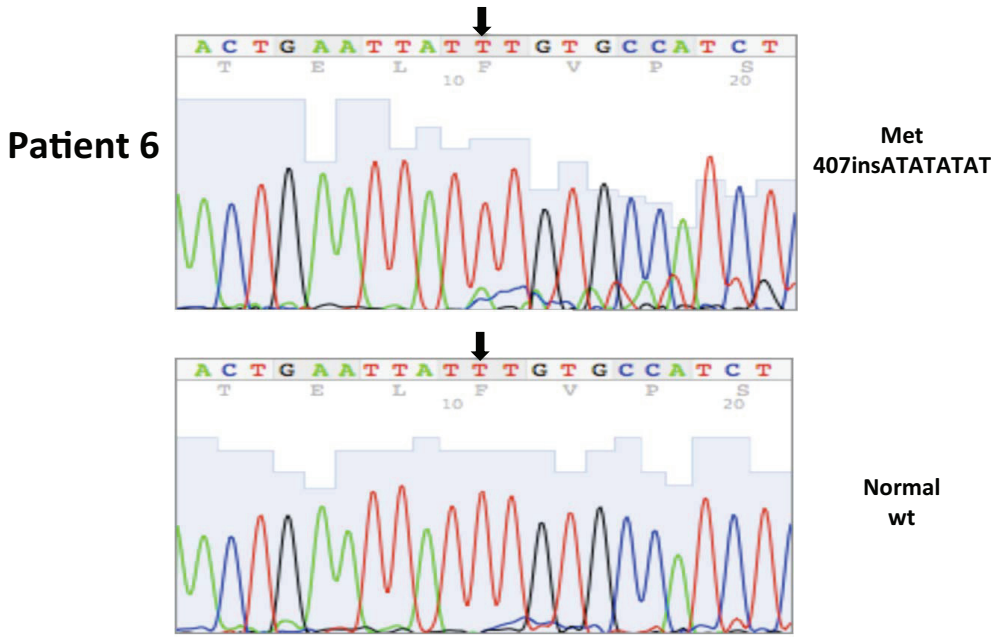

FIGURE A1 | Continued

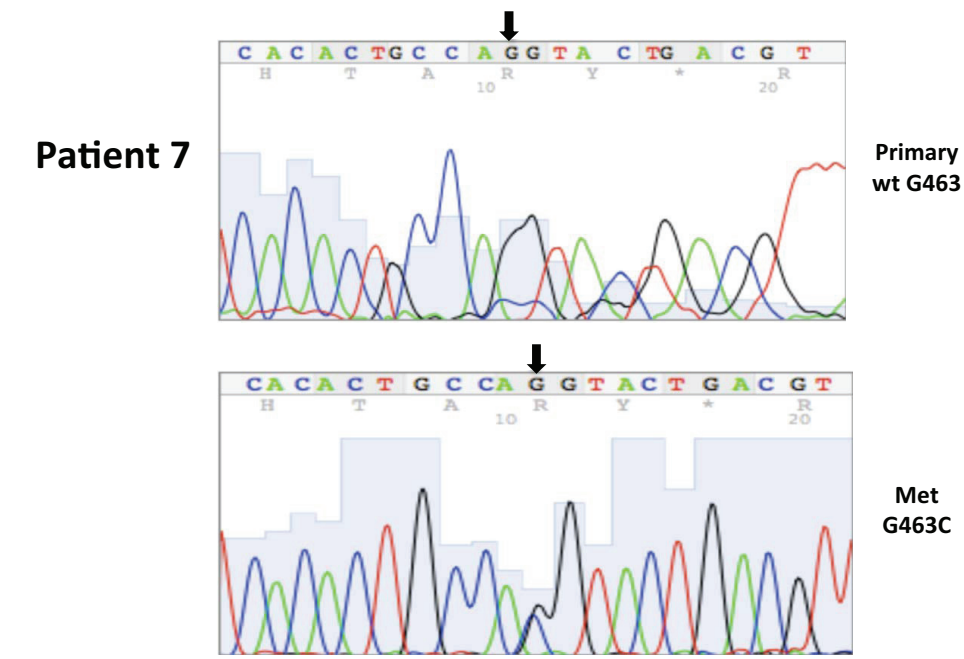

FIGURE A1 | Continued

Patient 9

Reverse complementary sequence for all tracings reported for patient 9

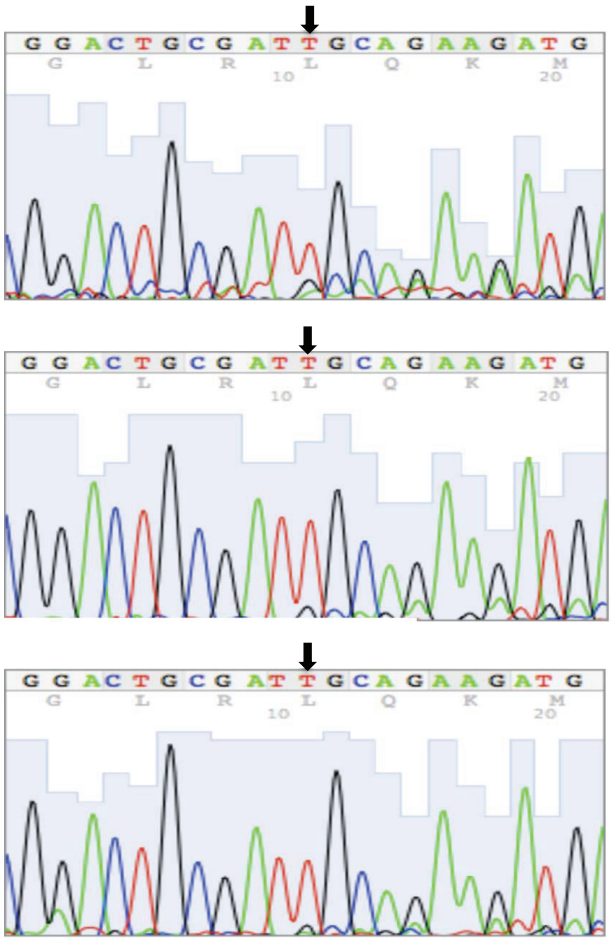

FIGURE A1 | Continued

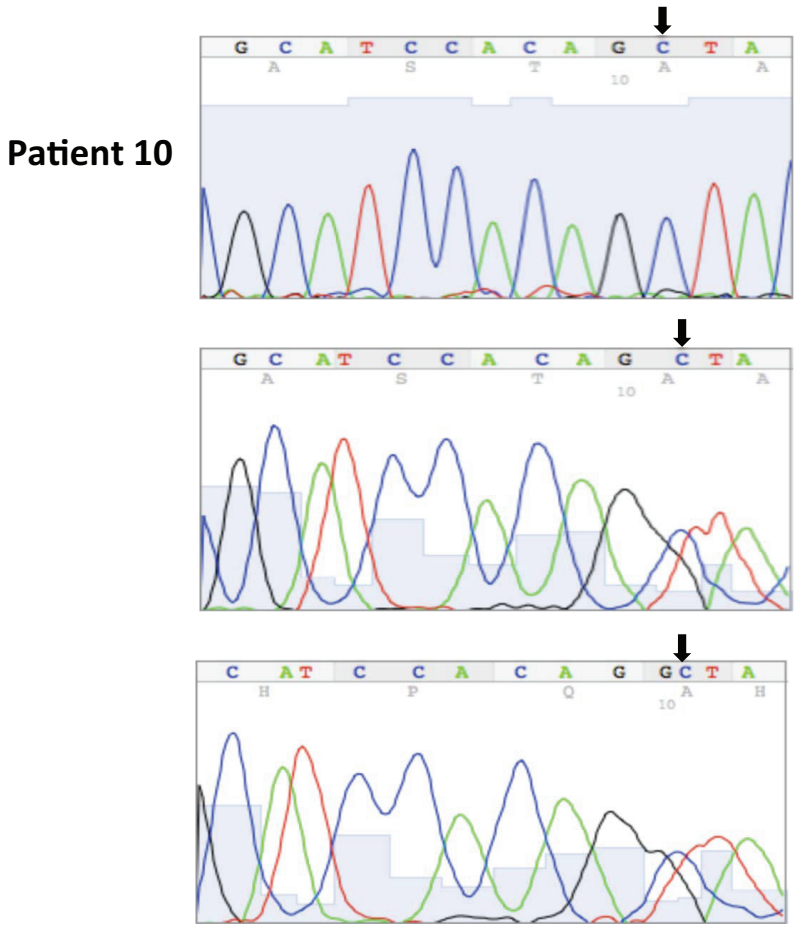

FIGURE A1 | Continued

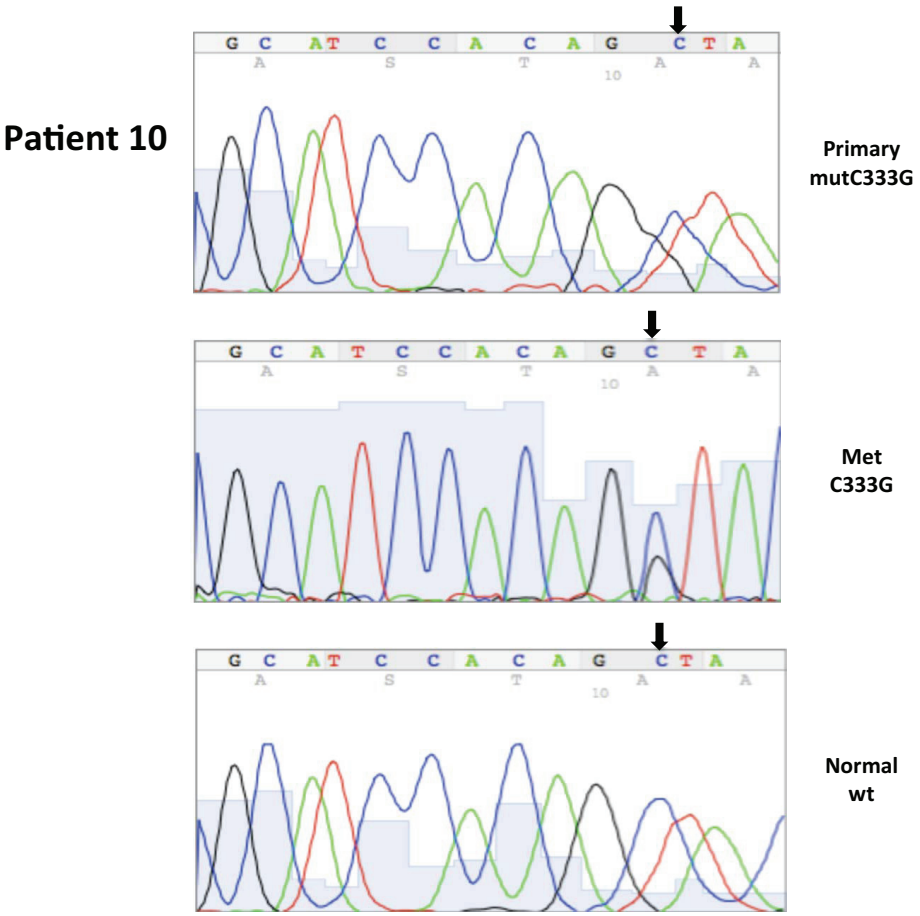

FIGURE A1 | Continued

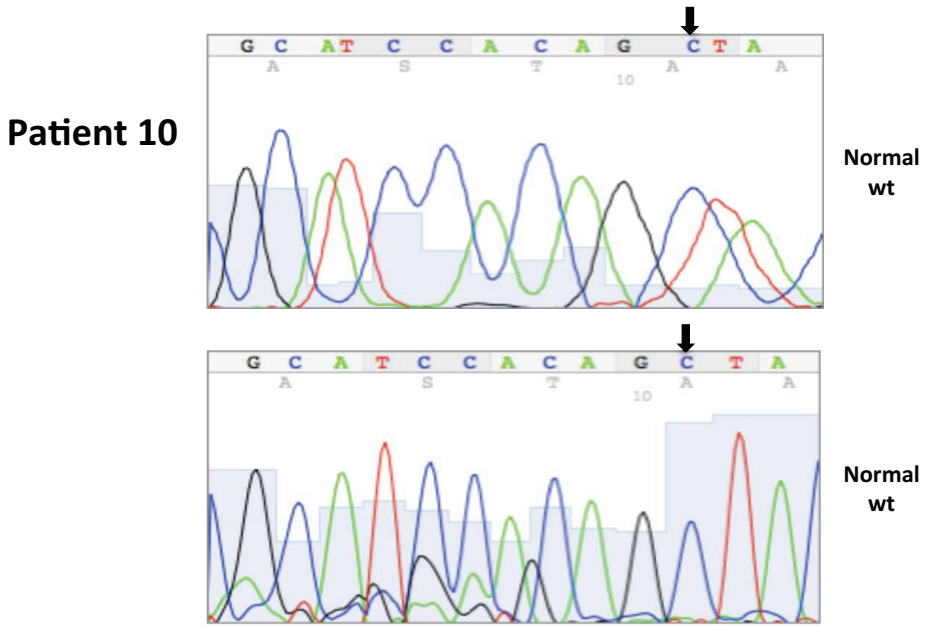

FIGURE A1 | Continued

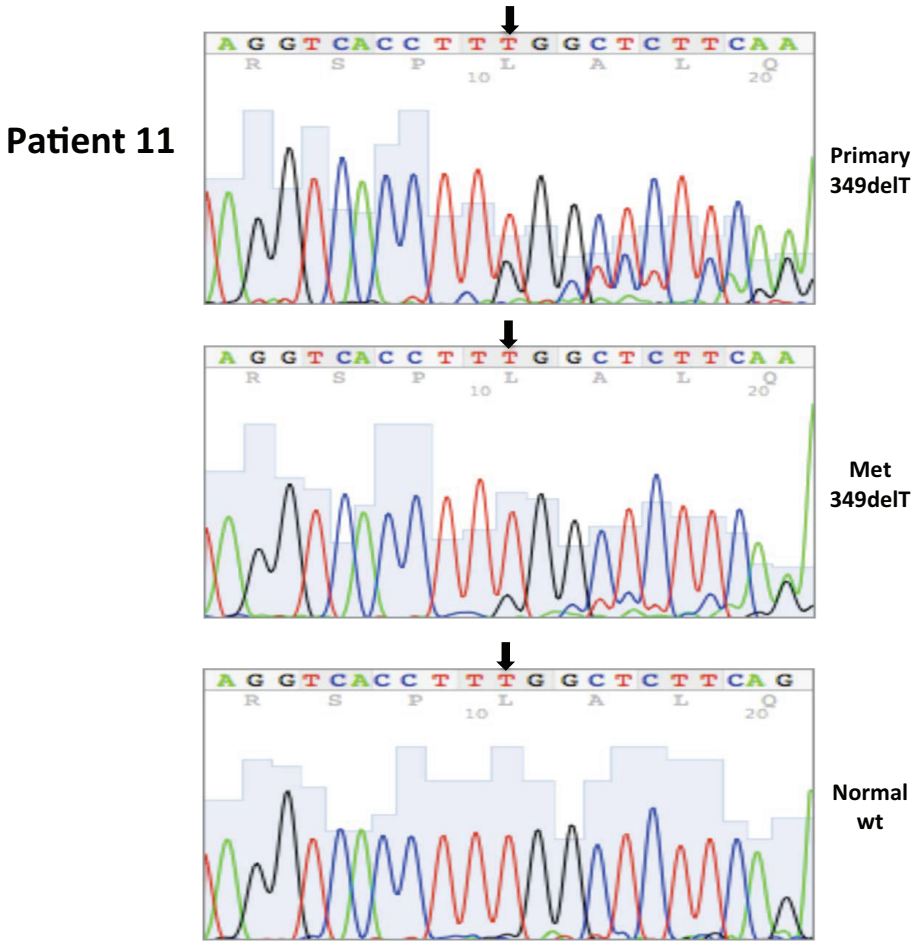

FIGURE A1 | Continued

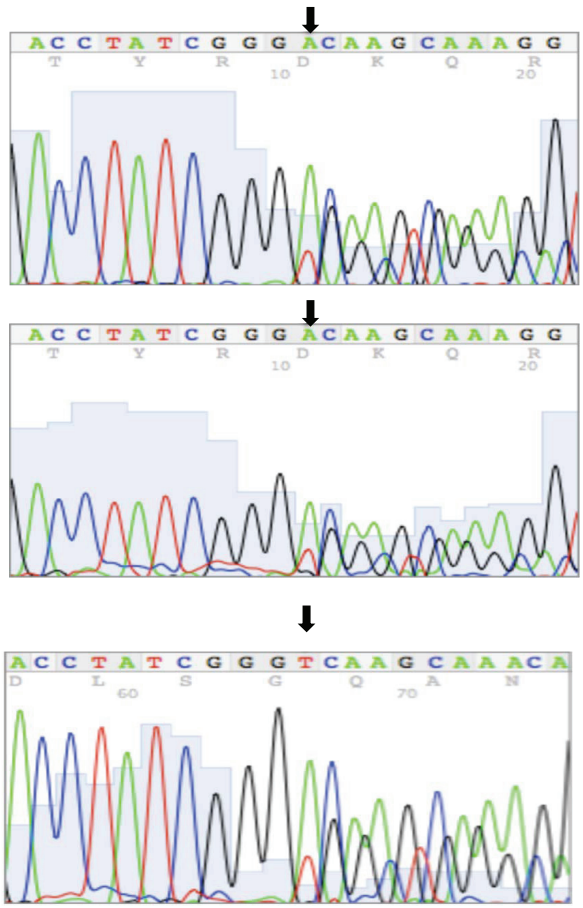

FIGURE A1 | Continued

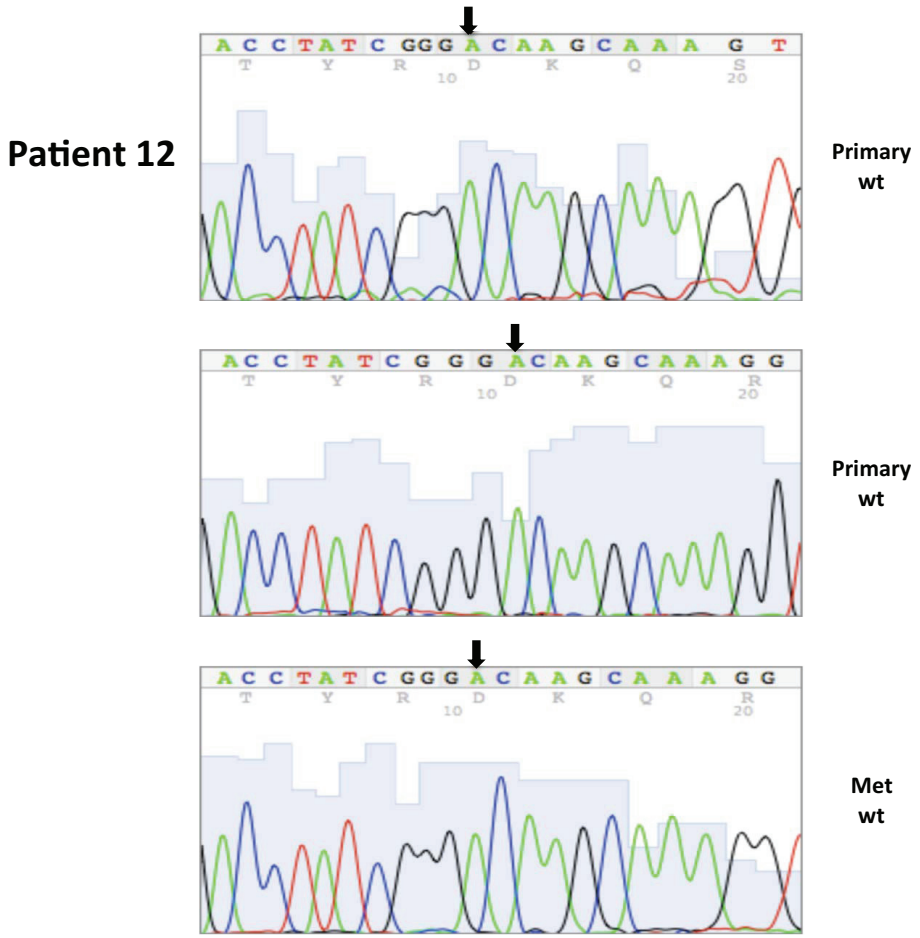

FIGURE A1 | Continued

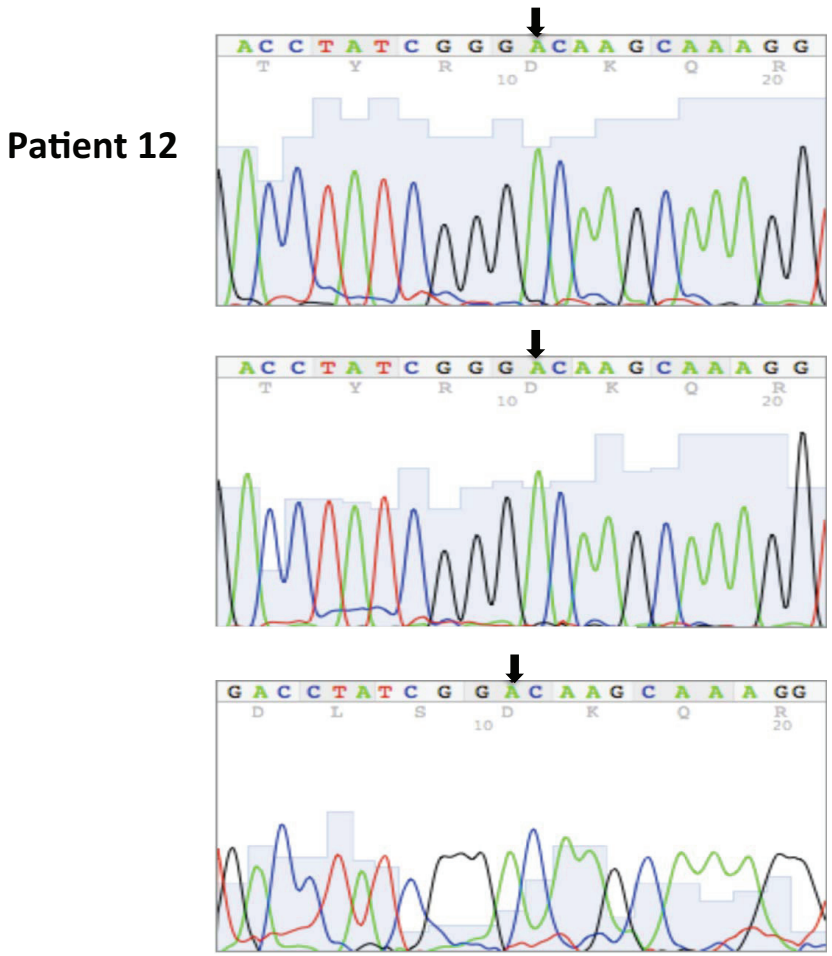

FIGURE A1 | Continued

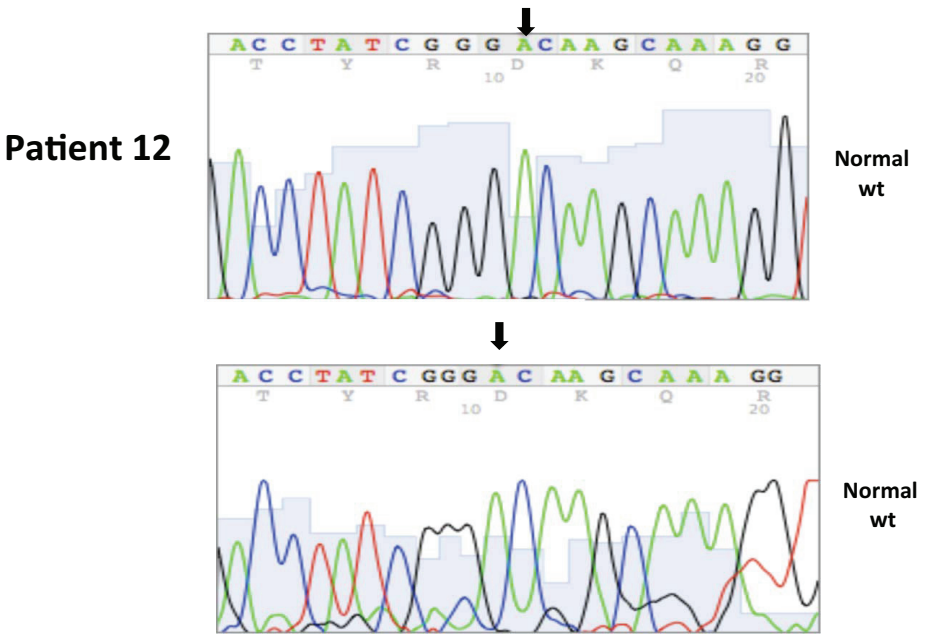

FIGURE A1 | Somatic VHL mutation sequencing in normal and tumor tissue of representative patients from Table 1.