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

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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.
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)         | Methylationed     | 20 80         |
| 8       | 7          | 2/2005       | Metastatic, adrenal (M)           | Methylationed     | 0 100         |
| 9*      | 8          | 8/2003       | Primary, left kidney (P)          | 232delA,ex1      | 10 90         |
| 9       | 9          | 8/2003       | Primary, left kidney (P)          |                  | 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)          | 407insATATATATAT, | 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)     | 407insATATATATAT, | 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)         | WT               | 90 10         |
| 10*     | 25         | 9/2002       | Primary, right kidney (P)         | C333G, ex1       | 10 90         |
| 10*     | 26         | 12/2003      | Primary, left kidney (P)          | C333G, ex1       | 45 55         |
| 10*     | 27         | 12/2003      | Primary, left kidney (P)          | 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)         | del31 bp, intron | 95 5          |

(Continued)
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 of 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

### 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)        | del31 bp in intron 1, 9nt before ex2 | 95            | 5            |
| 12     | 34        | 3/2004       | Primary, right kidney (P)        | del31 bp in intron 1, 9nt before ex2 | 95            | 5            |
| 12*    | 35        | 4/2004       | Primary, left kidney (P)         | WT           | 90           | 10           |
| 12     | 36        | 4/2004       | Primary, left kidney (P)         | WT           | 50           | 50           |
| 12*    | 37        | 3/2004       | Lung metastasis                  | WT           | 10           | 90           |
| 12*    | 38        | 3/2004       | Lung metastasis                  | WT           | 10           | 90           |
| 12     | 39        | 3/2004       | Lung metastasis                  | 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.*
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 only with kidney tumors and the aberrant signaling due to VHL. Although VHL mutations have 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 |
|------------|-----------|-----------------------------------|-------------------------------------------|
| 6Pa        | 407insATATATAT, ex2 | 412insATATATAT, ex2 |
| 6Mb        | wt        | wt                                |
| 6M         | wt        | 412insATATATAT, ex2 |
| 7P         | 407insATATATAT, ex2 | 412insATATATAT, ex2 |
| 7M         | wt        | G463C                             |
| 9P         | G463C     | G463C                             |
| 9M         | 232delA   | 232 delA                         |
| 10P        | 24        | C333G                             |
| 10P        | 25        | C333G                             |
| 10P        | 26        | C333G                             |
| 10P        | 27        | C333G                             |
| 12P        | 37        | C333G                             |
| 12M        | 38        | C333G                             |

*a Primary.

*b Metastatic.

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

f Relative signal intensity (RSI).
Patient 6

Primary
407insATATATAT

Met
wt

Met
wt

FIGURE A1 | Continued
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FIGURE A1 | Continued

Patient 6

Met
407insATATAT

Normal
wt

Patient 7

Primary
wt G463

Met
G463C

FIGURE A1 | Continued
Patient 9
Reverse complementary sequence for all tracings reported for patient 9

FIGURE A1 | Continued
FIGURE A1 | Continued
Patient 10

Primary mutC333G

Met C333G

Normal wt

FIGURE A1 | Continued
Patient 10

Normal wt

Normal wt

FIGURE A1 | Continued
Patient 11

Primary 349delT

Met 349delT

Normal wt

FIGURE A1 | Continued
Patient 12
Reverse complementary sequence for all tracings reported for patient 12

Primary del31nt 9nt before exon2

Primary del31nt 9nt before exon2

Primary del31nt 9nt before exon2

FIGURE A1 | Continued
FIGURE A1 | Continued

Patient 12

Primary wt

Primary wt

Met wt
Patient 12

FIGURE A1 | Continued
Patient 12

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