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Epigenetic sampling effects: nephrectomy modifies the clear cell renal cell cancer methylome

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Corresponding Author:	Christophe Van Neste Ghent University BELGIUM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Ghent University
Corresponding Author's Secondary Institution:	
First Author:	Christophe Van Neste
First Author Secondary Information:	
Order of Authors:	Christophe Van Neste
	Alexander Laird
	Fiach O'Mahony
	Wim Van Criekinge
	Dieter Deforce
	Filip Van Nieuwerburgh
	Thomas Powles
	David J Harrison
	Grant D Stewart
	Tim De Meyer
Order of Authors Secondary Information:	
Funding Information:	Chief Scientist Office (ETM37) — Dr. Grant D Stewart
	Pfizer UK — Dr. Thomas Powles
	Medical Research Council — Dr. Alexander Laird
	Royal College of Surgeons of Edinburgh — Dr. Alexander Laird
Abstract:	Currently, it is unclear to what extent sampling procedures affect the epigenome. Here, this phenomenon was evaluated by studying the impact of artery ligation on DNA methylation in clear cell renal cell cancer. To this end, DNA methylation profiles between vascularised tumour biopsy samples and devascularized nephrectomy samples from two individuals were compared. The relevance of significantly altered methylation profiles was validated in an independent clinical trial cohort. We found that six genes were differentially methylated in the test samples, of which four were linked to ischaemia or hypoxia (REXO1L1, TLR4, hsa-mir-1299, ANKRD2). Three of these genes were also found to be significantly differentially methylated in the validation cohort, indicating that the observed effects are genuine. Based on these results, we conclude that the impact of sampling procedures in clinical epigenetic studies should

	be considered, particularly after inducing hypoxia/ischemia, which occurs in many oncological surgery procedures through which tissues are harvested for translational research.
Response to Reviewers:	Dear editor, my sincerest thanks for your help in making this a better manuscript

The Editorial board of Cellular Oncology

Ghent, September 1, 2016

Dear Dr. Editor,

Please find enclosed our manuscript entitled "*Epigenetic sampling effects: nephrectomy modifies the clear cell renal cell cancer methylome*" for consideration for publication in *Cellular Oncology* as a short communication.

In a recently accepted paper (*Dynamic epigenetic changes to VHL occur with sunitinib in metastatic clear cell renal cancer*, Stewart et al, *Oncotarget*), we demonstrated an epigenetic change in the VHL gene upon clear cell renal cancer treatment. As pre- and post treatment conditions differed in the manner of sampling, we ran an additional study to analyse a possible sampling effect (biopsy vs. nephrectomy). Though this was not the case for VHL – subject of the previous paper - a clear sampling effect was observed for some loci and also appeared to be relevant in the actual treatment study. This implies that sampling procedure impacts results in cellular oncology studies. As the most likely underlying cause – hypoxia – is relevant in many such studies, and the sampling study is unique in its kind (biopsy immediately followed by nephrectomy), we are confident that this paper is of general interest to researchers in the field of cellular oncology.

Yours sincerely,

Prof. Dr. Tim De Meyer
Dept. of Mathematical Modelling, Statistics and Bioinformatics
Ghent University - Belgium

Short Communication

Epigenetic sampling effects: nephrectomy modifies the clear cell renal cell cancer methylome

Christophe Van Neste[1,2], Alexander Laird[3,4,5], Fiach O'Mahony[3,4], Wim Van Criekinge[6], Dieter Deforce[1], Filip Van Nieuwerburgh[1], Thomas Powles[7,8], David J. Harrison[4,9,*], Grant D. Stewart[3,4$] and Tim De Meyer[6$]

***Correspondence:**
david.harrison@st-andrews.ac.uk
9 School of Medicine,
University of St Andrews,
KY16 9AJ St Andrews, UK

[$] These authors contributed equally to this work

Affiliations:

[1] Laboratory of Pharmaceutical Biotechnology, Ghent University, Ottergemsesteenweg 460, 9000 Gent, Belgium.
[2] Center for Medical Genetics, Ghent University, De Pintelaan 185, 9000 Gent, Belgium.
[3] Edinburgh Urological Cancer Group, Institute of Genetics and Molecular Medicine, University of Edinburgh, EH4 2XU, Edinburgh, UK.
[4] Scottish Collaboration On Translational Research into Renal Cell Cancer (SCOTRRCC).
[5] MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, EH4 2XU Edinburgh, UK.
[6] Biobix: Laboratory of Bioinformatics and Computational Genomics, Department of Mathematical Modeling, Statistics and Bioninformatics, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.

[7] Renal Cancer Unit, The Royal Free Hospital, NW3 2QG London, UK.

[8] Centre for Experimental Cancer Medicine, Bart's Cancer Institute, Queen Mary University of London, EC1A 7BE London, UK.

[9] School of Medicine, University of St Andrews, KY16 9AJ St Andrews, UK.

Abstract

Currently, it is unclear to what extent sampling procedures affect the epigenome. Here, this phenomenon was evaluated by studying the impact of artery ligation on DNA methylation in clear cell renal cell cancer. To t–his end, DNA methylation profiles between vascularised tumour biopsy samples and devascularized nephrectomy samples from two individuals were compared. The relevance of significantly altered methylation profiles was validated in an independent clinical trial cohort. We found that six genes were differentially methylated in the test samples, of which four were linked to ischaemia or hypoxia (*REXO1L1*, *TLR4*, *hsa-mir-1299*, *ANKRD2*). Three of these genes were also found to be significantly differentially methylated in the validation cohort, indicating that the observed effects are genuine. Based on these results, we conclude that the impact of sampling procedures in clinical epigenetic studies should be considered, particularly after inducing hypoxia/ischemia, which occurs in many oncological surgery procedures through which tissues are harvested for translational research.

Keywords: Hypoxia; Cancer epigenetics; DNA methylation; Sampling effects

1 Introduction

The use of clinical samples in epigenetics research has become routine ~~(Ferraro, 2016; Taucher et al., 2016; Vitiello et al., 2015)~~[1–3]~~[refs. Cell. Oncol. 39, 295-318, 2016; Cell. Oncol. 39, 195-210, 2016; Cell. Oncol. 38, 17-28, 2015]~~. However, the nature of the sampling procedures may significantly compromise the resulting epigenetic profiles, leading to an “epigenetic observer effect". In renal cell carcinoma (RCC), dynamic molecular changes occur over time and with therapy, which require serial tissue samples for elucidation [4,5]~~[1, 2]~~. Despite the development of standard operating procedures for tissue acquisition and biobanking, less attention is paid to ensuring constant, robust pre-collection conditions, such as warm ischaemia time, than to post-collection handling and processing procedures, thus failing to avoid variation due to pre-analytical factors [6]~~[3]~~. There are recommended tissue sampling guidelines from organisations such as the Confederation of Cancer Biobanks, which advise that warm ischaemia time should be minimised as much as possible prior to freezing of fresh tissue samples, but this is very difficult to measure and standardise [7]~~[4]~~. Several studies have looked at the procurement conditions on RNA biomarker expression and the effect on previously identified cancer biomarkers [8]~~[5]~~, but so far there have been no such studies on DNA methylation.

Prolonged ischaemia leads to higher levels of tissue hypoxia, which has been shown to induce DNA demethylation in e.g. hepatoma cells [9]~~[6]~~. This demethylation has been shown to be induced by methionine adenosyltransferase 2A (*MAT2A*), of which the expression is positively regulated by *HIF-1α* [9]~~[6]~~, which in turn is negatively regulated by *VHL* [10]~~[7]~~. *VHL* plays a crucial role in RCC development [11]~~[8]~~. Hypoxia, methylation and RCC form, therefore, an intricate network of which the components cannot be studied separately. Yet, different RCC sampling procedures might obfuscate the results by influencing the hypoxic conditions. More than most tumours, RCC sampling is challenging as the majority of the extirpative procedures deal with minimally invasive approaches. It is well established that changes occur in mRNA levels with increasing time after renal artery clamping in RCC [12]~~[9]~~. Here, we assessed the effect of renal artery clamping on the RCC methylome.

Patient samples and methods

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Patient-matched sample sets were obtained from two patients who, at the time of open cytoreductive nephrectomy for metastatic clear cell RCC (ccRCC), had fresh ccRCC tumour biopsies taken prior to ligation of the renal artery after which matched fresh frozen tumour samples were harvested following ligation and division of the renal artery and removal of the kidney as per a previously described approach [5]~~[2]~~. A total of 12 samples was collected, i.e., 3 biopsy and 3 nephrectomy samples from each patient. These samples were obtained as part of the Scottish Collaboration On Translational Research into Renal Cell Cancer (SCOTRRCC) study (East of Scotland Research Ethics Service REC 1: 10/S1402/33). For validation purposes, but also to evaluate the potential impact on clinical epigenetics research, matched tumour samples taken at the time of diagnostic renal tumour biopsy and subsequent nephrectomy were obtained from 14 patients with metastatic ccRCC. Following primary tumour biopsy, these patients were treated with three cycles of sunitinib (18 weeks) followed by cytoreductive nephrectomy after 2 weeks of sunitinib as part of the Upfront Sunitinib (SU011248) Therapy Followed by Surgery in Patients with Metastatic Renal Cancer: a Pilot Phase II Study (SuMR; ClinicalTrials.gov identifier: NCT01024205) [13]~~[10]~~. All samples used in this study are listed in Supplementary file 1.

Extraction of genomic DNA was performed using a Qiagen DNeasy Blood and Tissue (Qiagen, UK) kit following the manufacturer's instructions. DNA methylation analyses using MBD sequencing was carried out as described previously [14]~~[11]~~. A MethylCap kit (Diagenode, Belgium) was used for capturing methylated fragments from 500 ng starting material. Massively parallel sequencing of these fragments was subsequently performed on an Illumina HiSeq 2000 machine (Illumina, San Diego, CA, USA).

Raw data files were mapped using BOWTIE to the human reference genome Hg19/GRCh37, and summarized using an in-house developed Map of the Human Methylome [15]~~[12]~~ consisting of a putative genome-wide overview of potentially methylated loci ("methylation cores"). Further data analyses were performed using Python 3.4.3 and R 3.2.1. The Bioconductor quantro software package (1.2) was used to assess quantile normalization assumptions [16]~~[13]~~, whereas the limma software package (3.24.15) was used to identify regions featured by differential methylation patterns. Prior to limma analysis, the samples were quantile normalized and transformed using the voom function [17]~~[14]~~. Only methylation cores that referred to annotated promoter regions (including exon1), and that had at least an average coverage of one mapped fragment per sample, were withheld for

analysis. Low coverage loci are featured by a too low power to be detected as differentially methylated, thereby unnecessarily inflating the amount of hypotheses tested, which justifies their unsupervised removal from the dataset. Regions with a False Discovery Rate (FDR) < 0.05 were selected for further assessment using the sample dataset [13]~~[10]~~. For the validation dataset, the FDR estimation (Benjamini-Hochberg) was based on the amount of loci to be validated.

Results and discussion

A quantro test was performed to check the suitability of the samples for quantile normalization, which was used for limma-voom. A *p*-value of 0.53 was obtained after 1000 permutations, implying that there were no global differences in the distributions between the non-ischaemic biopsy and ischaemic nephrectomy samples. A limma-voom data analysis was subsequently performed using quantile normalization. In Table 1 seven regions are listed that were found to be differentially methylated up to an FDR of 0.05 (Fig 1). Only two of these seven (*AC232323.1* and *ANKRD2*) exhibited a relative hypomethylation in the ischaemic nephrectomy samples compared to the non-ischaemic samples. In one of these (*AC232323.1*) a different region (8325757) exhibited a significant relative hypermethylation.

The differentially methylated regions with a FDR < 0.05 were subsequently subjected to validation using methylation data obtained from matched biopsy and nephrectomy samples from the SuMR clinical trial. Three of the seven regions identified in the test set, were again significantly altered at the same level within the validation cohort (*AC232323.1* region 8325757, *REXO1L1* and *OR6Q1*). A binomial test – using the FDR threshold as expected probability – rejected the null hypothesis that this would have occurred randomly ($p = 0.004$). We therefore hypothesise that the shared results between the two studies are caused by ischaemic conditions. Of the 7 loci identified in the test set, 6 were found to have the same fold change direction in the validation cohort. However, assuming a 50% random chance of having the same fold change direction, the number of similar direction of change was not found to be significant ($p = 0.125$). A lower FDR cut-off of 10% yielded 36 significant regions, yet this cut-off was deemed not sufficiently conservative as the fraction that could be validated in the validation cohort was too low to reject the null hypothesis of significant overlap ($p = 0.085$, data not shown).

Methylation meta-analyses have shown that DNA methylation is a critical event in tumorigenesis [18]~~[15]~~. It is, therefore, surprising that an analysis of the effect of tissue procurement on DNA methylation has so far not been performed, as it has in other molecular analyses. From our test and validation results it is clear that ischaemic conditions, induced as part of the surgical procedure, may lead to differential methylation.

The results of the study presented here indicate that the RCC methylome may be modulated following renal artery ligation. Global gene demethylation in samples was not observed, but consistent demethylation of at least one individual gene (*ANKRD2*) was found. These results hold considerable significance for translational methylation research for solid tumours obtained by extirpative surgery, especially where minimally invasive surgical approaches are used. The most significantly affected gene, *AC232323.1*, encodes a long non-coding RNA (lncRNA) product. According to LNCipedia [19]~~[16]~~ this lncRNA is linked to the second most significantly affected gene, *REXO1L1*, and has potential direct biological relevance (http://www.lncipedia.org/db/transcript/lnc-REXO1L2P-1:1). *REXO1L1* was found to be hypermethylated following renal artery clamping. *REXO1L1* deletions have been linked to increased apoptosis under certain conditions [20]~~[17]~~, such as intense hypoxia, which may underlie the results presented above [21]~~[18]~~. The third significantly affected gene, *TLR4*, plays a crucial role in kidney ischemia/reperfusion injury [22]~~[19]~~, and *Hsa-mir-1299* also has a possible role in apoptosis through interaction with *PIM1* [23]~~[20]~~. The *OR6Q1* gene encodes an olfactory receptor and is, therefore, an unlikely candidate, although it should be noted that there is evidence for a limited expression in other tissues as well [24]~~[21]~~. Finally, *ANKRD2* belongs to the conserved muscle ankyrin repeat protein (MARP) family. Expression of MARPs has been found to be induced in response to physiologic stress, injury, hypoxia and hypertrophy [25,26]~~[22, 23]~~ and the *ANKRD2* mRNA level has indeed been found to be upregulated under specific hypoxic conditions. Our results indicate that this upregulation may be brought about by demethylation of a specific methylation core.

Despite the test and validation cohorts used, this study should be considered as proof of principle with a necessarily low power, as it was deemed unethical to expose subjects to an extra pre-operative percutaneous biopsy procedure without clinical benefit. Also, in the validation cohort, patients were treated with sunitinib, the time between biopsy and nephrectomy was longer (20 weeks) and the sequencing depths were generally more shallow, implying that additional discrepancies between both datasets may have been caused by

treatment, temporal or other technical reasons.

Taken together, our data indicate that in both a test and a validation cohort renal artery ligation modulates gene methylation in a biologically relevant fashion. As with any research using surgically resected clinical samples, future methylation studies must be designed to include robust and well-documented sample procurement techniques in order to take these findings into account.

Author's contributions

Conception and design: DJH, AL, GDS, TDM, TP. Development of methodology: CVN, GDS, DJH, TP, TDM. Acquisition of data (acquired and managed patients, provided facilities, etc.): GDS, FOM, AL, DJH, TP. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): CVN, TDM, GDS. Writing, review, and/or revision of the manuscript: CVN, AL, FOM, WVC, DD, FVN, TP, DJH, GDS, TDM. Study supervision: GDS, DJH, TDM, WVC.

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Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Informed consent: Informed consent was obtained from all participants included in the study.

Legend tot the Figure

Figure 1 Sampling procedure differences. For each of the regions with a FDR < 0.05, boxplots of the methylation counts are shown. The methylation core region is shown under the gene label.

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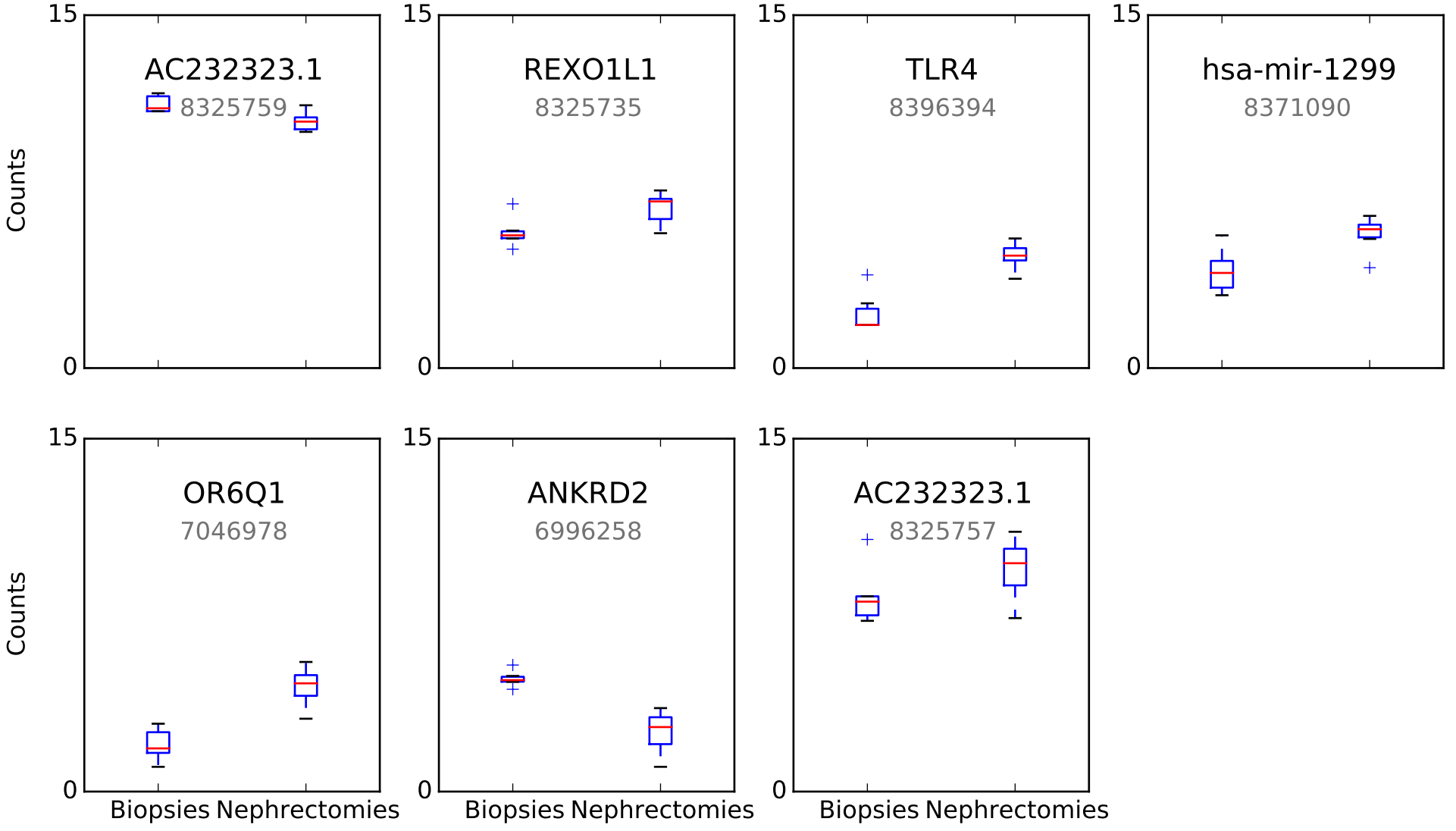
Table 1 Renal clamping differential results. One gene can have several methylation cores in the promotor region. 'AveExp' = average expression count. 'FDR' = Benjamini-Hochberg adjusted P-values, smaller than 0.05.

METHYLATION CORE ID	ANNOTATION	LOG-FC	AVE-EXP	P-VALUE	FDR
8325759	AC232323.1	0.731	10.84	1.256e-07	0.0055
8325735	REXO1L1	-1.184	6.28	1.842e-07	0.0055
8396394	TLR4	-2.483	3.55	8.634e-07	0.0173
8371090	hsa-mir-1299	-1.638	4.90	2.028e-06	0.0273
7046978	OR6Q1	-2.521	3.22	2.269e-06	0.0273
6996258	ANKRD2	2.249	3.66	3.262e-06	0.0327
8325757	AC232323.1	-1.242	8.88	4.777e-06	0.0411

figure 1

Click here to download line figure samplingProcedureDifferences.pdf



Supplementary table 1: Clinico-pathological characteristics of the two test patients.

Clinico-pathological Characteristic	Test patient 1	Test Patient 2
Age (years)	59	73
Gender	Male	Male
Fuhrman grade	2	2
T stage	3a	3a
M stage	1	1
Warm ischemia time* (mins)	55	60
Previous Treatment	3 cycles of Sunitinib 50mg once daily. Nephrectomy 4 days following cessation.	None

Warm ischemia time is the time from clamping of the renal artery to tumour sampling and snap freezing on dry ice.

Supplementary table 2: Clinico-pathological characteristics of the 14 validation cohort patients all with metastatic ccRCC and having tumour biopsy followed by sunitinib therapy and subsequent cytoreductive nephrectomy.

Age, median (range)	67 (52-78)
Male gender (%)	11 (78.6)
MSKCC prognostic risk (%)	
Intermediate	11 (78.6)
Poor	3 (21.4)
Metastatic sites (%)	
1-2	10 (71.4)
3+	4 (28.6)
Clear cell tumour grade, identified at nephrectomy (%)*	
1-2	7 (50)
3-4	7 (50)
Median PFS, months (95% CI)	17.5 (7-21)