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1 Short Communication

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

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## 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.

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

## 1 Introduction

The use of clinical samples in epigenetics research has become routine [1–3]. 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]. 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]. 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]. Several studies have looked at the procurement conditions on RNA biomarker expression and the effect on previously identified cancer biomarkers [8], 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]. This demethylation has been shown to be induced by methionine adenosyltransferase 2A (*MAT2A*), of which the expression is positively regulated by *HIF-1a* [9], which in turn is negatively regulated by *VHL* [10]. *VHL* plays a crucial role in RCC development [11]. 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]. Here, we assessed the effect of renal artery clamping on the RCC methylome.

## Patient samples and methods

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

90 biopsies taken prior to ligation of the renal artery after which matched fresh frozen tumour
91 samples were harvested following ligation and division of the renal artery and removal of the
92 kidney as per a previously described approach [5]. A total of 12 samples was collected, i.e., 3
93 biopsy and 3 nephrectomy samples from each patient. These samples were obtained as part
94 of the Scottish Collaboration On Translational Research into Renal Cell Cancer (SCOTRRCC)
95 study (East of Scotland Research Ethics Service REC 1: 10/S1402/33). For validation purposes,
96 but also to evaluate the potential impact on clinical epigenetics research, matched tumour
97 samples taken at the time of diagnostic renal tumour biopsy and subsequent nephrectomy
98 were obtained from 14 patients with metastatic ccRCC. Following primary tumour biopsy,
99 these patients were treated with three cycles of sunitinib (18 weeks) followed by
100 cytoreductive nephrectomy after 2 weeks of sunitinib as part of the Upfront Sunitinib
101 (SU011248) Therapy Followed by Surgery in Patients with Metastatic Renal Cancer: a Pilot
102 Phase II Study (SuMR; ClinicalTrials.gov identifier: NCT01024205) [13]. All samples used in this
103 study are listed in Supplementary file 1.
104 Extraction of genomic DNA was performed using a Qiagen DNeasy Blood and Tissue
105 (Qiagen, UK) kit following the manufacturer's instructions. DNA methylation analyses using
106 MBD sequencing was carried out as described previously [14]. A MethylCap kit (Diagenode,
107 Belgium) was used for capturing methylated fragments from 500 ng starting material.
108 Massively parallel sequencing of these fragments was subsequently performed on an Illumina
109 HiSeq 2000 machine (Illumina, San Diego, CA, USA).
110 Raw data files were mapped using BOWTIE to the human reference genome
111 Hg19/GRCh37, and summarized using an in-house developed Map of the Human Methylome
112 [15] consisting of a putative genome-wide overview of potentially methylated loci
113 (“methylation cores”). Further data analyses were performed using Python 3.4.3 and R 3.2.1.
114 The Bioconductor quantro software package (1.2) was used to assess quantile normalization
115 assumptions [16], whereas the limma software package (3.24.15) was used to identify regions
116 featured by differential methylation patterns. Prior to limma analysis, the samples were
117 quantile normalized and transformed using the voom function [17]. Only methylation cores
118 that referred to annotated promoter regions (including exon1), and that had at least an
119 average coverage of one mapped fragment per sample, were withheld for analysis. Low
120 coverage loci are featured by a too low power to be detected as differentially methylated,
121 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]. 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]. 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

154 analyses. From our test and validation results it is clear that ischaemic conditions, induced as
155 part of the surgical procedure, may lead to differential methylation.

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

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

184 Taken together, our data indicate that in both a test and a validation cohort renal
185 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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