Original Article
Prognostic utility of transrenal DNA for colorectal cancer patients with bone metastases

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Abstract: Purpose: The current study aims to address disease monitoring and prognosis for metastatic colorectal cancer (CRC) patients with bone metastasis using transrenal DNA. Methods: Transrenal DNA was isolated from urine specimens of CRC patients and matched to the tumor tissue profiles to gauge clinical relevance. KRAS mutations were analyzed in these samples. Monitoring of transrenal DNA quantity and KRAS mutations were performed at monthly intervals to track its variations between patients with or without bone metastases. For prognosis assessment, the Cox proportional hazards model was performed for all CRC patients and subsequently on patient subgroups with and without bone metastases. Results: The agreement in KRAS mutations between transrenal DNA and tumor tissues in CRC patients was more than 90%. Transrenal DNA quantities were significantly higher in CRC patients with bone metastases. In this patient cohort, quantities of transrenal DNA increased 2.58 folds during subsequent monitoring. Prognosis was poor for CRC patients who had bone metastases and higher transrenal DNA quantities. Hazard ratio was determined to be 2.11 (95% CI 1.58 to 4.04). Conclusion: Taken together, this demonstrated the usefulness of transrenal DNA for disease monitoring and prognosis of CRC patients with bone metastases. Our results showed that transrenal DNA carries KRAS mutations similar to tumor tissues and its quantity in urine specimen can be potentially useful to identify high-risk patients. This can lead to better disease management or tailored treatment.

Keywords: KRAS, colorectal cancer (CRC), disease monitoring, liquid biopsy, transrenal DNA

Introduction

Colorectal cancer (CRC) is one of the major diseases that affect millions worldwide [1]. Metastatic CRC is also a leading cause of death [2], and efforts have been placed on its early detection, at the stage where the disease is localized. Furthermore, investigating the epidemiology of the disease is challenging as CRC morphs quickly [3]. The most critical factors that contribute to disease relapse and survival are the extent of the cancer, lymph nodes involvement and its cellular differentiation. Metastatic spread in CRC has been extensively studied and bone metastasis is one common site accounting for 6-10% of the disease population [4]. The symptoms can be life threatening and are usually accompanied by considerable pain and may interfere with normal physical activities [5].

More importantly, the clinical outcomes for CRC patients with bone metastases are generally not encouraging [6]. Studies have shown that these patients have a poor prognosis [6] and metastatic lesions are usually abundant. It also invades the axial skeleton and proximal segments of the limbs [4]. With the progress in cancer treatments [7], patients have a wider selection of therapeutic options. It is imperative that close monitoring is needed to accurately profile the disease. Current methods for monitoring rely heavily on diagnostic imaging. Its primary purpose includes measuring the extent of secondary lesions and to evaluate treatment response [8]. Although these techniques are useful, it does not provide real-time disease updates to the molecular changes that can affect treatment efficacy. The molecular signatures are also important as many new treatments target unique mutations associated with the disease [9].

Alternative methods for CRC monitoring are critically needed. Circulating tumor DNA (ctDNA)
that resides in peripheral blood of cancer patients as a result of necrotic and apoptotic tumor cells [10] show good CRC correlations [11]. This has been performed in longitudinal studies to understand the pathogenesis of the disease [12, 13]. Siravegna et al. for instance tracked ctDNA in patients for use in genotyping and monitoring the molecular evolution of CRC. Patients involved in the trial were undergoing treatment with cetuximab and panitumumab that targets the epidermal growth factor receptor (EGFR). The dynamics of the disease was revealed and key genetic aberrations like KRAS mutations were highly correlative of treatment response [13]. The major drawback of the technique is the continual need for blood sampling of considerable volume, which limits the extent and frequency for measuring these events. Interestingly, ctDNA has been found within urine specimens as well [14]. Within the urinary system, ctDNA ends up in urine by diffusing through the kidney barrier during the waste removal process [15]. We hypothesize that the information that can be gathered will have practical clinical applications. Performing disease related testing in urine samples is appealing, as sample collection is non-invasive. It also exists in abundance to allow frequent collections and measurements.

In this current work, we aim to establish the clinical relevance of transrenal DNA from urine. Specifically, CRC patients that suffer from bone metastases were examined. We hypothesize that this will provide a sensitive capture of the disease dynamics that may aid in CRC management and prognosis. Serial specimen extractions at monthly intervals were conducted to gauge the variability in transrenal DNA. This is correlated directly to the overall survival of different patient groups, and measures the clinical significance of transrenal DNA in CRC.

### Materials and methods

**Colorectal patients characteristics and data collection**

A total of 150 patients were recruited for the present study. These patients were recruited when they sought treatment at the Xiangyang No.1 People’s Hospital or were patients who were transferred from other primary or secondary healthcare institutions. Participants of the trial provided informed consent, which was taken by their respective consulting doctor. The institutional review board (IRB) approved all patient recruitment procedures and sample recovery processes. Among the 150 CRC patients, 100 had confirmed bone metastases, established using magnetic resonance imaging (MRI) analysis. Details of the CRC patients are summarized in [Table 1](#). The remaining 50 patients without bone metastases were recruited as the experimental control and reference. Tissue biopsies used for profiling were mainly extracted by colonoscopy. The median age of the study cohort was 54 years old. The gender ratio was 1.08 (male/female). The baseline reference measurements were taken before treatment commencement. Serial urine samplings for transrenal DNA analysis were taken monthly for six

### Table 1. CRC patients and their characteristics

<table>
<thead>
<tr>
<th>Description</th>
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</tr>
</thead>
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<tr>
<td><strong>Age</strong></td>
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</tr>
<tr>
<td><strong>CRC Patients with Bone Metastases</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>53 (45-68)</td>
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<tr>
<td><strong>CRC Patients without Bone Metastases</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>56 (46-69)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
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<tr>
<td><strong>CRC Patients with Bone Metastases</strong></td>
<td></td>
</tr>
<tr>
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<td>53 (53%)</td>
</tr>
<tr>
<td>Female</td>
<td>47 (47%)</td>
</tr>
<tr>
<td><strong>CRC Patients without Bone Metastases</strong></td>
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<tr>
<td>Male</td>
<td>25 (50%)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (50%)</td>
</tr>
<tr>
<td><strong>PS at inclusion</strong></td>
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<tr>
<td>0</td>
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</tr>
<tr>
<td>1</td>
<td>75 (50%)</td>
</tr>
<tr>
<td>2</td>
<td>9 (6%)</td>
</tr>
<tr>
<td>ND</td>
<td>3 (2%)</td>
</tr>
<tr>
<td><strong>Locus of primary</strong></td>
<td></td>
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<tr>
<td>Colon</td>
<td>97 (65%)</td>
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<tr>
<td>Rectum</td>
<td>49 (33%)</td>
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<tr>
<td>ND</td>
<td>4 (3%)</td>
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<tr>
<td><strong>Molecular Profile (Tissue)</strong></td>
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<td><strong>CRC Patients with Bone Metastases</strong></td>
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<tr>
<td>Wildtype</td>
<td>37 (25%)</td>
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<tr>
<td>KRAS</td>
<td>63 (42%)</td>
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<tr>
<td><strong>CRC Patients without Bone Metastases</strong></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
<td>25 (17%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>25 (17%)</td>
</tr>
</tbody>
</table>
months consecutively. Comparisons were made with matched tumor profiles for KRAS mutation and transrenal DNA quantity variations were compared during treatment.

**Specimen retrieval and transrenal DNA purification**

Urine specimens were collected from each patient using the following criteria to ensure consistency. Participants collected approximately 50 ml of the midstream morning urine into a container with 0.5 M EDTA, pH 8.0. To ensure the integrity of the transrenal DNA, each sample was processed within 2 hours of collection. Retrieval of cell-free nucleic acids was done by centrifugation to remove all contaminating debris. A two-step centrifugation process was undertaken. The first centrifugation step was performed at 10,000 g for 15 minutes at 4°C and the supernatant carefully transferred to a new centrifuge tube. The second centrifugation step was performed at 10,000 g for 10 minutes at 4°C to ensure remaining contaminants were removed. For DNA purification, Qiagen’s QIAmp Circulating Nuclei Acid Kit (Qiagen Inc, USA) was used in accordance with the instructions by the manufacturer. 16 ml of each urine sample was processed and eluate of 20 μl in Tris-EDTA, pH 8.0 was recovered. These samples are then quantified using the Nanodrop 1000 (ThermoScientific, USA) and stored at -20°C before molecular analysis.

**Molecular profiling of transrenal DNA using digital droplet PCR**

Digital droplet PCR (ddPCR) was used to perform the KRAS molecular testing in the CRC patients. This technique is capable of detecting low-frequency mutations and has demonstrated its usefulness in various ctDNA detections [16]. Purified DNA from each urine specimen was processed through the QX100 ddPCR system (Bio-Rad Lab Inc., USA) using the ddPCR KRAS G12/G13 Screening kit (Bio-Rad Inc. USA). The laboratory procedures strictly followed manufacturer’s recommendations. Briefly, reaction master mixes and DNA templates were prepared in 20 μl reactions. PCR settings were as follows:

1. 95°C, 10 minutes; 2. 94°C, 30 s and 55°C, 1 minute (40 cycles); 3. 98°C, 10 minutes. Sample plate was held at 4°C thereafter.

For data analysis, each sample batch was evaluated using the QuantaSoft software (Version 1.6, Bio-Rad Lab Inc., USA).

**Statistical analysis**

The concordance between tumor tissue and transrenal DNA for KRAS mutations was tabulated to determine the clinical significance of transrenal DNA analysis. The Cohen’s Kappa that measures inter rater agreement was also computed. We compared the quantity of transrenal DNA among CRC patients using an unpaired Student t test. Patients in different groups were closely matched for demographic and other clinical parameters (such as age, gender and ratio of KRAS positive patients) as shown in Table 1. To ascertain the variability of transrenal DNA content in different patient groups, we computed the coefficient of variation (CV) among them. A repeated measure analysis of variance (ANOVA) was performed on the serial measurements of transrenal DNA quantities. To ascertain clinical relevance, Kaplan-Meier (KM) analysis was done by following up with patients and hazard ratios (HR) was achieved by the Cox proportional hazards regression. For all statistical computations, Microsoft Excel (Microsoft Inc, USA) or the Prism statistical software (GraphPad, USA) was used. In all tests, a *p*-value less than 0.05 was considered statistically significant.
Results

Experimental trial and assay validation

The prevalence of bone metastases in CRC is significant. Its incidence rate ranges from 6-10%, with median detection time approximately 11-21 months after primary tumor resection [4]. More importantly, bone metastases patients were also linked to lung and/or liver metastases [17]. This was consistent in the current trial, with more than 95% of the patients showing these secondary metastases. Studies have suggested that these patients have poor survival, and better clinical monitoring of this patient group can aid in their treatment selection to improve outcome. Transrenal DNA offers a relatively pain-free method for tumor material sampling and we hypothesize it can aid in the disease management of this group of advance stage CRC patients. We aim to measure the variations within transrenal DNA and ascertain its clinical relevance.

Besides quantifying the nucleic acid content in each urine specimen, we profiled the samples for KRAS mutations as well. This provided direct evidence of the clinical relevance of transrenal DNA in CRC. Using ddPCR, we included spiked conditions of 1000, 100 and 10 copies of mutant KRAS plasmids. It is important that the detection assay is sensitive to pick up low copy numbers of mutant DNA, as it is expected that clinical samples will be of low frequencies as well [18]. Figure 1 shows the result of the detection assay. Each data point was independently repeated six times and the data is shown in Supplementary Table 1. The results demonstrated good linearity within these input conditions and $r^2$ for the assay performance test was 0.978.

Transrenal DNA at baseline and agreement between tumor tissue for CRC

Baseline transrenal DNA content was measured in each CRC patient prior to treatment and the results are shown in Figure 2A. This is to compare if patients with bone metastases had different transrenal DNA quantity. At baseline, it was observed that the mean transrenal DNA recovered from CRC patients with bone metastases was 2.00 folds higher than patients without bone metastases. An unpaired Student t test for one tail showed that this was statistically significant ($p$-value < 0.05). Measurements of transrenal DNA content clearly showed a good separation of the patients, which might have further clinical implications. Comparisons were also made for the KRAS gene profile among CRC patients as illustrated in Figure 2B. This provided direct associative links of transrenal DNA to CRC, and the results were matched with the corresponding tumor biopsy KRAS profiles. Overall concordance between transrenal DNA and tumor tissue profiling was 89.3% ($\kappa = 0.788$; 95% CI 0.689 to 0.886) for all patients. A further breakdown of the different patient groups showed...
subgroups yielded an agreement of 87% and 94% for CRC patients with and without bone metastases respectively. It is interesting to note that for all patients with wildtype KRAS, the concordance rate was 100%. To gauge the inter-patient variability for different groups, the CVs were computed. The overall CV for CRC patients with bone metastases was 13.4%. In contrast, the CV for patients without bone metastases was 32.0%. A lower dispersion was observed with the former, indicating that transrenal DNA may be a good measurement parameter for this patient group. Taken together, the baseline transrenal analysis showed good agreement to the tissue profiles, which indicated a strong disease association. Variability measurements showed promising outcomes among different CRC patient groups to further use transrenal DNA for prognosis and disease monitoring.

**Serial sampling importance to capture the dynamic changes in CRC**

The attractiveness of transrenal DNA analysis is the ease of specimen collection. In subsequent serial measurements, the stability and variability of the assay were assessed. Patients were monitored for a total of six months where possible. Several interesting trends were observed. For CRC patients with bone metastases, we detected a clear monotonic increasing trend for mean transrenal DNA quantity over time as shown in Figure 3A. Using repeated measures ANOVA, the changes were significantly different with a p-value less than 0.05. The CVs at each time point was relatively similar and indicated that the dispersion of data points during serial monitoring was unaffected. This also ascertained the validity of the assay for use in CRC patients with bone metastases. We noted that the increasing trend could indicate changes to the disease and performed a case study using Figure 3.
imaging modalities as depicted in Figure 3B. This MRI scan showed a worsening condition and correspondingly a higher measured transrenal DNA quantity of 2.58 folds was registered compared with the baseline measurement. For the other patient group without bone metastases, the mean transrenal DNA quantities showed a less pronounced increase over time (Figure 3C). Comparing with baseline measurements, the maximum increase in mean transrenal DNA quantities was 1.23 folds. The repeated measures ANOVA analysis showed significant differences within the serial measurements but mean DNA quantity showed alternating trends. In the discordant cases detected at baseline, we noted several patients with positive detection during the six months monitoring period as shown in Figure 3D. A common trend that coexisted among them is the low concentration of detection mutant KRAS DNA using ddPCR. The positive identifica-

tions were also not stable throughout the entire monitoring period. This critically showed the importance of continual monitoring of patients.

Based on the monitoring results of nucleic acid quantity and KRAS profiling, we hypothesize that transrenal DNA is useful for clinical prognostication. KM analyses were performed on different subgroups of clinical patients. As disease severity could be directly related to the quantity of transrenal DNA, we rank the patients by the maximum measured transrenal DNA obtained during monitoring. Figure 4A shows the plot for all patients with mean detected maximum transrenal DNA quantity of 63.7 ng (95% CI 59.1 to 68.3 ng). Using a median split of the transrenal DNA quantity results, we observed that the median survival of the patient group with higher DNA content was 3 months lesser for all CRC patients. Using Cox regression, the hazard ratio was determined to be 1.17 (95% CI 0.83 to 1.74) as shown in Figure 4B. Next, we subdivided the analysis for CRC patients with and without bone metastases. Figure 4C shows the KM analysis using a median split of patients with bone metastases. The median survival for the patient cohort with higher DNA quantity was 4 months lesser. Using Cox regression, the hazard ratio was determined to be 2.12 (95% CI 1.58 to 4.04) with a p-value less than 0.05. With the same criteria for patient stratification, we analyzed the results of CRC patients without bone metastases. The hazard ratio was 1.90 (95% CI 1.18 to 4.94) with a p-value of 0.03 as shown in Figure 4D. The median survival of the patients’ cohort with higher transrenal DNA content was 4 months lesser. The results demonstrated the usefulness of transrenal DNA profiling within CRC patients and the ability to identify patients with a potentially worse outcome.
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Discussion

The need to address clinical monitoring of cancer is critical to enhance the survival of patients and offer timely therapeutic changes. Transrenal DNA provides alternative methods for cancer monitoring. The uses of urine specimens in various medical and forensic evidence gathering are common [19-21]. Urine sampling has proven to be valuable for extracting various markers, and in our study for disease profiling of CRC patients. The major benefits associated with sampling urine are that it has relatively abundant volume and is patient friendly for sample extraction. In the present study, we evaluated the clinical relevance in CRC patients with and without bone metastases. Patients with bone metastases are a critical group that will benefit from enhanced supervision. This can better complement current methods used in diagnostic imaging to profile the disease.

Our study was effective to show the strong links between CRC and transrenal DNA. In our baseline results, good agreements with transrenal DNA and matched tissue profiles had been made between patients with KRAS mutations and those of wildtype characteristics. Furthermore, we noted that serial monitoring of patient samples aided to reduce false negative results. Including the cases that were subsequently confirmed to be KRAS positive, this brought the overall concordance between transrenal DNA and tumor tissue to over 90%. Our results are consistent with several studies that had used ctDNA from plasma to demonstrate the close associations to cancer [16, 22-24]. The consolidated results from our serial monitoring data showed better agreement in KRAS mutations than studies on single time point measurements performed on plasma conducted by Guibert et al. [16]. It was registered that 78% of their patient population was positive for the KRAS mutations. An important deduction from their study was the close correlation of ctDNA to treatment response. In our case study, we noted a strong associative link with disease progression. Potentially, this suggests that transrenal DNA quantity could be a good treatment response marker. Our work provided the basis for future analysis to explore the use of transrenal DNA to monitor drug response and disease progression. CRC patients with bone metastases in our study registered higher quantities of transrenal DNA compared with the control wildtype group. Supposedly, this could also be used as a gauge of disease severity. In our analysis, we noted the transrenal DNA assay was specific, which is needed to reduce false positive results. For our tests of patients with wildtype KRAS characteristics, the profiles showed 100% agreement using transrenal DNA compared with tumor tissues.

Longitudinal examinations in cancer patients are an important task to accurately profile the changes during disease progression. For instance, CRC patients may develop secondary mutations [25] during treatment that affects the therapy efficacy. These changes may not be effectively captured during baseline molecular profiling. Repeat biopsies is a possible option [26] but the physical limitations associated with tissue extraction is a strong deterrence for patients. Transrenal DNA assays fill the gaps between medical imaging using CT and MRI scans. Our monitoring of CRC patients with bone metastases showed a monotonic increasing mean transrenal DNA quantity with relatively similar CV in between sampling periods. This highlighted the consistency and robustness of the assay. The increasing trend seen may be reflective of disease progression as shown earlier in Figure 3B. We also noted consistently higher mean quantities for CRC patients with bone metastases than patients without bone metastases that suggest distinct disparities in their disease manifestations. This led to the postulation that high transrenal DNA content could be a useful clinical parameter. KM curves showed that for the overall CRC population in this study, the group with higher transrenal DNA had worse outcomes. This is direct evidence that transrenal DNA can be useful to identify patients of higher-risk and is helpful in disease monitoring. In addressing the critical group for CRC patients with bone metastases, we observed that the hazard ratio was higher than examining the entire CRC population. We envision measuring transrenal DNA to better aid in providing critical care to high-risk patients and allow easier genetic profiling to determine suitable treatment options. A number of prior studies have investigated the use of alternative disease monitoring tools [23, 27, 28]. Similar work has been performed for lung cancer using plasma ctDNA by Mok et al. [29]. Our results are coherent with their studies to demonstrate clear benefits in clinical monitoring. Interestingly, serial measurements of key mutations...
for lung cancer patients at different treatment cycles correlated to disease outcome. We observed that by considering the maximum transrenal DNA quantity during serial measurements, this was a good parameter for patient stratifications. Our results are one of the first to address CRC patients using transrenal DNA, which may enable a faster turn-around for molecular diagnosis.

Future key questions that can be addressed with this assay include treatment response and clinical interventions. Transrenal DNA recovery and profiling open up new opportunities to better probe the disease. The limitations of the current study are insufficiently statistical power for tumor burden associations and this can be further expanded in future work. Concurrently, possibilities of clinical interventions based on transrenal DNA measurements can be investigated. We also observed that the sensitivity of the detection assay may be limiting as a number of cases had intermittent positive identification for KRAS mutations. This is due to low frequencies of mutant DNA within these urine specimens. The implications are possible false negative cases and this can be mitigated by having DNA pre-enrichment strategies [30].

Conclusions

Our study investigated the use of transrenal DNA for the assessment of CRC and its benefits for patients with bone metastases. The good agreement between tumor tissue samples and transrenal DNA highlighted the strong clinical associations for this urine based test. Results of the current serial monitoring suggest that this can be a useful clinical parameter to probe the disease. This can also be especially useful in cases where tumor tissue samples cannot be obtained. The technique is attractive as it is non-invasive and specimens are easy to obtain. This will ensure higher patient compliance and may enable faster turnover time for molecular diagnosis alongside conventional testing methods. Measuring transrenal DNA in our patients demonstrated strong prognostic abilities to identify high-risk patients and will help lead to better predictions for survival.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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**Supplementary Table 1.** Target inputs and recovered quantities using ddPCR performed on six independent runs

<table>
<thead>
<tr>
<th>Target Input (Copies)</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Run 5</th>
<th>Run 6</th>
</tr>
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