

# Liquid biopsies for surveillance and monitoring treatment response of bladder cancer

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## Introduction

Urinary bladder cancer is a frequent disease representing the ninth most common cancer worldwide with significant morbidity and mortality (1,2). Men are more often affected by bladder cancer than women, however for still unknown reasons women have a higher mortality rate (2). Besides external exposure to carcinogens such as tobacco, aromatic amines and polycyclic aromatic hydrocarbons inherent genetic predisposition is discussed as risk factor for bladder cancer (2,3). At initial diagnosis approximately 75% of patients have non-muscle-invasive bladder cancer (NMIBC) with a high recurrence rate of at least 50% and a progression rate to muscle-invasive bladder cancer (MIBC) of about 15% within 5 years (3,4). Urine cytology and cystoscopy are the gold standard for early bladder cancer diagnosis (5). However, to avoid inconvenience for patients and high costs associated with cystoscopy and to overcome low sensitivity of cytology to detect low grade tumors, the development of alternative non-invasive diagnostic methods is of utmost importance (6). Standard treatment of NMIBC comprises transurethral resection and intravesical injection of chemotherapy or immunotherapy followed by routine and long-term surveillance with cystoscopy and urine cytology (3,7). Naturally, MIBC requires more aggressive treatment by radical cystectomy or radiotherapy with or without chemotherapy (2). Because of the high morbidity and mortality rate of patients with MIBC, biomarkers applicable for (I) identification of patients at risk for recurrence and progression to MIBC; (II) early detection of MIBC; (III) monitoring treatment response are urgently needed. Moreover, development of biomarkers could contribute to a general decrease of the enormous

medical costs caused by intensive diagnostics, high rates of recurrence, long-term surveillance and repeated treatment of bladder cancer (8).

Considerable evidence points to the importance of liquid biopsies offering an easily implementable non-invasive approach for the detection of recurrences and early tumor progression (9). Several promising biomarkers for the management of bladder cancer have already been established mainly based on genomic (10), transcriptomic (11,12), epigenetic (13) and proteomic (14) approaches on tumor tissues or urine. However as Birkenkamp-Demtröder *et al.* claimed none of those biomarkers are sufficiently validated in the clinical situation or failed clinical application because of low sensitivity and specificity.

Within the European FP7 UROMOL project a predesigned assay combining *FGFR3*, *PIK3CA* and *RAS* mutational analysis and methylation specific Multiplex Ligation-dependent Probe Amplification (MLPA) as well as microsatellite approaches on tissue and at least three follow up urine samples after resection of an incident low grade, non-muscle invasive bladder tumor was tested on a large retrospective longitudinal cohort (6). Sensitivity of all tests for detection of recurrences was higher than that of urine cytology and the number of patients eligible for urine-based follow up investigations can be increased. The clinical value of these tests still remains to be elucidated in prospective clinical studies (6).

Because of its obvious clinical implications for personalized medicine, during the last years liquid biopsies focusing on the blood-based analysis of circulating tumor cells (CTC) and cell-free circulating tumor DNA of cancer patients have gained attraction (15-19).

In this regard, the prognostic relevance of CTC detected in peripheral blood was recently shown for patients with non-metastatic MIBC (20). Positivity for CTC was significantly associated with disease-recurrence, cancer-specific and overall survival (20). Thus, the presence of CTC in patients with non-metastatic MIBC treated with radical cystectomy is a powerful predictor for unfavorable outcomes and has the potential to support adjuvant chemotherapy decision-making and therapy monitoring. However, also for the usage of CTC as biomarker, integration of CTC analyses and validation in prospective clinical trials is still needed (21). Remarkably, also in 20% of patients with high risk NMIBC the presence of CTC significantly correlated to the time of first recurrence and progression-free survival. This suggests that CTC positivity can identify patients with T1 stage bladder cancer suffering already from systemic disease (22). Current research activities are directed to improve molecular and phenotypic characterization of CTC regarding the expression of therapeutically relevant targets (18). Hence, enumeration and characterization of human epidermal growth factor receptor 2 (HER2) expression of CTC is already used for the stratification of breast cancer patients to HER2-targeting therapies (23). This could be also of interest for bladder cancer where discordant results regarding the expression of HER2 between primary bladder cancer and CTC occurred in some cases which might have clinical implications (20).

### This study

The strength of the study by Birkenkamp-Demtröder *et al.* is the focus on the development of personalized assays for surveillance involving tumor tissue, blood, plasma and urine. The authors analyzed cell-free DNA from plasma and urine for the presence of genomic variants previously identified by three different next generation sequencing (NGS) techniques in corresponding and in some cases metachronous tumor tissues. This is a retrospective study on tumor tissue, blood, plasma and urine from 12 patients initially diagnosed with NMIBC. In total, 377 samples from 12 patients were collected between 1994 and 2015 and followed up to 20 years. Six patients experienced later progression to MIBC or metastatic disease during follow up of 4 to 20 years (PRO group) and 5/6 patients died of disease. The other six patients developed recurrences of NMIBC followed for 7 to 20 years, but were alive at time point of analysis (REC group). Applying three different NGS approaches (whole genome sequencing, whole exome

sequencing and Mate pair sequencing), the authors detected varying numbers of genomic variants in the analyzed tumor tissues. Subsequently, evaluation of somatic tumor-specific genomic variants and precise breakpoints were performed by PCR and Sanger sequencing comparing tumor and matched germline DNA. Genomic variants included deletions, insertions, inversions as well as intra- and interchromosomal translocations.

For designing 1 to 6 tumor-specific, highly sensitive personalized assays spanning the breakpoints, genomic variants were prioritized according to the following criteria: (I) detected in at least two metachronous tumors, supported by most read pairs or (II) affecting loci harbouring Catalogue of Somatic mutations in Cancer (COSMIC) cancer genes. DNA quantification in personalized assays was conducted by droplet digital PCR (ddPCR) designed to amplify short DNA fragments (68–125 bp) and using dual labeled fluorescent probes. These assays enabled detection of 1 copy of tumor DNA in a background of 6,000 copies of germline DNA and confirmed the patient-specific variants detected in the tumors at different visits.

Investigating plasma, Birkenkamp-Demtröder *et al.* detected circulating cell-free tumor-specific DNA in 10/12 patients by analyzing 115 samples collected at different visits. Interestingly, 4 of 6 PRO group patients were tumor DNA-positive already several months before clinical progression occurred. Although no differences were measured in the total amount of cell-free DNA between samples from the PRO and REC groups, the average circulating tumor DNA level during the course of disease was significantly higher in the PRO group than in the REC group (154 *vs.* 11 copies/mL). Focusing only on samples collected before diagnosis of progression to MIBC, the tumor DNA level was also higher in the PRO group than in the REC group (138 *vs.* 11 copies/mL). As demonstrated by the results of the personalized assays, different genomic variants could be released in the circulation by tumor cells of individual patients. One patient of the PRO group experienced high levels of tumor DNA at all time points during observation and in another PRO group patient a five-fold increase in tumor DNA amount over time could be observed during the course of the disease. For this patient an extraordinarily high number of tumor DNA copies (>2,000/mL plasma) could be measured 8 months after systemic therapy. As expected, this high copy number correlated with a relapse and disease progression. No tumor DNA or low levels below 60 copies/mL were determined for 5 REC patients with measurable tumor DNA at previous

visits.

However, there are also outliers. Thus, in the PRO group one patient did not display tumor DNA in plasma in one of the 6 personalized assays applied at 6 visits. Consecutive tumor tissues of this patient analyzed for genomic variants by NGS revealed large intratumoral heterogeneity with only 1 of 48 genomic variants of clonal origin. Here the authors suggest that in this case genomic variants different from those used in the personalized assays could be detected in the circulation. Importantly, in another PRO group patient with relapse after cystectomy and complete remission after systemic therapy, the personalized assays failed to detect tumor DNA in plasma 6.5 years after surgery.

In the second task, Birkenkamp-Demtröder analyzed 101 urine samples by the established NGS-based personalized assays. In total, they detected tumor DNA in 55/57 urine samples from the PRO group patients (96.5%). In contrast, only 22/44 samples (50%) of urine samples from the REC group patients exhibited tumor DNA. A very important output of the study by Birkenkamp-Demtröder is that all 6 PRO group patients exhibited high levels of tumor DNA and 5 of these patients even displayed >500 copies/mL. Importantly high levels of tumor DNA in urine were also observed in the two patients without or with only low tumor DNA levels in plasma, implying that a combination of plasma and urine assays has the potential to improve sensitivity and specificity to detect tumor DNA. In one PRO group patient, all genomic variants determined in the muscle-invasive tumor at the time point of progression were already detectable in the initial urine sample collected 4 years earlier. In contrast, in plasma samples of this patient no tumor DNA could be measured until 12 months after initial visit, but 7 months later 5/6 genomic variants could be identified. Hence, personalized tumor DNA determination can provide additional information to cystoscopy results already at very early non-invasive tumor stages in selected patients. Unexpectedly, two REC group patients also showed high copy numbers of tumor DNA in urine early in the course of the disease. Here, the authors hypothesized that due to the presence of high risk T1 tumors in these patients a considerable amount of DNA is released. After BCG instillation, however, in both patients tumor DNA was no longer detectable. Analysing all urine samples during the course of disease, PRO group samples had significantly higher average copy numbers than REC group samples at visits before diagnosis of progression to muscle-invasive tumors (1,242 *vs.* 31 copies/mL).

More than 80% of the PRO group patients displayed high levels of tumor DNA in urine samples several months before progression to muscle-invasive disease appeared (12–169 months).

In this retrospective pilot study on 377 samples including blood, plasma and urine from 12 patients, Birkenkamp-Demtröder *et al.* introduced sensitive and tumor-specific assays to early predict recurrences or progression to MIBC for patients initially diagnosed with NIMBC. To be able to establish these assays they analysed tumor tissues and matched germline DNA for genomic variants with different NGS methods enabling the detection of those variants even in very small tumor clones. Out of the 3 tested NGS methods whole exome sequencing has crystallized as best suited for standard clinical use. Highly tumor specific genomic variants absent in germline DNA and preferentially present in two metachronous tumors were identified, validated by Sanger sequencing and chosen for the development of personalized assays to examine plasma and urine samples. Finally, personalized assays were designed for sensitive ddPCR on plasma and urine samples. The number of these assays was dependent on the number of genomic variants identified by NGS. By using combinations of different markers as implemented in different personalized assays, in future studies higher numbers of patients should become eligible for follow up analyses using plasma and urine samples.

Since tumor DNA in plasma is supposed to reflect tumor burden the failure to detect tumor DNA in plasma samples of two patients with progressive disease remains unexplained. Heidary *et al.* also failed to detect adequate tumor DNA levels in a plasma sample from a patient with metastatic breast cancer who was detected CTC-positive with more than 100,000 CTC/7.5 mL blood. These authors speculated that differences in phagocytosis and DNA degradation mechanisms might contribute to variable genomic variant-bearing tumor DNA levels (16), further emphasizing to the necessity of combining tumor DNA analyses with enumeration and characterization of CTC. Moreover, future efforts have to be made to find out how Ta non-invasive tumors with intact basal membrane might release tumor DNA. Here, Birkenkamp-Demtröder *et al.* discuss a contribution of tumor interstitial fluid and transcapillary transport of DNA into the circulation (24,25).

Taken together, the pilot study of Birkenkamp-Demtröder *et al.* paved the way for the development of urgently needed personalized assays for bladder cancer surveillance. Clinical validation of the current findings

has to be performed in future prospective clinical trials on larger cohorts with defined endpoints.

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## Footnote

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