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Genomic profile of urine has high diagnostic sensitivity compared to cytology in non-invasive urothelial bladder cancer

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Abstract

Cytology is widely conducted for diagnosis of urothelial bladder cancer; however, its sensitivity is still low. Recent studies show that liquid biopsies can reflect tumor genomic profiles. We aim to investigate whether plasma or urine is more suitable for detecting tumor-derived DNA in patients with early-stage urothelial bladder cancer. Targeted sequencing of 71 genes was carried out using a total of 150 samples including primary tumor, urine supernatant, urine precipitation, plasma and buffy coat from 25 patients with bladder cancer and five patients with cystitis and benign tumor. We compared mutation profiles between each sample, identified tumor-identical mutations and compared tumor diagnostic sensitivities between urine and conventional cytology. We identified a total of 168 somatic mutations in primary tumor. In liquid biopsies, tumor-identical mutations were found at 53% (89/168) in urine supernatant, 48% (81/168) in urine precipitation and 2% (3/168) in plasma. The high variant allele fraction of urine was significantly related to worse clinical indicators such as tumor invasion and cytological examination. Although conventional cytology detected tumor cells in only 22% of non-invasive tumor, tumor diagnostic sensitivity increased to 67% and 78% using urine supernatant and precipitation, respectively. Urine is an ideal liquid biopsy for detecting tumor-derived DNA and more precisely reflects tumor mutational profiles than plasma. Genomic analysis of urine is clinically useful for diagnosis of superficial bladder cancer at early stage.

KEYWORDS

diagnosis, liquid biopsy, NGS, urine, urothelial bladder cancer

1 | INTRODUCTION

Bladder cancer (BC) is one of the most common cancers, and more than 90% of BC are urothelial cancer, arising from the urothelium of the bladder.[1] Approximately 75% of newly diagnosed cases are non-invasive BC,[2] and 40%-50% of non-invasive BC cases recur in the bladder and other parts of the urinary tract after complete resection. Therefore, long-term surveillance is mandatory for non-invasive BC

Hirotsu and Yokoyama contributed equally to this study.

patients. Cystoscopy is the gold standard for cancer diagnosis and follow up of BC; however, it is operator-dependent and highly invasive and can cause complications, such as dysuria, frequency, hematuria, and infection. Moreover, it is difficult to identify small or flat tumors such as carcinoma in situ (CIS) by cystoscopy. Although urine cytology is not invasive, its sensitivity is not as high, at 40%-60% of BC.[3] Soluble urinary protein biomarkers and exfoliated cell tests based on proteins and aneuploidy have been approved by the FDA; however, these strategies have not been widely adopted because of limited sensitivity and/or specificity. Thus, the development of accurate biomarkers for non-invasive diagnosis of BC is urgently required.

Recent studies have used genomic approaches to identify biomarkers for BC. Loss of chromosome 9 has been studied as an initiator of non-invasive BC, and genomic analysis frequently identified mutations in *FGFR3*, *PIK3CA*, *STAG2*, *CDKN2A*, and *KDM6A* in BC.[4-6] In addition, mutations in tumor suppressors such as *TP53*, *RB1*, *CDKN2A*, and *PTEN* have been estimated to play important roles in invasive BC. From the genomic profiles, many studies have been focused on identifying biomarkers for recurrence, prognosis and therapeutic targets of BC.[7-9] As the genetic profiling results of BC are applied to the clinical setting, a strategy for determining biomarkers in a non-invasive way will be required.

Liquid biopsy of blood and body fluid represents a non-invasive approach to obtain genetic information of tumors. Cell-free DNA (cfDNA) from apoptotic and/or necrotic tumor cells can be evaluated in liquid biopsy.[10] Liquid biopsies can thus provide a snapshot of the mutational profiles in tumors and detect drug-resistant mutations and predict treatment efficiency and tumor recurrence.[11-16] In general, plasma is commonly used for "liquid biopsy" in clinical research. However, it is difficult to find the tumor-identical mutation in early-stage cancer patients, because of the low amount of tumor DNA shed in plasma. To overcome this situation, we aimed to investigate which liquid biopsy is more suitable for detecting tumor-derived DNA. Previously, our data suggested that tumor-derived DNA was enriched in body fluids near the tumor tissue.[17] Thus, we focused on the urine in urothelial BC.

In the present study, we evaluated the genetic profiles of BC using various liquid biopsy samples, including urine supernatant, urine precipitation, and plasma, and compared these results with analyses from primary tumors. Our results showed that urine samples accurately reflect somatic mutations and could be an alternative strategy to conventional cytological examination.

2 | MATERIALS AND METHODS

2.1 | Patients and sample preparation

This study included 25 patients who were diagnosed with BC by transurethral resection of bladder tumor (TURBT), four patients with cystitis and one patient with inverted papilloma (25 males and 5 females; age 50-90 years) (Table S1). Informed consent was obtained from all patients. This study was approved by the Institutional Review Board at Yamanashi Central Hospital. Urine and blood samples were obtained before TURBT. Urine precipitate (cellular fraction) and urine supernatant (non-cellular fraction) were collected after centrifugation (Figure 1).[18]

DNA was extracted from the buffy coat and urine precipitation with the QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Hilden, Germany) and DNA concentration was determined using Nano Drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA was extracted from the urine supernatant and plasma with the MagMax

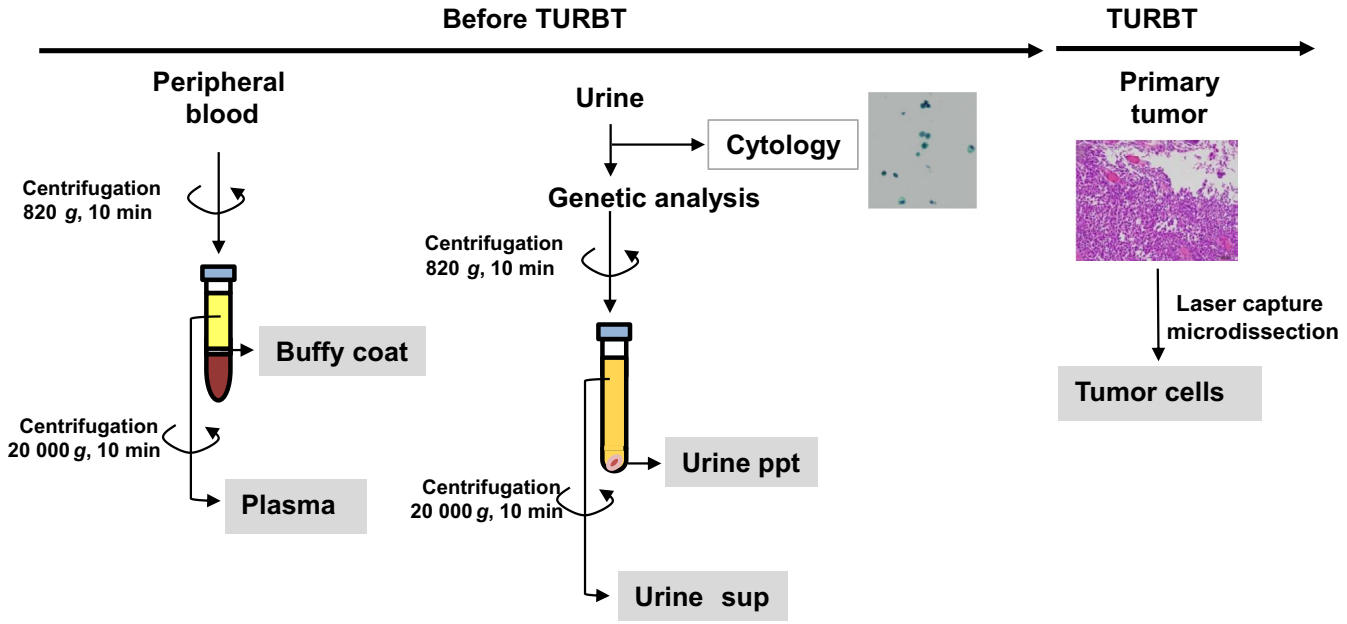

FIGURE 1 Scheme of sample preparation. Before transurethral resection of bladder tumor (TURBT), five different samples were obtained from each of the 25 patients with urothelial BC: buffy coat, plasma, urine precipitate (ppt), urine supernatant (sup), and primary tumor tissues. Urine cytology specimens were also prepared. DNA was extracted from these samples and analyzed by targeted sequencing

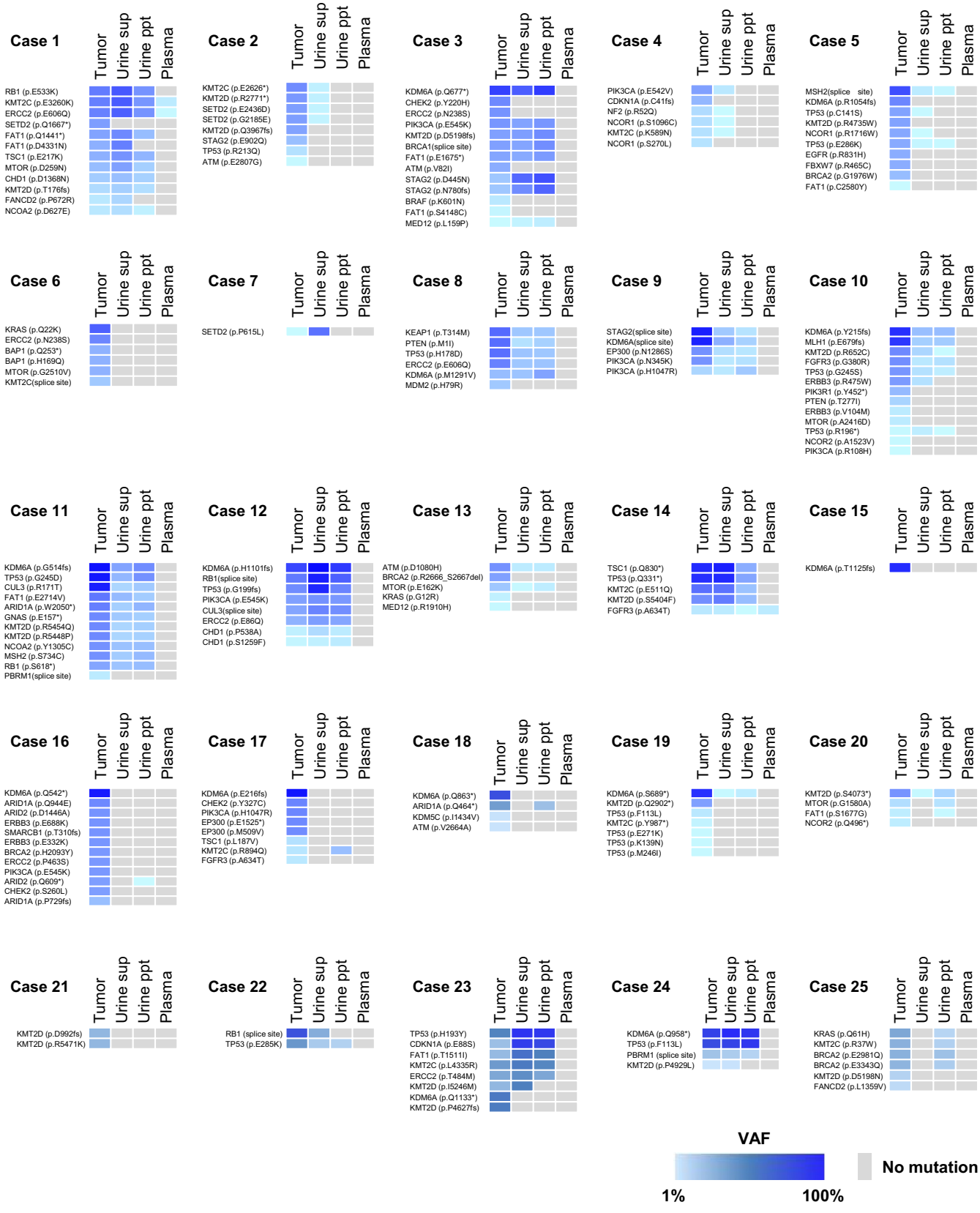

FIGURE 2 Mutation profiles identified in primary tumor, urine supernatant, urine precipitate, and plasma. Heat maps show identical mutations in the indicated samples corresponding with primary tumor mutations. Variant allele fraction values are shown in blue (high value) and light blue (low value) boxes. Grey boxes indicate no identified mutation in samples. ppt, urine precipitate; sup, urine supernatant

Cell Free DNA extraction kit and the KingFisher Duo Prime (Thermo Fisher Scientific).

2.2 | Tumor sample preparation and histology

Tumor samples were resected by TURBT and fixed using 10% buffered formalin.[19] Laser capture microdissection was carried out to enrich tumor purity as described previously.[13] Tumor DNA was extracted using the GeneRead DNA FFPE kit (Qiagen). Four cases diagnosed as cystitis had surgery several years prior and were previously diagnosed as BC, and genomic analysis was done using specimens resected at that time. Formalin-fixed paraffin-embedded (FFPE) DNA quality was analyzed as described previously.[20] Histological and cytological examinations were conducted by pathologist (T.O.) and cytologist (K.A.).

2.3 | Gene selection, targeted sequencing and data analysis

We searched The Cancer Genome Atlas (TCGA) data and literature[21-24] and selected 71 significantly mutated genes related to urological cancer (BC, kidney cancer and prostate cancer) (Table S2). A total of 3652 primer pairs were contained within the Urology Panel (covering 365.34 kb). Construction library for targeted sequencing was conducted as described previously.[25] The library concentration was determined using an Ion Library Quantitation Kit. Emulsion PCR and chip loading were carried out on the Ion Chef with the Ion PI Hi-Q Chef kit. Sequencing was done on the Ion Proton Sequencer (Thermo Fisher Scientific). Sequence data analysis was carried out as described previously.[25-27] Actionable mutations were referred to the OncoKB database (update: June 21, 2019) from the Memorial Sloan Kettering Cancer Center.[28]

2.4 | Statistical analysis

R package (version 3.1.2) was used for statistical analysis such as two-sample *t* test, Kruskal-Wallis rank sum test, and Pearson's chi-squared test.

3 | RESULTS

3.1 | Somatic mutation in primary tumor, urine and plasma

Twenty five patients with urothelial BC (18 non-invasive BC and 7 invasive BC) and five benign cases (cystitis [n = 4], inverted papilloma [n = 1]) were analyzed in this study (Table S1). Prior to TURBT, urine and peripheral blood samples were collected from all patients (Figure 1). A total of 150 samples were analyzed by targeted sequencing of 71 genes associated with urological cancer (Table S2). Corresponding buffy coats were used as normal controls to detect somatic mutations in each sample. In total, 168 somatic mutations were identified in the primary tumors in patients with urothelial BC (average 6.7 mutations per tumor). At least one mutation was identified in all primary tumors. The average number of mutations in tumor was not significantly different among clinicopathological features (Table S3, Kruskal-Wallis rank sum test).

To examine which liquid biopsy reflected tumor mutational profiles, we compared the genetic alterations of urine and plasma with those from the primary tumors (Figure 2). Of 168 mutations found in tumors, 89 mutations (53%) were also observed in urine supernatant, 81 (48%) were in urine precipitation. In plasma, only three (2%) mutations were identical to tumor mutations (Figure 2). At least one mutation identical to mutations in the corresponding primary tumor was observed in 72% (18/25) of patients by genomic analysis of urine supernatant, 76% (19/25) by urine precipitation, and 8% (2/25) by plasma (Figure 2). Analyses of both urine supernatant and precipitation detected tumor-derived DNA in 88% (22/25) of patients in total. Somatic mutations were not found in the five patients with cystitis and benign tumor. These results suggested that urine was an ideal "liquid biopsy" to detect tumor mutations in patients with urothelial BC.

To further investigate the relationship between genetic alterations in the liquid biopsy and clinical features, we analyzed the variant allele fraction (VAF) in each sample. Median VAF of tumor-identical mutation was 22.5% in urine supernatant, 22.6% in urine precipitation, and 8.7% in plasma (Figure S1). In addition, VAF was significantly correlated between urine supernatant and urine precipitation (R^2 = 0.7394) (Figure S2). In both urine supernatant and precipitate, VAF was significantly related to worse clinical indicators such as tumor invasion (median VAF = 20% vs 38% in supernatant, P = .03; 20% vs 35% in precipitate, P = .06) (Figure 3) and cytological examination (median VAF = 17% vs 32% in supernatant, P = 0.001; 20% vs 27% in precipitate, P = 0.005) (Figure 3). These results suggested that high VAF in urine would predict tumor aggressiveness.

3.2 | Clinical utility of urine genomic profiles

To examine the clinical utility of genome analysis using urine, we referred to the OncoKB database and searched the actionable mutations from genomic profiles. We observed 22, 21 and one actionable mutations in urine supernatant, urine precipitation and plasma, respectively (Table 1). Frequently identified actionable mutations had roles in the DNA repair pathway (*ERCC2*, *BRCA1*, *MLH1*, *MSH2*; 32%), chromatin remodeling (*KDM6A*; 32%), PI3K-AKT-mTOR pathway (PIK3CA, PTEN, TSC1; 32%), and the RTK-RAS-RAF pathway (FGFR3; 5%). Furthermore, actionable mutations were observed in 52% (13/25), 48% (12/25) and 4% (1/25) of patients using genomic profiles of urine supernatant, urine precipitate and plasma, respectively (Table 1). These results suggested that both urine supernatant and precipitate were superior in detecting actionable mutations compared with plasma.

3.3 | Tumor diagnostic sensitivity of urine compared to cytology

We next compared tumor diagnostic sensitivity between conventional cytology and urine DNA. Cytological examination showed

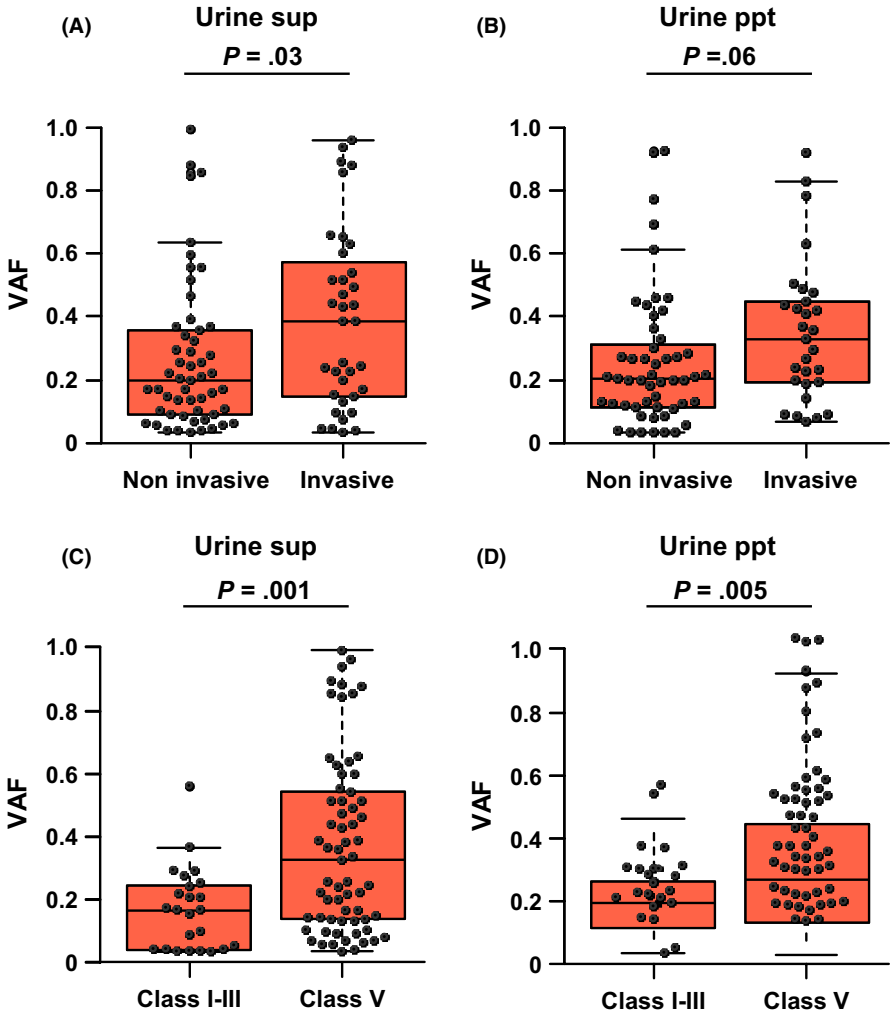

FIGURE 3 Variant allele fractions (VAF) of identified mutations in urine supernatant and urine precipitate are related to clinical indicators. Box plots show the VAF of identical mutations corresponding to tumor in urine (A-D). High VAF mutations were detected in invasive tumors compared to non-invasive tumors in urine supernatant (sup) (A) and precipitate (ppt) (B).High VAF mutations were observed in class V tumor determined by cytological examination compared to class I-III tumor in urine supernatant (C) and precipitate (D). *P*-value was calculated with Student's *t* test

tumor cells in 36% (9/25 patients) of total patients, in 22% (4/18) of patients with non-invasive tumors, and in 71% (5/7) of patients with invasive tumors (Figure 4). Of note, genomic profiles of urine supernatant and precipitate predicted 67% (12/18) and 78% (14/18) of non-invasive tumor and 86% (6/7) and 71% (5/7) of invasive tumor, respectively (Figure 4). Tumor diagnostic sensitivity of urine DNA was significantly higher than that of cytology, especially in patients with non-invasive tumor (urine supernatant vs cytology, $P = 0.018$; urine supernatant vs cytology $P = 0.002$; chi-squared test) (Figure 5). These results showed that urine DNA is clinically useful for the diagnosis of superficial BC.

4 | DISCUSSION

If genetic information in urine can provide a precise snapshot of the mutational profile of tumors, urine genomic analyses could serve as an approach that can greatly contribute to cancer diagnosis and treatment selection. Our results showed that genomic alterations in urine DNA could precisely reflect tumor mutations in urothelial BC. Mutations identical to those in primary tumors were more successfully detected using urine compared with plasma. Notably, using databases, mutation data obtained from urine could predict functionally relevant mutations and muscle invasiveness. Our results showed that urine can be considered a non-invasive "liquid biopsy" reflecting genetic alterations and that tumor-derived DNA shed in urine in patients with early-stage urothelial BC.

Plasma is a well-known liquid biopsy for analyzing the genetic status of tumors.[15,29] However, the abundance of cell-free tumor DNA in plasma is low, and therefore it is possible that mutations may not be detected in early-stage cancer. We previously showed that the concentration of cell-free tumor DNA is diluted from proximal to distal sites from tumor lesions.[17] Unlike blood circulation, urine accumulates in the bladder and thus cell-free tumor DNA may be more concentrated in urine. A recent study showed ultra-deep sequencing (median 10500-fold coverage depth) of plasma detected tumor-identical mutations in localized advanced BC.[30] To detect low VAF mutations in plasma cfDNA, high-coverage depth data would be need. Our data suggested that high VAF mutant could be detected in urine of urothelial BC, but not in plasma. Therefore, highly sensitive and specific detection could be achieved using urine DNA rather than plasma.

Urine cytology is routine clinical practice for tumor diagnosis during the follow-up period; however, low sensitivity, especially in

TABLE 1 Actionable mutations identified in urine and plasma

Case no.	Gene	Mutation	Urine sup	Urine ppt	Plasma	Drug
Case 1	*ERCC2*	p.E606Q	+	+	+	Cisplatin
Case 3	*KDM6A*	p.Q677*	+	+	−	Tazemetostat
	PIK3CA	p.E545K	+	+	−	Alpelisib + Fulvestrant, Buparlisib, Serabelisib, Copanlisib, GDC-0077, Taselisib + Fulvestrant, Alpelisib, Buparlisib + Fulvestrant, Taselisib
	BRCA1	Splice site	+	+	−	Olaparib, Rucaparib, Niraparib
Case 4	*PIK3CA*	p.E542V	+	−	−	Alpelisib + Fulvestrant, Buparlisib, Serabelisib, Copanlisib, GDC-0077, Taselisib + Fulvestrant, Alpelisib, Buparlisib + Fulvestrant, Taselisib
Case 5	*MSH2*	Splice site	+	+	−	Nivolumab, Pembrolizumab
Case 8	*PTEN*	p.M1I	+	+	−	GSK2636771, AZD8186
	ERCC2	p.E606Q	+	+	−	Cisplatin
Case 9	*KDM6A*	Splice site	+	+	−	Tazemetostat
	PIK3CA	p.N345K	+	+	−	Alpelisib + Fulvestrant, Buparlisib, Serabelisib, Copanlisib, GDC-0077, Taselisib + Fulvestrant, Alpelisib, Buparlisib + Fulvestrant, Taselisib
	PIK3CA	p.H1047R	+	+	−	
Case 10	*KDM6A*	p.Y215 fs	+	+	−	Tazemetostat
	MLH1	p.E679 fs	+	+	−	Nivolumab, Pembrolizumab
	FGFR3	p.G380R	+	+	−	AZD4547, BGJ398, Debio1347, Erdafitinib
Case 11	*KDM6A*	p.G514 fs	+	+	−	Tazemetostat
Case 12	*KDM6A*	p.H1101 fs	+	+	−	Tazemetostat
	PIK3CA	p.E545K	+	+	−	Alpelisib + Fulvestrant, Buparlisib, Serabelisib, Copanlisib, GDC-0077, Taselisib + Fulvestrant, Alpelisib, Buparlisib + Fulvestrant, Taselisib
	ERCC2	p.E86Q	+	+	−	Cisplatin
Case 14	*TSC1*	p.Q830*	+	+	−	Everolimus
Case 19	*KDM6A*	p.S689*	+	+	−	Tazemetostat
Case 23	*ERCC2*	p.T484M	+	+	−	Cisplatin
Case 24	*KDM6A*	p.Q958*	+	+	−	Tazemetostat

*, termination; +, detected; −, not detected; ppt, precipitate; sup, supernatant

non-invasive tumors, remains an unresolved problem. Our findings showed that genome analysis of urine DNA was superior to urine cytology as a diagnostic tool. In the present study, cytology could detect tumor cells in 71% of invasive cancer cases and in 22% of non-invasive cancer cases. However, the diagnostic rate using urine was 67%-77% in non-invasive urothelial BC and 71%-86% in invasive urothelial BC. Consistent with our observations, previous data also showed that analysis of urine DNA had higher diagnosis sensitivity in early-stage urothelial BC compared to cytological examination.[31] These findings indicate that detection of genomic alterations in urine could predict tumors in cases that cytology could not. Analysis of urine DNA in urothelial BC would help us to make diagnoses of cancer and monitor therapeutic effect.[14,32,33] Together, these results show that urine analysis can serve as an alternative and effective method for tumor diagnosis, even at an early stage.

Detection rates of urine supernatant and precipitate by genomic profile were almost the same. Therefore, examination of either urine supernatant or precipitate would be considered sufficient for detecting tumor-derived mutations. Alternatively, whole urine may also be suitable for genome analysis. Meanwhile, somatic mutations detected in the urine supernatant and precipitate were not necessarily the same. Although the precise reasons underlying this discrepancy are unknown, we discuss possible explanations. The first is contamination of inflammatory or normal cells. If there are many inflammatory cells in the urine, it is considered that lower tumor purity reduced the detection rate of mutations because a large number

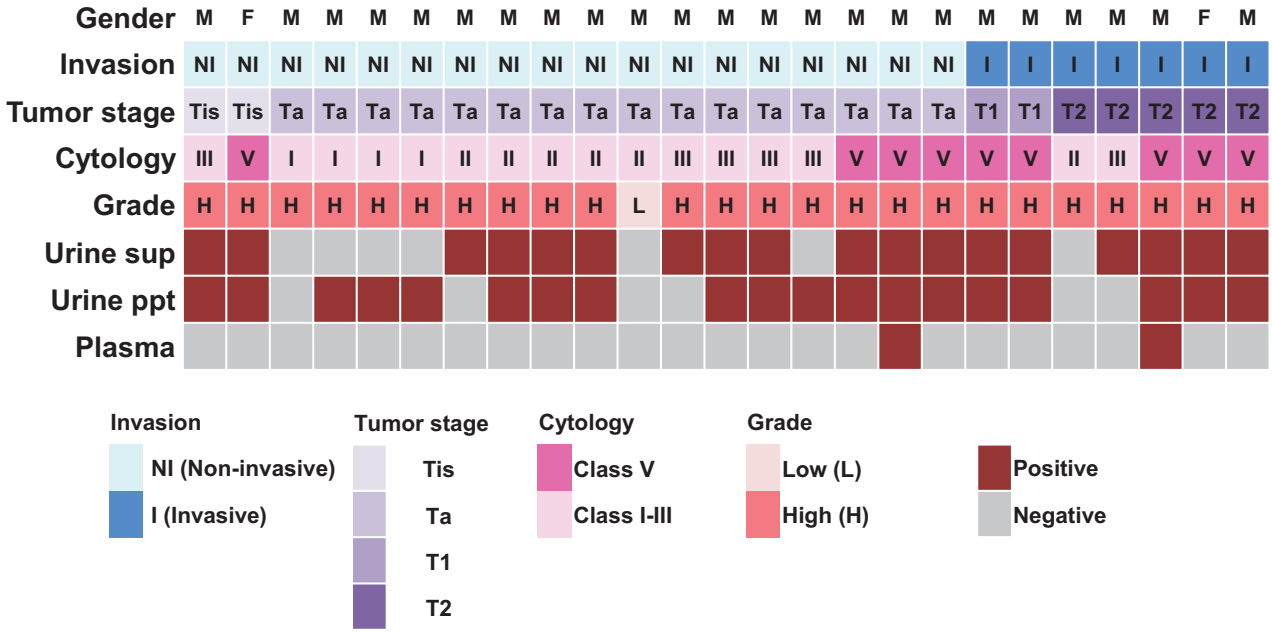

FIGURE 4 Tumor diagnostic sensitivity of conventional cytology and genetic analyses of urine and plasma samples. Comparison of the tumor diagnostic rate in each patient. Clinical information (gender, invasion, tumor stage, cytology, tumor grade) is shown at the top of the figure. Conventional cytology was used to classify tumors as class I-V. Class I, II and III indicates tumor negative (light pink) and class V indicates tumor positive (pink). Brown boxes indicate positive identical mutations corresponding to the primary tumor in urine supernatant (sup), urine precipitate (ppt), and plasma. No mutations are indicated by grey boxes. I, invasive tumor; NI, non-invasive tumor

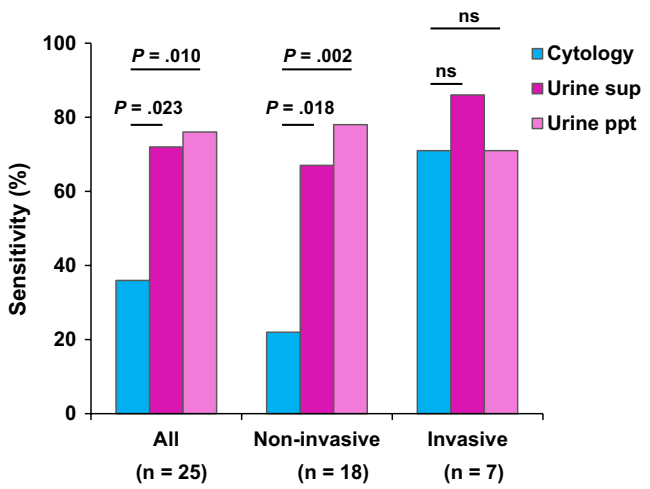

FIGURE 5 High diagnostic sensitivity of urine genomic profiles. Tumor diagnostic sensitivity of urine was higher than that of conventional cytology. Significant difference was observed in all tumor (n = 25) or non-invasive tumor (n = 18), but not in invasive tumor (n = 7). *P*-value was calculated with Pearson's chi-squared test. ns, not significant. ppt, urine precipitate; sup, urine supernatant

of normal alleles are contaminated in the urine precipitate. Second, there may be differences among individuals in the extent to which tumor cells collapse and tumor-derived DNA sheds into urine. Some cancer cells are susceptible to collapsing, whereas others are not. It is possible that cancer cell integrity determined whether mutant DNA is detected in urine supernatant or precipitate. Namely, if the tumor cells are susceptible to collapse (eg, apoptosis and necrosis), tumor-derived DNA can easily be detected in urine supernatant.

Although genomic information will provide invaluable information for precision medicine, tissue samples are not repeatedly obtained in clinical practice because of its invasiveness and cost of intervention. In contrast, urine samples have several advantages: (i) easily available and non-invasive; (ii) can be obtained frequently; (iii) can be scheduled at specific time points; and (iv) reflect the genetic heterogeneity of tumors. In the present study, we found that at least one tumor-identical mutation was identified in 72% of patients using urine supernatant and in 74% of patients using urine precipitate. Thus, urine liquid biopsy has the potential to contribute to treatment decisions and follow-up schedule without the requirement for invasive interventions.

In urothelial BC, FGFR3 fusion genes (eg, *FGFR3–TACC3*, *FGFR3–BAIAP2L1*, *FGFR3–ADD1*) were reported previously.[23,34,35] Our designed urology panel has a limitation for detecting fusion genes. In order to determine the therapeutic application for FGFR inhibition, it is important to construct an assay system that detects the fusion gene. Furthermore, detecting fusion genes will enable insight into the pathogenesis of urothelial BC.

In summary, we carried out a comprehensive mutational analysis of BC cases using "liquid biopsies" of urine and plasma compared with primary tumor samples. Although cytology showed tumor cells in 22% of patients with non-invasive tumors, tumor-identical mutations were detected in 67% of urine supernatant samples and in 78%

of urine precipitates. Our results suggest that urine-based genetic analysis is an effective method for BC diagnosis.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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