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Original Article

Usefulness of droplet digital PCR and Sanger sequencing for detection of *FGFR3* mutation in bladder cancer

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Abstract

Objective: The purpose of our research was to determine the usefulness of different methods for detecting Y373C mutation of gene *FGFR3*.

Patients and methods Total: 138 primary bladder cancer patients (71cases G1 and 67 cases G2-G3) were included in the study. Tumor tissue and urine samples were collected and kept frozen until the isolation of DNA. Sanger sequencing was applied for detecting mutation in cancer and ddPCR was utilized for urine assessment.

Results: ddPCR appears to be more effective and it identified *FGFR3* mutation (Y373C) in urinary sediment in 20.3% of cases whereas Sanger sequencing did in 15.5%. Only in 8/39 (20.5%) cases the mutation was observed both in urine and tissue. In 12/39 (30.8%) cases (5G1 and 7 G2-G3) we did not detect any *FGFR3* mutation in urine although it was confirmed by sequencing. We only found mutation in urine in 20/39 cases (15 G1, 5 G2-G3) (51.3%). The correlation between the presence of *FGFR3* mutations and better survival was confirmed. The Log-Rank test indicates a significant difference in the likelihood of survival for patients with the *FGFR3* mutation but without recurrence (Cox's *F*-test $P = 0.17006$; Log-Rank Test $P = 0.00059$).

Conclusion: ddPCR appeared to be more sensitive method for detection *FGFR3* gene mutation particularly for detecting low levels of tumor DNA amongst a large excess of nontumor DNA. It is significant as the implementation of such markers into routine practice could be beneficial. The prospective study in larger cohort is needed.

Keywords: Bladder cancer; *FGFR3* mutation; ddPCR; Sequencing

1. Introduction

Bladder cancer (BC) is a common type of cancer in men and women [1,2]. About 70% of newly diagnosed cases are a low-stage, low-grade, nonmuscle-invasive (NMI) disease. Transurethral resection is the standard treatment. Approximately 60% of the tumors will recur and 20% will progress to become muscle invasive (MI). Therefore, the European Association of Urology guidelines for **BC** recommend surveillance cystoscopy and urine cytology for NMI disease every 3 months for the first 1 to 2 years, repeated at longer intervals over the next 2 years, and annually thereafter [3]. This lifetime surveillance poses an enormous economic burden to health care systems [4,5]. Enormous care costs do not translate into the comfort of living for patients, who experience stress related to each hospital stay or to the risk of detection of the disease recurrence during each control examination.

Bladder tumors comprise a heterogeneous group in terms of both histopathology and clinical behavior [6,7]. Many genetic and epigenetic alterations have been identified to contribute directly or indirectly to the development of bladder tumors [8,9]. Thus, there is already some

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understanding of what combinations occur together most frequently and in which type of tumor. One of the most frequently observed genetic changes are gene *FGFR3* (Fibroblast growth factor receptor 3) alterations. The rate of *FGFR3* genomic alterations vary by stage and grade for urothelial carcinoma. The Y375C, S249C, R248C, and G372C are the most common in BC in the Caucasian population [11–13]. There are papers which confirm the higher rate of gene *FGFR3* alterations in NMI tumors (60%–85%) in relations to muscle invasive tumors (10% –54%) [14,15]. A similar analogy can be drawn for grade as more alterations can be observed in low-grade tumors than in high grade ones. Mutated *FGFR3* is constitutively activated and induces a number of oncogenic signaling pathways. It is associated with genetically more stable pTa and low-grade BC although the overexpression of invasive wild-type *FGFR3* tumors (detected by immunohistochemical technique) was also observed in even 40% of cases, suggesting an alternative mechanism of activation. [14–17]. The knowledge of the molecular alterations that are involved in the development and progression of BC will result in greater prognostic and predictive abilities and ultimately will lead to the development of new individualized therapies. The preclinical studies showed that alterations in *FGFR3* confer susceptibility to FGFR3 inhibitors, which suggests that applying a suitable treatment course could benefit patients in whom *FGFR3* alterations in tumor cells will be confirmed [18,19].

A number of methods can be applied in order to assess the presence of gene *FGFR3* alterations, including Sanger sequencing and droplet digital Polymerase Chain Reactions (ddPCR) [20–22]. The detection of *FGFR3* gene mutation in DNA isolated from cancer tissue or patients' urine ranged between 7% to 70% [23,24]. ddPCR is a relatively new method. Some papers indicated their usefulness in assessing rare events, particularly in assessing tumor samples in which mutations have to be detected in the background of wild type DNA. Partitioning into thousands of droplets is the main difference compared to the standard PCR reaction. Each droplet is an independent reaction. DNA amplified in each droplet is assessed for mutation via fluorescence signaling in the way that the number of positive and negative droplets can be counted. It allows assessing rare events at the lower limit of detection down to 0.005%, which is impossible for sequencing (20%) or other techniques of molecular biology (for real-time PCR 1%) [25,26]. The aim of our work was to compare the results obtained by means of Sanger sequencing in tumor tissue with the results of the ddPCR analysis conducted in DNA isolated from the urine samples of the same patients. Currently, there is no clinically relevant marker or a test for a prediction of progression in BC and an accurate method of assessing gene *FGFR3* alterations could enable introducing such a marker to the routine assessment.

2. Patients and methods

2.1. Patients' data

The group of 138 patients with urothelial **BC** have been examined: 28 women (20.3%) and 110 men (79.7%), aged 44 to 88 (the average 67). The detailed characteristic is presented in Table 1 and Figure 1. The group consisted of the patients from whom the stored material for testing, namely tumor tissues and urine sediment, was collected from 2006 to 2012 in 2 hospitals. All the patients signed the consent for using their samples in the scientific research, and the official approval of conducting analyses was obtained from the bioethical commission (RNN/294/13/KE). Tumors were graded according to 1973 WHO classification and staged using the TNM criteria [27–29].

2.2. DNA extraction and sequencing

DNA was extracted from frozen tissue as previously described [30,31]. Briefly tissues were homogenized in TRI REAGENT (guanidine thicyante/phenol, Molecular Research Center Inc. cat. no Tr-118) using ceramic beads (Roche MagNALyser Green Beads, Roche Applied Science, Mannheim, Germany, cat. no 3358941001), and Roche Magna Lyser (cat. No 03358976001). For exfoliated urinary cells, 200 to 400 mL of freshly voided urine was collected in Carbo-wax at diagnosis and urinary sediment was frozen in −20°C. DNA from homogenized tissue (section earlier verified under microscope) and from exfoliated urinary cells were isolated with a Sherlock AX Kit (A&A Biotechnology, Gdynia Poland) according to manufacturer's protocol. The DNA concentration and purity were determined using an ND-1000 spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific).The purity ratio was 1.8 or higher (λ 260/

Table 1
Characteristics of the patients.

Parameters	Number(%)
Mean age, y (range)	67 (44–88)
Median follow-up, mo	39 (5–48)
Gender	
Male	110 (79.7)
Female	28 (20.3)
Smoking (ex-or current)	106 (76.8)
Occupational exposure	73 (52.9)
Stage	
pTa	64 (46,4)
pT1	41(29.7)
pT2	27 (19.6)
pT3-4	6 (4.3)
Grade	
G1	71 (51.4)
G2	34 (24.6)
G3	33 (24)
Recurrence	105 (83.3)
Progression	32 (23.2)

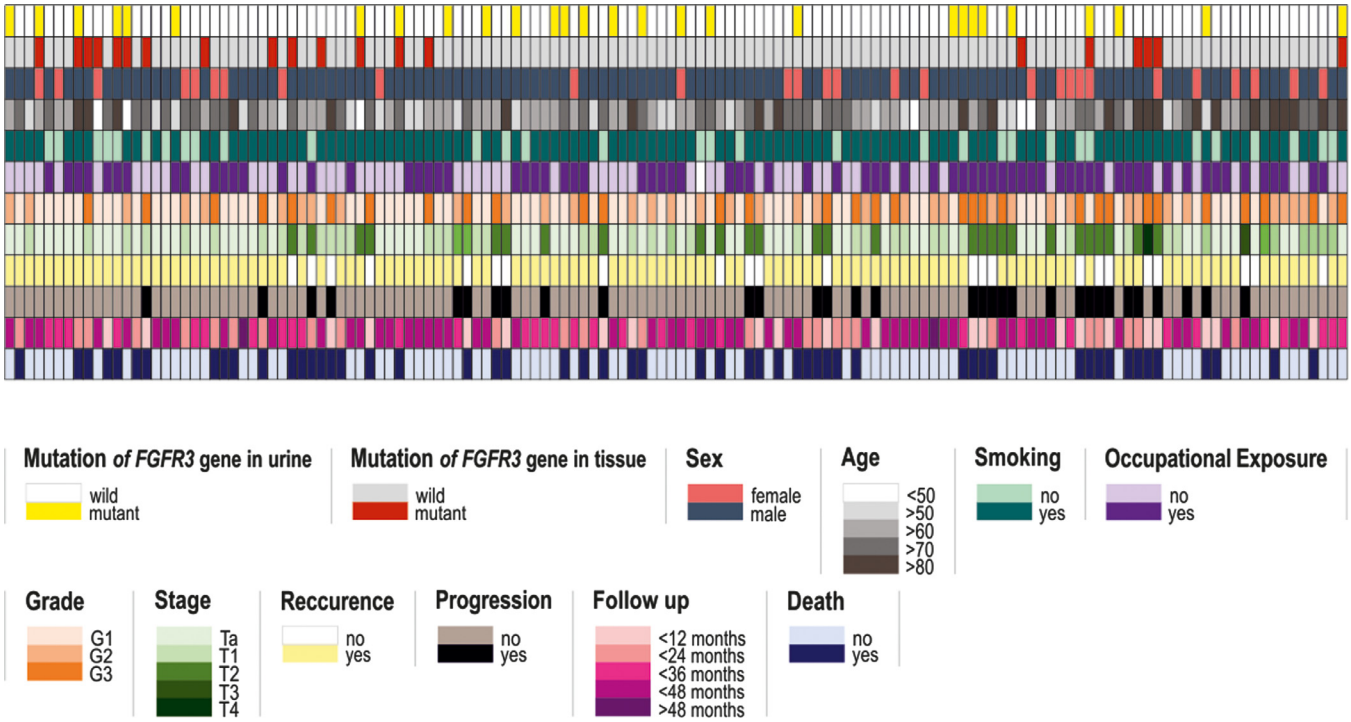

Fig. 1. Distribution of FGFR3 mutations as detected by droplet digital PCR (ddPCR) and Sanger sequencing as well as other clinical characterictics: the sex and age of the patients, smoking, occupational exposure, stage, and grade of the tumor tissue, information about recurrence, death, and follow-up (months).

280) and 1.8 to 2.2 (for λ 260/230). [32] Exon 10 of *FGFR3* gene was amplified using the following primers: 5'GCCGAGGAGGAGCTGGTGGAGGCT3'(forward), 5'CTTGAGCGGGAGATCTTG3' (reverse) in 20 μl volume reaction as previously described [33]. The PCR amplification (annealing 60˚C for 35 cycles) was conducted in BioRad thermocycler T-100 (Bio-Rad Hercules, cat no. 1861096). PCR products were directly sequenced using the Big Dye Sequencing kit (Applied Biosystems, Foster City, CA) on an ABIPRISM Genetic Analyser 3500.

2.3. ddPCR

ddPCR reactions begin by partitioning the PCR reaction mix containing DNA into aqueous droplets in oil via the BioRad QX100 Droplet Generator (cat no 1863005). BioRad reagents and consumables were used for the experiments including probes (Unique Assay ID: dHsaCP2000103: 1031246), ddPCR supermix for probes (cat no 1863026), dG8 Gasket (cat no 1864008), ddPCR plate (cat no 10023379). After transfer of droplet to a 96-well PCR plate, a 2-step thermocycling protocol (94˚C-30s, 60˚C-60s) followed by 1 cycle at 95˚C (2,5˚C/second ramp) for 10 minutes was carried out (40 cycles) in BioRad T100 device. The PCR plate was after that transferred to the BioRad XQ100 Droplet Reader (cat no 1863004) for automatic reading. A detailed description of the ddPCR method and workflow can be found in Hindson et al. 2011 [34].

2.4. Statistical analysis

The statistical analyses were performed using STATISTICA 10 (StatSoft, Inc., Tulsa, OK). *P* values lower than 0.05 were considered significant. Statistical association between clinicopathological features and presence of *FGFR3* (Y373C) mutation was compered by the $\chi 2$ or V^2 tests (in the table marked V or χ^2) with the Yates correction (in the table marked Y). Progression free survival was calculated with the use of Kaplan–Meier method with log-rank test (Fig. 4). Univariate and Multivariate analysis were conducted (results presented in Table 3 and 4). Progressive disease was defined as progression to stage T2 or higher stage of disease, development of nodal, or distant metastases.

3. Results

3.1. FGFR3 mutations assessment

Among the patients qualified for the tests there were 106 persons (including 17 women) who either smoked at the time or were ex-smokers. The connection between occupational exposure and **BC** development was identified in 73 examined persons (52.9%). Mutations were identified in 20.3% of cases with the use of droplet digital techniques, and in 15.5% by means of Sanger sequencing (results presented in Table 2, Fig. 2 and 3). In relation to urine samples, the group of patients with recognized mutation comprised

Table 2
Frequency of FGFR3 mutation (Y373C) detected using Sanger sequencing and ddPCR according to clinicopathological parameters of tumors.

Clinicopathological parameters		*FGFR3* ddPCR			*FGFR3* Sanger			*FGFR3* both methods		
		Wild *n*(%)	Mut *n*(%)	*P* value	Wild *n*(%)	Mut *n*(%)	*P* value	Wild *n*(%)	Mut *n*(%)	*P* value
Total	138									
Sex										
	Female	24 (17.4%)	4 (2.9%)		24 (17.4%)	4 (2.9%)		22 (15.9%)	6 (4.35%)	
	Male	87 (63.0%)	23 (16.7%)	0.4319 (V)	94 (68.1%)	16 (11.6%)	0.7904(Y)	77 (55.8%)	33 (23.9%)	0.3702(V)
Age at diagnosis										
	<60	21 (15.2%)	5 (3.6%)		22 (15.9)	4 (2.9%)		19 (13.8%)	7 (5.1%)	
	>60	90 (65.2%)	22 (15.9%)	0.9621 (V)	96 (69.6%)	16 (11.6%)	0.8683(Y)	80 (58.0%)	32 (23.2%)	0.8669(V)
Smoking status										
	Yes	84 (60.9%)	22 (15.9%)		90 (65.2%)	16 (11.6%)		74 (53.6%)	32 (23.2%)	
	No	27 (19.6%)	5 (3.6%)	0.523(V)	28 (20.3%)	4 (2.9%)	0.9371(Y)	25 (18.1%)	7 (5.1%)	0.3617(V)
Occupatinal exposure										
	Yes	58 (42.0%)	17 (12.3%)		66 (47.8%)	9 (6.5%)		53 (38.4%)	22 (16.0%)	
	No	53 (38.4%)	10 (7.2%)	0.3163	52 (37.7%)	11 (8.0%)	0.3658(V)	46 (33.3%)	17 (1.3%)	0.7601
Tumour stage										
	Ta-T1	84 (60.9%)	23 (16.7)		92 (66.7%)	15 (10.9%)		76 (55.1%)	31 (22.5%)	
	T2-T4	27 (19.6%)	4 (2.9%)	0.2901 (V)	26 (18.8%)	5 (3.6%)	0.9966(Y)	23 (16.7%)	8 (5.8%)	0.7313(V)
Grade										
	G1	55 (39.9%)	18 (13.0%)		62 (44.9%)	11 (8.0%)		51 (37.0%)	22 (16.0%)	
	G2	30 (21.7%)	4 (2.9%)		31 (22.5%)	3 (2.2%)		28 (29.3%)	6 (4.3%)	
	G3	26 (18.8%)	5 (3.6%)	0.2528	25 (18.1%)	6 (4.3%)	0.4741	20 (14.5%)	11 (8.0%)	0.2449
Recurrence										
	Yes	90 (65.2%)	25 (18.1%)		98 (71.0%)	17 (12.3%)		81 (58.7%)	34 (24.6%)	
	No	21 (15.2%)	2 (1.4%)	0.2495 (Y)	20 (14.5%)	3 (2.2%)	0.9138(Y)	18 (13.0%)	5 (3.6%)	0.4484(V)
Progression										
	Yes	27 (19.6%)	5 (3.62%)		28 (20.3%)	4 (2.9%)		24 (17.4%)	8 (5.8%)	
	No	84 (60.9%)	22 (15.9%)	0.523 (V)	90 (65.2)	16 (11.6%)	0.9371(Y)	75 (54.3%)	31 (22.5%)	0.6414(V)

Y-test chi-squared with Yeats corrections.
V-test V-squared.

14 patients in stage pTa, 7 patients in stage pT1, and 7 patients in pT2. Taking grade into consideration, there were 18 cases of cancers in stage G1, 7 cases in stage G2, and 3 cases in G3. The sequencing done in tumor tissue detected gene *FGFR3* alterations in 10 cases of pTa, 5 cases of pT1, 3 of pT2, and 2 of pT3-4. Total 10 of those cases were classified as G1, 3 as G2, and 5 as G3. The application of both methods led to obtaining 39 results that confirmed mutation (28.3%): 17 pTa (which constitutes 26.6%), 10 pT1 (24.4%), 9 pT2 (33.3%), and 3 pT3-4 (50%), and with regard to grade, 21 G1 (29.6%), 5 G2 (14.7%), and 13 G3 (39.4%). As the above comparison shows, most of the cases were in lower clinical stage (27 patients pTa-T1 vs. 12 pT2-4) and in lower grade. However, there was a small percentage of mutations detected in higher stage and grade. Mutation was detected by means of both techniques only in 8 out of 39 positive results (20.5%) (6 pTa, 1 pT1, 1 pT2). The ddPCR technique allowed for detecting more cases in lower clinical stage.

3.2. The association between FGFR3 *mutations, clinical parameters, and Progression free survival*

The analysis conducted to correlate clinical parameters with occurrence of mutation did not show any statistically significant differences between Sanger sequencing and ddPCR technique. Table 2 displays the detailed results. The survival analysis with the use of Kaplan–Meier curves showed no fundamental differences between the mentioned methods in the groups of the patients both with mutation of *FGFR3* (group 1) and without mutation (group 0). Only the survival analysis of the results obtained with the use of both methods simultaneously (all results indicating mutations) identified the difference in the group of the patients with mutation and without disease recurrence, who are more likely to survive. The Log-Rank test indicates a significant difference in the likelihood of survival for patients with the *FGFR3* mutation but without recurrence (Cox's F-test $P = 0.17006$; Log-Rank Test $P = 0.00059$). The results are demonstrated in Figure 4. In the univariate analysis it was observed that patients with recurrence have 10 times lower risk of having progression. The risk of occurrence of progression in patients with Ta stage proved to be 38 times higher than in other stages ($P = 0.000334$), while the risk of progression in patients with G1 grade was 50 times lower than in patients in other grade (results presented in Table 3 and Table 4).

4. Discussion

Gene *FGFR3* mutations are quite frequently detected in low grade and early stage of BC. According to the

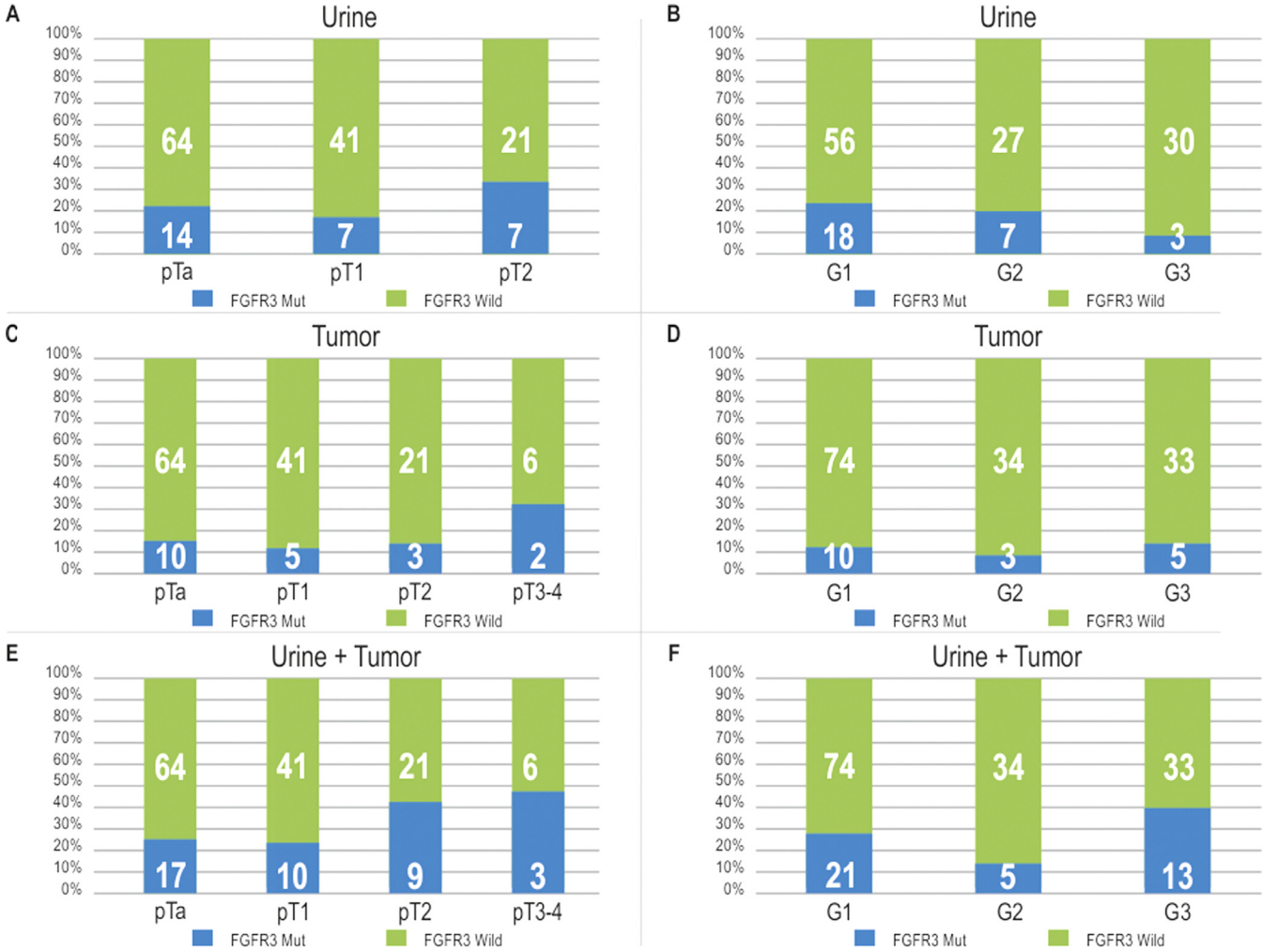

Fig. 2. FGFR3 mutation frequencies by stage (pT) and grade (G). The number of cases in each subgroups is indicated on the bars of the graph. Distribution of mutation in urine according to stage shows picture (A) and according to grade is presented on picture (B), distribution in tumor tissue according to stage shows picture (C), and according to grade is presented on picture (D) whereas total distribution for urine and tumor tissue is presented on pictures (E) and (F).

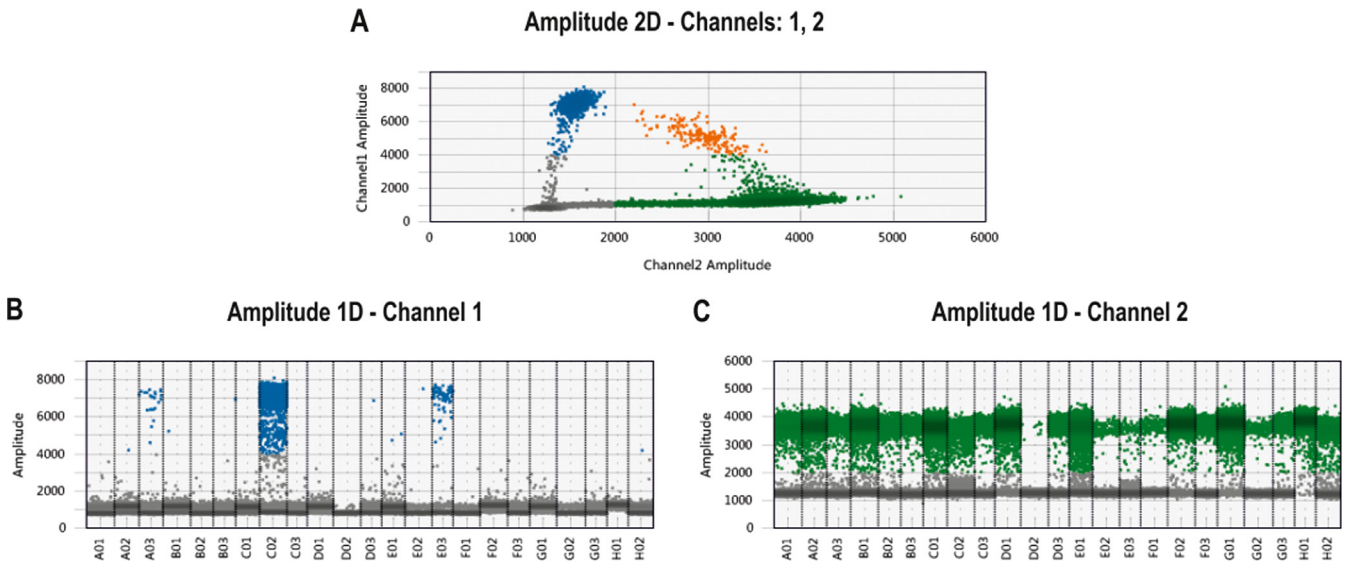

Fig. 3. FGFR3 mutation Y373C detected by ddPCR: (A) 2-dimentional plots of discordant FGFR3-mutant cells (blue and orange dots represents the presence of mutations (B) Mutant channel (FAM-blue) (C) Wild type probe (HEX-green).

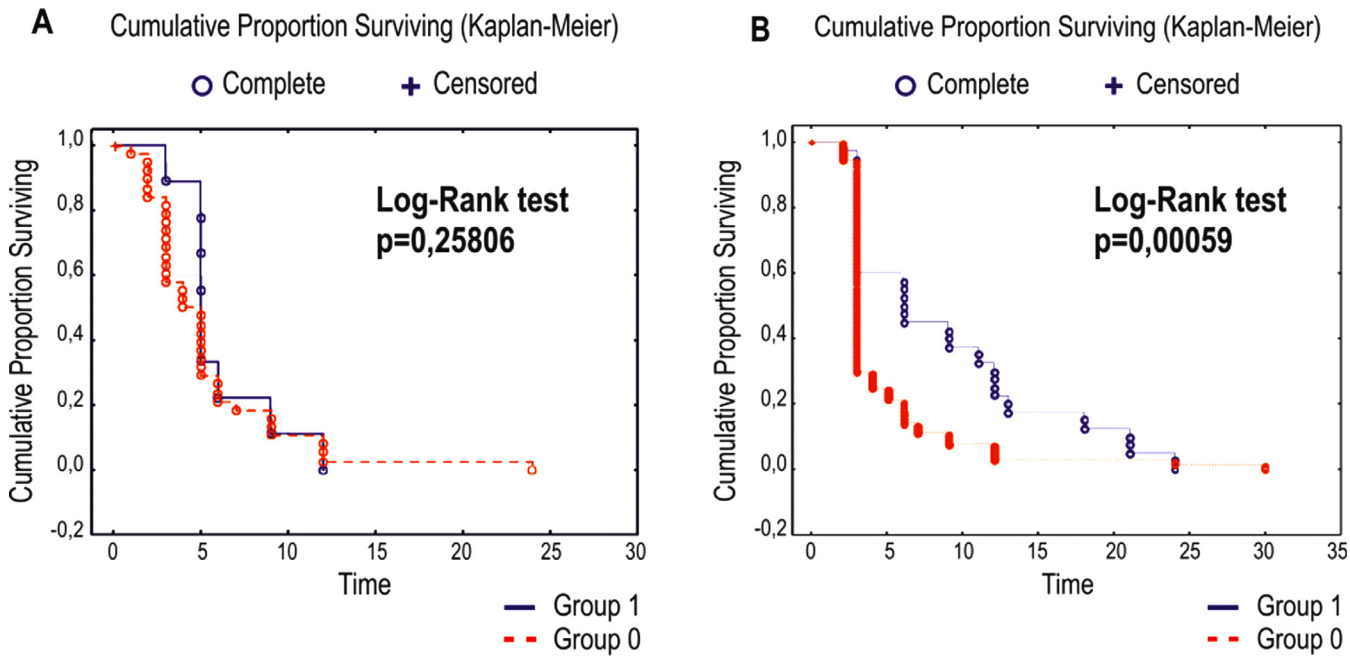

Fig. 4. Kaplan–Meier plots. Likelihood of survival without (A) progression (B) recurrence. Group 0 - without FGFR3 mutation, Group 1 - with FGFR3 mutation.

published and available data, frequency of gene *FGFR3* mutation decreases as stage and grade increase and it shows 65% in pTa, 30.2% in pT1, 11.5% in pT2-4, and 69.8% in G1, 68% in G2, and 18.6% in G3 [31–33]. As regards the results of our research, these data are 26.6% in pTa, 24.4% in pT1, 33.3% in pT2, and 50% in pT3-4 respectively and in terms of grade 29.6% in G1, 14.7% in G2 ,and 39.4% in G3. Higher numbers in higher stage and grade probably result from the small sizes of those groups but most of the cases were in lower clinical stage and in lower grade. The previous reports confirm that noninvasive low-grade BC tumors and invasive high grade ones are genetically and clinically different and are characterized by dissimilar biological potential and prognosis [8,10,28]. It has been found out that there is a higher percentage of disease recurrence in patients with gene *FGFR3* alterations and pointed to its favorable character in terms of prognosis [11]. This is in contrast to our research. The Log-Rank test indicates a significant difference in the likelihood of survival for patients with the *FGFR3* mutation but without recurrence (Cox's F-test $P = 0.17006$; Log-Rank Test $P = 0.00059$). The results are presented in Table 2 and Figure 3 and 4. In this study we concentrated on marking only 1 type of mutation (Y373C) whereas other kinds of mutations of this gene (e.g. R248C or S249C) are also recognized in BC [21]. Christensen et al. assessed 2 *FGFR3* mutations (Y373C and S249C) in tumor tissue in 363 nonmuscle-invasive bladder cancer (NMIBC) and 403 patients undergoing cystectomy. They only found mutation S249C in NMIBC cohort (112/368) whereas in the cystectomy cohort there were 6 patients with Y373C mutation and 8 patients with S259C mutation. This could be the explanation why the detection of mutationY373C in our study for NMIBC is not high. It has also been stated that the level of tumor DNA (tDNA) in urine supernatant is higher for tumors with later progression and that tDNA in plasma was significantly associated with later disease recurrence [35,36].

A number of the earlier analyses pointed to the presence of gene *FGFR3* mutation in patients with BC in both tissues and urine sediments [14,24,37]. None of these analyses, however, has been put into routine practice to date. Hence, the purpose of our research was to determine the usefulness of different methods for detecting Y373C mutation of gene *FGFR3*. The Sanger sequencing method has been in use for a long time. ddPCR has become available only recently; however, it offers much higher sensitivity, especially in detecting low levels of tDNA amongst a large excess of nontumor DNA [38,39]. That is an ideal technique for detecting rare alleles and simultaneously it allows for the absolute quantifications of the number of mutant alleles. Yet, it does not enable simultaneous assessment of many changes in the same sample, which is possible with the use of the sequencing technique. The discordance between results of sequencing and ddPCR mutations rate is not understood, but potential hypotheses include sample collection and tumor heterogeneity. Similar differences are also observed by other researchers [40,41]. In the research paper of McEvoy et al. there is a comparison of Sanger sequencing and pyrosequencing with the ddPCR method for *BRAF*, *NRAS*, and *TERT* mutation detecting in 40 formalin-fixed, paraffin-embedded melanoma tissue. High concordance between platforms was found only in tumors with high neoplastic cell content. The frequency of identified mutations of gene *BRAF* was different for different platforms and

Table 3
Univaraite Cox regression analysis of potential predictor variables and time to survival, progression or recurrence in patients group ($n = 138$).

	Beta	HR (95%CL)	p-value	p for Chi^2
Time to survival				
Gender	−0.39	0.67 (0.32−1.43)	0.306	0.285
Age at diagnosis	0.115	1.12 (1.08−1.16)	**0.000**	**0.000**
Stage				
Ta - another	1.26	3.53 (1.91−6.5)	**0.00005**	0.00001
Ta&T1 - T2&T4	1.52	4.61 (2.66−7.99)	**0.000**	**0.000**
Occupatinal exposure	0.033	1.03 (0.6−1.77)	0.903	0.903
Grade				
G1 - G2&G3	−1.29	0.27 (0.15−0.5)	**0.000025**	**0.00001**
Smoking status	−0.09	0.96 (0.48−1.69)	0.759	0.761
Recurrence	−1.97	0.13 (0.08−0.24)	**0.000**	**0.000**
Progression	1.64	5.17 (2.97−9.00)	**0.000**	**0.000**
Mutation	0.23	1.26 (0.71−2.23)	0.422	0.428
Time to progression				
Gender	−0.65	0.52 (0.18−1.49)	0.225	0.189
Age at diagnosis	0.026	1.03 (0.99−1.06)	0.138	0.132
Stage				
Ta - another	3.65	38.36 (5.23−281.28)	**0.000334**	**0.000**
Ta&T1 - T2&T4	3.14	23.24 (9.85−54.84)	**0.000**	**0.000**
Occupatinal exposure	−0.08	0.92 (0.46−1.84)	0.815	0.816
Grade				
G1 - G2&G3	−3.76	0.02 (0.003−0.17)	**0.000200**	**0.000**
Smoking status	−0.28	0.75 (0.25−1.63)	0.47	0.48
Recurrence	−2.48	0.08 (0.04−0.17)	**0.000**	**0.000**
Mutation	−0.23	0.79 (0.35−1.76)	0.572	0.565
Time to recurrence				
Gender	−0.14	0.86 (0.54−1.37)	0.535	0.529
Age at diagnosis	−0.009	0.99 (0.97−1.0007)	0.253	0.255
Stage				
Ta - another	−0.32	0.73 (0.5−1.05)	0.091	0.089
Ta&T1 - T2&T4	−1.21	0.298 (0.16−0.55)	**0.000**	**0.000**
Occupatinal exposure	0.05	1.05 (0.72−1.52)	0.786	0.786
Grade				
G1 - G2&G3	0.32	1.38 (0.95−2.01)	0.090	0.088
Smoking status	0.015	1.015 (0.65−1.57)	0.946	0.946
Progression	−0.68	0.5 (0.29−0.87)	**0.013**	**0.007**
Mutation	−0.23	0.79 (0.53−1.19)	**0.05071**	**0.0506**

Bold face representing P values <0.05.

these differences were significant: 57.5% ddPCR, 37.5% pyrosequencing, and only 20% if Sanger sequencing technique applied. The fact worth emphasizing is that Sanger sequencing was practical and effectual only in the samples with more than 55% neoplastic cells in content. Such a result is difficult to obtain in liquid biopsy of samples from urine or plasma. In our research all the tumor tissue samples which were subject to sequencing contained more than 55% tumor cells, and in 19 cases (13 pTa-T1 and 6pT2; 12G1 and 7 G2-3), despite being detected in urine, mutation was not confirmed in tumor.

Table 4
Multivariate Cox regression analysis of potential biomarker predictor variables and time to recurrence in all patients ($n = 138$).

Variables in model	HR (95%CI)	p
Stage Ta&T1-T2&T4	4.47 (1.92−10.39)	**0.00049**
Progression	1.38 (0.66−2.87)	0.39
Mutation	1.39 (0.92−2.11)	**0.0498**

Bold face representing P values <0.05.

5. Conclusions

The standardization of taking samples and the use of sensitive techniques like ddPCR which are resistant to big amounts of wild-type DNA in samples, can result in having repetitive and reliable analyses, on which treating patients and individualizing therapies can be based. The prospective validation of utility ddPCR for assessment of wider spectrum of *FGFR3* mutation is needed. Applying the ddPCR technique to do markers requires answering the question whether samples such as urine or plasma ones contain a sufficient amount of tumor DNA for detection, i.e. DNA in

exfoliated cells (in urine sediment) or cell-free DNA in urine supernatant/plasma. Finding mutation becomes an essential factor for the choice of a therapy because a few studies investigated the efficacy pazopanib (as *FGFR3* inhibitor) [19,20]. To our knowledge this is the first study showing discrepancies between these 2 methods for detection of mutations in DNA isolated from urinary sediment and frozen tumor tissue.

Conflict of interests

To my knowledge, the authors of that paper have no commercial financial incentive associated with publishing the article. In addition, all the sources of extra-institutional funding have been clearly indicated.

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