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RESEARCH ARTICLE

# Targeted Copy Number Variations Profiling of Non Muscle Invasive Bladder Urothelial Carcinoma Using BCA-1 Test on Urines Predicts High Grade Tumors

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ABSTRACT

**Introduction:** Urothelial carcinomas are the fourth leading cause of cancer in humans. Their incidence has increased by more than 50% in 25 years. Non Muscle Invasive Bladder Urothelial Carcinoma (NMI-TCC) represent 70% of newly diagnosed cases. Bladder NMI-TCC with high grades or pT1 stages require a close active surveillance regarding their significant risk of recurrence and progression to an invasive stage. From results obtained using DNA markers, It has been recognised that FGFR3 mutations were most associated to low grade and pTa NMI-TCC, while numerous CNV were associated to genetically instable tumors with high grade or pT1/pTis stage. The BCA-1 test provides a targeted copy number variations (CNV) profiling. So, we evaluated the predictive value of the FGFR3 mutations and BCA-1 test on urines in order to assess the tumoral grade and the stage of NMI-TCC.

**Method:** Urinary DNA was extracted from 50 patients with bladder NMI-TCC, with negative or non- informative urine cytology. The BCA1 test and identification of FGFR3 mutations (S249C; Y375C; G372C) was performed on urine samples. The molecular results were correlated with the tumoral pathological grade and stage.

Results: Each CNV locus was correlated to grade and stage. Gains at 8q, 11q and deletions at 9p 9q loci were the most informative CNV for high grade and pT1 prediction. A Bayesian model from molecular markers using urines CNV profiling predicts high grade and pT1 stage, with AUC respectively at 92% and 93%).

**Conclusion:** Our results showed that targeted CNV profiling using BCA-1 test on urines could be used as biomarkers during clinical management of bladder NMI-TCC, as a reliable predictor of tumors with high risk of recurrence and progression.

## Introduction

Nearly 180,000 new cases of bladder cancer are diagnosed each year in the European Union, with a ratio male/female of 2/3.The main risk factor is due to cigarettes smoking habits. At the time of diagnosis, around 70% of bladder tumors are Non-Muscle Invasive Transitional Cell Carcinoma (NMI- TCC) [1].

The stages and grades distribution of the bladder NMI-TCC newly diagnosed are respectively about 60% as pTa, 40% as pT1 and <5% pTis. Half of the distribution of bladder NMI-TCC is tumor with high grade. Urinary cytology usually performed in clinical practice has a low sensitivity for low- grade tumors and require experimented pathologist to perform this examination [2]. It is currently recognized than cytology has a sensitivity of 69–92% for high-grade tumors but this does not make it a good monitoring test [2]. One common feature to many tumors is the presence of chromosomal anomalies which can be detected by studying DNA markers [3]. This particular characteristic of the tumoral genome was first used in bladder cancers to detect tumoral cells by studying DNA extracted from patient urine with the help of microsatellite markers [4,5]. Several teams, including us, have since reported results where this technique has been used to diagnose or monitor such tumors [6,7]. We and other have, also, previously shown than molecular urine test using a panel of genomic markers identified with a high sensitivity and specificity (over 85%), recurrence of bladder NMI-TCC [4,8]. Since then, BCA1-test based on a targeted CNV panel (60,000 probes with 50-60 base pairs) performed on urine shown no false positives in a population of patients with normal cystoscopy, normal cytology and no history of bladder cancer, and showed a sensitivity of 95% for detecting positive cystoscopies in patients monitored for bladder cancer [9].

We have now extended our study to the prospective analysis of patients with superficial bladder cancer in order to identify the best CNV markers able to predict tumors grade and/or stage.

## Methods

A nested cohort of 50 patients with a bladder tumor was included in this study. The criteria for inclusion were firstly an indication of endoscopic bladder resection in the context of bladder NMI- TCC (<3cm) monitoring or recurrence; secondly, a negative or non-informative cytology; thirdly, no sign of **Upper Urinary Tract Tumors** (UUT-TCC) on CT-scan. All the patients gave their informed consent. The clinical data collected included age, sex, age on diagnosis, upper urinary tract tumor history, smoker status (active, former or non-smoker). The following anatomo-pathological data about the bladder tumor were also collected: the stage and grade, and the mutational status of the FGFR3 gene for S249C; Y375C; G372C mutations [10].

**Table 1**: Characteristics of the 50 patients included.

| | **Number (%)** |
|---|---|
| Men | 44 (88%) |
| Women | 6 (12%) |
| Median age on diagnosis (range) | 67 (35 – 90) |
| Non-smoker | 12 (24%) |
| Former-smoker | 29 (58%) |
| Active Smoker | 9 (18%) |
| UUT-TCC history | 5 (10%) |
| pTa | 36 (72%) |
| pT1 | 14 (28%) |
| High-grade | 22 (44%) |
| FGFR3 Mutation (S249C, Y375C or G372C) | 11 (22%) |

Bladder NMI-TCC (<3cm), negative or non informative cytology, no sign UUT-TCC on CT- scan

## Collection of Urine Samples

Patients include in the cohort, have sign a consent according to their participation to the protocols Protocole 2011/24 NICB : (IRB 00003835 Institutional Review Board) Comité de Protection des Personnes Ile de France IV - Saint Louis, 1, Avenue Claude Vellefaux 75010 PARIS). Urine samples (50 ml) were collected before each intervention. After centrifuging the urine, the QIAmp DNA Blood Mini kit (Qiagen) was used to extract the urinary DNA from the pellet obtained, complying with the supplier's recommendations. After validation of the quantity and quality of the extracted DNA, it was marked then hybridized on the BCA-Oligo array following the protocol described in Larré et al. [8].

Briefly, the BioPrime kit (Life Technologies Invitrogen) was used in accordance with the supplier's recommendations to mark the tested DNA (with Cyanine 5) and the reference DNA (with Cyanine 3) [11,12]. After double purification, the first with the Nucleospin Extract II kit (Macherey-Nagel) and the second with the Purelink® kit (Life Technologies), the two DNAs were mixed in equal quantity. The mixture was then incubated for 3 minutes at

97° C, then 30 minutes at 37° C in the presence of Cot-1 repeated DNA (1 mg/ml), the Agilent 2X Hybridization Buffer (Agilent Technologies) and the Agilent 10X Blocking Agent (Agilent Technologies). The mixture was then placed on the BCA-Oligo array for hybridization for 16 hours at 65° C.

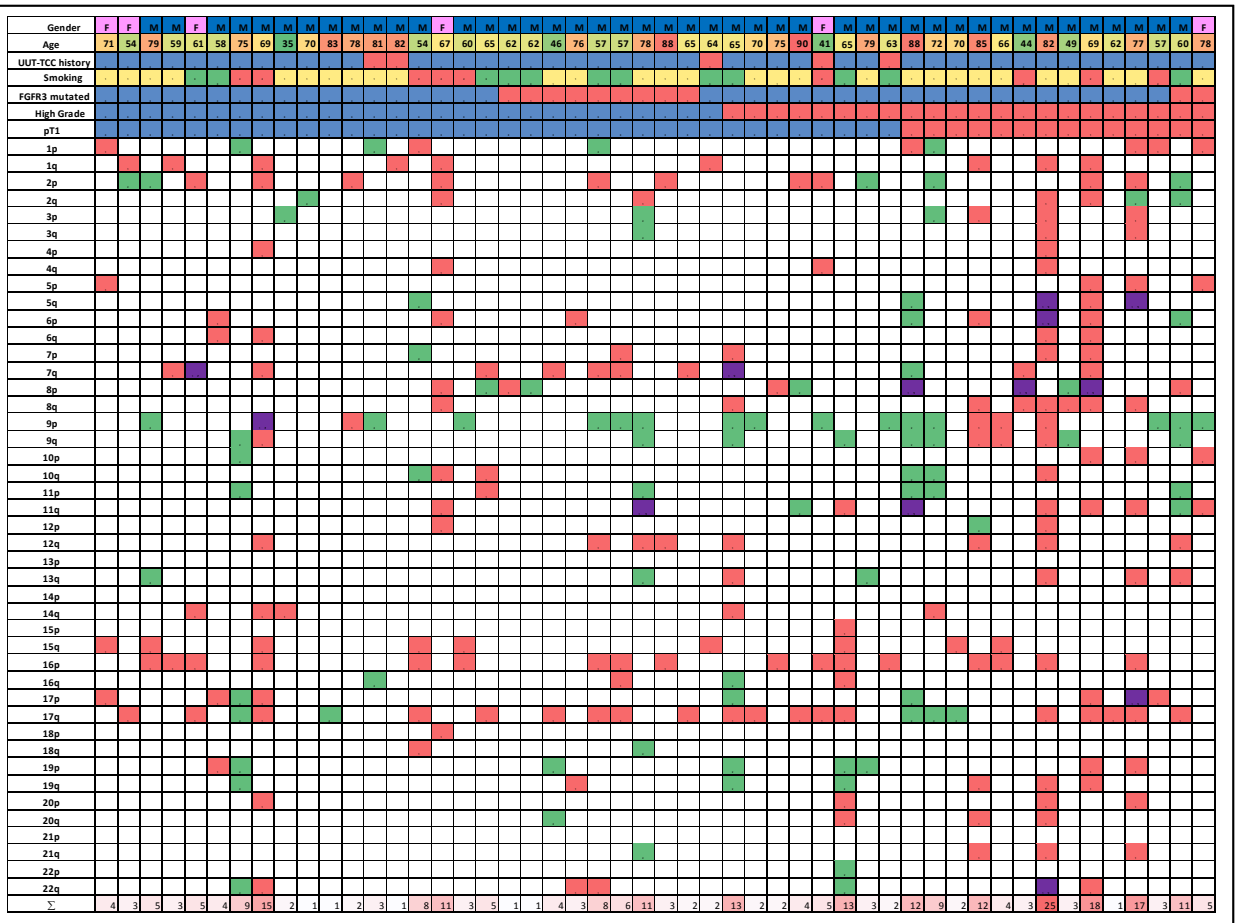


**Figure 1**: Individual results.
(Clinical data: Blue=No; Red= Yes; Smoking: green=No, yellow= former, red= active; CNV data: green= Deletion; Red=Gain; purple=Gain & Deletion on the same chromosomal arm)

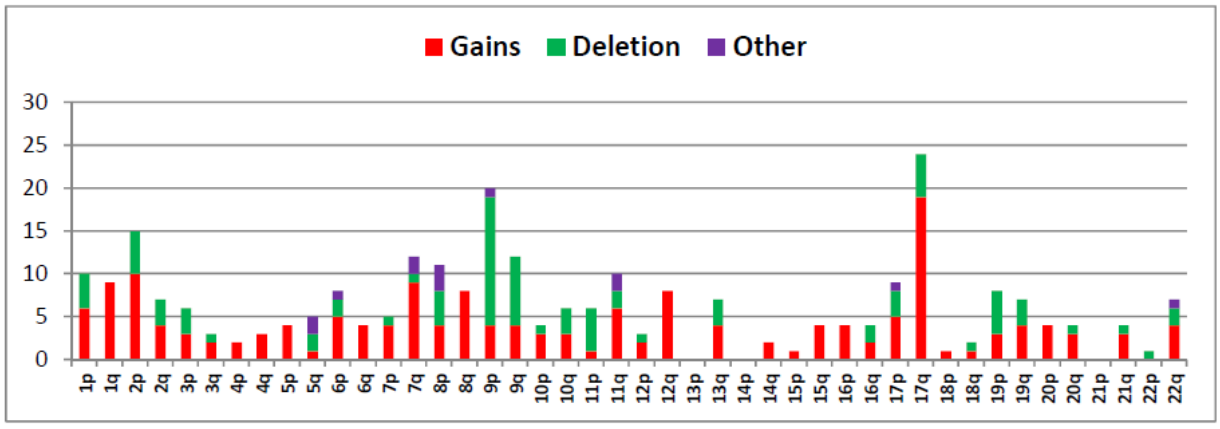


**Figure 2**: Distribution of Deletion (green), Gains (red) and association Gain+Deletion (Other: purple) by chromosomal arm.

After washing with the Wash Buffer washing solution (Agilent Technologies), the array was scanned using the Agilent High Resolution Scanner (Agilent Technologies). WorkBench software (Agilent Technologies) was used to analyze the results.

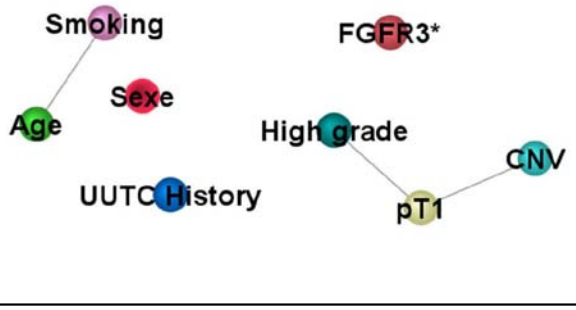


**Figure 3:** Significant correlations between clinical and molecular parameters.
(CNV: Copy Number Variations, UUT-TCC -history: Upper Urinary Tract History)

To interpret the outcome of the BCA-Oligo test, the median Log2 normalized fluorescence value was calculated for each oligonucleotide. An oligonucleotide was considered deleted or amplified if its normalized average Log2 ratio was lower or higher than -0.2 or 0.2 respectively. A chromosomal region was considered amplified or deleted if at least 6 successive oligonucleotides of the region were interpreted as gained or lost. If at least one chromosomal region was amplified or deleted, the test was considered positive for the study.

## Statistics

Descriptive statistics were performed using (XLS-STAT® 2017.4). Correlations, relative importance and predictive models were performed using naïve and supervised Baeysian networks analysis (BayesiaLab BayesiaLab® 6.0.8, Laval, France). Values of $p < 0.05$ were considered statistically significant. [13]

## Discussion

The management of bladder with a high grade or pT1/pTis stages require specific follow-up regarding the high risk (>50%) of recurrence or disease progression to muscle invasive stages [12,14]. Our study, using BCA-1 test on urine points out chromosomal locus, such as 8q or 11q or 9q with specific CNV related to high grade or pT1 bladder NMI-TCC. So, Zaharieva et al. [15] previously showed that the frequency of gains of the cMYC gene situated in 8q24 increases significantly with the grade and stage (frequency of 6% for grade G1, 12% for G2 and 20% for G3; 11% for pTa to 16% for pT1). In a study of 28 pTa and 28 pT1 tumors, Richter et al. [16] showed that the frequency of losses in 8p and gains in 3p, 8q and 10p was significantly higher in pT1 tumors. In another study of 64 Chinese patients with bladder tumors, the gains in 8q and 11q and the losses in 8p were more frequent in pT1 tumors than in pTa tumors [4].

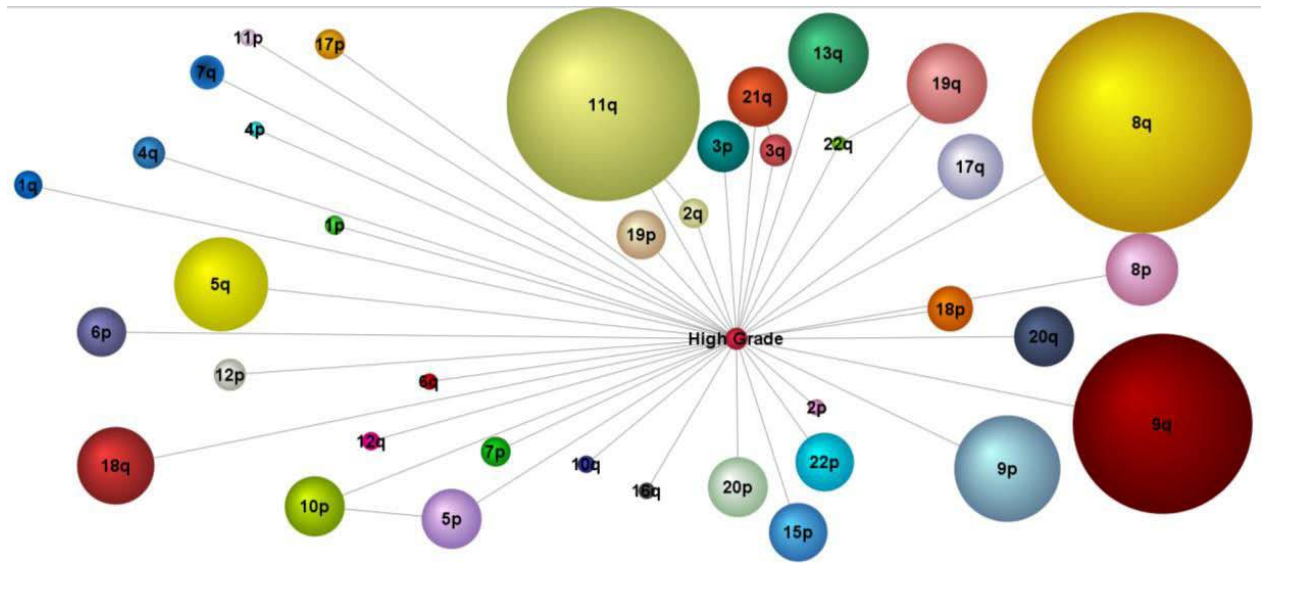


**Figure 4:** Bayesian network shows the cartography of relative importance of chromosomal arm alterations at each locus for the prediction of high grade bladder NMI-TCC. The size of each node (circle) is proportional to the influence of the chromosomal arm alteration on High grade prediction.

A study of 46 superficial tumors showed that the losses in 8p and amplifications in 8q, 5q and 10p were more frequent at the pT1 stage [17]. FGFR3 mutation were frequently (40%) associated to low grade disease, but not exclusively. The fact that FGFR3 mutation are inconstant in low grade, enhance the fact negative FGFR3 mutation is not a reliable markers if used alone [10]. These results suggest that the use of CNV to predict the risk of aggressiveness or evolution of bladder NMI-TCC from DNA extracted from urine samples could be easily done. However, our predictive model needs to be confirmed on an independent larger cohort of patients.

## Conclusion

As a complement to cytology, the identification of markers of aggressiveness in a patient can help the clinician to more easily take a decision on adjuvant therapy after resection of the tumor or to devise a personalized therapeutic strategy. Establishing distinct genetic profiles of patients with bladder NMI-TCC could help to improve care in terms of both diagnosis and prognosis assessment.

## Personal interests

Karim Sighar is an employee of Array Genomics, which helped to develop and market the BCA-oligo test (BCA-1). The other authors state that they have no personal interests.

## Results

The characteristics of the population studied were presented in the Table 1. Individual results were presented in (Figure 1). The distribution and the typology (Gains, Deletions, Others) of each targeted locus for CNV profile were shown on (Figure 2). The cartography of the relationship between clinical, pathological and molecular factors is represented on (Figure 3). Significant correlations are link between age and smoking habits (Pearson R: -0.7; p value<0.001) , high grade and stage pT1 (Pearson R: 0.7 ; p value<0.001) ; stage pT1 and CNV (Pearson R: 0.5 ; p value: 0.002). The informativeness each target locus for high grade was shown on Figure 4. The more informative (relative importance >0.3) markers were gains at 8q ,11q and deletions at 9q. From this cartography the predictive value of the Bayesian model give an AUC respectively at 92% and 93% for high grade and pT1.

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