Research Paper

Validation of Urine-based Gene Classifiers for Detecting Bladder Cancer in a Chinese Study

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Abstract

Background: Current standard methods used to detect and monitor bladder cancer (BC) are invasive or have low sensitivity. We have previously reported in an international European study four non-invasive tests for BC diagnosis based on the gene expression patterns of urine.

Objective: to validate the tests in an independent Asian cohort.

Design, setting and participants: Prospective blinded study in which consecutive voided urine samples from BC patients and controls (n=520) were collected in the Fudan University Shanghai Cancer Center from 2014-2016. Gene expression values were quantified using TaqMan Arrays. The same cut-off as previously reported for discrimination between tumours and controls was used in this validation study.

Results and limitations: Finally, a total of 257 tumour and 132 control urine samples were analysed. We found a high accuracy for the four gene classifiers in this independent Asian set, the classifiers composed of 5 and 10 genes achieved the best sensitivity (80.54% and 81.32%, respectively) maintaining a high specificity (91.67% and 85.61%, respectively). Sensitivity of 5-gene (GS_D5) and 10-gene (GS_D10) expression classifiers in recurrent BC cases (78 and 79%, respectively) is comparable to that of primary BC cases (82%). Cytology and NMP22 identified 67% and 40%, respectively, of tumours that have been diagnosed with our tests. In addition, influence of each studied gene was analyzed and showed similar gene rank between Chinese and Caucasian population.

Conclusions: Our study proves that our non-invasive diagnostic BC tests can be reproduced in independent cohorts and in an external laboratory. All the four gene classifiers have shown equal or superior performance to the current gold standard in the present and previously reported validation studies. Consequently, they may be taken for consideration as molecular tests applicable to clinical practice in the management of BC.

Patient summary: Our gene classifiers achieve sensitivities up to 90% in HR NMIBC and MIBC patients, while this achievement is comparatively lower in LR NMIBC ones.

Key words: Bladder cancer; Biomarkers; Gene expression; Gene classifiers; Non-invasive; Chinese

Introduction

As in other countries, urinary bladder cancer (BC) remains the second most frequent cause of mortality among genitourinary cancers in China, including approximately $4.8/10^5$ incidence and $2.2/10^5$ mortality rate in male in 2012 [1, 2]. Although this two rates are not as high as those in western

countries [3], the incidence and mortality rates of BC in China have increased gradually in the past few years [2].

The striking majority of malignant bladder tumours are urothelial cell carcinomas (UCC). Depending on the degree of tumour infiltration in the bladder wall, BC is classified as non-muscle invasive BC (NMIBC) accounting for 75% of tumours while the remainder are muscle invasive BC (MIBC) [4]. Although not typically life-threatening if detected early, NMIBC has up to 70% recurrence rate during the first two years after diagnosis, depending on the patient risk profile [5, 6]. This recurrence phenomenon means that NMIBC patients may undergo up to 15 invasive procedures during the first 5 years of follow up, depending on the patients risk profile [7].

Current approaches for detecting both primary and recurrent disease rely on invasive cystoscopy aided by voided urine cytology. Cytology is a noninvasive technique and has high specificity (90% to 96%) [8], but lacks sensitivity especially in low risk tumours (11% to 76%) [9]. Additionally, the inter-observer and intra-observer reproducibility of cytology is poor [10].

Invasive cystoscopy is associated with significant discomfort, possible infection and trauma. Moreover, cystoscopy misses up to 15% of the papillary and up to 30% of the flat lesions [11, 12]. In an effort to reduce the frequency of cystoscopies conducted, several noninvasive biomarkers such as nuclear matrix protein 22 (NMP22) test, have been approved by the Food and Drug Administration (FDA), albeit with performance rates remaining insufficient to replace or to guide current diagnostic methods [13]. Since the genetic nature of bladder tumours is heterogeneous, one possible reason of the lack performance of the assays is that they focus on a single or a limited number of biomarkers [14, 15]. In the last decade, several cancer-associated gene classifiers, obtained from voided urine, have been described with high diagnostic performances by different groups [14, 16-19]. Although the promising results presented, these multiple gene classifiers require large-scale prospective validations to prove its repeated efficiency and widespread application.

In this study, we have tested four gene expression classifiers previously developed and validated in Caucasian population [20], in an independent Asiatic cohort, to confirm its widespread clinical application. In addition, we have analyzed whether there is a difference in the influence of the genes included in the study to diagnostic performances between Chinese and Caucasian population.

Materials and Methods

Clinical sampling and processing

A total of 520 consecutive urine samples from patients with BC (336) and controls (184) were consecutively collected between September 2014 and March 2016 in the Fudan University Shanghai Cancer Center (FUSCC) and Shanghai 8th people hospital after obtaining Institutional Review Board approval and patients' informed consents. Of the 520 urine samples collected, 96 samples (49 from the cancer and 47 from the control group) were excluded from study because they did not fulfill RNA quality criteria (a Cq value of *GUSB*>23; see materials and methods). Twenty-seven samples were excluded for incomplete and incorrect clinical information and eight samples were excluded for repeated testing (Figure 1). Thus, 389 urine specimens were finally analyzed, including 257 samples from patients treated with transurethral resection of the bladder (TURB) for primary or recurrent BC who had histologically confirmed tumours and 132 from controls with non-neoplastic urological disease (Table 1). Grade and stage of the tumours were determined according to WHO criteria [21] and the TNM classification [22], respectively. Tumours were classified according to their risk in 3 categories, including low risk NMIBC: Ta and T1 LG without associated CIS, high risk NMIBC: Ta or T1 LG with associated CIS, Ta or T1 HG, or Tis and MIBC: T2, T3 or T4 LG and HG with or without associated CIS. Voided urine samples (20 to 100 ml) were collected in sterile containers containing 4 ml 0.5 M EDTA (pH 8.0). Urine samples were immediately stored at 4°C and processed within the next 24 hours. Samples were centrifuged at 1,000×g for 10 minutes at 4°C. Cell pellets were suspended in 1 ml TRIzol reagent and frozen at −80C until RNA extraction.

Gene Expression Quantification

RNA extraction, complementary DNA synthesis and gene expression quantification were performed in the FUSCC – Institut Merieux Laboratory as previously described [16, 17]. All the 16 target genes and two endogenous controls (*GUSB* and *PPIA*) analyzed in our previously reported studies were also analyzed in the present study [16]. Before gene expression quantification of the 16 target genes, an aliquot of 1 µl of preamplified cDNA was applied to verify the actual amount of endogenous control *GUSB* by quantitative PCR (qPCR) and standard reaction and amplification conditions. Those samples that provide *GUSB* cycle quantification (Cq) values lower than 18, were diluted with water to ensure a homogeneous amount of cDNA in all the samples and the correct quantification of mRNAs. Whereas those

samples with a Cq value higher that 23 were excluded from the study. Real-time quantitative PCR data was processed with SDS 2.4. Previous defined gene thresholds [16] were used for all genes to record Cq values.

Data Analysis

Relative expression values (DCq) for the genes contained in the four evaluated predictive models (GS_D2, GS_D5, GS_D10 and GS_D12; Supplementary Table 1S) [13] were used to calculate the risk for the sample of presenting BC. Raw data obtained from the qPCR platform was sent to Hospital Clinic (Barcelona, Spain) to be analized using a previously defined algorithm for each model which classify samples as tumours or controls All the researchers from the Hospital Clinic involved in this analysis of samples were blinded to the patients' clinical data, ensuring the reliability of the results. R-software was used for all calculations. Receiver Operating Characteristic (ROC) curves were generated using the Diagnosis Med (http://CRAN.R-project.org/packageZDiagnosisMed) and pROC package [23]. Gene influence analysis was performed using R package globaltest [17].

Results

Validation of Four Gene Expression classifiers

A total of 389 urine samples were finally analyzed (Figure 1; Table 1). The performances of four diagnostic classifiers in the Chinese set are listed in Figure 2A. All the four gene classifiers achieved high diagnostic accuracy (80.46%-84.32%; AUC=0.889-0.917). GS_D5 achieved the best diagnostic accuracy (84.32%; AUC=0.911), with 80.54% SN and 91.67% SP. GS_D10 has the best SN (81.32%), while GS_D12 has the best SP (92.42%). Similarly to that of European studies, SN increased through the BC risk groups. It was lower in low risk NMIBC (54%-58%), while SN are up to ~90% for high risk bladder cancer patients except for GS_D2 (85%) (Figure 2B). Therefore, the study in the Chinese cohort has confirmed the performance results previously obtained in European Studies.

Sensitivity in primary and recurrent BC cases

Among the 257 cancer patients, 67 of them are recurrent and 190 are primary tumours. GS_D10 showed the best sensitivity, both in primary and recurrent cases (Figure 3A). Interestingly, the sensitivity of GS_D10 in recurrent cases (79%) is comparable to that of primary tumours (82%). Furthermore, sensitivities of GS_D10, which showed best sensitivity among the classifiers, in LR NMIBC, HR NMIBC and MIBC patients for primary and

recurrent cases are shown in Figure 3B. There are no significant differences between primary and recurrent cases in all the comparisons (LR NMIBC: P=0.4233; HR NMIBC: P=0.3459; MIBC: P=0.2916).

Table 1. Clinical and histopathological variables for the patients and controls included in the study.

Variable	Tumor (N=257)	Control (N=132)
Sex (%)		
Male	211 (82.1)	105 (79.5)
Female	46 (17.9)	27 (20.5)
Age (yr)		
Mean	62.1	63.6
Range	24-89	29-90
Grade		
NMIBC LR	72	
NMIBC HR	146	
MIBC	39	
Urological condition		
Normal		10
BPH		73
Urinary tract infection		11
Calculus		27
Others		11

Table 2. Sensitivity comparison of 4 gene classifiers and cytology

Grade	Overall	LR	HR	MIBC
N	154/257	42	89	23
cytology	55%	19%	67%	74%
GS_D2	78%	57%	87%	83%
GS_D5	81%	60%	90%	87%
GS_D10	82%	60%	91%	87%
GS_D12	79%	57%	88%	83%

Cytology and NMP22 results BC samples

Cytology results were available for 154 (60%) of the 257 BC patients included in the study. We compared sensitivity of four gene classifiers to those of cytology (Table 2). In this subset of patients, overall sensitivity of cytology was 55%, much lower than that of the four gene classifiers (78%-82%). SN of the cytology in LR NMIBC, HR NMIBC and MIBC patients was 19%, 67% and 74% respectively, lower of those sensitivities of four gene models (Table 2). Further comparison analysis of 5-gene classifier (GS_D5) and cytology results showed that all positive cytologies were confirmed by the GS_D5 except in two cases (2%). On the contrary, of all the patients diagnosed GS_D5, cytology only detected BC in 66% of them (Figure 4A).

NMP22 test have been done for 109 (42%) of the 257 BC patients included in our study. Overall, sensitivity of NMP22 test in this subset of patients was 38% while SN of the gene classifiers ranged from 74% to 88%. SN of the NMP22 in LR NMIBC, HR NMIBC and MIBC patients was 21%, 45% and 42% respectively, more than half lower of those sensitivities of four gene models (Table 3). Furthermore, GS_D5 detects BC in 34 (83%) of all 41

positive NMP22 cases. On the contrary, of all the patients diagnosed by GS_D5, NMP22 tests only detect BC in 40% of them (Figure 4B).

Table 3. Sensitivity comparison of 4 gene classifiers and NMP22 tests

	Overall	LR	HR	MIBC
N	109/257	29	60	19
NMP22	38%	21%	45%	40%
GS_D2	76%	48%	88%	80%
GS_D5	78%	55%	86%	87%
GS_D10	74%	45%	86%	80%
GS_D12	76%	52%	85%	87%

Influence of each studied gene in Chinese population

IGF2, SLC1A6 and *CRH* are the most influential genes in both Caucasian and Chinese populations. All the high influent genes included in the top 8 genes are almost the same; except for *MAGEA3*, that has more influence in the European cohort, while *ANXA10* ranks higher in the Chinese validation set (Figure 4D). Those data were consistent with the good performances presented by the four gene classifiers on the Chinese validation cohort.
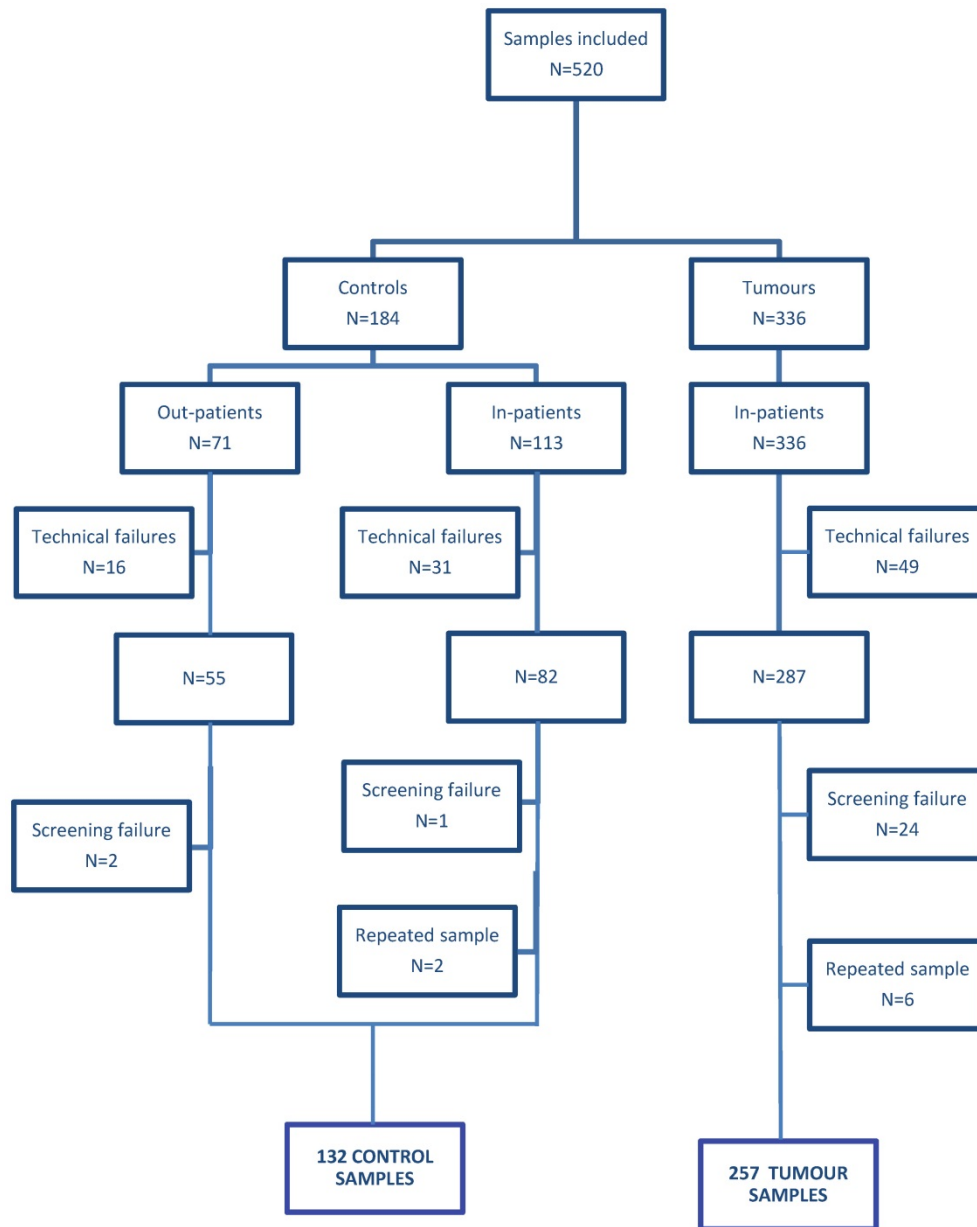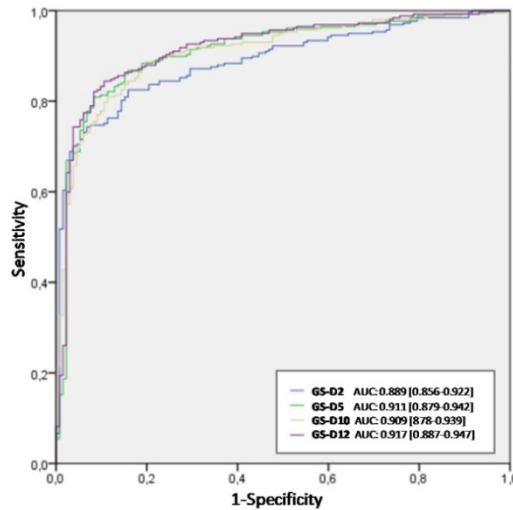

Figure 1: Flow diagram of participants satisfying the criteria for inclusion. Technical failure: samples that yielded insufficient RNA and samples that did not meet the *GUSB* RNA quality control; see material and methods. In-patients: patients samples collected in FUSCC; Out-patients: samples collected in Shanghai 8th people hospital.

A

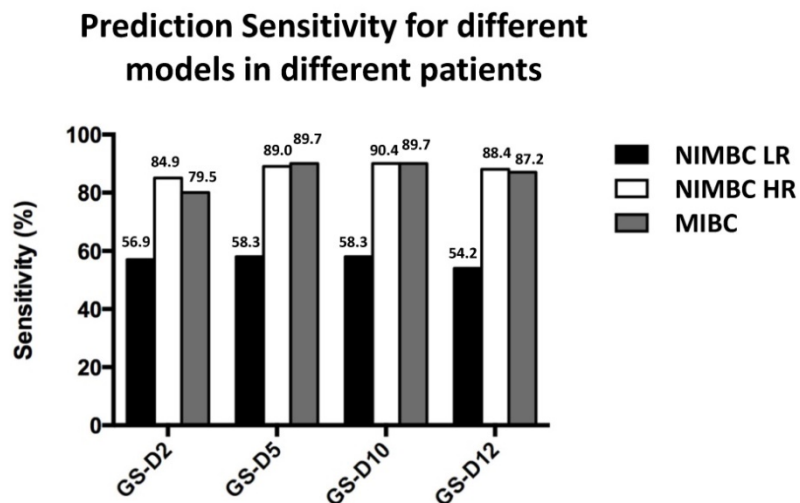| Models	Accuracy	Sensitivity	Specificity	PPV	NPV
GS_D2	80.46%	76.26%	88.64%	92.89%	65.73%
GS_D5	84.32%	80.54%	91.67%	94.95%	70.76%
GS_D10	82.78%	81.32%	85.61%	91.67%	70.19%
GS_D12	83.29%	78.60%	92.42%	95.28%	68.93%

B

Figure 2: Diagnostic performance of 4 gene expression classifiers in the Chinese validation cohort. A) ROC curves and overall diagnostic performances of the 4 diagnostic gene expression classifiers in the Chinese cohort. B) SN of 4 gene expression classifiers in BC risk groups. Abbreviation: AUC, area under curve. PPV, positive predictive value. NPV, negative predictive value

Discussion

Currently, cystoscopy is considered the gold standard method to diagnose and monitor BC, but misses up to 15% of the papillary and up to 30% of the flat recurrences [11, 12]. Furthermore, cystoscopy is expensive, invasive and bothersome to patients. Urine cytology, on the other hand, has a high specificity (SP=96%), but lacks sensitivity (SN=44%) especially in low risk tumours [24]. Additionally, the interobserver and intraobserver reproducibility of cytology is poor

[10]. The combination of both techniques achieves a high SN and SP in the diagnosis and monitoring of the disease (SN: 71%, SP: 96%) [25]. Nevertheless, the invasiveness of cystoscopy has led to the search for biomarkers in urine. This is especially important in the surveillance of BC patients. The high recurrence rate of BC leads to a life-time surveillance with frequent invasive procedures which are associated to significant pain, anxiety and financial cost to the BC patients. Our current and previous studies demonstrated that the non-invasive urine biomarkers
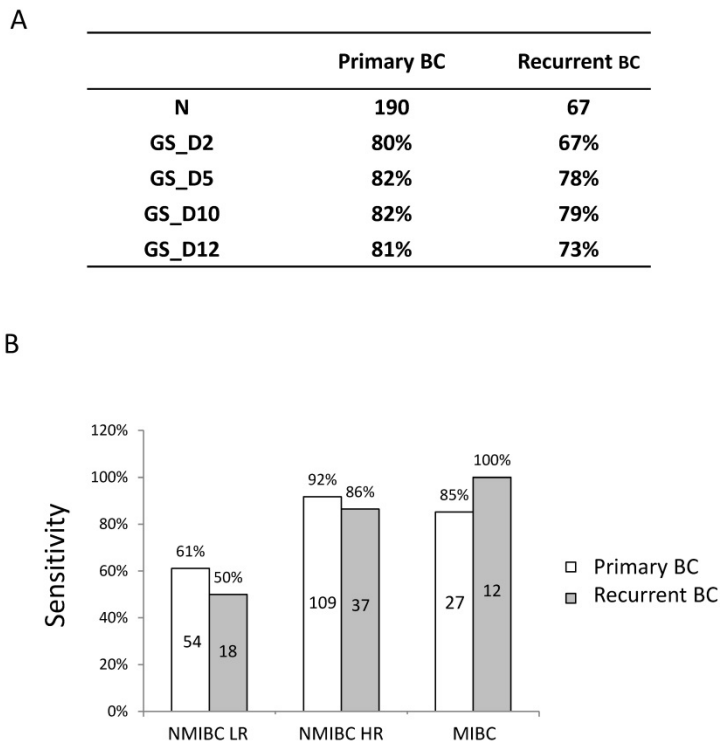
A

	Primary BC	Recurrent BC
N	**190**	**67**
GS_D2	**80%**	**67%**
GS_D5	**82%**	**78%**
GS_D10	**82%**	**79%**
GS_D12	**81%**	**73%**

B

Figure 3: Diagnostic performance of 4 the gene expression classifiers in primary and recurrent BC. A) SN of 4 gene expression classifiers in primary and recurrent BC. B) SN of the 10-gene expression classifier (GS_D10) between primary and recurrent cases in BC risk groups. Numbers in the boxes indicate the patient numbers for each group.

tests achieved accuracy values (SN up to 80% and SP up to 90%) for BC diagnosis in the range of that achieved for the gold standard. Furthermore, the good diagnostic performances were observed not only in BC patients with primary tumours but also on recurrent cases. Therefore, urine biomarkers tests could be a potential tool that aid to reduce the frequency of cystoscopies in selected patients.

A number of molecular tests have been developed to achieve this goal. Some of these tests include NMP-22 [25-28] bladder tumour antigen (BTA) [29], Survivin [30], RNA [19, 31] or MicroRNA profiling [32] and fluorescence in situ hybridization analysis for chromosomal abnormalities [9]. However, most of the biomarkers reported above have limited sensitivity, thus by themselves, have not proven to be accurate enough to replace cystoscopy or even cytology. In a meta-analysis of 57 studies none of them achieved a SN >69%, except 78% SN for ImmunoCyt (Scimedx, SP: 78%), with an overall moderate SP ranged of 74%~88% [9]. Consistently, in our study, the diagnostic performance of NMP22 (BladderChek, Alere) test is not sufficient to skip one cystoscopy resulting in a safe approach. In contrast to the moderate performance of one singe marker [33, 34], multiplex biomarkers panel usually show better performances [12-16, 18, 20, 31, 35, 36]. Urquidi et al reported a 14-gene panel in 2012 with 90% SE and

100% SP for BC diagnosis, although more high grade patients included in the cohort [19]. Recently, a DNA methylation signature (A 150 CpG loci biomarker panel) identified by high-throughput DNA sequencing showed a perfect performance (98% SE and 97% SP) on primary BC detection [37]. However, for routine clinical diagnostic use of those promising panels, they need to be tested further and prospective validated in heterogeneous patient populations. So far, our method is the first test validated in both Caucasian and Chinese populations.

Similar to previous results in European studies, the four gene classifiers showed comparatively lower SN (54 to 58%) in LR NMIBC patients than those of HR ones (80 to 90%). Actually, 30 of the 46 misclassified tumour samples by GS_D5, one of the best performer in all series, are LR NMIBC. However, the SN of four gene classifiers in LR NMIBC is still 40% higher than that of cytology and 30% higher than that of NMP22 tests. On the other hand, considering high recurrence rate and life threaten of HR NMIBC, the performance of the tests in this subgroup of tumours is of great importance. Our gene classifiers achieve sensitivities up to 90% in HR NMIBC and MIBC patients, that is 10-20% higher than those of cytology and 40-50% higher than those of NMP22 test in the present cohort. One limitation of our study is the lack of cytology and NMP22 test in the control group that does not allow for a direct calculation of the SP of cytology and NMP22 test in our own series. But total SP of our gene classifiers are in the range as that of reported for cytology [7].

Another limitation of the current study is that the prevalence of tumours in this cohort is higher than the disease prevalence in urologic practice [7], so evaluation of the validation study cohort is likely to provide an overly optimistic assessment of the positive predictive value (PPV) and an overly pessimistic assessment of the negative predictive value (NPV). For an estimated real prevalence of 10% in urologic practice, NPV would be above 90% for all gene classifiers.

Although these gene classifiers have been already validated in patients from multiple centers, this was the first time they were tested outside Europe. Thus, we wondered if there is any heterogeneity of BC gene expression between the two populations that could affect the performance of the gene classifiers. Therefore, we analyzed the influence of each gene included in the four classifiers (Figure 4C). Surprisingly, ranks of the gene influences on the
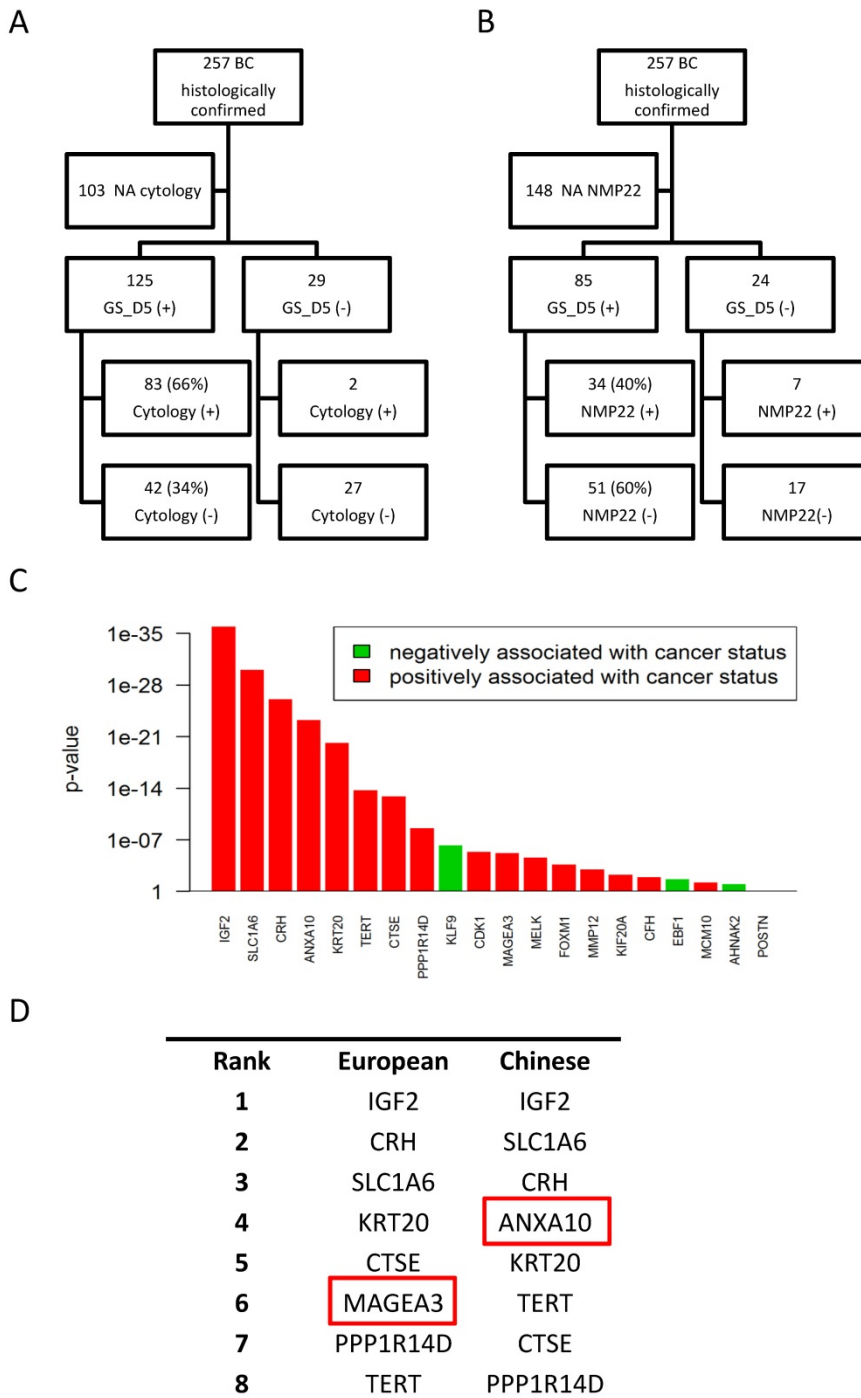
comparing to the GS_D5, GS_D10 and GS_D12 models, GS_D2 showed comparatively lower SN (75.49%) in the Chinese cohort. This minor discrepancy could be affected by the different enrolment of the two cohorts, such as BC grades, primary or recurrent cases distribution, or it could be explained by the heterogeneity of BC gene expression between the two populations. Further studies of the classifiers on Asiatic cohorts may be provide more evidence about it.

Taken together, this blinded and independent study proves that our non-invasive diagnostic BC tests can be reproduced in the Chinese cohort and in an external laboratory. All the four gene expression classifiers have shown equal or superior performance to the current gold standard in the present and previously reported validation studies. Consequently, they may be taken for consideration as a molecular test applicable to clinical practice in the management of BC.

Supplementary Material

Acknowledgements

Figure 4: Performance comparison of the 4 gene expression classifiers with cytology and NMP22 results. Comparison of the 5-gene classifier (GS_D5) results with cytology (A) and NMP22 results (B). C) Influence on BC diagnosis of each studied gene in the Chinese validation set. D) Comparison of influence top-ranked genes between Caucasian and Chinese populations.

BC diagnosis were very similar between Caucasian and Chinese population, although there are minor variations on the priority of those genes to predict BC between the two populations. We—found that *MAGEA3,* one of the two genes included in GS_D2, seems to be more important in Caucasian population, while *ANXA10* shows higher influence in Chinese population. This could possibly explain why when

Competing Interests

Lourdes Mengual, Juan José Lozano, and Antonio Alcaraz are inventors of patents WO 2008/113870 and WO 2014/118334 applied by Fina Biotech S.L. All other authors declared no conflicts of interest.

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