Gene Signatures for the Prediction of Response to Bacillus Calmette-Guérin Immunotherapy in Primary pT1 Bladder Cancers

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Abstract

Purpose: Intravesical Bacillus Calmette-Guérin (BCG) immunotherapy is effective in the prevention of recurrence and progression in many cases of nonmuscle invasive bladder cancer, but many patients fail to respond. The aim of this study was to identify gene sets of markers that could predict the response to BCG immunotherapy in primary pT1 bladder cancer using microarray gene expression profiling.

Experimental Design: We used 80 patients with primary pT1 bladder cancer treated with BCG immunotherapy as training (48) and test (32) sets. Microarray gene expression profiling was done in the training set to identify genes differentially expressed between responder and nonresponder to BCG immunotherapy according to the events (recurrence or progression). Using a real-time reverse-transcriptase PCR, our findings were validated in the test set.

Results: In the training set, 424 and 287 genes were significantly associated with recurrence- and progression-free survival, respectively. Functional annotation of these genes included cell-mediated immune response, inflammatory response, cellular growth, and proliferation. From these predictive gene signatures, 24 genes (12 in recurrence and 12 in progression) with the highest score of expression ratio were extracted for validation in the test set. In multivariate regression analyses, predictive gene signatures were the only independent predictors of recurrence (hazard ratio, 3.38; P = 0.048) or progression (hazard ratio, 10.49; P = 0.048) in the test set.

Conclusions: Predictive gene signatures have diagnostic value for determining the response to intravesical BCG immunotherapy in primary pT1 bladder cancer.

The prevention of disease recurrence and progression of nonmuscle-invasive bladder cancer (NMIBC) is a major clinical challenge. Intravesical instillation of Bacillus Calmette-Guérin (BCG) combined with transurethral resection (TUR) of bladder cancer is recognized as the best treatment option for the prevention or delay of recurrence and progression in high-risk NMIBC (1–6). However, many patients fail to respond to BCG therapy and are at higher risk of disease recurrence and progression (1, 2, 4, 5, 7).

Although the precise mechanism of BCG action is still unclear, molecular studies have suggested a multistep process involving immunologic response (4, 8–11). The advent of high-throughput microarray technology makes it possible to gain comprehensive insights into the molecular basis of numerous human diseases (12, 13). With this technology, hundreds or even thousands of genes in a tumor can be evaluated simultaneously and individual molecular targets or gene classifier sets that correlate with particular bladder cancer outcomes can be identified (14–17).

The determination of when to discontinue BCG and implement a more aggressive therapy is the hardest decision faced by urologists that manage high-risk NMIBC patients. The capability to predict the response to treatment before BCG instillation would be an invaluable tool in the selection of appropriate therapeutic modalities. The aim of the present study is to identify predictive gene signatures based on the microarray data analyses of tumors from patients with newly diagnosed pT1 bladder cancer with their response to BCG immunotherapy.
Subjects and sample collection. We used our previously published microarray data of bladder cancer specimens from the 165 primary bladder cancer patients histologically diagnosed with transitional cell carcinomas between 1995 and 2007 at our institute (18). Among these, 48 pT1 patients who received intravesical BCG immunotherapy were selected as an original cohort (training set). For the validation of the predictive models of microarray analyses data, consecutive 32 primary pT1 bladder cancer patients who had received intravesical BCG therapy were also chosen separately as an independent validation cohort (test set). To reduce confounding factors for affecting the analyses, any patients undergoing immediate postoperative single-dose mitomycin C, BCG maintenance therapy, or were diagnosed with a concomitant carcinoma in situ lesion were excluded from this study. In total, 80 primary pT1 bladder cancer patients receiving TUR of bladder cancer plus adjuvant 6-wk induction BCG immunotherapy were included in the study. Microarray gene expression data were used as the training set and these predictive models were validated using reverse transcription-PCR (RT-PCR) analyses in the independent test set.

All tumors were macrodissected within 15 min of surgical resection. Each bladder cancer specimen was confirmed by pathologic analysis of a part of the tissue sample in fresh frozen sections from TUR specimens, and then frozen in liquid nitrogen and stored at −80°C until use. The collection and analysis of all samples were approved by the local institutional review board and informed consent was obtained from each subject.

Tumors were staged according to the 2002 tumor-node-metastasis (TNM) classification and the 1973 WHO grading system (1). A second TUR was done 2 to 4 wk after the initial resection when a bladder cancer specimen did not include proper muscle or when a high-grade tumor was detected. All patients received six weekly intravesical administrations of 12.5 mg of Tice strain BCG in 50 mL of physiologic bacteriostatic-free saline solution within 2 to 4 wk after TUR (1, 3).

Response to treatment was assessed by cystoscopy and urinary cytology. Complete response was defined as normal cystoscopy and negative cytology. To histologically confirm relapse, all visible or suspicious lesions were removed by TUR for analysis. Patients who were free of disease 3 mo after treatment were assessed every 3 mo for the first 2 y and every 6 mo thereafter (1, 3). Recurrence was defined as the recurrence of primary NMIBC at a lower or equivalent pathologic stage (Ta/T1), and progression was defined as muscular invasion (tumor-node-metastasis stage T2 or higher) or metastatic disease.

Microarray gene expression profiling and RT-PCR analysis. Total RNA was isolated by Trizol reagent (Life Technologies), according to the manufacturer’s protocol. The quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under UV light. Five-hundred nanograms of total RNA were used for labeling hybridization according to the manufacturer’s protocol (Illumina HumanWG-6 BeadChip, version 2, Illumina, Inc.). Arrays were scanned with an Illumina Bead Array Reader confocal scanner (BeadStation 500GXDW; Illumina, Inc.) according to the manufacturer’s instructions. Initial microarray gene expression data were obtained using Bead Studio software with gene expression analysis module (version 3.1.3, Illumina, Inc.). The full microarray data of training set is available online5 under the data series accession number GSE19423.

For validation, RT-PCR was done with a Rotor Gene 3000 PCR system (Corbett Research) using SYBR Premix EX Taq (TaKaRa Bio, Inc.). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

Statistical analysis. The differences in continuous variables between the groups were assessed by two sample t test. Categorical variables were compared using the χ² test. To select genes differentially expressed between responder and nonresponder to BCG immunotherapy in microarray data, a separate univariate Cox regression analysis was done based on events (recurrence or progression). Using selected genes, each patient’s risk score for either event was calculated as the sum of the levels of expression of each gene multiplied by the corresponding regression coefficients (19–23). Receiver-operating-characteristic curves were used to determine the optimal cutoff point of each risk score that yielded

5 http://www.ncbi.nlm.nih.gov/geo/
the highest combined sensitivity and specificity for events. Using these values, patients were classified into good or poor predictive gene signature groups. To survey the spectrum of biological functions within our predictive gene signatures, we performed functional classification on the genes using the Ingenuity Pathways Analysis software. The significance of each function was estimated using the Fisher’s exact test method provided by the Ingenuity Pathway Analysis Tool (version 7.5). From the predictive gene signatures, we chose 24 genes (12 genes in recurrence and 12 genes in progression) with the highest score of expression ratio to validate our model with RT-PCR using tumor samples obtained from the independent validation cohort. The score of expression ratio in each gene was calculated as the number of samples that have at least 2-fold difference relative to median gene expression level across tissues (24). As with the microarray data, the same statistical analyses were applied to the validation cohort. Using the each 12 gene expression value of RT-PCR, patients in the test set were divided into good or poor predictive gene signature groups according to the calculated each patient’s risk score of event. The Kaplan-Meier method was used to estimate the time to recurrence or progression based on predictive gene signatures, and differences were assessed using log-rank statistics. Upon multivariate Cox proportional hazards regression models, the prognostic value of predictive gene signatures was evaluated and adjusted for the potential confounding effect of grade, tumor size, and multiplicity (1). Statistical analysis was done using R (version 2.8.1, available online)\(^6\) and the SPSS 12.0 software (SPSS, Inc.), and a \(P\) value of <0.05 was considered statistically significant.

### Results

**Baseline characteristics.** The baseline characteristics of training and validation cohorts are presented in Table 1. The mean follow-up after surgery among training and validation cohorts were 60.0 ± 35.0 months (median, 52.0; range 13.9-138.8) and 56.2 ± 47.8 months (median, 29.0; range 9.9-165.4), respectively. No significant differences were identified between two groups.

**Selection of predictive gene signatures and their clinical relevance.** Upon univariate Cox regression analysis of microarray data, candidate predictive genes that could predict recurrence (424) or progression (287) of bladder cancer following BCG therapy were selected, respectively (Supplementary Tables S1 and S2). Patients were divided into good or poor predictive gene signature groups according

\(^6\text{http://www.r-project.org/}\)
Patients in the poor predictive gene signature group had a significantly shorter recurrence or progression time than those in the good predictive gene signature group (log-rank test, $P < 0.001$, respectively; Fig. 1A and B).

**Functional classification of predictive gene signatures.** To determine whether our candidate genes were enriched in known biological functions, bioinformatic functional classification analyses of the 424 and 287 genes featured in the recurrence and the progression signatures, respectively, were carried out. This analysis revealed a series of recurrence- or progression-associated functional categories. The full functional classification list of statistically significant gene sets related to recurrence or progression is presented in Supplementary Tables S3 and S4. In the recurrence, we found that genes involved in the cellular growth and proliferation, cancer, cell cycle, and cell death were enriched, providing confidence in our results. We also found that genes involved in cell-mediated immune response, immunologic disease, inflammatory disease, and inflammatory response were significantly enriched. In the progression, genes involved in cell cycle, DNA replication and repair, cell-mediated immune response, immunologic disease, and immune cell tracking were enriched.

**Validation of predictive gene signatures in test set by RT-PCR.** Using 24 genes with the highest score of expression ratio in each candidate predictive gene groups, we validated our model in tumor samples obtained from the test set (Table 2). Risk scores of event were calculated separately and patients were classified into good or poor predictive gene signature groups based on their event. Patients with poor predictive gene signatures had shorter recurrence or progression times than those with good predictive gene signatures (log-rank test, $P < 0.05$, respectively; Fig. 2A and B). According to the Cox multivariate regression analysis, predictive gene signatures were significantly associated with recurrence [hazard ratio (HR), 3.38; $P = 0.048$] or progression (HR, 10.49; $P = 0.042$; Table 3).

**Discussion**

Although efficacy of BCG treatment has been clearly recognized, a significant number of patients fail to respond to treatment (1, 2, 4, 5, 7, 10). A method that accurately predicts the response to BCG therapy would allow physicians to determine appropriate alternative therapeutic modalities. A normal cell must undergo multiple genetic alterations to adopt a malignant and ultimately metastatic phenotype; thus, the simultaneous assessment of multiple markers might better characterize the biological phenotype of a particular cancer (25–27). Comprehensive insights into the molecular basis of human diseases have been achieved with microarray-based clinical research but the results should be validated using alternative techniques for quantification of RNA expression in an independent cohort to reduce false discovery rates (13, 28, 29). In these regards, the validation strategy for clinical relevance in different cohorts adds strength in the results of our analysis. We identified differentially expressed predictive gene signatures from the genome-wide analysis in responsive and nonresponsive bladder cancers following BCG treatment. Our findings suggest that the response to intravesical BCG instillation in patients with pT1 bladder cancer can be predetermined by predictive gene signatures. These predictive gene signatures can be used as significant independent indicators for prediction of prognosis, regardless of the pathologic characteristics of the cancer.

Numerous clinical assessments have been investigated as prognostic indicators, but none are able to sufficiently predict the response of cancer to BCG (5, 7–10). A wide array of clinical and pathologic risk factors is involved in the prognosis of NMIBC, including prior recurrence rate.
T category, carcinoma in situ, grade, and administration of intravesical therapy (1, 3, 6). In a majority of previous studies, heterogeneous study population characteristics have been included, such as treatment with a combination of drugs and therapeutic schedules (2, 5, 7–10). Specific studies that focus on prognostic factors in patients with homogeneous characteristics and treated by BCG with same the duration and dosage are needed. For these reasons, homogenous patient features of the present study strengthen the validity of our results. Moreover, urothelial cancers arise and evolve through divergent phenotypic pathways. Some tumors progress from urothelial hyperplasia to low-grade NMIBC. More aggressive variants arise either from flat, high-grade carcinoma in situ and progress to MIBC, or they arise de novo as MIBC. These two important phenotypic variants of urothelial cancers exhibit drastically different biological behaviors and prognoses. The low-grade papillary variant is often multifocal and tends to recur, but it infrequently progresses to muscle invasive stages, whereas most of the invasive variants develop into incurable metastases despite radical cystectomy (30). With these considerations, in the present study, separate analysis of predictive gene signature by their prognosis (recurrence and progression) is prudent.

Biological insights of our predictive gene signature should be mentioned. BCG evokes a complex immune

<table>
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<th>Table 2. 24 Predictive gene signatures with their associated functions</th>
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<td>Prognosis</td>
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*Upregulated or downregulated in poor prognosis group.
response in the bladder, beginning with its binding to fibronectin and integrin-mediated ingestion by urothelial cells with subsequent elaboration of various chemokines and proinflammatory cytokines (11). This induces a milieu of bioactive cytokines and activated immunoeffector cells that result in improved recognition and killing of the tumor by both nonspecific and specific mechanisms. Several gene functions (immune, inflammatory, etc.) are overlapped between our and previous studies (8–11). Nevertheless, the majority of genes found in our study were not found in others and we identified many additional genes not previously linked to the BCG response. This indicates that our knowledge of BCG response is still incomplete. Further, it suggests that a genome-wide systems biology approach could ultimately prove useful in understanding the mechanisms underlying genomic stability.

Although induction of BCG instillations are classically given according to the empirical 6-week schedule, many different maintenance schedules have been applied with up to 30 instillations given over 3 years (1). A possible limitation of the present study might be that the maintenance therapy was not done. If maintenance therapy were given, response rate might have been different. However, in our institute, only a small percentage of patients were able to complete the maintenance dosing schedule. The low compliance due to the BCG-related side effects makes it difficult to perform a study with enough number for analysis. The genome-wide analysis of patients treated with maintenance BCG is needed, and this is currently under study in our institute.

From a clinical point of view, the most promising applications for genetic markers are early detection, prediction of response to treatment, and indication of disease prognosis. The results presented here are considered promising because putative predictive gene signatures were selected from a genome-wide analysis and were validated by independent methods. In addition, the selected predictive gene signatures were independent predictors of the response to BCG therapy (Table 3). An

<table>
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<tr>
<th>Variables</th>
<th>Recurrence</th>
<th>Progression</th>
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<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
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<tr>
<td>Size (&lt;3 cm vs ≥3)</td>
<td>0.70 (0.23-2.10)</td>
<td>0.527</td>
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<tr>
<td>No. of tumor (single vs multiple)</td>
<td>0.99 (0.35-2.78)</td>
<td>0.986</td>
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<td>Grade</td>
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<tr>
<td>G1</td>
<td>1</td>
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<tr>
<td>G2</td>
<td>0.76 (0.16-3.66)</td>
<td>0.736</td>
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<tr>
<td>G3</td>
<td>0.62 (0.10-3.83)</td>
<td>0.609</td>
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<tr>
<td>Predictive gene signatures</td>
<td>3.38 (1.01-11.29)</td>
<td>0.048</td>
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Abbreviation: 95% CI, 95% confidence interval.
accurate prediction of the response to therapy and subsequent prognosis would aid in individual patient counseling, to determine the frequency and extent of monitoring and indicate the need for alternative therapy. Predictive gene signatures can help determine whether patients will benefit from adjuvant BCG treatment or may require more aggressive alternative therapies. Furthermore, the identification of a patient group that will maximally benefit from BCG therapy may be possible with predictive gene signatures in near future. This approach may lead to the development of personalized therapy, thereby significantly lowering the morbidity associated with NMIBC.

In conclusion, our results suggest that predictive gene signatures are independent indicators of the response to BCG treatment in pathologic T1 bladder cancer patients. The predictive gene signatures could constitute a promising technique for assessing the response to intravesical BCG therapy, which may allow for the formulation of individualized therapeutic modalities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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