Analysis of CXCL9, PD1 and PD-L1 mRNA in Stage T1 Non-Muscle Invasive Bladder Cancer and Their Association with Prognosis

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Received: 3 September 2020; Accepted: 24 September 2020; Published: 29 September 2020

Simple Summary: Non-muscle invasive bladder cancer (NMIBC) patients possess a high rate of recurrences and very long treatment times, which remains a major unresolved problem for them and the health care system. We analyzed the mRNA of three immune markers, CXCL9, PD1 and PD-L1, in 80 NMIBC by qRT-PCR. Lower CXCL9 mRNA appeared to be an independent prognostic parameter for reduced OS and RFS. Furthermore, low PD-L1 mRNA was an independent prognostic factor for DSS and RFS. In univariate Cox’s regression analysis, the stratification of patients revealed that low CXCL9 or PD1 mRNA was associated with reduced RFS in the younger patient group (<72 years). Low CXCL9 or PD-L1 was associated with shorter RFS in patients with higher tumor cell proliferation or without instillation therapy. In conclusion, the characterization of mRNA levels of the immune markers CXCL9, PD1 and PD-L1 differentiates NMIBC patients with respect to prognosis.

Abstract: Non-muscle invasive bladder cancer (NMIBC), which is characterized by a recurrence rate of approximately 30% and very long treatment times, remains a major unresolved problem for patients and the health care system. The immunological interplay between tumor cells and the immune environment is important for tumor development. Therefore, we analyzed the mRNA of three immune markers, CXCL9, PD1 and PD-L1, in NMIBC by qRT-PCR. Lower CXCL9 mRNA was associated with reduced RFS (RR = 2.08; p = 0.049), and PD-L1 mRNA was associated with reduced RFS (RR = 2.07; p = 0.044). Moreover, in univariate Cox’s regression analysis, the stratification of patients revealed that low CXCL9 or low PD1 mRNA was associated with reduced RFS in the younger patient group (≤71 years), but not in the older patient group (>71 years). In addition, low CXCL9 or low PD-L1
was associated with shorter RFS in patients with higher tumor cell proliferation and in patients without instillation therapy. In conclusion, the characterization of mRNA levels of immune markers differentiates NIMBC patients with respect to prognosis.

Keywords: CXCL9; PD1; PD-L1; stage T1 NMIBC; prognosis

1. Introduction

Urothelial bladder cancer (BCa) accounts for approximately 3% of global cancer diagnoses. It was recently reported to be the 10th most commonly diagnosed cancer and the 13th leading cause of cancer-related death worldwide [1]. Approximately 25% of BCas are categorized as muscle-invasive BCa (MIBC) and 75% as non-muscle invasive BCa (NMIBC) [2]. NMIBC treatment comprises transurethral resection of the bladder (TURB) and, depending on the risk of progression, instillation with bacillus Calmette-Guerin (BCG) or mitomycin [3–5]. However, high-risk NMIBC remains a challenge because 30% to 60% of patients with stage pT1 NMIBC develop local recurrence, and up to 20% experience disease progression to MIBC [6–8]. There is heterogeneity in stage pT1 NMIBC, and its risk stratification is based only on clinicopathological parameters that necessitate lifelong follow-up [9]. Altogether, bladder cancers, including NMIBC, impose the highest costs on society among cancers per patient from diagnosis to death [10]. However, bladder tumor markers cannot yet definitively replace cystoscopy in surveillance regimens [10]. Therefore, the continued search for biomarkers in bladder cancer is necessary.

The tumor biology of BCa, including NMIBC, is related to cell lineage and cell proliferation [11–13]. Therefore, we included an analysis of the mRNA of keratin 5 (KRT5; basal-like lineage), keratin 20 (KRT20; luminal-like lineage) and marker of proliferation KI67 (MKI67, KI67) in this study. Furthermore, studies conducted by other groups, as well as our own previous studies, showed that gene expression can differentiate NMIBCs into subsets that possess different risk profiles, and may impact treatment decisions in the future [14,15].

In the current study, we investigated the expression of genes associated with tumor immune status and their association with prognosis in stage pT1 NMIBC. Recently, we reported that a cytotoxic T-cell-related gene expression signature containing three genes (CXCL9, CD3 Z, CD8) correlates with immune cell infiltration, and predicts improved survival in MIBC patients after radical cystectomy and adjuvant chemotherapy [16]. All three immune signature genes were strongly associated with each other, which is why we chose only CXCL9 for the current analysis. Additionally, we chose programmed cell death 1 gene (PD1/PDCD1) and programmed cell death ligand 1 (PD-L1/CD274/B7-H1) since they are also very prominent in the immune response of MIBC, and represent therapeutic targets for MIBC [16–18]. CXCL9 (SCYB9/MIG) and CXCL10 (SCYB10) genes are located in chromosome band 4 q21 [19], and belong to the CXC family of chemokines [20]. CXCL9 encodes a T-cell chemoattractant that is significantly induced by interferon gamma, which mediates a T-cell-driven antitumoral immune response [21]. CXCL9 has not been previously studied in NMIBC. The PD1 gene has been mapped to the chromosome region 2 q37.3 by the Honyo group [22]. It encodes a cell surface receptor on T-cells and tumor-associated macrophages (TAMs), and is a member of the B7 superfamily involved in immunomodulation. PD1 acts as an inhibitory molecule on T-cells/TAMs after interacting with its ligand PD-L1 [23,24]. The PD-L1 gene is located on chromosome 9 p24.1 and codes for a costimulatory molecule that negatively regulates cell-mediated immune responses [23,25]. PD-L1 is expressed by both tumor cells and tumor-associated antigen-presenting cells [26]. Le Goux et al. [27] did not find an association between PD1 or PD-L1 gene expression and prognosis (RFS and progression-free survival) in NMIBC. We recently demonstrated in an NMIBC cohort that increased PD-L1 mRNA was an independent prognostic indicator for both RFS and DSS [28]. However, in that study, PD1 mRNA was not associated with prognosis [28].
In this study, we analyzed a new independent cohort of NMIBC patients with extended follow-up periods to reassess the long-term association of PD-L1 mRNA with disease prognosis, and to determine whether the two immune markers CXCL9 and PD1 are associated with survival.

2. Results

2.1. Correlations of CXCL9, PD1, PD-L1, KRT5 and KRT20 mRNA with Each Other and with Clinicopathological Parameters

CXCL9 mRNA negatively correlated with the incidence of recurrence (correlation coefficient; $r_s = -0.374$; $p = 0.001$) and with mRNA of KRT20 ($r_s = -0.305$; $p = 0.006$) and KRT5 ($r_s = -0.230$; $p = 0.040$), and is positively correlated with mRNA of PD1 ($r_s = 0.639$; $p < 0.001$) and PD-L1 ($r_s = 0.601$; $p < 0.001$) (Table 1). PD1 mRNA was negatively correlated with mRNA of KRT20 ($r_s = -0.253$; $p = 0.024$) and Ki67 ($r_s = -0.222$; $p = 0.047$), and positively correlated with time of RFS ($r_s = 0.298$; $p = 0.007$) and PD-L1 mRNA ($r_s = 0.459$; $p < 0.001$). PD-L1 mRNA negatively correlated with KRT20 ($r_s = -0.233$; $p = 0.038$) (Table 1).

Table 1. Bivariate correlations for mRNA of CXCL9, KRT20, KRT5, PD1, PD-L1 and Ki67 with clinicopathological parameters.

| Bivariate Correlations | KRT20 | KRT5 | PD1 | PD-L1 | Ki67 | Fu_Recurr | Recurr |
|------------------------|-------|------|-----|-------|------|-----------|--------|
| CXCL9                  |       |      |     |       |      |           |        |
| Correlation coefficient | -0.305| -0.230| 0.639| 0.601 | -0.136| 0.208     | -0.374 |
| Sig. (2-sided)         | 0.006 | 0.040| <0.001| <0.001| 0.228 | 0.065     | 0.001  |
| KRT20                  |       |      |     |       |      |           |        |
| Correlation coefficient | -0.042| -0.253| -0.233| 0.356 | -0.152| 0.116    |        |
| Sig. (2-sided)         | 0.714 | 0.024| 0.038|       | 0.001 | 0.178     | 0.304  |
| KRT5                   |       |      |     |       |      |           |        |
| Correlation coefficient | -0.212|       | 0.036| -0.070| 0.039 | 0.067     |        |
| Sig. (2-sided)         | 0.059 | 0.753|       | 0.537 | 0.733 | 0.557     |        |
| PD1                    |       |      |     |       |      |           |        |
| Correlation coefficient |       |       | 0.459| -0.222| 0.298 | -0.204    |        |
| Sig. (2-sided)         |       | 0.047|       | 0.001 | 0.007 | 0.070     |        |
| PD-L1                  |       |      |     |       |      |           |        |
| Correlation coefficient |       |       |       | 0.001 | 0.096 | -0.215    |        |
| Sig. (2-sided)         |       | 0.994|       | 0.397 | 0.055 |          |        |
| Ki67                   |       |      |     |       |      |           |        |
| Correlation coefficient |       |       |       |       | -0.152| 0.138     |        |
| Sig. (2-sided)         |       | 0.177|       |       | 0.222 |          |        |
| fu_recurr              |       |      |     |       |      |           |        |
| Correlation coefficient |       |       |       |       |       | -0.562    |        |
| Sig. (2-sided)         |       |       |       |       |       | <0.001    |        |

Abbreviation: fu recur—follow-up recurrence (time until occurrence of recurrence); recur.—recurrence. Bonferroni correction results in $\alpha = 0.00714$. Significance at the $\alpha$ level is marked in bold.

2.2. Association of CXCL9, PD1, PD-L1, KRT5 and KRT20 mRNA with NMIBC Prognosis

The association of mRNA in the 80 tumor samples with patient survival was examined by Kaplan–Meier analysis. As expected, age was associated with both OS and DSS ($p = 0.019$ and $p = 0.025$). However, CXCL9, PD1 and PD-L1 mRNA was not associated with OS or DSS (Table 2).

Interestingly, higher CXCL9 ($p < 0.001$), PD1 ($p = 0.023$) or PD-L1 ($p = 0.007$) mRNA were associated with increased RFS (all Kaplan–Meier analyses, Table 2; Figure 1).
Table 2. Kaplan–Meier analysis of the association of age, CXCL9, PD1 and PD-L1 mRNA with prognosis.

| Parameter | Kaplan–Meier Analysis |
|-----------|-----------------------|
|           | n | OS Months | p | n | DSS Months | p | n | RFS Months | p |
| Age       |   |           |   |   |            |   |   |            |   |
| ≤71 vs. >71 year | 40 vs. 40 | 124.8 vs. 84.5 | 0.019 | 40 vs. 40 | 170.2 vs. 108.3 | 0.025 | 40 vs. 40 | n.s. | n.s. |
| CXCL9 low vs. high | 32 vs. 48 | n.s. | n.s. | 25 vs. 55 | n.s. | n.s. | 32 vs. 48 | 38.7 vs. 87.4 | <0.001 |
| PD1 low vs. high | 40 vs. 40 | n.s. | n.s. | 40 vs. 40 | n.s. | n.s. | 53 vs. 27 | 62.0 vs. 99.5 | 0.023 |
| PD-L1 low vs. high | 24 vs. 56 | n.s. | n.s. | 46 vs. 34 | n.s. | n.s. | 46 vs. 34 | 58.6 vs. 102.7 | 0.007 |

Significant values are in bold face. Abbreviation: n.s., not significant.

Figure 1. Kaplan–Meier analysis of the association of CXCL9, PD1 or PD-L1 mRNA with RFS. Gene expression was significantly associated with RFS for the genes. (A): CXCL9 (p < 0.001). (B): PD1 (p = 0.023). (C): PD-L1 (p = 0.007).
In univariate Cox’s regression analysis, the clinicopathological parameters of histological grade, tumor stage (pT1 with/without presence of cis), intravesical therapy and gender, and the molecular parameters \( KI67 \), \( KRT5 \) and \( KRT20 \), were not associated with prognosis (OS, DSS, RFS), and therefore were not included in further multivariate Cox’s regression analysis (data not shown).

As expected, in univariate Cox’s regression analysis, higher age (RR = 2.29; \( p = 0.022 \)) was associated with an increased risk of shorter OS. Furthermore, higher age (RR = 3.44; \( p = 0.034 \)) was associated with increased risk of shorter DSS (Table 3).

| Parameter                  | Univariate Cox’s Regression Analysis |
|----------------------------|--------------------------------------|
|                            | n | OS RR | p   | n | DSS RR | p   | n | RFS RR | p   |
| Age ≤71 vs. >71 year CXCL9 | 40 vs. 40 | 2.29 | 0.022 | 40 vs. 40 | 3.44 | 0.034 | 40 vs. 40 | n.s. | n.s. |
| low vs. high CXCL9          | 32 vs. 48 | n.s. | n.s. | 25 vs. 55 | n.s. | n.s. | 21 vs. 59 | 3.30 | <0.001 |
| low vs. high PD-L1          | 40 vs. 40 | n.s. | n.s. | 40 vs. 40 | n.s. | n.s. | 53 vs. 27 | 2.31 | 0.027 |
| low vs. high PD-L1          | 24 vs. 56 | n.s. | n.s. | 46 vs. 34 | n.s. | n.s. | 46 vs. 34 | 2.51 | 0.009 |

Significant values are in bold face. Abbreviation: n.s., not significant.

In univariate Cox’s regression analysis, lower CXCL9 (RR = 3.30; \( p < 0.001 \)), lower \( PD1 \) (RR = 2.31; \( p = 0.027 \)) and lower \( PD-L1 \) (RR = 2.51; \( p = 0.009 \)) mRNA showed an increased risk for shorter RFS. However, age was not associated with an increased risk of shorter RFS (Table 3).

In multivariate Cox’s regression analysis (adjusted for age and the molecular parameters \( PD1 \), \( PD-L1 \) and \( CXCL9 \)), an association with OS was found for higher age (RR = 2.31; \( p = 0.021 \)) and lower CXCL9 (RR = 2.08; \( p = 0.049 \)) mRNA (Table 4). Multivariate analysis (adjusted for age and the molecular parameters \( PD1 \), \( PD-L1 \) and \( CXCL9 \)) revealed associations with DSS for higher age (RR = 4.47; \( p = 0.014 \)), lower CXCL9 (RR = 4.49; \( p = 0.006 \)) and lower \( PD-L1 \) (RR = 5.02; \( p = 0.042 \)) mRNA (Table 4).

| Parameter                  | Multivariate Cox’s Regression Analysis |
|----------------------------|---------------------------------------|
|                            | n | OS RR | p   | n | DSS RR | p   | n | RFS RR | p   |
| Age ≤71 vs. >71 year CXCL9 | 40 vs. 40 | 2.31 | 0.021 | 40 vs. 40 | 4.47 | 0.014 | 40 vs. 40 | n.s. | n.s. |
| low vs. high CXCL9         | 32 vs. 48 | 2.08 | 0.049 | 25 vs. 55 | 4.49 | 0.006 | 21 vs. 59 | 2.69 | 0.005 |
| low vs. high PD1           | 40 vs. 40 | n.s. | n.s. | 40 vs. 40 | n.s. | n.s. | 53 vs. 27 | n.s. | n.s. |
| low vs. high PD-L1         | 24 vs. 56 | n.s. | n.s. | 46 vs. 34 | 5.02 | 0.042 | 46 vs. 34 | 2.07 | 0.044 |

Significant values are in bold face. Abbreviation: n.s., not significant.

Furthermore, in the multivariate Cox’s regression analysis, associations with shorter RFS were found for lower CXCL9 (RR = 2.69; \( p = 0.005 \)) and lower \( PD-L1 \) (RR = 2.07; \( p = 0.044 \)) mRNA (Table 4).

Altogether, as expected, higher age was an independent prognostic factor for OS and DSS, but not for RFS. CXCL9 mRNA was as independent prognostic parameter for OS, DSS and RFS. In addition, \( PD-L1 \) mRNA was an independent prognostic factor for DSS and RFS.
2.3. Association of CXCL9, PD1, PD-L1, KRT5 and KRT20 mRNA with RFS Stratified by Clinicopathological Parameters or mRNA

2.3.1. Stratification by Age

Using the median age of 71 years as a cut-off to define the two age groups (≤71 vs. >71 years), age itself was not associated with RFS (Table 4). In the univariate Cox’s regression analysis in the younger age group, low CXCL9 (RR = 6.21; \( p < 0.001 \)) was associated with an increased risk of recurrence (Table 5). This finding is in accordance with the above mentioned results for all patients, but it indicates the greater relevance of CXCL9 mRNA in younger patients. Low PD1 mRNA was only associated with a risk of shorter RFS in the younger patient group (RR = 4.93; \( p = 0.035 \)). Altogether, the higher risks of recurrence for CXCL9 and low PD1 levels were only relevant to the younger age group (Table 5).

Table 5. Univariate Cox’s regression analysis for stratification by clinicopathological or molecular parameters: the association of CXCL9, PD1 and PD-L1 mRNA with RFS.

| Parameter by Stratification | Univariate Cox's Regression Analysis |
|-----------------------------|-------------------------------------|
|                             | \( n \) | RFS | RR  | \( p \) |
| **Strata age: young patients** |        |     |     |       |
| CXCL9 low vs. high          | 40     | 15 vs. 25 | 6.21 | <0.001 |
| \( PD1 \) low vs. high      |        | 27 vs. 13 | 4.93 | 0.035  |
| **Strata KRT5 low**         |        |     |     |       |
| CXCL9 low vs. high          | 40     | 13 vs. 27 | 3.76 | 0.004  |
| **Strata KRT5 high**        |        |     |     |       |
| CXCL9 low vs. high          | 40     | 19 vs. 21 | 3.33 | 0.013  |
| \( PD-L1 \) low vs. high    |        | 22 vs. 18 | 3.68 | 0.012  |
| **Strata KRT20 low**        |        |     |     |       |
| CXCL9 low vs. high          | 40     | 13 vs. 27 | 3.04 | 0.019  |
| **Strata KRT20 high**       |        |     |     |       |
| CXCL9 low vs. high          | 40     | 19 vs. 21 | 3.28 | 0.007  |
| \( PD-L1 \) low vs. high    |        | 25 vs. 15 | 4.23 | 0.009  |
| **Strata: KI67 high**       |        |     |     |       |
| CXCL9 low vs. high          | 40     | 19 vs. 21 | 4.54 | <0.001 |
| \( PD-L1 \) low vs. high    |        | 25 vs. 15 | 7.49 | 0.001  |
| **Strata: no intravesical** | 39     |     |     |       |
| CXCL9 low vs. high          | 15 vs. 24 | 10.33 | <0.001 |
| \( PD1 \) low vs. high      |        | 23 vs. 16 | 5.31 | 0.010  |
| \( PD-L1 \) low vs. high    |        | 22 vs. 17 | 4.36 | 0.022  |

Significant values are in bold face.

2.3.2. Stratification by KRT5 or KRT20 Expression

KRT5 or KRT20 mRNA is considered a characteristic feature for a basal or luminal lineage, respectively, in bladder cancer [11]. We utilized the expressions of both mRNA markers as proxies to define a more basal or more luminal-like gene expression pattern, respectively. The expression of both markers was separated by median expression into two groups with low/high KRT5 (≤36.78 vs. >36.78) or low/high KRT20 (≤37.47 vs. >37.47) mRNA level. In low and high KRT20 groups, CXCL9 mRNA was associated with a shorter RFS (RR = 3.04; \( p = 0.019 \) and RR = 3.28, respectively; \( p = 0.007 \)) (Table 5). Similarly, low CXCL9 mRNA was associated with a shorter RFS in the low and high KRT5 groups (RR = 3.76; \( p = 0.004 \) and RR = 3.33; \( p = 0.013 \), respectively; Table 5). These results were expected since they reflected findings for all patients. In the high KRT5 and high KRT20 groups, low PD-L1 mRNA was associated with shorter RFS (RR = 3.68; \( p = 0.012 \) and RR = 4.23, respectively; \( p = 0.009 \); Table 5), but this was not so in the low KRT5 or low KRT20 group.
2.3.3. Stratification by KI67

KI67 characterizes the proliferation activity of tumor cells [29]. KI67 expression was separated into two groups (low vs. high expression) by median mRNA (\(\leq 33.10\) vs. \(>33.10\)). In the high KI67 expression group, low CXCL9 (RR = 4.54; \(p < 0.001\)) mRNA and low PD-L1 (RR = 7.49; \(p = 0.001\); Table 5) mRNA were associated with a higher risk of shorter RFS, but these associations were not observed in the low KI67 group.

2.3.4. Stratification by Intravesical Therapy

Intravesical therapy was not associated with RFS in this study group. In the group with no intravesical therapy, low CXCL9 (RR = 10.33; \(p < 0.001\)), low PD1 (RR = 5.31; \(p = 0.010\)) and low PD-L1 (RR = 4.36; \(p = 0.022\); Table 5) mRNA was associated with the increased risk of shorter RFS, but no associations were observed with RFS in the intravesical group.

Altogether, CXCL9 mRNA was associated with RFS in all stratification approaches. Interestingly, the increased risk of shorter RFS in low CXCL9 mRNA patients was substantiated in the young patient group, the high KI67 group and in patients without instillation, but it showed no association with RFS in the older patient group, the low KI67 group or the instillation group.

In addition, the increased risk observed with low PD1 levels was assigned to the younger patient group and the no instillation group, with no association with RFS being observed in the older patient group or the instillation patient group.

For the third marker, PD-L1, an increased risk of shorter RFS with low PD-L1 mRNA was detected only in the high KRT5 and high KRT20 groups, but not in the low KRT5 or low KRT20 groups. In addition, this risk was found in the high KI67 and the no instillation group, but not in the low KI67 group or the instillation group.

3. Discussion

In this study, we investigated the mRNA of the immune markers CXCL9, PD1 and PD-L1. First, we correlated mRNA data with clinicopathological data and with each other. We observed that CXCL9 mRNA was positively correlated with transcript levels of PD1 and PD-L1, but negatively correlated with incidence of recurrence, as well as KRT5 and KRT20 mRNA. In addition, PD1 was positively correlated with PD-L1 mRNA and time to RFS, while being negatively correlated with KRT20 mRNA. PD-L1 mRNA was additionally negatively correlated with KRT20 mRNA.

Similar to Huang et al. we showed a correlation between the mRNA of PD-L1 and C-C chemokines (CCL2, CCL3, CCL8 and CCL18) [30,31]. A correlation between PD1 and PD-L1 mRNA was previously shown by both Huang et al. [31] and by us [28]. These correlations can all be explained by the common expression of these factors by immune cells, i.e., leukocytes such as T-cells and macrophages.

In this study, multivariate Cox’s regression analyses revealed that high CXCL9 mRNA was associated with longer OS and DSS, and high PD-L1 mRNA was correlated with longer DSS. In addition, the high mRNA of CXCL9 or PD-L1 was significantly associated with longer RFS. Huang and colleagues found that elevated PD-L1 mRNA was associated with reduced patient survival (OS, DSS), but they studied a mixed cohort of NMIBC and MIBC where the association could have been influenced by MIBC patients, and further, they did not examine RFS [31]. We previously found that increased PD-L1 mRNA expression was associated with longer DSS and RFS in pT1 NMIBC [28]. In this study, we confirmed the association of high PD-L1 mRNA with DSS and RFS. However, the impact of PD-L1 on OS, DSS and RFS need to be evaluated further in prospective studies.

PD1 was previously not described to be associated with RFS [28], but in this study, we observed an association between increased PD1 mRNA and longer RFS. Although both studies were performed in consecutive patients, in this study, observation time was longer (62 vs. 42 months), and the numbers of recurrences (51.3% vs. 33.4%) were higher than in the previous study, which may explain the differential results.
CXCL9 mRNA level has not been previously described in NMIBC to be associated with OS, DSS or RFS. The effect of an immune intravesical therapy with bacillus Calmette-Guérin (BCG) on CXCL9 mRNA was controversially discussed. BCG therapy upregulates the mRNA of different chemokines, including CXCL9, in an in vivo mouse model [32]. Interestingly, using an in vitro approach in established human BCa cell lines, Özcan et al. demonstrated that BCG treatment reduced CXCL9 mRNA [33]. This supports the assumption that the tumor microenvironment is responsible for the chemokine reaction following BCG therapy. A recent review reports that the CXCL9/CXCL10/CXCL11/CXCR3 axis is responsible for angiogenesis inhibition, and the activation and migration of immune cells such as cytotoxic lymphocytes and natural killer cells into the tumor microenvironment, to prevent tumor progression in BCa [34].

Next, we were interested in whether the association of CXCL9, PD1 and PD-L1 mRNA with RFS could be further stratified by clinicopathological parameter (age) or other parameters applied for lineage differentiation, such as KRT5 or KRT20 mRNA, proliferation activity (Ki67), or therapeutic application (instillation therapy). Interestingly, after separating patients by their median age (≤71 vs. >71 years), only in the younger age group (≤71 years) was higher CXCL9 or higher PD1 mRNA associated with longer RFS. This finding could be simply related to the fact that the immune system is more active in younger than in older persons, in whom immunosenescence has been reported [35]. Increasing multimorbidity affecting health status in elderly patients may also play a role in shorter RFS, although time to recurrence was not significantly different between the age groups (data not shown).

KRT5 and KRT20 are considered intrinsic markers for basal and luminal subtypes of muscle-invasive bladder cancer, respectively [11,36,37]. Interestingly, high PD-L1 mRNA was associated with longer RFS in both high KRT5 and high KRT20 groups, but not in the low KRT5 or low KRT20 groups. This finding suggests that high PD-L1 mRNA is favorable for longer RFS in both basal and luminal subtypes of NMIBC. We previously showed that high KRT20 mRNA was associated with shorter RFS [38]. In this context, PD-L1 mRNA further distinguishes the unfavorable RFS group (high KRT20) in patients with longer RFS (PD-L1 high) or shorter RFS (PD-L1 low).

Intravesical therapy with either BCG or cytostatic drugs, like mitomycin, is mostly standard therapy for intermediate or high risk NMIBC, but its application differs between several guidelines [3,5]. Interestingly, only in the no instillation group was high CXCL9, high PD1 or high PD-L1 associated with longer RFS compared to the instillation group. One explanation for this finding could be that BCG therapy affects the immune response of patients, and CXCL9, PD1 and PD-L1 reflect intrinsic immune status. In this way, both the expression of the immune markers and the intravesical therapy may influence each other. As mentioned above, the BCG exposure of established BCa cell lines devoid of any tumor microenvironment reduced CXCL9 mRNA in vitro [33]. Furthermore, increases in PD-L1 protein levels, which are considered a negative prognostic marker, have been reported after BCG therapy compared to before BCG treatment [39].

4. Material and Methods

4.1. Patients and Tumor Material

In this study, we retrospectively analyzed clinical and histopathological data from 80 patients treated with TURB at the Department of Urology and Pediatric Urology of the University Hospital Erlangen between 2000 and 2015 who were initially diagnosed with stage pT1 NMIBC (Table 6). All patients received a Re-TURB within six to eight weeks after the initial TURB. All patients were treated with a bladder-preserving approach. Tissue from formalin-fixed paraffin embedded (FFPE)
tumor samples from all patients was evaluated for pathological stage according to the 2010 TNM classification [40], and was graded according to the common grading systems [41,42] by two experienced uropathologists (M.E., A.H.). All specimens contained at least 20% tumor cells. All procedures were performed in accordance with the ethical standards established in the 1964 Declaration of Helsinki and its later amendments. All patients treated after 2008 provided informed consent. For samples collected prior to 2008, the Ethics Committee in Erlangen waived the need for informed individual consent. This study was approved by the Ethics Committee of the University Hospital Erlangen (No. 3755; 2008).

**Table 6. Clinicopathological and survival data.**

| Clinicopathological and Survival Parameters | Patients (Percentage) |
|--------------------------------------------|-----------------------|
| Total                                      | 80                    |
| Gender                                     |                       |
| female                                     | 19 (23.7)             |
| male                                       | 61 (76.3)             |
| Age (years)                                |                       |
| range                                      | 46.0–97.0             |
| mean                                       | 70.5                  |
| median                                     | 71.5                  |
| Tumor Stage                                |                       |
| pT1                                        | 52 (65.0)             |
| pT1 with cis                               | 28 (35.0)             |
| Tumor Grade 1973                           |                       |
| G1                                         | 3 (3.7)               |
| G2                                         | 28 (35.0)             |
| G3                                         | 48 (60.0)             |
| unknown                                    | 1 (1.3)               |
| Tumor Grade 2004                           |                       |
| low grade                                  | 3 (3.7)               |
| high grade                                 | 76 (95.0)             |
| unknown                                    | 1 (1.3)               |
| Intravesical Therapy                       |                       |
| yes                                        | 41 (51.3)             |
| no                                         | 39 (48.7)             |
| Survival/observation Time (months)         |                       |
| range                                      | 0–189.0               |
| mean                                       | 71.6                  |
| median                                     | 62.0                  |
| Overall Survival (OS)                      |                       |
| alive                                      | 44 (55.0)             |
| dead                                       | 36 (45.0)             |
| Disease-Specific Survival (DSS)            |                       |
| alive                                      | 64 (80.0)             |
| dead                                       | 16 (20.0)             |
| Recurrence-Free Survival Time (months)     |                       |
| range                                      | 0–149                 |
| mean                                       | 46.7                  |
| median                                     | 38.5                  |
| Recurrence-Free Survival (RFS)             |                       |
| without recurrence                         | 39 (48.7)             |
| with recurrence                            | 41 (51.3)             |
4.2. Assessment of mRNA by qRT-PCR

Tumor specimens were assessed by qRT-PCR as previously described [43]. In short, RNA was extracted from a single 10 μm curl of FFPE tissue and processed according to a commercially available bead-based extraction method (Xtract kit; Stratifyer Molecular Pathology GmbH, Cologne, Germany). RNA was eluted with 100 μL of elution buffer. DNA was digested, and RNA eluates were then stored at −80 °C until use.

The mRNA levels of CXCL9, PD1, PD-L1, KRT5, KRT20, Kl67 and the reference genes Calmodulin2 (CALM2) and Beta-2 microglobulin (B2 M) were determined by a one-step qRT-PCR using the SuperScript III RT-qPCR system (Invitrogen, Waltham, MA, USA) and gene specific primer-probe combinations (Stratifyer). Each patient sample or control was analyzed in duplicate in an ABI Step One PCR System (ThermoFisher, Darmstadt, Germany) according to the manufacturers’ instructions. Gene expression was quantified with a modification of the method by Schmittgen and Livak by calculating \( 40 - \Delta \text{Ct} \), whereas \( \Delta \text{Ct} \) was calculated as the difference in Ct between the test gene and the mean of the reference genes [38,44].

4.3. Statistical Methods

Correlations between the mRNA of CXCL9, PD1, PD-L1, KRT5, KRT20 and Kl67 and clinicopathological data were calculated using Spearman’s bivariate correlation. Optimized cut-off values for dichotomizing each marker with respect to survival were defined using Youden’s index on the receiver operating characteristic (ROC). Detailed information about the calculated optimal cut-off values, the associated area under the ROC curve and internal validation using bootstrapping are provided in Tables S1 and S2. Following standard practice in retrospective survival analysis, the common time point zero for all patients was the date of the first TURB. The associations of mRNA with recurrence-free survival (RFS), overall survival (OS) and cancer-specific survival (CSS) were determined by univariate (Kaplan–Meier analysis and Cox’s regression hazard models) and multivariate (Cox’s regression hazard models, adjusted for age and the molecular parameters PD1, PD-L1 and CXCL9) analyses. A \( p \)-value < 0.05 was considered statistically significant. Statistical analyses were performed with the SPSS 21.0 software package (SPSS Inc., Chicago, IL, USA) and R V3.2.1 (The R foundation for statistical computing, Vienna, Austria).

5. Conclusions

Altogether, we confirmed that high PD-L1 mRNA is associated with increased DSS and RFS. Furthermore, we demonstrated for the first time that CXCL9 mRNA is associated with a longer OS, DSS and RFS. Associations with RFS were also identified or further pinpointed to special groups, including the younger age group (CXCL9, PD1), the high KRT5 or high KRT20 group (CXCL9, PD-L1), the high Kl67 group (CXCL9, PD-L1) or the no instillation group (CXCL9, PD-L1).

An increased mRNA for PD1, PD-L1 and CXCL9 being associated with a better prognosis may mirror the host–tumor interaction. In this way, we suggest that the increased mRNA levels of all three genes may reflect the immune response of the host.

Our finding of associations between these immune markers and prognosis may aid in future therapeutic options and decisions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/10/2794/s1. Table S1: Optimized Ct cutoff values and internal validation and Table S2: Area under the ROC curve and internal validation.

Author Contributions: D.S., H.T., S.W., R.M.W. and B.K. designed the study. D.S., J.K., S.W., V.W., R.S., A.H. and B.W. acquired the clinical samples and patient information. A.H. and M.E. performed the pathological review of all cases. J.K. and A.N. performed qRT-PCR experiments. H.T., S.W., D.S. and J.K. performed statistical analyses, and H.T., S.W., J.K., D.S., M.E. prepared the tables and figures. H.T., S.W., D.S., B.W., M.E. and A.H. wrote the main manuscript. All authors reviewed the manuscript and approved the final version of the manuscript.
**Funding:** This study was funded by the ELAN Fund (ELAN 18’C08-18’C1-Sikic) and was supported by the Interdisciplinary Center for Clinical Research (IZKF) at the University Hospital of the Friedrich-Alexander University Erlangen-Nuremberg. We thank the Rudolf und Irmgard Kleinknecht-Stiftung for supporting H.T., and the Johannes und Frieda Marohn-Stiftung and the Wilhelm Sander-Stiftung for supporting S.W. and H.T.

**Acknowledgments:** The present work was performed in (partial) fulfillment of the requirements for obtaining the degree “Dr. med.” (M.D.) of the Friedrich-Alexander-Universität Erlangen-Nürnberg, Medizinische Fakultät for Jennifer Kubon. The authors thank Johannes Breyer (University of Regensburg) and Philipp Erben (Heidelberg University) for helpful discussion. We thank American Journal Experts for editing the manuscript. The authors also acknowledge support from Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg within the funding program Open Access Publishing.

**Conflicts of Interest:** The authors declare that there are no financial and/or nonfinancial conflicts of interest.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| BCa          | bladder cancer |
| CXCL9        | Chemokine, CXC motif, ligand 9 |
| DSS          | disease-free survival |
| Fu recur     | follow up recurrence |
| Ki67         | Proliferation marker Ki67 |
| KRT5         | Cytokeratin 5 |
| KRT20        | Cytokeratin 20 |
| MIBC         | muscle invasive bladder cancer |
| NMIBC        | non-muscle invasive bladder cancer |
| OS           | overall survival |
| n.s.         | not significant |
| n.d.         | not determined |
| PD1          | programmed cell death 1 |
| PD-L1        | programmed cell death ligand 1 |
| PFS          | progression-free survival |
| pT           | pathological tumor stage |
| pN           | pathological lymph node stage |
| qRT-PCR      | quantitative real-time PCR |
| RFS          | recurrence-free survival |

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