Identification of C16orf74 as a Marker of Progression in Primary Non-Muscle Invasive Bladder Cancer

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Abstract

Purpose: Methylation-induced silencing of PRSS3 has been shown to be significantly associated with invasive bladder cancer, and expression of the C16orf74 gene locus has been shown to correlate positively with PRSS3. The aim of the current study was to evaluate the relationship between C16orf74 expression level and progression in non-muscle invasive bladder cancer (NMIBC).

Materials and Methods: C16orf74 mRNA levels were examined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of 193 tumor specimens from patients with primary NMIBC. Expression data were analyzed in terms of clinical and experimental parameters. Kaplan-Meier curves and multivariate Cox regression models, respectively, were used to determine progression-free survival and to identify independent predictive parameters of progression.

Results: Analysis using Kaplan-Meier curves revealed prolonged progression-free survival of high-C16orf74-expressors as compared to low-expressors (p < 0.001). Multivariate Cox regression analysis revealed that low C16orf74 mRNA expression levels are a significant risk factor for disease progression in patients with primary NMIBC (HR: 10.042, CI: 2.699–37.360, p = 0.001).

Conclusions: Decreased expression of C16orf74 correlates significantly with progression in primary NMIBC. C16orf74 expression level represents a potentially useful marker for predicting progression in primary NMIBC patients.

Introduction

More than 90% of bladder cancers are transitional cell carcinomas, and most are papillary, well-, or moderately-differentiated non-muscle invasive bladder cancer (NMIBC) [1–2]. After endoscopic resection, cancer recurrence occurs in the majority (50–70%) of patients with NMIBC [3]. Approximately 20% of these patients subsequently experience disease progression to muscle invasive bladder cancer (MIBC) after appropriate treatment, including transurethral resection (TUR) and intravesical therapy with epirubicin, mitomycin-C, or Bacillus Calmette-Guerin (BCG) [1–2]. Thus, frequent recurrence after TUR and subsequent cancer progression are problematic for patients and urologists alike. Almost 25% of newly diagnosed bladder cancer patients have MIBC, and the vast majority of these cases are of high histological grade. Nearly 50% of patients with MIBC already have occult distant metastases at the time of diagnosis [1–2].

A number of potential tumor markers have been identified for bladder cancer, but few have demonstrated efficacy in terms of predicting disease recurrence and progression. However, several recent studies have suggested that the suppressor genes p53, RASSF1A, and PRSS3 are closely associated with the development and progression of bladder cancer [1–7]. Specifically, RASSF1A and PRSS3 promoter methylation is associated with advanced tumor stage [7], which suggests that these genes might be associated with bladder cancer progression. PRSS3 in turn has been shown to be positively associated with C16orf74 expression [8].

The C16orf74 (MGC17624) gene locus is on chromosome 16q24.1, and its function has yet to be characterized. The results of several genome-wide studies have indicated that C16orf74 is involved in inflammatory processes. Tumor necrosis factor (TNF)-α is a key regulator of the inflammatory cascade in chronic inflammatory diseases, and in patients with inflammatory disease, C16orf74 is strongly associated with an anti-TNF response [9]. C16orf74 is a hypoxia regulated gene [10–11]. Winter et al. [10] reported that C16orf74 median RNA expression level is an independent prognostic factor for recurrence-free survival in head
and neck cancer. C16orf74 has also been shown to be upregulated in lymph node-positive metastases in patients with oral tongue squamous cell carcinoma [12], and to correlate positively with PRSS3 expression in breast cancer [8].

Recently, we reported the identification of a progression-related gene classifier that had strong predictive value in terms of disease outcomes in NMIBC [13]. In that study, C16orf74 was one of eight candidate genes identified for predicting disease progression in NMIBC, suggesting a potential relationship between bladder cancer and C16orf74. In the current study, we assessed the relationship between C16orf74 and NMIBC outcomes using data from a previous study population as well as new cases, all with long-term follow-up.

Results

1. Baseline characteristics
The mean age of the 193 subjects with primary NMIBC was 64.1 ± 14.0 years, and the median follow-up period was 44.9 months. Seventy-one patients (36.8%) experienced recurrence and 20 (10.4%) experienced progression. Other baseline characteristics of the patients are presented in Table 1.

2. The value of C16orf74 mRNA expression level as a prognostic marker for progression
The relationship between C16orf74 mRNA expression level and time to progression was analyzed. Using a ROC curve, a cutoff value (11.7784) for progression with the highest combined sensitivity (53.2%) and specificity (85%) was determined. Time to progression was significantly different between the high and low C16orf74 mRNA expression groups, in that time to progression in the high C16orf74 expression group was significantly longer than the low expression group (p < 0.001) (Fig. 1). In univariate Cox regression analysis of several clinicopathological variables (age, sex, tumor size, number, grade, stage, intravesical therapy, and C16orf74 mRNA expression levels), age, intravesical therapy and C16orf74 mRNA expression levels were significant risk factors for progression (p = 0.031, p = 0.034 and p < 0.001, respectively). In multivariate Cox regression analysis, age and low C16orf74 mRNA expression levels were significant risk factors for progression-free survival in patients with primary NMIBC (HR: 1.049, CI: 1.005–1.094, p = 0.030; and HR: 10.042, CI: 2.699–37.360, p = 0.001, respectively) (Table 2). In multivariate Cox regression analysis in patients with intravesical therapy, age and low C16orf74 mRNA expression levels were significant risk factors for progression-free survival in patients with primary NMIBC with intravesical therapy (HR: 1.055, CI: 1.005–1.108, p = 0.031; and HR: 14.170, CI: 2.719–73.837, p = 0.002, respectively).

Discussion
Trypsin is a member of the serine protease family encoded by three trypsinogen genes including PRSS1, PRSS2 and PRSS3 encode trypsinogen I, trypsionogen II, and trypsinogen IV (also known as mesotrypsinogen), respectively [14–16]. This enzyme has been known as a potent proteolytic enzyme that can destroy tissue [17–18]. There are conflicting reports in the literature of the role of trypsin or PRSS3 in tumor progression, with some studies

Table 1. Baseline characteristics of primary non-muscle invasive bladder cancer patients.

| Variables | Incidence or mean value (%) |
|-----------|-----------------------------|
| Age (years) | 64.1 ± 14.0 |
| Median follow up periods (months) | 44.9 |
| Gender | | |
| Male | 157 (81.3) |
| Female | 36 (18.7) |
| Size | | |
| ≤ 3 cm | 109 (56.5) |
| > 3 cm | 84 (43.5) |
| Number | | |
| Single | 111 (57.5) |
| Multiple | 82 (42.5) |
| Grade | | |
| G1 | 67 (34.7) |
| G2 | 101 (52.3) |
| G3 | 25 (13.0) |
| Stage | | |
| Ta | 71 (36.8) |
| T1 | 122 (63.2) |
| Intravesical therapy | | |
| No | 80 (41.5) |
| Yes | 113 (58.5) |
| Recurrence | | |
| No | 122 (63.2) |
| Yes | 71 (36.8) |
| Progression | | |
| No | 173 (89.6) |
| Yes | 20 (10.4) |

Figure 1. Time to progression in primary NMIBC patients according to C16orf74 mRNA expression levels. doi:10.1371/journal.pone.0015260.g001
assigning a positive role [19–23], while others have reported that trypsin or PRSS3 plays a tumor suppressive role. The expression of PRSS3 is reduced in bladder, esophageal, and gastric cancers, and loss of PRSS3 expression is due to epigenetic silencing through promoter hypermethylation [7,24–25]. In particular, silencing of PRSS3 by promoter methylation has been significantly associated with invasive tumor stage in bladder cancer [7].

The expression of C16orf74 has been shown to correlate positively with PRSS3. Hockla et al. [8] reported that C16orf74 is down regulated by knockdown of PRSS3 and upregulated by mesotrypsin treatment. To date, there have been no reports of an association of C16orf74 with bladder cancer, except as indicated in C16orf74 [9,26].

In the current study, we investigated the mRNA expression levels of C16orf74 in human primary NMIBC tissues in a relatively large population with a long-term follow up period, along with several known clinical risk factors, including age, tumor size, number of tumors, T-category, tumor grade, and intravesical therapy [30–31]. These aspects of the study design lend strength to the results, and strongly suggest that C16orf74 may be a clinically useful predictor of progression in primary NMIBC.

In conclusion, decreased expression of C16orf74 was significantly associated with disease progression in primary NMIBC patients, suggesting that C16orf74 has a tumor suppressive role, similar to p53, RUNX3 and PRSS3, in disease progression. To date, the function of C16orf74 is unknown, and additional studies are needed to define the precise pathway by which C16orf74 influences progression in primary NMIBC.

Table 2. Multivariate Cox regression analysis for prediction of progression in NMIBC and in NMIBC with intravesical therapy.

| Variables            | Total Patients (N = 193) | Multivariate Cox regression | Intravesical Tx. Pt. (N = 113) |
|----------------------|--------------------------|-----------------------------|---------------------------------|
|                      | Univariate Cox regression | Multivariate Cox regression | Multivariate Cox regression     |
|                      | HR (95% CI) p-value       | HR (95% CI) p-value         | HR (95% CI) p-value             |
| Age (years)          | 1.043 (1.004–1.083) 0.031 | 1.049 (1.005–1.094) 0.030    | 1.055 (1.005–1.108) 0.031       |
| Sex (male vs. female)| 0.627 (0.145–2.706) 0.531 | 0.398 (0.078–2.035) 0.268    | 0.780 (0.140–4.352) 0.777       |
| Size (<3 cm vs. ≥3 cm)| 1.912 (0.781–4.680) 0.156 | 2.222 (0.808–6.109) 0.122    | 1.801 (0.560–5.793) 0.324       |
| Number (Single vs. multiple)| 1.885 (0.779–4.562) 0.160 | 1.391 (0.522–3.706) 0.509    | 1.424 (0.456–4.452) 0.543       |
| Grade                | 2.076 (0.989–4.360) 0.054 |                                | 0.586                           |
| G1                   | 1 - 1 -                  | 1 - 1 -                      |
| G2                   | 2.143 (0.698–6.574) 0.183 | 0.601 (0.130–2.785) 0.515    | 0.337 (0.042–2.683) 0.304       |
| G3                   | 4.279 (0.942–19.428) 0.060 | 0.735 (0.090–5.998) 0.774    | 0.317 (0.024–4.269) 0.386       |
| Stage (Ta vs. T1)    | 1.294 (0.470–3.565) 0.618 | 0.608 (0.168–2.195) 0.447    | 0.865 (0.145–5.151) 0.874       |
| Intravesical Tx. (No vs. Yes)| 3.765 (1.102–12.863) 0.034 | 2.840 (0.666–12.109) 0.158 | -                               |
| C16orf74 (high vs. low)| 8.940 (2.614–30.576) <0.001 | 10.042 (2.699–37.360) 0.001 | 14.170 (2.719–73.837) 0.002     |

Tx: Therapy; Pt: patients; HR: hazards ratio; CI: confidence interval.

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Materials and Methods

1. Ethics Statement

The Ethics Committee of Chungbuk National University approved this protocol, and written informed consent was obtained from each subject. Collection and analysis of all samples was approved by the Institutional Review Board of Chungbuk National University.

2. Patients and Tissue Samples

Primary NMIBC samples from patients with histologically-verified transitional cell carcinoma obtained at our institute were used for the current study. Patients with concomitant carcinoma in situ (CIS) or a short term follow-up period (less than 6 months), and those that underwent radical cystectomy or for whom there was incomplete data collection, were excluded to make the study population more homogeneous. A total of 193 primary NMIBC samples were analyzed.
All tumors were macrodissected, typically within 15 minutes of surgical resection. Each bladder cancer specimen was confirmed by pathological analysis of a part of the tissue sample in fresh frozen sections from TUR specimens, and was then frozen in liquid nitrogen and stored at −80°C until use. A second TUR was performed 2–4 weeks after the initial resection when a bladder cancer specimen did not include proper muscle or when high-grade tumor was detected [32]. Patients who had a T1 tumor, multiple tumors, large tumors ($\geq 3$ cm in diameter), or high grade Ta NMIBC received one cycle of intravesical treatment (BCG or mitomycin-C) [26,32]. If a patient refused intravesical therapy, it was not administered after TUR. Response to treatment was assessed by cystoscopy and urinary cytology. Patients who were free of disease within 3 months after treatment were assessed every 3 months for the first 2 years and then every 6 months thereafter [26,32]. Tumors were staged and graded according to the 2002 TNM classification and the 1973 WHO grading system, respectively [32–33]. Recurrence was defined as recurrence of primary NMIBC with a lower or the same pathological stage, and progression was defined as disease with a higher TNM stage upon relapse.

3. RNA extraction and construction of cDNA

RNA was isolated from tissue using 1 ml of TRIzol (Invitrogen, Carlsbad, CA) and homogenization in a 5-ml glass tube. The homogenate was transferred to a 1.5-ml tube and then mixed with 200 μl of chloroform. After incubation for 5 min at 4°C, the homogenate was centrifuged for 15 minutes (min) at 13,000 xg at 4°C. The upper aqueous phase was transferred to a clean tube and then 500 μl of isopropanol were added. The mixture was incubated for 60 min at 4°C, and then the tube was subjected to centrifugation for 8 min at 13,000 xg, 4°C. The upper aqueous phase was discarded and mixed with 500 μl of 75% ethanol, and then centrifuged for 5 min at 13,000 xg, 4°C. After discarding the upper aqueous layer, the pellet was dried at room temperature, dissolved in diethylpyrocarbonate (DEPC)-treated water, and then stored at −80°C until use. A second TUR was performed using 2 X SYBR premix EX Taq buffer, 0.5 μl each of 5' - primer (10 pmol/μl), and 1 μl of the sample cDNA. The product was purified with a QIAquick Extraction kit (QIAGEN, Hilden, Germany), quantified with a spectrophotometer (Perkin Elmer MMA2000, Fremont, CA), and then sequenced. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous RNA reference gene and gene expression was normalized to the expression of GAPDH.

4. Real-time PCR

Real-time PCR amplification was performed using a Rotor Gene 6000 instrument (Corbett Research, Mortlake, Australia) to quantify the expression of C16orf74. Real-time PCR assays were carried out in micro-reaction tubes (Corbett Research, Mortlake, Australia) using SYBR Premix EX Taq (TAKARA BIO INC., Otsu, Japan). The primers used for amplification were C16orf74 (179 basepairs) sense (5'-TAT GTG TGT CAG CAG CA-3') and anti-sense (5'-TTC CAT CAT GTG GGC AGC-3'). The PCR reaction was performed in a final volume of 10 μl consisting of 5 μl of 2 X SYBR premix EX Taq buffer, 0.5 μl each of 5' - and 3' - primer (10 pmol/μl), and 1 μl of the sample cDNA. The product was purified with a QIAquick Extraction kit (QIAGEN, Hilden, Germany), quantified with a spectrophotometer (Perkin Elmer MMA2000, Fremont, CA), and then sequenced. The PCR conditions were as follows: 1 cycle for 20 seconds (sec) at 96°C, followed by 40 cycles of 2 sec at 96°C for denaturation, 15 sec at 60°C for annealing, and 15 sec at 72°C for extension. The melting program was performed at 72–95°C with a heating rate of 1°C per 45 sec. Spectral data were captured and analyzed using Rotor-Gene Real-Time Analysis Software 6.0 Build 14 (Corbett Research, Mortlake, Australia). All samples were run in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as an endogenous RNA reference gene and gene expression was normalized to the expression of GAPDH.

5. Statistical analysis

To normalize the highly skewed distribution of mRNA expression levels of C16orf74, the data were natural log transformed and then back transformed for the interpretation of the results [34]. Receiver operating characteristics (ROC) curves were used to determine the optimal cutoff point of the mRNA level that yielded the highest combined sensitivity and specificity for progression. Using these values, patients were classified into high or low C16orf74 expression groups. The Kaplan-Meier method was used to estimate time to progression, and differences were assessed using log-rank statistics. The prognostic value of C16orf74 in terms of progression was analyzed using multivariate Cox proportional hazard regression models. Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, IL), and a p value of <0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: WTK SJY WJK. Performed the experiments: WTK SJY CP. Analyzed the data: WTK SJY. Contributed reagents/materials/analysis tools: IYK SKM TGK YHC. Wrote the paper: WTK SJY WJK. Supervised this study: IYK SKM TGK YHC.

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