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Original Article

Association Between Positive iNOS mRNA Expression and Recurrence-free Survival Among Patients with Non-muscle-invasive Bladder Cancer

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Abstract

Objective: Nitric oxide synthase is the key enzyme of the conversion of L-arginine to L-citrulline and nitric oxide. We conducted a study to investigate the association between positive inducible nitric oxide synthase (iNOS) mRNA expression and recurrence of bladder cancer.

Patients and Methods: Seventy-one patients with primary non-muscle-invasive bladder cancer were enrolled in this study. Tumor tissues were harvested during transurethral resection of bladder tumors. All operations were performed at the same hospital. Recurrence-free patients were followed up for at least 1 year unless tumors recurred during that time. The median intervals of follow-up were 34 months in the recurrence-free group and 12 months in the recurrence group. iNOS mRNA was detected using the RT-PCR-based method.

Results: Bladder cancer patients with positive iNOS mRNA expression had a higher recurrence risk (18.7% *vs.* 2.6%; $p=0.04$). After adjusting for other risk factors, a statistical significance remained ($p=0.04$; hazards ratio=13.27; 95% CI=1.07–164.36). The patients also had reduced recurrence-free survival ($p=0.019$).

Conclusion: The positive expression of iNOS mRNA may be a useful prognostic indicator of bladder cancer. (*Tzu Chi Med J* 2008;20(2):119–124)

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1. Introduction

The incidence of bladder cancer was ranked tenth among all cancer cases in Taiwan in 2000 (1). Significantly higher incidence rates of bladder cancer, 26.1% in men and 21.1% in women per 100,000 persons, have been observed in the endemic area of blackfoot disease (BFD) in southern Taiwan (2,3). Bladder cancer is a common urological malignancy with a recurrence rate of approximately 70% (4).

Nitric oxide synthase (NOS) is the key enzyme for the conversion of L-arginine to L-citrulline and nitric

oxide (NO) [5,6]. The NOS family consists of endothelial, neuronal, and inducible nitric oxide synthases (eNOS, nNOS, and iNOS, respectively) [7]. iNOS genes, located on the human chromosome 17, can be induced by lipopolysaccharide, cytokines in macrophages, or tumor-related immune reactions [8–10]. Swana et al reported that iNOS was detected in human bladder cancer tissues but not in normal bladder tissues, and that it was found in macrophages and neutrophils of bladder cancer tissues and some tumor cells [11]. To our knowledge, only one group of researchers has investigated the association between iNOS and bladder cancer recurrence, demonstrating a high but not significant risk of recurrence among patients with iNOS expression [12]. Therefore, the aim of this study was to elucidate the association between iNOS messenger ribonucleic acid (mRNA) expression and the recurrence of bladder cancer.

2. Patients and methods

2.1. Patients and tissue collection

From November 2000 through December 2003, patients with primary non-muscle-invasive bladder cancer were enrolled. These cases were newly diagnosed and pathology was confirmed. Patients who had combined urothelial tumors in the upper urinary tract were excluded. Tumor tissues were harvested by transurethral resection of bladder tumors (TURBT) performed at a single hospital. Specimens were immediately frozen in liquid nitrogen after TURBT, and were stored at –86°C in a tissue bank. Representative sections of each frozen block were embedded in paraffin and stained with hematoxylin-eosin. All specimens were graded according to a modification of the World Health Organization classification and pathological staging was based on the TNM pathological staging system [13,14]. All patients received weekly bacillus Calmette-Guerin (BCG) intravesical immunotherapy for 6 weeks. There was no perioperative intravesical chemotherapy. All patients underwent cystoscopy and urine cytology every 3 months for 24 months after the initial tumor resection, every 6 months for 2 years following that, and annually thereafter [15]. All recurrence-free patients were followed up for at least 12 months unless tumors recurred during that time. The study was approved by the Ethics Committee of the participating hospital.

2.2. RNA isolation

The size of each specimen was approximately 1 cm^3. The RNA isolation system (Biotecx Laboratories Inc., Houston, TX, USA) was applied as previously described [16]. RNA concentrations were determined using spectrophotometric analysis (1 OD of A260 equal to 40 μg/mL RNA). The purity of extraction was assessed using the A260/280 ratio, which was >1.7 in all specimens.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) with human iNOS primers

Single-strand complementary DNA (cDNA) was synthesized using oligo-dT priming of 2 μg of total RNA with 1 μL (200 U/L) Superscript II reverse transcriptase (Promega Inc., Madison, WI, USA) for 1 hour at 42°C, and then the reaction was stopped by denaturation at 70°C for 15 minutes. The primer pairs used were the sense primer 5′-ATTCACTCAGCTGTGCATCG-3′ and the antisense primer 5′-CAGCATACAGGCAAAGAGCA-3′. RT-PCR yielded 730-bp fragments. Controls for cDNA synthesis were β-actin specific primers and generated 307-bp of PCR product. Positive iNOS controls were from human liver cells [11]. As a negative control, PCR was performed in the absence of primers and in the absence of cDNA. An amplification reaction was carried out using one-tenth of the reverse-transcribed RNA, Taq polymerase buffer containing 200 μmol/L deoxynucleotide triphosphate (dNTP), 1 U DyNAzyme II DNA Polymerase (Finnzymes Inc., Finland), and 10 μM of a pair of iNOS primers. PCR was performed for 35 cycles with denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1 minute. Amplified products and 0.1 μg GeneRuler 100 bp DNA Ladder (MBI Ferments, Lithuania) were electrophoresed on 1.2% agarose gels, and stained with ethidium bromide. All gels were photographed with the ImageMaster VDS video imaging system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), and documented using LISCAP Image Capture Software, version 1.0 (Amersham Pharmacia Biotech Inc.). At least two repeated analyses were carried out for each tissue. Each gel picture was read by two researchers.

2.4. Statistical analysis

iNOS mRNA expression was denoted as positive or negative. The χ^2 and Fisher's exact tests were applied to calculate the association between the recurrence of bladder cancer and the risk factors (gender, age, endemic area of BFD, tumor grade, tumor stage, iNOS mRNA expression). Additionally, Kaplan-Meier product limit estimates of survival and log-rank tests were used to analyze the data between iNOS expression and disease-free months before recurrence. Multivariate analysis was done using Cox's proportional hazard regression. The level of statistical significance was set at $p<0.05$ (two-sided). SPSS statistical package version

9.0 (SPSS Inc., Chicago, IL, USA) was used for the analyses.

3. Results

At the endpoint of the study, a total of 78 specimens from seven cases with recurrence and 64 recurrence-free cases were obtained. Medians for the duration of follow-up were 12 months (range, 4–32 months) in the recurrence group and 34 months (range, 12–49 months) in the recurrence-free group. Overall age at the first diagnosis was 67.9±12.5 years (mean±standard deviation). Table 1 shows increased recurrence risks among the men (12.5% *vs.* 4.3%), the elderly (13% *vs.* 4%), those with high grade tumors (50.5% in high grade *vs.* 8.7% in low grade), and those with stage Ta (13.6% in Ta *vs.* 3.7% in T1), as well as those in whom iNOS mRNA expression was detected (18.7% *vs.* 2.6%). Positive iNOS mRNA expression was the only risk factor that reached a significant difference ($p=0.04$).

Among the 13 residents from the BFD endemic area, a high risk population for bladder cancer [3], only one (7.7%) developed recurrence of bladder cancer. No association was shown between being a resident of the BFD endemic area and recurrence of bladder cancer ($p=0.77$; Table 1).

Multivariate analysis revealed that patients with high grades of bladder cancer had higher recurrence risks ($p=0.057$; hazards ratio=17.28; 95% CI=0.92–325.23; Table 2).

iNOS mRNA expression was detected in 45.1% (32/71) of patients. Recurrence of bladder cancer was found in six (18.7%) patients with positive iNOS mRNA expression and one (2.6%) patient without positive results. A significant difference was shown between the two groups ($p=0.04$; Table 1). For each patient with recurrent cancer, the RT-PCR product levels were consistent during the first diagnosis and recurrent tumor tissues. After adjusting for age, gender, BFD, and tumor grade, a statistical significance was reached ($p=0.044$; hazards ratio=13.27; 95% CI=1.07–164.36; Table 2). In addition, patients with positive expressions of iNOS mRNA had reduced recurrence-free survival ($p=0.019$; Fig. 1).

Calculating the data from Table 1, the sensitivity of positive expression of iNOS mRNA to predict the recurrence of bladder cancer was 85.7%, and the specificity was 59.4%.

4. Discussion

The present study was designed to identify the association between positive iNOS mRNA expression and

Table 1 — Relationship between various risk factors and recurrent bladder cancer*

	Bladder cancer ($n=7$) Yes	Recurrence ($n=64$) No	p
Age (yr)			0.42
≥65	6 (13.0)	40 (87.0)	
<65	1 (4.0)	24 (96.0)	
Gender			0.41
Male	6 (12.5)	42 (87.5)	
Female	1 (4.3)	22 (95.7)	
Live in BFD endemic area			0.77
Yes	1 (7.7)	12 (92.3)	
No	6 (10.3)	52 (89.7)	
Tumor cell type			
Transitional	7 (100)	64 (100)	
Non-transitional	0 (0)	0 (0)	
Tumor stage			0.17
Tis	0 (0)	0 (0)	
Ta	6 (13.6)	38 (86.4)	
T1	1 (3.7)	26 (96.3)	
Tumor grade			0.19
Low	6 (8.7)	63 (91.3)	
High	1 (50.0)	1 (50.0)	
iNOS mRNA expression			0.04
Positive	6 (18.7)	26 (81.3)	
Negative	1 (2.6)	38 (97.4)	

*Data presented as n (%). BFD = blackfoot disease.

Table 2 — Multivariate analysis of the association of various risk factors and recurrence of bladder cancer

Variable	Classification	*p*	HR (95% CI)
Age (yr)	≥65 *vs.* <65	0.706	1.42 (0.17–14.29)
Gender	Male *vs.* Female	0.389	2.60 (0.30–22.75)
Live in BFD endemic area	Yes *vs.* No	0.604	0.50 (0.04–6.85)
Tumor grade	High *vs.* Low	0.057	17.28 (0.92–325.23)
iNOS mRNA expression	Positive *vs.* Negative	0.044	13.27 (1.07–164.36)

HR = hazards ratio, estimated by Cox's proportional hazard model and adjusted for age, gender, resident of BFD endemic area, tumor grade, and iNOS mRNA; CI = confidence interval; BFD = blackfoot disease.

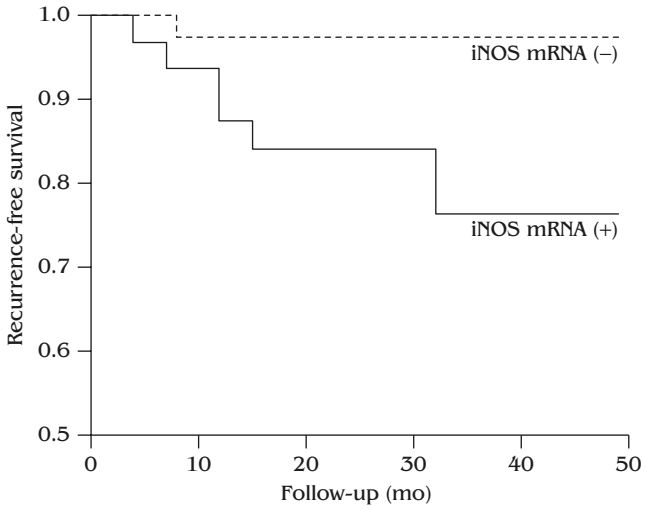

Fig. 1 — Recurrence-free survival of non-muscle-invasive bladder cancer patients with and without iNOS mRNA expression. A significant difference was reached (p=0.019, log-rank test).

the recurrence of bladder cancer. From our data, bladder cancer patients with positive iNOS mRNA expression had higher recurrence risks and reduced recurrence-free survival.

Some researchers reported that NOS was detected in malignant diseases, such as prostate cancer, gynecological cancer and breast cancer [17–19], and iNOS was not detected in those with colorectal cancer [20]. Only a few studies have provided evidence of the association between bladder cancer and iNOS or iNOS mRNA [10,11,21–24]. Moreover, only one published report included the recurrence of bladder cancer and iNOS [12].

Previously, researchers reported various detection rates of iNOS mRNA or iNOS in patients with primary bladder cancer, ranging from 50% to 100% [10–12, 22–24]. The results of our study showed that iNOS mRNA expression was detected in 45.1% of all bladder cancer patients. Most of the results from previously published studies had small sample sizes. In the study by Sandes and colleagues, 21 patients who were followed-up for 2 years had a rate of positive iNOS expression of 50% [12]. This detection rate was similar to our data.

The investigation by Sandes et al revealed that recurrences were found in 80% of subjects with positive iNOS and 27% of iNOS-negative patients [12]. In our study, recurrence rates (18.7% and 2.6%, respectively) were far lower than their's. However, we agree that patients with positive iNOS expression have markedly increased risks of recurrence of bladder cancer.

Based on the results of studies investigating bladder cancer and other malignancies, tumorigenesis of NO depends on the local concentrations [18,25–27]. Generally speaking, high concentrations of NO can cause tumor cell apoptosis. An example is provided by BCG immunotherapy for non-muscle-invasive bladder cancer patients. BCG can induce iNOS activity through certain cytokines in rat bladders [28]. For example, a patient who received BCG intravesical therapy produced a 30-fold increase of gaseous NO in his bladder [21,29].

On the other hand, low levels or deficiency of NOS activity can induce tumorigenic action such as neovascularity [10,17]. For example, iNOS is not found in patients with colon cancer [30]. Thomsen et al showed that the concentration of NO generated by NOS *in vivo* was too low to cause apoptosis and cytotoxicity [17]. The results of other studies pointed out that tumor angiogenic factors, such as vascular endothelial growth factor, requires NO/guanylate cyclase/cyclic guanosine monophosphate (cGMP) pathway to promote neovascular growth [31–35]. Lin et al reported higher microvessel density in bladder cancer tissues with positive iNOS than in tissues with negative iNOS [10]. These findings support that iNOS and NO promote tumor angiogenesis through the NO/cGMP pathway.

Becker found that tumor cells produced cytokines, such as interleukin (IL)-4 and IL-10, to suppress the NOS gene, and could be induced by interferon-γ, OK432 and lipopolysaccharide [36]. The hypothesis demonstrated that a low concentration of NO was modulated by tumor-related immunoactivity and inhibited apoptosis in tumor cells.

As shown in the discussion above, the presence of NOS activity caused anti- or pro-tumor action by its activity level. Based on our qualitative data of the RT-PCR product, we did not find evidence to clarify the relationship between tumor recurrence and levels of iNOS activity. Larger quantitative studies are necessary to

investigate the relationship. Additionally, because higher recurrence risks were observed in the group with positive iNOS mRNA expression, these cases will be intensively followed.

In the present study, the recurrence rate was 9.86% for patients with bladder cancer treated using TURBT and BCG intravesical immunotherapy. Previous researchers demonstrated that BCG reduced tumor recurrence rate to 11–27% in those with TURBT and BCG immunotherapy (37). However, the rate was not reliable in a small size study. Our lower recurrence rate (3.7%) in stage T1 may be attributed to the small size and to chance.

5. Conclusion

Bladder cancer patients with positive expression of iNOS mRNA had higher recurrence risks of bladder cancer and reduced recurrence-free survival. Positive iNOS mRNA expression may be a useful prognostic indicator of bladder cancer.

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