Pathological significance and prognostic implications of heme oxygenase 1 expression in non-muscle-invasive bladder cancer: Correlation with cell proliferation, angiogenesis, lymphangiogenesis and expression of VEGFs and COX-2

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Abstract. Heme oxygenase 1 (HO-1) is a stress-response protein and its expression is associated with malignant potential and poor prognosis in several types of cancer. The present study investigated the association between HO-1 expression levels and the pathological features, clinical outcomes and other associated factors in patients with non-muscle-invasive bladder cancer (NMIBC). HO-1 expression was evaluated using immunohistochemistry in 147 formalin-fixed tissue specimens. The proliferation index, microvessel density, lymph vessel density and expression of cyclooxygenase (COX)-2 and vascular endothelial growth factor (VEGF)-A, -C, and -D were also investigated. Correlations among variables were analyzed by multivariate analysis. Survival was assessed using Kaplan-Meier survival curves and multivariate statistics. HO-1 expression levels in high-grade and pT1 tumors were significantly higher compared with low-grade and pTa tumors, and were correlated with the proliferation index ($P<0.001$), lymph vessel density ($P=0.021$) and COX-2 expression levels ($P=0.003$). The proliferation index and COX-2 expression levels were also identified as independent contributing factors in multivariate models. Kaplan-Meier survival curves associated HO-1 expression with a poor prognosis in metastasis-free ($P=0.047$) and cause-specific survival ($P=0.017$), but not with urinary tract recurrence ($P=0.231$). Furthermore, HO-1 expression was identified by multivariate analysis to be a significant predictor for cause-specific survival (hazard ratio, 4.08; 95% confidence interval, 1.06-15.66; $P=0.004$). HO-1 has an important role in the malignant aggressiveness of NMIBC and its expression is associated with cause-specific survival. HO-1-associated activities are regulated by cancer cell proliferation, lymphangiogenesis and COX-2. The results suggest that HO-1 may be a potential therapeutic target and a useful predictive prognostic factor in patients with NMIBC.

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Introduction

Bladder cancer is a common malignancy in elderly individuals, particularly in industrialized countries. The standard treatment for early-stage bladder cancer, including non-muscle-invasive bladder cancer (NMIBC), is transurethral resection. However, these tumors have previously been observed to recur and progress to muscle-invasive phenotypes, which then require a radical cystectomy (1). Furthermore, ~50% of the patients who receive this procedure may subsequently experience local recurrence and metastasis, which are potentially lethal (2). Early detection of malignant potential is essential, as invasive and non-invasive tumors are treated, monitored and managed in different ways, and ~80% of patients present with non-invasive tumors at diagnosis (3).

At present, histopathological features, including grade and stage, are used as prognostic markers. In addition, various molecular and biological markers, such as survivin, interleukins, microRNAs, have also been reviewed (4,5). However, there are no clinically definitive markers for NMIBC, as tumor growth, invasion, metastasis and recurrence are regulated by complex underlying mechanisms. Tumor cell proliferation and vasculogenesis are associated with cancer progression and prognosis (6,7), as are various cancer-associated molecules, including cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) (8,9).

Heme oxygenase 1 (HO-1) is a microsomal enzyme that catalyzes the first, rate-limiting step in the degradation of heme (10). Although HO-1 has numerous functions under physiological conditions, a previous study demonstrated that it is induced in response to cellular stress and hypoxia (11).

Notably, HO-1 expression levels are elevated in various types of cancer (12-14) and have been demonstrated to regulate certain cancer-associated molecules (15,16). A previous study reported that HO-1 expression levels are significantly associated with malignant aggressiveness and prognosis in patients with bladder cancer (17). However, the prognostic value of HO-1 expression requires further investigation. Processes such as angiogenesis may be differently regulated in non-invasive and invasive bladder tumors (18).

The aim of the present study was to investigate and characterize the HO-1 expression profile in NMIBC cells, and compare it with the clinicopathological features, progression and outcomes of patients with NMIBC. In addition, the potential roles of HO-1 in other cancer processes, including cell proliferation, angiogenesis and lymphangiogenesis, in addition to its association with the expression levels of VEGF and COX-2, were investigated.

Materials and methods

Patients and tissue samples. Transurethral resection tissue samples from 147 patients with non-invasive bladder cancer, who were treated at Nagasaki University Hospital (Nagasaki, Japan) between 1993 and 2005, were formalin-fixed and paraffin-embedded, placed onto slides and analyzed. The patient population included 116 men (78.9%) and 31 women (21.1%), and their mean age at diagnosis was 68.6 years (standard deviation, 11.6 years). Patients who had previously received neo-adjuvant therapy were excluded, in addition to patients with carcinoma *in situ*, as these cases are challenging to analyze using immunostaining. Furthermore, cases of squamous cell carcinoma or adenocarcinoma were excluded. Patients were initially examined using chest radiography, ultrasonography, computed tomography of the urinary bladder and abdomen, and cystoscopy. In addition, computed tomography of the lungs or brain, magnetic resonance imaging, drip-infusion pyelography and bone scans were used as required. Stage and grade were assessed in accordance with the 2002 tumor-node-metastasis (TNM) classification (19) and the 2004 World Health Organization grading system (20). The median duration of follow-up was 51 months, with a range of 2-182 months.

The current study was conducted according to the Helsinki II declaration and was approved by the Ethics Review Committee of Nagasaki University Hospital (Nagasaki, Japan). Written informed consent was obtained from all patients prior to enrollment.

Immunohistochemistry. The following primary antibodies were utilized in the current study: Anti-HO-1 (Enzo Life Sciences Inc., Farmingdale, NY, USA; #ADI-SPA-896-F; dilution, 1:200); anti-Ki-67 (Dako, Glostrup, Denmark; #M7240; dilution, 1:100); anti-CD34 (Dako; #M7145); anti D2-40 (Dako; #M3619; dilution, 1:50); anti-VEGF-A (Santa Cruz Biotechnology Inc., Dallas, TX, USA; #sc-152; dilution, 1:120); anti-VEGF-C (Zymed; Thermo Fisher Scientific, Inc., Waltham, MA, USA; #18-2255; dilution, 1:70); anti-VEGF-D (R&D systems Europe, Ltd., Abingdon, UK; #MAB286; dilution, 1:100); and anti-COX-2 (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan; #18515; dilution, 1:40).

Tissue sections (thickness, 5 μm) were deparaffinized in xylene and rehydrated in graded solutions of ethanol. Antigens for the anti-Ki-67 antibody were retrieved at 121°C for 15 min in 0.01 M sodium citrate buffer (pH 6.0); all other antigens were retrieved at 95°C for 40 min. The tissue sections were immersed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The samples were then probed with the primary antibodies at 4°C overnight, then washed with 0.05% Tween 20 in phosphate-buffered saline. The tissue sections were labeled with peroxidase at room temperature for 60 min using Dako EnVision+™ Peroxidase (Dako; ready-to-use; anti-mouse, #K4001; anti-rabbit, #K4003). The peroxidase reaction was visualized with a 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Zymed; Thermo Fisher Scientific, Inc., Waltham, MA, USA; #00-2014) and the sections were counterstained with hematoxylin. Tumor sections stained with each antibody were examined using a Nikon E-400 bright-field microscope (Nikon, Tokyo, Japan).

A succeeding serial section from each paraffin-embedded tissue sample was processed without primary antibodies as a negative control. Immunohistochemical staining of the positive controls was performed as described previously for all antibodies (6,21-23). Positive control tissues (comprising resected specimens obtained from Nagasaki University Hospital) included samples from colon cancer (VEGF-A, -C and -D), renal cell carcinoma (COX-2), renal vein (CD34) and tonsil (D2-40 and Ki-67). Spleen tissue was used as positive control for HO-1 and the antibodies were used according to the manufacturer's instructions.

Evaluation and interpretation. To evaluate the expression levels of HO-1, immunostained tissue sections were scored semi-quantitatively, as previously described (24). Briefly, the specimens were assigned an immunoreactivity score, which was calculated by multiplying the staining intensity score (0, none; 1, weak; 2, moderate; 3, intense) with the score for the density of stained cells (0, <5.0%; 1, 5-25%; 2, 26-50%; 3, >51%). To determine the microvessel density (MVD) and lymph-vessel density (LVD), the tissue sections were stained with antibodies targeting CD34 and D2-40, respectively. For each tumor section, 3-5 fields with the highest density of stained vessels were evaluated, irrespective of their location in the tumor. MVD and LVD were defined as the number of stained vessels identified in each selected field at x200 magnification. The proliferation index (PI) was defined as the percentage of cells stained with anti-Ki-67. For these variables, scores greater than the median value were categorized as high, while scores below the median were considered low.

For the expression of VEGFs and COX-2, the staining intensity was graded as none, weak, moderate, or intense; moderate or intense staining was considered a positive reaction. When the percentages of positively stained cancer cells were higher than the median level, the specimen was consider to have high/positive expression for the purpose of survival analyses. Cells were counted in 3-7 randomly selected fields with ≥500 cancer cells.

Slides were examined on a computer-aided image analysis system (Win ROOF version 5.0; Mitani Corporation, Fukui, Japan) and were scored twice at various times by two

Table I. Association between HO-1 expression levels and clinicopathological features.

Category	All patients	HO-1 expression		P-value
		Negative	Positive	
Total number	147	75	72	-
Age, years; mean ± SD		66.9±13.0	70.5±11.5	0.077[a]
Gender, n (%)				0.377[b]
Male	116	57 (49.1)	59 (50.9)	
Female	31	18 (58.1)	13 (41.9)	
Grade, n (%)				0.040[b]
Low	76	45 (59.2)	31 (40.8)	
High	71	30 (42.3)	41 (57.7)	
pT stage, n (%)				0.031[b]
Ta	58	36 (62.1)	22 (37.9)	
T1	89	39 (43.8)	50 (56.2)	

[a]Analyzed by Student's t-test; [b]analyzed by χ^2 test. Samples with percentages of positively stained cancer cells ≤median and >median values were considered negative and positive, respectively. HO-1, heme oxygenase 1; SD, standard deviation; pT stage, pathological tumor stage.

independent pathologists who were blinded to the clinicopathological and survival data.

Statistical analysis. Normally distributed data are presented as the mean ± standard deviation, whereas the median value and interquartile range are presented for all other data. The Student's t-test and the Mann-Whitney U test were used to compare parametric and nonparametric continuous variables, respectively. A χ^2-test was used for the categorical comparison of normally distributed data. Survival was assessed using Kaplan-Meier analysis and a log-rank test. Variables that were statistically significant (P<0.05) in univariate analysis were subsequently subjected to multivariate analysis using Cox proportional hazards, and are reported as odds ratios with 95% confidence intervals and P-values. All statistical tests were two-sided and were performed using StatView software for Windows (version 5.0; Abacus Concepts, Berkeley, CA, USA). P<0.05 was considered to indicate a statistically significant result.

Results

HO-1 expression levels and clinicopathological features. In bladder cancer cells, HO-1 was expressed primarily in the cytoplasm (Fig. 1A). HO-1 expression was detected in fibroblast-like, tumor-infiltrating, and endothelial cells; however, there was no specific pattern regarding the distribution of these HO-1-positive cells (Fig. 1B). A total of 72/147 specimens (49.0%) were determined to have positive expression of HO-1. As indicated in Table I, the number of cells that were positively stained for HO-1 in high-grade tumors (41/71; 57.7%) was significantly higher (P=0.040) compared with low-grade tumors (31/76; 40.8%). Similarly, a positive association was demonstrated for the pathological tumor (pT) stage (P=0.031; Table I), however, HO-1 expression levels were not associated with patient age at diagnosis (P=0.077) or gender (P=0.377).
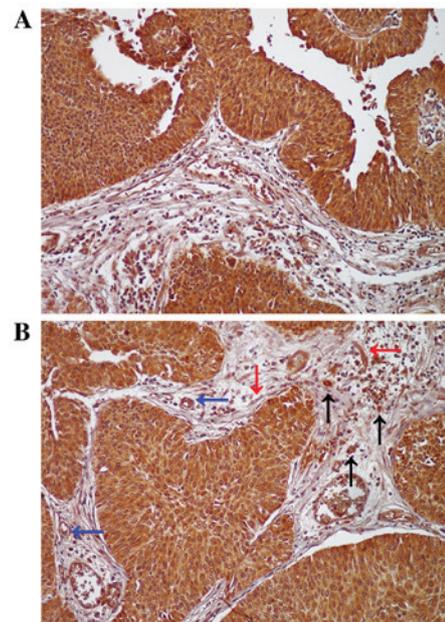

Figure 1. (A) In bladder cancer cells, HO-1 immunostaining was primarily detected in the cytoplasm. (B) In addition to cancer cells, HO-1 immunostaining was present in infiltrating cells (black arrows), fibroblast-like cells (red arrows), and endothelial cells (blue arrows). Magnification, x200. HO-1, heme oxygenase 1.

HO-1 expression and cancer-associated factors. The association of HO-1 expression levels with MVD, LVD and PI, and the expression profiles of COX-2, VEGF-A, -C, and -D, are depicted in Table II. HO-1 expression levels were associated with COX-2 expression (P=0.003), LVD (P=0.021) and PI (P<0.001), but not with VEGF-A (P=0.839) or VEGF-C (P=0.568). HO-1 expression levels were also associated with VEGF-D expression, although this was not determined to be significant (P=0.081). In a multivariate model that included pT stage and tumor grade, HO-1 expression was revealed to be

Table II. Association between HO-1 expression and cancer-associated factors.

Category	HO-1 expression			Multivariate analysis[b]		
	Negative	Positive	P-value[a]	OR	95% CI	P-value[c]
VEGF-A, %	30.8±13.0	31.2±12.9	0.839	-	-	-
VEGF-C, %	29.4±14.3	30.7±13.7	0.568	-	-	-
VEGF-D, %	29.6±12.9	33.3±13.3	0.081	-	-	-
COX-2, %	18.2±8.3	22.2±8.0	0.003	2.24	1.10-6.13	0.027
MVD, no./HPF	58.8±19.9	63.4±18.0	0.100	-	-	-
LVD, no./HPF	24.5±10.3	28.9±12.4	0.021	1.17	0.59-2.31	0.659
PI, %	19.8±7.0	24.1±7.9	<0.001	2.64	1.34-5.23	0.005

Data are presented as the mean ± standard deviation. [a]Analyzed by Student's *t*-test; [b]adjusted by pT stage and grade; [c]analyzed by Cox regression analysis. HO-1, heme oxygenase 1; OR, odds ratio; CI, confidence interval; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; MVD, microvessel density; LVD, lymph vessel density; PI, proliferation index; pT stage, pathological tumor stage; no./HPF, number per high-power field (x200 magnification).

Table III. Multivariate Cox regression analyses of clinicopathological variables with regard to metastasis and cause-specific survival.

Category	Metastasis			Cause-specific survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male vs. female)	2.88	0.37-22.61	0.314	1.98	0.25-15.77	0.517
pT stage (high vs. low)	1.32	0.40-4.47	0.649	2.63	0.70-9.93	0.153
Grade (high vs. low)	4.81	1.31-17.69	0.018	9.16	1.97-42.56	0.005
Adjuvant treatment (performed vs. not performed)	2.16	0.27-17.07	0.216	1.57	0.19-12.86	0.673
HO-1 (positive vs. negative)	2.17	0.66-7.11	0.217	4.08	1.06-15.66	0.040

HR, hazard ratio; CI, confidence interval; pT stage, pathological tumor stage; HO-1, heme oxygenase 1.

independently associated with COX-2 expression (P=0.027) and PI (P=0.005), but not with LVD (P=0.659; Table II).

HO-1 status and clinical outcomes. The Kaplan-Meier survival curves suggest that HO-1 expression levels were not associated with tumor recurrence in the urinary tract (P=0.231: Fig. 2A). However, patients with HO-1 positive tumors were identified to have a significantly increased risk of metastasis (P=0.047: Fig. 2B) and had poor cause-specific survival (P=0.017: Fig. 2C). In addition, HO-1 expression was demonstrated to be a significant and independent predictor of cause-specific survival (hazard ratio, 4.08; 95% confidence interval, 1.06-15.66; P=0.040), but not of metastasis, in a multivariate model that included clinicopathological features and adjuvant therapy (Table III).

Discussion

HO-1 was initially identified as a rate-limiting enzyme in the heme degradation pathway in microsomes (10). In addition to numerous physiological functions, HO-1 has important roles in various pathological conditions, including certain types of cancer. Increased expression levels of HO-1 were previously demonstrated to be associated with malignant potential, cancer cell dissemination and poor prognosis in renal cell, lung and gastric carcinomas (14,25,26). However, other studies have indicated that increased HO-1 expression levels are associated with favorable outcomes in colorectal (27) and tongue cancers (28). Therefore, the pathological significance and prognostic role of HO-1 are considered to be dependent on the type of cancer.

With regard to bladder cancer, a mouse xenograft model of bladder cancer demonstrated that inhibition of HO-1 expression decreased tumor size (29). In human tissues, HO-1 expression was revealed to be a significant marker of tumor recurrence and progression in patients with NMIBC (30). Other studies have also investigated the pathological role of HO-1 expression in patients with NMIBC (31,32); however, there are a number of undetermined features, including the association with vasculogenesis and the regulative mechanisms of HO-1-associated factors. Therefore, the current study focused on the association between HO-1 expression levels and angiogenesis, lymphangiogenesis and vasculogenesis-associated molecules, as these factors are crucial
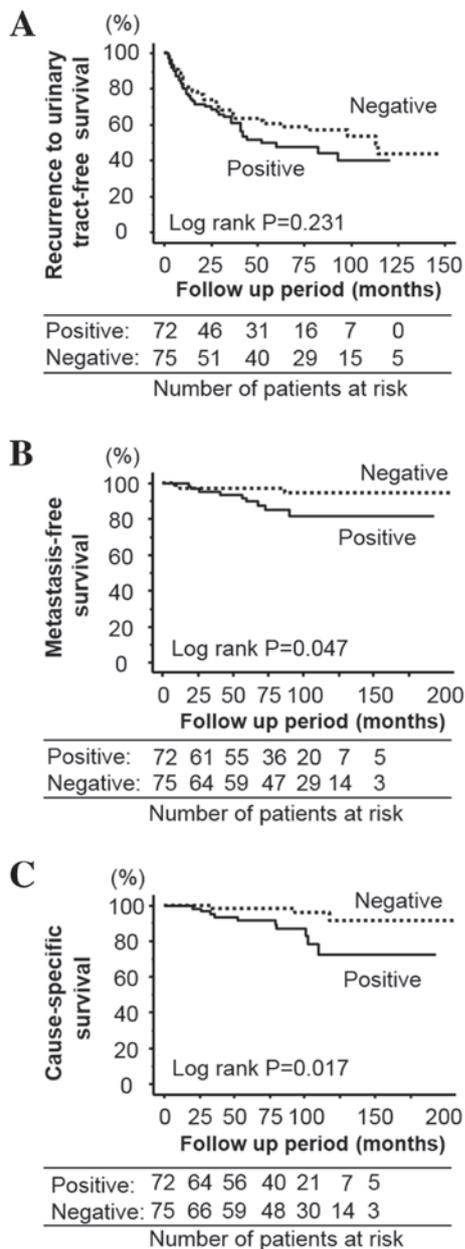
Figure 2. (A) Kaplan-Meier survival curves indicated that HO-1 expression was not significantly associated with urinary tract tumor recurrence-free survival rates in patients with non-muscle-invasive bladder cancer. However, positive expression of HO-1 was recognized as a predictive factor for poor (B) metastasis-free survival (log rank P=0.047) and (C) cause-specific survival (log rank P=0.017). HO-1, heme oxygenase 1.

for tumor growth, progression and prognosis in bladder cancer (6).

The results of the present study demonstrated that HO-1 expression patterns predict malignant potential and poor prognosis in patients with NMIBC. It was observed that HO-1 expression is associated with tumor grade and pT stage, concordant with the results of previous studies (31,32). In addition, the data indicate that HO-1 expression is a useful biomarker for progression-free survival, as was previously reported (31,32). To the best of our knowledge, the current study is the first to demonstrate that increased expression levels of HO-1 are significantly associated with poor cause-specific survival in patients with NMIBC.

Cell proliferation, angiogenesis and lymphangiogenesis were assessed in order to investigate the pathological mechanisms underlying the prognostic value of HO-1. The results demonstrated that HO-1 expression levels are significantly associated with cell proliferation, concordant with previous *in vivo* and *in vitro* studies (31,33). However, to the best of our knowledge, this study is the first to demonstrate that HO-1 expression levels are correlated with lymphangiogenesis. The association between HO-1 expression and lymphangiogenesis has only previously been investigated in bladder cancer (17), and further studies are required. The current study demonstrated that HO-1 expression levels are not correlated with angiogenesis, contradictory to the results of previous reports (29). This discrepancy may be due to the relatively small sample size (n=23) used in the study, or to the use of various markers to identify the endothelial cells.

Concordant with previous reports (17,34), HO-1 expression is significantly and independently associated with COX-2 expression, which was determined using a multivariate model that included various other pathological features. However, HO-1 expression was not observed to be associated with VEGF expression, as determined by univariate analysis. This result conflicts with the results of previous studies (17,35), which reported that VEGF, particularly VEGF-C and -D, were demonstrated to induce lymphangiogenesis. Therefore, HO-1 may stimulate lymphangiogenesis through mechanisms independent of VEGF signaling.

In conclusion, HO-1 expression is associated with higher grade and pT stage of NMIBC tumors, possibly due to the stimulation of cancer cell proliferation, lymphangiogenesis and COX-2 expression. In multivariate survival analysis, HO-1 expression was identified as a significant predictor for cause-specific survival, but not for metastasis. Therefore, HO-1 has exhibited prognostic value and may be a therapeutic target in patients with early-stage bladder cancer. However, further *in vivo* and *in vitro* studies are required to elucidate the underlying mechanisms of HO-1 in NMIBC.

Acknowledgements

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