The Prognostic Significance of Vasohibin-1 Expression in Patients with Upper Urinary Tract Urothelial Carcinoma

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Abstract

Purpose: Vasohibin-1 (VASH1) is a novel angiogenic molecule that is specifically expressed in activated vascular endothelial cells, and the status of VASH1 expression has been documented in cancer angiogenesis. The aim of this study was to address the prognostic value of VASH1 expression in upper tract urothelial carcinomas (UTUC).

Experimental Design: We retrospectively analyzed the clinical records of 171 patients with locally advanced UTUC (Ta-3N0M0). The median follow-up period was 3.8 years. We immunohistochemically examined the accomplished microvessels with anti-CD34 as microvessel density (MVD) and the microvessels with activated endothelial cells as VASH1 density. Then, we analyzed the association between immunohistochemical expression and clinical outcomes.

Results: Forty-two patients experienced tumor recurrence and of these 34 died of the disease during follow-up. VASH1 density was significantly associated with tumor grade, pathologic T stage, and MVD. The 5-year recurrence-free and cancer-specific survival rates were 66.1% and 72.8% in patients with VASH1 density (≥ 40/mm²) and 81.0% and 86.5% in their counterparts, respectively (P < 0.05). MVD was not an independent predictor of tumor recurrence or cancer-specific survival. Multivariate analyses revealed that high VASH1 density was an independent prognostic indicator of both tumor recurrence (P = 0.024, HR = 2.10) and cancer-specific survival (P = 0.031, HR = 2.23) as well as other standard prognostic factors including high tumor grade and lymphovascular invasion.

Conclusions: VASH1 density represents a clinically relevant predictor of patient prognosis in UTUC. The results suggest that VASH1 density could become a new biomarker and provide additional prognostic information in patients with UTUC. Clin Cancer Res; 18(15); 4145–53. ©2012 AACR.

Introduction

Upper urinary tract urothelial carcinoma (UTUC) is relatively rare, accounting for only 2% to 8% of all urothelial cancers (1). Although radical ipsilateral nephroureterectomy with excision of the bladder cuff remains the standard treatment in patients with localized UTUC, their prognosis is still poor due to local recurrence and distant metastasis following curative surgery (2). Many investigators have previously reported the possible predictors of tumor progression including local or distant recurrences of UTUC (3–6). The clinicopathologic parameters of UTUC, such as tumor stage, histologic grade, and lymphovascular invasion (LVI), have been reported to be independent predictors of clinical outcome following radical surgery (7). In addition to these standard predictors, several investigators evaluated relevant variables associated with tumor angiogenesis (8, 9). However, whether tumor angiogenesis could be a great predictor of UTUC outcome has not been fully evaluated yet.

Angiogenesis, that is the formation of new blood vessel networks, not only plays a role in human normal development, but also in pathophysiologic conditions such as inflammation and neoplasm. Angiogenic activity has been shown to clinically correlate with a greater incidence of metastasis and a poor prognosis for patients with neoplasm (10, 11). Angiogenesis is generally regulated by the balance between stimulatory and inhibitory factors. Angiogenic molecules such as CD34, von Willebrand factor, and vascular endothelial-cadherin, which are specifically expressed in vascular endothelial cells, could serve as biomarkers (12). However, those molecules are expressed in quiescent endothelial cells as well as in activated endothelial cells, and thus cannot fully reflect angiogenesis activity. One of the factors that provide information on angiogenesis activity in...
Angiogenesis is intimately involved in tumor growth and distant metastasis. Although several parameters evaluating tumor angiogenesis such as the microvessel density (MVD) have been investigated, it has not been fully characterized yet that the MVD reflects the activity of tumor angiogenesis or the predictor of cancer progression. Recently, we isolated a novel angiogenic molecule, vasohibin-1 (VASH1), which is an intrinsic factor specifically expressed in activated vascular endothelial cells. Previous studies found that the expression of VASH1 was restricted to endothelial cells of blood vessels in the tumor stroma. In this study, we evaluated the VASH1 expression of tumor microvessels in upper urinary tract urothelial carcinoma (UTUC) and revealed that VASH1 definitely becomes a clinically relevant prognostic indicator. This report suggests that VASH1 could become a new molecular biomarker and provide additional prognostic information in patients with UTUC.

In this study, we examined the expression of VASH1 and MVD in UTUC specimens acquired by primary surgery and retrospectively investigated whether VASH1 expression was related to tumor angiogenesis and clinical outcome in UTUC.

Materials and Methods

Patient selection

After obtaining Institutional Review Board approval, the medical records of patients operated on between 1983 and 2007 and archived at Keio University Hospital (Shinjuku-ku, Tokyo, Japan), were retrospectively reviewed. During this period, more than 200 patients underwent nephroureterectomy for UTUC at Keio University Hospital. Five patients with distant metastasis at diagnosis, 8 with pT4 and/or positive lymph node involvement, and 4 with concomitant muscle invasive bladder cancer were excluded from the analysis. After excluding 27 patients lost to follow-up within 6 months after surgery, we identified a total of 172 patients with locally advanced UTUC (pT4aN0M0) in our study population. One patient who began to receive neoadjuvant chemotherapy before nephroureterectomy was excluded; thus, 171 patients were included in the subsequent analyses. The median follow-up of the whole cohort was 3.8 years (0.7–20). One hundred and forty-three patients (83.6%) had undergone open nephroureterectomy and 28 patients laparoscopic nephroureterectomy. Regional lymph nodes were dissected in patients with enlarged nodes on a preoperative evaluation or who were suspected of having enlarged nodes at intraoperative inspection. Extended lymphadenectomy was not routinely conducted. Cisplatin-based adjuvant chemotherapy regimens were administered to 28 patients (16.4%). Patients with pT3 tumors and presence of LVI were generally recommended to receive adjuvant chemotherapies following nephroureterectomy in our institute during the study period. Postoperative adjuvant radiotherapy regimens were not routinely used. Patients were assessed by urine cytology and cystoscopy every 3 months for 2 years following nephroureterectomy, every 6 months for the next 3 years, and then every 6 to 12 months thereafter. Computed tomography and either MRI or excretory urography were conducted every 6 months for 5 years and annually thereafter. Disease recurrence was defined as any recurrence documented by radiograph or pathology-proven failure in nonbladder lesions such as contralateral kidney, operative site, regional lymph nodes, or distant metastasis. The cause of death was determined by the treating physicians. The independent variables included in the present study were age, gender, tumor location, pathologic factors (tumor grade, pathologic T stage, and the appearance of LVI), and the status of adjuvant chemotherapy. Tumor location was divided into 2 areas: the renal pelvis or ureter based upon the location of the dominant lesion.

Tissue samples

All the specimens were fixed in 10% formalin and embedded in paraffin. All pathologic specimens were reviewed again by genitourinary pathologists to unify the reproducibility of the diagnosis. As for the pathologic stage, all neoplasms were classified according to the 2002 tumor–node–metastasis staging system. Histologic grades were assigned according to the 3-tiered World Health Organization classification, namely low (G1 and G2) and high (G3) grades. Lymphovascular invasion was defined as the presence of tumor cells within an endothelium-lined space without underlying muscular walls.

Immunohistochemistry

We carried out immunohistochemical staining for VASH1 and CD34 (as a marker of vascular endothelial cells). All the tissue samples were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm thick sections and
placed on silane-coated glass slides. Tissue sections were deparaffinized in xylene, and hydrated through graded alcohols and to distilled water. Antigen retrieval was carried out in 10 mmol/L Tris buffer (Dako target retrieval solution pH 9.0, Dako) for VASH1, heated in autoclave at 121°C for 10 minutes. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxidase/methanol for 20 minutes at room temperature. The tissue sections were then incubated for 15 minutes at room temperature in a blocking solution of 6% dry milk in PBS. After that they were stained for 60 minutes at room temperature with primary antibodies, followed by staining for 30 minutes at room temperature with secondary antibodies. The primary antibodies were all mouse monoclonal antibodies (mAbs): anti-human VASH1 mAb diluted at 1:400 and anti-CD34 (Nichirei Biosciences) diluted at 1:200. We previously described a mouse mAb against a synthetic peptide corresponding to the 286 to 299 amino acid sequence of VASH1 (20). After washing with PBS, the tissue sections were incubated with secondary antibodies against mouse IgG conjugated to a peroxidase-labeled polymer (Histofine Simple Stain MAX PO (M), Nichirei Biosciences) for 30 minutes. Color was developed with 3,3′-diaminobenzamine in 50 mmol/L Tris-HCl (pH 7.5) containing 0.005% hydrogen peroxide. The sections were counterstained with hematoxylin. The positive control slide for CD34 antigen was prepared from paraffin-fixed bladder cancer tissue with high MVD. The appropriate negative control slides for CD34 antigen and VASH1 were prepared by substituting the primary antibody with the immune globulin fraction of nonimmune mouse serum at the same concentration in each staining run.

Evaluation of immunostaining

Two authors independently evaluated immunoreactivity. They were blinded to the clinical course of the patients and the average of the numbers counted by the 2 investigators was used for subsequent analyses. Olympus BH2 (Olympus) was used for the analysis. The number of microvessels was counted within the tumor. Microvessels were identified on the basis of their architecture, lumen lined by endothelial cells, complemented by positivity of the endothelial cells for anti-CD34 after scanning the immunostained section at low magnification (×40 and ×100). The areas with highest number of distinctly highlighted microvessels were selected and counted at high magnification (×200). We evaluated at least 6 areas in high-power fields and selected one area with the highest number of vessels. Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the “hot spot” as previously shown (14,23,24). The highest number of microvessels in the hot spot was counted for MVD. VASH1-positive signals were counted in the “hot spot” in which the highest number of vessels positive for anti-CD34 was identified. We regarded the number of VASH1-positive signals per mm² as “VASH1 density” (23,25,26). The median value of MVD and VASH1 density were 67.2 ± 3.8/mm² and 39.5 ± 3.3/mm², respectively. We used a median MVD ≥70/mm² and a VASH1 density ≥40/mm², as the cutoff levels.

Statistical analysis

The associations between clinicopathologic parameters and VASH1 density of the tumor were analyzed. These associations were validated by the χ² test or Mann–Whitney U test. Recurrence-free survival and cancer-specific survival rates were estimated using Kaplan–Meier method and were compared by the log-rank test. Survival time was calculated from the date of operation. Multivariate analysis was conducted using the Cox proportional hazard model with stepwise forward selection. Differences among groups were regarded as significant when P < 0.05. These analyses were conducted with the SPSS version 18.0 statistical software package.

Results

Patient characteristics

Table 1 shows the association of clinicopathologic characteristics with MVD or VASH1 density in our study population. The median age of the patients was 68 years (range: 36–89 years). One hundred and thirty-three patients (77.8%) were men. Pathologic analysis revealed 101 patients (59.1%) with tumors in the renal pelvis and 70 patients (40.9%) with tumors in the ureter. Tumor grade was low in 56 cases (32.7%) and high in 115 cases (67.3%). In 106 patients (62.0%), the disease was in ≥pT2 stage; 62 patients (36.3%) were positive for LVI. Cisplatin-based adjuvant chemotherapy was carried out in 28 patients (16.4%). During a median follow-up of 3.8 years, 42 patients (24.6%) experienced tumor recurrence, and of these 34 (19.9%) died of the disease during follow-up.

VASH1 expression in UTUC

To elucidate the biologic significance of VASH1 in UTUC, we examined the immunohistochemical expression of VASH1 in UTUC (Fig. 1). VASH1 staining of vascular endothelial cells was negative or negligible in superficial and low-grade UTUC (Fig. 1B). However, in other UTUCs with similar pathologic stage and tumor grade, strong VASH1 staining was observed in endothelial cells of microvascular vessels in the tumor lesion (Fig. 1D). In invasive UTUC, we observed low VASH1 density in some vessels (Fig. 1F) and high VASH1 density (Fig. 1H) in many. VASH1 staining of vascular endothelial cells was negative or negligible in large-size vessels of the tumor. The median MVD and VASH1 density (counts per mm²) were 67.2 ± 3.8 and 39.5 ± 3.3 in 171 patients, respectively (Table 1). Patients with high-grade tumors and ≥pT2 had significantly higher levels of VASH1 density. As it has been reported that VASH1 associates with CD34, we also investigated the relationship between VASH1 and CD34 expression. Using the Spearman correlation coefficient test, we detected a significant positive correlation between MVD and VASH1 density in microvessels in the tumor (ρ = 0.636, P < 0.001).
171 patients. High VASH1 density was significantly associated with tumor grade, pathologic T stage, and microvessel density, such as MVD and VASH1 density, as defined by cutoff values. Univariate and multivariate analysis were used to determine the indicators for subsequent tumor recurrence and death. The 5-year Kaplan–Meier recurrence-free survival rates were 66.1% and 72.8% in patients with high VASH1 density, compared with 81.0% (P = 0.017) and 86.5% (P = 0.042) in their counterparts (Fig. 2). High level of MVD was not an independent predictor of tumor recurrence (P = 0.216) and cancer-specific survival (P = 0.473; Fig. 3A and B).

**Prognostic significance of VASH1 expression in UTUC patients**

We conducted univariate and multivariate analysis to determine the indicators for subsequent tumor recurrence following surgery (Table 2). Univariate analysis revealed that high tumor grade, pT2 or greater, presence of LVI, adjuvant chemotherapy, and high VASH1 density (≥40/mm²) were significant predictors of tumor recurrence. Multivariate analysis showed that high tumor grade (P = 0.021, HR = 4.20), presence of LVI (P < 0.001, HR = 5.05), and high VASH1 density (P = 0.024, HR = 2.10) were also independent predictors of tumor recurrence.

We next conducted univariate and multivariate analysis to determine indicators for cancer-specific survival. Univariate analysis showed that high tumor grade, pT2 or greater, presence of LVI, adjuvant chemotherapy, and high VASH1 density were significant predictors of cancer-specific survival. Multivariate analysis confirmed that high tumor grade (P = 0.030, HR = 5.10), presence of LVI (P < 0.001, HR = 5.72), and high VASH1 density (P = 0.031, HR = 2.23) were also independent predictors of cancer-specific survival. Similar results could be obtained using other cutoff values of MVD and VASH1 density, such as MVD ≥ 90 or 80/mm² and VASH1 density ≥ 60 or 50/mm².

Table 3 shows the association between the level of VASH1 density and clinicopathologic characteristics in 171 patients. High VASH1 density was significantly associated with tumor grade, pathologic T stage, and microvessel density, whereas other characteristics such as patient age, gender, tumor location, LVI, and the status of adjuvant chemotherapy were not significantly different in these 2 groups. The 5-year Kaplan–Meier recurrence-free survival and cancer-specific survival rates were 66.1% and 72.8% in patients with high VASH1 density, compared with 81.0% (P = 0.017) and 86.5% (P = 0.042) in their counterparts (Fig. 2). High level of MVD was not an independent predictor of tumor recurrence (P = 0.216) and cancer-specific survival (P = 0.473; Fig. 3A and B).

**Risk stratification for UTUC according to prognostic factors, tumor grade, LVI, and VASH1 density**

We distributed the patients into 3 different groups according to tumor grade, LVI, and VASH1 density, which were the 3 statistically significant variables found by the multivariate Cox regression analysis (Fig. 3C and D). The relative risk of death could be calculated with the formula, \( \exp(1.434 \times \text{grade} + 1.619 \times \text{LVI} + 0.740 \times \text{VASH1 density}) \) for recurrence-free survival and \( \exp(1.630 \times \text{grade} + 1.744 \times \text{LVI} + 0.800 \times \text{VASH1 density}) \) for cancer-specific survival. In this equation, the grade equaled 1 if the tumor grade was high, and 0 if the tumor grade was low. LVI equaled 1 if LVI was present and 0 if absent. VASH1 density equaled 1 if VASH1 density was ≥40/mm² and 0 if <40/mm². On the basis of the relative risk of death, patients with UTUC were divided into 3 risk groups: low (relative risk of recurrence and death = 1), intermediate (2.10–21.2 for recurrence-free survival, 2.23–29.2 for cancer-specific survival) and high...
(10.6–44.4 for recurrence-free survival, 12.7–65.0 for cancer-specific survival). According to the risk stratification for UTUC based on prognostic factors, 31 patients (18.1%) were in the low-risk group (low tumor grade, LVI negative, and low VASH1 density), 31 patients (18.1%) in the high-risk group (any tumor grade, LVI positive, and high VASH1 density) and 109 patients (63.8%) in the intermediate-risk group (all others). The 5-year recurrence-free survival and cancer-specific survival rates were 96.4% and 96.4% in the low-risk group, 79.0% and 85.1% in the intermediate-risk group, and 30.7% and 41.3% in the high-risk group, respectively.

The differences among the groups were significant ($P = 0.015$ in recurrence-free survival and $P = 0.027$ in cancer-specific survival for low- vs. intermediate-risk group, $P < 0.001$ for low- vs. high-risk group and $P < 0.001$ for intermediate- vs. high-risk group in recurrence-free survival and cancer-specific survival).

Discussion

In the present study, we retrospectively evaluated the impact of VASH1 expression by immunohistochemistry in a series of patients with locally advanced UTUC treated...
in a single center. Our results suggested that VASH1 expression was a prognostic indicator in addition to other standard factors such as tumor grade and the presence of LVI. High VASH1 density was related to shorter patient survival. To the best of our knowledge, this is the first study evaluating prognostic value of VASH1 expression in patients with cancer.

Angiogenesis has a critical role in tumor growth and metastasis (11). Recent studies revealed significant roles for angiogenesis in the prediction of survival in patients with different malignancies (23, 27). One of the biomarkers that could reflect angiogenic aggressiveness was MVD (12–14). Several studies on urothelial carcinoma indicated that the status of MVD was associated with clinical outcomes, such as tumor grade and pathologic stage, and could be an independent prognostic factor of patient survival (13–15). However, to date evidence of the prognostic role of MVD in urothelial carcinoma is contradictory, suggesting the prognostic impact of MVD might be controversial (17–19). In the present study, we found no significant association between MVD or patient mortality and survival. One of the reasons might be because MVD corresponds to the number of accomplished vessels and includes vessels without the potential of neovascularization in tumors.

VASH1 has been isolated from VEGF inducible genes in endothelial cells present in newly formed blood vessels behind the sprouting front where angiogenesis terminates (28, 29). Recently, histologic evidence of VASH1 expression has been found in samples from patients with breast cancer (25), cervical carcinoma (23), and endometrial carcinoma (30). These reports indicated that VASH1 expression was associated with tumor grade and histologic type of carcinomas. Moreover, it was reported that VASH1 expression tended to be concordant with MVD although partial dissociation was observed in some patients with breast carcinoma. VASH1 expression was significantly higher in invasive breast carcinoma, although no significant difference was observed in the levels of MVD between patients with invasive disease or not. They also suggested that an evaluation of the number of VASH1-

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<th>Table 2. Univariate and multivariate analysis for recurrence-free survival and cancer-specific survival</th>
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<td>Lymphvascular invasion</td>
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<td>Adjuvant chemotherapy</td>
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<td>VASH1 density &lt;40/mm²</td>
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<td>Abbreviation: CI, confidence interval.</td>
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positive vessels may become one of the prognostic biomarkers for metastasis and prognosis (26). These results indicate that VASH1 could become a new molecular biomarker, which influences angiogenic heterogeneity of tumors.

Our study showed that UTUC with a higher number of VASH1-positive vessels tended to have a poor prognosis. We found a significant correlation among VASH1 density, tumor grade, and pathologic T stage. In multivariate analysis, high VASH1 density was an independent

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<th>Table 3. Clinicopathologic parameters in 171 patients according to the level of the VASH1 density</th>
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<td>Tumor location</td>
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Figure 2. Kaplan–Meier recurrence-free survival (A) and cancer-specific survival (B) of the patients after surgery for UTUC according to VASH1 density.
prognostic factor, suggesting the status of VASH1 density could serve as a biomarker of the malignant potential of tumor angiogenesis. These results suggest that the level of VASH1 expression may influence the clinical course of a disease.

Using VASH1 density and other independent indicators, we established a prognostic risk stratification for UTUC. Patients were stratified into 3 groups according to statistical modeling based on the relative risk associated with the prognostic indicators derived from multivariate analysis. As shown in Fig. 3C and D among patients with UTUC, the patients with positive LVI and higher VASH1 density showed a worse prognosis than other groups \((P < 0.001)\). This stratification made it possible to have a more accurate survival prediction, suggesting more appropriate follow-up including computerized tomography and the timing for aggressive treatments such as chemotherapy.

This study has several limitations. First, it was conducted in a retrospective manner and in a limited number of patients. Not all patients received adjuvant chemotherapy, which may have had an effect on subsequent tumor progression. Thus, a prospective study in a large population is warranted to clarify the prognostic role of VASH1 expression in UTUC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Miyazaki, T. Kosaka, E. Kikuchi, N. Tanaka, Y. Sato, M. Oya
Development of methodology: N. Tanaka, M. Oya
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Miyazaki, T. Kosaka, S. Mikami, N. Tanaka, T. Marda, M. Ishida, M. Oya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Miyazaki, T. Kosaka, S. Mikami, N. Tanaka, M. Ishida, M. Oya
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Study supervision: E. Kikuchi, N. Tanaka, K. Nakagawa, M. Oya

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