Biochemical and Biophysical Research Communications 459 (2015) 416–423

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Prospective validation of DACH2 as a novel biomarker for prediction of metastasis and prognosis in muscle-invasive urothelial carcinoma of the bladder

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ARTICLE INFO

Article history:
Received 19 February 2015
Available online 2 March 2015

Keywords:
Dachshund homolog 2 gene
Bladder cancer
Radical cystectomy
Prognosis
Immunohistochemistry

ABSTRACT

Metastasis is the main cause of death from muscle-invasive urothelial carcinoma of the bladder (UCB), and the metastatic potential of tumors is often unpredictable. The role of Dachshund homolog 2 gene (DACH2) in tumorigenesis remains unexplored. We aimed to investigate whether DACH2 can be used as a biomarker to predict metastasis and prognosis of muscle-invasive UCB in a sequential training and validation fashion. For the training set (n = 40), compared with UCB patients without lymph node (LN) metastasis, both DACH2 protein and mRNA expression were greatly increased in case-matched patients with LN metastasis. For the independent validation set (n = 243), patients with primary UCB that did not express DACH2 had a longer metastasis-free survival (MFS) and overall survival (OS) than did those with tumors expressing DACH2 (5-year MFS: 88% [95% CI 80–96] *versus* 19% [95% CI 7–31], $p < 0.001$; 5-year OS: 93% [95% CI 87–99] *versus* 37% [95% CI 23–51], $p < 0.001$). Multivariable analysis of DACH2 status showed hazard ratios of 7.34 (95% CI 3.15-11.87, $p < 0.001$) for MFS and 3.96 (95% CI 2.04-7.16, $p < 0.001$) for OS which were much higher than hazard ratios associated with other independent risk factors. Collectively, DACH2 is an independent prognostic marker that can be used at initial diagnosis of UCB to identify patients who have a high potential to develop metastasis.

1. Introduction

Bladder cancer is the second most common malignancy of the genitourinary tract. Incidence of this disorder has been rising steadily. About 74 690 new cases of bladder cancer were diagnosed in the USA in 2014, and about 15 580 patients will die from this disease [1]. Urothelial carcinoma of the bladder (UCB) is the most common bladder cancer, present in almost 90% of cases [2,3]. Radical cystectomy remains the reference standard treatment for those with muscle-invasive UCB [4–6]. However, despite this aggressive local approach, survival outcomes following radical cystectomy have not changed over decades [7,8]. Currently, prediction of prognosis and selection of patients for adjuvant treatment rely heavily upon histopathological evaluation [9]. However, because of substantial differences in the biological behavior of muscle-invasive UCB, the metastatic potential of primary tumors is difficult to predict [10]. Therefore, biomarkers that can accurately distinguish primary tumors with a high probability of metastasis from those that will remain indolent are urgently needed. Various molecular biomarkers and gene–expression profiles based on DNA

Abbreviations: CI, Confidence interval; DACH2, Dachshund homolog 2 gene; LN, Lymph node; UCB, Urothelial carcinoma of the bladder.

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http://dx.doi.org/10.1016/j.bbrc.2015.02.119
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microarray analysis have shown potential in predicting disease outcome in patients with UCB [11–13].

Dachshund homolog 2 gene (DACH2) is a member of the dachshund genes family that consists of DACH1 and DACH2. Although the DACH family members were first described in Drosophila, where they encode proteins involved in development of the eyes, limbs and genital disc [14,15]. They have been also found in mice, humans and chicken [16,17]. While the role of DACH2 in human tumorigenesis remains unexplored, alterations of DACH1 expression has been described in several kinds of cancer, e.g. gastric, prostate, breast, endometrial, and ovarian cancer [18–22]. To our knowledge, there is only one study investigating the expression of DACH2 in one cancer form (ovarian cancer) [23]. DACH2 expression in muscle-invasive UCB and the relation between DACH2 and tumor metastasis and prognosis after radical cystectomy are unknown.

In this study, we firstly assessed DACH2 being differentially expressed between muscle-invasive UCB patients with pelvic lymph node (LN) metastasis and those without LN metastasis in a training patient set, and then prospectively validated the prognostic role of DACH2 in an independent case group. The aim of this study is to investigate whether DACH2 could be used as an independent biomarker to predict postoperative metastasis and prognosis in patients with muscle-invasive UCB.

2. Materials and methods

2.1. Patients and tumor specimens

This study had sequential training and validation phases (Fig. 1). For the training set, a case-matched cohort of 40 representative tissue blocks was obtained from 40 patients with muscle-invasive UCB (tumor with LN metastasis, n = 20; tumor without LN metastasis, n = 20, Table 1) from the Third Affiliated Hospital of Sun Yat-sen University. Patients with stage ≥ pT2 UCB who underwent radical cystectomy and bilateral pelvic LN dissection, and with clinicopathological characteristics and follow-up information available, were included. We excluded patients if they had previous treatment with any anticancer therapy, had presented previously a tumor of the upper urinary tract, or presence of any tumor type other than urothelial carcinoma. Case matching was performed on a 1:1 basis in an automated fashion using SPSS v.19.0 and the following rules: the same gender, the same T stage, the same grade, and similar age (within 10 yrs).

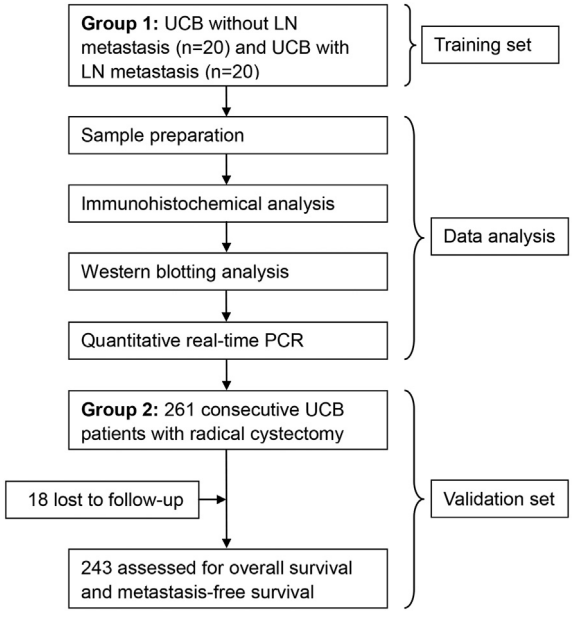

Fig. 1. Flowchart of sample processing and data analysis. UCB: Urothelial Carcinoma of the Bladder; LN: Lymph Node.

For the validation testing set, with the same including and excluding criteria as above, data were obtained from 261 patients from four hospitals (The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou; Foshan First Municipal People's Hospital, Foshan; The First Affiliated Hospital, University of South China, Hengyang; Foshan Hospital of Traditional Medicine, Foshan) in China between August 2007 and June 2014. All underwent open or laparoscopic radical cystectomy and bilateral pelvic LN dissection. The boundaries of LN dissection were as follows: the bifurcation of the common iliac artery proximally, the genitofemoral nerve laterally, the circumflex iliac vein and lymph node of Cloquet distally, and the hypogastric vessels posteriorly, including the obturator fossa [24]. Follow-up after radical cystectomy is generally recommended quarterly for the first 2 yr postoperatively, semi-annually for the next 2 yr, and annually thereafter for patients without evidence of metastatic disease. Oncologic evaluation consisted of history and physical examination, urine cytology, and imaging of the chest, abdomen, and pelvis. Bone scans were performed when clinically indicated.

All cases have undergone central pathological review before DACH2 analysis was performed. In detail, all pathologic slides were sent to, and reviewed by 2 dedicated uropathologists (D. He and C.-K. Shao) who were unaware of the original pathologic reports of each patient. Tumor stage and grade were assigned according to the TNM and WHO classification of malignant tumors of the urinary tract [25,26]. In cases in which the review diagnosis differed from the diagnosis at the source institution, the samples were further reviewed by another urological pathologist (Z.-L. Su), who acted as an arbiter. All review pathologists were unaware of the results of the DACH2 studies.

This study was approved by the ethics committees of all participating institutions, and all study participants provided written informed consent.

2.2. Immunohistochemical analysis

DACH2 protein expression was detected by immunohistochemistry according to the methods described by us previously [27,28]. In brief, 5 μm sections were processed for antigen retrieval in EDTA (pH 9.0) for 30 min in a microwave followed by 30 min of cooling in EDTA buffer. Slides were incubated with 1:100 of rabbit monoclonal DACH2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C, followed by chromogenic visualization using the EnVision system (DAKO, Glostrup, Denmark). Evaluation of DACH2 protein expression was scored using the following scale: (0) less than 10% positive staining cells; (1+) 10–25%; (2+) 25–50%; (3+) more than 50%. Cases with scores of 2 + or 3+ were designated as "positive," whereas cases with scores of 0 or 1+ were designated as "negative" [28]. Sections of ovarian carcinoma known to express DACH2 were used as positive controls. Every positive sample was evaluated further for the proportion of the cells that stained positively, and was scored as focal (≤30%) or diffuse (>30%).

In addition, to assess immunohistochemistry quantitatively, DACH2 protein expression for all cases was also assessed by using the automated Ariol imaging system (Genetix Corp, San Jose, CA). Briefly, the investigator set the color and shape characteristics to properly identify cells with positive staining. The software applied the color classifiers to identify regions of positive staining, excluding objects that were either too light or too dark. The

Table 1
Clinicopathologic characteristics of 40 patients with UCB included in the training set.

Case no.	Age (yr)	Gender	Operation	pT stage	Grade	IHC	WB	RT-PCR
UCB without lymph node metastasis								
1	48	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
2	52	Female	Radical cystectomy	pT2a	2	Yes	Yes	Yes
3	61	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
4	65	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
5	49	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
6	42	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
7	66	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
8	32	Male	Radical cystectomy	pT2a	1	Yes	Yes	Yes
9	50	Female	Radical cystectomy	pT2b	2	Yes	Yes	Yes
10	49	Male	Radical cystectomy	pT2b	2	Yes	Yes	Yes
11	69	Male	Radical cystectomy	pT2b	3	Yes	Yes	Yes
12	37	Male	Radical cystectomy	pT2b	2	Yes	Yes	Yes
13	49	Female	Radical cystectomy	pT3a	3	Yes	Yes	Yes
14	55	Male	Radical cystectomy	pT3a	2	Yes	Yes	Yes
15	62	Male	Radical cystectomy	pT3a	3	Yes	Yes	Yes
16	45	Male	Radical cystectomy	pT3a	2	Yes	Yes	Yes
17	32	Female	Radical cystectomy	pT3a	3	Yes	Yes	Yes
18	49	Female	Radical cystectomy	pT3b	3	Yes	Yes	Yes
19	41	Male	Radical cystectomy	pT3b	3	Yes	Yes	Yes
20	48	Male	Radical cystectomy	pT3b	3	Yes	Yes	Yes
UCB with lymph node metastasis								
21	56	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
22	43	Female	Radical cystectomy	pT2a	2	Yes	Yes	Yes
23	63	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
24	58	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
25	44	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
26	38	Male	Radical cystectomy	pT2a	2	Yes	Yes	Yes
27	59	Male	Radical cystectomy	pT2a	3	Yes	Yes	Yes
28	41	Male	Radical cystectomy	pT2a	1	Yes	Yes	Yes
29	45	Female	Radical cystectomy	pT2b	2	Yes	Yes	Yes
30	53	Male	Radical cystectomy	pT2b	2	Yes	Yes	Yes
31	61	Male	Radical cystectomy	pT2b	3	Yes	Yes	Yes
32	29	Male	Radical cystectomy	pT2b	2	Yes	Yes	Yes
33	53	Female	Radical cystectomy	pT3a	3	Yes	Yes	Yes
34	48	Male	Radical cystectomy	pT3a	2	Yes	Yes	Yes
35	54	Male	Radical cystectomy	pT3a	3	Yes	Yes	Yes
36	43	Male	Radical cystectomy	pT3a	2	Yes	Yes	Yes
37	40	Female	Radical cystectomy	pT3a	3	Yes	Yes	Yes
38	58	Female	Radical cystectomy	pT3b	3	Yes	Yes	Yes
39	36	Male	Radical cystectomy	pT3b	3	Yes	Yes	Yes
40	51	Male	Radical cystectomy	pT3b	3	Yes	Yes	Yes

Abbreviations: *IHC*, Immunohistochemistry; *WB*, Western Blotting; *RT-PCR*, Quantitative real-time PCR; *UCB*, Urothelial Carcinoma of the Bladder.

objective DACH2 protein expression level with Ariol system was defined as the ratio of "DACH2 staining area" to "analyzed tissue area." [27].

2.3. Western blotting analysis

Western blotting analyses were performed using previously reported methods [28,29]. Briefly, 60 μg of each protein sample was subjected to Western blotting analysis using anti-DACH2 (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). β-actin was used as a loading control in all blotting membranes (anti-β-actin, 1:5000; Sigma, St Louis, MO,USA). Immunoreactive proteins were then visualized using the ECL western blotting system (Pierce Biotechnology, Rockford, IL, USA). Images were scanned by the ImageMaster II scanner (GE Healthcare) and analyzed using ImageQuant TL v2003.03 (GE Healthcare). Background binding was subtracted out, and the band signals were expressed as relative protein amounts compared with β-actin.

2.4. Quantitative real-time PCR

We measured mRNA amounts of *DACH2* in UCB by using quantitative real-time PCR [28,29]. *GAPDH*-a housekeeping gene-was used as an internal reference. In brief, total RNA was extracted after homogenization of tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by DNase digestion (RNase-free DNase set; Qiagen, Valencia, CA, USA) and on-column clean up with the RNeasy MinElute kit (Qiagen). Total RNA (2 μg) was reverse transcribed with the Superscript II RNase H-reverse transcriptase kit (Invitrogen) for complementary DNA (cDNA) synthesis using random hexamer primers. Expression level and genomic copy number were evaluated by real-time PCR on an ABI PRISM 7000 Sequence Detector Thermocycler (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Sequences of PCR primers of DACH2 are as follows: forward:CCTAAGCGTTCTTTGGGAGTG,

reverse: TGATAAGTCCTGGCGATAAGAGG

All quantitative PCR reactions were performed in triplicate and repeated at least twice. The ΔCt for gene-specific mRNA expression was calculated relative to the Ct (threshold cycle) of *GAPDH*. Relative mRNA expression was calculated using the formula $2^{(-\Delta\Delta Ct)}$.

2.5. Statistical analysis

The overall survival was defined as time from the date of radical cystectomy to the date of death. Metastasis-free survival was defined as time from the date of radical cystectomy to the date of metastasis, based on histologic or radiologic evidence. Data for age,

gender, tumor size, tumor stage, tumor grade, LN status, and DACH2 status were obtained as baseline variables. The distribution of every baseline variable was compared for DACH2-positive and DACH2-negative subgroups, with the Wilcoxon rank sum test for continuous variables and the Fisher's exact test for categorical variables.

The overall survival and metastasis-free survival of patients were estimated by the Kaplan–Meier method and assessed by the use of log-rank test for univariate analysis. The Cox proportional-hazard model was used to assess the simultaneous contribution of baseline covariates in univariate and multivariable analyses. All statistical analyses were conducted using SPSS v.19.0 (SPSS Inc., Chicago, IL, USA), and a two-tailed p value less than 0.05 was considered statistically significant.

3. Results

3.1. Training cohort

We identified DACH2 protein in 17 (85%) of 20 UCB patients with LN metastasis, and 2 (10%) of 20 cases without LN metastasis ($p < 0.001$, Fig. 2). Of 19 DACH2-positive tumors, 10 had focal DACH2 positivity and 9 had diffuse positivity. No expression of DACH2 was observed in the benign bladder tissue adjacent to the tumors. Quantitative immunohistochemical analysis showed substantial differences in DACH2 staining values between positive and negative samples. The median arbitraray expression units were 0.176 in the DACH2-positive tumors and 0.025 in the DACH2-negative tumors ($p < 0.001$).

Western blotting results confirmed our immunohistochemistry findings that the expression of DACH2 was significantly higher in UCB patients with LN metastasis than in those without LN metastasis. Representative results are presented in Fig. 3A. Using quantitative real-time PCR, we also detected the expression of *DACH2* mRNA in 20 cases with LN metastasis and 20 without LN metastasis. The expression of *DACH2* gene was significantly increased in patients with LN metastasis compared with those without LN metastasis ($p = 0.016$, Fig. 3B). Results from the western blotting and quantitative real-time PCR of these 40 samples showed significant concordance between the tests (Spearman $R = 0.482$, $p = 0.002$, Fig. 3C).

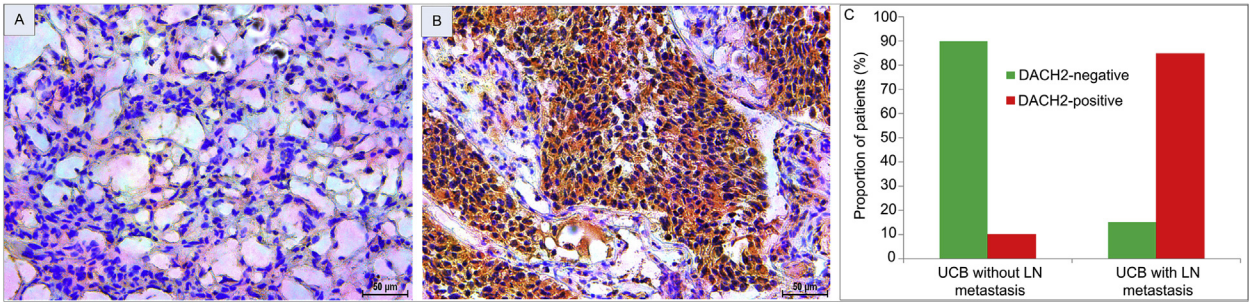

Fig. 2. DACH2 protein expression using immunohistochemical staining in UCB-Two representative tissue samples demonstrating negative DACH2 expression in UCB without LN metastasis **(A)**, and positive expression in UCB with LN metastasis **(B)**. The percentage of DACH2 positivity was significantly higher in UCB with LN metastasis than that in UCB without LN metastasis ($p < 0.001$, **C**). UCB: Urothelial Carcinoma of the Bladder; LN: Lymph Node.

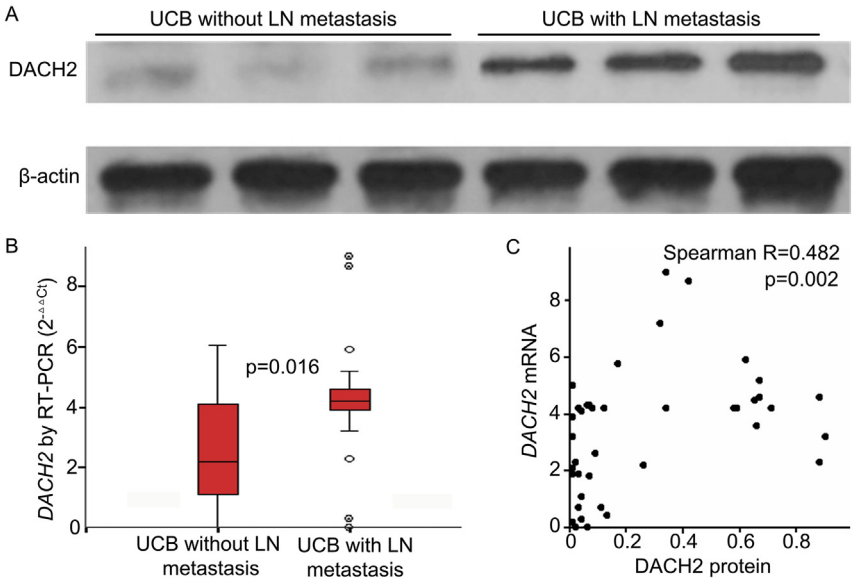

Fig. 3. Western blotting and quantitative RT-PCR of DACH2 expression in UCB. Western blotting analyses showing DACH2 expression was significantly upregulated in UCB with LN metastasis, ($p < 0.001$, compared with UCB without LN metastasis). There were three representative samples in each group. β-actin was used as the internal loading control **(A)**. Quantitative RT-PCR analyses showing the expression of *DACH2* gene was significantly increased in UCB with LN metastasis ($p = 0.016$, compared with UCB without LN metastasis). RT-PCR was carried out in triplicate and repeated twice to evaluate its repeatability and reproducibility **(B)**. Results from the western blotting and quantitative RT-PCR showed significant concordance (Spearman $R = 0.482$, $p = 0.002$) **(C)**. UCB: Urothelial Carcinoma of the Bladder; LN: Lymph Node.

3.2. Validation studies

Table 2 summarizes the relevant clinicopathologic characteristics of the 261 patients with muscle-invasive UCB in the validation cohort. Age and gender were not associated with DACH2-positive status ($p = 0.310$, $p = 0.202$, respectively). DACH2 expression was strongly associated with tumor stage, grade and LN status ($p < 0.001$, $p = 0.024$, $p < 0.001$, respectively), and was identified mainly in large tumors ($p = 0.006$). Only 15.9% of stage pT2 tumors expressed DACH2, whereas 44.6–77.8% of stage pT3-4 tumors expressed this protein. DACH2 was expressed mainly in high-grade (grade 2 and 3) tumors; only one grade 1 tumor was positive for this protein.

243 patients were included in follow-up. Median follow-up was 42 months (range 6–82 months). The proportion of samples with metastasis from muscle-invasive UCB after surgery differed substantially between patients who expressed DACH2 and those who did not. More patients with DACH2-positive tumors subsequently developed metastases than did those with DACH2-negative tumors (DACH2-positive, 51[73.9%] of 69 patients; DACH2-negative, 13 [7.5%] of 174, $p < 0.001$). Patients with DACH2-negative, muscle-invasive UCB had significantly longer metastasis-free survival and overall survival than those with DACH2-positive tumors. 5-year metastasis-free survival was 88% (95% CI 80–96) in patients with DACH2-negative tumors versus 19% (7–31) in those with DACH2-positive tumors (Fig. 4A). 5-year overall survival was 93% (87–99) in patients whose tumors did not express DACH2 versus 37% (23–51) in those whose tumors did express DACH2 (Fig. 4B). In patients with muscle-invasive UCB at stage pT2 (Fig. 4C and D), and pT3-4 (Fig. 4E and F), DACH2 expression was also associated with increased risk of metastasis and was strongly linked to poor overall survival.

In the univariate analysis, the hazard ratio of DACH2 expression was 8.65 (5.03–13.24) for metastasis-free survival and 5.3 8 (3.45–8.07) for overall survival (Table 3). Patients whose muscle-invasive UCB were DACH2-positive had a significantly lower 5-year metastasis-free survival than did those with tumors that were DACH2-negative for tumors of all stages (stage pT2, 32%[6-57] vs 91%[83-99]; stage pT3-4, 14%[2-26] vs 79%[65-93]). 5-year overall survival was also significantly lower in patients with DACH2-positive tumors than in those with DACH2-negative tumors (stage pT2, 57%[32-82] vs 97%[91-99]; stage pT3-4, 28%[10-46] vs 83%[67-99]). Table 4 shows the results of the multivariable analysis for metastasis-free survival and overall survival in these 243 patients with muscle-invasive UCB at the time of surgery. For these analyses, all factors shown in Table 2 were initially included in the model as potential risk factors. DACH2 expression in primary muscle-invasive UCB was a strong independent predictor of the patients' clinical outcome; hazard ratios were 7.34 for metastasis-free survival and 3.96 for overall survival, which were much higher than those associated with all other independent risk factors (Table 4). In addition to DACH2 status, tumor stage, grade and LN status were significant risk factors for overall survival, and tumor size, stage, grade and LN status were significant risk factors for metastasis-free survival (Table 4).

Interestingly, the DACH2-positive staining patterns (focal vs diffuse) in the primary muscle-invasive UCB did not affect the prognosis. There were no significant differences between patients with focal DACH2 staining and those with diffuse DACH2 staining in metastasis-free survival or overall survival ($p = 0.452$, $p = 0.268$, respectively, data not shown).

Table 2
Clinicopathologic characteristics of 261 patients with UCB included in the validation set.

	DACH2-positive tumors (n = 76)	DACH2-negative tumors (n = 185)	*p* Value
Age (years)			
Mean (SD)	55.6 (13.2)	57.8 (11.9)	0.310
Gender			0.202
Male	49 (26.8)	134 (73.2)	
Female	27 (34.6)	51 (65.4)	
Tumor stage			**<0.001**
pT2	24 (15.9)	127 (84.1)	
pT3	45 (44.6)	56 (55.4)	
pT4	7 (77.8)	2 (22.2)	
Tumor grade			**0.024**
G1	1 (11.1)	8 (88.9)	
G2	32 (23.4)	105 (76.6)	
G3	43 (37.4)	72 (62.6)	
Lymph node status			**<0.001**
Positive	39 (76.5)	12 (23.5)	
Negative	37 (17.6)	173 (82.4)	
Tumor size (cm)			
Mean (SD)	6.1 (2.8)	4.2 (1.9)	**0.006**

The *p* values less than 0.05 were marked in bold.
Abbreviations: *UCB*, urothelial carcinoma of the bladder; *SD*, standard deviation.

4. Discussion

To our knowledge, this is the first study to show that DACH2 is expressed much more in muscle-invasive UCB with pelvic LN metastasis than those without LN metastasis. We provide evidence that DACH2 expression in primary muscle-invasive UCB can predict whether the tumor is likely to metastasize and can provide important prognostic information in patients who undergo radical cystectomy.

Metastasis is the main cause of therapeutic failure and death in patients with muscle-invasive UCB. The protracted course and requirement for frequent monitoring is responsible for the huge costs involved with the care of patients with UCB [8]. Although tumor stage and grade provide some prognostic information, the metastatic potential of primary muscle-invasive UCB is often unpredictable. DACH2 shows several features that make it an attractive prognostic marker for muscle-invasive UCB. Firstly, the expression of DACH2 is associated with other known pathological indicators of aggressive muscle-invasive UCB. Our data showed that DACH2 expression was significantly associated with high tumor stage and grade, LN status, and with large tumor burden. Secondly, DACH2 expression is an independent predictor of tumor metastasis. Our results showed that DACH2 expression was significantly increased in patients with primary tumors who developed metastatic disease, compared with those who did not develop metastasis. In patients with stage pT3-4 disease, almost all patients with DACH2-positive tumors developed metastases after radical cystectomy. In the multivariable Cox analysis, patients with DACH2-positive primary tumors were more than seven times more likely to subsequently develop metastasis than were those with DACH2-negative tumors, after adjustment for other frequently used clinical variables, such as tumor stage, size, and grade. Thirdly, a reduced overall survival was strongly associated with metastasis in patients with DACH2-positive tumors. DACH2 expression in primary muscle-invasive UCB is independently associated with poor clinical outcome. Patients with tumors that expressed DACH2 were nearly four times more likely to die than those with tumors that did not express DACH2. Notably, the multivariable Cox analysis showed that the hazard ratio for death in patients with primary tumors expressing DACH2 was much higher than those associated with any other clinicopathologic predictors (e.g. age, gender, tumor size, LN status, stage and grade, etc.)

In addition, DACH2 immunohistochemical staining is a simple, cheap, and reliable assay. Our findings showed that pathologists can readily analyze DACH2 immunohistochemistry without

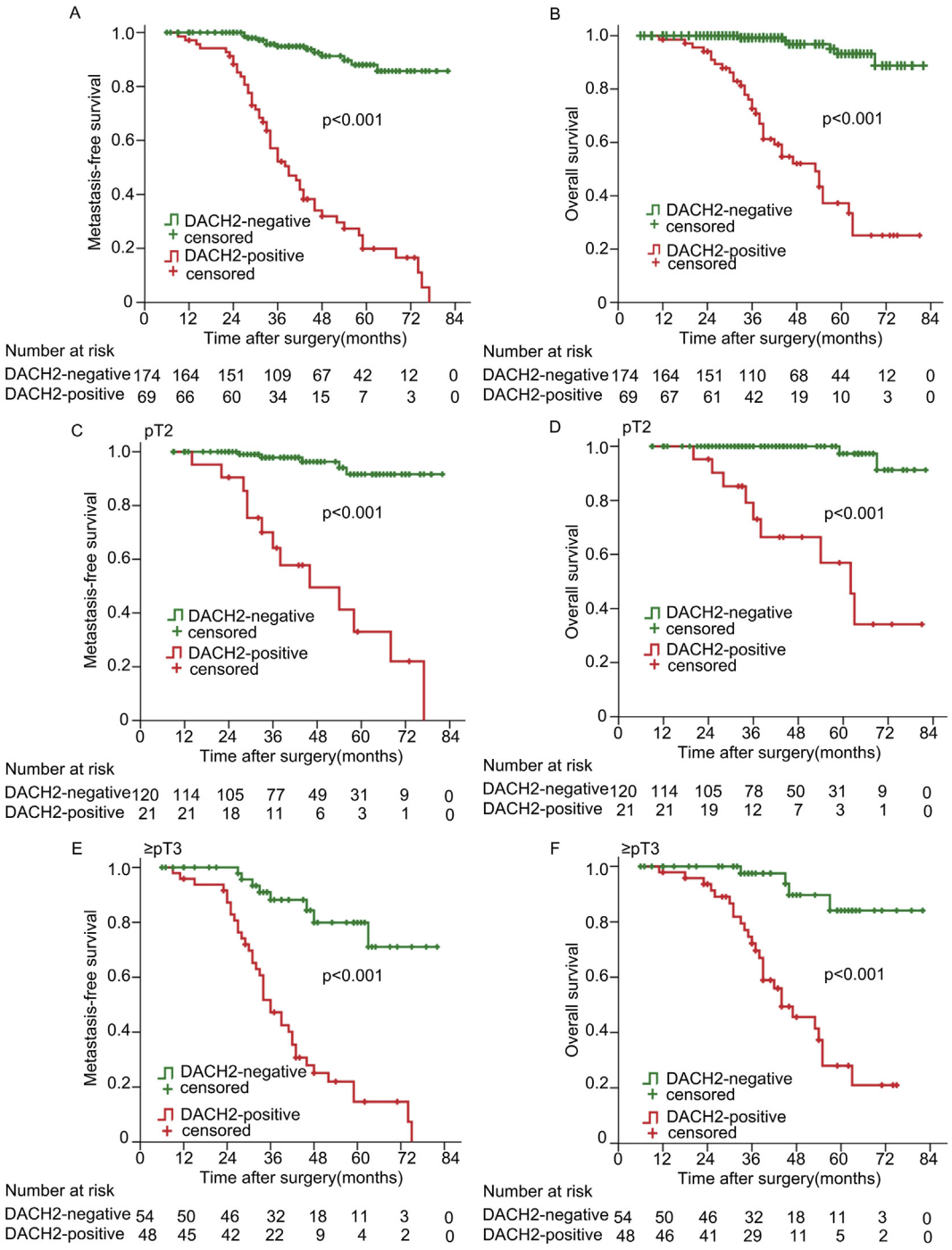

Fig. 4. Analysis of metastasis-free survival and overall survival based on DACH2 immunohistochemical protein expression. Kaplan–Meier analysis of metastasis-free survival and overall survival stratified by DACH2 immunohistochemical protein expression in all UCB patients (n = 243) in the validation set **(A, B)** as well as in patients at stage pT2 (n = 141) **(C, D)** and in patients at stage ≥ pT3 (n = 102) **(E, F)**. UCB: Urothelial Carcinoma of the Bladder.

variation between observers to assess positive and negative staining, which can be easily used in routine clinical practice in all patients who have had radical cystectomy. An automated Ariol imaging system for quantitative immunohistochemistry also confirmed the accuracy of the assessment of DACH2 immunostaining by pathologists. In this study, we focused on assessment of DACH2 immunostaining by pathologists and used Ariol imaging system as a confirmatory test. Further studies on investigating how well quantitative image analysis of immunostaining results correlates with clinical outcome have been ongoing in our centers.

The main strength of our study is that we prospectively validated that DACH2 could be used as an independent biomarker to predict postoperative metastasis and prognosis in muscle-invasive UCB in an independent case group from four different hospitals. The other strength is central pathological reviews. Apart from providing a first description of the expression and prognostic significance of DACH2 in muscle-invasive UCB, this is also, to our knowledge, the first report of DACH2 expression in any human cancer form except for ovarian cancer [23]. In their study, Nodin et al. [23] showed that DACH2 expression was considerably higher

Table 3
Univariate analysis for metastasis-free and overall survival.

	Metastasis-free survival		Overall survival	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
DACH2 status (positive vs negative)	8.65 (5.03–13.24)	**<0.001**	5.38 (3.45–8.07)	**<0.001**
Age	0.96 (0.61–1.32)	0.231	0.91 (0.59–1.29)	0.665
Gender (male vs female)	1.07 (1.01–1.09)	0.347	1.03 (1.00–1.32)	0.541
Tumor size	1.46 (1.05–2.18)	**<0.001**	1.38 (1.02–1.93)	**0.003**
Lymph node status (positive vs negative)	4.61 (2.13–9.20)	**<0.001**	3.27 (1.03–7.15)	**<0.001**
Tumor stage (≥pT3 vs pT2)	5.68 (2.91–9.66)	**<0.001**	4.79 (2.37–8.68)	**<0.001**
Tumor grade				
G2 vs G1	3.85 (1.75–7.82)	**0.004**	3.16 (1.47–6.92)	**0.015**
G3 vs G1	6.21 (2.33–9.18)	**0.026**	5.34 (1.94–7.67)	**<0.001**

The *p* values less than 0.05 were marked in bold.
Abbreviations: *HR*, hazard ratio; *CI*, confidence interval.

Table 4
Multivariable analysis for metastasis-free and overall survival.

	Metastasis-free survival		Overall survival	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
DACH2 status (positive vs negative)	7.34 (3.15–11.87)	**<0.001**	3.96 (2.04–7.16)	**<0.001**
Age	0.93 (0.49–1.28)	0.674	0.90 (0.47–1.11)	0.359
Gender (male vs female)	1.32 (1.05–2.14)	0.160	1.21 (1.02–2.09)	0.074
Tumor size	1.24 (1.00–1.87)	**0.002**	1.17 (1.01–1.94)	0.068
Lymph node status (positive vs negative)	2.94 (1.73–6.55)	**<0.001**	2.52 (1.05–5.88)	**0.027**
Tumor stage (≥pT3 vs pT2)	3.59 (1.83–7.57)	**0.008**	2.16 (1.08–6.34)	**<0.001**
Tumor grade				
G2 vs G1	2.34 (1.16–5.19)	**0.019**	1.43 (0.97–3.82)	0.092
G3 vs G1	2.56 (1.48–6.34)	0.064	2.78 (1.05–4.97)	**0.003**

The *p* values less than 0.05 were marked in bold.
Abbreviations: *HR*, hazard ratio; *CI*, confidence interval.

in the cisplatin resistant ovarian cancer cell line A2780-Cp70 compared to the cisplatin-sensitive ovarian cancer cell line A2780. Its expression correlated with several proteins involved in DNA integrity and proliferation, suggesting that DACH2 may be crucial in the regulation of cell proliferation. Our findings raise the possibility that DACH2 could have a critical role in the metastasis or more lethal behavior of muscle-invasive UCB. Herein, future studies are warranted to further elucidate the biological role of DACH2 in muscle-invasive UCB carcinogenesis, progression and metastasis.

Some limitations of this study should be noted. Firstly, only 56 patients finished the 5-yr follow-up, and the median is only 42 months. A longer follow-up may make it more convincing in estimated metastasis-free and overall survival rate. Secondly, the number of evaluated patients was not particularly large, which might limit the statistical power of the predictive value of DACH2. Consequently, further studies with larger patient populations and longer follow-up are needed to validate our current observations.

In conclusion, DACH2 is an independent prognostic marker for muscle-invasive UCB. Expression of this protein in primary UCB could identify patients who have a high potential to develop metastasis after radical cystectomy and die from this disease. These findings could have therapeutic implications in patients who might benefit from a different follow-up approach after surgery, and be used at initial diagnosis-the best time for considering early adjuvant treatment.

Conflict of interest

None.

Acknowledgments

This work was supported by Chinese Postdoctoral Fund (200801264, 20070420795 to L.Y.L) and the Fundamental Research Funds for the Central Universities (11ykpy39 to L.Y.L).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.119.

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