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The expression of IL-6 and STAT3 might predict progression and unfavorable prognosis in Wilms' tumor

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ABSTRACT

Purpose: To investigate the expression profiles of IL-6 and STAT3 in Wilms' tumor (WT) and their relationship with disease progression.
Methods: Immunohistochemistry was used to examine IL-6 and STAT3 expression in 58 primary tumors and 18 invasive/metastatic ones.
Results: Positive expression rate of IL-6/STAT3 was 39.7% (23/58)/29.3% (17/58) in primary WT tissues, while 61.1% (11/18)/33.3% (6/18) in associated invasive/metastatic tissues. The expression rate of IL-6 and STAT3 was higher in primary WT tumors of invasive/metastatic group than that of non-invasive/metastatic group ($P = 0.033$; $P = 0.012$). There was a positive correlation between IL-6 and STAT3 expression in 76 WT tissues ($P < 0.001$, $r = 0.444$). The expression of IL-6 /STAT3 between primary WT and matched invasive/metastatic tissues was concordance ($P = 0.727$; $P = 0.99$). IL-6 expression status and histopathological type were associated with disease-free survival (DFS) and overall survival (OS) ($P < 0.05$), while STAT3 was only correlated with DFS ($P < 0.05$).
Conclusions: IL-6 and STAT3 expression in WT might be correlated with progression and predict unfavorable prognosis, highlighting a new therapy target for invasive or metastatic WTs.

1. Introduction

Wilms' tumor (WT) is one of the most common malignant tumors in children with an incidence of approximately 1 in 10,000 [1]. The prognosis of the patients has greatly improved by the advancement of combination therapy. Nevertheless, about 10% patients with WT have poor survival suffering from metastasis or recurrence [2]. Although previous studies have showed some aberrant molecular events responsible for tumor progression such as p53 mutation, loss of E-cadhesion, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) overexpression [3–6], other biological factors governing Wilms' tumor invasion and metastasis remain largely unknown.

Research has suggested that deregulated inflammation is associated with most tumors [7]. Tumorigenesis and progression associated with inflammation are known to be influenced by multiple growth factors and cytokines including interleukin-6 (IL-6) [8]. The IL-6 signaling pathway is activated via binding to its receptor IL-6R resulting in downstream signal transmission through the JAK2/STAT3, RAS/MAPK and PI3K/AKT signal pathways and ultimately leading to induction of target genes that control cell proliferation, tumor invasion and metastasis [8–10]. In fact, an elevated serum IL-6 level has already been demonstrated to correlate with poor survival and unfavorable clinical outcome in some solid tissue cancers [11–14].

Among different transcriptional factors, activation of signal transducer and activator of transcription 3 (STAT3) by IL-6 leads to the increased expression of down stream target genes which could increase cell proliferation and survival, promote angiogenesis, tumor invasion and metastasis [7].

In view of IL-6/STAT3 signaling pathway involvement in tumorogenesis and progression, we conducted this retrospective research to evaluate the role of IL-6 and STAT3 in WT progression by immunohistochemistry for both primary tumor tissues and matched invasive/metastatic sites.

2. Materials and methods

2.1. Patients and samples

Patients with WT who underwent radical surgical resection were retrieved from the files of Department of Pediatric Surgery, Provincial Hospital affiliated to Shandong University during the period January 2003 to July 2011. Eligibility criteria included no

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prior chemo- or radiation therapy before surgery. A total of 58 cases with WT were enrolled in the research, of which 23 patients were diagnosed with invasive or metastatic disease by means of pathological detection after surgery or by imaging examination in the process of follow-up. Twenty-three invasive or metastatic sites included 4 perirenal adipose capsule (17.4%), 1 renal pelvis (4.3%), 2 ureter (8.7%), 5 lymph node (21.7%), 2 renal vascular (8.7%), 2 tumor embolus (8.7%), 4 postsurgical hematogenous metastasis (17.4%), 1 lymph node as well as renal vascular (4.3%), 1 lymph node as well as postsurgical hematogenous metastasis (4.3%), 1 tumor embolus formation as well as postsurgical hematogenous metastasis (4.3%). All the patients received postsurgical treatment according to NWTSG (National Wilms' Tumor Study Group) guidelines. Histopathological type and tumor stage were determined after pathological review. Clinicopathological factors including age, gender, stage and histopathological type. The paraffin- embedded tumor samples including invasive/metastatic specimens were retrieved from our hospital. The endpoint for disease-free survival (DFS) was the first documented day of recurrence distant metastasis or death from the disease. Overall survival (OS) was calculated starting from the first surgery to the date of death due to the disease or December 2012 for the surviving patients.

2.2. Immunohistochemistry

Briefly, 3-μm sections of tumor slides were deparaffinized with xylol. After rehydration, and washing in phosphate buffered saline (PBS), sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity at room temperature for 30 min. Then the antigen was retrieved in 0.01 M citrate buffer at pH 6.0 heated in a thermostat controlled water-bath at 97 °C for 15 min. Sections were incubated with 10% normal goat serum (Zhong Shan, China) to reduce nonspecific binding at room temperature for 30 min. After disposing of excess serum, the sections were incubated with the primary antibodies against IL-6 (1:100, mouse monoclonal (sc-130326), Santa Cruz, USA) and STAT3 (1:100, rabbit polyclonal (sc-7179), Santa Cruz, USA) over night at 4 °C. The secondary antibodies used were biotinylated goat anti-mouse IgG (SP-9002, Zhong Shan, China) and biotinylated goat anti-rabbit IgG (SP-9001, Zhong Shan, China) and were applied for 30 min at 37 °C. This was followed by the peroxidase-labelled streptavidin (Zhong Shan, China) applied for 30 min at 37 °C. Sections were stained with 3,3′diaminobenzidine substrate (ZLI-9033, Zhong Shan, China) for 3 min and counterstained with haematoxylin, dehydrated, and cover slipped. The negative control was carried out following the same steps but the antibody was replaced by normal serum from the same species of primary antibody. Cells were considered to be positive when stained for STAT3 in both the cytoplasm and nucleus and only stained in the cytoplasm in the case of IL-6 (Fig. 1). Immunohistochemical and pathological assessments were conducted by two investigators, completely blinded to any clinical information. Semi-quantitative immunoreactive score (IRS) was used as a scoring system [15]. Briefly, the IRS was calculated by multiplying the staining intensity(SI) (graded as: 0 = absent, 1 = weak, 2 = moderate and 3 = intense staining) and the percentage of positively stained cells (PP) (0 = no, 1 = 1–10% of stained cells, 2 = 11–50% of stained cells, 3 = 51–80% of stained cells and 4 = more than 80% of stained cells). Tumors were considered positive when IRS scoring at least 3.

2.3. Statistical analysis

Statistical analysis was performed using the SPSS 18.0 software package, with bilateral test $P < 0.05$ being considered significant. The association of IL-6 and STAT3 expression and clinicopathological factors was analyzed using Chi-square test. IL-6 and STAT3 immunostaining in primary and matched invasive/metastatic tissues were analyzed by the Mc Nemar's test. The survival analysis was calculated by the Kaplan–Meier method and checked using the log-rank test. Because of significant correlation between IL-6 and STAT3 expression and histopathological type, we did not perform multivariate analysis.

3. Results

3.1. Patients characteristics

Of the 58 patients, 20 were female (34.5%) and 38 were male (65.5%), with the median age of 31 months (range: 3–132 months). 45 patients (77.6%) had favorable histology (FH) while 13 patients (22.4%) had unfavorable histology (UH). The stage I–III and V occurred in 33 (56.9%), 11 (19.0%), 13 (22.4%) and 1 (1.7%) patients respectively. Five patients of the 23 patients diagnosed with invasive or metastatic disease mentioned above had no available tumor tissue samples. Subsequently, 18 pairs of primary tumors and matched invasive/metastatic tissues were collected for analysis. Thirteen patients suffered from relapse in which 10 cases died of the disease and the remaining 3 received secondary surgical therapy but were lost to follow up (Table 1).

3.2. IL-6 and STAT3 expression in primary WTs were correlated with invasion/metastasis and histopathological type

The overall positive rates of IL-6 and STAT3 were 39.7% (23/58) and 29.3% (17/58) in primary tissues, respectively. The expression rate of IL-6 in the group without invasion/metastasis was 28.6% (10/35) while 56.5% (13/23) in the invasive/metastatic group ($P = 0.033$). The expression rate of STAT3 in the group with and without invasion/metastasis was 47.8% (11/23) and 17.1% (6/35) ($P = 0.012$). IL-6 expression in patients with unfavorable histology (UH) and favorable histology (FH) was 69.2% (9/13) and 31.1% (14/45) respectively ($P = 0.013$). STAT3 expression in patients with unfavorable histology (UH) and favorable histology (FH) was 53.8% (7/13) and 22.2% (10/45) respectively ($P = 0.027$). STAT3 expression was also associated with staging and age ($P = 0.006$, $P = 0.032$) (Table 1).

3.3. Positive correlation between IL-6 and STAT3 expression in WTs

Spearman analysis showed that a positive correlation between the expression of IL-6 and STAT3 was observed in 76 WT tissues including 58 primary and 18 invasive/metastatic tissues ($r = 0.444$, $P < 0.001$) (Table 2).

3.4. Comparison of IL-6 and STAT3 staining between primary WTs and matched invasive/metastatic tissues

As shown in Table 3, a total of 18 primary WTs and matched invasive/metastatic tissues were collected. In these 18 cases, the expression rates for IL-6 and STAT3 in primary WTs were 50% (9/18) and 33.3% (6/18), respectively. IL-6 and STAT3 expression were found in 11 cases (61.1%) and 6 cases (33.3%) of matched invasive/metastatic tissues, respectively. Eight paired (8/18, 44.4%) primary and matched invasive/metastatic tumors were discordant in IL-6 expression ($P = 0.727$). For STAT3 expression, discordance was observed in 4 cases (4/18, 22.2%) between primary and matched invasive/metastatic tumors ($P = 0.99$).

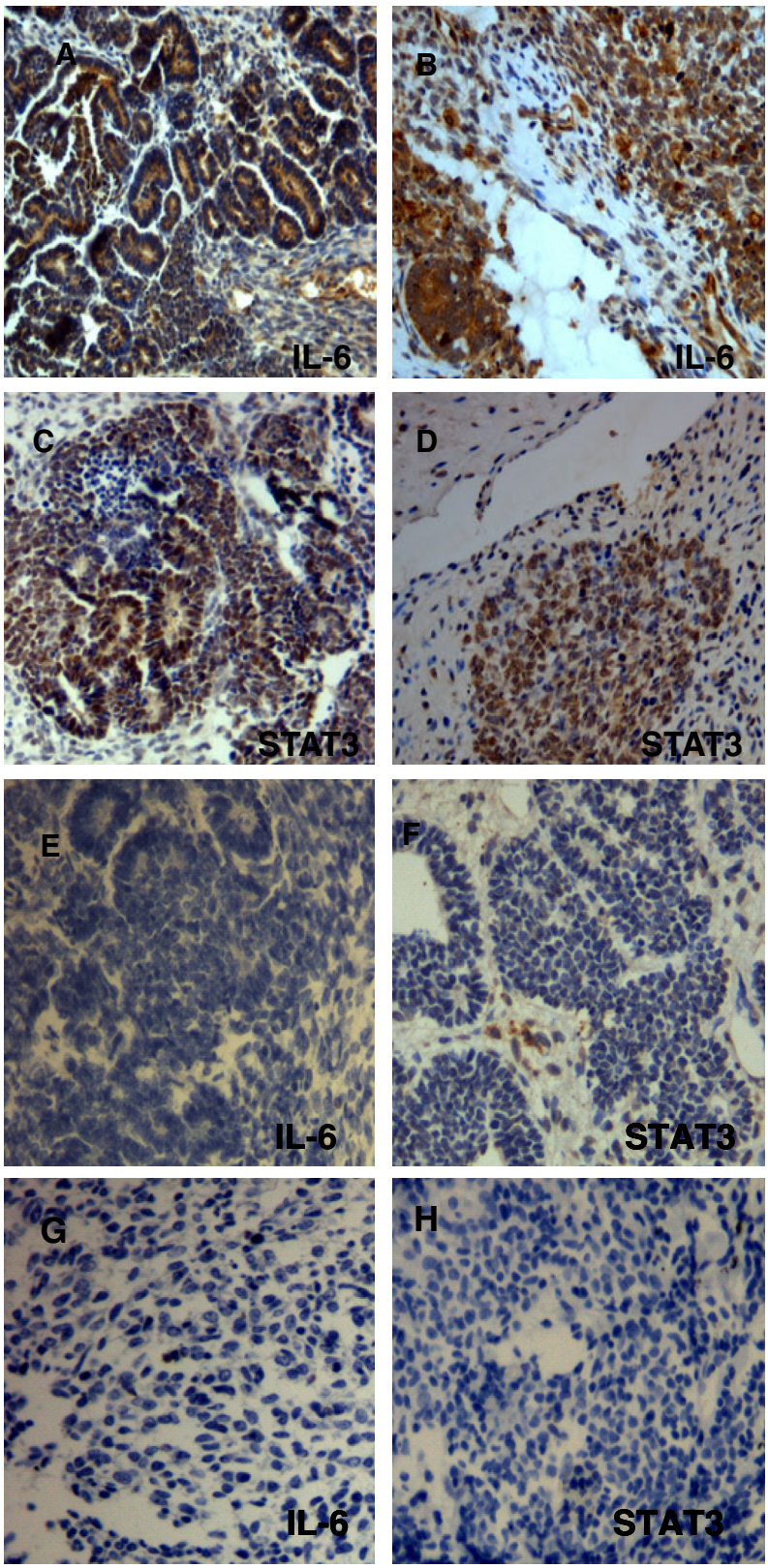

Fig. 1. The expression of IL-6 and STAT3 in WT tissues A–D shows the expression of IL-6 and STAT3 in primary WTs and their corresponding invasive/metastatic tissues from the same patient. A/C shows the positive expression of IL-6/STAT3 in primary WT, while B/D shows their positive expression in the matched renal pelvis invasive tissue. E/F shows the negative expression of IL-6/STAT3 in primary WTs, while G/H shows the result of negative control for the antibody IL-6/STAT3, respectively (all images, ×200).

3.5. Association of IL-6 and STAT3 expression with clinical outcome

Fourty-five cases were enrolled in survival analysis comprising 42 patients who have complete follow-up and 3 patients who received a secondary surgery after disease relapse but were lost to follow-up. Kaplan–Meier analysis was conducted to determine whether IL-6 and STAT3 expression and other clinical factors had predictive for therapy and prognostic value for survival. As shown

Table 1
The relationships between the expression of IL-6 and STAT3 in WTs and the clinicopathologic factors of the patients.

Clinicopathological factors	IL-6 expression		$\chi 2$	P	STAT3 expression		$\chi 2$	P
	Positive	Negative			Positive	Negative		
Gender								
Male	18	20	2.740	0.098	13	25	1.277	0.258
Female	5	15			4	16		
Age (months)								
>24	12	13	1.279	0.258	11	14	4.576	0.032
⩽24	11	22			6	27		
Stage								
I	10	23	6.899	0.075	4	29	12.279	0.006
II	8	3			6	5		
III	5	8			7	6		
V	0	1			0	1		
Invasion/metastasis								
Yes	13	10	4.531	0.033	11	12	6.307	0.012
No	10	25			6	29		
Relapse								
Yes	6	7	0.296	0.587	6	7	2.294	0.130
No	17	28			11	34		
Histopathological type								
Favorable	14	31	6.125	0.013	10	35	4.868	0.027
Unfavorable	9	4			7	6		

Table 2
The relationship between IL-6 and STAT3 expression in all tumor tissues.

STAT3 expression	IL-6 expression		$\chi 2$	P
	Positive	Negative		
Positive	18	5		
Negative	16	37	14.993	<0.001

Pearson's $R = 0.444$.

Table 3
The expression of IL-6/STAT3 in primary WT and their corresponding invasive/metastatic tissues (Inv/Meta).

Inv & Meta (IL-6/STAT3)	Primary WT tissues (IL-6/STAT3)		P
	Positive	Negative	
Positive	6/4	5/2	
Negative	3/2	4/10	0.727/0.99

in Fig. 2, the IL-6 expression status and histopathological type was associated with DFS and OS. Patients with IL-6 expression had a shorter DFS and OS compared with those with no IL-6 expression (28.8 months vs 65.5 months, $P = 0.025$; 34.5 months vs 70.2 months, $P = 0.037$). Patients with unfavorable histology (UH) had a median DFS and OS of 20.4 months and 29.9 months compared with 63.2 months and 69.9 months of those with favorable histology (FH), respectively ($P = 0.002$, $P = 0.006$). STAT3 expression was only correlated with DFS (26.4 months vs 68.2 months, $P = 0.004$), but not OS (35.2 months vs 66.9 months, $P = 0.102$).

4. Discussion

Tumor cell invasion is a very complicated and multistep process in which many molecules are involved. Malignant tumors shed large numbers of cells into the blood and lymph vessels, some of them developing in distant sites into metastases [16]. Therefore, the consensus is that searching for tools to allow effective assessment of invasive potential of tumors is a primary goal for cancer research.

Chronic inflammation is associated with tumor initiation and promotion. Inflammatory cells such as T cells, dendritic cells and tumor associated macrophages (TAM) can secrete cytokines and chemokines. Cytokines promote immune or inflammatory reactions and either favor antitumor immunity or enhance tumor progression. They also directly effect cancer cell growth and survival through activation of various downstream effectors such as nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and *Drosophila* mothers against decapentaplegic (SMAD) [17]. However, some clinical trials provide evidence that anti-inflammation drugs combined with other traditional therapy do not improve survival of certain solid tumors [18]. One explanation is that different cytokines can either promote or inhibit tumor development and progression at various stages of cancer development [17,19]. So it is meaningful for us to confer which type of cytokine act as a tumor initiator or promoter during oncogenesis. IL-6, secreted principally by lymphocytes and macrophages, is a pleiotropic cytokine with obvious tumor-promoting effects. Studies have revealed that IL-6 can actually be produced by the tumor cells and act by altering tumor cell function of target cells in an autocrine fashion [20]. In other solid tumors such as lung cancer, prostatic cancer and breast cancer, IL-6 has been demonstrated to play an important role at the stage of tumor progression but not that of tumor initiation [14,12]. STAT3 is the central partner of the IL-6/JAK2/STAT3 signaling pathway.

In this retrospective study, our results indicated IL-6/STAT3 signaling pathway might play an important role in the progression of WT. The expression of IL-6 and STAT3 was significantly associated with invasion/metastasis ($P = 0.033$; $P = 0.012$). Our data also suggested that IL-6 and STAT3 staining by IHC were good predictors for outcome after therapy. Kaplan–Meier survival analysis demonstrated that patients with either IL-6 or STAT3 expression had shorter DFS than those without expression. Survival analysis also showed that patients with IL-6/STAT3 had shorter OS than those without IL-6/STAT3 expression, although there was only a statistically significant difference for IL-6 expression. In all, our research suggested that IL-6/STAT3 signaling pathway might associate with disease progression and predict poor prognosis of WT. The therapy for targeting IL-6 and STAT3 might improve the outcome of some selective patients after surgery.

Previous study in other cancer showed that altered molecules happened in primary carcinomas may not predict the status in

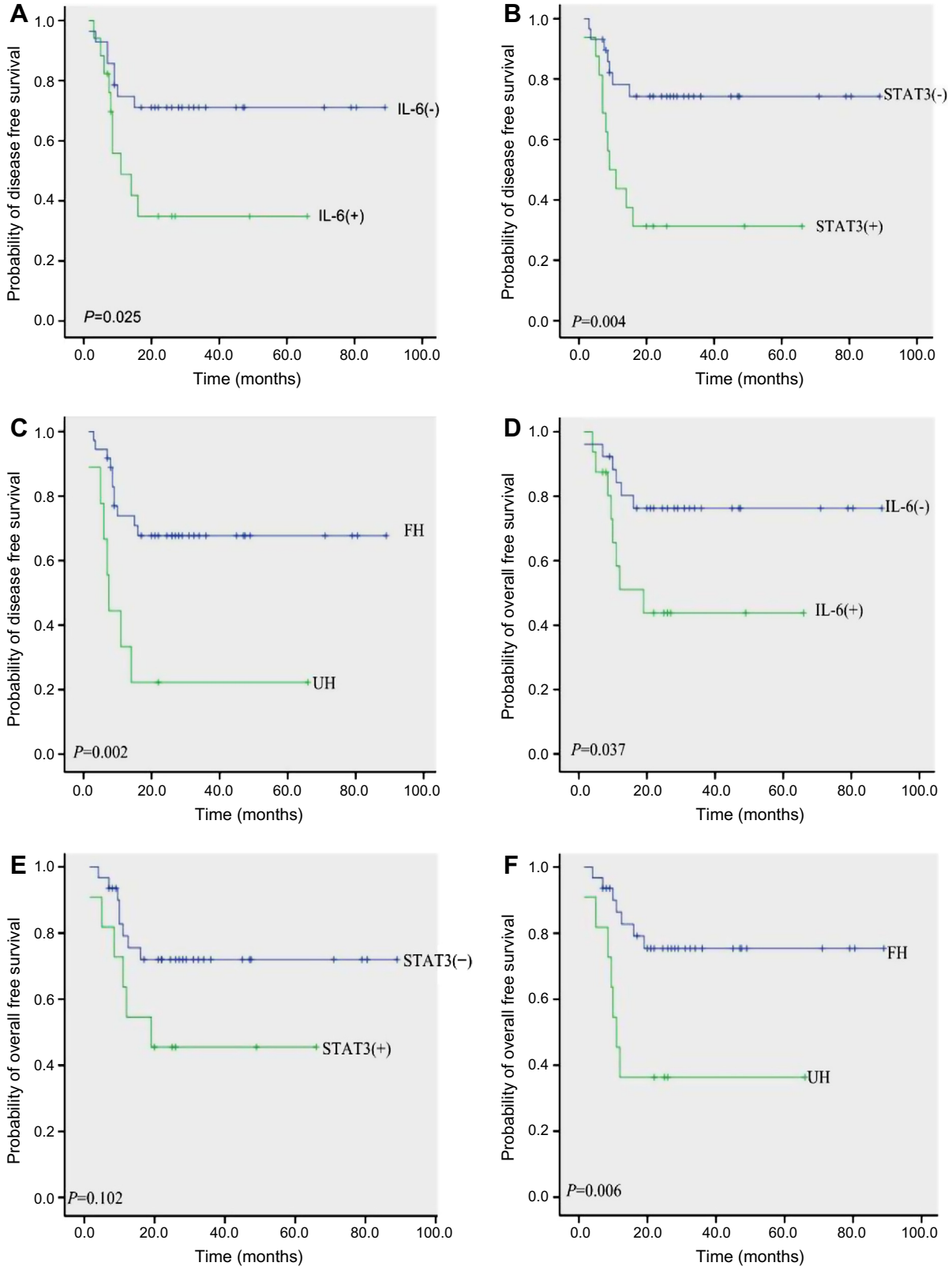

Fig. 2. Survival analysis. Kaplan–Meier curves of DFS and OS for IL-6/STAT3 expression and histopathological type. Kaplan–Meier curves reach statistical significance of disease free survival (DFS) in WT patients according to IL-6 expression (A), STAT3 expression (B) and histopathological type (C). Patients with IL-6 expression had shorter overall survival (OS) than those without IL-6 expression (D). Also compared with patients with unfavorable histology (UH), patients with favorable histology (FH) had longer OS (F). Although there was difference of OS between patients with and without STAT3 expression, It did not reach a statistically significance (E).

the matched metastases [21]. It is very important for us to explore common alterations both in primary carcinomas and metastases which may have important implications for molecular testing for targeted therapy. The comparison of IL-6/STAT3 expression showed a remarkable concordance between primary WTs and matched invasive/metastatic sites. Our research shows that cancer cells with IL-6 and STAT3 expression in primary tumors might easily migrate and colonize in a distant site. But we also noticed that a small fraction of cases had a discordance of these protein alterations between primary tumors and matched invasive/metastatic tissues. This discordance reflected the individual difference existing in patients with other mechanism involved in the process of metastasis.

Because of the important role of aberrant IL-6/STAT3 signaling pathway in cancer progression, IL-6 blocking antibodies have been tested preclinically either alone or combined with cytotoxic chemotherapies, showing tumor growth inhibition. An IL-6 ligand-blocking antibody (CNTO-328) is being tested in some phase I/II clinical trials in transplant-refractory myeloma and castrate-resistant prostate cancer [22,23]. To target STAT3, a number of STAT3 inhibitors have been designed and have demonstrated marked activity against tumors [24]. Preclinically, a STAT3 decoy was tested in head and neck squamous cell carcinomas expressing high levels of tyrosine phosphorylated STAT3 [25]. As we have observed a high expression of IL-6 and STAT3 in WT and potential roles in tumor invasion and metastasis, administration of IL-6 blocking antibodies or STAT3 inhibitors may be might be useful tools to improve prognosis of this type of cancer through detection of IL-6 and STAT3 expression in future.

In conclusion, this study suggests that IL-6/STAT3 might play an important role in WT progression and be associated with patients' poor prognosis. This research highlights a new therapeutic target for invasive and metastatic WT patients. Further studies should be conducted using WT cell lines to discover the mechanism of IL-6/STAT3 signal pathway in the progression of this tumor.

It is a single-institution pilot study. The number of patients is limited and the successful rate for treatment of these Wilms' tumor patients is a bit lower than multiple other centers' indeed. So, more conclusive results may be obtained with increased numbers of patients included.

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