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Molecular prediction of Wilms' tumor relapse risk

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Abstract: Background: Despite overall improved outcomes in Wilms' Tumor (WT), certain populations of patients continue to experience poor survival and increased rates of relapse. This article is dealing with studying the prognostic value of genetic marker and test its association with the risk of WT relapse. The measurements of b-FGF were made by RT-PCR and Immunohistochemical analysis. **Methods:** To study the discrepancy between patients with WT relapse and WT relapse-free, a prospective trial was carried out for 40 children; 20 patients had disease relapse and 20 remained relapse-free at last follow-up. Twenty patients' autologous normal renal tissue was used as control. Formalin-fixed, paraffin-embedded blocks of tumors and healthy renal tissue were used to obtain the total RNA. The expression of the b-FGF gene was assessed using quantitative real-time PCR (qRT-PCR) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. **Results:** The comparison between WT relapse and relapse-free, the b-FGF had significant difference (P value < 0.05) and strong FGFR3 expression was detected in the cytoplasm of tubules in relapsed patients 14(60.87)% compared to free relapsed patients 3(10.71)%. **Conclusions:** WT with the higher expression levels of b-FGF are associated with an increased risk of relapse disease which can serve as future prognostic predictors and help to support patients for treatment and follow-up regimen.

keywords: Wilms'Tumor, recurrence, Fibroblast growth factors, prognosis

1.Introduction

A Wilms' tumor (WT) is the most common pediatric renal tumor; approximately 95% are of favorable histology a childhood kidney tumor. Patients with this tumor have a 90% survival rate. However, 15% of patients experience a tumor recurrence [1]. According to theory, WT arises from metanephric tissue or nephrogenic rests. Only 35% of unilateral tumors have these aberrant metanephric cells, compared to up to 100% of bilateral Wilms cases [2]. Most of relapses occur in the first 2 years after nephrectomy, but late relapses may occur up to 13 years after nephrectomy. The lungs are the most common site of recurrence. Other sites include the liver, brain, contralateral kidney, original renal fossa and other intra-abdominal sites [3]. The risk factors for relapse include advanced stage, unfavorable histology, older age at diagnosis, larger tumor size, lymph node involvement, positive surgical margins, and local or diffuse tumor spills [4]. Although the cause of WT is not precisely known, it is thought to be caused by genetic changes that affect normal embryological development of the genitourinary system [5]. The basic fibroblast growth factor (b-FGF) plays a ubiquitous role in normal cell growth, survival, differentiation and formation of blood vessels, but it has also been implicated in tumor development [6]. Increased FGF receptor signaling effects on tumor cells as well as the stroma around them, including the vasculature. This dual activity plays an important role in tumor development [7]. The potential of FGFs

to promote tumor progression is highly dependent on specific FGFR signaling. b-FGF is particularly widespread, being overexpressed in many malignant tumors including bladder, melanomas, astrocytomas, breast, pancreatic, non-small lung cell, head and neck, prostate, and hepatocellular carcinoma [8].

Several studies had compared expression of b-FGF between tumor and control, but there are not enough to compare between WT relapsed and WT relapse-free. Therefore, this study was designed to investigate the oncogenic nature of b-FGF signaling replace network with pathway in Wilms 'tumor relapse.

2. Patients and methods:

Patients:

This prospective study was undertaken after Institutional Review Board (IRB) approval at Mansoura Urology and Nephrology center. The exclusion criteria were bilateral synchronous WT and syndromic cases. Patient files were reviewed for demographics, imaging studies, treatment received and histopathology reports. Follow-up data was queried for the occurrence of tumor relapse, time to relapse, relapse site and survival outcome. The study was performed on pathological specimens of 40 children treated for WTn, 20 patients had relapse disease and 20 remained relapse-free. Twenty autologous normal renal tissues were utilized as control.

Methods:

Quantitative reverse transcription PCR reaction

Using the RNeasy FFPE Kit (Qiagen, cat no. 73504), total RNA was extracted from formalin-fixed paraffin embedded blocks of tumors and normal renal tissues. mRNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, cat no.4368814). Quantitative PCR was performed with Maxima SYBR Green qPCR Master Mix (2X-Thermo Scientific, cat no.K0221) on the Roto-Gene real time PCR. The mRNA expression level of b-FGF gene in tumors that relapsed, tumors that remained free of relapse and adjoining normal renal tissue were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. The primer sequences that used in the present study is:

F: 5'AGCGGCTGTACTGCAAAAACGG3'

R: 5'CCTTTGATAGACACAACTCCTCTC3'

The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of the target gene. The expression levels of the examined gene were tested for association with WT relapse and survival outcomes.

Immunohistochemical study

Deparaffinized renal sections were heated for 20 min in citrate buffer pH 6.0 after being incubated for 30 min in 0.3% hydrogen peroxide (H_2O_2) and methanol, the endogenous peroxidase was blocked. Then, sections were incubated with primary human monoclonal antibody FGFR3 (Cat no. MAB766, R&D Systems, US) as much as needed to cover the tissue. The sections were washed in phosphate buffered saline (PBS). Diaminobenzidine tetrahydrochloride (DAB) as chromogen substrate solution was used to develop brown color. Slides were counterstained with hematoxylin, dehydrated and mounted. The staining intensity was graded as follows: 0, no staining; 1, weak stained; 2, moderately stained; 3, strong stained. The grade for the percentage of cells that displayed positive is scaled as follows: 1, 0–5%; 2, 6–25%; 3, 26–50%; 4, 51–75%; and 5, > 75%. Final scores were given to all tissue sections based on the multiplication of intensity and percentage scores. A final score of five or more was considered an overexpression and a score of less than five was considered low expression [9].

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 23.0 (Armonk, NY: IBM Corp). In the normally distributed variables, the chi-square test and ONEWAY ANOVA were used when appropriate P value $\leq$ 0.05 was considered statistically significant.

3. Results

40 WT patients (20 relapse and 20 relapse-free) treated at Mansoura Urology and Nephrology Center. **Table1** shows the patient demographic characteristics of the study groups.

Groups were matched in age, sex, body mass index (BMI), and renal function.

Most patients had lower tumor stage 30/40 (75%) stage I disease and with favorable histology 37/40 (92.5%). All patients were treated with radical nephrectomy and lymph node sampling through an open approach

Table 1: Demographic and Clinical characteristics of study groups

	Wilms' tumor relapse(N= 20)	**Wilms' tumor relapse-free (N = 20)**	**Control (N = 20)**	***P* value**
Age, years(mean ± SD)	3.03 ± 1.9	3.95 ± 2.3	3.55 ± 1.90	0.381
BMI(mean ± SD)	30.24 ± 43.7	26.58 ± 34.4		0.783
Gender, no. (%) – Male – Female	9 (15 %) 11 (18.3%)	11 (18.3 %) 9 (15 %)	10 (16.7%) 10 (16.7%)	0.819
Serum creatinine, mg/dL(mean ± SD)	0.97 ± 2.6	0.38 ± 0.1		0.248
Tumor stage, no. (%) – T1 – T2 – T3 – T4	16 (80%) 3 (15%) 1 (5%) 0 (0%)	14 (70%) 1 (5%) 3(15%) 2(10%)		0.247
Cell type final histology, no. (%) – Favorable – Un-favorable	19 (95%) 1 (5%)	(%85) 17 3 (15%)		0.115
* $p \leq 0.05$				

Gene expression analysis

Quantitative real-time PCR was carried out for all WT patients in formalin fixed paraffin embitted tissue (FFPE). **Table 2** shows comparison between b-FGF expressions in tumor relative to autologous renal tissue. Tissue expression levels of b-FGF gene was significantly increased in tumor tissue more than normal tissue with (p value < 0.05) **(Figure 1A).** The comparison between relapsed and free-relapse WT tissue show significantly higher among patients who had WT relapse relative to patients who remained free of relapse **(Figure 1B).** When comparing gene expression in patients who remained free of relapse to controls, WT patients who remained free of relapse had higher expression levels of b-FGF (p = 0.044).

Table 2: Expression level of b-FGF gene in FFPE:

		Mean gene expression levels ± SD	***p* value**
	Wilms' tumor tissue(n=40)	2.12 ± 0.87	< 0.001
	Control/ autologous renal tissue)n=20(	1.04 ± 0.57	
WT	WT relapse)n=20(	2.89 ± 0.4	< 0.001
	Relapse-free (n=20)	1.34 ± 0.3	

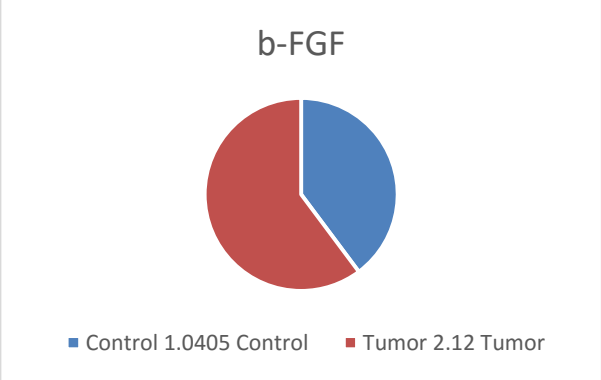


Fig 1A: Gene expression level of b-FGF in WT tissues among patients relative to their levels in autologous renal tissues

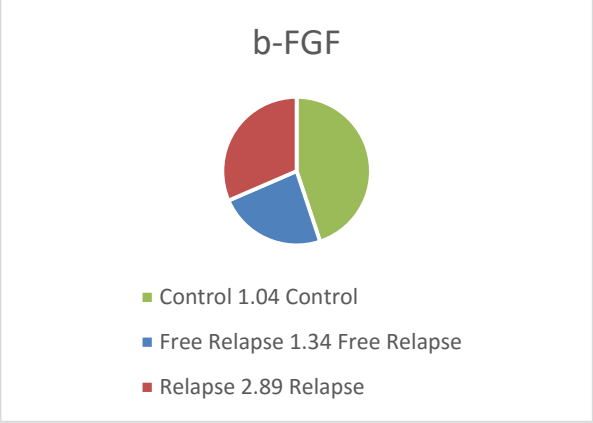
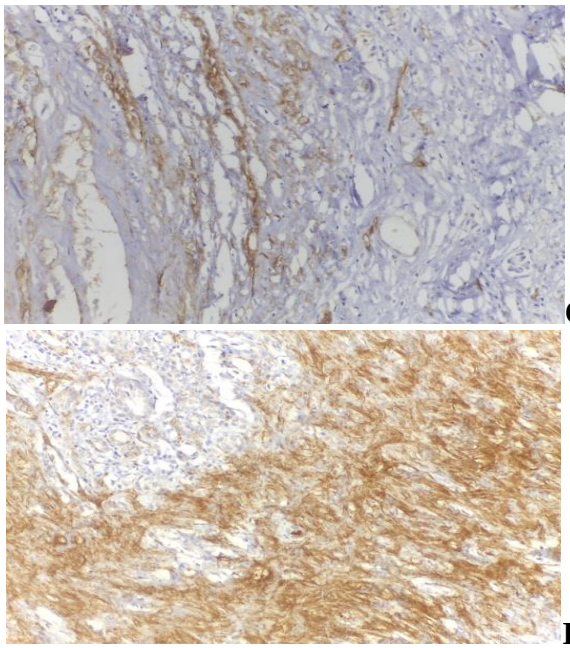


Figure 1B: Gene expression of b-FGF in WT tissues among patients who had disease relapse and those who remained free of relapse.

Immunohistochemically analysis

There was a significant increase in the immunoreactivity of FGFR3 antibodies in relapsed patients compared to free relapsed patients ($p < 0.001$).

Kidney tissue showed a very strong FGFR3 expression in the cytoplasm of tubules in relapsed patients compared to free relapsed patients (Figure2)



C

D

Fig2: Photomicrograph showing FGFR3 showing mild cytoplasmic staining in free relapsed patients and marked cytoplasmic staining in relapsed patients (x200) (C-D).

4. Discussion

A single institution analysis have been describe of all patients with Wilms tumor, including relapsed cases and relapse-free cases, a molecular study was required to clarify the difference between the two cases and to understand the behavior of each one of them.

In the current study, the molecular marker (b-FGF) and FGFR3 protein on both WT relapse and WT free relapse have been investigated and then compare with control as they play important role in cancer biology.

The basic fibroblast growth factor (b-FGF) have oncogenic role as it stimulate tumor progression, cell growth and angiogenesis. Expression of b-FGF and FGFR receptor in both tumors and their surrounding normal tissue was assessed.

In the present study the quantitative real-time PCR (qRT-PCR) was applied to evaluate the expression of b-FGF gene. In comparison to the adjacent normal, there are significant increments in the levels of b-FGF gene expression in relapsed WT patients than free relapsed and control, ($P < 0.001$). The present results is in consists with the results obtained by [10].

When b-FGF activity was compared between WT free-relapse and control, no significant differences were found between them ($P > 0.05$); ($P = 0.130$).

In relapsed WT, there were a significant increase in the immunoreactivity of FGFR3 antibodies compared to free relapsed patients ($P< 0.001$). This result agrees with study that reported FGFR3 was overexpressed in urothelial carcinoma; non-invasive tumors, and tumors with recurrences. It can also be used as a marker to determine the grade in difficult cases and the risk of recurrence [11]. In relapsed WT, FGFR3 was 14 (60.87%) strong cytoplasmic staining in compared to normal tissue and 6(21.44%) moderate cytoplasmic staining in free relapsed WT patients.

In the comparison between relapsed and free-relapsed, FGFR3 is overexpressed in relapsed than relapsed-free WT. The significant expression of b-FGF and FGFR3 protein in relapsed WT have demonstrated strong associations with the risk of disease relapse and overall survival.

5. Conclusion:

The present study indicates that there is a molecular difference between relapsed and relapse-free WT compare to normal tissue. This molecular difference may help in directing potential therapeutic interventions in hopes of improving WT treatment and decreasing risk of relapse.

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