Tissue factor expression predicts outcome in children with neuroblastoma: A retrospective study

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Abstract. Previous studies have revealed that the processes of tumor angiogenesis, metastasis and invasiveness are highly dependent on components of the blood coagulation cascade. Tissue factor (TF) is one of the key proteins in coagulation. Cumulative evidence suggested that in addition to its role in coagulation, TF regulates intracellular signaling pathways that serve an important role in angiogenesis, tumor development and metastasis. In the present study, TF expression in neuroblastoma as well as its association with tumor stage, pathology and outcome were assessed. A total of 40 formalin-fixed paraffin-embedded tissues were evaluated for TF expression by immunohistochemical analysis. Results revealed that TF expression was positive in 75% of the analyzed tumor tissues. No significant association between TF expression and sex, age, tumor stage or disease pathology was observed. MYCN proto-oncogene bHLH transcription factor (*MYCN)* was upregulated in 45% (n=18) of the study cases. Positive TF expression was observed in 94.4% of patients (n=17) with upregulated *MYCN*, while 59% of patients (n=13) with normal *MYCN* showed positive TF expression (P<0.05). TF expression was a significant outcome predictor for patients; 18/30 patients (60%) with positive TF expression succumbed to the disease during the study period. In conclusion, TF may be a promising prognosis indicator for neuroblastoma. Future studies to determine how TF affects the progression and outcome of neuroblastoma, as well as to investigate its potential role as a therapeutic target, are required.

Introduction

Neuroblastoma is the most common extracranial tumor in infants and accounts for 8-10% of all childhood tumors. Neuroblastoma resulted in ~15% of pediatric cancer-associated mortalities in 2010. The majority of cases with neuroblastoma (90%) are diagnosed before the age of 5 years and 30% of these cases are present within the first year. The median age of diagnosis is 22 months (1). Neuroblastoma is an embryonal tumor derived from precursor cells of the sympathetic nervous system. It is extremely heterogeneous with clinical presentations ranging from spontaneous remission to rapid tumor progression and mortality (2). Neuroblastoma prognosis varies greatly. Children with neuroblastoma exhibit marked variability in outcome when the disease is categorized by age, stage and biological characteristics (3). Patients with low- and intermediate-risk neuroblastoma, characterized by favorable stages and age at time of diagnosis <1 year, have a 5-year overall survival >90% following chemotherapy and surgical resection. By contrast, the 5-year overall survival for high-risk neuroblastoma, characterized by features such as metastasis, age at diagnosis >1 year and upregulated *MYCN*, is 40-50% despite intensive treatment protocols that include chemotherapy, surgery, radiation and stem cell transplantation (4,5). The biological behavior of neuroblastoma is influenced by specific factors which are also outcome predictors. These include patient-associated factors, including age at the time of diagnosis and tumor-associated factors, including histology, tumor stage, molecular and cytogenetic features (6). Tissue factor (TF) is the primary cellular initiator of blood coagulation and is a modulator of cancer angiogenesis (7). Hypercoagulability in cancer patients is closely associated with tumor progression (8). Cancer is associated with a 4-fold increase in thrombosis risk, with chemotherapy further increasing the risk (9). A number of risk factors. including leukocyte and platelet counts and circulating levels of tissue factor (TF), P-selectin and D-dimer are also implicated (10).

Cancer cells and their vascular stroma often exhibit procoagulant properties. An example of this is the deregulation of TF expression, as TF is an important coagulation factor in the extrinsic coagulation pathway (11). Previous studies revealed TF to be a contributing factor in intracellular signaling events through the TF cytoplasmic domain. TF activates protease-activated receptors (PAR) 1 and 2 via the TF/factor VIIa/factor Xa signalling pathway (12). Previous studies revealed a link between tumor cell-associated procoagulant function and cancer biology, as well as TF expression

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and poor patient outcome in hepatocellular, ovarian and gastric carcinoma (13-15). The present study investigated the association of TF expression with tumor pathology, stage and outcome in patients with neuroblastoma.

Materials and methods

Patients. A total of 40 patients with neuroblastoma treated at the Pediatric Oncology Unit of Zagazig University Hospital (Zagazig, Egypt) between January 2008 and December 2015 were enrolled in the current study. The mean follow-up period was 29.1 months. The mean age of patients was, 2.29±1.67 years (age range, 5 months to 5.5 years). There were 24 (60%) males and 16 (40%) females. All patients were treated according to the Children Oncology Group risk-based protocol (16). Relevant laboratory data, including complete blood count, liver and kidney function tests, serum electrolytes, lactate dehydrogenase, and vanillyl mandelic and homovanillic acid levels, were collected from patient medical records.

Clinical data. Data collected included patient age at diagnosis, sex, clinical symptoms and associated conditions at diagnosis. Data from computed tomography of the chest and abdomen, magnetic resonance imaging of the spine and bone and metaiodobenzylguanidine (^{131}1-MIBG) scans were used in the present study. Tumor data collected included tumor site, metastasis, stage according to the International Neuroblastoma Risk Group Staging System (INRGSS) (17), histopathology according to the International Neuroblastoma Pathology Classification (18) and *MYCN* expression status according to the recommendation of the European Neuroblastoma Quality Assurance Group (19).

Immunohistochemistry protocol. All samples were untreated biopsy specimens from primary tumors and were examined independently by two pathologists who were blinded to the collected data. Tissue samples were fixed in formalin and embedded in paraffin blocks according to standard procedures (20). Glass slides were cleaned with 95% ethanol, treated with subbing solution (0.5% gelatin in warm deionized water) and air-dried. Alternatively, pretreated slides were used. The tissue samples were cut into 4-6-μm thick sections and mounted onto slides. The sections were subsequently deparaffinized in xylene and rehydrated in a descending alcohol series: The sections were washed in 100% ethanol twice for 10 min each time, followed by two 10 min washes in 95% ethanol and one 1 min wash in deionized water with stirring. The heating temperature of the antigen retrieval step was 95-100˚C. The sections were incubated with 0.1% hydrogen peroxidase in deionized water for 5-10 min at room temperature and rinsed in distilled water. Blocking with 2-5% normal serum (Santa Cruz Biotechnology, Inc.) for 30 min at room temperature was performed to reduce background staining. Endogenous peroxidase activity was blocked using 0.5-3% hydrogen peroxide. Expression of TF was determined by incubating with an anti-TF mouse monoclonal antibody (cat no. sc-80952; 100 mg/ml; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Primary antibody incubation was followed by incubation with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:100; cat. no. HAF007; R&D systems, Inc.) for 1 h at room temperature. Sections were counterstained using hematoxylin for 30 sec at room temperature. Sections incubated with 2-5% normal mouse serum (Santa Cruz Biotechnology, Inc.) instead of the primary antibody were used as a control. Neoplastic cells were considered positive when they revealed cytoplasmic or membrane staining.

Immunohistochemistry interpretation. TF expression was visualized with an Axioskop 40 optical microscope (Carl Zeiss AG) at a magnification of x100, and analyzed using Image-Pro Plus image analysis software (version 7; Media Cybernetics, Inc.). Each slide was examined by two pathologists independently, and quantitative analysis of immunohistochemical expression of TF was performed using the method outlined by Sierko *et al* (20). This method pertains to cancer cell percentage with positive staining and staining intensity. Values from 0-4 were assigned to cancer cell percentage with positive staining. These values, referred to as the A value, were as follows: 0, no staining; 1, ≤10; 2, 11-50; 3, 51-75 and 4, >75%. Staining intensity values, referred to as the B value were assigned from 0-3 and were as follows: 0, no staining; 1, weak; 2, medium and 3, strong. The immunoreactive score (IRS) was calculated by multiplying the A and B values. The IRS value corresponded with TF expression and was assigned the following values: 1, negative; 2, weak; 3, medium and 4, strong. The IRS was assessed for cancer cells and tumor vascular endothelial cells that expressed TF as detected by optical microscopy (at least 20 high power fields/sample; magnification, x100).

Statistical analysis. SPSS software (version 15.0; SPSS, Inc.) was used for data handling and statistical analyses. Data are presented as the mean ± standard deviation for quantitative variables. Categorical variables are expressed as number (%). Variables were compared using Chi-squared test, Student's t-test, one-way ANOVA and Kruskal-Wallis tests, respectively. Fisher's least significant difference test was used as a post hoc test. The Kaplan-Meier method, log-rank, Breslow and Tarone-Ware tests were for survival curve analysis. The cumulative survival probability (S_t) is the proportion of patients surviving (or remaining event-free) past interval t and it is computed as follows. Firstly, the proportion of participants surviving past time 0 (the starting time) is defined as S_0=1 (all participants alive or event-free at time zero or the study start). The proportion surviving past each subsequent interval is computed using principles of conditional probability. The probability that a participant survives past interval 1 is $S_1=p_1$. The probability that a participant survives past interval 2 means that they had to survive past interval 1 and through interval 2: S_2=P (survive past interval 2)=P (survive through interval 2) x P (survive past interval 1), or $S_2=p_2$ x S_1. In general, $S_{t+1}=p_{t+1}$ x S_t. Death was used as the event of interest and the status at serial time (points of assessment during follow-up) was either 1=event of interest (death) or 0=censored. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data. Of the 40 patients, 22 (55%) were >18 months old, and 18 (45%) were ≤18 months old (mean, 2.29±1.67 years; range, 5 months to 5.5 years). A total of 24 patients (60%)

Table I. Clinical and pathological characteristics of the patients.

Variable	Number of patients (%)
Age	
Mean	2.29±1.67 years
Range	5 months-5.5 years
≤18 months	18 (45)
>18 months	22 (55)
Gender	
Males	24 (60)
Females	16 (40)
Primary tumor site	
Abdomen	34 (85)
Mediastinum	4 (10)
Neck	2 (5)
Metastatic sites	
Bone marrow	22 (55)
Bone	20 (50)
Eye	14 (35)
Liver	4 (10)
Stages	
Stage I	0 (0)
Stage II	4 (10)
Stage III	12 (30)
Stage IV	24 (60)
Pathology	
Favorable Shimada	20 (50)
Unfavorable Shimada	20 (50)
MYCN proto-oncogene bHLH transcription factor status	
Amplified	18 (45)
Non-amplified	22 (55)
Risk stratification	
Low	4 (10)
Intermediate	6 (15)
High	30 (75)
Tissue factor expression	
Positive (High)	6 (15)
Positive (Moderate)	10 (25)
Positive (Weak)	14 (35)
Negative	10 (25)

were male while 16 patients (40%) were female (Table I). The primary tumor sites included the abdomen (34/40; 85%), mediastinum (4/40; 10%) and neck (2/40; 5%). The bone marrow was the most common site of metastasis (22/40; 55%) followed by bone (20/40; 50%) and the eye (14/40; 35%) patients. Patients had >1 metastatic site. A total of 24 patients (60%) were diagnosed at stage IV, 12 patients (30%) at stage III and 4 patients (10%) at stage II. Histology was favorable for 20 out of 40 patients (50%). *MYCN* amplification was reported in 18 (45%) patients, while no amplification was observed in 22 (55%) cases. Risk stratification revealed that 30 (75%) cases

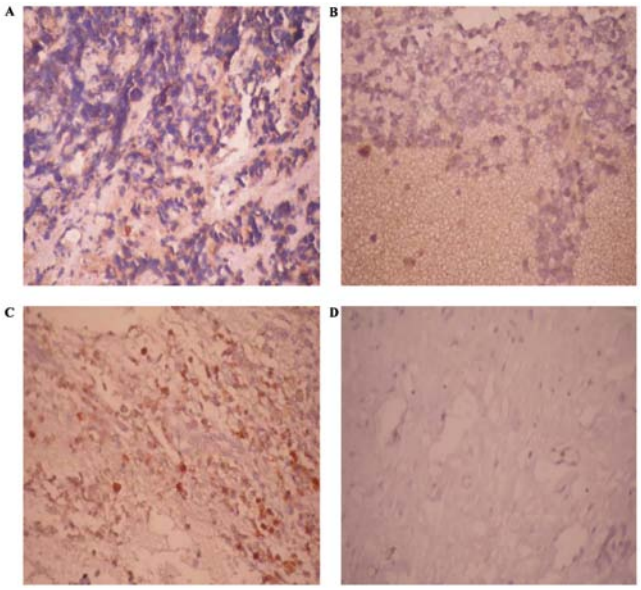

Figure 1. Representative images from 4 tumors, at a magnification of x100, showing immunohistochemical staining with anti-TF antibody. (A) Positive TF expression with strong immunoreactivity. (B) Positive TF expression with moderate immunoreactivity. (C) Positive TF expression with weak immunoreactivity. (D) Negative TF expression with no immunoreactivity. Non-linear adjustments were not applied to the images. TF, tissue factor.

were classified as high risk, 6 (15%) were intermediate risk and 4 (10%) were classified as low risk.

Immunohistochemical analysis. Varying levels of TF expression were observed in 30 out of 40 cases (75%), namely, weak intensity in 14 (35%), moderate intensity in 10 (25%) and high intensity in 6 (15%), while 10 (25%) cases showed no TF expression (Table I; Fig. 1). In the present study, 60% of unfavorable 'Shimada pathology' was detected in patients with positive TF expression, while only 20% was detected in patients with negative TF expression. This difference was statistically insignificant (P>0.05; Table II).

There was a significant association between TF expression and patient outcome. All patients with negative TF expression survived, whereas 18 (60%) patients with positive TF expression succumbed, 6 (20%) patients survived while another 6 (20%) were still undergoing treatment during the study period (P<0.05; Table II). The mean follow-up period was 29.1 months. The mean survival time for patients with negative TF expression was 59.8 months as opposed to 18.87 months for those with positive TF expression (P<0.05; Table III). All patients with negative tissue factor expression survived and completed their treatment successfully. The mean survival time is based on the last follow up point of the study and ranged from 28-95 months. Survival distributions for varying levels of TF expression were tested, and results revealed a significant difference between patients with and without TF expression (P<0.05; Table IV). There were no mortalities reported in patients with negative TF expression (Table IV; Fig. 2). In the present study, no association was observed between TF intensity staining and variables including sex, age, clinical symptoms, tumor stage, risk stratification, pathology, *MYCN* amplification and outcome (P>0.05; Table SI).

Table II. Association between tissue factor expression and tumor stage, pathology, risk stratification and patient outcome.

Variable	Tissue factor expression		P-value
	Negative (n=10) (%)	Positive (n=30) (%)	
Stage			
II	0 (0.0)	4 (13.3)	0.64
III	4 (40)	8 (26.7)	
IV	6 (60)	18 (60)	
Risk stratification			
Low	0 (0.0)	4 (13.3)	0.16
Intermediate	4 (40)	2 (6.7)	
High	6 (60)	24 (80)	
Pathology			
Unfavorable	2 (20)	18 (60)	0.3
Favorable	8 (80)	12 (40)	
Outcome			
Mortality	0 (0.0)	18 (60)	0.002
Cured	10 (100)	6 (20)	
Under treatment	0 (0.0)	6 (20)	
Age			
≤18 months	6 (60)	12 (40)	0.79
>18 months	4 (40)	18 (60)	
Gender			
Male	8 (80)	16 (53.3)	0.59
Female	2 (20)	14 (46.7)	
MYCN proto-oncogene bHLH transcription factor status			
Amplified	1 (10)	17 (56.7)	0.01
Non-amplified	9 (90)	13 (43.3)	

Chi-square test was used for these comparisons.

Similarly, no significant link was identified between patient outcome and sex, age, clinical symptoms, tumor stage, risk stratification, pathology and *MYCN* amplification (P>0.05). Patients who succumbed to the disease were found to have a higher percentage of *MYCN* amplification, however, this did not reach a statistically significant level (P=0.08). The present study revealed a significant association between TF expression and *MYCN* amplification. Only 10% of cases with negative TF expression showed *MYCN* amplification, as opposed to 56.7% of cases with positive TF expression (P=0.01; Table II).

Discussion

TF is an evolutionary conserved glycoprotein that serves an important role in the pathogenesis of cancer. TF affects a variety of pathological processes, including tumor-associated angiogenesis, thrombogenicity, tumor growth and metastasis (21). Additionally, high levels of TF expression were associated with increased expression of vascular endothelial growth factor and vascularity as development of blood vessels in tumors is required for tumor growth and spread (22).

Previous studies revealed that TF expression was higher in patients with KRAS proto-oncogene GTPase and tumor protein 53 mutations, suggesting that TF expression is a poor prognostic factor (23,24). TF upregulation parallels the expression of several mutant oncogenes including epidermal growth factor receptor, erb-b2 receptor tyrosine kinase 2 and promyelocytic leukemia-retinoic acid receptor α. Previous studies have revealed that the upregulation of TF is associated with the upregulation of the aforementioned oncogenes in colorectal cancer cells, mammary glands, cutaneous tissue, astrocytes and blood (23,24). Although upregulation of TF is often detected on the surface of tumor endothelial cells, determining its effect on prognosis and patient survival remains challenging (25). To the best of our knowledge, the present study is the first relating immunohistochemical analysis of TF expression in children with neuroblastoma and its role in tumor pathology, stage and patient outcome. The current study revealed increased TF expression in 30/40 (75%) patients. Similarly, Maciel *et al* (26), reported increased TF expression in 38/41 (88.3%) cases of nephroblastoma. In a previous study by Abdulkadir *et al* (27), 73% of patients diagnosed with prostate cancer had positive TF expression, which was positively correlated with preoperative serum prostate specific antigen levels. As demonstrated by Callander *et al* (28), TF upregulation is a characteristic marker of certain neoplasms. At least 60% of epithelial neoplasms,

Table III. Mean survival time for patients with and without tissue factor expression.

Tissue factor expression	n	Mean (months)	Standard deviation (months)	Median (months)	Minimum (months)	Maximum (months)	t-test	P-value
Negative tissue factor expression	10	59.80	24.087	61.00	28	95	4.64	<0.001
Positive tissue factor expression	30	18.87	24.176	9.00	1	90		
Total	40	29.10	29.845	13.00	1	95		

Table IV. Tests of equality of survival distributions for the upregulated and normal expression levels of tissue factor.

Test	Chi-square test	Degrees of freedom	P-value
Log rank (mantel-cox)	10.048	1	0.002
Breslow (generalized wilcoxon)	8.860	1	0.003
Tarone-ware	9.523	1	0.002

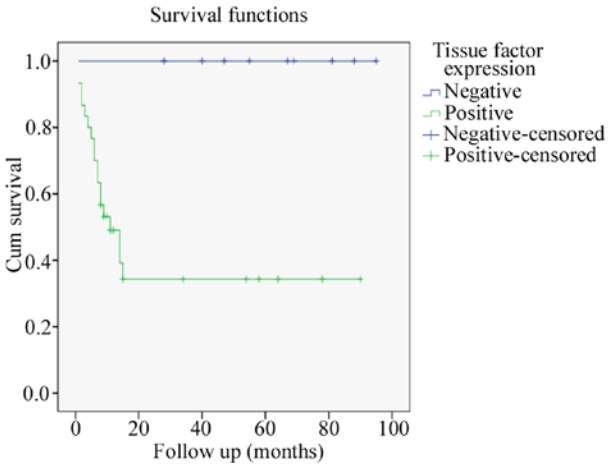

Figure 2. Kaplan-Meier survival curves in patients with and without TF expression. There is a significant difference between patients with and without TF expression as regarding survival (P=0.002). No mortality was reported in patients with negative TF expression.

and up to 100% of gastrointestinal carcinomas, express high levels of TF. TF expression has been detected in breast cancer, lung carcinomas, colon cancer and gliomas (29-32). In the aforementioned malignancies, TF is expressed by the tumor cells or adjacent stromal cells. This has been correlated with tumor grading, metastasis and poor prognosis. TF upregulation has been associated with tumorigenesis with high procoagulant activity observed in tumor cells (29-32).

TF is involved in hematogenous metastasis where TF levels in metastatic cells may be 1,000-fold higher than in non-metastatic cells in leukemia and melanoma (33,34). TF enhances metastasis by inducing a fibrin coating on malignant cells, trapping the cells in the microvasculature, thereby aiding metastasis. Additionally, thrombin activates platelets, leading to the formation of platelet-fibrin thrombi in the microvasculature (35). Clot formation around the tumor cells in circulation prevents the removal of tumor cells by natural-killer cells (35). TF expression by the tumor and host cells initiates direct or indirect signaling events that support tumor development by distinct mechanisms. TF supports cell migration and cellular trafficking by binding to the protein filamin-A, resulting in reorganization of actin filaments and cell migration (36). Regulation of cell motility by binding to its ligand factor VIIa is another pathway by which TF may directly influence tumor metastasis (37). The present study revealed TF expression in 18/24 (75%) patients with stage IV neuroblastoma, and 8/12 (67%) patients with stage III neuroblastoma. However, results revealed no significant statistical association between TF expression and neuroblastoma stages. The present study was based on a small patient number with a high stage IV percentile (60%).

Previous studies have revealed an association between TF expression and metastases in various types of cancer including prostate cancer, human glioma, colorectal cancer, breast cancer, hepatocellular carcinoma and pancreatic duct carcinoma (27,32,38-41). Additionally, it has been shown that TF serves a role in tumor-associated angiogenesis and its expression levels are associated with the metastatic potential of numerous hematological malignancies (42). In the present study, a significant association between TF expression and age and sex of the patients was not observed. These results are similar to previous studies in human non-small cell lung carcinoma, colorectal carcinoma, hepatocellular carcinoma and pancreatic ductal adenocarcinoma (43-46). As pathological symptoms of neuroblastoma have been used to further analyze these tumors, Shimada *et al* (18), initially classified these tumors as favorable and unfavorable by combining age with the extent of tumor differentiation, presence of schwannian stroma components and cellular information from the mitosis-karyorrhexis index. In the present study, 60% of unfavorable 'Shimada pathology' was detected in patients with positive TF expression, while only 20% was detected in patients with negative TF expression. This difference was statistically insignificant (P>0.05).

Previous studies have shown no significant association between TF expression and favorable or unfavorable

nephroblastoma histology (26). Similarly, no association between pathological grade and TF expression in prostatic cancer has been found (47). However, a number of studies have revealed an association between the histological grading of a tumor and TF expression. Kakkar *et al* (48) reported that TF expression was associated with histological grade in pancreatic cancer and a significant linear trend was detected with stronger immunoreactivity observed in poorly differentiated tumors. Similar results have been documented in human glioma and breast cancer (29,32).

The *MYCN* gene was the first oncogene revealed to be amplified in solid malignancies and the only consistently amplified gene in neuroblastoma (49). The *MYCN* gene has been used by The Children's Oncology Group to assign newly diagnosed neuroblastoma patients to a high-risk group requiring intensive multimodal therapy (50). In the present study, all patients were tested for *MYCN* amplification. It was amplified in 18/40 (45%) patients and was not amplified in 22/40 (55%) patients. There was an association between *MYCN* amplification and TF expression, whereby only 10% of those with negative TF expression had amplified *MYCN*, while 56.7% of those with positive TF expression had amplified *MYCN*. The link between TF and *MYCN* expression may improve the value of *MYCN* as a prognostic indicator in children with neuroblastoma.

In the present study, TF expression was significantly associated with patient outcome. All patients with negative TF expression survived, whereas 18/30 (60%) cases with positive TF expression succumbed to the disease. These results were similar to a previous study where an association between TF expression and prognosis in patients with nephroblastoma and increased immunohistochemical detection of TF expression was the most important risk factor for recurrence and mortality according to bivariate and multivariate analysis (26). Furthermore, immunohistochemical TF expression was an important independent predictor of mortality in a cohort of patients with clear cell renal cell carcinoma (51). Rao *et al* (23) reported that TF expression was markedly higher in patients with poor differentiation in colorectal carcinoma based on Dukes' staging system, whereas patients with low TF expression had longer disease-free survival and overall survival compared with patients with high TF expression (23). Akashi *et al* (47) revealed that in patients with metastatic prostate cancer treated with androgen-withdrawal therapy, TF expression was not associated with therapeutic response; however, patients with TF positive tumors had a shorter survival rate compared with patients with TF negative tumors. Hamada *et al* (32) did not demonstrate a correlation between TF expression and patient outcome in human glioma; however, an association with malignancy grade was observed. In the present study, no significant association was found between TF staining intensity and pathology, stage and age of the patients. Nitori *et al* (46) reported that patients with pancreatic duct carcinoma with negative TF expression had markedly improved survival time compared with patients with positive TF expression. Furthermore, increased levels of TF expression are an independent risk factor for poor survival in patients with solid tumors such as breast cancer, colorectal carcinoma, hepatocellular carcinoma and pancreatic duct carcinoma (29,38,44-46). However, more extensive and disease-specific clinical analysis is warranted to establish whether tumor-associated TF levels as well as changes in circulating TF, possess independent prognostic and predictive utility (52). In summary, TF is a unique molecule that may be explored further and utilized in various ways in order to treat human cancer.

The present study had a number of limitations. A limited number of patients were enrolled, however, this should be read in light of the rarity of the disease. Additionally, the present study focused on TF and its relationship with outcome and other well-known risk factors. Future larger studies with a wide scope that take into consideration all variables that may impact outcome are required. In conclusion, the results of the present study revealed that TF expression may be useful for the prognosis of patients with neuroblastoma. The mechanisms underlying the effects of TF expression on the progression of neuroblastoma and patient outcome, as well as its potential for the development of novel therapeutic strategies, require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LMS and THH designed the study. LMS participated in the data analyses, manuscript writing and revision. THH performed the statistical analysis and participated in manuscript writing and revision. MZ, MF and MAB collected the clinicopathological data, performed statistical analysis and participated in manuscript writing. EAE performed the histopathological examinations and analyzed *MYCN* upregulation and TF expression. AE performed routine laboratory work for the patients. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Faculty of Medicine, Zagazig University (Zagazig, Egypt). The study was performed according to the Helsinki Declaration as revised in 2000. Written informed consent was obtained from the legal guardians of the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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