CORRELATION BETWEEN SOME BIOLOGIC AND OTHER PROGNOSTIC MARKERS OF CHILDHOOD NEUROBLASTIC TUMORS

Ebtesam M. El-Gezawy*, Osama B Sedik*, Marwa Abdelgawad**, Ahmed M Morsy*** and Samy Al Gizawy**

ABSTRACT

Background: The mutated Anaplastic lymphoma kinase (ALK) gene has been identified as a potential and major predisposition oncogene in human neuroblastosomas (NBLs). However, the frequency of mutation is only 5-8%. Purpose: The present study was performed to determine the level of ALK mRNA gene expression in primary neuroblastoma and to assess its relation to other prognostic factors of neuroblastoma. Methods: Quantitative real-time RT-PCR was applied to examine the expression level of ALK mRNA in seventy nine primary neuroblastoma patients, and its prognostic value in those patients. Immunohistochemical staining was used to check the expression level of ALK proteins. Results: In analysis of 79 patients with sporadic primary neuroblastoma, we found that high expression level of ALK mRNA was significantly associated with Shimada’s pathological classification (p<0.001), patient’s age (p<0.001), MYCN amplification status (p<0.001), tumor stage (p<0.001) and low TrkA expression level (p=0.0390), all these factors are known to be associated with poor prognosis in neuroblastoma. Of interest, immunohistochemical study revealed positive ALKin ALK-amplified tissues. Furthermore, mutation results showed that ALK mutation represented about 4.6% of cases and ALK amplification represented about 1.5% of cases. So that mutations not only occur among unfavorable cases with low ALK but also in favorable cases with high ALK expression. Conclusion: Our findings suggested that, high expression of ALK gene is associated with poor prognosis of NBL so it can be used as a prognostic factor in NB in clinical practice. Keywords: ALK, neuroblastoma, N-MYC, TrkA

INTRODUCTION

Neuroblastoma (NBL) is one of the most common solid tumors in childhood; it accounts for 7-10% of childhood cancer and around 15% of childhood cancer mortality; it originates from the sympathoadrenal lineage of the neural crest. Clinical courses are highly variable, ranging from spontaneous regression to therapy resistant progression(30). Clinical and biological features, such as age at diagnosis, disease stage, DNA ploidy and structural chromosomal alterations are associated with patient outcome(9,22,25).

It was demonstrated that heritable mutations of anaplastic lymphoma kinase (ALK) gene are the main cause of familial neuroblastoma(21). It was described NBL patients with high levels of ALK expression without ALK gene mutations(23). They showed that regardless of mutation status, high ALK levels were strongly correlated with poor prognosis. Moreover, it was demonstrated that RNA interference (RNAi)-based knockdown of ALK, regardless of its genetic status, showed reduced proliferation and increased apoptosis in NBL cells and inhibited NBL tumor growth as well as prolonged survival in vivo(19).

ALK is a 220 kDa transmembrane receptor tyrosine kinase that belongs to the insulin receptor superfamily. It was first described as part of an oncogenic fusion tyrosine kinase, nucleophosmin (NPM)-ALK. It is dominantly expressed in the neural system and the gene encoding it is located on the short arm of chromosome 2 (2p23.2) proximal to the N-MYC amplicon on 2p24.1(7,15).

For years, it was considered that N-MYC (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog) is a Protein Coding gene. It was the only oncogene known to be involved recurrently in ~22% of tumors, and the N-MYC protein is highly overexpressed by copy number gains of the gene in neuroblastic tumors with advanced stages and aggressive clinical behavior(27). Analysis of N-MYC remains an essential component of disease evaluation for newly diagnosed NBL patients and serves as a paradigm for the utility of molecular information in cancer treatment stratification(29). N-MYC is proved to be vital for proliferation, migration, and stem cell homeostasis, whereas decreased levels are associated with terminal
neuronal differentiation. However, downregulation of N-MYC may lead to decreased proliferation and differentiation, emphasizing the importance of N-MYC signaling in NB biology (27).

Tropomyosin receptor kinase A (TrkA) is a neurotrophic tyrosine kinase receptor that is activated by binding to the nerve growth factor (NGF). Activation of TrkA promotes the growth and differentiation of neural cells. Previous studies have shown that TrkA expression is associated with good prognosis of NB; in fact, low or absent TrkA expression is correlated with poor outcome (10).

The purpose of our study was to determine ALK expression level in human NBL tissue samples to evaluate its role in disease progression and prognosis.

**MATERIALS AND METHODS**

Seventy nine primary neuroblastoma patients were included in this study, the tumor specimen were obtained during surgery or biopsy after informed consent from the parents of the children with neuroblastoma who were referred to Department of Clinical Oncology, Faculty of Medicine and Department of Pediatric Oncology, South Egypt Cancer Institute, Assiut University, Egypt, during the period from January 2008 to November 2015. The samples were stored at -80°C.

Staging work up including computed tomographic scan of the neck to the pelvis, bone scan and bone marrow biopsy before and after treatment were mandatory. Patients were diagnosed clinically as well as pathologically and tested for DNA ploidy, MYCN amplification and TrkA expression. Tumors were staged according to the International Neuroblastoma Staging System criteria (INSS) (19). Patients were stage 1, (11 patients) were stage 2, (19) patients were stage 3, (25) patients were stage 4, and (5) patients were stage 4S.

The patients were treated following the protocols proposed by the Pediatric Oncology Group for Treatment of Advanced Neuroblastoma (20). Regular follow up data should be available for every patient. Our present study was approved by the ethics committee of the Faculty of Medicine, Assiut University. The clinical follow-up ranged from 3 to 93 months, with a median of 42.0 months.

Patient samples: The tumor specimens were sent to Department of Clinical Pathology, Faculty of Medicine, Assiut University, Egypt, after informed consent from the parents of the children. The selection of samples was solely based on the availability of a sufficient amount of tumor tissue, from which DNA and mRNA could be prepared for analyses described below.

RNA extraction: Total mRNA was prepared from Fresh-frozen tissues of primary neuroblastoma by using (Qiagen extraction kit, Valenica, CA, USA).

The RNA quality was verified by gel electrophoresis. Two µg of total RNA was used as started material for complementary DNA (cDNA) synthesis by employed with SuperScript III Reverse Transcriptase (200U/µl, Invitrogen, Carlsbad, Calif.). After reverse transcription reaction was finished, a total volume of 40 µl cDNA was obtained. One µl resulting cDNA was used for further PCR reactions.

Reverse transcription was carried out using random primers and SuperScript II (Invitrogen) following the manufacture’s instructions. Following the reverse transcription, the resultant cDNA was subjected to PCR-based amplification using the following primer sets- and annealing temperatures (Ta):

**Human GAPDH,** 5’ACCTGA CCTGC-CGTCTAGAA-3’ (sense) and 5’ TCCACCACCCCTTGGTGA-3’ (antisense), Ta, 58°C; human ALK, 5’AGGACCAGGATGTAAC-CAAC-3’ (sense) and 5’- CTTGTGCAACTC- CGAAGGAG-3’ (antisense), Ta, 58°C;

Quantitative RT-PCR: Total RNA prepared from primary neuroblastomas was reverse transcribed into cDNA (SuperScript II kit) and subjected to the real-time PCR. The expression level of GAPDH was measured in all samples to normalize ALK expression according to the manufacturer’s instructions (14) (Applied Biosystems, 7500 fast real-time PCR System, CA, USA). Oligonucleotide primers and TaqMan probes, which were labeled at the 5’end with the
reporter dye 6-carboxyfluorescein (FAM) and at the 3’end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA), were provided by Applied Biosystems for ALK.

FISH Technique: The tissue samples were tested for MYCN amplification and duplication and TrkA expression using FISH in paraffin-embedded tissue sections.

The MYCN amplification probe was used to detect MYCN gene amplification and copy number changes (duplication). This probe contains two differently labeled probes: a 140 kb N-MYC probe labeled with red covers the N-MYC region at 2p24 and the LAF gene probe, 201 kb and labeled with Spectrum Green, located at 2q11, acts as the control.

Interphase fluorescence in situ hybridization (FISH) was performed on 4 um-thick paraffin sections of the specimens. Briefly, the tissue sections were deparaffinized and heated in 8% sodium thiocyanate for 3 min. The sections were then digested in 0.1% of pepsin solution at 37°C for 20 min and the probe was applied onto the appropriate tissue areas. The slides were incubated at 73°C for 6 min and at 37°C for 16 h, followed by washing in gradient SSC solutions and counter-staining with anti-fade solution containing DAPI. The slides were examined using an Axioscope 2 mot plus fluorescence Microscope (Zeiss, NY, USA) and diagnosed using image system (Leica dg software, Leica, Manhiem, Germany).

In each case, around 100–200 nuclei from at least five to eight areas were examined. Nuclei with apparent overlapping or truncation were excluded from analysis. The cut-off value was established on 16 paraffin slides of the control bone marrow tissue, and was calculated as the mean (8%) plus 3 SD of nuclei counted. All the cut-off values of the probes were less than 11%.

Immunohistochemistry: Human neuroblastoma tissue samples were stained with immunoperoxidase method using anti-ALK antibody. Neuroblastoma specimens were fixed in 10% buffered formalin and embedded in paraffin, and 3 μm sections were applied to the immune staining using the DAKO, ALK1 monoclonal. Before incubation with anti-ALK antibody, the sections were treated with 0.05% Pronase in 0.05 mol/L Tris-HCl (pH 7.6) for 5 minutes. The sections were incubated with anti-ALK antibody, which was diluted to 1:200 at ˚C overnight. The biotin-streptavidin method (Nichirei, Tokyo, Japan) was done, and the sections were visualized with diaminobenzidine solution. The nuclei were counterstained with hematoxylin.

Statistical analysis:

Student’s t tests were used to explore possible associations between ALK expression and other factors, the difference between high and low levels of ALK expression was based on the mean value obtained from quantitative real-time PCR analysis. Kaplan-Meier survival curves were compared using the log-rank test. Multivariate analysis using a Cox proportional hazards regression model was used to assess the independent predictive importance of ALK expression for survival. Statistical significance was declared if P<0.05. The statistical analysis was done using SPSS Statistical Software Release 20.0.

RESULTS

Patients characteristics (Table 1):

We collected data from 79 patients with NBL who met study criteria, 46 of them were favorable and 33 unfavorable according to Shimadas’ classification, 34 below one year of age and 45 above one year, 19 patients were stage 1, 11 were stage 2, 19 were stage 3, 25 were stage 4, and 5 were stage 4S. The clinical follow-up ranged from 3 to 93 months, with a median of 42.0 months.

ALK expression and other biomarkers Assay:

The tissue samples of all (79) NBL patients were examined. Expression levels of ALK were examined by the quantitative real-time RT-PCR. High ALK expression was reported in 46 patients (58.22%). Other biomarkers assay included 57 patients with a single copy of MYCN and 22 with amplified copies of MYCN, 33 with high TrkA 1 expression and 46 with low TrkA 1 expression.

Correlation between ALK Expression and other biologic and clinical prognostic factors in all patients (Table 1 and Figures 1 & 2):

We found significantly high expression of ALK mRNA in patients of age >1 year in com-
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In comparison to patients <1 year (p<0.001) with mean value (108.7±25.1) and (35.4±12.5) respectively, unfavorable NBLs histology (p<0.001) with mean value (108.8±20.0) for unfavorable cases and (2.3±1.0) for favorable cases. Regarding other biologic biomarkers, we recorded that significantly high expression of ALK mRNA in patients with amplified copies of MYCN (p<0.001) with mean value (84.8±17.7) as compared with patients with a single copy of MYCN (19.8±10.3), and low TrkA 1 expression level (P =0.0390) with mean value (91.4±16.9) for patients with low TrkA 1 expression and (54.3±15.2) for patients with high TrkA 1 expression.

On the other hand, we reported significantly high expression of ALK mRNA in patients with the tumor stages 3 and 4 (p<0.001) with mean value (101.2±25.5) for patients with stage 3 and 4 and (39.6±12.0) for patients with stage 1, 2 and 4s. Also, we found that low expression of ALK mRNA is significantly related to stage 4s tumor when compared to whole other stages (p<0.001) with mean value (224.4±102.4) for stage 4s and (50.2±11.0) for stage 1, 2, 3 and 4, which may suggest a relationship between low and high expression of ALK mRNA and metastasis of neuroblastoma.

There was no association between high expression of ALK mRNA and DNA index (p=0.4) or tumor origin (p=0.1). These results suggest that high expression of ALK is well associated with conventional markers indicating the unfavorable outcome of neuroblastoma.

**High expression of ALK and other biomarkers associated with poorer survival in NBL:**

As shown in fig. (2), high expression of ALK was significantly associated with unfavorable survival (p=0.04). The univariate analysis demonstrated that Shimadas’ histology (p=0.0003), age (p=0.0227), tumor stage (p=0.0030), MYCN amplification (p<0.0001), TrkA 1 expression (p=0.0120) and DNA index (p=0.0134) were of prognostic importance, supporting the results of log-rank test. On the other hand, the multivariate analysis showed that ALK expression was not significantly associated with survival suggesting that ALK expression was not an independent prognostic factor from the other factors, (Table 2).

**Immunohistochemical findings in NBLs tissues:**

Immunohistochemical staining was applied to examine the ALK protein expression in neuroblastoma human tissue samples Immunohistochemical study revealed positive ALK only in ALK-amplified tissues as shown in fig.(3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>ALK expression (Mean ± SEM)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt; 1 year</td>
<td>34</td>
<td>108.7 ± 25.1</td>
<td>P&lt;0.001</td>
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<tr>
<td>≥ 1 year</td>
<td>45</td>
<td>35.4 ± 12.5</td>
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<tr>
<td><strong>Shimadas histology</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Favorable</td>
<td>45</td>
<td>108.8 ± 20.0</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>34</td>
<td>2.3 ± 1.0</td>
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<tr>
<td><strong>INSS stage</strong></td>
<td></td>
<td></td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1,2,4e (non metastatic)</td>
<td>35</td>
<td>101.2 ± 25.5</td>
<td></td>
</tr>
<tr>
<td>3,4 (metastatic)</td>
<td>44</td>
<td>39.6 ± 12.0</td>
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<td><strong>Tumor origin</strong></td>
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<tr>
<td>Non adrenal</td>
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<td><strong>MYCN</strong></td>
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<td>Single copy</td>
<td>57</td>
<td>84.6 ± 17.7</td>
<td>P=0.001</td>
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<td>Amplified</td>
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<td>19.8 ± 10.3</td>
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<td>Low expression</td>
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<td>54.3 ± 15.2</td>
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<tr>
<td>High expression</td>
<td>24</td>
<td>91.4 ± 16.9</td>
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<tr>
<td><strong>DNA Ploidy</strong></td>
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<td>Diploid/tetraploidy</td>
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<td>60.7 ± 16.8</td>
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<tr>
<td>Hyperdiploidy</td>
<td>28</td>
<td>82.5 ± 28.3</td>
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</table>

Table(1): Relation between the expression of ALK and prognostic factors of NBL.
The evidence that ALK has an important role in both familial and sporadic NBL pathogenesis has been provided by several studies (4,6,13,16,21). ALK is a dependence receptor that homodimerizes in the presence of ligand leading to activation of its tyrosine kinase domain by transphosphorylation and mediating a decrease of apoptosis through various signaling pathways (1,24).

Our results indicate that high expression level of ALK is significantly associated with poorer survival and patient outcome. Moreover, we found that the high expression level of ALK is significantly correlated with other prognostic factors of NBL including patient age, Shimada's pathological classification, tumor stage, MYCN amplification status and TrkA expression levels.

Contrary to our findings, it was described by Lamant, et al. (2000) in 19 neuroblastoma cases using Table (2) Cox regression model using ALK expression:

- Tumor stage (p=0.0030), MYCN amplification (p <0.0001), TrkA expression (p=0.0120) and DNA index (p=0.0134) were of prognostic importance, supporting the results of log-rank test. On the other hand, the multivariate analysis showed that ALK expression was not significantly associated with survival suggesting that ALK expression was not an independent prognostic factor from the other factors, (Table 2).

Immunohistochemical findings in NBLs tissues:

Immunohistochemical staining was applied to examine the ALK protein expression in neuroblastoma human tissue samples. Immunohistochemical study revealed positive ALK only in ALK-amplified tissues as shown in Fig.(3).

Figure(1): Expression levels of ALK mRNA examined by the quantitative RT-PCR and relations to other prognostic factors.
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Our results indicate that high expression level of ALK is significantly associated with poorer survival and patient outcome. Moreover, we found that the high expression level of ALK is significantly correlated with other prognostic factors of NBL including patient age, Shimada’s pathological classification, tumor stage, MYCN amplification status and TrkA expression levels. Contrary to our findings, it was described by Laman, et al. (2000) in 19 neuroblastoma cases using Western Blotting that there is no correlation between the level of ALK expression and the known neuroblastoma prognostic factors, but this may be due to small number of cases, different methodology and different population (18).

Consistent with our results, earlier studies clearly showed that aggressive and metastatic NBLs exhibited a significantly higher expression level of ALK mRNA compared with localized and favorable NBLs, supporting the oncogenic role of ALK in this disease (3,8,11,23).

The molecular mechanisms how ALK induces aggressive NBL have not yet been fully elucidated. Schonheer et al. (2011), showed that both wild-type and gain-of-function ALK mutants were able to stimulate transcription at the MYCN promoter through the activation of a downstream molecule, ERK, and initiate mRNA transcription of MYCN in both neuronal and NBL cells. Furthermore, Berry et al., 2012 demonstrated that the F1174L mutation of ALK enhanced MYCN protein stabilization and found that endogenous Mycn mRNA was upregulated in the tumors of MYCN/ALKF1174L transgenic mice (3).

**DISCUSSION**

Figure (2): Kaplan-Meier survival curves of patients with NBLs based on higher or lower expression of ALK according to the mean value of ALK mRNA expression in 79 primary neuroblastoma patients, relative expression level of ALK mRNA was determined by calculating the ratio between GAPDH and ALK. To evaluate whether a significant relationship could be observed between the expression of ALK in primary neuroblastoma and the patients’ survival.

**Figure (3): Immunohistochemistry for ALK in neuroblastoma tissues**
Correlation between Some Biologic and Other Prognostic Markers of Childhood

MYCN amplification occurs in approximately 25% of primary NBLs and is one of the most reliable prognostic factors identified to date\(^{(12,14)}\). It is significantly associated with advanced disease stages, rapid tumor progression and poor prognosis. However, the molecular mechanism show MYCN induces aggressive NBL have not yet been fully elucidated. In our present, findings clearly provided the evidence that MYCN-mediated ALK induction promotes cell proliferation, migration and invasion and this is in consistent with Hasan et al. (2013).

In our study, Immunohistochemical study revealed positive ALK expression in ALK-amplified tissues. Previously it was reported that ALK is a dependence receptor, this type of receptors, defined by its functional rather than structural similarity, creates cellular states of dependence on their respective ligands by inducing or favoring apoptosis when unoccupied by ligand, but inhibiting apoptosis in the presence of ligand\(^{(1)}\).

Hasan et al. (2013), found that ALK expression was significantly high in NBL clinical samples with amplified MYCN and in developing tumors of MYCN-transgenic mice demonstrating its role as a key molecule for MYC proteins to exert influence towards oncogenesis. They suggested that a therapy targeting ALK should be considered in combination with more conventional agents to treat NBLs with high expression of ALK\(^{(14)}\). They revealed that ALK is a direct transcriptional target of MYC proteins and a key molecule for MYC proteins to exert influence towards oncogenesis. On the basis of the observation of Chen et al., 2008 that N-MYC and ALK loci were localized in nuclei as separate amplicons for a single case, it was reported that ALK amplifications contribute actively toward the pathogenesis of NB rather than being secondary passenger events of MYCN amplification\(^{(6)}\). Regarding the results of TrkA, our study showed that high expression of Alk gene was associated with low TrkA expression and this is indication for poor prognosis and this is parallel to the study of Gao1, et al., 2015 who indicated that the increase in TrkA expression precedes the growth arrest and morphological differentiation were associated in part with the increase in TrkA expression\(^{(12)}\).

In our study, Immunohistochemical study revealed positive ALK expression in ALK-amplified tissues and cell lines. Previously it was reported that ALK is a dependence receptor, this type of receptors, defined by its functional rather than structural similarity, creates cellular states of dependence on their respective ligands by inducing or favoring apoptosis when unoccupied by ligand, but inhibiting apoptosis in the presence of ligand\(^{(1)}\).

Collectively, our findings suggest that high expression of ALK is associated with poor prognosis of NB, also the expression level of ALK gene might also have some function in cell growth as well as differentiation in neuroblastoma. Also our study revealed that the ALK gene in association with N-MYC amplification in NB are highly expressed. The ALK gene may be a good target for ALK inhibitors in the treatment of NB.

REFERENCES

1- Allouche M. 2007 ALK is a Novel Dependence Receptor Potential Implications in Development and Cancer [Cell Cycle 6:13, 1533-1538, 1 July]; Landes Bioscience.


17- Khalafer MR, Zaki EM, Darwish AM, El-Naggar MG 2015: Frequency of the ALK gene and its prognostic value in neuroblastoma by FISH, Egyptian Journal of Hematology, April 09.,


