

ALK/MYCN Coamplification in Neuroblastoma: A Short Review

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Abstract: Neuroblastoma remains one of devastating childhood cancer that plagues clinical researchers worldwide. Although, key oncogenes have been discovered, the prognostic significance of these remains to be confirms. The discovery of ALK in 2008 has opened new therapeutic area for this childhood cancer. Although ALK amplification only occurs in 1-2% of whole population sample, coamplification of ALK/MYCN in neuroblastoma suggests a correlation between these two genes. This review discusses how coamplification of ALK/MYCN results in oncogeneity in neuroblastoma. In addition, this review also highlights the recent discoveries of ALK/MYCN pathway and how this co-existence may lead to high-risk neuroblastoma and treatment resistance in patients with this genetic alteration.

Key words: Neuroblastoma · ALK · MYCN · Coamplification · Oncogeneity

INTRODUCTION

In Malaysia, neuroblastoma is a rare pediatric cancer and accounts to approximately for 10% of childhood mortality. Malaysian Cancer Statistic-Data at 2007 reported that neuroblastoma was one of the sixth most common histological type for brain and nervous system cancer with an increase to 5.1 percentage compare to 0.9% in year 2003-2005 in Malaysia [1, 2]. Due to commonly like symptoms such as fever, loss of appetite and weight loss, it often results in difficulty to diagnose at an early stage. Hence, by the time the patients are taken to hospital, the disease may have already progressed to high risk neuroblastoma which occasionally leads to poor prognosis. Nonetheless, this disease sparks research interest as it has the mechanism to spontaneously regress from malignant to benign, particularly in younger infants [3]. Anaplastic Lymphoma Kinase in neuroblastoma has only been identified in less than a decade ago. In the last few years, intense research has been ongoing to prove the relationship of ALK in neuroblastoma with poor prognosis. This review will emphasis on how amplification of both genes in chromosomal locus two, which is adjacent to each other, when co-amplified promotes on Neuroblastoma oncogenesis.

Neuroblastoma: Neuroblastoma is listed as the third most common pediatric cancer and is responsible for approximately 15% of all childhood cancer deaths. Neuroblastoma is one of the diseases that showed wide clinical and genetic heterogeneity. There was also slight male predominance in neuroblastoma patients and with median age of 22 months [3]. This common solid extracranial neoplasm that arises from postganglionic sympathetic nervous system was first described more than a century ago by three physicians who recognized the neural nature of the tumor [3, 4]. Neuroblastoma may be graded into several stages that has different prognostic outcome, most patients with localized disease (stages 1 and 2) are infants (less than 1 year) has excellent survival after surgical treatment only, whereas most older children (1 year) have widespread metastasis (stages 3 and 4) and need additional chemotherapy and hematopoietic stem cell rescue. However, stage 3 and 4 are lethal for older patient age 4 to 5 years old. In contrast, stage 4S tumors predominantly occur in infants and may regress into ganglioneuroma and disappear without treatment. There are two important clinical parameters; age and clinical stage that determine the prediction of treatment outcome patient survival. Recent clinical treatment also includes biological parameter to improve

patients that require intensive treatment such as patient with MYCN amplification [5, 6]. Recently, ALK has been identified as a major predisposing gene as well as a potential therapeutic target for neuroblastoma [7].

Furthermore, according to Marta and colleagues, it appears that ALK is a crucial oncogene whose regulation status influences the clinical evolution of neuroblastoma. Their study also had shown that individuals with ALK-related neuroblastoma susceptibility (*i.e.*, heterozygous for ALK mutation) are at risk of developing neuroblastic tumors. Aberrant copy number or mutations in ALK gene and over expression of its protein tyrosine-kinase receptor have been related to poor prognosis of this disease, although a great degree of discrepancy exists regarding the clinical validation of these alterations [8]. Another study also demonstrated that ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma [9].

Anaplastic Lymphoma Kinase (ALK): ALK is one of protein tyrosine kinase gene that is located on chromosome 2p23.

Since the original discovery of ALK in 1994 resulted directly from investigations into the frequently observed t(2;5)(p23;q35) chromosomal rearrangement in anaplastic large cell lymphoma (ALCL), which fuses the cytoplasmic domain of ALK to the N-terminal portion of the nucleolar phosphoprotein, NPM [10-12], similar studies have also investigated the role of ALK in neuroblastoma. Moreover, earlier researchers stated that ALK is preferentially expressed in the central and peripheral nervous system [13, 14].

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor that affects a number of biological and biochemical functions through normal ligand-dependent signaling. It has oncogenic functions in a number of tumors including non-small cell lung cancer (NSCLC), anaplastic large cell lymphoma and neuroblastoma when altered by translocation or amplification or mutation [15].

Many other examples of aberrant ALK activation due to chromosomal rearrangement have been described in human cancers, including non-small-cell lung carcinomas (NSCLC), inflammatory myofibroblastic tumors (IMTs), diffuse large cell B-cell lymphomas and esophageal squamous cell carcinomas [16].

ALK has the typical structure of a transmembrane RTK, with a large extracellular domain, a lipophilic transmembrane segment and a cytoplasmic tyrosine kinase domain. It is a single-chain 1620 amino acid (aa) transmembrane protein consisting of a 1030-aa

extracellular domain, a 28-aa transmembrane-spanning domain and a 276-aa intracellular tyrosine kinase domain. Ligand binding leads to receptor dimerization and activation via *trans*-autophosphorylation of tyrosine residues within kinase domain A-loop (Activation loop) segments. Phosphorylation of sites outside the A-loop within the cytoplasmic domain serve as docking sites for downstream SH2 (Src homology 2) and PTB (Phosphotyrosine-binding domain) domain-containing effector and adapter proteins involved in the signal transduction cascade [17].

N-linked glycosylation of ALK leads to a glycoprotein that migrates in SDS gels at about 210 kD. The 177-kDa polypeptide encoded by the human ALK gene undergoes post-translational modifications, such as N-glycosylation, to generate a mature protein doublet of 220 and 190 kDa. ALK migrates as two protein isoforms: the 220-kDa full-length receptor and the truncated 140 kDa protein that results from extracellular cleavage [18].

The cytoplasmic portion contains 563 residues and includes the kinase catalytic domain. This full-length form is implicated in malignancies where ALK promotes tumorigenesis via activation by autocrine and paracrine growth-promoting loops involving the putative endogenous ALK ligands PTN (Pleiotrophin) and MK (Midkine) [19, 20].

Genetic abnormalities or disease states leading to ALK hyperactivation manifest in oncogenic transformation from overstimulation of downstream STAT (Signal transducer and activator of transcription), Ras/Raf/MEK [MAPK (Mitogen-activated protein kinase)/ERK (Extracellular-signal-regulated kinase) kinase], PI3K (Phosphoinositide 3-kinase)/Akt and PI3K/PLC (Phospholipase C)- γ pathways involved in cell survival, differentiation and apoptosis [21].

Iwahara and colleagues independently clone the human and mouse ALK receptor which was shown to code for a protein with a predicted molecular mass of 180 kD. Their study also observed that the alk gene is dominantly expressed in specific parts of the central nervous system (CNS) and the peripheral nervous system (PNS). Its level of expression is especially high at the neonatal stage. Their data shows there's a linkage between ALK function in neuroblastoma pathogenesis [22].

Thus based on previous findings, activation of ALK may regulate cellular proliferation, differentiation and apoptosis via a number of different signaling pathways, including PI3K/AKT, RAS/ MAPK and STAT3, although its precise physiologic role remains elusive [23].

ALK Amplification: Neuroblastoma is one of paediatric cancer examples that possesses heterogeneity in which the ALK oncogene can either arise from germline mutation by mutation in tyrosine kinase domain or spontaneously mutated. This discovery provides the evidence that activation of ALK in tyrosine kinase domain as major cause of hereditary neuroblastoma [7, 24]. ALK aberration has been identified since, its discovery in 2008 that includes missense mutation on both sporadically and hereditary mutation ALK gene, chromosomal translocation and least but not rare is ALK amplification [7, 24, 25]. However, ALK amplification and mutation are seldom observed within the same tumor [26]. According to Lamant *et al.* [27], initial studies identified ALK protein over expression both in primary neuroblastoma and cell lines as a consequence of gene amplification. Hassan and colleagues proven the hypothesis postulates by Chen and colleagues, that ALK amplification actively contributed to the pathogenesis of neuroblastoma rather than being secondary passenger events of MYCN amplification [9,24]. Recent study demonstrated that ALK expression was significantly correlated with MYCN amplification in primary neuroblastoma due to overexpression of MYCN induced promoter activity of ALK gene that resulted high level ALK expression in neuroblastoma cells [9].

As previously reported, ALK amplification is about 2% of all neuroblastoma cases and a previous studies have shown that solitary ALK amplification is rare, however ALK amplification is found to occur almost exclusively with MYCN amplification [26, 28, 29]

George *et al.* [29] also stated that consistent but less frequently occurring high level ALK amplification was detected. In addition, constitutive and increased kinase activity of mutated ALK protein was shown in their studies. Inhibition of some mutant forms of ALK in human neuroblastoma cell lines resulted in apoptosis and impaired cell proliferation, demonstrating ALK as a potential therapeutic target for this disease.

Oncogeneity Relationship Between ALK and MYCN:

In addition, earlier report established the direct role of MYCN in the transcriptional regulation of ALK in neuroblastoma which afterward indirectly proposes the possibility of positive cooperation link between these two oncogenes ALK and MYCN. This is also supported by previous finding which showed that ALK amplification occurs almost exclusively with MYCN amplification and may affect as many as 7-15% of primary neuroblastomas with an amplified MYCN gene [26, 29].

MYCN oncogene amplification is an establish marker indicating aggressive tumor progression of high-risk neuroblastoma [30]. The MYCN gene is located on the distal short arm of chromosome 2 (2p24). The MYC family of proto-oncogenes belongs to the basic helix-loop-helix leucine-zipper class of transcription factor. MYC protein (MYCN and c-myc) shares several regions of homology and similar cellular functions that target proliferative pathways vital for cancer progression. MYCN is an important gene for proliferation, migration, stem cells homeostasis while decrease levels are associated with terminal neuronal differentiation [31, 32]. High-risk tumors without MYCN amplification frequently display increased c-MYC expression and/or activation of MYC signaling pathways while down regulation of MYCN leads to decreased proliferation and differentiation, emphasizing the importance of MYC signaling in neuroblastoma biology [33].

Since the genomic region amplified with MYCN is large, usually on the order of 500 to 1,000 kb. Therefore, it has been postulated that additional genes located near MYCN may contribute to the ultimate tumor phenotype when co-amplified. High-resolution restriction mapping of the MYCN locus has shown that a 130-kb core domain is the consistent target of amplification [34].

An earlier study by Schonherr [35] also showed that both wild type and gain of function ALK mutants were able to stimulate transcription at MYCN promoter through the activation of downstream molecule ERK and initiate mRNA transcription of MYCN in both neuronal and neuroblastoma cells. Their findings also showed co-expression of the constitutively active ALK mutants and MYCN results in increased transformation as compared with the MYCN, ALK F^{1174L} or ALK R^{1275Q}. They also hypothesized that ALK is a component responsible for increased MYCN protein levels and this maybe of relevance for patients with chromosome 2 amplification harboring either wild type ALK or activating ALK variant, which showed extremely poor clinical outcome. Their study also proved that, when there were copresence of ALK and MYCN, a robust transformation could be observed compared to an activated ALK alone. This indicates that, when both genes are co-expressed together it will result oncogenicity that provides the driving force for neuroblastoma development and progression.

According to Hassan *et al.* [9] reported that MYCN-mediated ALK induction promotes cell proliferation, migration and invasion. ALK expression was

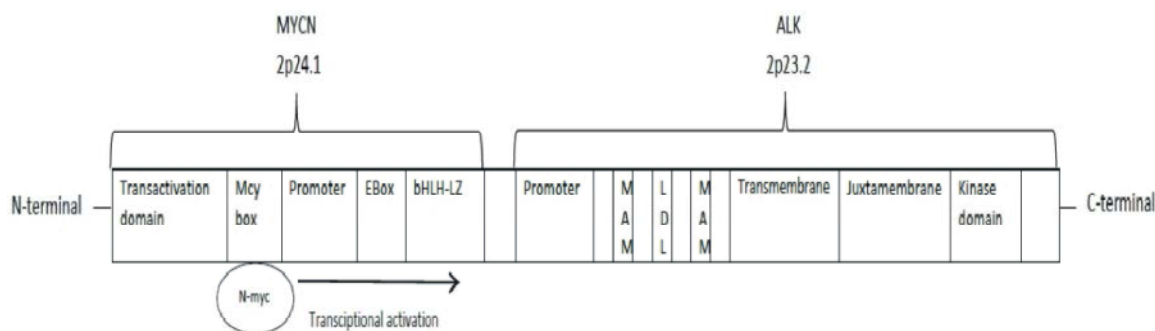


Fig. 1: Amplification of MYCN leads to increase MYC protein. This later on activates the MYCN promoter transcriptional target and MYCN amplification in neuroblastoma induced promoter of ALK gene, leading to a high level of ALK expression in neuroblastoma cells. It is postulated that transcription of ALK kinase domain also been enhanced by increasing amount of MYC protein that is regulated by MYCN indirectly affect the ALK gene in tyrosine kinase domain. (Adapted from Maris and Mathay [3] and Anna M. Azarova [10])

significantly correlated with MYCN amplification in primary neuroblastoma, while over expression of MYCN induces promoter activity of ALK gene, leading to a high level of ALK expression in neuroblastoma. The promoter region of ALK gene contains a non-canonical E-box located upstream of the transcription initiation site and MYC proteins bind onto the promoter region and regulates its transcription. Moreover, wild type ALK functions as a modulator of proliferation as well as cell migration and invasion. They performed promoter study by constructing luciferase reporter constructs containing -2056 bp to +30 bp fragments of ALK gene by using NBL (mutated ALK cell line; SH-SY5Y and wild-type ALK cell line; SK-N-AS) and non-NBL (U2OS and HeLa) cells, which showed co-related regulation of ALK gene with MYCN or c-MYC expression increased in luciferase activity with pGL4.17 ALK (-2056 BP) was enhanced by co-expression of increasing amount of MYCN or c-MYC expression vector. Their data also demonstrated ALK as a direct target of MYC proteins due to high level of ALK expression that were observed in a 2 week old MYCN-hemizygous mice, which grew spontaneous arising ALK expressed neuroblastoma in early stage of tumor development driven by MYCN [9].

Ogawa *et al.* [36] states that ALK plays a positive role during the initiation and promotion of neuroblastoma even though established tumors may or may not depend on the ALK activity. A schematic diagram shows the potential role of ALK in neuroblastoma (Fig. 1).

When amplification of MYCN gene locus occurs, MYC proteins will bind onto the promoter regulating its transcription. This will activate continuous transcriptional activation on the MYCN gene. As both MYCN and ALK

gene are located on chromosomal locus 2, continuous transcriptional activation will consequently activate the growth promoting gene. Thus, over expression of MYCN induces promoter activity of ALK gene which eventually leads to a high level of ALK expression in neuroblastoma as ALK is also involved in cell proliferation. Thus, we postulates that whenever MYCN gene is amplified activation of ALK genes also occurs resulting in coamplification. This leads to poor prognosis in patients with neuroblastoma although the exact mechanism is still unclear.

Retinoic Acid (RA) and ALK/MYCN Resistance: RA is one of the potent differentiation inducers for human neuroblastoma in vitro. Upon exposure of RA, various neuroblastoma cell lines cease proliferation, differentiate into neuronal like cells, or undergo apoptosis [37]. RA is well known MYCN suppressor and the addition of RA to proliferating neuroblastoma cells halts their division and leads to either differentiation or apoptosis [28, 37, 38, 39]. However, according to Hassan *et al.* [9], their recent study suggested that ALK expression partially prevented the effect of RA on cell proliferation. Hassan *et al.* [9] also analyzed RA treatment experiment using MYCN amplified cultured human neuroblastoma cell line known as GOTO, which displayed wild type ALK allele and showed sensitivity to RA. In addition, the proliferation as well as the number and size of colony of neuroblastoma cells were significantly suppressed after treatment with RA as described by Futami [40]. Moreover, after treatment with RA, MYCN showed decreased expression and ALK expression was also down regulated. However, both wild-type and F1174L mutated ALK over expressing cells

had a higher number of live clones compared with the control cells following the treatment with RA, which suggested that expression of ALK partially prevented the retinoic acid effect on cell proliferation [9]. Cheung *et al.* [41] stated that, there was no correlation seen between resistance to RA and the level of RAR or RXR expression. However, higher expression of RAR β has been associated with good outcome in neuroblastoma and over expression of that gene by transfection appears to increase the responsiveness of some neuroblastoma cell lines to RA [44].

CONCLUSION

Neuroblastoma remains a fascinating subject of research as it has unique heterogeneity in its pathogenesis pathway. MYCN amplification is known to result in poor prognosis of neuroblastoma patients as well as ALK amplification as ALK gene alteration may result in tumors resistance towards therapy. Thus, this review emphasizes on co-amplification between MYCN and ALK gene when activation of both of these genes results in a robust transformation of cell proliferation. On the other hand, although retinoic acid is known as MYCN suppressor, again when both of these genes are coexpressed, ALK expression partially prevents the effect on cell proliferation. However, the exact mechanism should further be studied.

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