MYCN, neuroblastoma and focal adhesion kinase (FAK)

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1. ABSTRACT

   Neuroblastoma is the most common extracranial solid tumor of childhood. This tumor is characterized by poor survival, especially when it features amplification of the MYCN oncogene. The ability for human cancers to propagate is marked by their ability to invade and metastasize to distant sites. Focal adhesion kinase (FAK) is a key tyrosine kinase involved in the survival and metastasis of a number of human tumor types. We have shown that FAK is present in human neuroblastoma and that its expression in neuroblastoma is related to the MYCN oncogene. We have also demonstrated that inhibition of FAK in neuroblastoma leads to decreased tumor cell survival. The current review addresses the relationship between the MYCN oncogene, focal adhesion kinase and neuroblastoma.

2. INTRODUCTION

   In the following review, we discuss the role of focal adhesion kinase in human neuroblastoma and its relationship to the MYCN oncogene in these tumors.

3. MYCN ONCOGENE

   The MYCN oncogene plays a major role in human tumorigenesis. Previous data indicate that the primary function of MYCN is as a transcription factor, known to bind to the specific DNA E-box sequence, CACGTG (1, 2). Recent evidence supports a dual role for MYCN. Murphy et al recently showed that MYCN more commonly binds to the CATGTG E-box sequence, and MYCN binding correlates with DNA hypermethylation, thereby, also functioning as a mediator of chromatin...
structure (3). Normal expression of MYCN is limited to embryonic brain and kidney tissues, and lymphocytes early in their differentiation (4, 5, 6). The importance of MYCN in embryogenesis is demonstrated by the fact that in mice, homozygous MYCN null is an embryonic lethal mutation, with these embryos showing a significant decrease in mature neurons of neural crest origin (7). Abnormal expression of MYCN is associated with neuroblastoma, the most common extracranial solid tumor of childhood. The exact function and gene targets of MYCN have not been completely defined (8), and a thorough investigation of which target genes are required for the proliferation and tumorigenesis of neuroblastoma have not been published (9). However, there is significant interest in this arena and some progress is being made with studies showing in vitro (10, 11, 12), and in vivo (9, 13) binding of MYCN to the genes that it regulates. For example, MYCN has been shown to bind to the gene promoters for various oncogenes involved in cellular proliferation, differentiation, and survival and to increase the expression of these targets. These genes include the proto-oncogenes high mobility group A1 (HMGAI) (14) and Pax-3 (11), the multidrug resistance-associated protein gene (MRPI) (12), ATP-binding cassette (ABC) transporters that mediate efflux of chemotherapeutic agents from cancer cells (15), and the proteins nestin (10) and livin (16). There are also a number of negative transcriptional targets for MYCN in neuroblastoma including the growth-inhibitory gene Ndrg1 (17) and leukemia inhibitory factor (LIF) (18). The MYCN protein has a number of functional regions including a basic helix-loop-helix leucine zipper motif and two highly conserved areas, Myc box I and II. These regions are important for the function of the MYCN protein however, the specific regions required for transcriptional regulation are segregated and appear to vary with different target genes (19).

4. MYCN ONCOGENE AND NEUROBLASTOMA

Neuroblastoma is the most common extracranial solid tumor of childhood. Despite many advances in both medical and surgical care, the overall survival for children presenting with advanced stage disease remains less than 30%. The strongest adverse prognostic indicator in human neuroblastoma is gene amplification of greater than ten copies of the MYCN oncogene (20). Amplification of this gene occurs in about 20% of neuroblastomas (8, 20) and is associated with both increased recurrence of disease and decreased patient survival. Numerous studies have demonstrated the importance of MYCN in neuroblastoma tumorigenesis. The level of MYCN expression has been shown to correlate with the growth and proliferation of neuroblastoma cells in vitro (4, 21, 22, 23), and the interruption of MYCN induces cellular differentiation in neuroblastoma cells. Nara and colleagues showed that after blocking MYCN, neuroblastoma cells developed multidirectional neurite extension and increased cellular and nuclear size, findings consistent with differentiation (24). Downregulation of MYCN with antisense oligonucleotides resulted in a decrease in both cellular proliferation and anchorage independent growth in the cells (4, 23). Other authors have utilized small interfering RNAs (siRNAs) to silence MYCN. Woo et al demonstrated that MYCN interference resulted in a decrease in the number of neuroblastoma cells in the S-phase of the cell cycle (25). In addition, other investigators utilizing this method to silence MYCN have shown decreased neuroblastoma cell growth and increased neuroblastoma cell apoptosis (26). Finally, transgenic mice with MYCN over-expression develop spontaneous neuroblastomas (27) that have the same histological features seen in human tumors (28).

5. FOCAL ADHESION KINASE (FAK)

Focal adhesion kinase (FAK) is a nonreceptor, cytoplasmic 125kDa protein tyrosine kinase. Initial studies revealed that both the transcription of FAK mRNA (29) and the expression of FAK protein is significantly increased in primary and metastatic breast, colon, and thyroid tumors and hepatocellular carcinoma when compared normal tissues (30-37), and that these changes occur early in tumorigenesis. Real-time PCR analysis of colorectal carcinoma and liver metastasis with matched normal colonic tissues demonstrated increased FAK mRNA abundance in the tumors and metastatic tissues compared to control tissues (38), suggesting that the increased FAK expression in human tumors occurs at the level of transcription. FAK controls a number of cell signaling pathways including motility, proliferation, viability and survival (39-42). Silencing FAK expression with small interfering RNAs resulted in decreased migration of lung cancer (43) and glioblastoma cells (44), and decreased tumor growth in ovarian (45) and prostate cancer models (46). The inhibition of FAK with antisense oligonucleotides had been shown to cause decreased growth in tumor cells (47) and FAK inhibition with a dominant-negative FAK protein (FAK-CD), inhibited cell growth in human melanoma cells (29), and in human breast cancer cell lines (30, 48).

6. MYCN, NEUROBLASTOMA AND CELL ADHESION

An association between MYCN amplification and tumorigenicity through cell adhesion, motility, and invasiveness has been implied in some studies. MYCN amplified human neuroblastoma cells have both increased cellular motility and invasiveness (49) and decreased attachment (50) compared to MYCN non-amplified cell lines, but no mechanistic explanation for these observations has been elucidated. To this end, Ma and others recently demonstrated that MYCN amplification correlates with levels of microRNA-9 (miR-9) which is associated with increased cell motility and invasiveness by regulating E-cadherin in breast cancer cells (51). More specifically to neuroblastoma, Akeson and Bernards showed that rat neuroblastoma cells transfected with a MYCN expression vector have significant reductions in mRNA and protein expression of neural cell adhesion molecule, a specific cell-cell adhesion molecule (52). In addition, other researchers have shown that the expression of integrin subunits α3 and β1 are inversely related to overexpression of the N-Myc protein in neuroblastoma (53, 54). MYCN overexpression in neuroblastoma cells in vitro also results in decreased
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Figure 1. FAK protein detection in formalin-fixed, paraffin embedded human neuroblastoma specimens. Antibody to p125FAK was utilized to detect FAK staining in 70 human neuroblastoma specimens. A. This photomicrograph (40×) shows a MYCN amplified INSS stage 4 neuroblastoma specimen with significant staining for p125FAK. B. This photomicrograph (40×) shows a MYCN non-amplified INSS stage 4 neuroblastoma specimen with no significant p125FAK staining.

expression of α1 integrin, leading to decreased attachment and increased migratory activity (55). In addition, Wu and others have demonstrated that FAK activity is required to promote integrin stimulated neuroblastoma motility through α5β1 but not for α4β1 integrin (56). These data provide evidence that MYCN is involved in neuroblastoma cell adhesion.

7. MYCN, NEUROBLASTOMA AND FOCAL ADHESION KINASE (FAK)

The association between MYCN amplification and cellular motility and invasiveness in neuroblastoma implies a potential relationship between focal adhesion kinase (FAK) and MYCN, since FAK is a key protein involved in cellular motility.

7.1. FAK in human neuroblastoma

Early studies relating neuroblastoma and FAK primarily involved the use of SH-SY5Y neuroblastoma cells (non-amplified MYCN) to investigate the relationship between insulin-like growth factor -1 (IGF-1) and FAK activation in their role in neuronal morphology during cellular differentiation (57, 58). These investigators showed that IGF-1 stimulated cell motility was mediated through the phosphorylation of FAK. In these studies, they utilized SH-SY5Y cells, not because of their cancer properties, but because these cells can be induced to differentiate, and are thereby a good experimental model for neuronal differentiation studies. In more recent studies, Kim and Feldman began investigating the effects of FAK dephosphorylation in neuroblastoma cell survival (59, 60). In the first study they showed that treatment of SH-EP neuroblastoma cells with mannitol resulted in the loss of FAK phosphorylation and increased cellular detachment and apoptosis which were reversible by IGF-1 (59). In a subsequent study, they reported that okadaic acid treatment of SH-EP cells resulted in the loss of FAK phosphorylation and resultant apoptosis that was not reversible with the treatment of IGF-1 (60). However, until recently, there were no data demonstrating the expression of FAK in human neuroblastoma specimens, and none to clearly show that FAK and MYCN were related in neuroblastoma. An examination of 70 human neuroblastoma specimens with various INSS stage and MYCN amplification status revealed an increase in the expression of FAK protein, as detected by immunohistochemistry, in MYCN amplified human neuroblastoma specimens compared to those tumors that were not MYCN amplified (Figure 1, panels A, B) (61). Although FAK expression did not prove to be an independent prognostic indicator in that study, FAK expression was strongly associated with those advanced stage tumors that had amplification of the MYCN oncogene (61).

7.2. MYCN regulates FAK expression in neuroblastoma

Investigations into the mechanisms of FAK regulation in neuroblastoma have been ongoing. The FAK promoter has been cloned (62) and evaluations of this promoter have demonstrated a number of binding sites present for various oncogenes, such as p53. In addition, examination of the FAK promoter has revealed E-box sequences on this promoter that are potential binding sites for MYCN (Figure 2, panel A). Further studies including electrophoretic mobility shift, chromatin immunoprecipitation (ChIP), and dual luciferase assays have shown that MYCN does bind to the FAK promoter both in vitro and in vivo, resulting in an upregulation of FAK expression (63) (Figure 2, panel B). Developing a better understanding of the effects of MYCN upon the FAK promoter in neuroblastoma impacts our evaluation of the role of FAK in tumorigenesis in other tumor types. For example, MYCN is reported to be amplified in human melanoma and sarcomas (64) and is associated with poor outcomes in these tumor types (65). FAK has also been
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Figure 2. MYCN regulates the FAK promoter. A. Nucleotide sequence (GenBank Accession Number AY323812) of the 5'-flanking region of the FAK gene, with the transcription initiation site marked as +1. The putative E-box binding site for MYCN is underlined. B. Dual luciferase assays are utilized to detect FAK promoter activity. Graph showing fold change in FAK promoter activity in MYCN– and MYCN+ isogenic neuroblastoma cell lines. There is a significant increase in FAK promoter activity with the P-280 construct in the MYCN+ cell line compared to the MYCN– cells (*P<0.01). This is the construct that contains the E-box binding sequence for MYCN. When the E-box binding site is mutated (∆P-280), the FAK promoter activity in the MYCN+ neuroblastoma cell lines significantly decreases compared to the wild type (P-280) promoter construct (†P<0.01).

shown to be overexpressed in human sarcoma and melanoma tumors (66, 67), and downregulation of FAK results in decreased survival in human melanoma cells (68). These data provide evidence that MYCN is potentially a transcriptional regulator of FAK in these tumor types as well, but as of yet the role of MYCN here has not been fully investigated.

7.3. FAK as a target in neuroblastoma

The biological relevance of increased FAK expression by MYCN amplified neuroblastoma cell lines has begun to be explored. In an initial study, an isogenic MYCN neuroblastoma cell line with a repressible MYCN vector (SHEP Tet-21/N) (69) was utilized. Treatment of these cell lines with siRNA to FAK resulted in a significant increase in cellular apoptosis in the MYCN overexpressing cells compared to their MYCN non-expressing counterparts (63). Additional studies using AdFAK-CD, an adenoviral construct that functions as a dominant-negative of FAK, for FAK inhibition in the same isogenic MYCN neuroblastoma cell lines resulted in decreased cellular attachment and proliferation and increased cellular apoptosis in the neuroblastoma cells that had MYCN overexpression compared to the non-overexpressing cell line (70).

Recent advances have been made in the development of small molecule inhibitors of FAK. For instance, TAE226, a small molecule FAK inhibitor, has been reported to decrease cell survival in human glioma cells (71) and to decrease metastasis and enhance survival in xenograft models of pancreatic and breast cancer (72, 73). TAE226 has been studied in neuroblastoma and found to decrease tumor cell survival (74). FAK inhibition with TAE226 in a MYCN amplified neuroblastoma cell line [SK-N-BE(2)] results in significantly decreased cell viability at low concentrations with minimal effects upon a MYCN non-amplified cell line (SK-N-AS) (Figure 3). Other small molecule FAK inhibitors are also being examined. Golubovskaya et al have reported their results with 1,2,4,5-benzenetetraamine tetrahydrochloride, a small molecule directed towards the site of autophosphorylation on FAK (75). FAK inhibition using 1,2,4,5-benzenetetraamine tetrahydrochloride resulted in decreased breast cancer cell survival both in vitro and in vivo. Other investigators have
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Figure 3. FAK inhibition leads to decreased cell viability in MYCN amplified neuroblastoma cell lines. FAK inhibition is achieved with TAE226, a specific FAK kinase inhibitor. Neuroblastoma cells, SK-N-BE(2) (amplified MYCN) and SK-N-AS (non-amplified MYCN) are treated with TAE226 for 24 hours and viability is measured using Alamar Blue Assay. After 24 hours, cellular viability is significantly decreased in the MYCN amplified neuroblastoma cell line, SK-N-BE(2) (P<0.01), compared to that in the non-amplified, SK-N-AS, cell line, demonstrating the biologic importance of FAK in MYCN amplified neuroblastoma cells.

Figure 4. FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride has minimal effects upon normal ganglion cells. Normal ganglion cells are treated with a small molecule FAK inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, for 24 and 48 hours. Viability is measured using Alamar Blue Assay. The cellular viability in these normal ganglion cells is essentially unaffected, even after 48 hours of treatment, by FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride.

Figure 4. FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride has minimal effects upon normal ganglion cells. Normal ganglion cells are treated with a small molecule FAK inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, for 24 and 48 hours. Viability is measured using Alamar Blue Assay. The cellular viability in these normal ganglion cells is essentially unaffected, even after 48 hours of treatment, by FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride.

demonstrated the efficacy of this small molecule in treating pancreatic cancer cell xenografts (76). Use of 1,2,4,5-benzenetetraamine tetrahydrochloride in human neuroblastoma has also been reported (77). Treatment with 1,2,4,5-benzenetetraamine tetrahydrochloride resulted in decreased cellular attachment, viability and increased apoptosis in vitro, and decreased neuroblastoma tumor growth in vivo, in a number of human neuroblastoma cell lines. As seen in previous studies, neuroblastoma cell lines with MYCN amplification were more sensitive to FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride than those that were not MYCN amplified (77). Notably, further studies with 1,2,4,5-benzenetetraamine tetrahydrochloride have shown no effect upon the viability of normal ganglion cells (Figure 4). These studies suggest that FAK is a potential therapeutic target for the treatment of human neuroblastoma, and may be most effective when directed toward aggressive, MYCN amplified tumors.

8. SUMMARY AND PERSPECTIVE

Neuroblastoma is a common childhood malignancy that continues to have a poor response to the current available therapies. It is clear that novel interventions will be required to treat this disease, especially in the advanced stages. Fairly good evidence has been generated to show that in neuroblastoma, focal adhesion kinase is correlated with MYCN oncogene amplification, and that inhibition of FAK in these amplified tumors results in decreased tumor cell survival. These findings underscore the potential for FAK inhibition in the
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development of new therapeutic strategies for neuroblastoma.

9. REFERENCES


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**Abbreviations:** FAK: focal adhesion kinase

**Key Words:** MYCN, neuroblastoma, FAK, Pediatric, SHEP-21/N, SK-N-AS, SK-N-BE(2), Review

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