MOLECULAR CHARACTERIZATION
OF PEDIATRIC SPINDLE CELL TUMORS

by

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(Department of Pathology and Laboratory Medicine)

We accept this thesis as conforming
to the required standard

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2000

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Date **April 5/00**
Congenital fibrosarcoma (CFS) is a cellular, mitotically active neoplasm of soft tissues. It affects infants less than two years of age, has a low metastatic rate and a relatively high propensity for local recurrence. One of the predominant clinical issues surrounding CFS is its distinction from other histologically identical and virtually indistinguishable pediatric spindle cell tumors including adult-type fibrosarcoma (ATFS) and infantile fibromatosis (IFB). ATFS is a malignant lesion that is treated more aggressively than CFS, while IFB is a benign lesion which is treated less aggressively. Reliable distinction between these entities is therefore clinically very important. We therefore wanted to identify a diagnostic tool to distinguish CFS from other fibroblastic tumors such as ATFS and IFB. Cytogenetic analysis of CFS cases has shown a nonrandom gain in chromosomes 8, 11, 17, and 20 with trisomy for chromosome 11 being present in most cases. Cytogeneticists at the Department of Pathology of B.C.C.H. recently identified recurrent cytogenetic alterations involving chromosome 12p13 and 15q25 in three CFS cases, which were not present in ATFS, IFB, and aggressive fibromatosis. Cloning of the chromosomal breakpoints revealed a novel fusion between the ETS transcription factor member, \textit{ETV6}, and the gene encoding the neurotrophin-3 cell surface receptor, \textit{NTRK3}. This fusion results in the juxtaposition of the HLH dimerization domain of ETV6 to the protein tyrosine kinase (PTK) domain of NTRK3. We hypothesized that this molecule acts as an aberrant PTK signaling molecule in which the HLH domain mediates ligand independent dimerization resulting in
constitutive PTK activation. The fusion protein exists as a 70-80 kDa doublet and was found to undergo homodimerization as well as heterodimerization with ETV6. Furthermore, we were able to show that the ETV6-NTRK3 protein acts as a PTK that was capable of interacting with PLCγ1, but not with other known NTRK3 interactors including SHC, SH2Bβ, GRB2 and PI3K. Moreover, ETV6-NTRK3 was shown to localize mainly in the cytoplasm. Our data support the notion that CFS is a biologically distinct entity, and ETV6-NTRK3 detection provides a diagnostic screening tool potentially useful in the clinical evaluation of children with spindle cell tumors.
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<td>refractory anemia with excess blasts (with basophilia)</td>
</tr>
<tr>
<td>RB</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RMS</td>
<td>rhabdomyosarcoma</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>SH3</td>
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<tr>
<td>SNT</td>
<td>sucl-associated neurotrophin factor target</td>
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<tr>
<td>Sos</td>
<td>Son of Sevenless</td>
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<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TAD</td>
<td>transactivation domain</td>
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CHAPTER I

INTRODUCTION

1.1 SYNOPSIS AND RATIONALE FOR THE THESIS

The studies to be described in this thesis were initially performed on a series of congenital fibrosarcoma cases, which belong to the family of spindle cell lesions of childhood. This family also includes infantile fibromatosis, aggressive fibromatosis and adult-type fibrosarcoma, all of which appear histologically similar under the microscope. Pediatric spindle cell lesions of early childhood pose significant diagnostic challenges for the pathologist, as they are difficult to differentiate from one another due to their similar morphologic appearance. Differentiating these tumors is of great importance clinically as they show different clinical behaviors and require distinct treatment protocols. We were therefore interested in finding a specific recurring genetic anomaly which would provide the pathologist with a molecular tool for accurately diagnosing these tumors. We studied and identified a recurrent t(12;15)(p13;q25) in congenital fibrosarcoma, which lead to the identification of a novel gene fusion between ETV6 (also known as TEL) and NTRK3 (also known as TRKC) from chromosomes 12 and 15, respectively. Further studies showed that the fusion gene encoded a chimeric tyrosine kinase protein which we hypothesized functioned by dysregulating normal signaling pathways within the malignant cell. Given these findings, the remainder of this chapter will deal with pediatric spindle cell tumors (with an emphasis on congenital fibrosarcoma and other morphologically similar lesions), general aspects
of cancer biology and genetics, normal and abnormal growth (signal transduction and cell cycle), pediatric solid tumors, as well as signal transduction as it relates to tyrosine kinase receptors.

1.2 **PEDIATRIC SPINDLE CELL SARCOMAS**

Human malignant tumors can be categorized into sarcomas, carcinomas and hematopoietic (including lymphoid) malignancies. Sarcomas are tumors which have arisen from mesenchymal tissue while carcinomas derive from epithelial tissue. Hematopoietic malignancies arise from blood forming and lymphoid cells which originate from mesoderm. During embryogenesis there are three primary germ layers; endoderm, mesoderm and ectoderm. The ectoderm gives rise to the epithelium, the entire nervous system, the lens and retina of the eye as well as a broad range of structures in the head and pharynx. The mesoderm gives rise to mesenchyme which is responsible for forming the skeletal muscles, vertebrae and skull, connective tissues and blood vessels of the body wall, the skeletal elements of the body wall, girdles and limbs, the smooth muscles and connective tissue of the digestive tract, the heart, the blood vessels of the viscera and blood. The endoderm gives rise to the epithelium of the digestive tract and to the epithelioid components of all organs that arise as evaginations from the embryonic foregut, midgut, or hindgut. Sarcomas are malignant mesenchymally-derived tumors which exhibit local recurrence and metastatic behavior, and have a high proliferative rate. Sarcomas are categorized on the basis of cell of origin. For example, there are rhabdomyosarcomas (derived from skeletal muscle precursor cells),
leiomyosarcomas (smooth muscle cells), chondrosarcomas (cartilage cells), liposarcomas (fat cells), hemangiosarcomas (blood vessels), fibrosarcomas (fibroblasts), osteosarcomas (bone cells), synovial sarcomas (synovial cells), and sarcomas of unknown origin. Several of the sarcomas mentioned above can also have spindle cell morphology. The word “spindle” refers to the shape of the cells, being long and drawn out in the shape of a spindle. Some examples of spindle cell tumors include fibrosarcoma, leiomyosarcoma, synovial sarcoma and some forms of rhabdomyosarcoma.

One of the predominant issues in tumor pathology is the difficulty in differentiating malignant sarcomas from each other as well as from benign lesions such as fibromatoses (benign lesions involving fibroblast cells). These benign lesions can be virtually indistinguishable from their malignant counterparts by morphological criteria, but have no metastatic behavior, have a low recurrence rate, and are less aggressive than sarcomas. A variety of approaches commonly used by the pathologist in diagnosing tumors are discussed below.

1.2.1 Pathologic Workup of Pediatric Sarcomas

Histology is the primary method for evaluating tumors and is based on the study of the morphology of the various tumors. Briefly, the tumor specimen is fixed in formalin and embedded in paraffin. A 5μm section of the tumor is then placed onto a microscope slide and stained with hematoxylin and eosin. This is known as an H&E section and the characteristic staining patterns it produces is the primary diagnostic modality used in evaluating tumors.
Immunohistochemistry helps determine the cell of origin. A thin section of the tumor is placed onto a slide and is then incubated with antibodies against specific antigens such as neuron-specific enolase (NSE), Leu7, neurofilament triplet protein (NFTP), desmin, muscle-specific actin, vimentin, S100, keratin, leukocyte common antigen (CD45), and the surface antigen MIC2. For example, a neural tumor would be positive for NSE, Leu7, and NFTP, while myogenic tumors would be positive for desmin, muscle-specific actin, and MYOD (a transcription factor that appears early in myogenesis and activates later gene expression). Therefore, the combination of positive and negative staining for the above antigens helps the pathologist form an accurate diagnosis.

Another analytical technique often used is electron microscopy. The electron microscope is used to evaluate ultrastructural features. Neuroblastomas, for example, contain dense core granules, rhabdomyosarcomas contain myofilaments, and acute megakaryocytic leukemia contains platelet granules each of which can be identified by electron microscopy.

Cytogenetics is a technique which involves the analysis of chromosomes from short term cultures of tumors. Recurrent chromosomal abnormalities can be used as a diagnostic tool, especially in pediatric tumors where there are many examples of recurring chromosomal deletions, translocations and whole chromosome gains and losses (discussed in further detail below).

The results from histologic, immunohistochemistry, electron microscopic and cytogenetic analysis provide the pathologist with useful diagnostic information. This information needs to be correlated with the clinical history and
radiological features of the tumor. In summary, histology and other pathologic modalities, coupled with the clinical history (including radiological results) is the initial route taken in the accurate diagnosis of many soft tissue pediatric tumors. Often however, it is extremely difficult if not impossible to distinguish different sarcomas from each other or sarcomas from benign lesions as they may be virtually identical morphologically. This has lead to the relatively new field of molecular pathology, in which the tumor is further analyzed by cytogenetic and molecular techniques to make an accurate diagnosis (discussed in further detail below).

1.2.2 Congenital Fibrosarcoma

Congenital (infantile) fibrosarcoma (CFS) arises from fibroblasts and is a mitotically active spindle cell lesion affecting soft tissue. CFS acquired its name because of its histologic similarity to adult fibrosarcoma [1]. Fibrosarcomas tend to be categorized into two distinct age groups (the upper and lower limits of which are poorly defined): those occurring before the age of 2 years (most under one year of age), known as CFS, and those occurring in patients aged 10 years or older, known as adult-type fibrosarcoma (ATFS). Correlating with these age groups are distinct differences in clinical behavior. While CFS has a metastatic and recurrence rate of 10% and up to 40%, respectively [2-4], it is unique among human sarcomas for its excellent prognosis with an 80-90% overall survival rate [5, 6]. On the other hand, ATFS is an aggressive lesion with a poor prognosis similar to that of adult fibrosarcoma [7].
Under the microscope, CFS is morphologically similar to other fibroblastic tumors such as aggressive fibromatosis (AFB), ATFS and infantile fibromatosis (IFB) and has, historically, been difficult to diagnose [8-10]. This is evidenced by CFS having been misdiagnosed in the past for a lymphatic malformation [11, 12], hydrops fetalis [13], or congenital hemangioma [11, 14]. CFS was also postulated as being histogenetically related to infantile myofibromatosis (MFB) and congenital hemangiopericytoma [1, 15, 16].

CFS is predominantly found in the extremities (71%) and is primarily treated by surgical excision [17], often with adjuvant preoperative and postoperative chemotherapy as well as combination chemotherapy [8, 18-22]. The propensity for CFS tumors to metastasize seems to depend on the primary tumor site: 7-8% of primary CFS tumors located in the extremities metastasize while 26% of those located within the abdomen, pelvic and chest area metastasize [9, 23].

The benign counterpart to fibrosarcoma is infantile fibromatosis (IFB). IFB is a lesion that arises from fibroblasts and/or myofibroblasts and exhibits lower cellularity and mitotic activity than fibrosarcomas [1]. Infantile fibromatosis (IFB) primarily occurs in children aged 2 years or younger and, in addition to the above characteristics, contains more collagenous matrix than CFS. A variant of IFB known as aggressive fibromatosis (AFB) is clinically more aggressive than IFB and is inclined to increased local invasion and recurrence [1].

Cytogenetic analyses of CFS cases to date have shown non-random whole chromosome gains for chromosomes 2, 8, 11, 17 and 20 [6, 24-26]. Other tumors which share some of these specific chromosomal abnormalities include: +20 and +8
in desmoid tumors [27-29], +8 in Dupuytren's contracture, Peyronie's disease and hematologic malignancies [30-33], +20, +17, +11, and +8 in congenital mesoblastic nephroma (CMN) [34, 35], and +11 in acute myeloid leukemia (AML) [36]. Currently, there have been no reports of any recurring chromosomal abnormalities for IFB and ATFS. As will be discussed in later chapters, we have now identified a recurrent t(12;15)(p13;q25) translocation in CFS.

1.3 GENERAL ASPECTS OF NORMAL GROWTH REGULATION

Understanding the mechanisms of oncogenesis (see section 1.4 below), requires the familiarization with the way an extracellular "message" is transferred from the cell membrane into the nucleus. Once in the nucleus the initial message results in the activation or suppression of transcription of certain genes required either for growth and progression through the cell cycle or differentiation. A cell maintains a normal rate of growth by a variety of important biological mechanisms. The following discussion will briefly cover three mechanisms pertaining to normal growth regulation:

1. Signal Transduction involved in Cell Proliferation: the concept of how a cell receives an extracellular "message" and the intracellular (including nuclear) consequences.

2. Signal Transduction Involved in Limiting Cell Growth: the concept of how a cell "knows" when to stop growing in the context of its environment.
3. Cell Cycle: the nuclear machinery responsible for determining when it is appropriate for a cell to proliferate and divide.

1.3.1 Signal Transduction Involved in Cell Proliferation

In general, signal transduction refers to the specific molecular interactions and subsequent modifications in response to an external stimulus, such as growth factors and cytokines. Messages from extracellular growth regulatory molecules are transferred into the cell via receptors that bind these molecules. There are several different classes of receptors including growth factor receptors (e.g., fibroblast growth factor receptor), steroid receptors (e.g., glucocorticoid receptor), neurotransmitter receptors (e.g., acetylcholine receptor), seven transmembrane-spanning or serpentine receptors (e.g., gastrin releasing peptide receptor), B-cell receptors (e.g., B cell antigen receptor (BCR)), and T-cell receptors (e.g., TCR). Since our studies resulted in the discovery of a chimeric fusion protein containing a portion of a tyrosine kinase receptor (a member of the growth factor receptor family), the remainder of this discussion will focus on signal transduction as it pertains to the protein tyrosine kinase family of growth factor receptors.

Many of the signals responsible for inducing proliferation or differentiation act through growth factors binding to cell surface receptor tyrosine kinases (RTKs). The process of transducing a signal from RTKs on the cell surface to the nucleus can be divided into 4 steps (other receptors, such as the steroid receptors, contain a slightly different series of events):
1. ligand binding

2. receptor dimerization and PTK activation

3. phosphorylation of cytoplasmic proteins

4. phosphorylation of nuclear proteins (i.e. transcription factors responsible for the activation or inhibition of genes involved in growth control)

These steps will now be discussed below in the context of the PDGF receptor and the well established RAS pathway [37-39].

1.3.1.1 The PDGF receptor and RAS pathway

The way many extracellular hormone or growth factors deliver their messages to the intracellular environment of a cell is by binding to a specific protein tyrosine kinase (PTK) receptor. Each PTK molecule has an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain [37]. The ligand (e.g. EGF, FGF or PDGF), will bind to the appropriate extracellular portion of the receptor, thus beginning the process of signal transduction. An example of this is the platelet derived growth factor (PDGF) binding to PDGFR.

Upon ligand binding, the PDGF receptor undergoes homodimerization [37] bringing the kinase domains in close proximity. This interaction results in the auto- or cross-phosphorylation of specific tyrosine moieties within the intracellular domain of the receptor. The structure responsible for this is known as the activation loop and consists of tyrosine residues within the kinase domain. Phosphorylated tyrosine residues outside the tyrosine kinase domain act as anchors for downstream molecular interactions (see below) [40, 41]. In addition to these
tyrosine residues are tyrosines which do not get phosphorylated and act as structural amino acids. Other types of receptors which undergo ligand induced dimerization (and even oligomerization) include B-cell receptors, T-cell receptors, hormone and cytokine receptors [42].

Once the ligand interaction has taken place, the PDGF/PDGFR complex acts as a tyrosine kinase and phosphorylates interacting proteins (including other tyrosine kinases) which can then interact with other molecules and phosphorylate them [37]. The PDGF receptor contains several different phosphorylated tyrosines responsible for its interaction with associating molecules such as Src [43], phosphatidylinositol-3 kinase (PI-3K) [44], GAP [45], Syp [46], SHC [47], growth factor receptor-bound protein 2 (GRB2) [48, 49] and PLC-γ1 [44]. These downstream interacting molecules possess an SH2 domain (for Src homology 2) which allows for the specific interaction with phosphorylated tyrosines [50]. The specificity for a particular phosphorylated tyrosine is determined by the 3 dimensional shape of the SH2 domain in the downstream molecule and the 3 dimensional context of the phosphorylated tyrosine moiety on the receptor. Another domain which has recently been implicated in phosphorylated tyrosine binding is known as the phosphotyrosine binding domain (PTB) [51, 52]. GRB2 is the 217 amino acid homologue of drk in Drosophila and Sem-5 in Caenorhabditis [53, 54]. It contains an SH2 domain responsible for interacting with one of the phosphorylated tyrosines on the intracellular portion of the activated EGF receptor and a SH3 domain which interacts with proline rich sequences on downstream molecules such as Sos [55]. GRB2 interaction with Sos (the 1596-residue product of the Son of Sevenless gene,
so named because Sos interacts with the *Sevenless* gene product, a PTK receptor that regulates the development of the R7 photoreceptor cell in the *Drosophila* compound eye [42]) results in the relocalization of Sos to the plasma membrane where it can convert inactive RAS (RAS-GDP) into active RAS (RAS-GTP) [56, 57]. One of the pathways affected by RAS is the mitogen activated protein kinases (MAPK) pathway which is a series of cytosolic serine/threonine kinases [58]. One of the molecules which leads to the activation of the MAPK pathway is RAF1, which is a serine/threonine protein kinase (also known as MAP kinase kinase kinase (MAPKKK)) [59]. Phosphorylation of multiple serine and threonine residues activates RAF1 allowing it to interact with and phosphorylate MEK (MAP kinase/ERK-activating Kinase or MAP kinase kinase (MAPKK)) [39, 60]. Activated MEK then interacts with and phosphorylates a family of proteins, known as the MAP kinases or ERKs (extracellular-regulated kinases) which require the phosphorylation of both serine/threonine and tyrosine residues in order for proper activation.

Before proliferation or differentiation can occur, certain genes involved in growth regulation need to be turned on or off. One mechanism involves the phosphorylation of particular transcription factors found in the nucleus by activated cytoplasmic kinases that have migrated into the nucleus. In the RAS pathway, activated (phosphorylated) ERK migrates from the cytoplasm into the nucleus and phosphorylates various transcription factors, such as Jun/AP1, Fos, Myc, and Sap1 which subsequently turn their respective target genes on or off [60-62]. These target genes are most likely responsible for controlling the cell cycle and
proliferation associated signaling pathways, but more research is needed to elucidate the exact targets and functions of these transcription factors (see Figure 1).

In addition to controlling the RAF kinase cascade, RAS has been shown to either directly or indirectly interact with a number of other molecules including PI-3K [63], Bcl-2 [64-66], protein kinase Cζ (PKCζ) [67, 68], Rin, the guanine nucleotide exchange factors for Ral, AF6 and p120GAP which can result in a variety of cellular responses including cell survival, mitogenesis, and differentiation [69]. Since the purpose of this introduction is to familiarize the reader with the important mechanisms involved in growth regulation, a brief discussion of the PI-3K pathway will now be included as it is important in activating cell survival pathways.

1.3.1.2 The phosphatidylinositol-3 kinase and protein kinase B pathway

PI-3K is a heterodimeric enzyme composed of a p85 and a p110 subunit (other isoforms exist for both of these molecules) [70]. RAS can protect a cell from detachment-induced programmed cell death (apoptosis) and other forms of apoptosis through the activation of the PI-3K pathway [57]. RAS activation of PI-3K is mediated through the p110 subunit of PI-3K [63]. The PI-3K pathway is implicated in apoptosis, cell motility, and vesicle trafficking and secretion [71]. Its role in apoptosis will be discussed further.

Activated PI-3K (either through the interaction with an activated tyrosine kinase receptor such as PDGF receptor or through RAS) leads to the phosphorylation of phosphatidylinositol (PtdIns) [71]. In addition, activated PI-3K can phosphorylate PtdIns 4-P and PtdIns 4, 5-P₂ [71]. Yao and Cooper found that the
FIGURE 1. RAS signaling in the eukaryotic cell. The initial event, which ultimately results in the activation of nuclear transcription factors, is ligand induced dimerization of the cell surface receptor. This results in the autophosphorylation of specific tyrosine residues of which only a few are involved in attracting cytosolic molecules to the receptor. An adaptor molecule, GRB2, attaches itself to one of these phosphotyrosines and is responsible for recruiting Sos (son of sevenless) to the plasma membrane, which facilitates the dissociation of GDP from RAS, thus activating RAS. This process is reversed by another molecule known as GTPase activating protein (GAP). RAS activation can result in the activation of mitogen activated protein kinase pathways like RAF, MEK (mitogen activated, ERK activating protein), and ERK (extracellular signal-regulated protein kinase). Phosphorylated ERK can phosphorylate and thus activate nuclear transcription factors such as Elk, Myc and c-Jun, which results in physiological changes in the cell (ie. Cell cycle activation).
PI-3K inhibitor, wortmannin, caused apoptosis in PC12 cells suggesting that the PI-3K pathway is involved in cell survival [72]. One of the downstream interactors for PI-3K responsible for cell survival is the protein kinase AKT (also known as "protein kinase B" (PKB)) [73].

AKT is homologous to the protein kinase A and C families and to the retroviral oncogene v-akt and encodes a serine/threonine protein kinase that is ubiquitously expressed [74]. AKT differs from PKA and PKC in that the amino terminal portion contains an AKT homology domain part of which is related to the Pleckstrin homology domain. This domain is found in a number of signaling molecules including the phospholipase family of tyrosine kinases (see chapter 6) and are thought to mediate protein-lipid and/or protein-protein interactions [75, 76]. Serine and threonine phosphorylation of AKT by PI-3K activated PDK1 activates AKT. This leads to the phosphorylation by AKT of the Bcl-2 antagonist of cell death (BAD). BAD may also be phosphorylated by an as of yet unknown kinase which does not involve PI-3K or AKT [77]. In its unphosphorylated form, BAD can interact with the Bcl family members Bcl-XL and Bcl-2 inducing apoptosis. There has been a report on non-BAD mediated apoptosis, however further elucidation of the pathways involve are required [78]. Once phosphorylated by AKT however, BAD associates with another protein called 14-3-3 (a group of scaffolding proteins that bind to phosphorylated serine residues and are thought to be implicated in cell cycle control and various signal transduction pathways) and is no longer able to interact with Bcl-XL or Bcl-2 and apoptosis is abrogated [79, 80]. Expression of BAD
is not ubiquitous however, suggesting that other cell survival proteins must exist for AKT [81].

Recently, Brunet et al. showed that AKT can directly phosphorylate and inactivate the forkhead in rhabdomyosarcoma-like 1 transcription factor (FKHRL1) [82]. FKHRL1 is a member of the forkhead family of transcription factors and is thought to transcribe genes responsible for cell death. Phosphorylation causes FKHRL1 to associate with 14-3-3 proteins [83, 84]. This association causes FKHRL1 to remain in the cytoplasm thus inhibiting its transcriptional activity. In the absence of survival factors, the FKHRL1 transcription factor becomes dephosphorylated, translocates to the nucleus and transactivates target genes critical for cell death, such as the tumor necrosis factor ligand superfamily member 6 (TNFSF6) gene [85].

1.3.2 Signal Transduction Involved in Limiting Growth

Cells need to know when to stop growing. It is thought that when a normal cell is in contact with a neighboring cell, certain proteins on the surface of the cell recognize similar proteins on the neighboring cell, sending signals to the nucleus to stop proliferation. Adherens junctions (AJ or zonula adherens) mediate adhesion between cells, communicate a signal that neighboring cells are present (contact inhibition), and anchor the actin cytoskeleton. Beta-catenin (β-catenin) is an AJ protein which is critical for the establishment and maintenance of epithelial layers, such as those lining organ surfaces [86]. AJs are therefore responsible for regulating normal cell growth and behavior.
The AJ is a multiprotein complex assembled around calcium-regulated cell adhesion molecules called cadherins [86]. Cadherins are membrane spanning proteins that mediate interactions with neighboring cells also containing cadherins. The intracellular domain of the cadherin molecule transmits the adhesion signal resulting in the anchoring of the AJ to the actin cytoskeleton. The cytoplasmic proteins responsible for transmitting the signal include the α-, β-, and γ-catenins [87].

Korinek *et al.* and Morin *et al.* showed that the adenomatosus polyposis coli (APC) gene (mutated in adenomatosus polyposis of the colon), is a negative regulator of beta-catenin signaling [88, 89]. The APC protein normally binds to β-catenin, which interacts with the Tcf and Lef transcription factors. Studies by Korinek *et al.* showed nuclei of APC -/- colon carcinoma cells contained a stable β-catenin/Tcf4 (T-cell transcription factor-4) complex that was constitutively active. Reintroduction of APC removed beta-catenin from Tcf4 and ablated transcriptional activation. They concluded that APC loss of function leads to constitutively activated Tcf4 and may be an important step towards early transformation of colonic epithelium. Some colorectal tumors have been found to contain intact APC genes, while the β-catenin gene contains an activating mutant. These studies suggest that regulation of β-catenin is critical for normal growth, that the APC gene acts as a growth inhibitor (tumor suppressor gene; see below), and that mutations in either APC or β-catenin can lead to tumorigenesis (see Fig. 2).
FIGURE 2. Partial schematic of signaling mechanisms involved in growth control. In order for a cell to become tumorigenic and/or malignant, the cell must acquire immortality, increase its growth rate, and even develop motility in order to become metastatic. A number of these requirements are met by the constitutive activation of RAS. RAS can become constitutively activated by a point mutation or by another aberrantly regulated molecule which could lead to increased proliferation through the activation of certain MAPK pathways. RAS can also activate NFκB (responsible for the expression of anti-apoptotic proteins) and PI-3K, which can activate AKT which stimulates BAD and ultimately results in the activation of anti-apoptotic pathways. Activated AKT can phosphorylate and block the activity of another molecule, namely GSK3 (glycogen synthase kinase-3) to block either gene transcription mediated through β-catenin or motility regulated by APC (adenomatous polyposis of the colon). Some of these pathways are duplicated by the cell surface receptor itself. For example, when a ligand binds to the extracellular ligand binding domain (ECD), the protein tyrosine kinase domain (PTK) can activate PI3-K or RAS. Moreover, the activated cell surface receptor can activate α-catenin, which is involved in cytoskeletal modifications.
1.3.3 Cell Cycle

Since many of the pathways discussed above converge on the cell cycle, regulation of the cell cycle will now be summarized. The process of cell division and differentiation is essentially determined by the impact of external stimuli (e.g., growth factors, lack of nutrients, stress, DNA damage) on the cell cycle machinery within the cell [90]. The cell cycle machinery is composed of cyclins, cyclin dependent serine/threonine kinases (CDKs) and their regulatory kinases and phosphatases. Briefly, the cell cycle consists of four phases known as \( G_1 \), \( S \), \( G_2 \), and \( M \). \( G_1 \) (GAP1) refers to the first growth phase of the cell cycle and represents the time frame during which the various growth factors can act upon the cell. The synthesis of DNA occurs during \( S \) phase, where the normal diploid content, \( 2n \), becomes tetraploid, \( 4n \) [90]. \( G_2 \) (GAP2) follows \( S \) phase and represents the termination of DNA synthesis and the continuation of cell growth (organelles and proteins). Mitosis (\( M \) phase) is the point when the cell divides and distributes the newly synthesized DNA into two identical daughter cells. Another phase exists in which the cell is static. There is neither growth nor differentiation and this is known as \( G_0 \). When nutrients are in short supply or the cells are touching one another (contact inhibition), the cell enters \( G_0 \). Surgical removal of tissue, on the other hand, will cause the surrounding cells to re-enter the cell cycle and begin the regeneration process until growth is arrested due to contact inhibition (see Fig. 3).

The cell cycle is tightly controlled by cyclins (regulatory subunits) [91], cyclin dependent kinases (CDKs) (enzymatic subunits) [91, 92], and cyclin-dependent kinase inhibitors (CDKIs) [93, 94]. Briefly, the level of expression of cyclins and their rapid
Figure 3. The cell cycle. The chart (top panel) displays the variation of expression of some of the cyclins which are important in regulating the transition from one phase to another. There are 4 phases in the cell cycle (bottom) including G1, S, G2 (which comprise interphase) and M (mitosis). The amount of time spent in each phase and the amount of DNA present within a phase is also shown. Quiescent cells are in a phase known as G0 and can re-enter the cell cycle with certain stimuli such as growth factors.
degradation, determines when a transition from one phase to another will occur. In addition, the cyclins can interact with CDK proteins to form complexes whose phosphorylation status determines if it is active or not, thus augmenting the control of the cell cycle [90].

Phosphorylation and dephosphorylation play a major role in the control of the cell cycle. The G_{1} to S transition is controlled by the retinoblastoma gene product, RB [95]. When RB is hypophosphorylated, it can interact with E2F, a transcription factor which can activate transcription of a number of genes [96-98]. When the RB protein is hyperphosphorylated by the cyclin-D/CDK 4/6 complex, it no longer has the ability to interact with E2F. Hyperphosphorylation of RB needs to take place in order for the transition from G_{1} to S to occur [99-102]. This is commonly referred to as the G_{1} to S checkpoint.

Once the signal which initiated the phosphorylation is terminated, the phosphate groups need to be removed in order to terminate downstream activations [103-107]. This is carried out by proteins known as phosphatases such as CDC25. An additional level of control is achieved by directly inhibiting CDK molecules. CDK inhibitors, such as p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p19^{INK4D}, p21^{CIP1, WAF1, SDI1, CAP20}, p27^{KIP1}, and p57^{KIP2} are responsible for directly coupling with CDK molecules and inhibiting their regulative role in the cell cycle [93]. The INK4 (cyclin-dependent kinase inhibitor) proteins are responsible for inhibiting Cyclin D/CDK4/6 complexes, while the CIP/KIP (CDK-interacting proteins) molecules inhibit Cyclin A, B, and E/CDK2 complexes [93].
Another important gene which is involved in the regulation of the cell cycle is p53. Like RB, p53 is also a tumor suppressor gene whose activity increases when there is damage to DNA [108-111]. p53 is a nuclear phosphoprotein with two DNA binding domains [112], two SV40 large T-antigen binding sites [113, 114], a nuclear localizing signal [115], an oligomerization domain [116, 117], and several phosphorylation sites [118]. It is thought to act as a transcription factor for other growth regulatory genes either activating or inhibiting their transcription [109, 119].

In addition to acting as a transcription factor, p53 can interact with proteins such as p21 (CIP1) [120-122]. CIP1 inactivates G₂ cyclin/CDK complexes and in addition, binds to the DNA polymerase cofactor, proliferating cell nuclear antigen (PCNA), thus preventing DNA replication, but allowing for DNA repair [123]. This event effectively blocks the G₁ to S transition and again acts as a checkpoint within the cell cycle. Bunz et al. demonstrated that upon DNA damage, cells enter a sustained arrest in the G₂ phase only when p53 was present in the cell and capable of transcriptionally activating the cyclin-dependent kinase inhibitor CIP1 [124]. After disruption of either the p53 or the CIP1 gene, gamma-radiated cells progressed into mitosis, but failed to undergo cytokinesis. The cells therefore exhibited a G₂ (tetraploid) DNA content. In addition, Shieh et al. showed that DNA damage leads to the phosphorylation of p53 and that this event reduces the ability of p53 to interact with MDM2. MDM2 is a negative regulator of p53 that normally binds to p53 inhibiting its function [125]. Furthermore, they demonstrated that the phosphorylation of p53 by purified DNA-dependent protein kinase (DNA-PK) impairs the ability of MDM2 to inhibit p53-dependent transactivation.
Finally, p53 has been implicated in apoptosis [126-128]. One group, Polyak et al., examined in detail the transcripts induced by p53 expression before the onset of apoptosis [129]. Of the 7,202 transcripts identified, only 14 (0.19%) were found to be markedly increased in p53-expressing cells compared with controls. Strikingly, many of these genes were predicted to encode proteins that could generate or respond to oxidative stress. p53 levels are normally very low, but have been shown to rapidly increase after DNA damage or viral infection. It is known that p53 induces BAX transcription, a member of the Bcl-2 gene family [130]. The exact mechanism of p53 associated apoptosis, however, is not known and further research is needed to elucidate this pathway.

1.4 MECHANISMS OF ONCOGENESIS

The activation or inactivation of specific molecules or cellular pathways, such as those discussed above, can result in the transformation of normal cells into oncogenic ones. For example, inactivating mutations of p53 results in the tolerance of DNA damage throughout the cell cycle leading to increased genetic instability and possibly an oncogenic advantage. Tumor cell metastasis is thought to be a result of a disrupted β-catenin pathway where contact inhibition is no longer present thus allowing the cell to continue growing in the presence of neighboring cells and invade other tissues in the body [131]. Viral oncoproteins (such as the SV40, adenovirus and papillomavirus) have the capability to bind RB and p53 and thus disrupt their ability to inhibit growth [132-134]. It is important to note that cancer is not due to a single aberration, but the disruption of multiple pathways. An increase
in the expression of an oncogene or the loss of tumor suppressor factor expression alone, for example, is insufficient to produce a malignant tumor. Genes which are responsible for enhancing the proliferative rate of a cell are known as oncogenes while growth inhibiting genes are known as tumor suppressor genes (discussed in further detail below).

1.4.1 Oncogenes

Oncogenes are mutated forms of their normal cellular counterparts, proto-oncogenes. Briefly, these proto-oncogenes can be converted to an oncogene by a chromosomal rearrangement, proviral insertion, gene amplification or a point mutation (discussed in further detail below). Proto-oncogenes have been found at virtually every level of the proliferation associated signal transduction pathway and include: growth factors, growth factor receptors, guanine nucleotide like proteins, guanine nucleotide exchange factors (GNEFs), cytoplasmic serine/threonine or tyrosine protein kinases, or nuclear proteins (transcription factors). Oncogenes were first discovered as the transforming elements in the RNA viruses responsible for causing sarcomas in fowl [135]. The following discussion will detail some of the mechanisms by which a proto-oncogene is converted into an oncogene.

1.4.1.1 Proviral Insertion

When a retrovirus inserts itself adjacent to a proto-oncogene, it places the expression of the proto-oncogene under the control of the enhancer elements of the retrovirus. This type of conversion (proto-oncogene to oncogene) was first identified with avian leukemia virus-induced bursal lymphomas where the level of
transcription of \textit{c-myc} was 50 to 100 fold higher than normal due to proviral insertion upstream of the \textit{c-myc} proto-oncogene locus [136].

1.4.1.2 Gene Amplification

Genomic amplification has been seen in many solid tumors and represents another mechanism by which an oncogene may be overexpressed [137]. The actual mechanism by which this amplification occurs is not clear but the end result may be seen cytogenetically as structures referred to as double minutes (dmins) and homogenously staining regions (HSRs) [138, 139]. dmins are small extrachromosomal, circular structures that occur in pairs and contain specific chromosomal regions, but lack telomeres [140]. These regions (amplicons) contain a number of genes (typically proto-oncogenes) and the number of dmins per cell determines the level of amplification of the genes involved. Tumorigenesis usually results when one or more of the genes amplified are responsible for cell proliferation [141, 142]. HSRs are dmins which have integrated into the genome. They appear as homogenously staining regions (hence the name) within a chromosome. The mechanism(s) by which a cell acquires dmins and HSRs is not fully understood. The fact that both forms of gene amplification may be seen in a particular cancer is interesting. For example, one cell (or subline) may have an HSR while another cell (or subline) within the same tumor may have a dmin. It has been postulated that the dmins represent the unstable form of HSRs [143]. These dmins however, will integrate and form HSRs in culture [144].

One of the most extensively studied tumors where gene amplification plays an important role in tumorigenesis is neuroblastoma. The \textit{NMYC} oncogene is
located on chromosome 2p24.1 and is amplified 25- to 700-fold in human neuroblastomas by means of dmins and HSRs [145-149]. The high levels of NMYC protein production is thought to deregulate transcription and lead to a proliferative advantage for the cell. NMYC amplification in neuroblastoma is associated with advanced stage disease and a poor prognosis [146, 150, 151].

A recent review has summarized the various reports of recurrent DNA sequence copy number amplifications in human neoplasms detected by comparative genomic hybridization (CGH) [152]. CGH is a technique similar to FISH which allows for the detection of deletions, duplications and amplifications. Amplicons have been identified for almost every chromosome. One of these regions, namely 12q13-q21, encompasses the GLI, CHOP, SAS, WNT1, WNT10b, CDK2, MDM2 and CDK4 genes, and has been shown to be amplified in a variety of tumors. Portions of this region have also been shown to be amplified in a number of tumors including osteosarcoma (12q13-q14, specifically involving CDK4, MDM2 and SAS), chondrosarcoma (12cen-q15 and 12q24.1), liposarcoma (12q14-q21 specifically involving the CDK4 and MDM2 genes), synovial sarcoma (12q15), embryonal and alveolar rhabdomyosarcoma (12q13-q15), breast carcinoma (12q15), hereditary ovarian cancer (12q13-q21), colon carcinoma (12q13), bladder carcinoma (12q13-q15), diffuse large cell lymphoma (12q13-q14), follicular lymphoma (12q13-q14, specifically involving the GLI gene), neuroglial tumors (12q13-q15), non-small cell lung cancer (12q14-q21), and squamous cell carcinomas of the head and neck (12q13-q14) [153-176].
1.4.1.3 Point Mutations

Another mechanism by which a proto-oncogene can be converted to an oncogene is by single base pair substitutions (mutations), which can have drastic effects on the translated protein. These mutations can arise due to replication errors, or from direct DNA damage such as ultraviolet radiation [42]. The RAS gene family (HRAS, KRAS, and NRAS) have all been found to contain point mutations in various malignancies [177-179]. These point mutations result in the constitutive activation of the RAS molecule by ablating its need for a guanine nucleotide exchange factor thus mimicking a constitutively activated growth factor receptor, such as EGFR or PDGFR [177-182]. Sekiya et al. found a point mutation in the second exon of the HRAS1 gene substituting an adenine residue to a thymine residue in a melanoma [183]. Activation of the NRAS gene by point mutation occurs in about 15% of all human melanomas [184]. In these cases, mutated NRAS was found to contribute to tumor growth by enhancing cellular proliferation and by blocking apoptosis. Other changes observed include: gly12val (bladder carcinoma), gly12asp (mammary carcinosarcoma), gln61leu (lung carcinoma), and gln61 to arg (renal pelvic carcinoma) for the HRAS gene, and gln61 to arg (lung carcinoma) for the NRAS gene [185].

1.4.1.4 Chromosomal Rearrangements

Finally, proto-oncogene activation can arise when a chromosomal rearrangement, such as a translocation, places a proto-oncogene downstream of a promoter of an IgG locus. This results in the constitutive expression of an otherwise tightly regulated gene. An example of this is seen with the MYC gene and
its translocation to an IgG locus in Burkitt's lymphoma due to a t(8;14) [186]. Alternatively a translocation can produce a fusion gene where part of gene A is fused to part of gene B (this topic will be discussed in further detail below). Table 1 summarizes the more well known oncogenes.

1.4.2 Tumor Suppressor Genes

Generally, tumor suppressor genes are responsible for inhibiting cellular growth. Multiple molecular approaches that have typically been used to identify tumor suppressor genes, including: cytogenetic analysis to determine the extent of chromosomal loss (deletions) or rearrangements; linkage analysis to determine which region-specific markers are linked to the disease (which helps lead to the identification of the gene); loss of heterozygosity (LOH) analysis, which detects the loss of an allele or other molecular marker. Oncogenic transformation of a cell can also occur if both copies of a tumor suppressor gene are inactivated. Inactivation of tumor suppressor genes can occur via a variety of mechanisms including loss of the gene (deletion) or mutation (point, missense or nonsense) and must include both copies. Some well characterized examples of tumor suppressor genes include APC (discussed above), RB1, and p53. A brief discussion on their inactivation will now be discussed using RB1 and p53 as examples.

1.4.2.1 RB1

The retinoblastoma gene, RB1 (see cell cycle above), is a tumor suppressor since it is responsible for controlling the transition from G1 to S in the cell cycle. Retinoblastoma occurs when both copies of the RB1 gene have been inactivated.
TABLE 1. Various classes of oncogenes and their mode of action within tumors. From Vogelstein, 1998 [90].

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Mechanism of Activation</th>
<th>Neoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth Factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-sis</td>
<td>DNA transfection studies</td>
<td>Glioma/fibrosarcoma</td>
</tr>
<tr>
<td>KS3</td>
<td>DNA transfection studies</td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>HST</td>
<td>DNA transfection studies</td>
<td>Stomach carcinoma</td>
</tr>
<tr>
<td><strong>Tyrosine Kinases: Integral Membrane Proteins, Growth Factor Receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Amplification</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>v-fms</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-kit</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-ras</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>TRK</td>
<td>Rearrangement</td>
<td>Colon carcinoma</td>
</tr>
<tr>
<td>NEU</td>
<td>Point mutation</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td></td>
<td>Amplification</td>
<td>Carcinoma of breast</td>
</tr>
<tr>
<td><strong>Tyrosine Kinases: Non-receptor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC</td>
<td></td>
<td>Colon carcinoma</td>
</tr>
<tr>
<td>v-yes</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-fgr</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-fes</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>BCR/ABL</td>
<td>Chromosome translocation</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td><strong>Membrane Associated G Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-RAS</td>
<td>Point mutation</td>
<td>Colon, lung, pancreas carcinoma</td>
</tr>
<tr>
<td>K-RAS</td>
<td>Point mutation</td>
<td>AML, thyroid carcinoma, melanoma</td>
</tr>
<tr>
<td>N-RAS</td>
<td>Point mutation</td>
<td>Carcinoma, melanoma</td>
</tr>
<tr>
<td><strong>GEF Family of Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dbl</td>
<td>Rearrangement</td>
<td>Diffuse B-cell lymphoma</td>
</tr>
<tr>
<td>Ost</td>
<td></td>
<td>Osteosarcomas</td>
</tr>
<tr>
<td><strong>Serine/Threonine Kinases: Cytoplasmic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-mos</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-RAF</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>Pim-1</td>
<td>Proximal insertion</td>
<td>T-cell lymphoma</td>
</tr>
<tr>
<td><strong>Nuclear Protein Family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-myc</td>
<td></td>
<td>Carcinoma myelocytomatosis</td>
</tr>
<tr>
<td>N-MYC</td>
<td>Gene amplification</td>
<td>Neuroblastoma: Lung carcinoma</td>
</tr>
<tr>
<td>L-MYC</td>
<td>Gene amplification</td>
<td>Carcinoma of lung</td>
</tr>
<tr>
<td>v-myb</td>
<td></td>
<td>Myeloblastosis</td>
</tr>
<tr>
<td>v-fos</td>
<td></td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>v-jun</td>
<td></td>
<td>Sarcoma</td>
</tr>
<tr>
<td>v-ski</td>
<td></td>
<td>Carcinoma</td>
</tr>
<tr>
<td>v-rel</td>
<td></td>
<td>Lymphatic leukemia</td>
</tr>
<tr>
<td>v-ets</td>
<td></td>
<td>Myeloblastosis</td>
</tr>
<tr>
<td>v-erbA</td>
<td></td>
<td>Erythroid blastosis</td>
</tr>
</tbody>
</table>
Patients with germline mutations in one allele of \textit{RB1} are predisposed to other malignancies including osteosarcomas, soft tissue sarcomas and melanoma later in life [90]. Lohmann \textit{et al.} investigated a series of isolated unilateral retinoblastomas from 119 patients for the frequency and nature of germline \textit{RB1} gene mutations [193]. Of the 119 patients studied, 99 (83\%) contained mutations for the \textit{RB1} gene. The types of mutations found included large deletions (15\%), translocations (26\%), and base substitutions (42\%).

1.4.2.2 \textit{p53}

The \textit{p53} protein exists as a tetramer and acts as a tumor suppressor gene because it can inhibit tumor growth when introduced into a variety of transformed cells by blocking cells from entering the S phase of the cell cycle [194-197]. Other evidence has shown that \textit{p53} also blocks the transition from G2 to M via binding to CIP1 [128, 198-200].

Mutations in the \textit{p53} gene represent the most frequently encountered genetic aberrations in human malignancies [90, 201-204]. Vogelstein and Kinzler outlined 5 mechanisms for \textit{p53} inactivation [109]. Deletion of one or both \textit{p53} alleles reduces the expression of tetramers, resulting in decreased expression of growth inhibitory genes such as \textit{CIP1}. Nonsense or splice site mutations that result in truncation of the protein inhibit oligomerization, thus resulting in a similar reduction of \textit{p53} tetramers. Mutations of this type are fairly common in lung [205], esophagus [206], and other cancers [207]. A third mechanism involves missense mutations resulting in dominant-negative effects with an even greater reduction of functionally active tetramers. Such missense mutations are common in colon [208, 209], brain [210],
lung [205], breast [207], skin [211, 212], and bladder cancers [213]. A fourth mechanism by which p53 is involved in oncogenesis is common in cervical cancers where the expression of the E6 gene of human papillomavirus (HPV) results in the functional inactivation of p53 through binding and degradation [214]. Patients with germline p53 mutations are predisposed to breast cancers, sarcomas, brain tumors, lymphomas and Li-Fraumeni syndrome [215-217].

The p53 pathway may also be disrupted by alteration of its negative regulator, MDM2 (see above). This gene was originally identified by virtue of its amplification in a spontaneously transformed mouse cell line [218]. The MDM2 gene is amplified (seen cytogenetically as HSRs and dmins) in a significant fraction of the most common human sarcomas and the consequent overexpression of MDM2 is likely to interfere with p53 activity [219]. Table 2 summarizes the tumor suppressor genes discussed above as well as a few others including their function and localization within cells.

1.5 GENETIC ASPECTS OF PEDIATRIC SOLID TUMORS

The presence of activated oncogenes and the deletion of tumor suppressor genes as discussed above has been reported in many pediatric solid tumors. Pediatric solid tumors are known to contain various chromosomal aberrations, many of which can be detected by conventional cytogenetics. These aberrations include whole chromosome losses and gains (aneuploidy), translocations, HSRs and dmins (discussed above), deletions, duplications, ring chromosomes, inversions and marker chromosomes. Aneuploidy, amplifications (dmins and HSRs) and
Table 2. Tumor suppressors and the tumors affected by their loss. From Vogelstein, 1998 [90].

<table>
<thead>
<tr>
<th>SYNDROME</th>
<th>GENE</th>
<th>TUMORS</th>
<th>LOCALIZATION</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma</td>
<td>RB1</td>
<td>Ret, Ost</td>
<td>Nucleus</td>
<td>TF/cell cycle control</td>
</tr>
<tr>
<td>Li-Fraumeni</td>
<td>p53</td>
<td>Sar, breast and brain tumors</td>
<td>Nucleus</td>
<td>TF</td>
</tr>
<tr>
<td>Familial adenomatous polyposis</td>
<td>APC</td>
<td>Adenomatous polyps, CC</td>
<td>Cytoplasm</td>
<td>Possible β-catenin regulator</td>
</tr>
<tr>
<td>NF Type I</td>
<td>NF1</td>
<td>Neurofibromas, sar, gli</td>
<td>Nucleus</td>
<td>p21RAS-GTPase activator</td>
</tr>
<tr>
<td>NF Type II</td>
<td>NF2</td>
<td>Schwannomas, meningiomas</td>
<td>Cytoplasm,</td>
<td>Cytoskeleton membrane link</td>
</tr>
<tr>
<td>Familial breast cancer</td>
<td>BRCA1</td>
<td>Breast and ovaries</td>
<td>?Nucleus</td>
<td>DNA repair</td>
</tr>
<tr>
<td>Wilms' tumor</td>
<td>WT1</td>
<td>Nephroblastoma</td>
<td>Nucleus</td>
<td>DNA repair</td>
</tr>
</tbody>
</table>

Abbreviations. Ret; retinoblastoma, Ost; osteosarcoma, TF; transcription factor, sar; sarcoma, gli; glioma, NF; neurofibromatosis, CC; colon cancer, ?= not known.
translocations are the most common recurrent abnormalities associated with pediatric soft tissue tumors. Since gene amplification was discussed in section 1.4.1.2 above, the following discussion will concentrate on aneuploidy and translocations in pediatric solid tumors. Table 3 summarizes the common recurring abnormalities found in various soft tissue tumors.

1.5.1 Aneuploidy

Every cell in the human body (except for sperm and ova) contains 46 chromosomes. There are 22 autosomes (1 through 22) and two sex chromosomes (X and Y). Every normal cell contains two copies of each of the autosomes (44 chromosomes) and either a pair of X chromosomes (female) or an X and a Y chromosome (male). Since there are two copies of each chromosome (except in male individuals who have only one X and one Y chromosome) the DNA content is said to be diploid or 2n (n equals the haploid content of the cell). Aneuploidy refers to the abnormal amount of genetic material on the chromosome level [90, 144]. Whole chromosomes or their arms may be lost or gained and this is easily detected using conventional cytogenetics. Some examples of soft tissue tumors that display aneuploidy are embryonal rhabdomyosarcomas (gains in chromosomes 2, 8, 12, 13 and 20 as well as a loss of material from 11p15.5) [220], leiomyosarcomas (frequent losses in 10q and 13q and frequent gains in 17p) [221], mesotheliomas (frequent deletions of specific regions within chromosome arms 1p, 3p, 6q, 9p, 15q and 22q) [222], prognostically poor neuroblastomas (deletions in the short arm of
TABLE 3. Summary of the various recurring chromosomal abnormalities found in pediatric soft tissue tumors. Adapted from Enzinger, 1988 [1].

<table>
<thead>
<tr>
<th>HISTOLOGY</th>
<th>CYTOGENETICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell sarcoma</td>
<td>t(12;22)(q13;q12), t(7;18)(p11.2;q21.3), +der(7)t(7;18)(p11.2;q21.3), +8, +der(8;17)(q10;q10), t(12;22)(q13;q12.2-12.3)</td>
</tr>
<tr>
<td>Dermatofibrosarcoma protuberans</td>
<td>t(17;22)(q22;q13), ring derived from t(17;22)</td>
</tr>
<tr>
<td>Ewing sarcoma</td>
<td>t(11;22)(q24;q12), t(1;16)(q11;q11).1, t(21;22)(q22;q12), t(7;22)(p22;q12)</td>
</tr>
<tr>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>t(9;22)(q22-31;q11-12), -Y</td>
</tr>
<tr>
<td>Infantile fibrosarcoma</td>
<td>+2, +8, +11, +17, +20</td>
</tr>
<tr>
<td>Hemangiopericytoma</td>
<td>Translocation at 12q13</td>
</tr>
<tr>
<td>Intraabdominal desmoplastic small round cell tumor</td>
<td>t(11;22)(p13;q12)</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>Deletion of 1p</td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma</td>
<td>Complex</td>
</tr>
<tr>
<td>High grade</td>
<td>Ring Chromosomes</td>
</tr>
<tr>
<td>Myxoid</td>
<td></td>
</tr>
<tr>
<td>Malignant peripheral nerve sheath tumor</td>
<td>Complex</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>del(1)(p32-36), der(1)t(1;17)(p36?), dmins and HSRs (amplification of NMYC)</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>t(2;13)(q35;q34), t(1;13)(p36;q14)</td>
</tr>
<tr>
<td>Alveolar</td>
<td>+2q, +8, +20</td>
</tr>
<tr>
<td>Embryonal</td>
<td></td>
</tr>
<tr>
<td>Schwannoma</td>
<td>-22</td>
</tr>
<tr>
<td>Synovial Sarcoma</td>
<td>t(X;18)(p11;q11)</td>
</tr>
<tr>
<td>Desmoid tumor</td>
<td>+8, +20</td>
</tr>
<tr>
<td>deletion of 5q</td>
<td></td>
</tr>
<tr>
<td>Lipoblastoma</td>
<td>t(7;8)(p31;q13)</td>
</tr>
<tr>
<td>Lipoma</td>
<td>t(1;12)(p33-34;q13-15), t(2;12)(p22-23;q13-15), t(3;12)(q27-28;q13-15), t(5;12)(q33;q13-15), t(11;12)(q13;q13-15), t(12;21)(q13-15;q21), t(11;22)(q24;q12), del(12)(q13q15), del(13)(q12-q22)</td>
</tr>
<tr>
<td>Liposarcoma (myxoid)</td>
<td>t(12;16)(q13;p11)</td>
</tr>
<tr>
<td>Uterine leiomyoma</td>
<td>t(12;14)(q15;q24), deletion of 7q, +20</td>
</tr>
</tbody>
</table>

**Abbreviations.** Del= deletion and der= derivative.
chromosome 1), desmoid tumors (gains in chromosomes 8 and 20) [27-29] and uterine leiomyomas (deletions of the long arm of chromosome 7) [223].

One possible role of an extra copy of a chromosome is to introduce an extra copy of a growth related gene (growth factors or their receptors) or oncogenes (such as RAS, PDGF, and MYC), leading to an increase in the proliferative rate of the cell. Loss of a chromosome or a chromosome region could lead to a proliferative advantage for a cell if tumor suppressor genes (such as APC, p53, and RB1) were deleted. In addition to the examples provided above, there are other tumors that may have many extra copies of several chromosomes. An example of this is uterine leiomyosarcoma which can have up to 8 copies (8n) of almost every chromosome in contrast to two copies (2n) in normal cells [224].

1.5.2 Tumor Specific Translocations

A translocation refers to the exchange of chromosomal material between two chromosomes. A translocation can involve either a part of a chromosome arm or the entire arm itself. Robertsonian translocations result in the fusion of the long arms of two acrocentric chromosomes (chromosomes 13-15 and 21 and 22) with the subsequent loss of the short arms [144].

Translocations can occur in either transcriptionally active or inactive regions. The translocation results in the formation of two derivative chromosomes (if the translocation is reciprocal). A derivative chromosome is a structurally rearranged chromosome generated either by a rearrangement involving two or more chromosomes or by multiple aberrations within a single chromosome.
The majority of reciprocal translocations result in derivative chromosomes that give rise to no visible phenotypic change [225]. This can be explained by the fact that the breakpoint may fall within DNA that does not contain any genes or is not expressing any genes. The expression of the genes around the breakpoint therefore, are not affected. Alternatively, there are two possibilities where the translocation can give rise to derivative chromosomes associated with a phenotypic change.

One possibility involves the breakpoint occurring in transcriptionally inactive DNA, as above, except that this region is responsible for the expression of nearby genes. The derivative chromosomes now contain a part of chromosome 'A' fused to a part of chromosome 'B'. Since the translocation will affect the expression of nearby genes, the function of these genes will determine the viability of this rearrangement. For example, if the translocation results in the overexpression of nearby genes and one of these genes is an oncogene (e.g., MYC, PDGF or RAS) then the cell will most likely have an increased proliferative rate. On the other hand, if the expression of the genes is suppressed and one of the genes involved is a tumor suppressor gene (e.g., APC, p16 or p53), then the proliferative rate may increase as above. If the gene(s) involved in the latter case is involved in cell survival such as the glucose-6-phosphate molecule (necessary for glucose metabolism), then the phenotype may be lethal (see Fig. 4a). The most interesting result of a translocation occurs when the breakpoints occur within expressed sequences (see Fig. 4b). This type of translocation is relatively common in soft tissue tumors [90, 224, 226, 227], where a chimeric gene fusion is formed due to the translocation and will now be discussed in more detail below.
FIGURE 4. The possible outcomes of chromosomal translocations.  

a) Translocations can occur in either transcriptionally inactive or active DNA. The phenotype of the former is usually nothing, unless the transcriptionally inactive region is involved in regulating the expression of nearby genes. The latter possibility can result in a deletion of a gene, the introduction of a stop codon, a non-functional protein or a functional chimeric protein which fuses part of gene one to part of gene 2.  

b) Chimeric gene fusions can form as a result of an inter-exonic gene fusion or an intra-exonic gene fusion. The fusion gene can be oncogenic if the regulation of specific domains involved in the fusion are lost.
A

Breaks in heterochromatin

Deletion of gene 1 or gene 2

Stop codon in gene 1 or 2 sequence

Non-functional protein 1 or 2

Functional in-frame gene fusion
Inter-exonic gene fusion

5'-methyl cap - 3' Poly A tail

Chimeric transcript

Intra-exonic gene fusion

Chimeric oncoprotein
1.5.3 Tumor Specific Translocations Result in Functional Gene Fusions in Solid Tumors

Hematopoietic tumors helped pave the way to understanding chimeric genes as a result of specific chromosomal translocations [228-230]. These translocations almost always result in a proliferative advantage for the malignant cell and this is accomplished by one of two mechanisms.

The first possibility is that the translocation splices an oncogene to a positive regulatory element of the partner gene. The result is overexpression of the oncogene leading to an increased growth rate for the malignant cell. An example of this is seen in Burkitt’s lymphoma due to the t(8;14) which places the MYC oncogene under the control of the IgH locus promoter [229, 231, 232]. A similar situation exists in the solid tissue tumor dermatofibrosarcoma protuberans (DFSP). In this tumor the expression of the PDGF-B chain molecule is placed under the control of the COL1A1 gene promoter [233]. The COL1A1 product is a major constituent of the connective tissue matrix. The increased expression of PDGF-B contributes to tumorigenesis since it has transforming activity and is a potent mitogen for a number of cell types [234-237]. Its role in the oncogenic process however is not fully understood.

The second possibility involves the juxtaposition of a portion of one gene to a portion of another gene. The breakpoints can occur within an intron or an exon, but the end result is usually the in frame fusion between the exons of two genes. These chimeric gene fusions contain functional domains from both genes and are largely responsible for the increased proliferation seen in many soft tissue tumors.
and leukemias [238]. Many sarcomas express chimeric transcription factors resulting from gene fusions [229]. Since our studies have identified a gene fusion in congenital fibrosarcoma as a result of a chromosomal translocation, the remainder of this chapter will concentrate on a few examples of solid tumors and their associated gene fusions.

1.5.3.1 Ewing's Sarcoma Family of Peripheral Primitive Neuroecto-Dermal Tumors

Ewing sarcoma, peripheral neuroepithelioma and Askin tumor are a group of malignancies which are poorly differentiated and belong to the family of peripheral Primitive Neuroectodermal Tumors (pPNETs) [5, 239]. Ewings sarcomas (ES) and peripheral neuroepithelioma contain a t(11;22)(q12;q24) which juxtaposes the FLI1 gene from chromosome 11q12 to the EWS gene from chromosome 22q24 in approximately 85% of cases [240-246]. The FLI1 gene is a member of the ETS family of transcription factors and is known to contain at least two functional domains [247, 248]. One of the domains, a helix loop helix domain (HLHD), is responsible for protein-protein dimerization while the other domain, the ETS DNA binding domain, is responsible for binding to DNA. The EWS gene contains an RNA binding domain and a transcriptional activation domain [249, 250]. The translocation results in der(22) giving rise to a fusion gene containing the transcriptional activation domain from EWS as the 5'end of the fusion gene and the ETS DNA binding domain from the FLI1 gene as the 3' end (see Fig. 5) [244, 251]. Studies have shown the fusion product to be a transcriptional activator due to the swapping of the RNA binding domain from the EWS gene with the DNA binding
FIGURE 5. Schematic representation of EWS-ETS fusions. The EWS gene (top panel) contains an RNA binding domain and a transcriptional activation domain. Ewing sarcomas are characterized by a translocation resulting in a der(22), giving rise to a fusion gene containing the transcriptional activation domain from EWS as the 5’end of the fusion gene and the ETS DNA binding domain from an ETS family transcription factor (such as FLI1) as the 3’ end (bottom panel). The arrow indicates the approximate location of the breakpoint region within each of the genes. The ETS genes are transcription factors and are known to contain a HLH dimerization domain as well as an ETS DNA binding domain (middle panel). Studies have shown the fusion product to be a transcriptional activator since the DNA binding domain from FLI1, for example, is now regulated by the promoter elements of the EWS gene.
domain from the *FLI1* gene [252]. This places the DNA binding domain from FLI1 under the promoter elements of the EWS gene, which effectively disrupts the normal activity of the DNA binding domain. It is therefore thought that this altered DNA binding activity somehow gives rise to an increased proliferative rate. One possibility is that the chimeric fusion protein may be able to activate c-MYC, since both EWS/FLI1 and FLI1 are capable of activating the c-MYC promoter [253]. The elucidation of the exact mechanism of pathogenesis, however, will require further investigation.

The majority of the remaining 15% of pPNETs appear to harbor a variant t(21;22)(q22;q12), which has been shown to express an *EWS-ERG* chimeric transcript [247, 254, 255]. The fusion protein acts as a transcriptional activator and requires the amino terminal domain of EWS [256]. In addition, since the clinical behavior of the pPNETs with an *EWS-FLI1* or an *EWS-ERG* fusion appear similar [254, 257], it is thought that the fusion proteins may be acting along similar oncogenic pathways. Another rare variant translocation has been discovered in Ewing sarcoma where a t(7;22)(p22;q12) results in the fusion of the 5' portion of the *EWS* gene to the 3' DNA binding domain (ETS domain) of the *ETV1* gene, another member of the ETS family of transcription factors. It is still not known whether this variant functions in similar ways as do the EWS-FLI1 and EWS-ERG chimeras. The *EWS* gene has been involved in at least two other translocations involving the *ATF1* and *WT1* genes, however these fusions occur in malignant melanoma of soft parts and intra-abdominal desmoplastic small round cell tumor, respectively.
1.5.3.2 Myxoid Liposarcoma

Liposarcomas are malignant tumors of fat cells that primarily occur in the extremities and retroperitoneum of adults [1]. Of the various types of liposarcomas, myxoid liposarcoma (myxoid LPS) is the most common. Several independent groups characterized the t(12;16)(q13;p11) in myxoid LPS and found that the \( FUS \) gene from chromosome 16p11 is fused to the \( CHOP \) gene from chromosome 12q13 [258-262]. In addition, round cell liposarcoma, a poorly differentiated form of myxoid LPS, was also found to harbor the same gene fusion [263].

The \( FUS \) gene (also known as \( TLS \) for “translocated in liposarcoma”) is a nuclear RNA-binding protein with extensive homology to \( EWS \) [259]. Moreover, the amino terminus of FUS appears to contain a strong transcriptional activation domain as found in EWS [264]. Åman et al. found that the size of the \( FUS \) gene is 11 kb and consists of 15 exons [261]. The \( CHOP \) gene is a nuclear protein and acts as a dominant-negative transcription factor inhibitor for C/EBP and LAP [265]. \( CHOP \) stands for “C/EBP-homologous protein”, but it has also been referred to as \( DDIT3 \) and \( GADD153 \) for “DNA damage-inducible transcript 3” and “growth arrest and DNA damage-inducible gene”, respectively. \( CHOP \) inhibits the transcriptional activity of C/EBP and LAP by heterodimerizing with them through the leucine zipper domain, thus interfering with their DNA binding capabilities. This interaction is mainly seen in response to cellular stress, such as DNA damage [265]. In addition, \( CHOP \) is thought to function in pathways of terminal differentiation and growth arrest in fat cells [266].
The t(12;16) results in the fusion of the FUS amino terminus (FAT) to the entire CHOP coding region [258, 259]. Transformation of NIH3T3 cells requires both the FAT portion as well as the entire CHOP region [264]. Furthermore, the carboxy terminal leucine zipper domain from CHOP was essential for transformation. Based on these results it would be reasonable to assume that the genes regulated by C/EBP and LAP are tumor suppressor genes and the constitutive inhibition of their expression by FUS-CHOP could lead to oncogenesis.

In addition to the FUS-CHOP fusion, the FUS gene was found to be rearranged in an acute myeloid leukemia with a t(16:21)(p11;q22) [267, 268]. The fusion partner was identified as ERG, seen previously in Ewing sarcoma as part of an EWS-ERG gene fusion. ERG expression in both acute myeloid leukemia and in Ewing sarcoma is therefore under the control of either the FUS or EWS promoter elements, respectively [269]. Åman et al. states that although the N-terminal ends of FUS and EWS are different, they share extensive homology and are distinct from the N-terminal regions of other ribonucleoprotein (RNP)-carrying proteins [269].

1.5.3.3 Alveolar Rhabdomyosarcoma

Rhabdomyosarcomas (RMS) are a heterogeneous group of malignant tumors and are the most common soft-tissue sarcoma of childhood [220]. There are three subtypes: embryonal RMS (ERMS) (~ 65% of RMS cases), alveolar RMS (ARMS) (~ 20% of RMS cases), and the less well-defined undifferentiated sarcomas (~15% of RMS cases) [5, 270]. Differentiation between these subtypes as well as other poorly defined sarcomas poses a problem to the pathologist, once again illustrating the need for more accurate tools for diagnosing these tumors.
Alveolar RMS cases harbor either a t(2;13)(q35;q14) (~60% of ARMS cases) or a t(1;13)(p36;q14) (~10-20% of ARMS cases) which fuses the paired box (PB) and homeobox (HB) DNA binding domains from either the PAX3 or PAX7 genes, respectively, to the acidic and proline rich domain of the FKHR gene [271-273]. The PAX3 and PAX7 genes are members of the PAX family of transcription factors which means the fusion gene is a chimeric transcription factor. The PAX genes appear to be important for myogenic differentiation during embryonic development [274-276]. FKHR belongs to another transcription factor family known as the forkhead family of transcription factors [277]. FKHR is known to contain a DNA binding domain as well as a carboxy terminal transactivation domain. The DNA binding domain of FKHR is of the winged-helix type. The fusion product between PAX3 or PAX7 and FKHR has the DNA binding domain from PAX3 or PAX7 attached to the transactivation domain from FKHR. The overexpression of PAX genes leads to oncogenic transformation [278]. This has lead researchers to hypothesize that the transcriptional disruption of normal PAX target genes due to the PAX-FKHR gene fusions plays a major role in ARMS oncogenesis.

1.5.3.4 Synovial Sarcoma

Synovial sarcoma is an aggressive soft tissue malignancy occurring predominantly in the extremities of adolescents and young adults [1]. A recent case was misdiagnosed as a desmoplastic small round-cell tumor providing further evidence for the need of a molecular based approach to diagnosing morphologically similar tumors [279]. The recently characterized t(X;18)(p11.2;q11.2) is found in approximately 90% of human synovial sarcomas [280-282]. This results in a der(X)
chromosome which gives rise to the fusion of the chromosome 18 SYT gene (also known as SSXT) located at 18q11.2, to either of 2 distinct genes (at least 2Mb apart from each other at the Xp11.2 locus) from Xp11.2, SSX1 or SSX2 [283-286]. Recent studies have shown that the SYT protein contains a transcriptional co-activator domain [287]. The N-terminus of the SYT protein has a novel conserved 54 amino acid domain (SNH domain) which has been observed in a wide variety of species. The C-terminal domain is rich in glutamine, proline, glycine and tyrosine (the QPGY domain), which harbors transcriptional activator sequences. Mutagenic analysis of the SYT gene has shown an increase in transcriptional activation along with the deletion of the SNH domain, suggesting that this domain acts as an inhibitor of the activation domain [287]. The mouse homologue of SYT, Syt, was isolated and sequenced in full by de Bruijn et al. [288]. They found that during early embryogenesis, mouse Syt is ubiquitously expressed. Later, expression becomes confined to cartilage tissues, specific neuronal cells, and some epithelium-derived tissues and in primary spermatocytes.

The SSX1 and SSX2 proteins are 81% homologous and are rich in charged amino acids [289]. Due to the high homology between the molecules, it is likely that the function of SYT-SSX1 and SYT-SSX2 is similar. The SSX molecules contain a transcriptional co-repressor domain, known as the Kruppel-associated box (KRAB), at their N-terminus [290-292]. KRAB domains have been previously identified only in Kruppel-type zinc finger proteins, e.g., zinc finger protein-117 and -83 (ZNF117 and ZNF83).
The translocation in synovial sarcomas fuses the transcriptional activating domain from the SYT gene to the transcriptional repressor domain from the SSX gene. Crew et al. found these transcripts in 29 of 32 (91%) synovial sarcomas, thus providing pathologists with a useful diagnostic tool [286]. Further analysis of the breakpoints lead to the identification of 2 distinct fusion junctions for each of SYT-SSX2 and SYT-SSX1 fusion transcripts [286]. Since then, additional variants have been characterized, differing in the placement of the breakpoint [293, 294]. In addition, subcellular localization studies have shown that SYT and SYT-SSX proteins co-localize with the human homologue of the SNF2/Brahma protein BRM in the nucleus. The function of SNF2 in mammals is unknown, but evidence in S. cerevisiae and D. melanogaster suggests that it may act as a global activator of transcription [295]. In vitro studies have further shown that these molecules interact with each other [287]. This implies that the SYT-SSX fusion protein may have a role in activating or inhibiting the normal role of human homologue of SNF2.

1.6 AIMS AND OBJECTIVES

We focussed on the genetics of congenital fibrosarcoma (CFS) and adult-type fibrosarcoma (ATFS) for three reasons. Firstly, CFS and ATFS are considered to be malignant lesions. Secondly, CFS and ATFS (but particularly CFS) are very difficult to differentiate from benign fibroblastic proliferations of childhood such as infantile fibromatosis (IFB) and aggressive fibromatosis (AFB) due to significant phenotypic overlap and similar age distributions. Lastly, they appear to differ markedly from
each other in behavior and response to therapy, making a reliable marker for their
distinction extremely important from a clinical perspective. With this in mind, our
goal was to identify and characterize recurrent genetic alterations in cellular
fibroblastic tumours of childhood that would be useful for the pathologic and
prognostic classification of members of this tumor subgroup. Cytogenetic analysis of
CFS cases to date have only shown non random abnormalities involving whole
chromosomes, such as trisomy of chromosome 11. The cytogeneticists at B.C.
Children’s Hospital identified an apparently non-random abnormality involving
the chromosomal regions 12p13 and 15q25.

The specific objectives of the work described in this thesis were to:

1. Characterize, at the molecular cytogenetic level, the consequences of
the 12p13 and 15q25 aberrations by fluorescence in situ hybridization.

2. Clone the chromosomal breakpoints and characterize the genes
involved.

3. Characterize the ETV6-NTRK3 gene fusion at the cDNA level.

4. Characterize the ETV6-NTRK3 protein product (molecular weight,
hetero- and homodimerization status, and phosphorylation status)

5. Elucidate the downstream protein interactions of ETV6-NTRK3.
CHAPTER II
MATERIALS AND METHODS

2.1 CLINICAL FEATURES OF INDEX CASE

A term baby girl (TB) was found at birth to display a quarter-sized discoloration in the left lumbar region. Over the course of four weeks the mass grew in size to 7 x 5 cm. Initial biopsy was diagnosed as a congenital fibrosarcoma by a dermatologist. This diagnosis was confirmed by pathologist review. A CT scan and ultrasound confirmed the absence of any deep tissue extension. Surgery revealed a fleshy grey colored mass which was resected with overlying bluish tinged skin. The deep margin was at the posterior superior iliac spine and was negative for tumor. There was no further treatment administered post surgery. TB was followed yearly and has since shown no recurrence at the local site and chest X-rays have been clear.

2.1.1 Pathology of Index Case

The gross specimen consisted of a large tan-brown mass with minimal evidence of hemorrhage or necrosis. Histologic analysis revealed a cellular spindle cell lesion in which there was nuclear pleomorphism and moderate mitotic activity. Cells showed no obvious evidence of specific differentiation other than having the appearance of possible fibroblastic origin. There was positive immunohistochemical staining for vimentin, but not for muscle specific actin, desmin, S100, histiocytic markers, or endothelial markers. Ultrastructural analysis
showed no rhabdomyoblastic differentiation, but also suggested a possible fibroblastic origin. Based on these features, the specimen was diagnosed as a congenital fibrosarcoma (see Fig. 6a). This appearance is contrasted with infantile fibromatosis (see Fig. 6b), which shows similar, but less pleomorphic cells, lower mitotic activity and increased cellular matrix between cells.

2.1.2 Cytogenetic Analysis of Index Case

Cytogenetic analysis of cultured tumor tissue of the initial CFS case showed an abnormal karyotype in all cells examined as indicated in Figure 7. All cells examined showed additional copies of chromosome 11, with a few cells including chromosomes +20 and +2. There were structural abnormalities including additional material on the long arm of chromosome 1, apparent deletion of the distal portion of the short arm of chromosome 12, and a rearrangement of the long arm of chromosome 15. The final cytogenetic assessment of 24 metaphases of this case was as follows: 46-49, XX, -X, add(1)(q43), +2, +11, del(12)(p13), add(15)(q25), +20.

2.2 CLINICAL SAMPLES, TISSUE CULTURE TECHNIQUES AND CYTOGENETIC ANALYSIS

All CFS, CMN, ATFS, and IFB cases analyzed in this study were collected from either British Columbia’s Children’s Hospital, Childrens Hospital of Los Angeles, National Wilms’ Tumor Study Group (NWTSG) tumor bank, or Cooperative Human Tissue Network (CCHTN) at Columbus Children’s Hospital, Columbus, Ohio and presented during the period 1988 to the present. The cases
FIGURE 6. Histologic analysis of CFS and IFB. A, 40X magnification of a CFS case stained with hematoxylin and eosin (H&E). Note the characteristic highly cellular spindle morphology (bluish-purple), mitotic figures and nuclear pleomorphism. B, 40X magnification of an IFB case stained with H&E. Note the lower density of spindle cells (bluish-purple) and an increase in the amount of extracellular collagenous matrix (pink) relative to CFS.
FIGURE 7. G-banded karyotype of index case. A total of 24 metaphases were analyzed by B.C. Children's Hospital's cytogeneticists. The following karyotype displays 48 chromosomes, XX, add(1)(q43), +11, del(12)(p13), add(15)(q25), and +20. Three of four CFS cases analyzed showed recurring anomalies including addition of chromosome 11 (vertical arrow), apparent deletion of 12p13 (angled arrow), and a rearrangement of 15q25-qter (horizontal arrow).
were analyzed by short term culturing and cytogenetic analysis by cytogeneticists at
either B.C. Children's Hospital or Childrens Hospital of L.A. according to
established protocols [296, 297]. Briefly, excised tumor tissue was minced in
collagenase (200 units/ml, Sigma) and incubated for 2 hours. Washed cells were
incubated in 60 mm plastic petri dishes in RPMI 1640 medium with L-glutamine
(Gibco BRL) supplemented with 15 or 20% fetal bovine serum (FBS, Sigma), 5%
antibiotic-antimycotic solution (Gibco BRL), and maintained in this medium at
37°C in a 5% CO₂ incubator. Short-term cultures used for cytogenetic analysis were
arrested in metaphase with Colcemid (1 ng/ml final concentration, Gibco BRL) for 3
to 4 hours prior to harvesting. After swelling the cells in a hypotonic solution, the
cells were fixed in a 3:1 solution of methanol to acetic acid and dropped onto glass
slides. G-banding techniques were used to stain metaphases previously fixed, dried,
and treated overnight at 60°C on the petri dish surface. Cells were harvested at
various passages and were used for cytogenetic analysis and as a source of DNA or
RNA. Frozen primary tumor specimens for molecular studies were stored at -70°C
prior to analysis. Karyotypes are described in accordance with the International
features for the 4 CFS cases initially analyzed cytogenetically are summarized in
Table 4.

Cell lines expressing NTRK3 (kindly provided by Drs. B. Nelkin and D.
Kaplan) were grown in RPMI 1640 supplemented as above with the addition of 200
**TABLE 4.** Summary of cytogenetic analysis of initial BCCH CFS cases. Initial karyotype refers to the initial cytogenetic assessment of the cases while final karyotype refers to the cytogenetic interpretation after FISH analysis. A final cytogenetic assessment on case 2 was not possible due to the absence of material for FISH.

<table>
<thead>
<tr>
<th>CASE</th>
<th>INITIAL CYTOGENETICS</th>
<th>FINAL CYTOGENETICS</th>
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<tr>
<td>3</td>
<td>48, XY, +11, +8, t(4;12)(p10;q10), add (15)(q25), t(12;15)(p13;q26) [cp 21]</td>
<td>48, XY, +11, +8, t(4;12;15)(q12;p13;q25)</td>
</tr>
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**Abbreviations.** cp= number of cells evaluated, NA= not available, add= addition, del= deletion.
μg/ml of G418 (Gibco/BRL).

2.3 **YAC AND COSMID PROBES**

Yeast artificial chromosomes (YACs), mapping within specific areas of interest were kindly provided by Dr. S. Scherer from the Canadian Genome Centre YAC Core Facility at the Hospital for Sick Children, Toronto, Ontario. These included 890_E_3, 854_E_6, 924_H_12, 817_H_1, 954_G_10, 738_B_11 from chromosome 12p13, and 882_H_8, 895_H_10, 932_F_12 (chimeric), and 802_B_4 from chromosome 15q25-26. All YACs were either previously confirmed to be non-chimeric [298, 299], or were confirmed to be non-chimeric by FISH analysis of normal metaphases in our laboratory. Cosmid contig probes spanning the *ETV6* locus, including 179A6, 171H6, 45E12, 163E7, 54D5, and 148B6 [300], were generous gifts of Dr. Peter Marynen, University of Leuven. YACs and cosmids were grown and maintained in our laboratory using standard methods [301]. Probes were labeled with either biotin or digoxygenin using a commercially available kit according to the manufacturer’s instructions (Gibco/BRL).

2.4 **DNA AND RNA ISOLATION**

Primary CFS, IFB, or CMN tumor tissue, normal fibroblasts and NIH3T3 cells infected with *ETV6-NTRK3* retroviral constructs (kindly provided by Daniel Wai in our laboratory) were used as sources of DNA or RNA. Several other previously established cell lines were used as either positive or negative controls including the leukemia cell lines *K562* [302] and *Jurkat* [303], the neuroblastoma cell line *SAN2* [304], a Ewing tumor cell line *TC-71* [305], and the rhabdomyosarcoma cell line *Birch*
(established at St. Jude's Hospital, Memphis, TN, Piper S, unpublished data). DNA was extracted from these cells using standard methods and an Applied Biosystems DNA Extractor, Model 340A [306]. Total RNA was extracted using the acid guanidium thiocyanate phenol/chloroform method [307].

DNA used for FISH was isolated from either YAC or cosmid clones (see section above). DNA from cosmids was extracted using a Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. DNA from YACs was isolated by previously established methods (D. Ward, personal communications). Briefly, an AHC plate was inoculated with a YAC clone and allowed to grow at 30°C for at least two days or until colonies were visible. One red colony (the red colony indicates the presence of insert within the YAC) was then used to inoculate either 50 ml of minimal media or yeast extract, peptone, dextrose (YPD) media, which was subsequently shaken overnight at 30°C at 225 rpm. Cells were then centrifuged at 2000 rpm for 3 minutes and the pellet was resuspended in 0.5 ml of 1M sorbitol (Difco)/0.1M Na₂EDTA (pH 8) and 20 μl of Sigma Lyticase (Sigma)(12.5 mg/ml of Sigma Lyticase, prepared fresh, in 1M sorbitol/0.1M Na₂EDTA (pH 8)). The solution was then transferred to an eppendorf tube, incubated at 37°C for 60 min, and centrifuged for 1 minute. The pellet was resuspended (gently with a pipet) in 0.5 ml 50 mM Tris-Cl (pH 7.4)/20 mM Na₂EDTA (pH 8) before the addition of 50 μl 10% SDS. This mixture was incubated at 65°C for 30 minutes. The SDS was removed by adding 0.2 ml 5M potassium acetate, incubating on ice for 60 minutes and then centrifuging at 14,000 rpm at 4°C for 5 minutes. The supernatant was then transferred to a fresh eppendorf tube and the DNA was precipitated with
isopropanol according to previously established methods [306]. The DNA was RNase treated prior to hapten labeling for use as a FISH probe.

2.5 SOUTHERN AND NORTHERN BLOT ANALYSIS

Southern and Northern blot analyses were performed as previously described [306]. For Southern analysis, 10μg of genomic DNA from each case was digested with HindIII, BamHI, EcoRI, or XhoI. For Northern analysis, at least 20μg total RNA was used. Briefly, a 0.8% agarose gel was used for Southern analysis, while a 1.2% formaldehyde-agarose gel was used for Northern analysis. DNA or RNA was transferred onto nylon membrane filters (HYBOND) by capillary blotting and fixed to the membrane by baking at 80°C for 2 hours. Probes included: full length ETV6 cDNA (a generous gift of Dr. Peter Marynen, University of Leuven); ETV6-5/6, consisting of ETV6 exons 5 and 6 (nt 825-1137) and generated by digesting ETV6 full length cDNA with BamHI and PvuI; full length NTRK3 cDNA (a generous gift of Dr. Barry Nelkin, Johns Hopkins University); and NTRK3-PTK, consisting of NTRK3 nt 1740-2715 (including the PTK region), and generated by XbaI digestion of full length NTRK3 cDNA. Probes were radiolabeled with [α-32P]dCTP (50μCi) using random primer extension (Oligo Labeling Kit, PHARMACIA) followed by nick-column purification (PHARMACIA). The DNA and RNA membranes were hybridized overnight with the radiolabeled probes after which they were washed and autoradiographed at -70°C using standard methods [306]. To control for equal loading of RNA for Northern analysis, membranes were stripped and reprobed with a β-actin DNA probe.
2.6 FLUORESCENCE IN SITU HYBRIDIZATION (FISH) STUDIES

Cell suspensions of normal cultured lymphocytes, fibroblasts, and primary CFS tumor cells were processed according to standard cytogenetic procedures [296] and stored at -20°C in methanol/acetic acid fixative (3:1) until used. Metaphase chromosome spreads and interphase nuclei were prepared on glass microscope slides [296]. Slides for FISH were prepared by applying a drop of fixed cells onto a slide from approximately 1 foot above the slide, which was held at an angle of 45°. The slide was allowed to air dry and then aged by storing the slide in a dessicating chamber overnight at room temperature. Prior to use, the slides were passed through a series of room temperature ethanol washes (2 minutes each in 70%, 90% and 100% to dehydrate the slides). The chromosomes were denatured by submerging them in 70% Formamide (Sigma)/2X SSC (pH 7.0) for 2 minutes. The slides were then immediately run through a -20°C ethanol series as above. The slides were then allowed to air dry before the probe was applied.

The DNA probes for FISH (YAC, cosmid, and/or α-centromeric) were labeled with either biotin-14-dATP (Gibco/BRL) or digoxigenin-11-dUTP (Boehringer Mannheim) using BioNick Labeling Kit (Gibco/BRL) and purified by ethanol precipitation according to the manufacturer’s instructions. α-centromeric probes were purchased prelabeled with biotin or digoxigenin (Oncor). The addition of 5 Units of DNA Polymerase I (New England Biolabs) per labeling reaction was necessary for proper labeling of YAC and cosmid DNA. The purified probe (500 μg of labeled YAC or 50 ng of cosmid probe) was then dissolved in Hybrisol VII (Oncor), denatured at 75°C for 10 minutes and allowed to preanneal at 37°C for 30
minutes (for cosmids) or 1 hour (for YACs). The preannealed probe was then applied directly to the denatured slides, sealed with a coverslip and rubber cement (Canadian Tire) and allowed to hybridize for at least 16 hours at 37°C.

After hybridization, the slides were washed in 50% formamide/2X SSC (pH 7.0) at 45°C for 5 minutes, and another wash in 2X SSC at 45°C for 5 minutes. Detection of the signal then proceeded according to the manufacturer's instructions (Oncor). The chromosomes were counter-stained in diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (2μl of a 200mg/μl stock in 200μl of antifade; 20μl of this solution was applied to each slide) and visualized using a 100X oil immersion objective through a Zeiss Axioplan Universal epifluorescence microscope. Images were captured with a COHU High Performance camera with PSI Scientific Systems software (League City, TX). Images were converted to TIFF files and then processed through Adobe Photoshop 5.0 prior to printing.

2.7 3' AND 5' RAPID AMPLIFICATION OF cDNA ENDS (RACE)

Two micrograms of total RNA were used as starting material for 3'-RACE. ETV6 primers 541 and 701 [308] were used sequentially as sense primers in 3'-RACE experiments performed according to the manufacturer's instructions (3'-RACE System; Gibco/BRL). PCR conditions for both primers were as follows: 94°C for 1.5 minutes followed by 34 cycles of 94°C for 45 seconds, 58°C for 2 minutes, and 72°C for 3 minutes, and a final extension of 72°C for 10 minutes. Products were analyzed on agarose gels, cloned using the Invitrogen TA Cloning System (Version 1.3), and
sequenced on an ABI Applied Biosystems 373A DNA Sequencer. Sequences were analyzed using DNASTAR Sequence Analysis software.

To determine if the 3' end of the \textit{ETV6} gene (the ETS DNA binding domain) was involved in a gene fusion, 5'-RACE was utilized using five micrograms of RNA as starting material. Primers used included:

- \textbf{TELOUT:} 5'-GCTGGGTAGTTTGTCTAAGGTGC-3'
- \textbf{TELMID:} 5'-TGGTCTGCAAGAGAAGTGTCCCT-3'
- \textbf{TELIN:} 5'-CAGGGCTCTGGACATTTTCTCATA-3'

Products were analyzed as described above.

To determine whether the 5' end of the \textit{NTRK3} gene (extracellular ligand binding domain including the transmembrane domain) was involved in a gene fusion, 3'-RACE was utilized using 2 \( \mu \)g of RNA as starting material and primers:

- \textbf{TRKC1044:} 5'-GGAGTCCAAGATCATCCATGTGG-3'
- \textbf{TRKC1329:} 5'-TGCTGCTTTTGCCTGTGTCCTG-3'

Products were analyzed as described above.

\section*{2.8 RT-PCR ANALYSIS OF TUMOR SAMPLES}

Total RNA (2\( \mu \)g) was isolated from primary tumor samples and used to make cDNA as previously described [309]. Oligonucleotide primers for PCR included:

- \textit{ETV6} primers 541 and 701 [308]
- 352 (5'-GGTGATGTGCTCTATGAAGCTCC-3')
- 199 (5'-ATTACTGGAGCAGGGATGAC-3')
used in combination with NTRK3 primer:

NTRK3-2 (TRKC nt 1816-1838: 5'-CCGCACACTCCATAGAACTTGAC-3').

PCR conditions were as follows: 94°C for 1.5 minutes, followed by 33 cycles of 94°C for 45 seconds, 60°C for 1 minute and 72°C for 1 minute and a final extension of 72°C for 10 minutes. Products were analyzed as described above on agarose gels.

The presence of amplifiable RNA in all samples was confirmed by RT-PCR using β-Actin primers as a control. All samples were confirmed for the presence of the breakpoint by Southern blot analysis as described above using a DNA oligo spanning the breakpoint:

5'-GGGAGAATAGCAGATGTGCAGCAC-3'.

2.9 PREPARATION OF PROTEIN LYSATES FOR IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Cells infected with the various retroviral constructs including ETV6-NTRK3, along with the individual mutants (see Table 5) were grown until 80-90% confluent at which point the media was decanted and the cells were rinsed twice with ice-cold PBS. Briefly, 1ml of Lysis Buffer (1.5 mM MgCl₂ (Fisher), 150 mM NaCl (Fisher), 50 mM Hepes (Sigma), 10 mM NaF (Sigma), 10 mM Na₃PO₄ (Sigma), 2 mM Na₃VO₄ (Sigma), 2 mM ethylene-diamine-tetraacetic acid (EDTA) (Fisher), 2 mM NaMoO₄ 2H₂O (Sigma), 10% Glycerol (Fisher), 0.5-1.0% Nonidet P-40 (Fisher), Leupeptin (1:1000 dilution of 2 mg/ml stock made in H₂O)(Sigma), Aprotinin (1:1000 dilution of 10mg/mL stock made in H₂O)(Sigma), Phenylmethylsulfonyl Fluoride (PMSF)
TABLE 5. Summary of the various constructs used to transfect NIH3T3 cells. The vector used to make these constructs was MSCVpac and contained the various mutants listed below. Many of the mutations involved replacing a tyrosine residue (Y) with a phenylalanine (F). The Kinase Dead mutant (KD) consisted of a mutation replacing a lysine residue to an arginine. The ΔHLH mutant contained a deletion encompassing nucleotides 191-347 of ETV6-NTRK3. Corresponding residues in NTRK3 are given for comparison.

<table>
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<th>CONSTRUCT</th>
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<tr>
<td>MUTATION IN ETV6-NTRK3</td>
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<tr>
<td>CORRESPONDING RESIDUE IN NTRK3</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>ETV6-NTRK3</td>
</tr>
<tr>
<td>ΔHLH</td>
</tr>
<tr>
<td>ALD</td>
</tr>
<tr>
<td>KD</td>
</tr>
<tr>
<td>PLCγQ</td>
</tr>
<tr>
<td>PLCγT</td>
</tr>
<tr>
<td>PLCγE</td>
</tr>
</tbody>
</table>

Abbreviations. Δ = deletion, Y = tyrosine, F = phenylalanine, K = lysine, N = asparagine, Q = glutamine, T = threonine, and E = glutamate.
(1:200 dilution of a 100mM solution made in dimethyl sulfoxide) (Sigma)) was then added to the rinsed cells and incubated for 15 minutes on ice [310, 311]. Lysates were then centrifuged at 12000 rpm for 10 minutes, at which point the supernatant was transferred into a fresh tube for further analysis.

2.10 IMMUNOPRECIPITATION

One milliliter of lysate was incubated with gentle agitation for two hours at 4°C with either NTRK3 antibody (20µl) (Santa Cruz Biotechnology) or α-ETV6:HLH (3µl) (generous gift of Dr. P. Marynen) along with 10µl of Protein A-Sepharose (Pharmacia). The tubes were centrifuged at 2500 rpm for 5 minutes and the supernatant discarded. The pellet was washed 2 to 3 times in Lysis Buffer (except the concentration of Nonidet P-40 was 0.1% instead of 1%), boiled in Laemmli buffer [306], loaded onto a Protean II/xi Cell electrophoresis system (Bio-Rad) and electrophoresed on a 7.5, 10 or 15% polyacrylamide gel overnight at 70 – 100 Volts according to standard methods [306].

2.11 IMMUNOBLOTTING

Transfer of the proteins from the gel to Immobilon-P (Millipore) was accomplished with the Bio-Rad Trans-Blot SD Semi-Dry Transfer cell at 25 volts for 45 minutes using Towbin Transfer Buffer (25 mM Tris (Fisher), 192 mM glycine (Fisher), 20% methanol (Fisher). The membranes were blocked with Blocking Buffer (1X TBS, 5% Skim Milk Powder (Safeway), 0.05% Tween-20 (Fisher)) or (1X TBS, 1% BSA, 0.05% Tween-20) (for the RC-20 anti-phosphotyrosine antibody
(Transduction Laboratories)) for one hour at room temperature with gentle agitation. The membrane was then incubated with one of the following antibodies: anti-TrkC (C14) [1μg/ml] (Santa Cruz Biotechnology), RC20-Horse Radish Peroxidase conjugated [1:2500], anti-SHC [1:250], anti-PI3-K [1:5000], anti-GRB2 [1:5000], anti-PLCγ [1:1000] (Transduction Laboratories) (see Table 6). The membrane was washed three times for 5 minute intervals in TBS/0.05% Tween-20 and then incubated in the secondary antibody: anti-mouse-horse radish peroxidase conjugated [1:7000] or anti-rabbit-horse radish peroxidase conjugated [1:7000] (Transduction Laboratories) for one hour with gentle agitation. The blot was then washed as above prior to visualization by enzymatic chemiluminescence (ECL) (Amersham/Pharmacia) according to the manufacturer’s instructions. After ECL detection, the membrane was placed in between two individual Saran wrap sheets and placed into an X-ray cassette and exposed to a XAR-5 film for 10 seconds up to 20 minutes.

2.12 GENERATION OF GST-ETV6-NTRK3 FUSION PROTEINS

The BaculoGold Starter Package (Pharmingen) was used for the production and purification of recombinant virus encoding the fusion GST-ETV6-NTRK3 gene and subsequent infection of SF9 cells for the production of large quantities of recombinant protein. All materials mentioned were supplied in the kit unless otherwise stated. The recombinant protein was purified by affinity chromatography. Briefly, the lysate (prepared according to the manufacturer’s instructions) was applied to a 0.8 x 4 cm Poly-Prep Chromatography Column (Bio-Rad) equipped with a 2-way Stopcock (Bio-Rad) containing 1 ml of glutathione beads. The beads were
TABLE 6. Summary of various antibodies used for immunoblotting, their source and required concentrations.

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>MANUFACTURER</th>
<th>CONCENTRATION OR DILUTION FACTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ETV6-HLH</td>
<td>Dr. Peter Marynen</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-TrkC (C14)</td>
<td>SCBT</td>
<td>2.0 µg/ml</td>
</tr>
<tr>
<td>Anti-TrkC</td>
<td>Dr. David Kaplan</td>
<td>1:2500</td>
</tr>
<tr>
<td>RC20-HRPO</td>
<td>TL</td>
<td>1:2500</td>
</tr>
<tr>
<td>Anti-Rabbit-HRPO</td>
<td>TL</td>
<td>1:7500</td>
</tr>
<tr>
<td>Anti-Mouse-HRPO</td>
<td>TL</td>
<td>1:7500</td>
</tr>
<tr>
<td>Anti-GRB2</td>
<td>TL</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-PI3K</td>
<td>TL</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-SHC</td>
<td>TL</td>
<td>1:250</td>
</tr>
<tr>
<td>Anti-PLCγ</td>
<td>TL</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-SH2Bβ</td>
<td>Dr. Lyiangyou Rui</td>
<td>1:15000</td>
</tr>
</tbody>
</table>

Abbreviations. SCBT= Santa Cruz Biotechnology, TL= Transduction Laboratories, Inc., HRPO= horse radish peroxidase.
washed with washing buffer and the protein was eluted with 3 ml of elution buffer (both of which are supplied in the kit). The concentration of protein was determined using the BioRad Protein Determination Assay Kit according to the manufacturer's instructions. The fusion protein was then aliquoted and frozen at −20°C until use.

2.13 IN VITRO PROTEIN ASSOCIATION STUDIES

To determine whether there is heterodimerization between ETV6-NTRK3 and wild type ETV6, both ETV6 and ETV6-NTRK3 were co-translated with the TNT T7/T3 Coupled Reticulocyte Lysate System (Promega). The ETV6-NTRK3 construct was contained within pBluescript II KS (kindly provided by Daniel Wai in the laboratory) and required the T3 promoter for the production of mRNA, while the ETV6 construct was contained within the pQE9 vector (generous gift of Dr. P. Marynen) and requiring the addition of 1 Unit of RNA Polymerase, E. coli (Boehringer Mannheim) for proper mRNA production. The in vitro translated materials were then immunoprecipitated with the NTRK3 (C-14) antibody (Santa Cruz Biotechnology), electrophoresed on 10% polyacrylamide gels, blotted onto Immobilon-P and immunoblotted with the ETV6:HLH antibody, as described above.

To determine if the ETV6-NTRK3 protein had homodimerization properties, 5μg of the GST-ETV6-NTRK3 recombinant protein (see above) was mixed with 35S-Methionine (Amersham) labeled in vitro translated ETV6-NTRK3 (labeled according to the instructions provided with the TNT T7/T3 Coupled
Reticulocyte Lysate System), immunoprecipitated with glutathione beads, and electrophoresed as described above. The resolving gel was then dried using a Gel Drier (Labconco) for 1.5 hours prior to exposing the gel to XAR-5 film for 4 to 24 hours.

2.14 SUBCELLULAR LOCALIZATION BY IMMUNOFLOUORESCENCE

Green fluorescent protein constructs of ETV6-NTRK3, HLH, and KD were made using the pEGFP-N3 vector (Clonetech). Since the GFP portion was placed on the 3' end of the various constructs, the stop codon was replaced with a glycine codon to allow for the continued translation of GFP. This was accomplished using the QuikChange™ Site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Primers used to mutate the stop codon (TAG) to a glycine (GGG) for the various constructs were:

5'-GAC ATT CTT GGC GGG TGG TGG CTG GTG GTC-3' (forward)
5'-GAC CAC CAG CCA CCA CCC GCC AAG AAT GTC-3' (reverse)

NIH3T3 cells were then transfected with 2 μg of the purified construct and LipofectAMINE™ (Gibco/BRL) according to the manufacturer's instructions. During transfection, the cells were grown in OptiMEM™ I Reduced Serum Media (Gibco/BRL). Two days post-transfection, the cells were passaged 1:10 into selective medium (RPMI 1640 with L-Glutamine, 10% calf serum, 1xPSF, 400 μg Geneticin (Gibco/BRL)) and grown in this medium for 5 days. The cells were then seeded onto Fisherbrand Superfrost/Plus (Fisher) glass slides and allowed to grow in regular growth medium (RPMI 1640 with L-Glutamine, 10% calf serum and 1xPSF)
until 50-80% confluent. The slides were then quickly rinsed with PBS and fixed in 4% paraformaldehyde (pH 7.4) (Sigma) in PBS for 10 minutes at room temperature. The slides were then washed in PBS (3 x 5), counterstained with DAPI (as above for FISH studies) and briefly rinsed in PBS. One drop of VectaShield™ was then applied and the slide was covered with a coverslip and visualized by confocal microscopy using a Bio-Rad MRC-600 Laser Scanning Confocal Microscope.

In addition, NIH3T3 cells infected with the various constructs including NIH3T3 cells transfected with vector alone (for use as a negative control) (provided by D. Wai in our lab) were analyzed by immunofluorescence. Cells were grown on Fisherbrand Superfrost/Plus (Fisher) glass slides as described above until the confluency reached approximately 80%. The slides were then rinsed quickly with PBS and fixed in 4% paraformaldehyde (pH 7.4) in PBS for 10 minutes at room temperature and permeabilized with 0.1% TritonX-100 (Sigma) for an additional 10 minutes at room temperature. An antibody towards the carboxy terminal portion of NTRK3 was used as a primary antibody [1:2500] (a generous gift of Dr. D. Kaplan). The slide was incubated with the primary antibody for 1 hour at 37°C, at which point the slide was run through a series of four 5 minute washes in 1X phosphate-buffered saline (PBS). Rhodamine anti-rabbit (Boehringer Mannheim) was used as the secondary antibody at a concentration of 30 μg/ml and the slide was incubated at 37°C for 30 minutes. The slides were then run through another PBS series, counterstained, mounted and visualized by confocal microscopy as described above.
CHAPTER III

A NOVEL t(12; 15)(p13: q25) IN CONGENITAL FIBROSARCOMA

3.1 INTRODUCTION

Subtle chromosomal translocations, inversions, and other rearrangements are often missed by conventional cytogenetics [312]. Molecular cytogenetics has overcome these barriers by providing more sensitive techniques offering higher resolution [297]. Fluorescence in situ hybridization (FISH), for example, is a powerful molecular tool, which can be used to determine if a certain chromosomal region has been deleted or rearranged or to finely map a newly discovered gene. Conventional cytogenetics along with molecular cytogenetics has, therefore, permitted scientists to investigate genetic alterations in cancer cells, thus allowing for the detection of recurrent genetic abnormalities in certain tumors [238, 313].

The diagnosis of CFS has historically been very challenging for the pathologist due to its morphologic overlap with other childhood spindle cell tumors including ATFS, IFB, myofibromatosis (MFB), and AFB [314, 315]. We therefore performed cytogenetic analysis on a group of CFS cases at BCCH, as well as a series of ATFS, IFB, MFB, and AFB cases in an attempt to identify recurrent genetic markers of these entities. Of these cases, only the CFS cases displayed a recurring chromosomal abnormality involving chromosome 12p13 and 15q25. To determine if this aberration was a translocation, whole chromosome FISH of a CFS case using a chromosome 12 painting probe was performed. This revealed a
portion of chromosome 12 on a smaller acrocentric chromosome. We therefore wanted to elucidate the exact consequences of this rearrangement.

3.2 RESULTS

3.2.1 Cytogenetic Analysis

To screen for recurrent genetic markers of CFS, cytogenetic analysis was performed on a series of CFS (n=4), IFB (n=15) and ATFS (n=2) cases diagnosed in patients either from BCCH or CHLA. CFS Cases 1-3 each demonstrated abnormal metaphases with a subtle rearrangement of chromosome 15q25-26. Two of these cases had additional abnormalities of chromosome 12p13. Cytogenetic analysis of ATFS (n=2) and IFB (n=15) cases showed no rearrangements involving either chromosome 12 nor 15 (see Fig. 7 (chapter 2)).

3.2.2 Fish Analysis Identifies a Common Derivative Chromosome

We next performed fluorescence in situ hybridization (FISH) analysis of 12p13 and 15q25-26 alterations using the FISH probes depicted in Fig. 8. Only CFS cases 1 and 2 had tumor metaphases available for FISH. To map each breakpoint, we screened CFS metaphases using a series of non-chimeric 12p13 and 15q25-26 yeast artificial chromosomes (YACs) to identify those spanning the breakpoints. Initially, we started with YACs representing the telomeric portions of 12p and 15q. To test for a translocation of 12p material to chromosome 15q, we used either YAC 890_E_3 or 854_E_6 (from 12p telomere) along with an α-centromeric chromosome 15 probe. Conversely, we tested either YAC 882_H_8 or 895_H_10...
FIGURE 8. Mapping of chromosome 12p13 and 15q25-26 breakpoints in CFS. Chromosomes 12p13 and 15q25-26 are schematically represented at the top and bottom, respectively, of the diagram. The positions of the \textit{ETV6} (TEL) and \textit{KIP1} loci are indicated by boxes. Relative YAC positions are indicated by solid lines; neither chromosome or YAC sizes are to scale. The \textit{ETV6} locus is drawn to scale, and exons are indicated by numbers and letters above the solid line. Cosmid positions are indicated by open lines. The position of the \textit{NTRK3} (TRKC) locus relative to 802B4 is based on the present study.
(from 15q telomere) along with an α-centromeric chromosome 12 probe. When the telomeric 12p YAC 890_E_3 and the chromosome 15 α-centromeric probe were used in a dual colored metaphase preparation, one of the chromosomes hybridized both probes (see Fig. 9a). This study demonstrated that the rearrangement involving 12p represented a translocation of material to 15qter. On the other hand, material from 15q25 did not translocate back to 12pter, but rather to another chromosome (later identified as chromosome 1 by DAPI banding) (see Fig. 9b). FISH using the above YACs together with a series of chromosome-specific probes demonstrated complex three-way translocations for both cases in which material from distal 12p was translocated to distal 15q and material from distal 15q was translocated to either 1q43 or 4q10, respectively.

3.2.3 Identification of the Breakpoint Region by YAC Mapping

To narrow down the breakpoint, we "walked" along chromosome 12p and 15q using various YAC clones until we found one which spanned the breakpoint. We found that the 12p13 YAC, 817_H_1, and the 15q25-26 YAC, 802_B_4, were each split in CFS cells, giving 3 signals as opposed to only 2 in IFB and other controls. When these YACs were used together in dual-coloured FISH, a derivative chromosome hybridizing a fusion signal was detected in both CFS cases (see Fig. 10a). This derivative was shown to represent a der(15)t(12;15) as it simultaneously hybridized both an α-centromeric chromosome 15 probe and 817_H_1 by dual-color FISH (data not shown). Similar experiments on 3 ATFS cases and 6 IFB cases
FIGURE 9. Dual-coloured FISH of CFS. A, A metaphase from a CFS case was used in a dual colored FISH experiment using the 12p telomeric YAC 890_E_3 (green), and a 15 α-centromere (red). A der(15) chromosome hybridizing both probes shows the translocation of material from 12pter to 15qter. B, Another metaphase preparation from the same CFS case was subjected to dual colored FISH using the 15qter YAC, 882_H_8 (green), along with a 12 α-centromere (red), however, material from 15qter, as seen by the 15qter YAC, was translocated to another chromosome, later identified as chromosome 1 by computer generated DAPI banding.
FIGURE 10. FISH analysis for CFS breakpoints. A, Dual-coloured FISH of CFS case 2 metaphase using the 12p13 breakpoint-spanning YAC, 817_H_1 (green), and the 15q25-26 breakpoint-spanning YAC, 802_B_4 (red). The arrowhead shows a yellow fusion signal indicating the der(15)t(12;15)(p13;q25-26). B, Dual-coloured FISH of CFS case 1 interphase cell using ETV6 exon 1-containing cosmid 179A6 (green) and ETV6 exon 8-containing cosmid 148B6. The arrowhead shows a fusion signal representing one copy of normal chromosome 12 in each cell, while the arrows show separate signals indicating disruption of the ETV6 gene.
revealed normal FISH patterns only with these probes. FISH using 802_B_4 together with an α-centromeric 12 probe failed to detect reciprocal der(12)t(12;15) chromosomes in either case 1 or 2 (data not shown). In each case, FISH identified derivative chromosomes that had not been previously detected cytogenetically (see Table 7).

3.2.4 Micromapping the Breakpoint with Cosmid Probes

The 12p13 breakpoint was narrowed to the telomeric end of 817_H_1 as the distal overlapping 12p13 YAC, 924_H_12, was also split in CFS (data not shown). This region contains the ETV6 gene (TEL), a member of the ETS family of transcription factors and has been involved in a variety of translocations which have given rise to gene fusions in human leukemias [308, 316-320]. To test for the involvement of ETV6 in CFS, we performed dual-coloured FISH using 802_B_4 together with either cosmid 179A6, which contains ETV6 exon 1, or cosmid 148B6, containing ETV6 exon 8 [300]. This revealed a fusion signal with 179A6 but not with 148B6 (data not shown). Dual-coloured FISH of CFS using 179A6 and 148B6 together revealed one fusion signal as expected for normal chromosome 12, as well as two widely separated signals (see Fig. 10b). This indicates that the breakpoint lies between exon 1 and 8 of ETV6 in CFS, and that the telomeric portion of ETV6 is translocated to chromosome 15q25-26. Further mapping using cosmids 171H6, 45E12, 163E7, and 54D5 (see Fig. 8), localized the ETV6 breakpoint to the region between exons 5 and 7 of ETV6. Fusion signals were not detected by FISH using
Table 7. Summary of *ETV6* rearrangements in human neoplasia.

<table>
<thead>
<tr>
<th>Gene Fusion</th>
<th>Translocation</th>
<th>Protein (ETV6 – Partner Gene)</th>
<th>Phenotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ETV6-MDS/EVI1</em></td>
<td>t(3;12)(p26;p13)</td>
<td>NFD – ND</td>
<td>MDS</td>
<td>[319]</td>
</tr>
<tr>
<td><em>ETV6-JAK2</em></td>
<td>t(9;12)(p24;p13)</td>
<td>HLH – PTK</td>
<td>preB cALL, aCML</td>
<td>[320, 321]</td>
</tr>
<tr>
<td><em>ETV6-PDGFB</em></td>
<td>t(5;12)(q33;p13)</td>
<td>HLH – PTK</td>
<td>CMML</td>
<td>[316]</td>
</tr>
<tr>
<td><em>ETV6-STL</em></td>
<td>t(6;12)(q23;p13)</td>
<td>HLH, DBD–ND</td>
<td>B-cell ALL cell line</td>
<td>[322]</td>
</tr>
<tr>
<td><em>ETV6-ABL</em></td>
<td>t(9;12)(q34;p13)</td>
<td>HLH – PTK</td>
<td>AML, ALL, aCML</td>
<td>[318]</td>
</tr>
<tr>
<td><em>ETV6-CBFA2</em></td>
<td>(AML1) t(12;21)(p13;q22)</td>
<td>HLH – DBD, cALL</td>
<td>TAD, AML</td>
<td>[308, 323-326]</td>
</tr>
<tr>
<td><em>ETV6-MNN1</em></td>
<td>t(12;22)(p13,q22)</td>
<td>DBD – ND</td>
<td>AML and MDS</td>
<td>[317]</td>
</tr>
<tr>
<td><em>ETV6-CDX2</em></td>
<td>t(12;13)(p13;q12)</td>
<td>NFD – ND</td>
<td>AML</td>
<td>[327, 328]</td>
</tr>
<tr>
<td><em>ETV6-ACS2</em></td>
<td>t(5;12)(q31;p13)</td>
<td>ND</td>
<td>RAEB, AML, AEL</td>
<td>[329]</td>
</tr>
<tr>
<td><em>ETV6-BTL</em></td>
<td>t(4;12)(q11-q12;p13)</td>
<td>ND</td>
<td>AML</td>
<td>[330]</td>
</tr>
<tr>
<td><em>ETV6-ARG</em></td>
<td>t(1;12)(q25;p13)</td>
<td>HLH-SH2, SH3, AML</td>
<td>TAD, PTK</td>
<td>[331]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(7;12)(q22;p13)</td>
<td>ND</td>
<td>MDS</td>
<td>[332]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(7;12)(q36;p13)</td>
<td>ND</td>
<td>AML</td>
<td>[332]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(9;12)(q11;p13)</td>
<td>ND</td>
<td>ALL</td>
<td>[332]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(10;12)(q24;p13)</td>
<td>ND</td>
<td>MDS</td>
<td>[333]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(6;12,17)(p21;p13;q25)</td>
<td>ND</td>
<td>AML</td>
<td>[328]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(7;12)(p14;p13)</td>
<td>ND</td>
<td>AML</td>
<td>[328]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(12;17)(p13;q11)</td>
<td>ND</td>
<td>B-NHL</td>
<td>[328]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(7;12)(p12;p13)</td>
<td>ND</td>
<td>AML</td>
<td>[328]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(X;12)(q28;p13)</td>
<td>ND</td>
<td>MDS</td>
<td>[334]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(1;12)(q21;p13)</td>
<td>ND</td>
<td>AML</td>
<td>[334]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(9;12)(p23-24;p13)</td>
<td>ND</td>
<td>ALL</td>
<td>[334]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(12;14)(p13;q11)</td>
<td>ND</td>
<td>ALL</td>
<td>[335]</td>
</tr>
<tr>
<td><em>ETV6-</em></td>
<td>t(7;12)(q35;p13)</td>
<td>ND</td>
<td>ALL</td>
<td>[335]</td>
</tr>
</tbody>
</table>

Abbreviations. AML = acute myelogenous leukemia, CMML = chronic myelomonocytic leukemia, B-NHL = B-cell non-Hodgkin's lymphoma, SH2(3) = Src homology 2(3), MDS = myelodysplastic syndrome, ALL = acute lymphoblastic leukemia, cALL = childhood acute lymphoblastic leukemia, aCML = atypical chronic myeloid leukemia, ? or ND = not determined, NFD = no functional domain, HLH = helix loop helix domain, PTK = protein tyrosine kinase domain, TAD = transactivation domain, DBD = ETS DNA binding domain, RAEB = refractory anemia with excess blasts (with basophilia), AEL = acute eosinophilic leukemia.
802_B_4 in conjunction with 148B6, confirming the absence of the der(12)
t(12;15)(p13;q25-26) in tumor cells.

3.3 DISCUSSION

In this report we describe for the first time the association of a
\(t(12;15)(p13;q25-26)\) translocation with congenital fibrosarcoma. All CFS cases with
abnormal karyotypes in this study demonstrated alterations of the distal long arm
of chromosome 15 (15q25-26), while 2 also showed subtle alterations involving
chromosome 12p13. The normal karyotype of case 4 likely represents in vitro
overgrowth by normal fibroblasts. In contrast, similar abnormalities were not
detected in a series of IFB, MFB, or AFB cases. FISH analysis using region-specific
YAC probes confirmed the presence of a der(15)t(12;15)(p13;q25-26) in 2 of 2 CFS
cases with metaphases available for FISH studies. Moreover, when we screened
with a series of 12p13 and 15q25-26 specific YACs against CFS metaphases, we found
that the same region-specific YAC was split in both CFS cases, i.e. YAC 817_H_1
spanning the chromosome 12p13 breakpoint and YAC 802_B_4 spanning the 15q25-
26 breakpoint. Therefore the breakpoints in each chromosome are contained
within identical region-specific YACs in both of the CFS cases tested, providing
further evidence that this represents a non-random rearrangement in CFS. We
also tested 3 ATFS, 3 IFB, and 3 AFB cases using the identical probes. None of the 9
non-CFS cases demonstrated similar findings, suggesting that the
der(15)t(12;15)(p13;q25-26) is specific for CFS. We are currently accumulating and
testing additional cases of CFS, ATFS, IFB, and AFB to more rigorously test this hypothesis.

Finer FISH mapping of the breakpoint using cosmid probes localized the breakpoint within the \textit{ETV6} gene. This is the first known involvement of the \textit{ETV6} gene fusion in solid tumors, as such rearrangements were previously observed only in leukemias [308, 316-320, 333].

The \textit{ETV6} (ETS variant gene 6) gene is located on chromosome 12p13 and was cloned as a result of a t(5; 12)(q33; p13), fusing it to the platelet-derived growth factor \(\beta\) receptor (\textit{PDGFB}\(\beta\)) gene in chronic myelomonocytic leukemia (CMML) [316]. \textit{ETV6} (also known as TEL: translocation ETS leukemia) is a member of the large family of transcription factors known as the E26 transformation-specific (ETS) transcription factors first discovered as part of the E26 avian erythroblastosis virus genome [336]. The ETS family of transcription factors recognize the core motif C/A GGA A/T [337].

The \textit{ETV6} gene is approximately 240 kb in size and is made up of 8 exons (with one alternatively spliced exon) which are oriented from telomere to centromere (see Fig. 8) [300]. It encodes a nuclear phosphoprotein with a helix loop helix dimerizing domain (HLHD) encoded within exons 3 and 4 and a DNA binding domain (DBD) encoded within exons 6-8 [300, 338]. The ETS DNA binding domain is known to contain the nuclear localization signal and sequence specific DNA binding activity [339]. The mRNA encoding the ETV6 protein contains two possible initiation sites and results in two species of transcripts. One of the ETV6 proteins migrates around 50 kDa and the other around 57 kDa. Both contain the HLHD and
the ETS DBD, but only the full length species encodes a MAPK consensus site (amino acids 20-23), which is phosphorylated in vivo [338]. This suggests that the regulation of expression of these two proteins are different. Poirel et al. noted that most of the cell lines they examined showed greater expression of the higher molecular weight ETV6 species [338].

Since its discovery in 1994, the *ETV6* gene has been implicated in a large number of hematopoietic malignancies. Fluorescence *in situ* hybridization (FISH) analysis of the 12p chromosomal region has shown numerous translocations involving the *ETV6* gene, with some cases showing a deletion in addition to the translocation [308, 324, 340-343]. Approximately 50% of the rearrangements involving the *ETV6* gene within these neoplasms have been characterized (see Table 7). Cytogenetic and molecular genetic analysis of these rearrangements has led to the discovery of chimeric gene fusions involving specific exons from the *ETV6* gene as a result of chromosomal translocations. In CMML, for example, the helix loop helix dimerization domain (HLH) from the *ETV6* gene is fused to the protein tyrosine kinase domain (PTK) from the *PDGFβR* gene [316]. The HLH domain acts as a protein-protein dimerizing domain and constitutively activates the PTK domain by ligand-independent dimerization. The t(12;22)(p13;q11) in myeloproliferative disorders (MDS) results in the fusion of nearly the entire MN1 protein to the ETS DNA binding domain from the *ETV6* gene [317]. In this case, it is thought that oncogenesis is due to the altered transcriptional activity of the *ETV6* gene. The *ETV6*-CDX2 and the *ETV6*-STL gene fusions in AML and a B-cell ALL cell-line, respectively, only contain the first two exons from the *ETV6* gene fused to
unknown 3' sequences from the partner gene [322, 327]. There are no known functional domains in the first two exons of ETV6 and it is thought that the STL and CDX2 genes are under transcriptional control from the ETV6 promoter. The well characterized ETV6-CBFA2 (TEL-AML1) is usually accompanied by a deletion of the residual ETV6 gene on the normal chromosome [308]. It is possible that the normal ETV6 protein dimerizes with the ETV6-CBFA2 protein interfering with its oncogenic potential or that ETV6-CBFA2 functions as a dominant negative inhibitor of normal ETV6 function. Protein analysis led to the discovery of up to 5 different species due to alternative translational initiation and postranslational modification [338, 344]. Other ETS family members are known to be phosphorylated and it has been shown that these postranslational modifications play an important role in the function of the protein [345-348].

The mechanism by which a chromosome participates in a translocation is still unclear. In a recent study, however, the ETV6-CBFA2 breakpoint was cloned at the genomic level and almost all breakpoints in the ETV6 gene were found near a purine/pyrimidine rich sequence within intron 5 of ETV6 (most breakpoints involving ETV6 occur in intron 5) [349]. This suggests that this region may be susceptible to DNA breakage and re-ligation, including translocations. Figure 11 summarizes the known breakpoints within the ETV6 gene as well as the corresponding fusion genes.

ETV6 expression is seen in almost all tissues during development and in the adult. ETV6 has recently been shown to be important in angiogenesis and its transcription is downregulated by an angiogenic growth factor, vascular endothelial
FIGURE 11. Schematic representation of the cDNA for ETV6 as well as some of the more common rearrangements involving the ETV6 gene. The ETV6 gene is located on chromosome 12p13 and consists of 8 exons. The top panel shows the coding sequence (cDNA) of ETV6 along with the nucleotide position of each exon (exons are separated by a vertical line with the nucleotide position shown below). A number of translocation breakpoints have been found in the ETV6 gene resulting in its in frame fusion to other genes such as PDGFβR (in CMML), ABL (in AML, ALL, and aCML), AML1 (in cALL) and MN1 (in AML and MDS). The fusion proteins generated juxtapose either the HLH dimerization domain from exons 3 and 4 of ETV6 to the protein tyrosine kinase (PTK) domain of PDGFβR, ABL and the runt and transactivation domain (TAD) of the AML1 gene or the ETS DNA binding domain (ETS domain) from exons 6, 7 and 8 to the MN1 region of the MN1 gene (shown as protein structures in the bottom 5 panels). The location of some of the more common breakpoints found in various hematologic malignancies are shown in the cDNA sequence (arrows) (see Table 7 for more information).
growth factor (VEGF) [350]. Angiogenesis refers to the process by which new vascular elements emerge from pre-existing vasculature. This group, however, found that only the lower molecular weight species was expressed in human umbilical vein endothelial cells (HUVE). In addition, they were able to demonstrate the loss of one of the mRNA \textit{ETV6} species upon treatment of the cells with VEGF. The precise role of \textit{ETV6} in blood vessel formation and maturation is presently under investigation. It has been suggested that the \textit{ETV6} protein may act as a transcriptional repressor (Golub \textit{et al.}, unpublished data). Since VEGF promotes vascular growth and inhibits \textit{ETV6} transcription, it is possible that the transcription of genes inhibited by \textit{ETV6}, are involved in blood vessel formation and maturation. \textit{ETV6} knockout mice showed embryonic lethality and failed to maintain yolk sac blood vessel formation. Furthermore, studies have shown \textit{ETV6} to be necessary for bone marrow hematopoiesis but not essential for liver hematopoiesis [351].

We observed complex three-way translocations in both CFS cases studied by FISH (see Table 4 (chapter 2)), suggesting that these alterations might be favored in CFS. Three-way translocations generating typical \textit{EWS-FLI1} gene fusions are described in Ewing tumors [352, 353], as are complex translocations in \textit{ETV6-CBFA2} positive leukemia cell lines [354]. Moreover, \textit{ETV6-CBFA2} positive cases with the t(12;21) show a high frequency of variant derivative 21 chromosomes and absence of \textit{CBFA2-ETV6} expression, while other \textit{ETV6-CBFA2} positive cases commonly delete the normal \textit{ETV6} allele [355]. This suggests that reciprocal fusion products or normal \textit{ETV6} itself may inhibit functional \textit{ETV6} chimeric oncoproteins. Further
rarrangements of the der(12)t(12;15) in CFS may therefore be selected for as a mechanism of eliminating expression of an inhibitory NTRK3-ETV6 molecule.

In summary, cytogenetic analysis coupled with detailed FISH mapping of two CFS cases with available metaphases resulted in the identification of a complex three-way rearrangements for both cases, interpreted as t(1;12;15)(q44;p13;q25-26) and t(4;12;15)(q10;p13;q25-26), respectively [356]. We now report an apparently non-random association between the presence of a der(15)t(12;15)(p13;q25-26) chromosome and the diagnosis of CFS. This rearrangement was not detected in cases of ATFS, IFB, MFB nor in AFB, and therefore may be useful in the differentiation of these entities from CFS. Furthermore, our findings provide additional evidence that CFS is biologically distinct from fibrosarcomas occurring in older children. These data suggest that CFS may be characterized by an ETV6 gene fusion; the identification of the partner gene is the topic of the next chapter.
CHAPTER IV

CLONING AND CHARACTERIZATION OF THE t(12;15) IN CFS

4.1 INTRODUCTION

Translocations leading to specific gene fusions are a common event in cancer [238]. Molecular analysis of these gene fusions has led to the discovery of chimeric oncogenes. Most of these chimeras appear to act as aberrant transcription factors, likely functioning in transformation by dysregulating, ablating or introducing new gene expression profiles within the cell [238]. Bone and soft tissue sarcomas of childhood have provided an abundant resource for studying various types of gene fusions [238, 357]. The detection of these fusion transcripts in tumor specimens, specific for given tumor subtypes, has become an extremely useful diagnostic tool for the pathologist. Childhood sarcomas tend to be extremely primitive in appearance and therefore very difficult to differentiate from each other morphologically [5]. Accurate diagnosis of two morphologically similar, yet distinct, tumors is extremely important since initial diagnosis often determines which treatment protocol a patient is enrolled in. Accurate pathologic classification is, therefore, a critical prognostic factor for these patients.

The identification of the breakpoint within the ETV6 gene led us to believe there may be a gene fusion involving this gene in CFS. The ETV6 gene has only been rearranged in leukemias to date (see Table 7 in Chapter 3), but this does not rule out its potential involvement in a solid tumor as well. ERG and FLI1 are two other ETS family members found to be fused with EWS or TLS/FUS as a result of
chromosome translocations in human solid tumors and leukemias [254, 258, 259, 268].

We therefore hypothesized that the t(12;15) rearrangement may similarly give rise to an oncogenic gene fusion in CFS. Because the der(15)t(12;15)(p13;q25-26) was common to both CFS cases analyzed by molecular cytogenetics, we further reasoned that a functional gene fusion, if present, might be expected to be expressed from this derivative chromosome and would involve the ETV6 gene.

4.2 RESULTS

4.2.1 Cloning the t(12;15) Breakpoint in CFS

The 8 exon ETV6 locus is oriented in a telomere to centromere direction, with exons 3 and 4 encoding a helix-loop-helix (HLH) protein dimerization domain and exons 6-8 contributing to the ETV6 ETS DNA binding domain [300, 316]. Because the ETV6 5'-HLH region is fused to other partner genes in human leukemias [308, 316-320], we performed 3'-rapid amplification of cDNA ends (RACE) using ETV6 exon 5 primers 541 and 701 along with a poly-dT-linked primer (see Chapter 2). RACE using primer 701 generated similar ~1.5 kb fragments in 3/3 CFS cases but not in 3 ATFS, 3 IFB, or other control cases. Cloning and sequencing of these fragments revealed that 333 bp of ETV6 sequence were fused in-frame to 1115 bp of unknown sequence. Database analysis revealed this to represent the terminal 1115 bp of the human NTRK3 gene encoding the neurotrophin-3 surface receptor [358-360] (see Fig. 12a). The fusion point in all 3 cases was after nt 1033 of ETV6,
FIGURE 12. *ETV6-NTRK3* gene fusions in CFS. **A,** Junctional nucleotide (small case) and single letter amino acid sequence of PCR fragments generated by 3′-RACE of cDNA from CFS cases 1-3, using sense primers 541 or 701 from *ETV6* exon 5 in combination with a poly-dT primer. Sequence analysis revealed an in-frame fusion after *ETV6* nt 1033 with nt 1601-2715 of the human *NTRK3* gene. **B,** RT-PCR using *ETV6* primer 541 and primer *NTRK3*-2 demonstrates a 731 bp fragment in CFS (lanes 3-5) but not in normal fibroblasts (lanes 1 and 2), IFB (lanes 6-8), ATFS (lane 9), or the *Jurkat* leukemia cell line (lane 10).
which is the last nt of *ETV6* exon 5 [300]. The *ETV6* breakpoints therefore appear to be localized to intron 5. The *NTRK3* portion originated at *NTRK3* nt 1601 and included the entire protein tyrosine kinase (PTK) domain and remaining C-terminus of *NTRK3* [358-360].

4.2.2 Reciprocal Fusions were not Detected

5'-RACE with 5'-*NTRK3* and 3'-RACE with 3'-*ETV6* ETS region primers failed to detect fusion transcripts that might be encoded by functional der(12)t(1;12) or der(1)t(1;15) chromosomes, thus ruling out additional *ETV6* or *NTRK3* gene fusions involving the 3'-ETS region and the 5'-*NTRK3* extracellular ligand binding domain, respectively (data not shown).

4.2.3 RT-PCR Analysis of CFS and Other Morphologically Similar Tumors

RT-PCR using *ETV6* primer 541 and the TRKC-2 primer from the *NTRK3* PTK region amplified the expected 731 bp *ETV6*-*NTRK3* fusion transcripts in all 3 CFS cases, while ATFS, IFB, and other controls were negative (see Fig. 12b). RT-PCR using *ETV6* primer 114 located 5' to the HLH region, together with TRKC-2 generated the expected 1158 bp product only in CFS samples, and sequencing of this product confirmed the presence of the entire *ETV6* HLH region in fusion transcripts (data not shown).
4.2.4 Northern and Southern Blot Analysis

Northern blot analysis using a full length cDNA probe for ETv6 hybridized to three transcripts with sizes of 6.2, 4.3, and 2.4 kb and was found to be ubiquitously expressed in CFS, ATFS, IFB, and other control cells. When we used a NTRK3 cDNA or a partial cDNA probe including the NTRK3 PTK motif, only the 4.3 kb transcript in CFS cells hybridized (see Fig. 13). Southern blot analysis of genomic DNA isolated from CFS primary tumor tissue showed the disruption of both ETv6 and NTRK3 genes when hybridized with the ETv6 5/6 and NTRK3-PTK probes (see Fig. 14). The multiple bands seen in the CFS lane are due to the chromosomal rearrangement involving the ETv6 gene.

4.3 DISCUSSION

By cloning the chromosome breakpoints we show that the rearrangement fuses the ETv6 (TEL) gene from 12p13 with the 15q25 NTRK3 neurotrophin-3 receptor gene (TRKC). Analysis of mRNA revealed the expression of ETv6-NTRK3 chimeric transcripts in 3 of 3 CFS tumors. These were not detected in ATFS or infantile fibromatosis (IFB), a histologically similar but benign fibroblastic proliferation occurring in the same age group as CFS.

The NTRK3 gene (also known as TRKC and neurotrophin 3 receptor) is the third member of the TRK family of tyrosine kinase receptors. Lamballe et al. found the NTRK3 gene to contain up to 97 and 98% homology to the rat and porcine Trk sequences, respectively [358, 359]. The human NTRK3 gene was cloned and
FIGURE 13. Northern analysis of CFS cases. Blots were probed sequentially with full length *ETV6* cDNA (top panel), a 3' *NTRK3* partial cDNA probe encoding the PTK domain (*NTRK3*-PTK; middle panel), and a β-actin cDNA probe (bottom panel) to test for equal loading. The *ETV6* cDNA probe detected previously described 6.2, 4.3, and 2.4 kb transcripts in multiple samples (9), including CFS, while *NTRK3*-PTK detected a 4.3 kb species only in CFS cases (lanes 3 and 4; arrow). Identical results were obtained using full length *NTRK3* cDNA (data not shown). Lanes 1 and 2, normal fibroblasts; 5-7, three IFB cases; 8 and 9, leukemia cell lines *K562* and *Jurkat*, respectively; 10, neuroblastoma cell line *SAN2*; 11, rhabdomyosarcoma cell line *Birch*. 
FIGURE 14. Southern analysis of CFS cases. HindIII digests probed with ETV6-5/6 (left panel) and NTRK3-PTK (right panel) revealed rearranged bands in CFS case 3 and 1 (lanes 1 and 2, respectively), indicated by arrows. Germline bands only were observed in IFB (lane 3), SAN2 (lane 4), Birch (lane 5), and normal fibroblasts (lane 6).
mapped to chromosome 15q25 by McGregor et al. [360]. Expression of NTRK3 in adult tissues is predominantly restricted to the central and peripheral nervous systems, but detection of transcripts in non-neural cells including intestinal glandular cells, adrenal medullary cells, ovarian granulosa and thecal cells, kidney tubular cells, as well as skeletal muscle, lung, testis, prostate, and heart has also been reported [361, 362]. Fetal tissues show strong expression in brain, kidney, lung, and heart tissues; however, the role of NTRK3 in non-neural tissues is not presently known. Studies of NT-3 knockout mice showed multiple heart defects as well as the ablation of proliferation and survival of neural crest cells suggesting an important role for NT-3 mediated pathways in cardiogenesis and neurogenesis [363]. Additional studies have shown that mechanical injury to the hippocampus results in the increased expression of NTRK3 and inducible transcription factors (ITFs) such as Fos, c-Jun, and Krox-24 [364, 365]. The results of this study suggest that the expression of NTRK3 may be controlled in part by ITFs.

The human NTRK3 gene is organized with its 20 exons from telomere to centromere [366]. The extracellular domain consists of a signal peptide, 2 cysteine clusters, a leucine rich motif, and 2 Ig-like domains. These structures are encoded by exons 1 through 8. The transmembrane domain is encoded by exons 11-13 and the protein tyrosine kinase domain (intracellular domain) by exons 13-18. Exons 9 and 16 encode the alternative inserts found in the extracellular and kinase domains and exons 13b and 14b encode the terminal domain of the truncated isoform [361]. The truncated and full length isoforms have a carboxy terminal tail and a 3' UTR which are encoded by exons 14b and 18, respectively. The isoform without any
inserts represents the active tyrosine kinase receptor, while the isoforms which are truncated or have inserts within the kinase domain are inactive tyrosine kinase receptors [367-369]. The function of the inserts is not yet known, however, there is evidence that the truncated NTRK3 receptors are important in the modulatory development of certain neural cell populations [370]. Palko et al. found that overexpression of a truncated form of the NTRK3 receptor lacking the tyrosine kinase domain, resulted in a phenotype which closely resembled NT-3 deficient cells, suggesting that the truncated isoforms serve a role as NT-3 sequestering molecules preventing the activation of the functional NTRK3 isoform [370]. Our studies showed no evidence of truncated forms of NTRK3 nor of inserts in the NTRK3 portion of ETV6-NTRK3.

Studies of knockout mice defective for the NTRK3 gene displayed numerous anomalies in their neuroanatomical development [371, 372]. There was a 20% loss of neural cells from dorsal root ganglia, 100% loss of Ia muscle afferents from the spinal cord, 50% loss of myelinated axons from the spinal cord/dorsal roots and 30% loss of various fibers from the spinal cord/ventral roots. In addition, these mice displayed highly unusual behavioral characteristics mainly involving the positioning of their limbs in relation to their trunk. This is suggestive of a faulty development in proprioception and can be mostly attributed to the faulty development of the nervous system. Finally, these mice have a relatively short lifespan (most die by the third week after birth (P21)) suggesting that these mice may have additional neural defects.

Upon NT-3 activation, NTRK3 molecules dimerize with each other which
leads to the autophosphorylation of tyrosine moieties within the intracellular domain [372-374]. The phosphotyrosines flanking the tyrosine kinase domain act as anchors for downstream signaling molecules including SNT, SHC, PLC-γ1, rAPS, and SH2Bβ. These molecules, in turn, activate other molecules ultimately leading to the activation or suppression of certain genes within the nucleus. These signaling pathways are blocked by the binding of monoamine activated α-2 Macroglobulin (MA-α2M) to NTRK1, NTRK2 or NTRK3 [375]. MA-α2M is a ubiquitously expressed glycoprotein and may be involved in neuronal regulation and certain neuropathologic conditions.

*NTRK1* and *NTRK3* have been the only two members of the NTRK family found to be involved in a variety of human cancers. NTRK1 is activated and has been implicated as a causative factor in human prostate [376, 377], breast [378], thyroid [379-381], and colon cancer [382-385]. In colon cancer, for example, the NTRK1 molecule is activated due to a chromosomal rearrangement fusing the coiled coil domain from the tropomyosin 3 (*TPM3*) gene from chromosome 1q22 to the NTRK1 kinase domain [385]. Papillary thyroid carcinomas, however, have been found to contain rearrangements of *NTRK1* resulting in its fusion of the kinase domains to either *TPR* on chromosome 1q25 [381, 386], or *TPM3* [387]. Alternatively, expression of either *NTRK1* or *NTRK3* is a marker of favorable prognosis marker in neuroblastomas and medulloblastomas (reviewed in [388]).

The predicted ETV6-NTRK3 chimeric protein consists of the ETV6 HLH protein dimerization domain fused to the PTK domain of the NTRK3 nerve
growth factor receptor (see Fig. 15). The ETV6-NTRK3 fusion is similar to the TPR-NTRK1 fusion where the coiled coil protein dimerization domain from TPR is fused to the protein tyrosine kinase domain of NTRK1 [381]. In human leukemias, the ETV6 HLH domain is fused to the PTK domains of PDGFB receptor, ABL, and JAK2 [316, 318-320]. Resulting chimeric proteins have constitutively active PTK domains that stimulate corresponding PTK-mediated signal transduction pathways in leukemic cells [318, 321]. Receptor PTKs, including NTRK3, require ligand-mediated cell surface oligomerization leading to autophosphorylation of cytoplasmic tyrosine residues and consequent kinase activation [37]. In ETV6-NTRK3 fusions, the NTRK3 ligand binding domain is replaced by the ETV6 HLH domain, potentially resulting in HLH-mediated dimerization and ligand-independent activation of the NTRK3 protein tyrosine kinase domain. Also, since ETV6 is widely expressed in mammalian tissues while NTRK3 expression is primarily restricted to neuronal cells, an additional role of ETV6 may be to provide an active promoter driving ectopic expression of NTRK3-induced signal transduction. In fact, while ETV6 was expressed in normal fibroblasts, there was no evidence of NTRK3 expression in these cells (see Fig. 13). Our studies indicate that a chimeric PTK is expressed in CFS that may contribute to oncogenesis by dysregulation of NTRK3 signal transduction pathways. We have identified previously unrecognized rearrangements giving rise to CFS-specific ETV6-NTRK3 gene fusions. This is the first known involvement of the NTRK receptor family in human oncogenesis. It is also the first ETV6 gene fusion described in solid tumors, as such rearrangements were previously observed only in leukemias. These data
FIGURE 15. Schematic representation of the predicted ETV6-NTRK3 protein. The signal peptide (SS), extracellular ligand binding domains (ECD-LB), and transmembrane (TM) domain of NTRK3 are replaced by the helix-loop-helix (HLH) domain of the ETS transcription factor ETV6, resulting in a chimeric PTK with putative dimerization activity. Vertical arrows show approximate locations of fusion points and half arrows indicate the positions of the ETV6 primers 114, 541 and 701, and the NTRK3 primer TRKC-2 used in RACE RT-PCR experiments.
therefore provide a new example of a fusion gene partner implicated in both leukemogenesis and solid tumor formation. Our data support the notion that CFS is a biologically distinct entity, and ETV6-NTRK3 detection provides a diagnostic screening tool potentially useful in the clinical evaluation of children with spindle cell tumors.
CHAPTER V

ETV6-NTRK3 GENE FUSIONS AND TRISOMY 11 ESTABLISH A HISTOGENETIC LINK BETWEEN MESOBLASTIC NEPHROMA AND CONGENITAL FIBROSARCOMA

5.1 INTRODUCTION

Congenital mesoblastic nephroma (CMN) is a renal spindle cell tumor that occurs predominantly in newborns and very young infants, with most cases being diagnosed before three months of age [224, 389]. This tumor is subdivided into so-called classical and cellular forms based on histologic features. Classical CMN consists of a moderately cellular proliferation of loosely arranged bland fibroblastic cells, while cellular (or atypical) CMN is characterized by high cellularity, numerous mitoses, and cellular pleomorphism [224]. Mixed forms are also known to occur, and it has been suggested that cellular CMN may arise from classical CMN. Despite the infiltrative growth patterns seen in all forms of CMN, these tumors are generally thought to have an excellent prognosis with surgery alone being curative [390]. However, there are several reports of local recurrences and metastatic spread, and these are almost exclusively associated with the cellular variant [391, 392]. It therefore remains to be determined whether cellular morphology is predictive of a more aggressive course.

The histogenesis of CMN is unknown. Several lines of evidence point to a derivation from primitive nephrogenic mesenchyme and a possible relationship to
other pediatric kidney tumors [393]. A link to Wilms’ tumor (WT) has been proposed based on similar patterns of loss of heterozygosity (LOH) involving chromosome 11p13-15 in WT and CMN [394, 395]. However, other studies failed to detect LOH of this region in CMN [396]. Moreover, the observed pattern in CMN of abundant expression of insulin-like growth factor II (IGFII) coupled with lack of Wilms’ tumor gene 1 (WT1) expression is distinct from the documented expression of both transcripts in WT [396, 397]. In fact, the pattern of expression of these genes in CMN is reminiscent of that observed in clear cell sarcoma of the kidney (CCSK), a highly aggressive pediatric renal neoplasm [398] and it has been proposed that CCSK may be the malignant counterpart of CMN [224].

Cytogenetic analysis of classical and cellular CMN has led to an alternate hypothesis for the derivation of these tumors. The most consistent non-random karyotypic finding in CMN is trisomy 11, with additional copies of chromosomes 8, 10, 17, and 20 being less commonly reported [34, 395, 399-401]. Moreover, trisomy 11 appears to correlate with the cellular phenotype [34, 400, 401], whereas classical CMN cases are only rarely associated with this finding [34, 400]. This is highly reminiscent of the pattern of trisomy 11 and other trisomies in congenital fibrosarcoma (CFS), a malignant tumor of fibroblasts that occurs in patients aged 2 years or younger that has striking morphologic similarity to cellular CMN [402]. CFS is characterized by local recurrence but, like cellular CMN, has an excellent prognosis and a very low metastatic rate [402]. Its benign counterpart, infantile fibromatosis (IFB), occurs in the same age group as CFS but, like classical CMN, lacks trisomy 11 [6]. This, together with ultrastructural similarities, has led to the
proposal that classical and cellular CMN are the renal counterparts of IFB and CFS, respectively [403].

As described in chapters 3 and 4, we have recently identified a novel t(12;15)(p13;q25) translocation in CFS, and have shown that this rearrangement fuses the ETV6 (TEL) gene from 12p13 with the 15q25 neurotrophin-3 receptor gene, NTRK3 (TRKC) [356]. ETV6-NTRK3 fusion transcripts encoding the helix-loop-helix (HLH) protein dimerization domain of ETV6 fused to the protein tyrosine kinase (PTK) domain of NTRK3 were identified in CFS tumors but not in adult-type fibrosarcoma or IFB. The CFS cases studied also showed trisomy 11 [404]. Several previous reports have described alterations of chromosomes 12 and/or 15 in CMN [399, 401, 405], including a t(12;15)(p13;q25) [401]. We therefore screened a series of classical and cellular CMN cases for both ETV6-NTRK3 gene fusions and trisomy 11. We found that cellular CMN was strongly correlated with ETV6-NTRK3 expression and trisomy 11, but that classical CMN was negative for both findings. These results suggest that cellular CMN is distinct from classical CMN and is histogenetically related to CFS.

5.2 RESULTS

5.2.1 Clinical History and Cytogenetics

The clinical features of the 15 CMN cases analyzed in this study are summarized in Table 8. These included 9 cellular CMNs, 2 mixed CMNs, and 4 classical CMNs in 9 males and 6 females. The diagnosis for each case was based on
**Table 8.** Clinical characteristics and molecular genetic findings in CMN cases.

<table>
<thead>
<tr>
<th>Case</th>
<th>CMN Subtype</th>
<th>Age (months)</th>
<th>Sex</th>
<th>ETV6-NTRK3 (RT-PCR)</th>
<th>Trisomy 11 (FISH)</th>
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*+, present; -, absent; ND, not determined*
standard pathologic criteria [224] and was confirmed by NWTSG or CHTN pathologic review. All cases were in patients 3 years of age or younger, and the majority were in patients younger than 3 months as expected for CMN [224].

Two of the cellular CMN cases from this study (case 1 and 2 in Table 8) had previous cytogenetic analysis performed on tumor metaphases. Case 1 was previously published as having a t(12;15)(p13;q25) in addition to trisomy 11 and other trisomies [401]. Case 2 had a similar karyotype, with a t(12;15)(p13;q24.1), trisomy 11, and other trisomies (data not shown). These findings, coupled with known morphologic similarities between cellular CMN and CFS, prompted us to screen the cohort of CMN cases for CFS-associated ETV6-NTRK3 gene fusions [404].

5.2.2 RT-PCR Analysis of CMN Cases

We performed RT-PCR to detect ETV6-NTRK3 fusion transcripts using a previously described assay [356]. As shown in Fig. 16, 8/9 cellular CMNs and 2/2 mixed CMNs were positive for the expected 731 bp ETV6-NTRK3 fusion transcript, while all 4 classical CMNs were negative. Sequencing of the amplification products demonstrated identical fusion sequences as those described for CFS ([356]; data not shown). We also screened primary tumor tissue from 12 cases of CCSK as well as one case of predominantly spindle cell monomorphic WT in a 16 month child. These cases were uniformly negative for identical ETV6-NTRK3 fusion transcripts (data not shown).
Figure 16. **ETV6-NTRK3** detection in CMN. RT-PCR using **ETV6** primer 541 and **NTRK3-2** demonstrates a 731 bp fragment in 8 of 9 cellular CMN (lanes 2-10) and 2 of 2 mixed CMNs (lanes 11 and 12). Four of four classical CMNs were negative (lanes 13-16).
5.2.3 Northern Blot Analysis

To confirm our results, we performed Northern blot analysis of a cellular and classical CMN using ETV6 and NTRK3 probes. Both samples demonstrated 6.2-, 4.3-, and 2.4-kb ETV6 transcripts (data not shown), as expected for this ubiquitously expressed gene [316]. However, only the cellular CMN expressed a 4.3-kb transcript also hybridizing either a full length NTRK3 cDNA probe or a probe for the NTRK3 PTK region (see Fig. 17), as is observed for CFS [356]. These data indicate that cellular CMN, but not classical CMN, CCSK, or WT, expresses identical ETV6-NTRK3 fusion transcripts as those detected in CFS.

5.2.4 FISH Analysis

We next wanted to determine whether there was a correlation in CMN between the expression of the ETV6-NTRK3 gene fusion and trisomy 11 as we had previously observed for CFS. We therefore prepared touch preparations of each CMN case and probed them with an α-centromeric chromosome 11 probe. As shown in a representative example in Fig. 18, trisomy for chromosome 11 was observed in every case which expressed ETV6-NTRK3 fusion transcripts. Trisomy 11 was never observed in CMN cases lacking this gene fusion (see Table 8), including the cellular CMN case which was RT-PCR negative.

5.3 DISCUSSION

Congenital mesoblastic nephroma (CMN) is a renal, spindle cell tumor of infancy which is subdivided into a cellular, mixed, and classical forms based on
Figure 17. Northern analysis of CMN cases. Blots were probed with a 3' NTRK3 partial cDNA probe encoding the PTK domain (NTRK3-PTK; top panel), and a β-actin cDNA probe (bottom panel) to test for equal loading. The ETV6 cDNA probe detected previously described 6.2, 4.3, and 2.4 kb transcripts in multiple samples (data not shown), including CFS and CMN cases, while NTRK3-PTK detected a 4.3 kb species only in CFS and cellular CMN cases (lanes 1 and 3; arrow). Lane 2, classical CMN; 4, Ewing's TC71; 5, SAN-2; 6, human brain RNA.
Figure 18. FISH analysis for trisomy 11. The presence of an extra copy of chromosome 11 was determined by FISH analysis on touch preparations made from primary tissue specimens. An α-centromeric 11 probe was used to probe touch preparations of all CMN cases. Shown above is a cellular CMN case with three copies of chromosome 11.
mitotic activity and degree of cellularity. Histologic and cytogenetic evidence has suggested that CMN and CFS are histogenetically related. This prompted us to screen CMN cases for the t(12;15)(p13;q25)-associated ETV6-NTRK3 gene fusion previously reported in CFS. Two of two mixed and 8 of 9 cellular CMNs were positive for the ETV6-NTRK3 gene fusion while all 4 classical CMN cases tested were negative for this alteration. We also found a striking correlation between trisomy 11 and fusion gene expression, with all CMN cases harboring the ETV6-NTRK3 gene fusion displaying an extra copy of chromosome 11 by FISH. This included two cases (cases 1 and 2, Table 8) with cytogenetically proven extra copies of chromosome 11.

Our findings strongly support the notion that cellular CMN and CFS are histogenetically related. The data do not support a relationship with CCSK or WT as has been previously proposed [224, 394, 395]. Molecular testing for ETV6-NTRK3 gene fusions therefore provides a potential modality for the diagnosis of cellular CMN. Our data also suggest that classical and cellular CMN are genetically distinct entities, as no cases with classical morphology displayed either ETV6-NTRK3 gene fusions or trisomy 11. It is tempting to speculate, as have others [403], that cellular and classical CMN represent the renal counterparts of CFS and IFB, respectively, particularly given the overlapping age ranges of these lesions. The fact that both mixed CMN cases tested in this study expressed ETV6-NTRK3 fusion transcripts lends support to the intriguing possibility that the mixed form represents a transitional stage in which distinct regions within classical CMN have acquired the
chromosomal aberrations found in cellular CMN. Tissue microdissection experiments may be useful to address this question.

It remains unclear as to how ETV6-NTRK3 expression confers a proliferative advantage to tumor cells. The gene fusion links the HLH dimerization domain of the ETV6 ETS family transcription factor to the PTK domain of NTRK3 [356]. NTRK3 is a member of the NTRK family of receptor PTKs and binds neurotrophin-3 (NT-3) with high affinity [358, 359]. NT-3 binding induces receptor dimerization and autophosphorylation of PTK tyrosine residues. These residues serve as anchors for downstream signal transduction molecules such as SHC, phospholipase Cγ1 (PLCγ1), and PI-3K [358, 359]. We have hypothesized that the ETV6 HLH domain induces ligand-independent dimerization and constitutive activation of NTRK3 signaling.

The finding that all fusion positive CMN and CFS cases demonstrate trisomy 11 suggests that this alteration also contributes to tumorigenesis. The IGFII gene, a paternally expressed member of a cluster of imprinted genes localized to chromosome 11p15.5, encodes an insulin-like growth factor expressed in certain human tumors and overgrowth syndromes [406]. It is therefore possible that some form of complementarity or synergism occurs between ETV6-NTRK3 and IGFII signaling pathways that is required for CFS or CMN tumor cells to proliferate, as has been observed for other oncogenes [407]. Further studies will be necessary to elucidate the comparative roles of these alterations in oncogenesis and to determine if this relationship is unique to tumors of very young children.
CHAPTER VI

MOLECULAR STUDIES OF THE ETV6-NTRK3 FUSION PROTEIN

6.1 INTRODUCTION

Tyrosine kinase receptors are activated through a process known as ligand mediated receptor dimerization. Briefly, after a ligand, such as a growth factor, has attached itself to the extracellular ligand binding domain of a tyrosine kinase receptor, the receptor undergoes a conformational change favoring its interaction with another similar receptor [37]. This interaction induces the cross phosphorylation of certain tyrosine moieties within the intracellular domain, thus activating the receptor-dimer complex leading to further interactions with cytoplasmic substrate proteins. The deactivation of the receptor-dimer complex by specific protein tyrosine phosphatases (PTPs) is equally important in the regulation of signal transduction [408].

When a cell acquires a chromosomal translocation, a rearrangement may be generated which produces a fusion gene (discussed in the previous chapters). This fusion gene consists of part of one gene fused to a part of another gene. In the case of ETV6 gene fusions, we saw how a translocation can result in the fusion of the HLH domain from ETV6 to the protein tyrosine kinase domain from either PDGFβR, ABL or JAK2 [316, 318-320, 409]. These fusion proteins lack the extracellular ligand binding (regulatory) domains for the tyrosine kinase receptors; instead, these domains are replaced by the HLH domain encoded by the ETV6 gene.
The HLH domain is known to induce dimerization, and therefore, acts to ablate the necessity for ligand induced activation resulting in the constitutive activation of the tyrosine kinase domain [339, 410, 411]. The downstream pathways affected by the activated tyrosine kinase domain are, therefore, constantly activated.

Other studies have shown fusion genes involving the ETV6 gene are able to heterodimerize with normal ETV6 as well as the ability to homodimerize giving rise to the concept that the introduction of constitutively activated signaling pathways or the disruption of normal ETV6 function, or a combination of both may be the source of oncogenesis [321, 412, 413].

We therefore wanted to determine the specific characteristics of the ETV6-NTRK3 gene product, including downstream interactions (similar to the ones that interact with NTRK3), dimerization status (homo- and heterodimerization) and phosphorylation status.

6.2 RESULTS

6.2.1 Expression and Phosphorylation Status of ETV6-NTRK3 and ETV6-NTRK3 Mutant Proteins in NIH3T3 Cells

Analysis of the ETV6-NTRK3 nucleotide sequence using Lasergene Navigator software (DNASTAR) estimated the molecular weight of the fusion protein to be approximately 74,300 Da. Immunoprecipitation of lysates derived from primary CFS tumor cells grown in culture, in vitro translated ETV6-NTRK3 as well as NIH3T3 cells infected with an ETV6-NTRK3 retroviral construct (supplied by D. Wai in our lab) with either the α-ETV6:HLH or α-NTRK antibodies
followed by immunoblotting analysis using the opposite antibody detected a doublet (due to the presence of two initiation sites within ETV6) in the 70-80 kDa range, confirming the presence of ETV6-NTRK3 proteins (see Fig. 19).

We were interested in determining which domains of the ETV6-NTRK3 protein (HLH domain, PTK domain, ATP binding domain, etc.) were important for transformation and what these domains were responsible for. We therefore generated a series of constructs (see Table 5 in chapter 2) which were used to infect NIH3T3 cells including: ETV6-NTRK3 (as the positive control), Vector (NIH3T3 cells transfected with MSCVpac vector alone containing no insert, as the negative control), ΔHLH (we deleted the HLH dimerization domain in ETV6-NTRK3 to test for its significance in oncogenesis), PLCγQ, PLCγT, PLCγE (the tyrosine residue specific for PLCγ1 binding was replaced with a glutamine (Q), threonine (T), or a glutamate (E) residue, respectively, to ablate the ability of PLCγ1 to bind to ETV6-NTRK3 and test for its significance in oncogenesis), Activation Loop Dead (ALD) (the three tyrosines known to be essential for autophosphorylation of NTRK3 were mutated in order to determine their importance in the oncogenic process), and KinaseDead (KD) (the ATP binding site was mutated so that tyrosine phosphorylation of the ETV6-NTRK3 protein would be ablated in order to determine the significance of tyrosine phosphorylation in the oncogenic process) [414, 415]. Of these constructs, only ETV6-NTRK3 and the PLCγQ, PLCγT, PLCγE mutants were able to transform NIH3T3 cells while cells infected with Vector,
FIGURE 19. Western blot analysis of NIH3T3 cells expressing ETV6-NTRK3 and various mutants using NTRK3 (C-14) antibody. Left panel: ETV6-NTRK3, lane 1; ALD, lane 2; PLCγQ, lane 3; PLCγT, lane 4; PLCγE, lane 5; KD, lane 6; and Vector, lane 7. Right panel: Vector, lane 1; ETV6-NTRK3, lane 2; and ΔHLH, lane 3.
ΔHLH, ALD and KD constructs appeared morphologically normal. These cells were subsequently analyzed by immunoprecipitation and immunoblotting, as described above for cells expressing ETV6-NTRK3 (see Fig. 19).

To determine the tyrosine phosphorylation status of the ETV6-NTRK3 protein and the various mutants, we immunoprecipitated lysates from various NIH3T3 cells expressing either ETV6-NTRK3 or one of the mutant constructs with anti-NTRK3 antibody and subsequently immunoblotted with the anti-phosphotyrosine antibody, RC-20. We were able to show tyrosine phosphorylation for ETV6-NTRK3, ALD, and PLCγQ, PLCγT, PLCγE, while Vector, ΔHLH, and KD failed to show any signs of tyrosine phosphorylation (see Fig. 20).

6.2.2 ETV6-NTRK3 Homodimerizes and Heterodimerizes with ETV6

To examine the possibility that ETV6-NTRK3 is capable of homodimerization, we took advantage of the high affinity between glutathione-S-transferase (GST) and glutathione-agarose beads [416]. A vector coding for a 5'-GST protein was used to generate an GST-ETV6-NTRK3 construct, which was used to transfect SF9 insect cells for the production and subsequent purification of GST-ETV6-NTRK3 protein.

We checked for homodimerization by mixing purified GST-ETV6-NTRK3 fusion protein with in vitro translated ETV6-NTRK3 radiolabeled with 35S-Methionine and glutathione beads. ETV6-NTRK3 was pulled down with the GST-ETV6-NTRK3 protein by the addition of glutathione beads, but not with glutathione beads alone suggesting that ETV6-NTRK3 is able to homodimerize (see
FIGURE 20. Immunoprecipitation and Western blot analysis demonstrating ETV6-NTRK3 tyrosine phosphorylation. A, Whole cell lysates were prepared from NIH3T3 cells expressing ETV6-NTRK3 and various mutant constructs and immunoprecipitated with NTRK3 antibody and subsequently immunoblotted with the anti-phosphotyrosine antibody, RC-20. Immunoprecipitates from cells expressing ETV6-NTRK3, ALD, PLCγQ, PLCγT, PLCγE constructs (lanes 1, and 4-7 respectively) demonstrated tyrosine phosphorylation of 73 and 68 kD bands (arrows). NIH3T3 cells expressing Vector, ΔHLH and KD (lanes 2, 3 and 8, respectively) showed no tyrosine phosphorylation. B, Presence of constructs was confirmed by reprobing the blot in panel A with an ETV6:HLH antibody (panel B) or NTRK3 antibody (not shown).
Fig. 21a). Since dimerization is thought to act through the HLH domain we tested the ability of the ΔHLH mutant (ETV6-NTRK3 lacking the HLH domain) to dimerize with either ETV6 or ETV6-NTRK3. The ΔHLH mutant did not dimerize with ETV6 nor the ETV6-NTRK3 protein (see Fig. 21b). To determine if ETV6-NTRK3 was able to heterodimerize with ETV6, we co-in vitro translated ETV6-NTRK3 and ETV6, immunoprecipitated with anti-NTRK3 antibody and immunoblotted with anti-ETV6:HLH antibody. Figure 21c shows ETV6 coimmunoprecipitating along with ETV6-NTRK3 protein suggesting that the ETV6-NTRK3 is able to heterodimerize with ETV6.

6.2.3 Downstream Interactors Affected by the ETV6-NTRK3 Molecule

NTRK3 is known to interact with specific cytoplasmic tyrosine kinases including SHC, GRB2, SH2Bβ, rAPS, PI3K, and PLCγ1 [310, 311, 417, 418]. We were interested in determining if these molecules were able to interact with the ETV6-NTRK3 fusion protein. We were interested in determining which regions of the ETV6-NTRK3 protein were important in downstream interactions. Sequence analysis of the ETV6-NTRK3 chimera determined that the SHC and PI3K interaction site were lost as a result of the position of the breakpoint, but the PLCγ1 site was retained [356]. We therefore wished to elucidate the downstream interactors with ETV6-NTRK3 by testing SHC, SH2Bβ, GRB2, PI3K, and PLCγ1 (rAPS was not included due to the lack of antibody). We lysed cells expressing the ETV6-NTRK3 protein, immunoprecipitated with anti-NTRK3 antibody, and subsequently immunoblotted with antibodies toward either SHC, GRB2,
**Figure 21.** Immunoblot analysis demonstrating H1H-domain dependent homodimerization and heterodimerization of ETV6-NTRK3. A. Metabolically labeled in vitro-translated ETV6-NTRK3 (shown in lane 1 (arrows)) was able to interact with purified GST-ETV6-NTRK3 protein and glutathione beads (lane 2), but not with glutathione beads alone (lane 3). B. Metabolically labeled in vitro-translated ΔH1H mutant protein (shown in lane 1 (arrows)) could not interact with purified GST-ETV6-NTRK3 fusion protein plus glutathione beads (lane 2) nor with glutathione beads alone (lane 3). C. Metabolically labeled ETV6-NTRK3 and wild-type ETV6 were co-in vitro translated (shown in lane 1 (arrows)) and immunoprecipitated with a-TrkC antibody and protein A-Sepharose beads (lane 2). D. Wild-type ETV6 was shown to interact with ETV6-NTRK3. E. In vitro translated ΔH1H was unable to interact with ETV6 when immunoprecipitated with NTRK3 antibody and protein A beads (lane 4). F. Protein A beads alone (lane 5). G. ΔH1H was unlabeled in these experiments due to size overlap with ETV6 (doublet in lane 6).
SH2Bβ (data not shown), PI3K or PLCγ1 (see Fig. 22). Of these molecules, only PLCγ1 coimmunoprecipitated with ETV6-NTRK3 as well as with the ALD mutant. Similar analysis of the other ETV6-NTRK3 mutants, including Vector, PLCγQ, PLCγT, PLCγE, ΔHLH, and KD failed to coimmunoprecipitate PLCγ1 (see Fig. 23). None of the constructs, including ETV6-NTRK3, were able to coimmunoprecipitate SHC, GRB2, PI3K or SH2Bβ.

6.2.4 Subcellular Localization

We were interested in the subcellular localization of the ETV6-NTRK3 fusion protein as this would help explain its mechanism of action as an oncogenic molecule within the cell. Cells infected with ETV6-NTRK3, ΔHLH, KD, or Vector constructs were fixed in paraformaldehyde, incubated with anti-NTRK3 antibody followed by another incubation with rhodamine-anti-rabbit and subsequently analyzed by confocal microscopy. Our preliminary results suggest that ETV6-NTRK3 is mainly localized within the cytoplasm with low amounts in the nucleus. Similar results were obtained for ΔHLH and KD, while Vector showed relatively little fluorescence and was used as the negative control (see Fig. 24).

6.3 DISCUSSION

The ETV6 gene has been shown to be involved in numerous translocations giving rise to various gene fusions in human leukemias (see Table 7, Chapter 3).
FIGURE 22. ETV6-NTRK3 interacts with PLCγ but not with SHC, GRB2, or PI-3K p85 subunit. Whole cell lysates were prepared from a human medullary thyroid carcinoma cell line overexpressing wild-type NTRK3 (lane 1), as well as from NIH3T3 cells expressing ETV6-NTRK3 (lane 2), Vector (lane 3), or KD (lane 4). Immunoprecipitation was performed with antibodies against the NTRK3 PTK domain followed by immunoblotting with antibodies directed against SHC, GRB2, PI-3K, or PLCγ as indicated. Only wild-type NTRK3 was found to associate with SHC, GRB2, and PI-3K (Lanes 1 of top three panels), while both NTRK3 and ETV6-NTRK3 bound PLCγ (lanes 1 and 2 of bottom panel).
FIGURE 23. Analysis of PLCγ binding mutants for ability to associate with PLCγ1. Whole cell lysates were prepared from control human medullary thyroid carcinoma cells overexpressing wildtype NTRK3 (lane 1), or NIH3T3 cells (lanes 2-9) expressing Vector (lane 2) ΔHLH (lane 3), ETV6-NTRK3 (lane 4), ALD (lane 5), PLCγQ, PLCγT, and PLCγE (lanes 6-8 respectively), and KD (lane 9). The arrow shows the position of PLCγ1.
FIGURE 24. Confocal microscopy of ETV6-NTRK3 and ΔHLH expressing NIH3T3 cells. NIH3T3 cells expressing either ETV6-NTRK3 (panels A and B) and ΔHLH (panels C and D) were probed with either α-NRTK3 antibody (panels A and C) or with α-ETV6:HLH antibody (panels B and D). When probed with α-NTRK3 antibody, confocal microscopy showed localization of ETV6-NTRK3 and ΔHLH to the cytoplasm with relatively low amounts in the nucleus. Probing with α-ETV6:HLH antibody detected wild type ETV6 in addition to ETV6-NTRK3 and as a result showed more intense nuclear staining.
Most of the gene fusions encoding the HLH domain of *ETV6* as the 5' end, possess sequences encoding a tyrosine kinase domain as the 3' end [320, 409, 419]. This is expected to result in constitutive activation of the PTK domain due to the ligand independent dimerizing capabilities of the HLH domain.

The recently characterized *ETV6*-NTRK3 gene fusion in CFS and cellular CMN was identified in our studies predominantly as a protein doublet in lysates derived from NIH3T3 cells infected with either an ETV6-NTRK3 construct or one of the mutants. This can, in part, be explained by the fact that the *ETV6* gene has two transcription initiation sites. Following the translation of these two species, post-translational modifications (primarily tyrosine phosphorylation of the NTRK3 portion) can further divide each of the two bands producing a total of four bands (two unphosphorylated and two phosphorylated). A study analyzing the ETV6 and ETV6-CBFA2 fusion protein identified this fusion protein as several different species due to alternative initiation sites in the *ETV6* gene in addition to the post-translational modification of these proteins [338, 344]. Further studies are still needed to clarify the role of each species in the oncogenic process.

The ΔHLH and KD mutants were unable to transform NIH3T3 cells and did not show any sign of being tyrosine phosphorylated. The only difference between KD and ETV6-NTRK3 is a single amino acid (lysine 380 to an arginine), that blocks ATP binding which is crucial for PTK activity. Previous attempts to inactivate the ATP binding site in NTRK molecules showed similar results as the NTRK molecule was no longer able to tyrosine phosphorylate (even after ligand-induced dimerization) and interact with downstream molecules [420]. This indicates that an
intact PTK domain is essential for transformation. The ΔHLH mutant lacks most of the helix loop helix domain from exons 3 and 4, which has been shown to be important in dimerization. Dimerization is essential for bringing the tyrosine kinase domains in close proximity so that they can phosphorylate each other, suggesting that the HLH domain is critical in the process of activation of the tyrosine kinase domain in the ETV6-NTRK3 fusion. The ΔHLH and KD mutants, therefore, provide evidence that dimerization and tyrosine phosphorylation are both required for transformation of NIH3T3 cells.

We were able to show that the ETV6-NTRK3 can homodimerize as well as heterodimerize with ETV6. ETV6-NTRK3 heterodimerization with ETV6 might ablate the normal function of ETV6 by interfering with its DNA binding potential. There has been some evidence suggesting that ETV6 may actually be a tumor suppressor gene [421-423]. Furthermore, we were able to show that the ETV6-NTRK3 fusion protein was predominantly localized within the cytoplasm with lower amounts in the nucleus. Further studies are necessary to determine the role of dimerization and oncogenesis in these tumors.

Our results suggest that most of the known signaling interactors with NTRK3 (namely, SHC, GRB2, PI3-K, and SH2Bβ) do not associate with the ETV6-NTRK3 molecule. Phospholipase C-γ1 (PLCγ1) was, however, able to interact with the ETV6-NTRK3 fusion protein. PLCγ1 is a member of the family of inositol phospholipid phosphodiesterases [424]. There are a total of three members to this family including β, γ, and δ. PLCγ is activated by the intracellular tyrosine kinase
domains of its respective receptors [425]. Activation of PLCγ leads to the production of intracellular second messengers inositol (1, 4, 5)-triphosphate (InsP$_3$) and sn 1, 2-diacylglycerol (DAG) [424].

At the amino terminus of the PLC molecules is a structure known as the pleckstrin homology domain (PH) responsible for facilitating binding of PLCγ to PtdInsP$_2$ and other inositol phosphates [426]. The PLC molecules also contain an EF Hand domain which is thought to bind Ca$^{++}$, as these molecules are Ca$^{++}$ dependent enzymes [427]. Deletion of the EF Hand abrogates activity [428]. The two most highly conserved structures in mammalian PLC molecules are the X and Y boxes. These structures are essential for activity and may also determine which substrates and subsequent reactions the molecule can support [429]. On the tertiary level, these boxes form a structure known as a TIM barrel. Within the TIM barrel are PH, SH2 and SH3 domains responsible for the specific interactions with substrate molecules such as PtdInsP$_2$ (SH2 domains were first discussed in Chapter 1 as structures essential for the interaction with phosphorylated tyrosines) [430]. The last domain in PLCγ (conserved and present in PLCβ and δ) is the C2 domain, which is Ca$^{++}$ dependent and seems to act as an interface between the EF Hand and the TIM barrel catalytic domain [431, 432].

PLCγ1 is a ubiquitously expressed tyrosine kinase substrate responsible for the control of intracellular levels of Ca$^{++}$ and DAG [433]. After tyrosine phosphorylation of PLCγ1, the molecule translocates to the membrane where the PH domains recognize PI 4, 5-P$_2$ and Ins 1, 4, 5-P$_3$ as ligands. The deactivation by
phosphatases is evident, but unclear. In addition to tyrosine phosphorylation, the PLCγ1 molecule undergoes serine-threonine phosphorylation, but the kinases and phosphatases of this system have not been characterized yet. The main reaction catalyzed by PLCγ1 is the conversion of PtdIns 4, 5-P$_2$ to DAG and Ins 1, 4, 5-P$_3$. Ins 1, 4, 5-P$_3$ is responsible for stimulating the secretion of Ca$^{++}$ from the endoplasmic reticulum [433]. Ins 1, 4, 5-P$_3$ is responsible for tethering protein kinase C (PKC) to the membrane while DAG acts as a potent activator of PKC [433]. There has been some evidence suggesting that PKC then activates the MAPK pathway through RAF and can ultimately result in an increase in cellular proliferation (in undifferentiated cells) and other cellular responses such as contraction, secretion and membrane conductance (in differentiated cells) [434, 435].

Our studies showed that there is no loss in the transformation capabilities of non-PLCγ1 binding ETV6-NTRK3 mutants, suggesting that PLCγ1 signaling may not contribute to ETV6-NTRK3 transformation. Our results failed to show an association between ETV6-NTRK3 and known NTRK3 interactors such as SHC, GRB2, PI3-K, and SH2BB. These results, however, do not rule out that these molecules are involved indirectly in ETV6-NTRK3 transformation activity. Other adaptor molecules may link ETV6-NTRK3 to known NTRK3 signaling pathways. These molecules must be assayed directly to assess their possible roles in ETV6-NTRK3 signaling. Alternatively, a completely novel pathway may be involved. The increase in proliferation and transformation seen in ETV6-NTRK3 infected NIH3T3 cells can be explained by the fact that some other molecule is able to
associate with ETV6-NTRK3 and activate proliferative and/or cell survival signal transduction pathways. The possibility that the ETV6-NTRK3 fusion is interacting with normal ETV6 and dysregulating it is unlikely, since the KD mutant is capable of dimerizing with normal ETV6 (data not shown), but is non-transforming. In view of this, the ΔHLH mutant is non-transforming because of its inability to dimerize and activate the tyrosine kinase domain and not because of its inability to dimerize and interfere with normal ETV6 function. Further studies such as yeast two hybrid screening will be necessary (and are currently being performed in our laboratory) for characterizing new and essential downstream interactors with the NTRK3 portion (and possibly the ETV6 portion) of the ETV6-NTRK3 chimeric fusion.
CHAPTER VII
SUMMARY AND CONCLUSIONS

The dilemma in finding a cure for cancer is that there will not be one cure for all cancers, but rather a specific conglomerate of approaches for each individual cancer. Developing treatment protocols for a specific cancer would require a number of factors. The first requirement, which would be crucial in determining the course of action for a particular tumor, is the ability to accurately differentiate and diagnose that tumor from other tumors. Secondly, an in-depth knowledge of the molecular basis for tumorigenesis of the specific tumor is essential. Once the tumor has been characterized at the molecular level, the knowledge on how it has evolved and maintains itself needs to be integrated in order to develop a treatment strategy. The treatment protocol would need to target tumor cells while leaving normal cells unharmed.

We have accomplished in part the first two tasks with congenital fibrosarcoma (CFS), a soft tissue pediatric spindle cell lesion. CFS is difficult to diagnose because of its histologic similarity with adult type fibrosarcoma, aggressive fibromatosis and infantile fibromatosis. Cytogenetic analysis on a series of CFS, ATFS, AFB and IFB cases revealed a recurring rearrangement involving chromosomes 12 and 15 only in CFS. Whole chromosome FISH of a CFS case using a chromosome 12 painting probe revealed a portion of chromosome 12 on a smaller acrocentric chromosome. This rearrangement was not seen in normal
fibroblastic tissues. This warranted the characterization of the rearrangement on a molecular level, in hopes of finding a recurring molecular marker of CFS which would be an invaluable tool for the pathologist.

7.1 IDENTIFICATION OF A RECURRING t(12;15) in CONGENITAL FIBROSARCOMA

Cytogenetic analysis revealed a rearrangement involving chromosomes 12p13 and 15q25-q26. FISH analysis using a combination of α-centromeric chromosome specific probes and region specific YAC probes identified a translocation of material from chromosome 12p13 to chromosome 15q26. One of the YACs, namely 817_H_1, was found to be split, indicating that it spanned the breakpoint and contained the gene involved. This YAC was known to harbor the ETV6 gene, which has been rearranged in numerous hematopoietic malignancies. High resolution FISH mapping using cosmids specific for certain exons of the ETV6 gene placed the breakpoint within the ETV6 gene thus confirming its involvement. ETV6 belongs to the ETS family of transcription factors and has been shown to be involved in gene fusions in hematopoietic malignancies. The involvement of a gene in both hematopoietic malignancies and sarcomas has been described previously, as the ERG and FLI1 genes (other ETS family members) are found to be fused with two similar genes, EWS and TLS/FUS, as a result of chromosome translocations in human solid tumors and leukemias.
7.2 **THE ETV6-NTRK3 GENE FUSION CHARACTERIZES CONGENITAL FIBROSARCOMA**

We hypothesized that ETV6 was involved in a gene fusion in CFS. The ETV6 gene is known to be oriented in a telomere to centromere fashion on chromosome 12p13. Therefore the t(12;15) in CFS is expected to result in the translocation of 5' ETV6 material to chromosome 15. We therefore performed 3' rapid amplification of cDNA (3'RACE) using known ETV6 sequence primers to amplify and clone the breakpoint. Sequence analysis of RACE products revealed 5' ETV6 sequence until nucleotide 1033, which corresponds to the last nucleotide in ETV6 exon 5. The remaining sequence was compared to public databases and was 100% homologous to a portion of the NTRK3 gene. The rearrangement was confirmed by Southern analysis and the presence of an ETV6-NTRK3 gene fusion was confirmed by Northern analysis and RT-PCR. As this is one of two recurring chromosomal abnormalities along with trisomy 11, it was hypothesized that this fusion gene is etiologic in CFS oncogenesis.

7.3 **TRISOMY 11 AND THE ETV6-NTRK3 GENE FUSION LINK CONGENITAL FIBROSARCOMA TO CONGENITAL MESOBLASTIC NEPHROMA**

Congenital mesoblastic nephroma (CMN) is an infantile spindle cell tumor of the kidney which has an excellent prognosis similar to that of CFS. CMN is subdivided into classic and cellular forms depending on the degree of cellularity and mitotic activity of the spindle cells. The cellular variant is virtually identical histologically and cytogenetically to CFS, and this morphologic overlap has led to
the hypothesis that these tumors are histogenetically related. Cytogenetic studies have reported common trisomies in CFS and cellular CMN, particularly of chromosome 11.

We analyzed CMN cases and found that the t(12;15)(p13;q25) rearrangement in CFS is also present in cellular CMN and may underlie the distinctive biological properties of these two tumors. Analysis of mRNA revealed the expression of ETV6-NTRK3 chimeric transcripts in 8 of 9 cellular CMN cases as well as in 2 of 2 mixed CMN cases. Four of four classical CMN cases were negative for the ETV6-NTRK3. In addition, we found trisomy 11 to be strongly correlated with the presence of the ETV6-NTRK3 gene fusion.

Our studies therefore indicate that congenital fibrosarcoma and cellular congenital mesoblastic nephroma are histogenetically related. Table 9 summarizes the results of RT-PCR analysis on a series of CFS, CMN, ATFS, IFB, and AFB primary tumor samples to date. Briefly, the ETV6-NTRK3 fusion was found in 100% of CFS cases (n=15), 90% of cellular CMN cases (n=10) and 100% of mixed CMN cases (n=2) analyzed. Fusion transcripts were not detected in ATFS (n=10), IFB (n=12), and AFB (n=5) cases, nor in classical CMN cases analyzed (n=4). Only one of ten cellular CMN cases was negative for the fusion transcript.

7.4 MOLECULAR STUDIES OF THE ETV6-NTRK3 FUSION PROTEIN

Understanding the oncogenic process requires the familiarization with the complex biochemical interactions within the tumor cell. We generated a full length ETV6-NTRK3 construct encoding the helix-loop-helix (HLH) dimerization

<table>
<thead>
<tr>
<th>TUMOR</th>
<th>NUMBER ANALYZED</th>
<th>ETV6-NTRK3 FUSION POSITIVE</th>
<th>ETV6-NTRK3 FUSION NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>ATFS</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>IFB</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>AFB</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CMN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Classical</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>2. Cellular</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>3. Mixed</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

**Abbreviations.** CFS, congenital fibrosarcoma; ATFS, adult-type fibrosarcoma; IFB, infantile fibrosarcoma; AFB, aggressive fibrosarcoma; CMN, congenital mesoblastic nephroma.
domain of ETV6 fused to the protein tyrosine kinase (PTK) domain of NTRK3. NIH3T3 cells were infected with recombinant retroviral viruses carrying either the full-length ETV6-NTRK3 cDNA or one of the mutants (kindly provided by Daniel Wai in our laboratory). Cells expressing the ETV6-NTRK3 construct exhibited a transformed phenotype and formed macroscopic colonies in soft agar. We hypothesized that chimeric proteins mediate transformation by dysregulating NTRK3 signal transduction pathways via ligand-independent dimerization and PTK-autophosphorylation. To test this hypothesis, a series of different mutants were generated to help determine which regions of the ETV6-NTRK3 fusion protein were necessary for oncogenesis. We showed that ETV6-NTRK3 homodimerizes and is capable of forming heterodimers with wild-type ETV6 \textit{in vitro}. The HLH domain of ETV6 in the ETV6-NTRK3 fusion was deleted in order to investigate the role of protein dimerization in transformation (ΔHLH mutant). The ΔHLH mutant was not able to associate with ETV6, nor with ETV6-NTRK3. Cells expressing this mutant protein were morphologically non-transformed and failed to grow in soft agar. To investigate the role of the NTRK3 PTK domain, NIH3T3 cells were transfected with a variety of ETV6-NTRK3 mutants with activation loop amino acid substitutions (ALD) as well as a kinase inactive mutant unable to bind ATP (KD). The three PTK activation-loop tyrosines mutated (ALD) to phenylalanines still became tyrosine phosphorylated but were unable to transform NIH3T3 cells. The KD mutant failed to autophosphorylate and lacked transformation ability. Of a series of signaling molecules well known to bind to wild-type NTRK3, only PLCγ1 was found to associate with and become tyrosine
phosphorylated by ETV6-NTRK3. Interestingly, several PLCγ1 binding mutants were unable to bind PLCγ1, but were still capable of transforming NIH3T3 cells suggesting that another pathway is being activated and is responsible for the transforming abilities of ETV6-NTRK3. In addition, preliminary subcellular localization studies showed that the ETV6-NTRK3 fusion protein localizes mainly in the cytoplasm, with limited presence in the nucleus. Our studies confirm that ETV6-NTRK3 is a transforming protein that requires both an intact dimerization domain and a functional PTK domain for transformation activity.

7.5 GENERAL COMMENTS

The discovery of the ETV6-NTRK3 gene fusion in CFS and cellular CMN lead us to screen other cancers for the same gene fusion. Interestingly, we were able to detect the ETV6-NTRK3 gene fusion in a breast carcinoma from a 6 year old patient. Cytogenetic analysis on this case confirmed the presence of the t(12;15)(p13;q25) but failed to show further evidence of any other chromosomal abnormalities including trisomy chromosome 11. Our transfection studies have shown that the ETV6-NTRK3 gene fusion product has transformation ability, which supports the notion that the breast carcinoma may have arisen solely due to the gene fusion. Another group has recently identified the ETV6-NTRK3 gene fusion in an adult acute myeloid leukemia [436]. The ETV6 gene has been implicated in numerous hematopoietic rearrangements, but this is the first report of NTRK3 involvement with a leukemia. This makes the ETV6-NTRK3 gene fusion the only documented gene fusion to date which has been involved in both a
solid tumor as well as a leukemia. The ETV6-NTRK3 gene fusion may therefore have a wider spectrum of involvement in human malignancies. Further studies are required to test this possibility in detail.

Recent evidence suggests that the ETV6 central domain (previously thought to contain no known domains) mediates transcriptional repression by associating with SMRT and mSin3A, while the ETV6 HLH domain represses gene transcription through a mechanism that is independent of known corepressors [437]. This domain is a part of the ETV6-NTRK3 gene fusion. Further studies are therefore needed to determine if there are other molecules interacting with ETV6-NTRK3 which are responsible for oncogenesis. This is currently being explored in our laboratory by the use of yeast-2-hybrid screening.

Future studies, some of which are being explored currently in our laboratory, include elucidating the signal transduction pathways which are being utilized by ETV6-NTRK3. To look for other interactors with ETV6-NTRK3, yeast-2-hybrid screening is currently being used to identify novel interactors. To help increase the resolution of the yeast two hybrid approach, the ETV6-NTRK3 has been divided into two so that interactors with the ETV6 portion and the NTRK3 portion can be studied independently. Specific inhibitors of signaling pathways (e.g., wortmannin specifically inhibits PI-3K; PD98059 inhibits MEK1, and U73122 inhibits PLC molecules) should be tested to determine their impact on tumor growth and whether or not they can be used to treat CFS and cellular CMN in patients. Similarly further research is needed to see if NTRK specific inhibitors could be used to treat CFS and cellular CMN (e.g., K252a and CEP-751 are effective NTRK tyrosine
kinase inhibitors [438, 439]). To explore the possibility that the dimerization domain of ETV6 is dimerizing with other molecules or contributing other functions to the oncogenic process, the 3'-portion of NTRK3 involved in the CFS translocation has recently been fused to an inducible dimerization domain, FKBP$_{36v}$ [440, 441], in our laboratory. This will allow us to control dimerization and therefore the oncogenic activity of the NTRK3 portion of ETV6-NTRK3 in NIH3T3 cells and will provide valuable information on the proliferative and transformation process. For example, if the introduction of the artificial dimerization domain results in the failure of transformation, then a possible explanation might be that the ETV6 portion of ETV6-NTRK3 contributes an important domain that is required for proper transformation ability. Finally, some of the most useful information can be derived from in vivo models. Currently our laboratory is exploring the oncogenic activity of ETV6-NTRK3 in transgenic mice.
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