Assessing the prevalence of a novel oncogene, EML4-NTRK3, in archival fibrosarcoma samples

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Review of Literature

The Role of Chromosomal Translocations in the Transformation of Cells and the Initiation of Tumorigenesis

Chromosomal rearrangements are mechanisms that can lead to abnormalities in cells. These rearrangements vary in both size and complexity and encompass distinctive varieties of events including chromosomal translocations (Liu, et al., 2011). The chromosomal translocation is defined as the interchange of parts between two non-homologous chromosomes (Nambiar, et al., 2008). While a selected amount of these rearrangements are benign, others may lead to altered expression of proteins and the development of cancers (Liu, et al., 2011). Myriad researchers thus hypothesize that the potential cause of malignancy results from gene fusions due to chromosomal translocations (Hashemi, et al., 2013).

Chromosomal translocations have been identified in a plethora of cancer types, including congenital fibrosarcoma. Congenital fibrosarcoma, also known as infantile fibrosarcoma, is a rare soft tissue tumor (Hashemi, et al., 2013). This pediatric spindle cell tumor often occurs in infants less than two years of age (Fisher et al., 1996) and accounts for 10% of all sarcomas in the adolescent population (Hashemi, et al., 2013). This disease rarely metastasizes (less than 10%) (Obermeier et al., 1993a) (Fisher et al., 1996), has a good prognosis (Grier, et al., 2006), and the survival rate is approximately 80%-90% (Lambelle et al., 1993).

The chromosomal translocation has been identified as the potential cause of malignancy in infantile fibrosarcoma. One chromosomal translocation that has been

identified in a multitude of patients with infantile fibrosarcoma is ETV6-NTRK3 t(12;15)(p13;q25) (Knezevich, et al., 1998).

The ETV6 gene is located on chromosome twelve, on band 12p13 (E. De Braekeleer, et al., 2014) and is a member of the ETS family, which is a large family of transcriptional factors (Seth et al., 1992). It has been hypothesized that these transcriptional factors are thought to influence angiogenesis and early hematopoiesis (Baens et al., 1996) (Edel, 1998) (Kwiatkowski et al., 1998) (O'Connor et al., 1998) (Poirel et al., 1997) (Wang LC et al., 1997). The ETV6 protein is a 452 amino acid polypeptide (Mavrothalassitis G, et al., 2000) that contains two major domains. Exons three and four include the HLH (helix-loop-helix) domain, and exons six through eight include the ETS domain (De Braekeleer et al., 2012). Recent studies have concluded that the ETV6 gene is vital for standard development and for sustaining blood vessel integrity (Wang LC, et al., 1997). Studies have also detected 48 chromosome bands that are involved in ETV6 translocations, and scientists have molecularly characterized 30 ETV6 partner genes as well (De Braekeleer et al., 2012). There are a variety of genes that have been spliced to be in frame with the ETV6 gene on particular chromosomes. Some rearrangements include ETV6 fusions with other genes such as ABL (Papadopoulos et al., 1995), MN1 (Buijs et al., 1995), JAK2 (Lacronique et al., 1997) (Peeters et al., 1997a), and NTRK3 (Wai, et al., 2000).

The NTRK3 gene is located on chromosome 15q 25 and is encoded by 20 exons (Knezevich, 2004) (ETV6 Gene, 2014). The domains for the NTRK3 gene consist of various regions. Beginning at the amino terminus is the Signal Sequence. This region is reliable for leading the newly translated protein to the cell surface (Knezevich, 2004). The next region is the Extracellular Ligand Binding Domain (Knezevich, 2004).

Following that area is the Transmembrane Domain (Knezevich, 2004). Lastly, the Intracellular Domain consists of the Protein Tyrosine Kinase (PTK), which has been identified as an activator of molecule down-streaming (Knezevich, 2004). This gene encodes for the transmembrane protein receptor for neurotrophin-3 and is involved in cell growth and the development of the central nervous system (Barbacid, 1995a) (Conover and Yancopoulos, 1997) (Ebadi et al., 1997) (Ichaso et al., 1998) (Lamballe et al., 1991) (McGregor et al., 1994). This gene, in addition, has been detected to fuse with other genes. One of the most common protein fusions associated with NTRK3 is its fusion with ETV6 in infantile fibrosarcoma cases (Henno, et al., 2003).

The ETV6-NTRK3 chromosomal translocation fuses the N-terminal SAM domain of EVT6 to the C-terminal PTK domain of NTRK3 (Lannon, et al., 2005). Several in-vitro and in-vivo studies have been performed to confirm this chromosomal rearrangement's role in tumor transforming activity. Fluorescent in Situ Hybridization (FISH) assays have been performed to localize the breakpoint. In a study conducted, results indicated that ETV6 was disrupted between exon one and eight and that exon one was translocated to chromosome 15q25 (Knezevich, et al., 1998). Another FISH analysis study also confirmed the detection of ETV6-NTRK3 fusion signal in congenital fibrosarcoma cell samples (Watanabe et al., 2002). Reverse Transcription Polymerase Chain Reaction (RT-PCR) was also conducted that detected the ETV6-NTRK3 fusion transcript (Knezevich, et al., 1998) (Watanabe et al., 2002). Lastly, to determine this rearrangement's transforming activity, studies were conducted including soft agar assays, in-vitro experiments, Western blots, and analysis of tumor formation in experiments involving live mice (Wai et al., 2000). In one study described, NIH3T3 cells were infected with

recombinant retroviral vectors carrying the full length ETV6-NTRK3 cDNA (Wai et al., 2000). The results of this study, as confirmed by Western blot analysis, revealed protein expression of ETV6-NTRK3 in infected cells. In the soft agar assay involving the NIH3T3 cells infected with the gene of interest, ETV6-NTRK3, it was shown that macroscopic cell colonies had grown. Severely Immuno-deficient mice that had been injected with the ETV6-NTRK3 chromosomal translocation revealed tumor growth, which further confirmed the hypothesis that this chromosomal rearrangement leads to a malignant phenotype (Wai et al., 2000). In other experiments, this fusion protein has been confirmed to transform other types of cells including fibroblastic cells (Wai, et al., 2000), hematopoietic cells (Liu et al., 2000), and breast epithelial cells (Tognon et al., 2002).

The ETV6-NTRK3 translocation is prevalent in many congenital fibrosarcoma cases (Triche et al., 2002) and may explain the distinct characteristics and properties of tumors in this disease (Knezevich, et al., 1998). Over the past few years, research has been able to identify this translocation as a diagnostic marker that leads to oncogenesis and exhibits a transformed phenotype of cells.

A prevalent chromosomal translocation that plays a major role in pathogenesis of solid tumors is the EML4-ALK fusion gene (Mano, 2008). Soda, et al. identified this credible driver mutation in clinical specimens using direct sequencing (Soda, et al., 2007) (Robesova, et al., 2014) (Horn, et al., 2009). The EML4 gene is located on chromosome 2p21 and ALK is located on chromosome 2p23 (Perner, 2008). The inversion of chromosome 2 fuses the ALK gene with the EML4 gene. Variant one is described as the most frequent fusion, occurring 49.6% of all positive cases (Robesova, et al., 2014). Variant one fuses EML4 (exon 13) to ALK (exon 20). Other variants described include

variable truncations of ALK (exon 20) to EML4 (exons 2,6,13,14,15,18,20) (Horn et al., 2009) (Robesova, et al., 2014). This rearrangement has been detected in and is novel to non-small cell lung cancers (NSCLCs) (Robesova, et al., 2014) (Horn, et al., 2009) (Perner, 2008). NSCLCs harboring the EML4-ALK fusion have been confirmed to show oncogenic transformation both in-vivo and in-vitro studies (Alí, 2013). In a clinical study, the EML4-ALK fusion gene was detected in 4% of individuals with NSCLC (Choi, et al., 2008). In addition, another case study identified 33 out of 96 specimens (34%) with NSCLC, harboring the EML4-ALK rearrangement (Robesova, et al., 2014). An in-vivo assay of tumorigenicity confirmed the expression of the gene fusion and transformation potential of the translocation in nu/nu mice (Choi, et al., 2008). An established report of this translocation reveals the potent oncogenic activity of the EML4-ALK fusion.

This research aims to identify other chromosomal translocations that lead to tumor formation. Confirmation of these oncogenic rearrangements will lead to advanced studies to classify the phenotypes of cancer tumors and the mechanisms that contribute to oncogenesis (Mercado, et al., 2006).

Abstract

In a case study involving a patient with non-classical infantile fibrosarcoma, molecular characterization revealed a novel somatic t(2;15)(2p21;15q25) chromosomal translocation, fusing EML4 and NTRK3. Six months after complete surgical resection of the primary tumor, bulky bilateral pulmonary metastasis of the lesion was detected (Tannenbaum et al., 2015). The patient was subsequently treated with a full course of chemotherapy and radiation. The patient was responsive to the treatment and is currently in long-term remission.

In an RT-PCR assay, 25 fibrosarcoma patients' tumor specimens were studied for the presence of EML4-NTRK3. Preliminary assessment was hypothesized to reveal recurrent prevalence of EML4-NTRK3 in patients presumably diagnosed with fibrosarcoma. After analysis of five RT-PCR assays, results presented likely detection of the fusion gene in selected specimens, but inconsistent results ensued incomplete conclusions in this portion of the study. This fusion is hypothesized to be a potent oncogenetic driver responsible for the malignant phenotype of cells in this disease. Since detection is probable in fibrosarcoma cases evaluated in this study, EML4-NTRK3 seems to be a likely marker for patients with similar diagnosis to receive a potentially alternate chemotherapy-based treatment course, divergent from standard surgery to increase their chances of survival.

Methods

Clinical Samples

25 de-identified fibrosarcoma patients' tumor specimens were used in this study. Samples were stored in cool conditions until used. RNA was extracted by Qiasymphony FFPE protocol (100 µl elution for all samples except #25- 50 µl elution) and miRNeasy FFPE kit. Specimens were prepared for performance of RTPCR.

cDNA Synthesis for 25 Patient Archival Samples

RNA was defrosted and placed on ice while cDNA Synthesis Master Mix was made for 26 samples. Each component of the Master Mix was centrifuged briefly (20 seconds) before use. To prepare cDNA synthesis master mix, 52µl of 10X RT Buffer was combined with 104µl 25mM $MgCl_2$, 52µl 0.1M DTT, 26µl RNase Out, and 26µl Superscript III RT. After preparation, the cDNA Synthesis Master Mix was chilled on ice until further use. Next, the RNA mix was prepared for each of the 25 samples. For sample FS1, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 6.3µl of RNA, and 0.7µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS2, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 3.8µl of RNA, and 3.2µl of DEPC-treated water, were combined in a 0.2 ml tube. For sample FS3, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 5.5µl of RNA, and 1.5µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS4, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 5.1µl of RNA, and 1.9µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS5, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 2.0µl of RNA, and 5.0µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS6, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.1µl of RNA, and 2.9µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS7,

2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.1µl of RNA, and 2.9µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS8, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.3µl of RNA, and 2.7µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS9, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 2.8µl of RNA, and 4.2µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS10, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.5µl of RNA, and 2.5µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS11, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 3.3µl of RNA, and 3.7µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS12, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 1.6µl of RNA, and 5.4µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS13, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 1.1µl of RNA, and 5.9µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS14, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 3.6µl of RNA, and 3.4µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS15, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 1.6µl of RNA, and 5.4µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS16, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.7µl of RNA, and 2.3µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS17, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 4.9µl of RNA, and 2.1µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS18, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 7.3µl of RNA, and 0µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS19, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 3.5µl of RNA, and 3.5µl of DEPC-treated

water were combined in a 0.2 ml tube. For sample FS20, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 1.4µl of RNA, and 5.6µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS21, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 1.2µl of RNA, and 5.8µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS22, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 2.1µl of RNA, and 4.9µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS23, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 2.6µl of RNA, and 4.4µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS24, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 5.4µl of RNA, and 1.6µl of DEPC-treated water were combined in a 0.2 ml tube. For sample FS25, 2µl of 50ng/µl random hexamers, 1µl of 10mM dNTP mix, 0.4µl of RNA, and 6.6µl of DEPC-treated water were combined in a 0.2 ml tube. All of the 0.2 ml 25 specimen tubes of RNA mix were denatured at 65ºC for 5 minutes in the Thermo Cycler. 10 µl of cDNA Synthesis Master Mix was added to each of the 25 samples after denaturation. All the samples were then placed in the Thermo Cycler at 20ºC for 10 minutes, 50ºC for 50 minutes, 85ºC for 5 minutes, and 4ºC until the samples were taken out. The reactions were collected and were briefly centrifuged. 1µl of RNase H was added to each of the 25 tubes and all were incubated for 20 minutes at 37ºC. All samples were put in -20ºC and stored.

PCR for 25 Archival Samples

Platinium Blue PCR Supermix and all 25 samples were removed from the -20ºC freezer. In addition, Forward 1 Primer and Reverse 2 Primer were removed from the 4ºC freezer and were defrosted. Next 22µl of PCR Master Mix, 1µl of Reverse 2 Primer, 1µl of Forward 1 Primer, and 1µl of cDNA was combined in a 0.2 ml PCR tube (repeated for

each of the 25 specimens). After, PCR ran on the Thermo Cycler for 2 minutes at 94ºC, 45 seconds at 94ºC, 45 seconds at 58ºC, 1 minute for 68ºC, 7 minutes at 68ºC, and ∞ at 4ºC for a total of 32 cycles. When PCR program terminated, all 25 samples were placed on ice.

Gel Preparation & Sample Loading

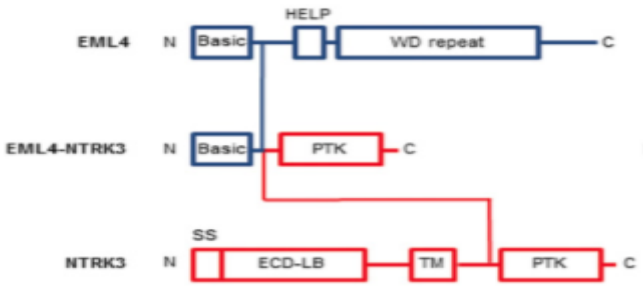
1% agarose gels were prepared and placed in gel cassettes. TAE buffer was added and gels were fully submerged. Next, 9µl of EtBr was added to buffer. 11µl of Trackit 1kb Plus DNA ladder (St.) was loaded into the first well. 15µl of each of the 25 samples (P1-P25) were loaded into wells. 15µl of each positive control was loaded into consecutive wells- (L-1) and (HBM). 15µl of each negative control was loaded into consecutive wells- (W), (S.M.), (S.B), (ASPS), (SKNEP), (SKNMC), and (EWS). The gels ran on 100V for approximately 30 minutes. The gels were then removed from the cassette and placed on a UV box for analysis of results.

Discussion/Results

Primer Selectivity

An optimal primer sequence for the EML4-NTRK3 genetic code (1290 bp) was designed and utilized for maximal specificity in PCR. For successful amplification, primers NTRK3 Reverse 2 and EML4 Forward 1 were selected. In this PCR assay, annealing occurred between the primers and complementary DNA sequences in the EML4-NTRK3 template, which encompassed approximately 960 bp.

Figure 1A: EML4-NTRK3 Gene Structure



(Tannenbaum et al., 2015)

Figure 1B: EML4-NTRK3 Coding Sequence (1290 bp)

Red Sequence = EML4 (Exons 1-2)
Blue Sequence = NTRK3 (Exons 14-19)

ATGGACGGTTTCGCCGGCAGTCTCGATGATAGTATTTCTGCTGCAAGTACTTCTGATGTTCAAGATCGCCTGTCAGCTCT
TGAGTCACGAGTTCAGCAACAAGAAGATGAAATCACTGTGCTAAAGGCGGCTTTGGCTGATGTTTTGAGGCGTCTTGCAA
TCTCTGAAGATCATGTGGCCTCAGTGAAAAAATCAGTCTCAAGTAAAGGTCCCGTGGCTGTCATCAGTGGTGAGGAGGAC
TCAGCCAGCCCACTGCACCACATCAACCACGGCATCACCACGCCCTCGTCACTGGATGCGGGGCCCGACACTGTGGTCAT
TGGCATGACTCGCATCCCTGTCATTGAGAACCCCCAGTACTTCCGTCAGGGACACAACTGCCACAAGCCGGACACGTATG
TGCAGCACATTAAGAGGAGAGACATCGTGCTGAAGCGAGAACTGGGTGAGGGAGCCTTTGGAAAGGTCTTCCTGGCCGAG
TGCTACAACCTCAGCCCGACCAAGGACAAGATGCTTGTGGCTGTGAAGGCCCTGAAGGATCCCACCCTGGCTGCCCGGAA
GGATTTCCAGAGGGAGGCCGAGCTGCTCACCAACCTGCAGCATGAGCACATTGTCAAGTTCTATGGAGTGTGCGGCGATG
GGGACCCCCTCATCATGGTCTTTGAATACATGAAGCATGGAGACCTGAATAAGTTCCTCAGGGCCCATGGGCCAGATGCA
ATGATCCTTGTGGATGGACAGCCACGCCAGGCCAAGGGTGAGCTGGGGCTCTCCCAAATGCTCCACATTGCCAGTCAGAT
CGCCTCGGGTATGGTGTACCTGGCCTCCCAGCACTTTGTGCACCGAGACCTGGCCACCAGGAACTGCCTGGTTGGAGCGA
ATCTGCTAGTGAAGATTGGGGACTTCGGCATGTCCAGAGATGTCTACAGCACGGATTATTACAGGGTGGGAGGACACACC
ATGCTCCCCATTCGCTGGATGCCTCCTGAAAGCATCATGTACCGGAAGTTCACTACAGAGAGTGATGTATGGAGCTTCGG
GGTGATCCTCTGGGAGATCTTCACCTATGGAAAGCAGCCATGGTTCCAACTCTCAAACACGGAGGTCATTGAGTGCATTA
CCCAAGGTCGTGTTTTGGAGCGGCCCCGAGTCTGCCCCAAAGAGGTGTACGATGTCATGCTGGGGTGCTGGCAGAGGGAA
CCACAGCAGCGGTTGAACATCAAGGAGATCTACAAAATCCTCCATGCTTTGGGGAAGGCCACCCCAATCTACCTGGACAT
TCTTGGCTAG

NTRK3REV2: ACTCTCTGTAGTAGTGAACTTCCGG
TGAGAGACATCATCACTTGAAGGC
CGGAAFTTCACTACRACAGAGAGT

EML4For1: AGATCGCCTGTCAGCTCTT

Positive & Negative Controls

2 positive controls and 7 negative controls were used to verify amplification of the desired EML4-NTRK3 fusion gene. The positive controls in this assay included RNA from a murine tumor (mice that were injected with NIH3T3 cells stably expressing the EML4-NTRK3 gene) and Mesenchymal Stem Cells derived from (pediatric) human bone marrow transduced with the lentivirus encoding the EML4-NTRK3 fusion. The negative controls in this experiment included water, RNA from skeletal muscles, RNA from tumor tissue (case of Alveolar Soft Part Sarcoma), RNA isolated from two Ewing sarcoma cell lines, and RNA isolated from a metastatic tumor located at the lung (a patient diagnosed with EWS-like sarcoma).

Patient Archival Samples

25 de-identified fibrosarcoma patient archival samples were used in this assay to assess harboring frequency of the novel chromosomal translocation. One of the 25 samples is known to be the original patient, diagnosed with the novel EML4-NTRK3 fusion. It is thus expected that all five PCR assays will detect the EML4-NTRK3 transcript in one of the 25 samples, known to be the primary patient.

Figure 2A: Abbreviations Table

Abbreviation	cDNA Resources	Sample Type
St.	DNA standard	-
W	Water	Negative control
L-1	RNA from murine tumor (mice were injected with NIH 3T3 cells stably expressing EML4-NTRK3)	Positive control
HBM	Mesenchymal Stem Cells delivered from (pediatric) human bone marrow transduced with lentivirus encoding EML4-NTRK3	Positive control
S.M.	RNA from skeleton muscles	Negative control
S.B	RNA from Bone Marrow (patient diagnosed with Ewing Sarcoma)	Negative control
ASPS	RNA from Tumor tissue (case of Alveolar Soft Part Sarcoma)	Negative control
SKNEP	RNA isolated from Ewing sarcoma cell line	Negative control
SKNMC	RNA isolated from Ewing sarcoma cell line	Negative control
EWS	RNA isolation from metastatic tumor located at lung (patient diagnosed with EWS-like sarcoma)	Negative control
P1-P25	Random RNA from patients diagnosed with Fibrosarcoma	Sample to test

In all five PCR assays, it was concluded that the positive samples were positive for the EML4-NTRK3 fusion, and negative samples were negative for the fusion gene.

In the first assay, patients 1-25 were used for analysis, and samples from 5 and 11 samples were repeated twice. The fusion gene was detected for patients 2 and 4. In addition, the EML4-NTRK3 fusion gene was detected in both repeated samples for patients 5 and 11 (figure 3A). In the second PCR assay, the fusion gene transcripts were detected in the samples for patients 2, 5, 6, 7, 14, 23, and 24. In the third assay, the EML4-NTRK3 fusion gene was detected for patients 2, 6, 7, 9, 11, 17, 18, and 24. In the fourth assay, the fusion gene was detected for patients 2, 9, and11. In the last PCR assay, the EML4-NTRK3 fusion gene was detected for patients 2,6,7,17, 18, and 24.

In this project, patient 2 had the highest frequency of detection for the EML4-NTRK3 fusion transcripts (5/5). Patient 11 had the second highest frequency of EML4-NTRK3 detection (4/5), while patients 6, 7, and 24 revealed to have the third most frequent detection of the fusion gene (3/5). In patients 9, 10, 17, and 18, the fusion gene was detected 2 out of 5 PCR assays, and patient 4, 14, and 23 revealed detection of the EML4-NTRK3 fusion gene in 1 out of 5 PCR assays. Patients 1, 3, 5, 8, 12, 13, 15, 16, 19, 20, 21, 22, and 25 revealed no detection of the fusion gene at all (0/5).

In this, it is likely that patient 2 was the primary patient with the sequenced, detected EML4-NTRK3 novel chromosomal translocation. It is probable that patient 11 and possibly patients 4, 6, 7, 9, 10, 24, 17, 18, 23, and 24 might contain the fusion gene as well, as these samples revealed detection in at least one completed PCR assay. Due to potential primer annealing to non-complementary sequences and amplification of those products, the results of this portion of the project cannot be certain. The inconsistencies

of results from these PCR assays indicate that further analysis and repetition of the experiment are needed for conclusions.

Figure 3A: First PCR Assay Performed

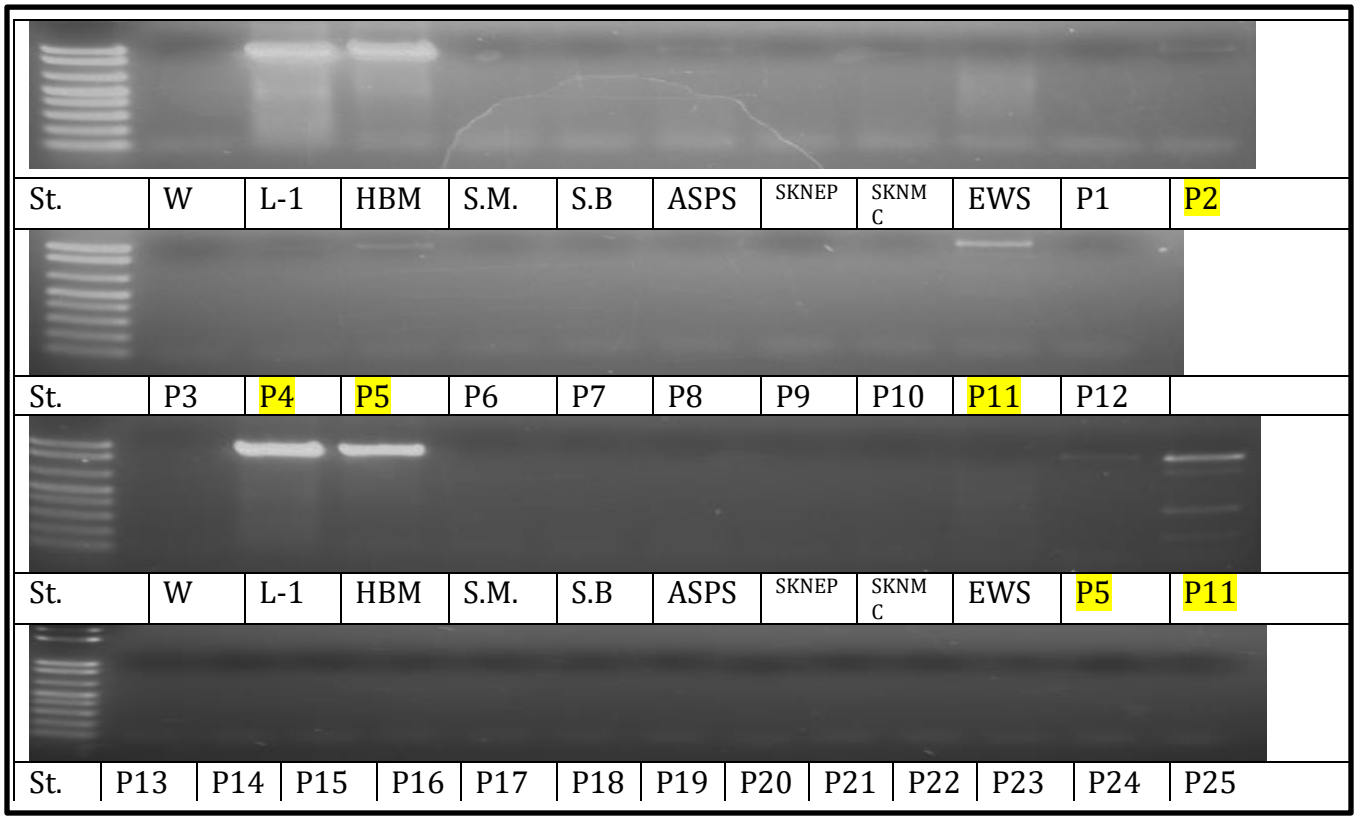
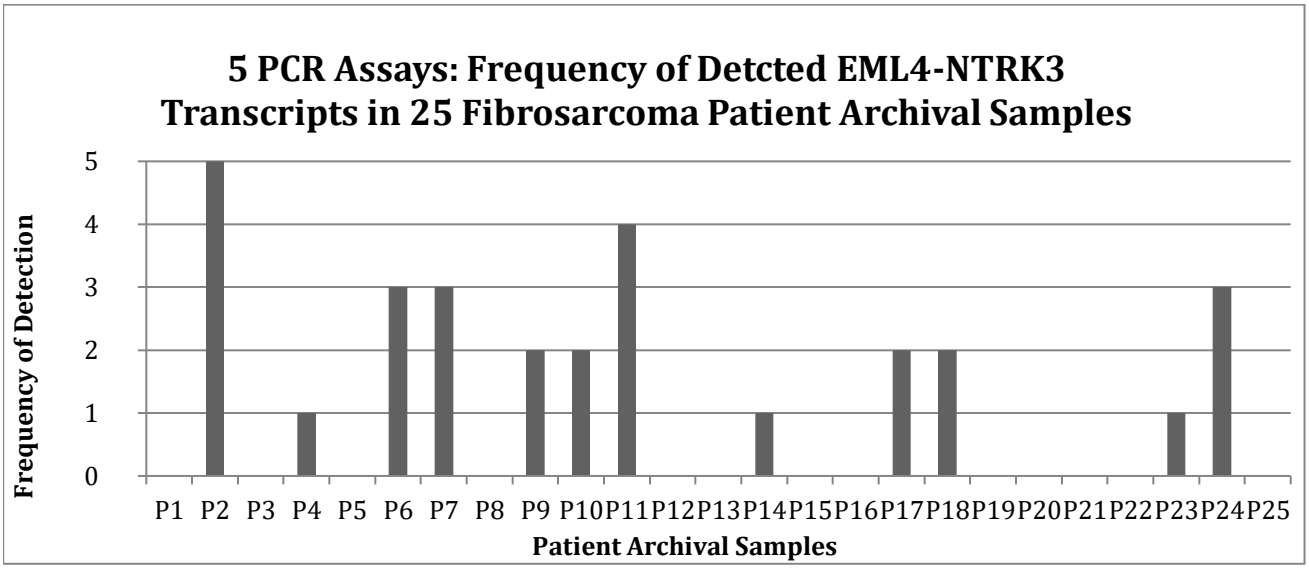


Figure 3B: Graph: Frequency of Detected Gene in Patient Archival Samples



Conclusion

Presentation of a case of non-classical congenital fibrosarcoma led to the sequencing of the patient's DNA and identification of somatic t(2;15)(2p21;15q25) novel chromosomal translocation EML4-NTRK3. The novel fusion gene is hypothesized to be a potent oncogenetic driver and prevalent in patients presumably diagnosed with fibrosarcoma. The performance of 5 PCR assays revealed that this novel chromosomal translocation might be common in other patients with similar uncharacteristically, aggressive, clinical course cancers. Analysis presented likely detection of the fusion gene in selected specimens, but inconsistent results yielded incomplete conclusions in this portion of the study. Detection of EML4-NTRK3 gene is probable in some of the cases evaluated in this study, and future research will include the repetition of these PCR assays or gene fusion detection with more advanced technologies. The EML4-NTRK3 fusion gene presumes to be a likely marker for patients with similar diagnosis to receive a multimodal therapy (chemoradiation and surgery), similar to the original patient who had responded extremely well to treatment and, currently, remains clinically well and free of disease.

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