Summer 8-19-2016

# Genetic Landscape of Pediatric Myelodysplastic Syndromes

Jennifer E. Grove
*University of Nebraska Medical Center*

# GENETIC LANDSCAPE OF PEDIATRIC MYELODYSPLASTIC SYNDROMES

By

**Jennifer E. Grove**

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Medical Sciences Interdepartmental Area Graduate Program

(Pediatrics)

**Under the Supervision of Professor Bhavana J. Dave**

University of Nebraska Medical Center Omaha, Nebraska

June 2016

Supervisory Committee:

Bhavana J. Dave, Ph.D.

Tanner Hagelstrom, Ph.D.

James Eudy, Ph.D.

Gregory Bociek, M.D.

## ACKNOWLEDGEMENTS:

My deep gratitude goes first to Prof. Bhavana J. Dave, who guided me throughout these years of my graduate career. Her unwavering dedication to her students and their work and her willingness to mentor throughout this process was unfailing. I will forever be grateful for her expert guidance, understanding, and encouragement.

A special acknowledgement to the late Dr. Warren Sanger who served on my committee until his untimely departure. Without Dr. Sanger's support and encouragement in the early years, I would never have made it to this point. I am also thankful to Dr. Tanner Hagelstrom, Dr. Gregory Bociek, and Dr. James Eudy for agreeing to serve on the committee of a non-traditional student. Their comments and questions were greatly valued.

I would also like to extend my appreciation to my colleagues in the Human Genetics Laboratory at UNMC. I would not have accomplished this research without their expertise and experience in the area of cytogenetics and microarray. I would like to specifically thank my fellow technologist and classmate, Rachel Utter. She has been my advocate every step of the way. I am lucky to call her an equal and a friend.

Last, but certainly not least, I would like to thank my family especially my husband Mike. His encouragement and support for me during this very long process contributed to my success. His willingness to take care of our children, the house, and our responsibilities, by himself at times, allowed me to accomplish my goals. I would never have completed this without him. I would also like to thank my young children, Tristan and Alice, for giving me the motivation I needed to fulfill this dream. Their understanding for all those evenings when Mommy had work to do will never be forgotten. Finally, I would

like to thank my parents and in-laws who supported me over these years and provided the extra hands to our family whenever and wherever we needed it.

# GENETIC LANDSCAPE OF PEDIATRIC MYELODYSPLASTIC SYNDROMES

Jennifer E. Grove, Ph.D.

University of Nebraska Medical Center, 2016

Supervisor: Bhavana J. Dave, Ph.D.

## ABSTRACT:

Myelodysplastic syndromes (MDS) are acquired heterogeneous hematopoietic clonal disorders primarily seen in the adult and elderly populations that presents a variety of cellular morphologies in cell lineages, varying prognoses, and differences in overall survival (OS) between individual patients. The occurrence of MDS in the pediatric and young adult population, or those between the ages of 0 and 29, is slowly on the rise. Pediatric and elderly cases exhibit diverse cytogenetic findings with differences in OS. The characterization of the genetic landscape of pediatric MDS is limited and most studies detailing genetic changes have been conducted in adult MDS cases. In order to aid in therapeutic stratification for pediatric cases, the key genes involved in hematopoietic transformation must be deciphered. This study utilized comprehensive analysis including cytogenetic karyotyping, FISH, and high-resolution microarray techniques. With the use of multiple techniques, this study confirmed the rarity of MDS in the pediatric population, characterized the frequencies of hallmark cytogenetic abnormalities, and identified key aberrations observed at the genetic level. With the use of microarray, we were able to detect genomic aberrations in 33 genes including novel copy number changes in more than one case in the *PRDM16*, *IRF4, MYH11, ALK, CDKN2B, PAX5, EXT2,* and *ERCC4* genes. The results from this study prove the importance of comprehensive testing utilizing a variety of techniques in distinguishing the most accurate genetic landscape of pediatric

MDS. This information can be used to better equip the medical community in accurately diagnosing and providing prognostic implications for therapy and treatment.

# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** ..... **i**

**ABSTRACT** ..... **iii**

**TABLE OF CONTENTS** ..... **v**

**LIST OF FIGURES** ..... **viii**

**LIST OF TABLES** ..... **xii**

**LIST OF ABBREVIATIONS** ..... **xiii**

**INTRODUCTION** ..... **1**

**Hematopoiesis** ..... 4

**Pathogenesis of MDS** ..... 7

**Epidemiology of MDS** ..... 11

**Classification of MDS** ..... 13

***Refractory Cytopenia of Childhood (RCC)*** ..... 14

***Refractory Anemia with Excess Blasts and Refractory Anemia with Excess Blasts in Transformation (RAEB and RAEB-T)*** ..... 15

***Secondary MDS: Treatment Related and Occupational Exposure*** ..... 15

***Inherited Bone Marrow Failure Disorders (IBMF)*** ..... 17

***Aplastic Anemia (AA)*** ..... 18

**Treatment for MDS** ..... 18

**Genetic Testing in Myelodysplastic Syndromes** ..... 19

***Cytogenetics in Adult Populations*** ..... 21

***Cytogenetics in Pediatric Populations*** ..... 21

**Prognostic Implications of Cytogenetics** ..... 22

**Utility of Microarray Studies** ..... 23

**Hypothesis and Specific Objectives** ..... 25

**MATERIALS AND METHODS** ..... **27**

**Specimen Collection and Handling** ..... 28

**Conventional Cytogenetic Studies** ..... 29

**Fluorescence *in situ* Hybridization (FISH)** ..... 35

**Microarray** ..... 40

***DNA Extraction*** ..... 40

***DNA Quantification*** ..... 41

***CytoScan® HD Array Assay Technique*** ..... 41

***OncoScan® FFPE Assay Technique*** ..... 51

**RESULTS** ..... **69**

**Patient Demographics** ..... 70

***Pediatric Patient Demographics*** ..... 70

**Conventional Cytogenetic and FISH Analyses** ..... 74

**Microarray Samples** ..... 82

**Microarray Results** ..... 86

***Case Studies*** ..... 91

**Comprehensive Testing Results** ..... 128

**DISCUSSION** ..... **131**

**Demographics** ..... 132

**Cytogenetics and FISH** ..... 133

**Prognostic Implications in MDS** ..... 137

**Microarray** ..... 138

**Current Trends in Adult MDS Array Analysis** ..... 139

**Current Trends in Pediatric MDS Array Analysis** ..... 145

**Limitations of this Study** ..... 146

**Gene Involvement in Pediatric MDS** ..... 147

**Comprehensive Testing** ..... 165

**SUMMARY AND FUTURE DIRECTIONS ..... 168**

**Summary** ..... 169

**Future Directions** ..... 171

**BIBLIOGRAPHY ..... 174**

**Appendix A. Reagent names and manufacturers ..... 193**

**Appendix B. Equipment product names and manufacturers ..... 198**

**Appendix C. Software product name and manufacturers ..... 199**

# LIST OF FIGURES

Figure 1a. Schematic diagram of normal hematopoiesis ..... 5

Figure 1b. Schematic diagram of abnormal hematopoiesis..... 6

Figure 2. Workflow of Cytogenetics and FISH .....30

Figure 3. Workflow of cytogenetic culture setup.....32

Figure 4. Workflow of FISH studies .....36

Figure 5. MDS FISH Panel .....38

Figure 6. Restriction enzyme digestion CytoScan® protocol.....43

Figure 7. Ligation CytoScan® protocol .....44

Figure 8. PCR CytoScan® protocol .....45

Figure 9. Verification of the PCR product.....47

Figure 10. Fragmentation CytoScan® protocol .....49

Figure 11. Verification of the fragmentation product.....50

Figure 12. Labeling CytoScan® protocol .....52

Figure 13. Hybridization CytoScan® protocol .....53

Figure 14. Anneal OncoScan® protocol.....55

Figure 15. Gap fill OncoScan® protocol.....56

Figure 16. First PCR OncoScan® protocol .....58

Figure 17. Second PCR OncoScan® protocol .....60

Figure 18. Verification of the first PCR product .....61

Figure 19. HaeIII Digestion OncoScan® protocol .....62

Figure 20. Verification of the HaeIII digestion product.....64

Figure 21. Hybridization OncoScan® protocol .....65

Figure 22. Smooth signal examination using ChAS .....67

Figure 23. Demographic distribution of MDS .....71

Figure 24. Percentage of MDS specimens per age group.....72

Figure 25. Demographic distribution of pediatric and young adult MDS. .....73

Figure 26. Hallmark cytogenetic and FISH findings of MDS.....75

Figure 27. Cytogenetic/FISH Findings of 2353 specimens from adult MDS (≥30 years of age) cases.....76

Figure 28. Cytogenetic/FISH findings of pediatric and young adult MDS .....79

Figure 29. Cytogenetic/FISH findings of pediatric MDS .....81

Figure 30. Cytogenetic/FISH findings of young adult (19-29 years) MDS .....83

Figure 31. Representative Images depicting normal cytogenetic karyotypes .....87

Figure 32. Representative normal MDS FISH images .....88

Figure 33. Microarray findings of pediatric and young adult MDS cases .....89

Figure 34. Microarray results for Case 1.....92

Figure 35. Microarray results for Case 4.....94

Figure 36. FISH results for Case 5 .....95

Figure 37. Microarray results for Case 5 ..... 96

Figure 38. Microarray results for Case 6 ..... 98

Figure 39. Cytogenetic and FISH results for Case 7 ..... 99

Figure 40. Microarray results for Case 7 ..... 100

Figure 41. Cytogenetic and FISH results for Case 11 ..... 103

Figure 42. Cytogenetic and FISH results for Case 12 ..... 106

Figure 43. Microarray results for Case 12 ..... 107

Figure 44. Cytogenetic and FISH results for Case 13 ..... 108

Figure 45. Microarray results for Case 15 ..... 110

Figure 46. Microarray results for Case 16 ..... 111

Figure 47. Cytogenetic and FISH results for Case 17 ..... 112

Figure 48. Microarray results for Case 19 ..... 114

Figure 49. Microarray results for Case 20 ..... 115

Figure 50. Microarray results for Case 21 ..... 116

Figure 51a-b. Microarray results for Case 22 ..... 118

Figure 51c-d. Microarray results for Case 22 ..... 119

Figure 52. FISH results for Case 24 ..... 120

Figure 53. Microarray results for Case 25 ..... 122

Figure 54. Microarray results for Case 26 ..... 123

Figure 55. Cytogenetic and FISH results for Case 27 .....124

Figure 56. Microarray results for Case 28.....126

Figure 57. Cytogenetic or FISH results in cases with abnormal microarray analyses..141

# LIST OF TABLES

Table I. Characterization of the MDS-related chromosomal abnormalities detected by cytogenetic and FISH analyses in adult MDS .....78

Table II. Characterization of the MDS-related chromosomal abnormalities detected by cytogenetic and FISH analyses in the pediatric/young adult population .....80

Table III. Comparison of MDS-related abnormalities observed in the pediatric and young adult MDS populations .....84

Table IV. List of pediatric and young adult MDS specimens for microarray studies after karyotyping and FISH analyses .....85

Table V. Characterization of genetic aberrations detected by microarray .....90

Table VI. Microarray results for Case 10.....102

Table VII. Microarray results for Case 11.....105

Table VIII. Characterization of the cases with the *PRDM16* gene aberration .....127

Table IX. Characterization of results by cytogenetics and/or FISH analyses.....129

Table X. Characterization of results from comprehensive testing.....130

Table XI. Details of abnormalities observed in pediatric MDS cases by microarray ....140

Table XII. List of the most frequent gene alterations in adult MDS.....143

Table XIII. List of genes that were detected in more than one case .....148

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Aplastic anemia |
| ALL | Acute lymphoblastic lymphoma |
| AML | Acute myeloid leukemia |
| AN | Anemia |
| APL | Acute promyelocytic leukemia |
| ChAS | Affymetrix® chromosome analysis suite |
| CML | Chronic myelogenous leukemia |
| CNV | Copy number variations |
| DAPI | 4,6-diamidino-2-phenylindole |
| dbVAR | NCBI database for genomic structural variants |
| DGV | Database of genomic variants |
| DIR | Direct culture |
| DON | Direct overnight |
| ET | Essential thrombocythemia |
| FA | Fanconi's anemia |
| FBS | Fetal bovine serum |
| FISH | Fluorescence in situ hybridization |
| G-banding | Giemsa banding |

| | |
|---|---|
| HBSS | Hank's balanced salt solution |
| HL | Hodgkins lymphoma |
| HSCs | Hematopoietic stem cells |
| HSCT | Hematopoietic stem cell transplant |
| IBMF | Inherited bone marrow failure disorder |
| IPSS-R | Revised international prognostic scoring system |
| ISCN | International system for human cytogenetic nomenclature |
| IST | Immunosuppressive therapy |
| JMML | Juvenile myelomonocytic leukemia |
| KCL | Potassium chloride |
| LOH | Loss of heterozygosity |
| MDS | Myelodysplastic syndromes |
| MIP | Molecular inversion probe |
| ML-DS | Myeloid leukemia of Down syndrome |
| MPD | Myeloproliferative disorders |
| MPN | Myeloproliferative neoplasms |
| NCBI | National Center for Biotechnology Information |
| NEU | Neutropenia |
| NHL | Non-Hodgkins lymphoma |

| | |
|---|---|
| NP-40 | Nonidet P-40 |
| OMIM | Online Mendelian Inheritance in Men® |
| OS | Overall survival |
| PCP | Pancytopenia |
| PCR | Polymerase chain reaction |
| PMF | Primary myelofibrosis |
| PV | Polycythemia Vera |
| RA | Refractory anemia |
| RAEB | Refractory anemia with excess blasts |
| RAEB-t | Refractory anemia with excess blasts in transformation |
| RBC | Red blood cell |
| RCC | Refractory cytopenia of childhood |
| SDS | Shwachman-Diamond syndrome |
| SNP | Single-nucleotide polymorphisms |
| SSC | Sodium chloride and sodium citrate |
| TCP | Thrombocytopenia |
| TdT | Terminal deoxynucleotidyl transferase |
| THC | Trypsin, hypotonic salts, and colcemid |
| UCS | Unknown clinical significance |

| | |
|---|---|
| WBC | White blood cell |
| WHO | World Health Organization |

# INTRODUCTION

## INTRODUCTION

Myelodysplastic syndromes (MDS) are acquired hematopoietic clonal disorders primarily seen in the adult and elderly populations with an overall estimate of incidence at 14,000 new cases per year (Siegel, Ma et al. 2014). This group of heterogeneous bone marrow syndromes are characterized as stem-cell disorders with varying degrees of overall reduction in blood cell production. The heterogeneous nature of MDS presents as a variety of cellular morphologies in a number of myeloid cell lineages, varying prognoses, and differences in overall survival (OS) between individual patients. The majority of patients present a normocellular or hyperplastic bone marrow, however, up to 20% of patients have shown hypoplastic and mylofibrotic bone marrow (Aul, Bowen et al. 1998). The overall numbers of myeloid cell lineages vary and morphological aberrations are observed in the clonal origin of hematopoietic cells. Hypercellular bone marrow displays morphological dysplasia and ineffective hematopoiesis in at least one of the three myeloid lineages (Aul, Bowen et al. 1998, Tefferi, Vardiman 2009, Whichard, Sarkar et al. 2010). Cellular bone marrow is unable to produce and deliver adequate numbers of mature cells to the peripheral blood during ineffective hematopoiesis.

Even though MDS is predominantly a disease of older populations, the frequency in the pediatric and young adult population, or those between the ages of 0 and 29, is slowly on the rise. Myelodysplastic syndromes in pediatric cases present diverse cytogenetic findings and differs in OS in comparison with the elderly. The amount of information on this rare group is limited. In order to aid in therapeutic stratification for pediatric patients more information is needed (Glaubach, Robinson et al. 2014, Ganapathi, Schafernak et al. 2015) . The use of high-resolution techniques including microarray can help with deciphering possible key aberrations that are observed at the genetic level.

Current molecular genetic studies have detailed key genes involved in adult MDS and the present study will be useful to compare similarities and differences between the elderly and pediatric populations (Silva, Maschietto et al. 2013, Shih, Abdel-Wahab et al. 2012, Bejar 2014).

The onset of this disease can be relatively benign in the form of refractory anemia (RA), typically observed as a decrease in red blood cells, but causes a decrease in the production of healthy platelets, red and white blood cells (Aul, Bowen et al. 1998, Tefferi, Vardiman 2009, Corey, Minden et al. 2007, Akhtari 2011). Red blood cells (RBC) transport oxygen to the rest of the body and brings carbon dioxide to the lungs. Having too few RBC, anemia, leaves the patient feeling tired and weak and can cause shortness of breath. White blood cells (WBC) are important as a line of defense against infection. The two major types are lymphocytes, which make antibodies, and granulocytes that destroy bacteria. Having too few WBC leads to severe infections in the body, or neutropenia. The small fragments of the megakaryocyte that enter the blood stream are called platelets. These are essential for blood clotting and without them can result in thrombocytopenia, which causes abnormal bleeding, and bruising (Brunning, RD. Bennett, JM. Flandrin, G. Matutes, E. Head, D. Vardiman, JW. 2001).

Myelodysplastic syndromes can be diagnosed as primary, or *de novo* MDS, or secondary MDS from past chemo- or radiation therapy or exposure to certain chemicals and heavy metals. Both result in dysplastic blood and bone marrow cells and cytogenetic abnormalities are observed in over 50% of MDS cases (Corey, Minden et al. 2007, Flandrin 2002). Hallmark genetic aberrations detected by conventional karyotyping or fluorescence *in situ* hybridization (FISH) include -5/del(5q), -7/del(7q), +8, or del(20q) (Cherian, Bagg 2006). The classification of these genetic findings has prognostic

implications and helps to stratify a more individualized treatment plan for MDS patients (Haase, Germing et al. 2007).

## Hematopoiesis

Normal hematopoiesis gives rise to progressively more differentiated progenitor cells, which eventually differentiate into mature blood cells (Lobo, Shimono et al. 2007, Orkin, Zon 2008). The fundamental properties of hematopoiesis include proliferation, loss, and differentiation resulting in the development of over 500 billion blood cells per day. Normal adult hematopoietic stem cells (HSCs) are the common ancestors of all blood cells and are needed to maintain or repair their host tissue. These cell types have two functions of division that include symmetrical division, which yields two stem cells or two differentiated daughter cells, and asymmetrical division into either another stem cell or a more specialized cell. Self-renewal of HSCs produces a replicate stem cell that typically has the same development and replication fate. The production of specialized daughter cells is decided from biochemical signals and transcription factors and the HSC has the potential to generate cell types of each lineage (Kondo 2010, Whichard, Sarkar et al. 2010, Wilson, Laurenti et al. 2008).

During normal hematopoiesis the blood stem cell, or immature blast cells, make up 5% or less of the cells in the bone marrow and will develop into one of the three healthy blood cells (red blood cells, platelets, or white blood cells) from a homeostatic balance between proliferation, differentiation, and apoptosis (Figure 1a). The normal system efficiently replenishes the body when hematological stresses are encountered which includes infection and blood loss (Passegue, Wagers et al. 2005). Hematopoietic disorders, including MDS, are acquired when normal development is disrupted and blast cells lose proper function the way they should and die in the bone marrow or soon after

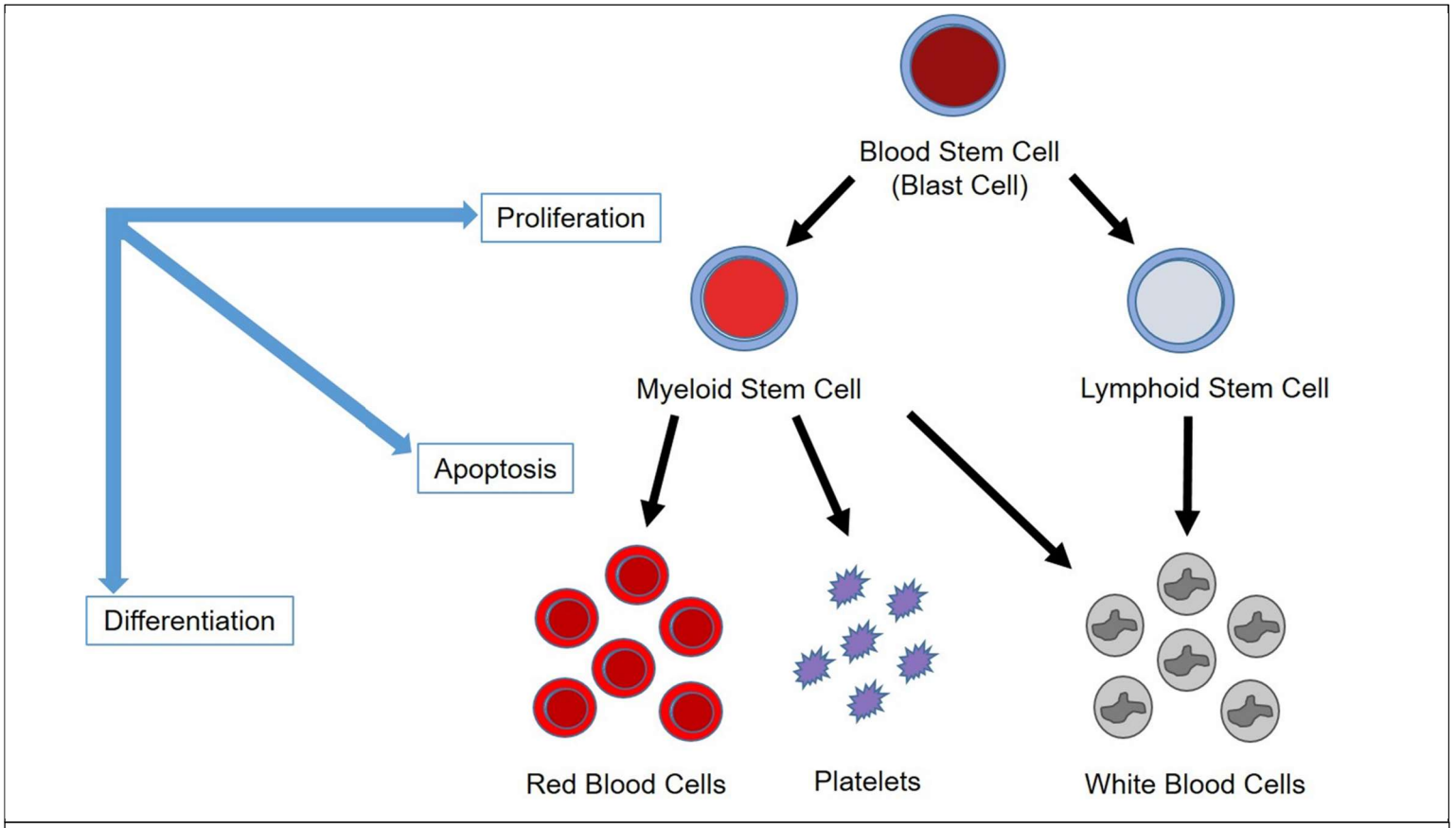



**Figure 1a. Schematic diagram of normal hematopoiesis**. Normal hematopoiesis results in the development of one of the three healthy blood cells, red blood cells, platelets, or white blood cells, from an immature blast cell. This process occurs from a homeostatic balance between proliferation, differentiation, and apoptosis.

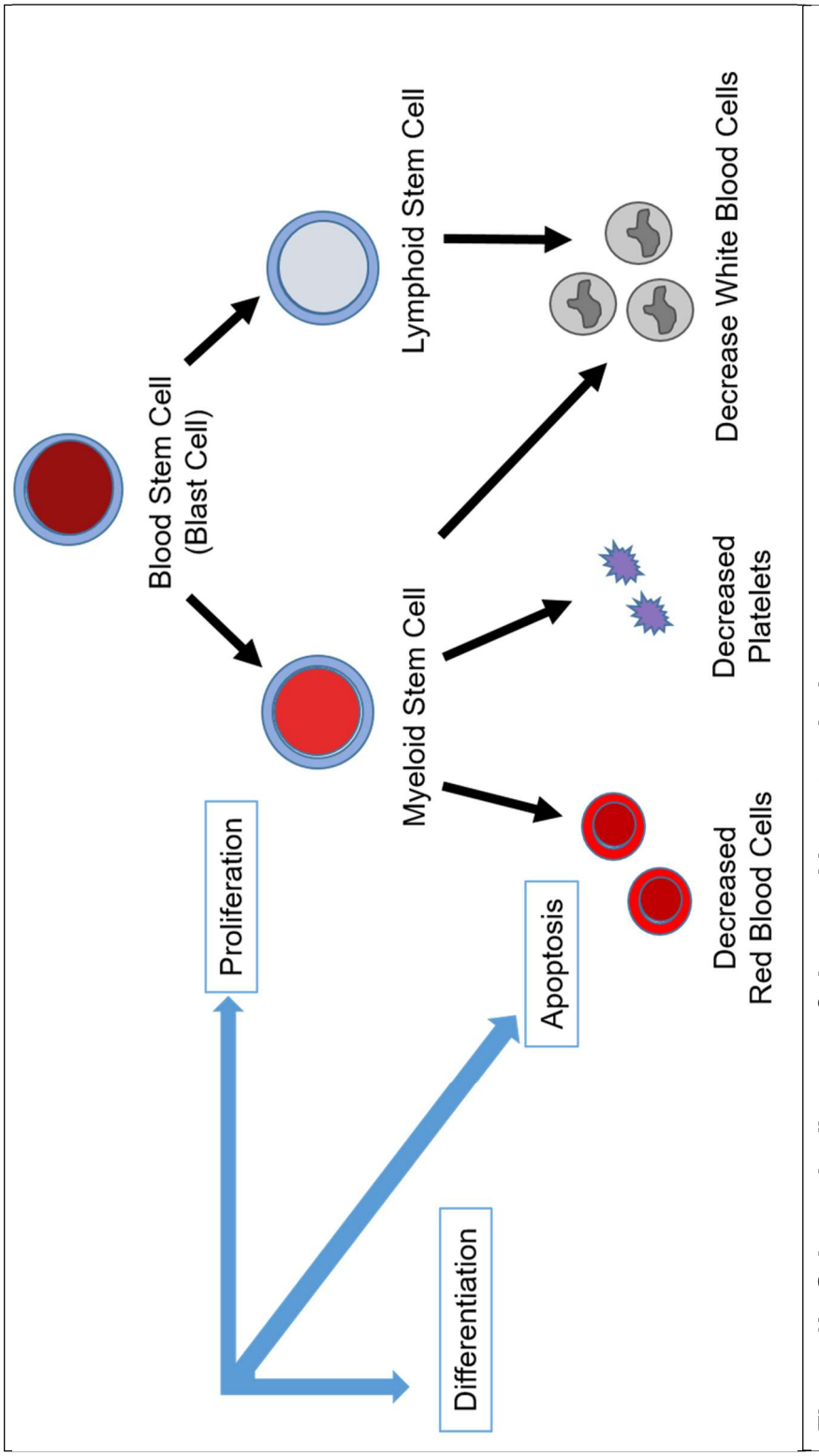



**Figure 1b. Schematic diagram of abnormal hematopoiesis.** In hematopoietic disorders, normal development of one of the three blood cells are disrupted by malfunctioning blood stem cells. Bone marrow is unable to produce and deliver the adequate number of mature cells to the peripheral blood from an unbalance in proliferation, differentiation, and apoptosis.

entering the blood. Cellular bone marrow is then unable to produce and deliver adequate numbers of mature cells to the peripheral blood. Ineffective hematopoiesis presents a loss of homeostatic balance between proliferation, differentiation, and apoptosis leading to cell death of many precursor cells giving an overall unbalance in cell production. A varying degree of cellular dysplasia and an overall reduction in RBC, platelets, and WBC is produced (Figure 1b). This results in a higher propensity of infection, anemia, bleeding and the evolvement to acute myeloid leukemia (AML) (Corey, Minden et al. 2007, Tefferi, Vardiman 2009, Akhtari 2011).

## Pathogenesis of MDS

Even though morphological variation exists, the clonal disorder of MDS progresses through the same multistep process of tumorigenesis of initiation, promotion, and malignant transformation. A single or even multiple initial genetic insults occur to the predominantly quiescent HSCs and initiates the cell into an early lesion state (Wilson, Laurenti et al. 2008, Fozza, Longinotti 2013). The exact nature of the initial insult has yet to be deciphered. Promotion or progression of the abnormal clones occur with abnormally high rates of apoptosis on the normal cells and leads to transformation. The transformation stage has an apoptotic paradox of excessive cell death during initiation and promotion stages followed by a loss of apoptotic function that increases cell survival and proliferation of malignant cells. Over time, MDS has the ability to progress to a malignant transformation and ultimately evolve to AML (Davids, Steensma 2010, Kulasekararaj, Mohamedali et al. 2013).

### *Initiation and Promotion*

The cancer stem cell theory originated from studies showing that some aspects of normal cell growth and differentiation are observed in tumor growth. Malignant cells arise

from long-lived stem cells with the capability of self-renewal but unlike normal expansion, uncontrolled growth is not prevented (Lobo, Shimono et al. 2007). It was once believed that cancer stem cells were derived from normal stem cells but new studies show that progenitor cells can also gain normal stem cell attributes of long life and self-renewal. Leukemic stem cells have been shown to originate from progenitor cells with additional mutations that gives them the ability to self-renew. A single or multiple initial genetic insult event to either type of stem cell leads to the tumor promotion stage of clonal expansion of abnormal clones. These clones have abnormally high rates of apoptosis downstream in later progenitors due to their longevity and therefore their ability to accumulate genetic changes (Wang, Dick 2005, Chatterjee, Choudhry 2013).

The exact mechanism for tumorigenesis is still unknown but many studies have suggested that the microenvironment plays a role (Raaijmakers 2012). The microenvironment of bone marrow elements has been shown to be disorganized and all lineages in MDS patients can become affected. These elements subject stem cells to a range of stimuli including cell-cell interactions, contact to extracellular matrix molecules, and exposure to growth stimulatory and inhibitory cytokines. These growth factors not only stimulate proliferation but also support cell survival, and their loss presents an increase in apoptosis (Arai, Hirao et al. 2005, Aul, Bowen et al. 1998). Mutations of the genes within the stem cell niche causes failure of bone marrow stem cell maintenance and a thorough understanding of these aberrations can aid in potential targeted therapies for MDS patients (Wilson, Trumpp 2006).

### *Transformation*

After the stages of initiation and promotion, MDS can then progress to a malignant transformation increasing the amount of leukemic blast cells and ultimately evolving to

AML. Clonal expansion of MDS show changes of dysplasia, ineffective hematopoiesis, cellular dysfunction, and defective differentiation (Aul, Bowen et al. 1998).

Dysplasia in MDS can lead to morphological changes of the immature blast cell, which leads to changes in colony formation and the ability to produce a healthy adherent cell layer. Poor colony formation results in an abnormal increase in cluster formation. Dysplasia from MDS also causes abnormal megakaryopoiesis, or platelet production, which leads to the presence of micromegakaryocytes, small platelet precursors. These are associated with large atypical platelets and is often associated with myeloproliferative disorders (MPD) (Howe, Porwit-MacDonald et al. 2004, Sun, Konoplev et al. 2011, Orazi, Germing 2008).

In ineffective hematopoiesis, apoptosis and cell death of many cell precursors occurs, limiting production of healthy blood cells. Furthermore, increased cell proliferation concurrently with reduced cell differentiation leads to a net increase in the number of precursor cells despite higher rates of apoptosis (Ginzburg, Rivella 2011). Mutations in cell division and signaling systems that regulate specialization leads to abnormal proliferations and typically results in the aberrant cell being eliminated but cancers can occur when these mutations escape destruction and accumulate. Typically, short-lived cells, like differentiated progeny, are less prone to these mutations in comparison to the longer-lived stem cell populations (Lobo, Shimono et al. 2007).

Cellular dysfunction has been attributed to the progression of abnormal MDS clones slowly replacing normal stems cells through excessive apoptosis. Defective maturation including delayed maturation leads to an increase in non-erythroid lineages and an increase in early myeloid cells. In spite of normal and hypercellular bone marrow, persistent premature cell death has been shown in bone marrow biopsies. Numerous

DNA breaks and increased engulfment by macrophage has been observed in bone marrow and leads to an ineffective production of red blood cells and the suppression of normal stem cell differentiation (Aul, Bowen et al. 1998). In some MDS subtypes, cytotoxic T-cells have been found to be the primary inhibitor of hematopoietic precursors by increasing the frequency of apoptosis (Fozza, Longinotti 2013). Malignant transformation of MDS to AML is caused from an apoptosis paradox. The excessive cell suicide during initiation and promotion stages, now leads to the uncontrolled growth of malignant cells with increased cell survival and proliferation. Cells from AML cases have been shown to contain significantly reduced apoptotic behaviors especially when certain chromosome aberrations are present. For example, in 5q deletions, tumor aggressiveness is increased through the inactivation of the tumor suppressor gene *IRF-1* found in the 5q critical region which has been attributed to the reduction or complete loss of apoptotic function (Pitchford, Hettinga et al. 2010, Mallo, Arenillas et al. 2008, List, Dewald et al. 2006, Aul, Bowen et al. 1998).

Lastly, defective differentiation suppresses normal stem cell differentiation. Normal differentiation uses unique hematopoietic cells that are able to self-renew as well as generate all cells of the hematopoietic system. They give rise to multipotent progenitors and lineage-restricted progenitors that have a limited capacity to divide. On the other hand, leukemic stem cells have been hypothesized to originate from immature hematopoietic progenitors or even the HSC and will give rise to clonogenic leukemic progenitors that differentiate into leukemic blasts and more differentiated progeny (Tan, Park et al. 2006).

## Epidemiology of MDS

The onset of MDS can be relatively benign in the form of refractory anemia and can show signs of fatigue, shortness of breath, pale skin, and easy bruising or bleeding. Diagnosis of MDS consists of blood and bone marrow tests. Lower than usual numbers of red blood cells, neutrophils, and platelets is frequently seen in MDS. Bone marrow aspirates are biopsied and sent for more extensive genetic tests and diagnosis of MDS occurs when an excessive amount of blast cells are present, chromosomal abnormalities typical for MDS are observed, and/or changes in the structure or form of the bone marrow cells (Brunning, Bennett, et al. 2001, Cherian, Bagg 2006).

Myelodysplastic syndromes are typically described as a geriatric disorder due to the gradual accumulation of random genetic damage from endogenous and exogenous carcinogens over a lifetime. The risk of MDS increases with age; 3.5 to 12.6 adults per 100,000 over the age of 60; 15 to 50 per 100,000 over 70 years; and 89 per 100,000 for the over 80 populations with men having a slightly higher risk than women with a 1.2 ratio (Corey, Minden et al. 2007, Rollison, Howlader et al. 2008). As of 2014, the estimates from Medicare claims show that MDS is one of the top 10 adult neoplasms with at least 40,000 to 50,000 patients per year being diagnosed (Steensma, Komrokji et al. 2014, Goldberg, Chen et al. 2010).

Even though MDS is one of the most prevalent bone marrow disorders in the older populations; it is less frequently diagnosed in those under the age of 29 years. The incidence in the pediatric population, or those between the ages of 0 and 18, is 1.8 to 4 cases of MDS per million children per year (Rau, Shreedhara et al. 2012, Hofmann 2015a). Since this malignancy is less frequently observed, there is limited information regarding the diagnostic criteria for MDS in the younger population and in turn even less

information on the specific subtypes and prognostic implications in this group (Glaubach, Robinson et al. 2014, Hofmann 2015b). Younger patients presenting MDS are on the rise and typically present diverse features in comparison to the adult population including a more unfavorable prognosis and a higher propensity to evolve to malignant neoplasms including AML (Aul, Bowen et al. 1998, Tefferi, Vardiman 2009, Corey, Minden et al. 2007, Akhtari 2011, Mandel, Dror et al. 2002, Hofmann 2015a, Gohring, Michalova et al. 2010). Until the 2003 World Health Organization (WHO) Pediatric modification, children were categorized with the adult population. This was due to the limited information on any real differences between the two populations that resulted in the younger population receiving the same treatments and similar therapy strategies were utilized as in the older population (Hasle, Niemeyer et al. 2003).

With limited cases and a lack of consensus on when to diagnose MDS in pediatric patients, classifying new and individualized diagnostic and prognostic criteria for these individuals was quite difficult. With more research, some key differences between the adult and pediatric MDS populations have been determined. Refractory anemia with ring sideroblasts is quite common in adults and is very rare in younger patients and isolated anemia is one of the major presentations in adults while thrombocytopenia (TCP) and neutropenia (NEU) are the major presentations in pediatric populations (Chatterjee, Choudhry 2013). The most frequent cytogenetic abnormality of isolated deleted 5q observed in the adult population is rarely seen in pediatric MDS. In addition, the median age of MDS presentation in adults is 70 with primary MDS being the most common while in the pediatric population the median age is 7 years and the most common type is secondary or therapy-related MDS (Glaubach, Robinson et al. 2014, Niemeyer, Baumann 2008).

Since the etiology of MDS has been linked to age related alterations, including certain epigenetic factors like methylation, criteria on the vast number of older patients with this disease is understood (Shih, Abdel-Wahab et al. 2012). The reasons for occurrence of MDS in pediatric and young adult patients, or those between 0 and 29 years of age, has yet to be fully understood. Studies show a strong correlation to certain genetic disorders that are associated with a future MDS occurrence including trisomy 21 (Down syndrome), Fanconi's anemia, and inherited bone marrow failure disorder; yet biological and clinical features are different in non-Down children with MDS and not all pediatric MDS patients have one of these constitutional disorders (Rau, Shreedhara et al. 2012, Stary, Baumann et al. 2008, Glaubach, Robinson et al. 2014, Cantor 2015). Therefore, more research is needed in order to decipher what triggers the ineffective hematopoiesis. When we can accurately diagnose and determine the true characteristics of pediatric MDS, we will then be able to prepare a more individualized therapeutic strategy for the younger population.

## Classification of MDS

Currently, MDS in the pediatric and adolescent populations is classified as either primary MDS, or *de novo* MDS, and secondary MDS (Rau, Shreedhara et al. 2012). Primary MDS is subdivided into refractory cytopenia of childhood (RCC), refractory anemia with excess blasts (RAEB) and RAEB in transformation (RAEB-t) (Niemeyer, Baumann 2011). Diagnosis is typically determined by morphological criteria, karyotype analysis, and molecular genetic techniques. These tests help in risk-assessment and determining the most effective therapeutic approaches (Aul, Bowen et al. 1998, Stary, Baumann et al. 2008).

### *Refractory Cytopenia of Childhood (RCC)*

The most common pediatric and young adult subtype of primary MDS is RCC and can be difficult to distinguish from other disorders due to the overlapping morphological findings, which includes a variety of viral infections, vitamin deficiencies, and metabolic disorders. This subtype is seen in more than half of the childhood MDS cases and affects both males and females equally. These patients frequently present thrombocytopenia and neutropenia with a hypocellularity of bone marrow cells occurring in 75% of the patients and less frequently presents anemia. Refractory cytopenia of childhood affects the bone marrow and blood and is rarely observed in the lymph nodes, spleen, or liver (Germing, Aul et al. 2008, Chatterjee, Choudhry 2013). The most common signs and symptoms of RCC in children are malaise, bleeding, infection, and fever with over 20% of cases being asymptomatic. Less than 2% blasts are found in the peripheral blood and less than 5% in the bone marrow. Bone marrow aspirates present dysplastic changes including non-lobulated nuclei, micromegakaryocytes, and abnormally separated nuclear lobes with an increase in erythropoiesis resulting in increased numbers of mitoses (Koh, Cho et al. 2013). Most RCC bone marrow aspirates will show a normal karyotype or a loss of chromosome 7 as the most common abnormality but karyotypes that are more complex have also been observed. Cytogenetic karyotyping is the most important tool to determine the progression of RCC into more advanced subtypes of MDS. The consequence of loss of chromosome 7 has an increased chance of progression than a normal karyotype or other aberrations with a median progression time of 1.9 years. A gain of chromosome 8 or a normal karyotype may result in longer OS and represents a more stable disease. Hematopoietic stem cell transplant (HSCT) is the treatment of choice in the early stages of RCC when monosomy 7 or a complex karyotype is observed and presents a favorable OS after treatment. Immunosuppressive therapy (IST) is a potential treatment for patients

with infections, presenting severe cytopenia, or lack the appropriate transfusion requirements from T-cell immunosuppression of hematopoiesis (Niemeyer, Baumann 2011, Chatterjee, Choudhry 2013).

### *Refractory Anemia with Excess Blasts and Refractory Anemia with Excess Blasts in Transformation (RAEB and RAEB-T)*

Refractory anemia with excess blasts, also called oligoblastic myelogenous leukemia, consists of 2-19% blasts in the peripheral blood or 5-19% blasts in the bone marrow. In children, RAEB is typically slow progressing and shows stable blood counts for extended periods (Greenberg, Cox et al. 1997). Refractory anemia with excess blasts in transformation may behave more like MDS and lacks the typical clinical features of AML including the responses to AML-type therapies (Chatterjee, Choudhry 2013). An increased number of blast cells at about 20 to 30% are observed in the bone marrow in RAEB-T. Typically, RAEB and RAEB-t present with AML associated cytogenetic aberrations of t(15;17), t(8;21), inv(16), t(9;11), or complex karyotypes consisting of three or more aberrations (Hasle, Niemeyer et al. 2003). This subtype is the interface between MDS and *de novo* AML. The most appropriate treatment for RAEB and RAEB-t is unknown but hematopoietic stem cell transplantation can increase overall survival of the patient (Chatterjee, Choudhry 2013, Hasle, Niemeyer et al. 2003).

### *Secondary MDS: Treatment Related and Occupational Exposure*

Secondary MDS occurs at a younger onset age after chemotherapy or radiation therapy for a prior disease, after acquired aplastic anemia, or as a result of an inherited bone marrow failure disorder (IBMF) or familial disease (Niemeyer, Baumann 2011, Hasle, Niemeyer et al. 2003). The exposure to chemotherapeutic alkylating agents following

treatment for Hodgkins lymphoma (HL), Non-Hodgkins lymphoma (NHL), and acute lymphoblastic lymphoma (ALL) have been shown to be related to secondary MDS in younger populations (Stone 2009, Armitage, Carbone et al. 2003, Rubin, Arthur et al. 1991). The damage to hematopoietic stem cells during drug therapy causes an increase in the frequency and severity of thrombocytopenia, increases AML transformation, and decreases OS. Ionizing radiation effects leukemia progression but is dose and duration dependent. For example, the total dose exposure in HL treatment is directly proportional to the development of secondary MDS (Aul, Bowen et al. 1998).

Occupational and environmental carcinogens including heavy metals, fumes, exhaust gases, pesticides and cigarette smoking can lead to secondary MDS. Occupational exposures are less observed in the younger populations than the adult population but include exposures to copper, welding fumes and hydrogen peroxide. Other potential occupational causes of MDS later in life include associations with degreasing agents, nickel, exhaust gases and radio transmissions (West, Stafford et al. 2000). In smaller case-controlled studies, plant and machine operation exposures to pesticides, exhaust fumes, fertilizers, and store dust have implicated etiologies for secondary MDS. Another risk for MDS includes cigarette smoking due to the number of carcinogens, specifically the presence of benzene (West, Stafford et al. 2000). Benzene has been shown to cause a high incidence of morphological dysplasia and severity of thrombocytopenia, increases AML transformation, and decreases OS. Benzene in the workplace including petroleum plants has stricter laws lowering the amount of benzene exposure due to the negative effects later in life (Aul, Bowen et al. 1998).

### *Inherited Bone Marrow Failure Disorders (IBMF)*

Inherited bone marrow failure disorders have overlapping morphological features with RCC including macrocytosis of the red blood cells on blood smears and elevated hemoglobin F. These disorders need to be excluded using extensive past medical and family history evaluations in order to differentiate between IBMF and RCC (Niemeyer, Baumann 2011). These acquired bone marrow disorders only consist of a small fraction of MDSs in the population. Familial MDSs come from hereditary disorders that cause defective DNA repair and include Fanconi's anemia and Bloom's syndrome. Fanconi's anemia begins in childhood with bone marrow failure; 4-7% progress to MDS and AML in childhood and over 40% progress by the age of 40.

Cytogenetically, these patients typically present monosomy 7 or gain of 3q26, which has been shown to have an adverse risk factor for AML progression due to the overexpression of *EVI1* (Bernasconi, Cavigliano et al. 2003, Seif 2011, Niemeyer, Baumann 2011). On the contrary, a gain of chromosome 1q, observed cytogenetically can lead to an extended amount of years without progression. Neurofibromatosis, a genetic disorder of the nervous system, Shwachman-Diamond syndrome (SDS), and Down syndrome have also been attributed to MDS. Patients with SDS have a 10-25% risk of MDS progression and cytogenetically show an increase in the incidence of isochromosome 7q leading to a potential clonal marker for the SDS in the *SBDS* gene located at 7q11.2 (Cantu, Proytcheva 2015). Fortunately, cytogenetic correlations have shown a stable course for many years with i(7q) or del(20) in SDS patients (Chatterjee, Choudhry 2013, Niemeyer, Baumann 2011).

An association with Down syndrome is seen in 20-25% of the pediatric MDS cases and these patients have a 10 to 20-fold increased risk of AML progression in comparison

to non-Down syndrome patients. However, this type of myeloid leukemia is unique to this population and is classified into its own category of myeloid leukemia of Down syndrome (ML-DS) (Chatterjee, Choudhry 2013, Germing, Aul et al. 2008, Seif 2011, Cantor 2015).

### *Aplastic Anemia (AA)*

Aplastic anemia (AA) displays aplasia of all three hematological cells with an increase in lymphocytes, plasma cells and mast cells. This form of anemia has been shown to have an immune-mediated pathogenesis and typically follows immunosuppression with antithymocyte globulin, an antibody administered against human T-cells for drug therapy (Fu, Xue et al. 2015). These agents have an incidence of MDS evolution at 9.6% and those that evolve to MDS typically display monosomy 7 or trisomy 8 by conventional cytogenetic analysis (Aul, Bowen et al. 1998, Niemeyer, Baumann 2011, Ohara, Kojima et al. 1997).

## Treatment for MDS

The only potential cure for MDS consists of allogenic HSCT. The goals of the transplant are to restore the body's ability to make healthy blood cells once chemotherapy has been administered and to kill any remaining MDS cells. When benefits exceed the risks for the patient, this in an option when a stem cell donor is available. Unfortunately, HSCT is not a favorable therapy in the older populations due to the dangers of advanced age and other comorbidities (Tilak, Sookmane et al. 2008, Smith, Christiansen et al. 2013).

The current treatment of MDS primarily in the elderly populations includes the use of one of three FDA approved chemotherapeutic drugs azacitidine, decitabine, and lenalidomide (Zou, Fink et al. 2007, List, Dewald et al. 2006). These drugs are approved for both low- and high-risk patients. In the adult populations, MDS has well recognized

entities including cytogenetic findings that result in specific treatments and therapy (Giagounidis 2006). For example, the most frequent cytogenetic abnormality in the adult population, about 30% of the abnormal adult MDS cases, consists of loss of chromosome 5 or more frequently the loss of the long arm of 5 and the hypomethylating agents of azacitidine and decitabine are approved and used for these patients with a high success rate. Without the observance of a complex karyotype, this treatment gives the most favorable prognosis for survival with a higher median year of OS (Greenberg, Tuechler et al. 2012, Haase 2008, Haase, Germing et al. 2007, Steensma, Komrokji et al. 2014).

Unfortunately, these same therapies have yet to be deemed as a successful treatment in the pediatric population; without a bone marrow transplant, OS of pediatric MDS patients is low (Ohara, Kojima et al. 1997, Silva, Maschietto et al. 2013, Steensma, Komrokji et al. 2014). Since HSCT is the most favorable treatment for patients with less advanced age, it is ideal for the pediatric MDS population. This treatment is most successful when performed early in the disease and is one reason why an accurate and timely diagnosis criterion needs to be established. The sooner we can diagnose, the sooner we can treat, and the higher probability that a cure can occur (Germing, Aul et al. 2008, Smith, Christiansen et al. 2013). With the numerous techniques currently available, it is crucial to continue to contribute to the scientific understanding of pediatric MDS, so early diagnostic testing is made available.

## Genetic Testing in Myelodysplastic Syndromes

The heterogeneity of MDS is not only observed in cellular morphology but is also seen at the genetic level. Cytogenetic abnormalities by conventional karyotyping or FISH is observed in over 50% of MDS cases; yet a single cytogenetic abnormality is not considered a genetic hallmark of the disease (Haase, Germing et al. 2007, Cherian, Bagg

2006, Rigolin, Bigoni et al. 2001). Variable abnormalities including the hallmark cytogenetic abnormalities for adult MDS of monosomy 5 or a del(5q), monosomy 7 or del(7q), trisomy 8, and del(20q) are described in MDS (Haase 2008, Pitchford, Hettinga et al. 2010). Cytogenetic techniques are an essential tool in accurate diagnosis and provide important prognostic impact including therapeutic stratification. These strategies are used for determining disease clonality and to determine probabilities of AML progression (Costa, Valera et al. 2010, Bernasconi, Cavigliano et al. 2003, Valent, Horny 2009). The presence or absence of specific aberrations form the basis of very good-, good-, intermediate-, poor-, or very poor-prognosis designation in the adult population which has a profound impact on patient survival and leukemic transformation (Germing, Aul et al. 2008, Greenberg, Tuechler et al. 2012).

Cytogenetic abnormalities of MDS typically consist of an unbalanced aberration in the form of deletions or monosomies leading to the assumption that tumor suppressor genes have lost function or have become inactivated. Balanced translocations and inversions along with random abnormalities are rarely observed. The presence of hallmark aberrations, either as a sole anomaly or in a more complex karyotype with more than one change, contributes to the overall prognostic score. The occurrence of these abnormalities have shown distinct differences between the adult and pediatric populations. To date, we have already determined distinct features in the pediatric population that are different from the adult patients including the occurrence and frequency of these hallmark cytogenetic abnormalities and their prognostic implications. Refinement of the prognosis of chromosomal findings has occurred as recently as 2012 in the revised international prognostic scoring system (IPSS-R) and provides insight into the patient's OS and the frequency of MDS transformation into AML (Greenberg, Tuechler et al. 2012, Kulasekararaj, Mohamedali et al. 2013, Gohring, Michalova et al. 2010, Greenberg 2015).

### *Cytogenetics in Adult Populations*

The most frequent cytogenetic abnormality among the adult population, over 30%, consists of the loss of chromosome 5 or a variable deletion of the long arm of chromosome 5 (-5/del5q) but always spanning the chromosome region of q31 (Haase, Germing et al. 2007). This abnormality presents the most favorable prognosis in the older populations with specific therapies and treatments that present favorable OS. The least favorable prognosis occurs with the loss of chromosome 7 or a deletion of the long arm of 7 (-7/ del7q) and is observed in about 21% of adult MDS (Jhanwar 2015). Loss of 7 or del(7q) occurs more often as part of a complex karyotype and is associated with severe refractory cytopenia and a proneness to infections. Drug therapy for these patients has been unsatisfactory and the best treatment is allogeneic stem cell transplantation, when possible (Deeg, Scott et al. 2012). Trisomy 8 (+8) in the adult population is only observed in about 16% of MDS and is observed as a sole anomaly 46% of the time. This abnormality falls under an intermediate prognosis of a median month of survival at or above 23 months (Haase 2008). A deletion of the long arm of chromosome 20 (del20q) is the least frequent abnormality observed at about 7% and gives a favorable prognosis of OS above 23 months (Haase 2008, Haase, Germing et al. 2007, Mallo, Arenillas et al. 2008).

### *Cytogenetics in Pediatric Populations*

In the pediatric population, 55% of the primary MDS cases contain a karyotypic abnormality and 76% of the secondary MDS cases contain an abnormality that can be observed using conventional techniques (Gohring, Michalova et al. 2010, Silva, Maschietto et al. 2013). The differences between the adult and younger populations is seen in the occurrence and frequency of hallmark cytogenetic abnormalities. The most distinct difference between the adult and pediatric population occurs with the hallmark

abnormality of -5/del(5q). In the pediatric population, -5/del(5q) is virutally never seen, and when observed, presents the most unfavorable prognosis (Hofmann 2015a). The younger patients with this aberration have the highest occurrence of AML transformation, a lowered OS, and the treatment protocol remains undefined.

The pediatric MDS population exhibits -7/del(7q) most frequently amongst the four hallmark abnormalities in about 30% of the patients (Niemeyer, Baumann 2008, Kardos, Baumann et al. 2003). This chromosomal aberration is seen as a sole abnormality, presents the most favorable prognosis in young patients, and correlates with a longer-term survival. The moderate frequency, 16%, of a +8 in the younger population is comparable to the frequency observed in the adult population. In both populations, this abnormality falls under an intermediate prognosis of a median month of survival at or above 23 months. Lastly, the observance of del(20q) in the pediatric population is comparable to the adult population in about 10%. This abnormality presents a prognosis of survival above 23 months in both populations (Glaubach, Robinson et al. 2014, Rau, Shreedhara et al. 2012).

## Prognostic Implications of Cytogenetics

According to the most recent IPSS-R, prognostic variables are given scores based upon the number of blasts in the bone marrow, number of platelets, absolute neutrophil count, hemoglobin numbers, and cytogenetic findings. Cytogenetic findings are needed to determine specific anomalies and the number of aberrations present to determine the prognostic implications for MDS (Steensma, Komrokji et al. 2014). The score values for these factors will predict median survival in years without treatment and the potential of AML transformation without therapy. A prognostic score value of very good, good,

intermediate, poor, and very poor is given for specific cytogenetic abnormalities being observed.

In adult MDS cases, a score of 'very good' is given to MDS cases with a loss of the Y chromosome (-Y) and a deletion of the long arm of chromosome 11, del (11q). The loss of Y aberration has often been associated with advanced age in males. Myelodysplastic syndrome is typically a disease of the advanced age, hence loss of Y would be common in MDS and prognostic correlations show that it leads to a favorable or neutral prognosis (Goldberg, Chen et al. 2010, Flandrin 2002). A score of 'good' is given with normal karyotypes, del(5q), del(12p), del(20q), and double abnormalities with del(5q). Cytogenetic findings of del(7q), +8, +19, i(17q), and the presence of any other single or double independent clones leads to an 'intermediate' score. A 'poor' score consists of -7, inv(3)/t(3q)/del(3q), double abnormalities including -7/del(7q), and complex karyotypes with three abnormalities. A 'very poor' score is given to adult MDS cases with more than three cytogenetic abnormalities (Greenberg 2015, Greenberg, Tuechler et al. 2012).

In general, these prognostic scores are meant as a point of reference for MDS cases and caution should be used when pediatric management and therapeutic strategies are utilized. Even though the IPSS is a useful therapy guide in adults, a validated and useful prognostic guide for the pediatric group has not been established to date and is needed for therapeutic success in this group of patients.

## Utility of Microarray Studies

Understanding the genetic characteristics unique to the pediatric group is the first step to decipher the unknowns in this group of MDS. Conventional techniques are able to detect chromosomal abnormalities in over 50% of primary MDS and 80% of secondary

MDS cases but in order to detect aberrations that may be too small or that are missed by these techniques; we need to utilize higher resolution array techniques. This technique is a reliable and efficient tool to identify genomic alterations in MDS from bone marrow DNA (Maciejewski, Tiu et al. 2009). When deletions and gains are observed cytogenetically, this suggests haplosufficiency, a loss of tumor suppressor genes, or oncogene activation and microarray can shed light onto these molecular alterations.

Microarray will also be useful in suboptimal cases with little or no growth of cells and for those studies that present normal karyotypes and FISH analyses (Visconte, Selleri et al. 2014). With rare diseases that have limited information, we are in need of a starting point and we can achieve this through the utilization of resources and techniques that are available to us. Copy number variations (CNV), microdeletions and single-nucleotide polymorphisms (SNPs), and specific gene disruptions that alter epigenetic regulators can be detected using microarray techniques. High-resolution microarray can improve the detection of genomic aberrations in MDS by identifying key alterations in copy number gains or losses in DNA from MDS samples. Microarray studies do not require chromosome preparations to determine copy number changes and they can identify very small deletions and duplications that would otherwise be undetected by conventional cytogenetic techniques (Silva, Maschietto et al. 2013, Ismael, Shimada et al. 2012).

Current novel studies have determined that the majority of adult MDS patients, over 80%, have a detectable somatic alteration among genes involved in various epigenetic regulatory pathways (Shih, Abdel-Wahab et al. 2012, Bejar 2014). Over 60 genes have been identified and play a role in chromatin modification, DNA methylation, transcriptional regulation, DNA repair/tumor suppressor, signal transduction, and cohesion complexes. RNA splicing mutations play a major role in determining clinical

features of the disease including the morphological features and OS of the patient (Zhang, Padron et al. 2015, Jhanwar 2015, Greenberg 2015, Visconte, Selleri et al. 2014, Papaemmanuil, Gerstung et al. 2013). However, these somatic mutations have yet to be included into the current prognostic scoring systems and most studies detailing these genetic changes have been conducted on adult MDS cases. The continued identification of key genes is a crucial step in deciphering players involved in hematopoietic transformation in the pediatric MDS group. This information can be used to better equip the medical community in accurately diagnosing and providing prognostic implications for therapy and treatment (Bejar 2014).

## Hypothesis and Specific Objectives

Since MDS is less frequently observed in the younger populations, there is limited information available in comparison to the elderly population including diagnostic criteria and prognostic implications. The younger population is relatively less exposed to the environmental and occupational risk factors, and for a shorter time span, therefore, it is more likely that a genetic alteration may be playing a role in disease causation.

Genetic disorders are closely associated with a predisposition to MDS including Down syndrome, Bloom syndrome, and IBMF syndromes but not all pediatric MDS patients share one of these constitutional syndromes. More research is needed in order to decipher the genetic causes of the disease. Our overall working hypothesis is that *there are distinct genetic differences between pediatric/young adult and elderly MDS populations; and in-depth investigations can improve the understanding of the pathophysiology, refine diagnostic categorization and therapeutic stratification using comprehensive analysis of multiple genetic techniques*.

Inconsistence and imprecise classification of pediatric MDS causes differences in the diagnosis and reporting of this rare group. We propose to perform cytogenetic comparisons in pediatric/young adult and adult/elderly MDS samples. A comprehensive genetic analysis using microarray in a subset of pediatric/young adult MDS cases will be conducted. The significance of altered genes in various pathways will be determined. This study uses one of the largest cytogenetically characterized cohorts of MDS specimens in order to accurately detect frequencies of specific abnormalities. A small subset of pediatric/young adult cases will be used for high-resolution array studies to determine cryptic abnormalities not observed by conventional cytogenetic techniques. A comparison to existing reports helps determine the genomic alterations that specifically influence the classification and pathway changes in MDS cases. Even though current studies have determined a set of recurrent abnormal gene aberrations in MDS cases, the vastly different populations of adult and pediatric has not been well characterized at the molecular level (Papaemmanuil, Gerstung et al. 2013, Zhang, Padron et al. 2015).

Currently, there are no known targeted therapies based upon the genetic landscape in the younger populations. This study will give insight on any differences and similarities between MDS from the two age groups and will allow us to better understand the key genetic and prognostic differences that can subsequently aid in the development of treatments specific for the pediatric and young adult MDS populations (Mandel, Dror et al. 2002).

# MATERIALS AND METHODS

## MATERIALS AND METHODS

This study was comprised of conventional cytogenetics, FISH, and Affymetrix CytoScan® or OncoScan® array analysis of MDS specimens that were referred to the Human Genetics Laboratory at the University of Nebraska Medical Center from January 1, 2003 to December 31, 2015. Bone marrow aspirations and unstimulated peripheral blood samples were used for cytogenetic and FISH analyses and when available, DNA was extracted from leftover whole specimen for microarray analysis. This study was approved by the institutional review board.

### Specimen Collection and Handling

From January 1, 2003 through December 31, 2015, bone marrow and peripheral blood specimens with a clinical diagnosis of MDS were analyzed using conventional cytogenetic techniques including karyotyping and/or FISH. These specimen types have the capability of spontaneous proliferation and the manner in which the samples are collected and handled upon arrival into the laboratory greatly influences the quality of analyses.

Bone marrow aspirates and peripheral blood samples collected from 3992 consecutive samples obtained from 2948 MDS cases and analyzed for cytogenetic and/or FISH studies performed at the Human Genetics Laboratory at the University of Nebraska Medical Center from 1990 to 2015 were examined. Over the given time span, a variable number of specimens ranging from 1-13 were analyzed from each patient, thus explaining a total of 3992 consecutive specimens from 2948 MDS cases received in our laboratory.

***Sample Requirements for Blood and Bone Marrow:*** Peripheral blood samples and bone marrow aspirate collection methods were essentially the same. The specimens

were collected in sterile syringes or vacuum tubes containing preservation-free sodium heparin. Typically, the first few milliliters of bone marrow is the optimum sample with the highest amount of cells. In order to achieve the highest quality of results, 0.5 to 2.0mL of specimen was collected and transported at room temperature and processed for genetic studies in our laboratory.

## Conventional Cytogenetic Studies

Traditional cytogenetic studies utilizes actively dividing cells; bone marrow and peripheral blood contain proliferating cells but when dividing cells are few in numbers, additives are used to stimulate mitosis. Successful cytogenetic cultures had specific requirements for initiation, maintenance, and cell harvesting for optimum results. Prepared slides were stained using Giemsa banding (G-banding) techniques and analysis was performed using brightfield microscopes and fluorescence microscopes for FISH analyses in conjunction with a computerized imaging system for classical karyotyping (CytoVision® Image Analysis System, Leica Biosystems, Buffalo Grove, IL) (Figure 2).

***Specimen Culturing:*** Peripheral blood and bone marrow specimens were grown and maintained in a liquid growth medium containing essential components for optimal cell growth that produced high quality banded karyotypes rapidly. Chang Medium® BMC (Irvine Scientific, Irvine, CA) was used for human bone marrow and peripheral blood specimens for cytogenetic testing of hematological disorders. Chang Medium® BMC consists of RPMI Medium 1640 with fetal bovine serum (FBS), essential for good cell growth; HEPES buffer to maintain the proper pH; L-glutamine, an essential amino acid, a component for maximum cell growth; gentamicin sulfate, used to inhibit microbial growth in the medium; and giant cell tumor extract a specially formulated growth factor for bone marrow cultures.

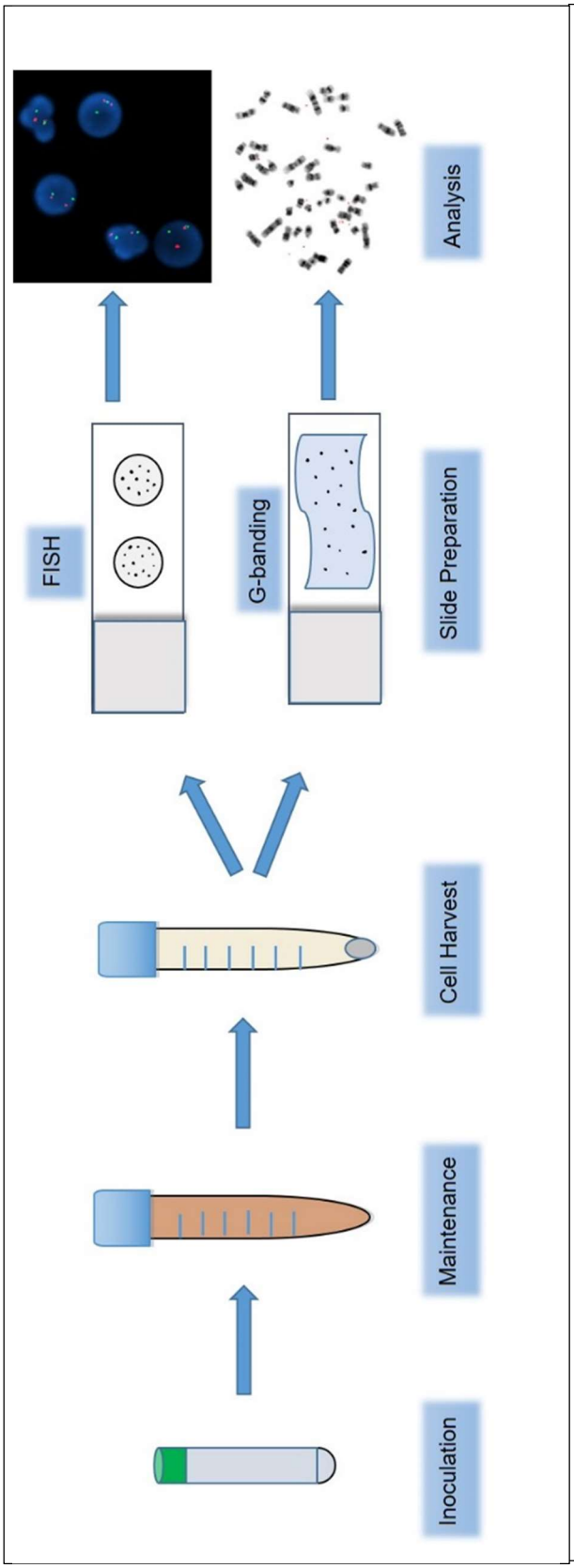



**Figure 2. Workflow of Cytogenetics and FISH**. Conventional cytogenetic studies included specimen inoculation and culture maintenance. After cell harvest, fixed cell pellets were used for FISH studies or cytogenetic karyotyping.

Chang Medium® BMC has been optimized to support efficient cell attachment and growth of bone marrow cells for cytogenetic analysis. These cultures were grown in suspension using sterile 15mL screw cap centrifuge tubes in a slant rack placed within a 37°C incubator with 0.2 to 0.5mL of whole specimen added aseptically. As described in Figure 3, multiple cultures were initiated and incubated for 24 and 48-hour time periods. In one culture, Colcemid® (Irvine Scientific, Irvine, CA) was added to block mitotic cells at metaphase. For specimens collected and received on the same day in the laboratory, a direct culture (DIR) was set up which nearly eliminates cell culture time by treating the bone marrow aspirate with a solution containing 1XTrypsin-EDTA (Irvine Scientific, Irvine, CA), hypotonic salts, and 0.08ug/ml Colcemid® (THC). The Trypsin-EDTA in this solution breaks up cell clusters and alters cell membranes, which facilitates better spreading of chromosomes during the slide preparation stage. A hypotonic salt solution containing 0.4% potassium chloride (KCL) was used to induce cell swelling and the simultaneous events of Trypsin-EDTA and KCL acted to "prime" the cells for the remaining steps of chromosome preparation. The immediacy of the THC treatment, especially with regard to the action of Colcemid® in the hypotonic solution, gives a better representation of the mitotic index and proportion of various cell types in the marrow at the time of aspiration. These cultures were incubated at 37°C in a slant rack for 20 minutes and were immediately harvested after the incubation time. A direct overnight culture (DON) was initiated for specimens received in the laboratory at least one day after collection. These cultures were incubated for 24 hours with Chang Medium® BMC and 10µl of Colcemid® (Dave, Wiggins et al. 2005, Dave, Hess et al. 1999, Higgins, Soe et al. 1993, Gersen, Keagle 2013).

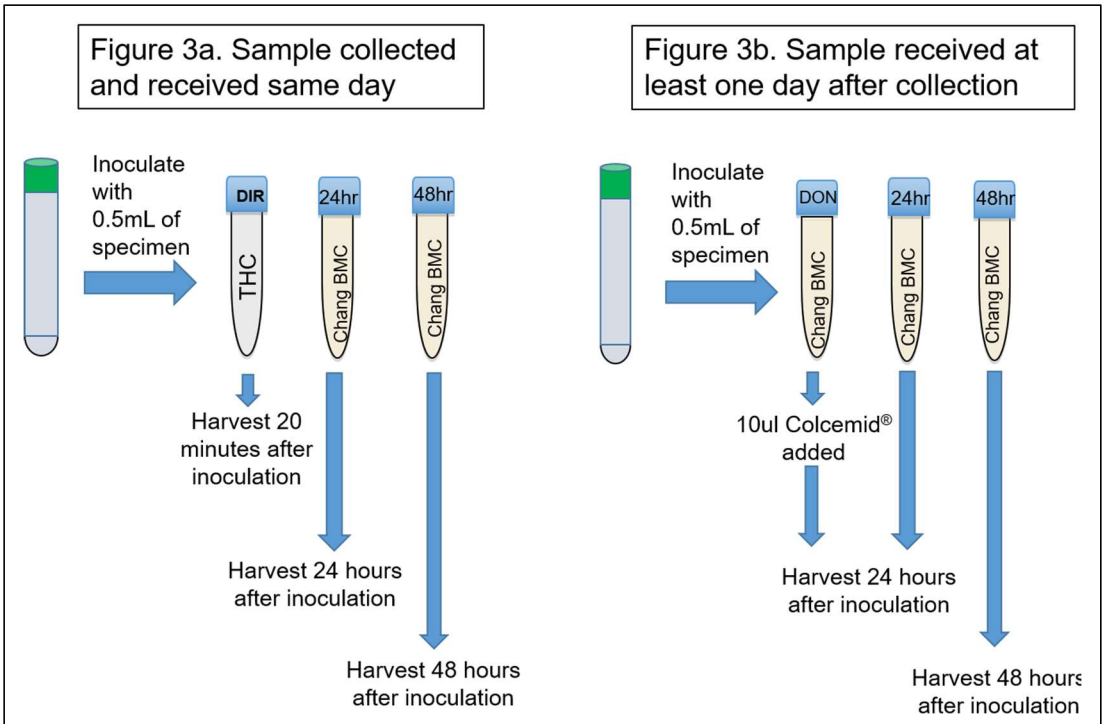



**Figure 3. Workflow of cytogenetic culture setup.** Culture setup for conventional cytogenetic studies based upon collection and received date of specimen. (a) When specimens were collected and received on the same day, two cultures were inoculated with 0.5mL of specimen in Chang BMC media and incubated for 24 and 48 hours before harvesting. One culture was inoculated with 0.5mL of specimen in THC (1XTrypsin-EDTA, hypotonic salts, and 0.08ug/ml Colcemid®), incubated for 20 minutes, and harvested. (b) When specimens were collected and received on different days, two cultures were inoculated with 0.5mL of specimen into Chang BMC media and incubated for 24 and 48 hours before harvesting. One culture had 0.5mL of specimen, Chang BMC media, and Colcemid® added, to block mitotic cells in metaphase, and was then incubated for 24 hours before harvesting.

***Specimen Harvesting:*** The harvesting of bone marrow and cancer blood samples was performed after the appropriate initial incubation period (20 minutes, 24 hours, or 48 hours). On the day of harvest, cultures were centrifuged for 6 minutes at 1500 rpm, the supernatant was aspirated off to 0.5-1.0 cm above the cell pellet, and 10mL of THC was added. Cultures were incubated in a slant rack for 20 minutes at 37°C. After the incubation time, suspension cultures were prefixed using 2mL of freshly prepared 3:1 methyl alcohol glacial acetic acid (Mallinckrodt Pharmaceuticals, St. Louis, MO) fixative for a gentle initial lysing of red blood cells. Specimen tubes were gently inverted to mix fixative and specimen culture and then spun at 1500 rpm for 6 minutes. Supernatant of the fixed cell pellet was aspirated 0.5-1.0cm above the cell pellet and 6mL of fixative was added, inverted to mix, and spun for 6 minutes. After aspirating the supernatant, 4mL of fixative was added, mixed with the culture, and spun for another 6 minutes. This step was repeated until the supernatant was clear of lysed red blood cells. After each fixation, the supernatant was removed closer to the cell pellet, approximately 0.5-1.0cm above to just above the cell pellet (Higgins, Soe et al. 1993, Howe, Umrigar et al. 2014).

***Slide Preparation***: After specimen culturing and harvesting, slide preparations were prepared from fixed cell pellets free of lysed red blood cells. Fixed cells were dropped onto pre-cleaned cold, wet slides with a micropipette at a 45° angle. To obtain optimum chromosome spreading and morphology, a temperature and humidity controlled environmental chamber was utilized during the slide preparation and drying process. The Thermatron Drying Chamber CDS-5® (Venturedyne, Ltd., Holland, MI.) was set at 27°C and 47% relative humidity, which is ideal for metaphase spreads for bone marrows and peripheral bloods (Howe, Umrigar et al. 2014, Dave, Wiggins et al. 2005, Higgins, Soe et al. 1993).

***Slide Staining:*** To produce high-contrast permanent banding of chromosomes, G-banding techniques were utilized. Unstained slides were aged by incubation in a 100ºC hot oven for 25 minutes before staining reagents were applied to enhance the absorption of stain and to produce a more consistent banding pattern. Slides with metaphase spreads were placed in 1X Trypsin-EDTA with Hanks Balanced Salt Solution (HBSS) (Irvine Scientific, Irvine, CA) for 6 seconds and then stained for approximately 50 seconds with Wright's working stain, prepared by adding one-part Wright's Stock Solution (Sigma-Aldrich, St. Louis, Missouri) to two parts of pH 6.8 Gurr's Buffer (BDH Laboratory, Poole, England). After the appropriate time, slides were immediately rinsed with cold fresh water and air dried before analyses.

***Cytogenetic Analysis:*** Giemsa-banded chromosomes were analyzed using bright-field microscopes (Olympus BX models). Image capture and karyotypic analysis was performed using CytoVision® Image Analysis System (Leica Biosystems, Buffalo Grove, IL, USA). Karyotypes were described and documented according to the most recent International System for Human Cytogenetic Nomenclature (ISCN 2013) (Shaffer, McGowan-Jordan et al. 2013). A minimum of 20 metaphases from at least two independently established cultures were examined, when possible, for each case. The cells selected for analysis had a band-level resolution greater than or equal to 400 and a range of chromosome morphology for unbiased results. A normal karyotype was defined as the lack of an identifiable abnormal clone in the metaphase cells analyzed. An abnormal clone was defined, according to the ISCN guidelines, when three cells containing the same loss of chromosome and two cells showing the same gain of chromosome or structural abnormality was observed. Independent clones, clonal evolution with one or more subclones, and complex karyotypes containing three or more aberrations were also reported according to ISCN guidelines. Conventional chromosome

analysis is used at time of diagnosis as a prognostic tool and can be especially beneficial to track clonal evolution and progression of the disease (Dave, Wiggins et al. 2005, Valent, Horny 2009, Valent, Horny et al. 2007).

## Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization techniques allow for the rapid and precise detection of specific nucleic acid sequences on interphase cells from fixed cells pellets following specimen initiation, maintenance, and harvesting as described previously. This technique was applicable for the detection of aneuploidy, translocations, and the identification of small marker and derivative chromosomes. This technique was a valuable addition to conventional cytogenetic analysis for diagnostic evaluation of hematologic disorders especially when metaphases were unavailable or suboptimal (Dave, Wiggins et al. 2005). Figure 4 depicts the flow diagram describing the technical steps involved in the FISH process. Fixed cells were dropped on clean slides specifically for FISH studies and probe mixtures were prepared and applied. A series of steps including co-denaturation, hybridization, and counter staining were performed before FISH analysis was carried out. This technique utilized a defined panel of probes specific for characteristic chromosome aberrations observed in MDS patients (Pitchford, Hettinga et al. 2010). The panel consisted of commercially available probes: LSI® EGR-1 (5q31)/D5S23, D5S721 (5p15.2) DNA Probe, the D7S486 (7q31)/CEP 7 (D7Z1) DNA Probe, and either the LSI® D20S108 (20q12) DNA Probe (Abbott-Vysis) or the Cytocell Del(20q) (20q12 and 20q13.12) Deletion Probe (Cytocell, Cambridge, UK) cocktailed with the CEP 8 (D8Z1) DNA Probe (Abbott-Vysis, Abbott Park, IL) (Figure 5) (Dave, Wiggins et al. 2005, Dave, Hess et al. 1999, Rigolin, Bigoni et al. 2001, Costa, Valera et al. 2010, Bernasconi, Cavigliano et al. 2003, Mallo, Arenillas et al. 2008).

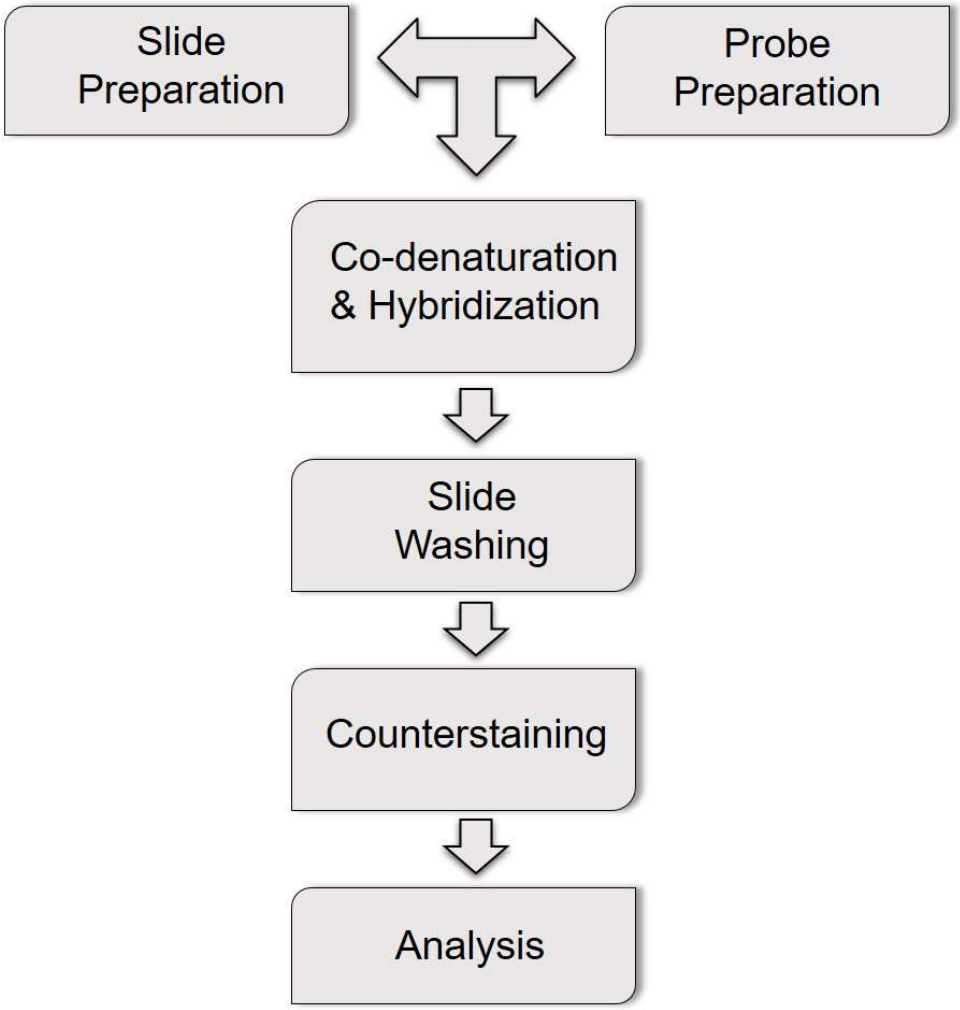



**Figure 4. Workflow of FISH studies**. Slides and probes were prepared before co-denaturation and hybridization occurred. Slides were then washed and counterstained before analysis.

***Slide Preparation***: Fixed cells from cultured bone marrow were used to prepare slides specifically for FISH analyses. Using the Thermatron Drying Chamber® provided a consistent environment for optimal results of 25°C temperature and 47% relative humidity during dropping. On each slide, two areas were utilized for cell dropping. Typically, 10µL of resuspended cells were placed on each hybridization area. Once the slides were dry, the slides were examined under a phase contrast microscope to verify that an adequate number of interphase cells were present. If inadequate numbers of cells were present, more cells were dropped on the specific hybridization area before proceeding with aging the cells in a hot oven at 100°C for two minutes (Dave, Hess et al. 1999).

***FISH Probe Preparation:*** The preparation of probe and buffer mixtures from commercially purchased probes were performed after slide preparation. Probes for the MDS specific abnormalities were mixed in a solution that contained 1µL of probe with 7µL of probe specific LSI or CEP hybridization buffer and 1µL of sterile, deionized water. Only 3µL of the probe mixture containing the specific probe, buffer, and water was applied to the hybridization area on the pre-warmed slide. Each hybridization area was coverslipped with 12mm round coverslips and then sealed with rubber cement.

***Co-Denaturation and Hybridization:*** Interphase cells and probes applied to the slide were co-denatured and hybridized using a HYBrite™ or a ThermoBrite™ instrument (Abbott-Vysis, Abbott Park, IL). The co-denaturing and hybridization of both the target DNA and the specific probe set was performed at 78°C for 3 minutes and then 39°C overnight when using the HYBrite™ instrument. When using the ThermoBrite™ instrument, the program consisted of 75°C for 1 minute and overnight at 37°C.

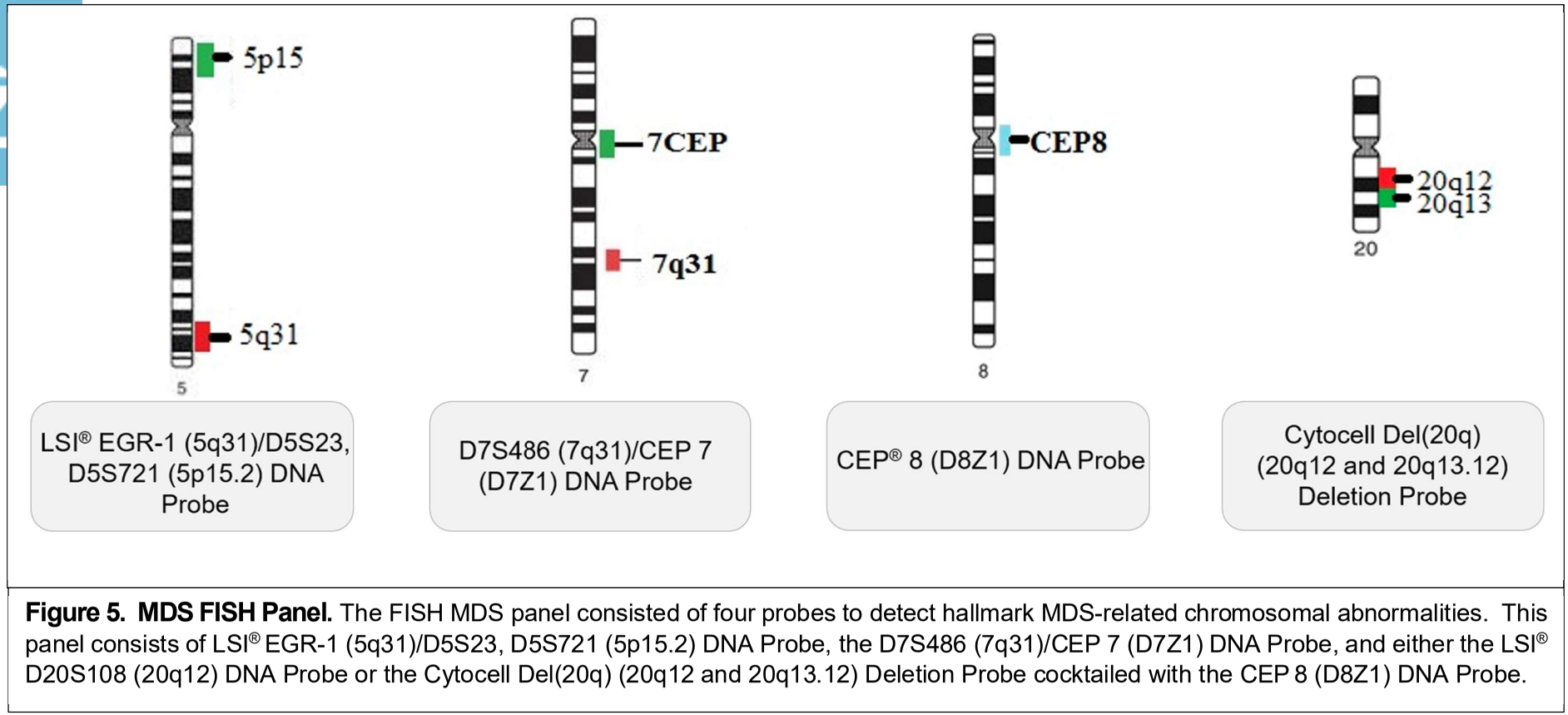



**Figure 5. MDS FISH Panel.** The FISH MDS panel consisted of four probes to detect hallmark MDS-related chromosomal abnormalities. This panel consists of LSI® EGR-1 (5q31)/D5S23, D5S721 (5p15.2) DNA Probe, the D7S486 (7q31)/CEP 7 (D7Z1) DNA Probe, and either the LSI® D20S108 (20q12) DNA Probe or the Cytocell Del(20q) (20q12 and 20q13.12) Deletion Probe cocktailed with the CEP 8 (D8Z1) DNA Probe.

***Slide Washing:*** Following hybridization, the rubber cement and round coverslips were removed and slides were washed with 0.4% sodium chloride and sodium citrate (SSC) (Sigma-Aldrich, St. Louis, MO) containing 0.3% Nonidet P-40 (NP-40) (Abbott-Vysis, Abbott Park, IL). The wash solution was at a temperature of 72°C and was performed for 2 minutes. Slides were then placed in 2XSSC with 0.1% NP-40 for 1 minute at room temperature. The slides were removed and air-dried before counter-staining.

***Counter Staining***: Slides containing interphase nuclei were counterstained with 10µL of 4,6-diamidino-2-phenylindole (DAPI II) in Antifade solution (Abbott-Vysis, Abbott Park, IL) proceeding co-denaturation, hybridization, and slide washing. After the application of DAPI II, a 22x50mm coverglass was applied for slide protection and for microscopic analysis.

***FISH Analysis:*** The prepared slides were analyzed using Olympus BX61 or Leica DM6000B fluorescence microscopes equipped with appropriate filters for individual colors: red, green, aqua, and DAPI as well as dual color red/green and DAPI/red/green filters. Hybridization signals, when available, were assessed in 50-200 interphase nuclei per probe. A deletion or loss of DNA region was determined by the absence of probe signals specific for the region of interest with the presence of applied controls. A gain was defined as the presence of greater than two individual probe signals specific for the region of interest. Abnormal ranges were established for each specific probe and included 5-100% of total interphase cells observed for probes detecting -5, -7, +8, and concurrent deletions of 20q12-20q13.12; for deletions of 5q31 and 7q31 the abnormal range was 7-100%; and for a deletion of 20q12 the abnormal range was 10-100%. Images were acquired, analyzed, and archived using the Leica Biosystems capture software, CytoVision® Image

Analysis System (Leica Microsystems, Buffalo Grove, IL) (Dave, Wiggins et al. 2005, Dave, Hess et al. 1999, Rigolin, Bigoni et al. 2001).

## Microarray

Microarray is a reliable and powerful molecular tool that can be used in conjunction with traditional cytogenetics to identify genomic alterations in MDS from bone marrow DNA. This technique aids in providing an overview of DNA sequence copy number changes including losses, gains, and amplifications for the whole genome. Unlike other molecular methods, microarray allows for a complete analysis of every chromosome in the genome using extracted DNA from whole bone marrow and peripheral blood (Bejar, Stevenson et al. 2011, Shih, Abdel-Wahab et al. 2012, Ismael, Shimada et al. 2012, Orazi, Germing 2008). This method utilized the Affymetrix CytoScan® HD Array (Affymetrix, Santa Clara, CA) protocol that included a series of specific steps of DNA digestion, ligation, PCR, purification, fragmentation, labeling, hybridization, array washing, scanning, and analysis using the Affymetrix Chromosome Analysis Suite (ChAS) software. For three cases, we utilized the Affymetrix OncoScan® FFPE Assay (Affymetrix, Santa Clara, CA) protocol to validate the use of whole bone marrow specimen on this platform. This protocol contained a series of stages including annealing, gap filling, two rounds of PCR, digestion, hybridization followed by washing, staining, and scanning.

***DNA Extraction***: Genomic DNA was isolated from whole bone marrow and peripheral blood samples when sufficient specimen was available after conventional cytogenetic and FISH analyses. DNA was extracted using the Qiagen QIAcube® automated robot and Qiagen spin column kits (Qiagen, Redwood City, CA). The Qiagen QIAcube protocol utilized advanced technology to enable a completely automated, low-throughput sample preparation of DNA purification from a small amount of original specimen (400µl). The

automated robot lysed the red blood cells of bone marrow and peripheral blood and bound the DNA to the Qiagen spin columns. The bound DNA was washed and then eluted from the column leaving a total of 100µl of high quality DNA.

***DNA Quantification***: DNA samples that presented high quality, based upon spectrophotometry results, were used for microarray. DNA quantification was determined using the Qubit™ 3.0 Fluorometer instrument (ThermoFisher Scientific, Waltham, MA). The Qubit® fluorometer uses commercially purchased standards and a small amount of purified DNA that enables a greater sensitivity and accuracy than UV absorbance measurements. This analytical assay determined DNA quality of 40 to 400 µg/ml for 26 of the 28 bone marrow and peripheral blood specimens collected for microarray studies.

### *CytoScan® HD Array Assay Technique*

The Affymetrix CytoScan® HD Array offered high-density resolution of the entire genome, covering promotor and miRNA regions, for detection and reporting of abnormalities. It provided maximum coverage for precise mapping of the whole genome for detecting copy number variations and copy neutral loss of heterozygosity (LOH) in addition to detecting novel submicroscopic deletions/duplications. This assay included 750,000 million SNPs with over 99% accuracy to detect chromosomal aberrations across the genome. The CytoScan® Assay protocol was optimized for processing 8 to 24 samples in parallel and followed eight stages of DNA digestion, ligation, PCR, PCR purification, PCR quantification, fragmentation, labeling, and hybridization. After the arrays were hybridized, the chips were then washed and stained and scanned using the Affymetrix GeneChip® Scanner 3000 7G, controlled by the Affymetrix GeneChip® Command Center. The exported array results were analyzed using the Affymetrix Chromosome Analysis Suite (ChAS) software.

***CytoScan® HD Array Digestion:*** Restriction enzyme digestion of the purified DNA sample utilized Nsp I enzyme. A master mix of Affymetrix CytoScan® chilled nuclease-free water, 10X Nsp I buffer, 100X BSA, and the Nsp I enzyme was prepared on ice (Figure 6a). Only 14.75µL of this master mix was aliquoted to 5.0µL of genomic DNA (50ng/µL) and 5.0µL of a negative control containing low EDTA TE buffer in a 96-well plate. The plate was placed into a preheated GeneAmp™ PCR System 9700 thermocycler (Applied Biosystems, Waltham, MA) and the CytoScan® Digest program was set for approximately 140 minutes before proceeding to stage two (Figure 6b).

***CytoScan® HD Array Ligation:*** After digestion with Nsp I, adaptors were ligated using a master mix composed of Affymetrix CytoScan® 10X T4 DNA ligase buffer, 50µM Nsp I adaptor, and T4 DNA ligase (Figure 7a). A fraction of the ligation mixture, 5.25µL, was added to the digested sample plate for a total reaction volume of 25µL. The ligation plate was placed into a preheated thermocycler and the CytoScan® Ligate program ran for approximately three and a half hours (Figure 7b).

***CytoScan® HD Array Polymerase Chain Reaction (PCR):*** Before PCR could proceed, the ligated samples were diluted with Affymetrix nuclease-free water. The 25µL ligated DNA was diluted with 75µL of Affymetrix nuclease-free water to a final volume of 100µL. The PCR reaction utilized a Titanium™ DNA amplification kit (Clontech Laboratories, Takara Bio Company, Mountain View, CA) and water from Affymetrix CytoScan® reagents. Only 10µL of the diluted samples were then transferred to four empty wells on a 96-well plate. A PCR master mix was prepared with chilled Affymetrix nuclease-free water, 10X Titanium™ *Taq* PCR buffer, GC-melt reagent, dNTP mixture (2.5mM each), PCR primer (002), and 50X Titanium™ *Taq* DNA polymerase was added immediately before aliquoting into the diluted ligated sample wells (Figure 8a).

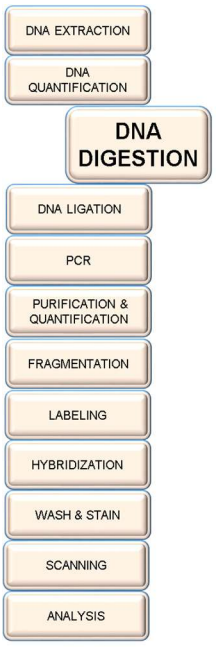



**Figure 6a. Affymetrix CytoScan® Restriction Enzyme Digestion Master Mix**

| Reagent | 1 Sample | 8 Samples |
|---|---|---|
| Chilled Affymetrix® Nuclease-Free Water | 11.55µL | 110.9µL |
| 10X Nsp I Buffer | 2.00µL | 19.2µL |
| 100X BSA | 0.20µL | 1.9µL |
| Nsp I | 1.00µL | 9.6µL |
| Total | 14.75µL | 141.6µL (20% overage) |

**Figure 6b. CytoScan® Digestion Thermocycler Program**

| Temperature | Time |
|---|---|
| 37°C | 2 hours |
| 65°C | 20 minutes |
| 4°C | Hold |

**Figure 6. Restriction enzyme digestion CytoScan® protocol.** (a) The restriction enzyme digestion master mix utilized Affymetrix CytoScan® chilled nuclease-free water, 10X Nsp I buffer, 100X BSA, and the Nsp I enzyme. (b) The digestion reaction was performed in a thermocycler for 140 minutes. The process involves specific temperature changes as mentioned.

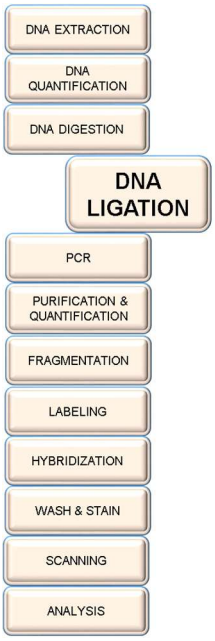



**Figure 7a. Affymetrix CytoScan® Ligation Master Mix**

| Reagent | 1 Sample | 8 Samples |
|---|---|---|
| 10X T4 DNA Ligase Buffer | 2.50µL | 25.0µL |
| 50µM Adaptor, Nsp I | 0.75µL | 7.5µL |
| T4 DNA Ligase | 2.00µL | 20.0µL |
| Total | 5.25µL | 52.5µL (25% overage) |

**Figure 7b. CytoScan® Ligation Thermocycler Program**

| Temperature | Time |
|---|---|
| 16°C | 3 hours |
| 70°C | 20 minutes |
| 4°C | Hold |

**Figure 7. Ligation CytoScan® protocol**. (a) The ligation master mix utilized Affymetrix CytoScan® 10X T4 DNA ligase buffer, 50µM Nsp I adaptor, and T4 DNA ligase. (b) The ligation reaction was performed in a thermocycler for approximately 3 ½ hours at various temperatures.

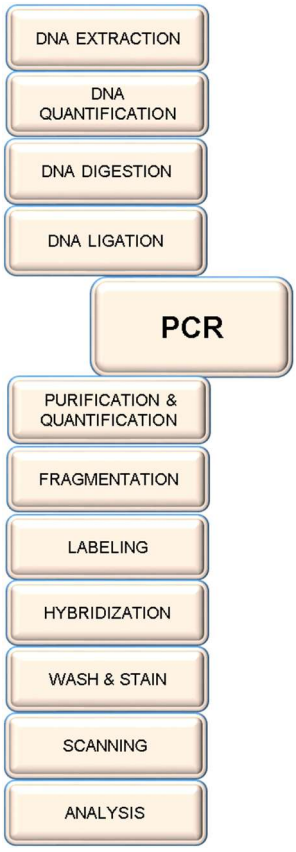



**Figure 8a. Affymetrix CytoScan® PCR Master Mix**

| Reagent | 1 Sample | 8 Samples |
|---|---|---|
| Chilled Affymetrix® Nuclease-Free Water | 39.5µL | 1453.6µL |
| 10X Titanium™ *Taq* PCR Buffer | 10.0µL | 368.0µL |
| GC-Melt Reagent | 20.0µL | 736.0µL |
| dNTP Mixture (2.5mM each) | 14.0µL | 515.2µL |
| PCR Primer, 002 | 4.5µL | 165.6µL |
| 50X Titanium™ *Taq* DNA Polymerase | 2.0µL | 73.6µL |
| Total | 90µL | 3312.0µL (15% overage) |

**Figure 8b. CytoScan® PCR Program for GeneAmp® PCR System 9700**

| Temperature | Time | Cycles |
|---|---|---|
| 94°C | 3 minutes | 1X |
| 94°C<br>60°C<br>68°C | 30 seconds<br>45 seconds<br>15 seconds | 30X |
| 68°C | 7 minutes | 1X |
| 4°C | Hold | |

**Figure 8. PCR CytoScan® protocol**. (a) The PCR master mix utilized chilled Affymetrix nuclease-free water, 10X Titanium™ *Taq* PCR buffer, GC-melt reagent, dNTP mixture (2.5mM each), PCR primer (002), and 50X Titanium™ *Taq* DNA polymerase. (b) The PCR reaction was performed in a thermocycler for approximately 55 minutes.

The PCR plate was placed into a preheated thermocycler in the post-PCR area to avoid contamination. A total of 90µL of the PCR master mix was added to each of the four 10µL ligated and diluted DNA samples. The CytoScan® PCR program required approximately 55 minutes for completion (Figure 8b).

After the PCR reaction, 3µL of the product quality was determined by running it on a 2% TBE (Lonza Group, LTD, Switzerland) precast gel against 5µL of USB PCR marker 50-2000bp (Affymetrix, Santa Clara, CA) ladder for 20 minutes at 5V/cm. This gel contained ethidium bromide to visually observe the PCR product distribution. The PCR product was confirmed as good quality and ready to proceed to PCR purification when the average product distribution was between 150 and 2000bp (Figure 9).

***CytoScan® HD Array PCR Purification***: Before the PCR product was purified, the aliquots of each sample were combined for a total of 397 µL (100µL from each well, minus 3µL for the PCR gel). After the samples were pooled, 720µL of Affymetrix CytoScan® purification beads were added to bind to the DNA in the PCR sample. The DNA was separated using magnetic stands (MagnaRack, Life Technologies, Carlsbad, CA) that allowed the magnetic beads with bound DNA to be pulled aside. The supernatant was removed and the pellet of DNA and beads was washed with 1mL of Affymetrix CytoScan® purification wash buffer. The supernatant was then removed again with the use of magnetic stands. DNA was eluted from the magnetic beads using 52µL of Affymetrix CytoScan® elution buffer and beads were pulled away from the DNA with the magnetic stand; 47µL of the eluted sample was removed and transferred to a fresh 96-well plate.

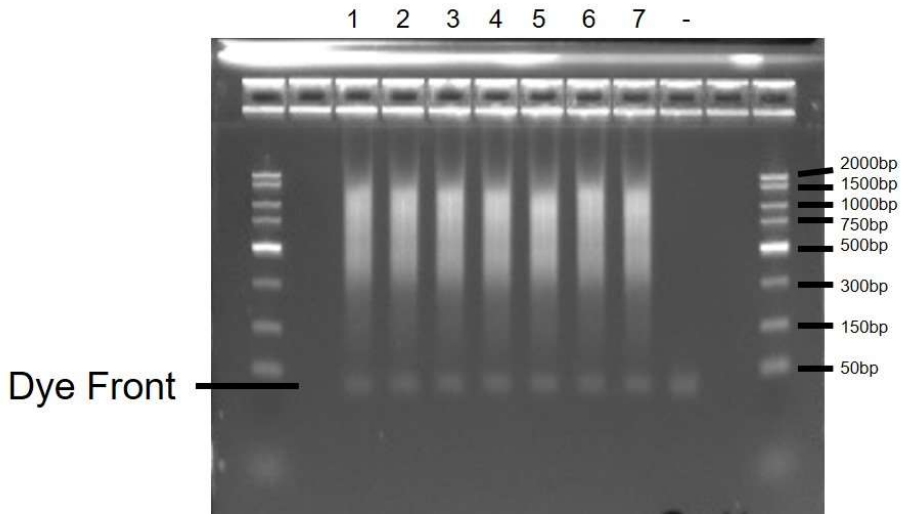



**Figure 9. Verification of the PCR product**. The product was of good quality when the average product distribution was between 150 and 2000bp on a 2% TBE gel.

***CytoScan® HD Array PCR Quantification***: After the PCR product was purified, the quantification of the DNA was determined using a NanoDrop® Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE). The DNA sample was first diluted using 18µL of Affymetrix nuclease-free water and 2µL of product. The NanoDrop® was blanked with water and 2µL of the diluted sample was measured at OD260/OD280. The DNA yield was within the acceptable range of 250ng/µL and above in order for fragmentation to be initiated.

***CytoScan® HD Array Fragmentation:*** The fragmentation stage has critical temperature sensitivities and in order to ensure uniform, reproducible fragmentation this step was performed rapidly on ice and in a plate centrifuge at 4°C. The fragmentation master mix was made using Affymetrix CytoScan® reagents with a concentration of 2.25U/µL. The master mix included chilled Affymetrix nuclease-free water, 10X fragmentation buffer, and fragmentation reagent (Figure 10a). An amount of 10µL of the fragmentation master mix was added directly to the purified PCR products in a 96-well plate on ice. The plate was transferred to a preheated thermocycler and the CytoScan® fragment program was performed for approximately 50 minutes (Figure 10b).

Once the fragmentation program was completed, the sample was removed and the products were checked on a 4% TBE (Lonza Group, LTD, Switzerland) precast gel at 5V/cm for 19 minutes. The finished product was diluted using 4µL of the finished fragmented sample with 28µL of water and a further dilution of 8µL of the original dilution added to 12µL of water. The final diluted fragmentation sample was loaded onto the gel against the TrackIt™ 25bp DNA ladder (Life Technologies, Carlsbad, CA) to determine the distribution of the product. The majority of fragmented PCR products were between 25 to 125bp and were ready to proceed to labeling (Figure 11).

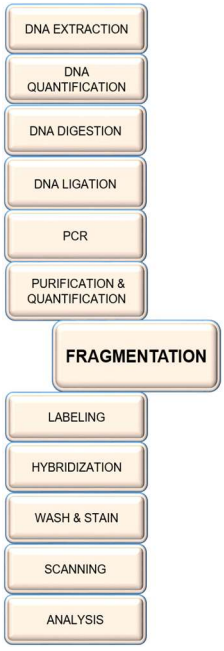



**Figure 10a. Affymetrix CytoScan® Fragmentation Master Mix**

| Reagent (2.25U/µL Concentration) | Amount |
|---|---|
| Chilled Affymetrix® Nuclease-Free Water | 123.2µL |
| 10X Fragmentation Buffer | 158.4µL |
| Fragmentation Reagent | 6.4µL |
| Total | 288.0µL |

**Figure 10b. CytoScan® Fragmentation Thermocycler Program**

| Temperature | Time |
|---|---|
| 37°C | 35 minutes |
| 95°C | 15 minutes |
| 4°C | Hold |

**Figure 10. Fragmentation CytoScan® protocol**. (a) The fragmentation master mix utilized chilled Affymetrix CytoScan® reagents with a concentration of 2.25U/µL and included Affymetrix nuclease-free water, 10X fragmentation buffer, and fragmentation reagent. (b) The fragmentation reaction was performed in a thermocycler for approximately 50 minutes.

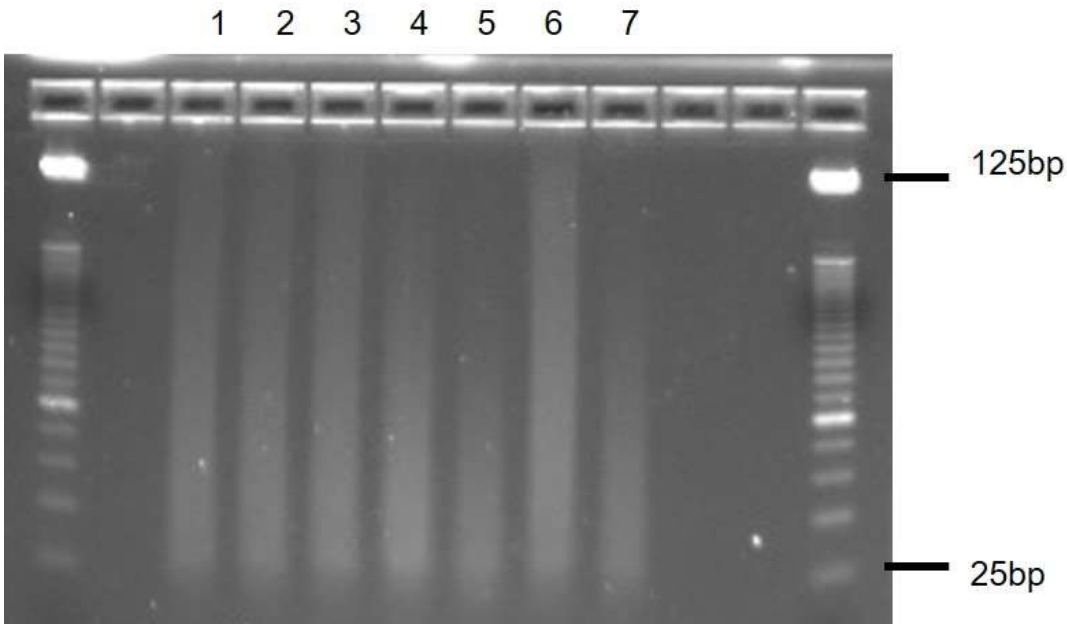



**Figure 11. Verification of the fragmentation product**. The product was of good quality when the majority of the product distribution was between 25 and 125bp on a 4% TBE gel.

***CytoScan® HD Array Labeling***: The fragmented PCR product was labeled using terminal deoxynucleotidyl transferase (TdT) enzyme. The master mix solution was composed of Affymetrix CytoScan®5X TdT buffer, 30mM DNA labeling reagent, and TdT enzyme (Figure 12). A portion (19.5 µL) of the labeling mix was added to the remaining 51.0µL of fragmented DNA; 4.0µL was used for the fragmentation gel analysis. The plate was transferred to a preheated thermocycler and the CytoScan® label program was performed for approximately 4 hours and 15 minutes (Figure 12b).

***CytoScan® HD Array Hybridization***: Target hybridization was performed using the Hybridization Oven 645® at a temperature of 50°C at a rotation speed of 60rpm. Arrays for each patient were recorded and entered into the Affymetrix GeneChip® Command Center® 3.2 and then left at room temperature until they were loaded. A hybridization master mix was made using the Affymetrix CytoScan® Hyb buffer part 1, Hyb buffer part 2, Hyb buffer part 3, Hyb buffer part 4, and oligo control reagent 0100 (Figure 13a) and 190µL of the mix was added to the labeled samples. The plate was placed into the thermocycler and the CytoScan® Hyb program ran for about 10 minutes (Figure 13b). When the thermocycler reached 49°C, the samples were injected into the septa of the array and covered with ½" Microtube Tough-Spots (Diversified Biotech, Boston, MA). The arrays were placed into an oven tray evenly spaced and allowed to rotate at the appropriate temperature and speed for 16 to 18 hours.

### *OncoScan® FFPE Assay Technique*

The OncoScan® Assay utilized the Molecular Inversion Probe (MIP) assay technology for genome wide copy number and LOH profiles. This test was developed to perform well with highly degraded DNA. This protocol is typically reserved for fixed formalin paraffin embedded (FFPE) preserved tumor samples of various ages and with

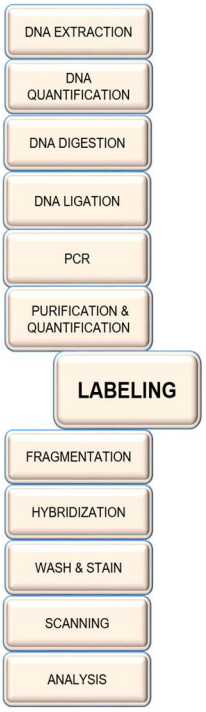



**Figure 12a. Affymetrix CytoScan® Labeling Master Mix**

| Reagent | 1 Sample | 8 Samples |
|---|---|---|
| 5X TdT Buffer | 14.0µL | 134.4µL |
| 30mM DNA Labeling Reagent | 2.0µL | 19.2µL |
| TdT | 3.5µL | 33.6µL |
| Total | 19.5µL | 187.2µL (20% overage) |

**Figure 12b. CytoScan® Labeling Thermocycler Program**

| Temperature | Time |
|---|---|
| 37°C | 4 hours |
| 95°C | 15 minutes |
| 4°C | Hold |

**Figure 12. Labeling CytoScan® protocol**. (a) The labeling master mix utilized Affymetrix CytoScan®5X TdT buffer, 30mM DNA labeling reagent, and TdT enzyme. (b) The labeling reaction was performed in a thermocycler for approximately 4 hours and 15 minutes.

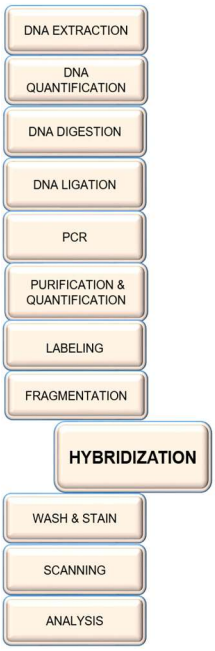



**Figure 13a. Affymetrix CytoScan® Hybridization Master Mix**

| Reagent | 1 Sample | 8 Samples |
|---|---|---|
| Hyb Buffer Part 1 | 165.0µL | 1584.0µL |
| Hyb Buffer Part 2 | 15.0µL | 144.0µL |
| Hyb Buffer Part 3 | 7.0µL | 67.2µL |
| Hyb Buffer Part 4 | 1.0µL | 9.6µL |
| Oligo Control Reagent 0100 | 2.00µL | 19.2µL |
| Total | 190µL | 1824µL (20% overage) |

**Figure 13b. CytoScan® Hybridization Thermocycler Program**

| Temperature | Time |
|---|---|
| 96ºC | 10 minutes |
| 49ºC | Hold |

**Figure 13. Hybridization CytoScan® protocol**. (a) The hybridization master mix utilized Affymetrix CytoScan® Hyb buffer part 1, Hyb buffer part 2, Hyb buffer part 3, Hyb buffer part 4, and oligo control reagent 0100. (b) The labeling reaction was performed in a thermocycler for approximately 10 minutes.

<100 ng DNA of starting material, which makes it the ideal assay for clinical cancer research. This laboratory validated the use of this assay on DNA extracted from whole bone marrow specimen for future research use. The OncoScan® Assay protocol was optimized for processing 6 to 24 samples in parallel and followed five stages of annealing, gap fill through first PCR, second PCR, HaeIII digestion, and hybridization. After the arrays were hybridized, the chips were then washed and stained and scanned using the Affymetrix GeneChip® Scanner 3000 7G, controlled by the Affymetrix GeneChip® Command Center. The exported array results were analyzed using the Affymetrix Chromosome Analysis Suite (ChAS) software.

***OncoScan® Annealing:*** Genomic DNA was normalized to 12ng/µL using 1X TE (pH 8.0) with reduced EDTA (0.1Mm EDTA) before annealing of probe mixes occurred. A master mix of Affymetrix OncoScan® buffer A, copy number probe mix 1.0, and somatic mutation probe mix 1.0 was prepared on ice (Figure 14a). Only 3.4µL of this master mix was aliquoted to 6.6µL of DNA leaving a total volume of 10µL. The OncoScan® positive and negative control included in the reagent kit was also added to the 96-well plate. The plate was placed into a GeneAmp™ PCR System 9700 thermocycler (Applied Biosystems, Waltham, MA) and the OncoScan® Anneal program ran overnight, for approximately 16-18 hours (Figure 14b).

***OncoScan® Gap Fill through first PCR:*** After the overnight anneal program, the plate was removed and the OncoScan® Gap Fill program was started. Once the temperature reached 58°C, the program was paused. The gap fill master mix was created and contained Affymetrix OncoScan® nuclease-free water, buffer A, SAP recombinant (1U/µL), and gap fill enzyme mix (Figure 15a). The mix was made on ice and 14.0µL was


**Figure 14a. Affymetrix OncoScan® Anneal Master Mix**

| Reagent | 1 Reaction | 7 Reactions (20% Overage) |
|---|---|---|
| Buffer A | 1.53µL | 17.1µL |
| Copy Number Probe Mix 1.0 | 1.37µL | 15.3µL |
| Somatic Mutation Mix 1.0 | 0.5µL | 5.6µL |
| Total Volume | 3.40µL | 38.1µL |

**Figure 14a. OncoScan® Anneal Master Mix**

| Temperature | Time |
|---|---|
| 95ºC | 5 minutes |
| 58ºC | 16-18 hours |
| 58ºC | Hold |

**Figure 14. Anneal OncoScan® protocol**. (a) The anneal master mix utilized Affymetrix OncoScan® buffer A, copy number probe mix 1.0, and somatic mutation mix 1.0. (b) The annealing reaction was performed in a thermocycler overnight for 16-18 hours.

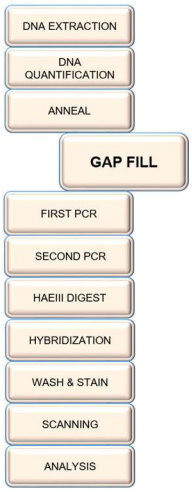



**Figure 15a. Affymetrix OncoScan® Gap Fill Master Mix**

| Reagent | 1 Reaction | 7 Reactions (20% Overage) |
|---|---|---|
| Nuclease-free Water | 10.58µL | 89µL |
| Buffer A | 1.18µL | 9.9µL |
| SAP, Recombinant (1U/µL) | 0.84µL | 7.1µL |
| Gap Fill Enzyme Mix | 1.40µL | 11.8µL |
| Total | 14.0µL | 117.6µL |

**Figure 15b. OncoScan® A/T and G/C Master Mix**

| Reagent | 1 Reaction | 7 Reactions (20% Overage) |
|---|---|---|
| Nuclease-free Water | 3.93µL | 113µL |
| dNTPs (A/T) | 0.07µL | 2.1µL |
| Total | 4.0µL | 115µL |

| Reagent | 1 Reaction | 7 Reactions (20% Overage) |
|---|---|---|
| Nuclease-free Water | 3.93µL | 113µL |
| dNTPs (G/C) | 0.07µL | 2.1µL |
| Total | 4.0µL | 115µL |

**Figure 15c. OncoScan® Cleavage Master Mix**

| Reagent | 1 Reaction | 14 Reactions (25% Overage) |
|---|---|---|
| Cleavage Buffer | 25.0µL | 438µL |
| Cleavage Enzyme | 0.2µL | 3.5µL |
| Total | 25.2µL | 441.0µL |

**Figure 15d. OncoScan® Gap Fill Thermocycler Program**

| Temperature | Time |
|---|---|
| 58ºC | 11 minutes |
| *Paused for dNTP addition* | |
| 58ºC | 11 minutes |
| *Paused for Exo Mix addition* | |
| 37ºC | 20 minutes |
| 95ºC | 10 minutes |
| *Paused for Cleavage Mix addition* | |
| 37ºC | 15 minutes |
| 95ºC | 15 minutes |
| 4ºC | Hold |

**Figure 15. Gap fill OncoScan® protocol.** a) The gap fill master mix utilized OncoScan® nuclease-free water, buffer A, SAP recombinant, and gap fill enzyme. b) The dNTP master mixes utilized nuclease-free water and the specific dNTP mixes (AT and GC). c) The cleavage master mix utilized cleavage buffer and enzyme. d) The gap fill reaction was performed in a thermocycler for approximately 90 minutes.

added to each reaction for a final volume of 24.0µL and left on ice while the first PCR plate was created.

The first PCR step required that the AT mix and GC mix be created independently in order to avoid contamination. Each mix contained Affymetrix OncoScan® nuclease-free water and the specific dNTPs (A/T or GC) (Figure 15b). A fresh 96-well plate, labelled *first PCR*, was used and designated rows for AT and GC were labeled. From the anneal plate, 10µL of the gap fill reactions were aliquoted into the wells of the two designated rows on the first PCR plate. The plate was loaded on the thermocycler and the Gap Fill program was resumed at 58ºC for 11 minutes. After this time, the program was paused and the first PCR plate removed to add 4µL of the A/T mix to each of the wells in the A/T designated row. The same amount of G/C mix was added to each of the wells in the G/C designated row on the first PCR plate. The plate was loaded back onto the thermocycler and the program was resumed for another 11 minutes. The program was paused before the cycler ramped down to 37ºC. The first PCR plate was removed and 3µL of Affymetrix OncoScan® Exo Mix reagent was added to each reaction for a final volume of 17µL. The plate was loaded back onto the cycler and the program was resumed. During the last 5 minutes of the program at 95ºC, the cleavage master mix was created using Affymetrix OncoScan® cleavage buffer and cleavage enzyme (Figure 15c). The program was paused when the thermocycler reached the start of the 37ºC step and 25µL of the cleavage mix was added to each reaction. The thermocycler program was resumed and finished after 30 minutes (Figure 15d).

The first PCR master mix was created during the last 5 minutes of the Gap Fill program and consisted of Affymetrix OncoScan® PCR mix and *Taq* polymerase (Figure 16a). Once the Gap Fill program was completed, the plate was removed and 25µL of the

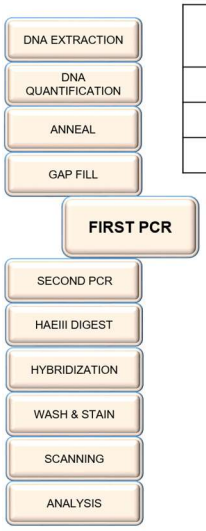



**Figure 16a. Affymetrix OncoScan® First PCR Master Mix**

| Reagent | 1 Reaction | 14 Reactions (25% Overage) |
|---|---|---|
| PCR Mix | 24.4µL | 428µL |
| Taq Polymerase | 0.56µL | 9.8µL |
| Total | 25.0µL | 437µL |

**Figure 16b. OncoScan® First PCR Thermocycler Program**

| Temperature | Time | Cycles |
|---|---|---|
| 60ºC | 30 seconds | 1X |
| 95ºC | 1 minute | 1X |
| 95ºC<br>60ºC<br>72ºC | 20 seconds<br>10 seconds<br>10 seconds | 20X |
| 72ºC | 5 minutes | 1X |
| 4ºC | Hold | |

**Figure 16. First PCR OncoScan® protocol**. a) The first PCR master mix utilized OncoScan® PCR mix and *Taq* polymerase. b) The first PCR reaction was performed in a thermocycler for approximately 20 minutes.

PCR master mix was added to each reaction. The OncoScan® First PCR program on the thermocycler was started and once the cycler reached 60°C the plate was loaded and ran for approximately 20 minutes (Figure 16b).

***OncoScan® Second PCR***: The second PCR stage occurred in the post-PCR lab. A fresh 96-well plate was labeled as the *second PCR plate* and designated AT and GC rows were marked. The second PCR master mix utilized OncoScan® PCR mix and *Taq* polymerase and 25µL of this mix was aliquoted to each of the designated wells (Figure 17a). From the first PCR plate, 2µL were removed and added to the corresponding wells on the second PCR plate. This plate was loaded onto the thermocycler and the OncoScan® Second PCR program ran for approximately 20 minutes (Figure 17b).

A quality control gel was used to check the first PCR product. From the first PCR plate, 8µL from each well were loaded onto a 3% precast agarose gel with a 50bp ladder (New England Biolabs, Ipswich, Massachusetts). The gel ran for 15 minutes at 150V/cm and the PCR products were determined of good quality when they were approximately at 120bp (Figure 18).

***OncoScan® Digestion:*** Smaller DNA fragments were generated to improve the sample hybridization onto the array during the *HaeIII* digestions. A fresh 96-well plate labeled *HaeIII* was used and the appropriate AT and GC rows were designated. The digestion master mix utilized OncoScan® buffer B, *HaeIII* enzyme, and exo enzyme (Figure 19a). From the master mix, 20µL was aliquoted to the appropriate wells of the *HaeIII* plate with 10µL of the second PCR amplified reaction. The plate was loaded on the thermocycler and the OncoScan® *HaeIII* program ran for approximately 100 minutes (Figure 19b).

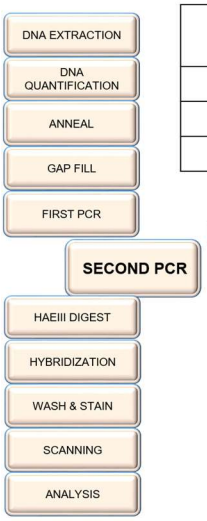



**Figure 17a. Affymetrix OncoScan® Second PCR Master Mix**

| Reagent | 1 Reaction | 14 Reactions (25% Overage) |
|---|---|---|
| PCR Mix | 24.4µL | 428µL |
| Taq Polymerase | 0.56µL | 9.8µL |
| Total | 25.0µL | 437µL |

**Figure 17b. OncoScan® Second PCR Thermocycler Program**

| Temperature | Time | Cycles |
|---|---|---|
| 60ºC | 30 seconds | 1X |
| 95ºC | 1 minute | 1X |
| 95ºC<br>60ºC<br>72ºC | 20 seconds<br>10 seconds<br>10 seconds | 15X |
| 72ºC | 5 minutes | 1X |
| 4ºC | Hold | |

**Figure 17. Second PCR OncoScan® protocol**. a) The second PCR master mix utilized OncoScan® PCR mix and *Taq* polymerase. b) The second PCR reaction was performed in a thermocycler for approximately 20 minutes.

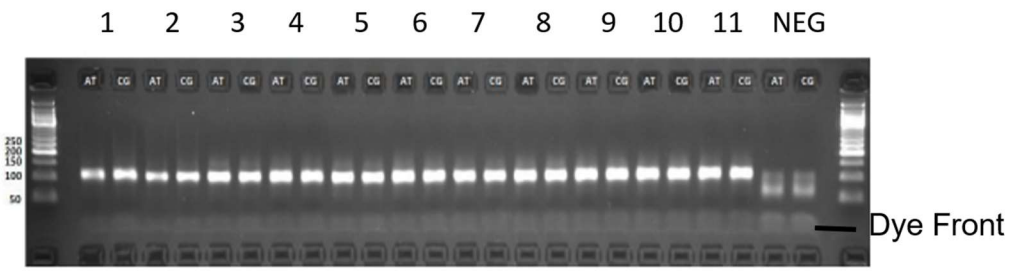



**Figure 18. Verification of the first PCR product**. The product was of good quality when product was at 125bp on a 3% TBE gel.

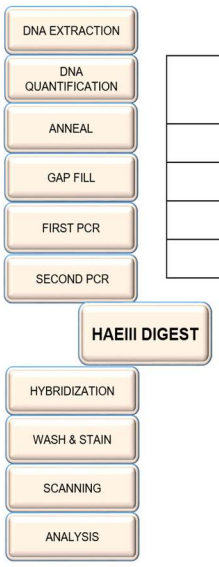



**Figure 19a. Affymetrix OncoScan® *HaeIII* Digestion Master Mix**

| Reagent | 1 Reaction | 14 Reactions (20% Overage) |
|---|---|---|
| Buffer B | 19.10µL | 321µL |
| HaeIII enzyme | 0.40µL | 6.7µL |
| Exo enzyme | 0.50µL | 8.4µL |
| Total | 20.0µL | 336µL |

**Figure 19b. Affymetrix OncoScan® *HaeIII* Thermocycler Program**

| Temperature | Time |
|---|---|
| 37°C | 88 minutes |
| 37°C | 2 minutes |
| 95°C | 10 minutes |
| 4°C | Hold |

**Figure 19. HaeIII Digestion OncoScan® protocol**. a) The *HaeIII* digestion master mix utilized OncoScan® buffer B, *HaeIII* enzyme, and exo enzyme. b) The *HaeIII* digestion reaction was performed in a thermocycler for approximately 100 minutes.

The quality of the *HaeIII* digestion was determined by running a 3% precast agarose gel. The thermocycler was paused after the 88 minutes at 37°C and 4µL of the digest reaction was removed from each well. The thermocycler program was resumed and the gel was loaded with 4µL of the reaction and 3.5µL of a 50bp ladder and ran for 15 minutes at 150V/cm. The gel was examined and determined of good quality when doublet bands around 40-70bp were observed (Figure 20).

***OncoScan® Hybridization***: Target hybridization was performed using the Hybridization Oven 645® at a temperature of 49°C at a rotation speed of 60rpm. Two arrays for each patient (AT and GC) were recorded and entered into the Affymetrix GeneChip® Command Center® 3.2 and then left at room temperature until they were loaded. A hybridization master mix was made using the Affymetrix OncoScan® nuclease-free water and hybridization mix (Figure 21a). To a new 96-well plate labeled *Hyb*, 190µL of the mix was added to the appropriate wells for each reaction. From the *HaeIII* plate, 22µL of the digestion reaction was aliquoted to the hybridization plate. The plate was loaded into the thermocycler and the OncoScan® Hybridization program ran for about 15 minutes (Figure 21b). When the thermocycler reached 49°C, the samples were injected into the septa of the array and covered with ½" Microtube Tough-Spots (Diversified Biotech, Boston, MA). The arrays were placed into an oven tray evenly spaced and allowed to rotate at the appropriate temperature and speed for 16 to 18 hours.

***Array Washing and Staining:*** The hybridized arrays (CytoScan® and OncoScan®) were removed from the hybridization oven between 16 and 18 hours from the time of incubation. Tough-Spots were removed from the septa on the arrays before being inserted into the array-designated modules of the GeneChip® Fluidics Station 450. The fluidics station was primed with Affymetrix GeneChip® Wash A and Wash B before

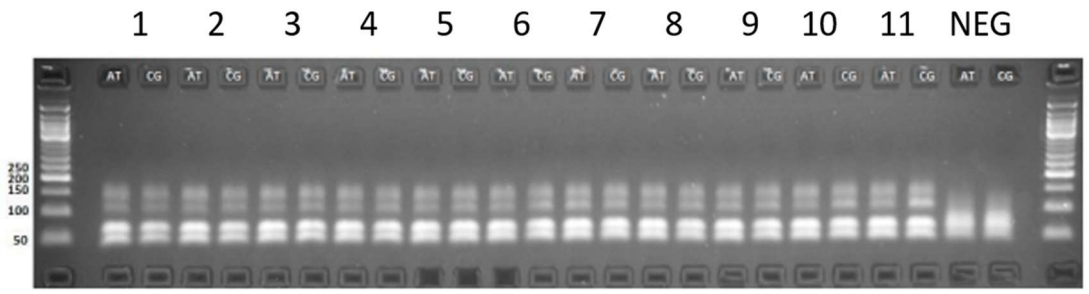



**Figure 20. Verification of the HaeIII digestion product**. The product was of good quality when doublet bands were observed at 40-70bp on a 3% TBE gel.

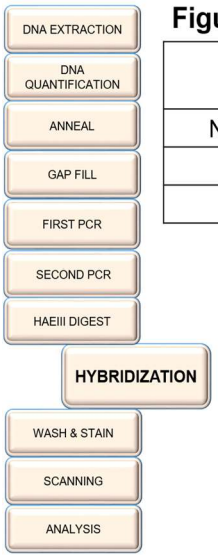



**Figure 21a. Affymetrix OncoScan® Hybridization Master Mix**

| Reagent | 1 Reaction | 14 Reactions (25% Overage) |
|---|---|---|
| Nuclease-free Water | 30.0µL | .504mL |
| Hybridization Mix | 118.0µL | 1.9824mL |
| Total | 148.0µL | 2.4864mL |

**Figure 21b. OncoScan® Hybridization Thermocycler Program**

| Temperature | Time |
|---|---|
| 95°C | 10 minutes |
| 49°C | 5 minutes |
| 49°C | Hold |

**Figure 21. Hybridization OncoScan® protocol**. a) The hybridization master mix utilized OncoScan® nuclease-free water and hybridization mix. b) The hybridization reaction was performed in a thermocycler for approximately 15 minutes.

proceeding with the washing and staining stage. In the position labelled 1, 500µL of stain buffer 1 was placed into a 1.5mL microfuge tube. Position 2 contained a 1.5mL microfuge tube containing 500µL of stain Buffer 2 and 800µL of array holding buffer was aliquoted into a 1.5mL microfuge tube and placed into position 3. The fluidics protocol was started using the Affymetrix GeneChip® Command Console and ran for approximately 1 hour.

***Array Scanning:*** When the wash and stain procedure was finished, arrays were removed and checked for bubbles or air pockets and returned to the fluidics station if present. Once air was removed, the septa on the arrays were closed using ½" Microtube Tough-Spots and placed into the GeneChip® Scanner 3000 7G and scanned.

***Microarray Analysis:*** Analysis was performed with the Affymetrix Chromosome Analysis Suite (ChAS) software. The classification of the microarray results was based on the reference ranges established in validation for the array test type performed by this laboratory. The detection rate for mosaicism was determined above 20-25% by validation. The size threshold for gains was set at 5Kb and losses set at 10Kb. The threshold for LOH was set at 3120Kb. Analysis of each chromosome was performed using the software, aberrations were flagged by the system, and the size of the anomaly was determined. Further investigation of each chromosome was performed by a manual examination of the smooth signal for peaks and dips to determine gains or losses not flagged by the ChAS software (Figure 22). When these aberrations were observed, the location and size was manually determined. A benign classification was determined when no genes were seen, an overlap with the Database of Genomic Variants (DGV) or NCBI Database for Genomic Structural Variants (dbVAR) was present, or when an overlap with a recurring del/dup region or CNV based upon the Fullerton and Cooper overlap maps occurred. Unknown clinical significance (UCS) was determined with greater than 10Kb

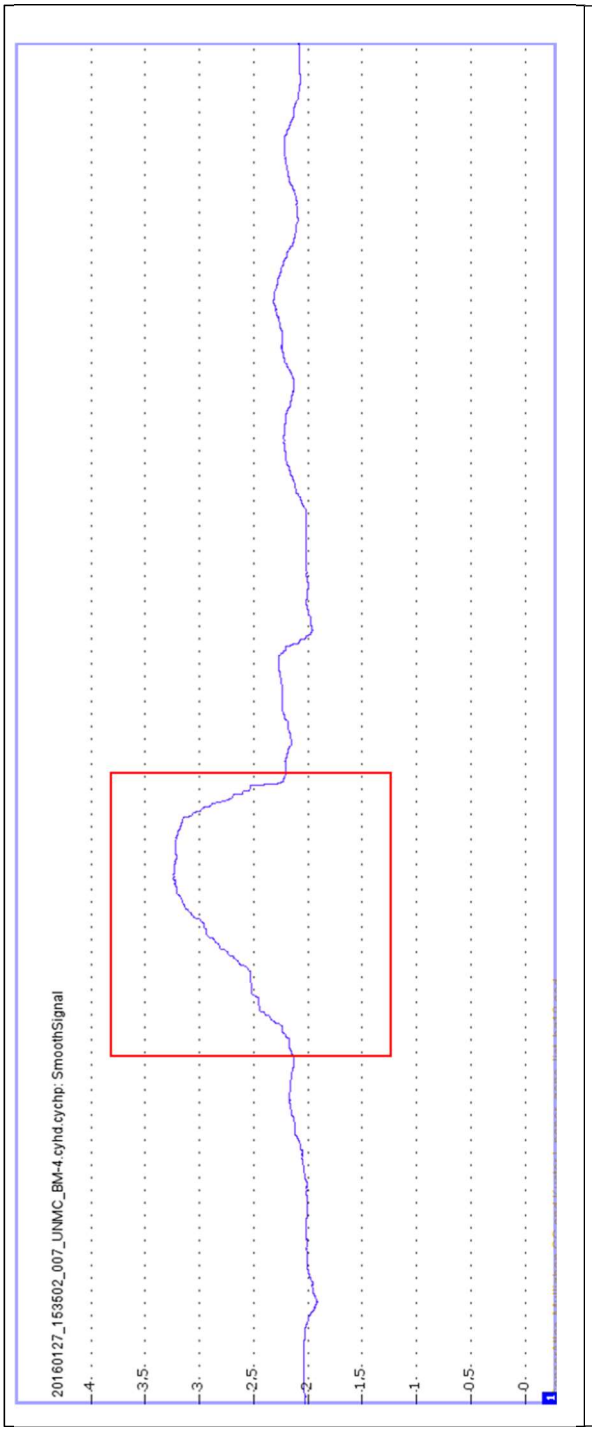



**Figure 22. Smooth signal examination using ChAS**. Manual examination of the peaks and dips of the smooth signal in ChAS on each chromosome for each case to determine aberrations that were not flagged by the software.

backbone, greater than 5Kb intragenic involving exons or a single gene and at the time of the analysis there was not sufficient evidence available for a determination of clinical significance. A designation of pathogenic was given when the abnormality was cytogenetically visible, when overlapped with a known cancer gene was observed, or when it had been documented as pathogenic in multiple peer-reviewed publications.

# RESULTS

## Patient Demographics

From January 1, 1990 to December 31, 2015, bone marrow aspirates and peripheral blood samples were collected for conventional cytogenetic and/or FISH analysis on cases with a clinical diagnosis of MDS. A total of 3992 specimens from 2948 individual cases were studied. The 2948 cases included 1666 males and 1282 females with an age range of 0 to 99 years with the highest prevalence in the 7$^{th}$ and 8$^{th}$ decade of life (Figure 23). This data coincides with the current knowledge that MDS is frequently observed in the elderly population. Of the 3992 total number of specimens received into the laboratory, 306 specimens (7.6%) were from pediatric or young adult cases, or those between the ages of 0 and 29. Further delineation of the younger population shows 205 (5.1%) specimens were from pediatric only patients aged 0 to 18 years and 101 (2.5%) specimens were from young adults or those between 19 to 29 years of age (Figure 24).

### *Pediatric Patient Demographics*

From the given time span, our laboratory analyzed 306 bone marrow or peripheral blood samples with a clinical diagnosis of MDS from pediatric and young adult cases, or those between the ages of 0 and 29 years. These samples were from 177 cases. Over the given time span, a variable number of specimens ranging from 1 to 12 were analyzed from each patient, thus explaining a total of 306 consecutive specimens. The younger population of MDS cases consisted of 95 males and 82 females with the most prevalent age of occurrence in the first year of life (Figure 25). Of these cases, 205 specimens were collected from 119 pediatric cases rangin16g from 0-18 years of age. The specimens from pediatric cases were from 65 males and 54 females. The young adult population, 19-29 years of age, consisted of 101 specimens from 58 cases with the most prevalent age of MDS at 19 years and consisted of 30 males and 28 female cases.

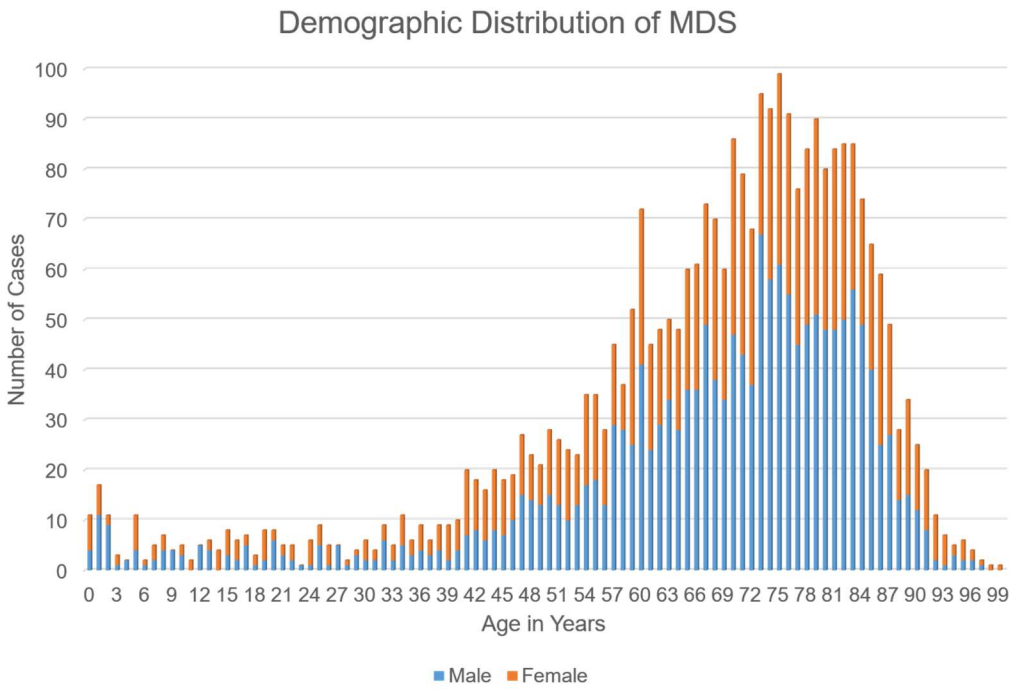



**Figure 23. Demographic distribution of MDS**. The 2948 bone marrow or peripheral blood cases with a clinical diagnosis of MDS over the time span of 1990-2015 included 1666 males and 1282 females. The age ranged from 0 to 99 years of age with the highest prevalence in the 7$^{th}$ and 8$^{th}$ decade of life.

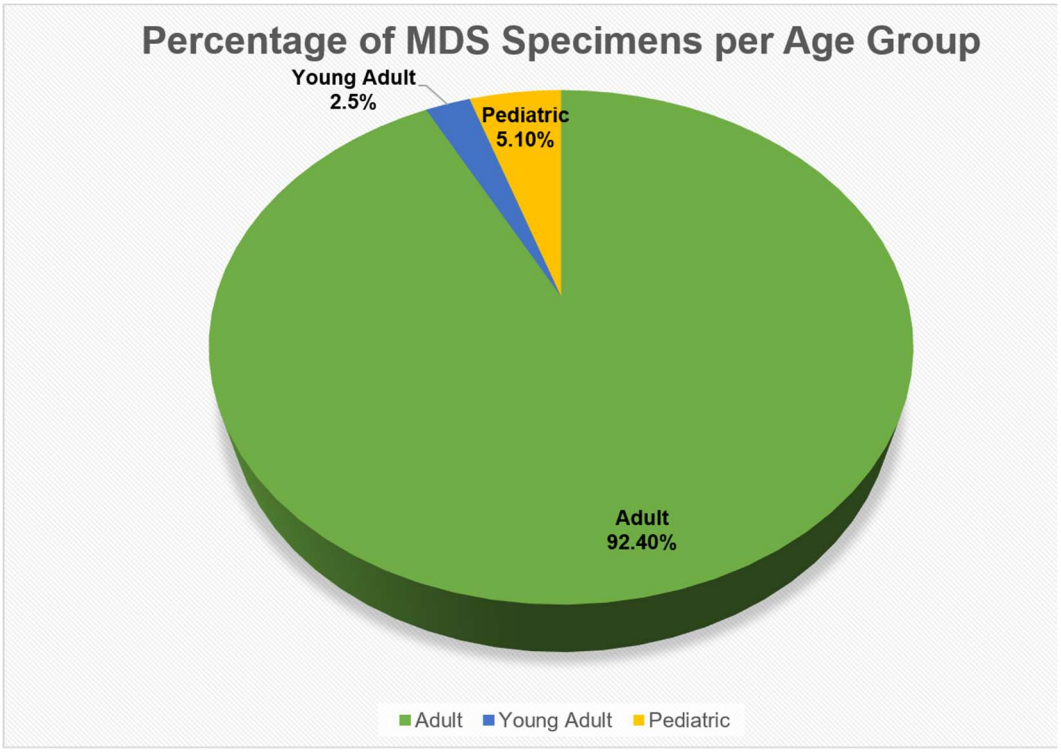



**Figure 24. Percentage of MDS specimens per age group.** From the 3992 total number of specimens received in our laboratory over the given time span of 1990-2015, 205 (5.1%) specimens were from the pediatric population aged 0 to 18 years and 101 (2.5%) specimens were from young adult samples (19 to 29 years). The majority of the specimens, 3686 (92%), were from the adult population, aged 30 years and above.

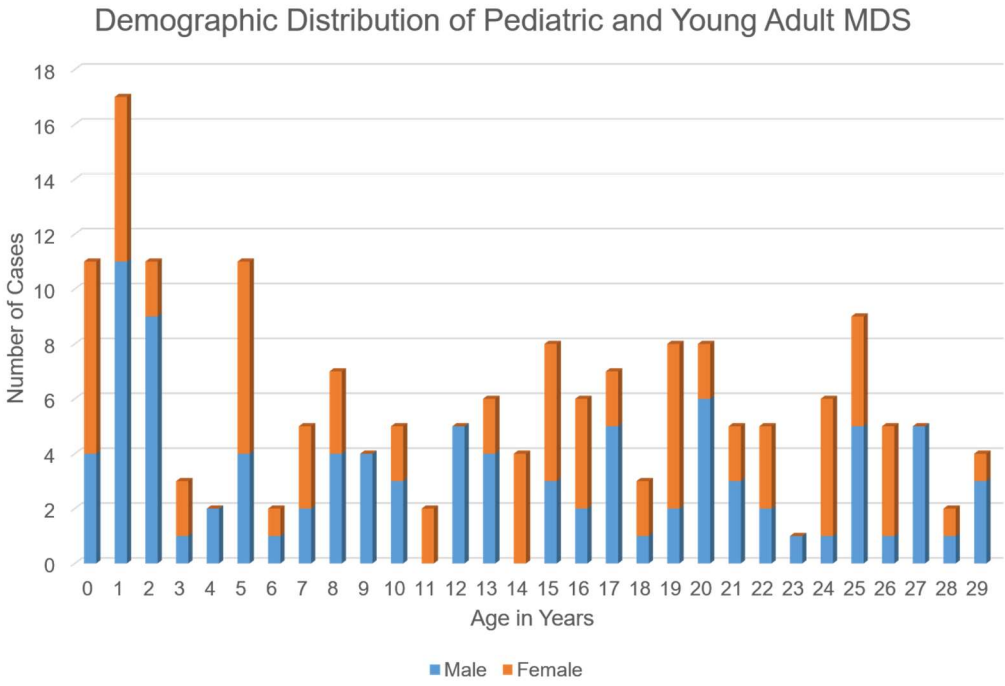



**Figure 25. Demographic distribution of pediatric and young adult MDS.** From 1990-2015, this laboratory analyzed 306 bone marrow or peripheral blood samples with a clinical diagnosis of MDS from 177 pediatric and young adult cases. The pediatric cases consisted of 95 males and 82 females with the most prevalent ages of occurrence in the first year of life.

## Conventional Cytogenetic and FISH Analyses

The cytogenetic and FISH data comparison was performed on the total number of specimens received into the laboratory. Over the given time span of 1990 to 2015, a total of 3992 MDS specimens from 2948 individual cases were studied in our laboratory. Cytogenetic and FISH analyses were performed on each specimen in order to detect chromosome aberrations and to accurately determine disease clonality and monitor disease progression.

Of the 3992 MDS specimens, 2353 (58.9%; 2353 / 3992) presented a normal cytogenetic karyotype and FISH results. A total of 1639 (41.1%; 1639 / 3992) samples presented abnormal findings by conventional cytogenetics and/or FISH analyses. One of the hallmark chromosome abnormalities commonly observed in MDS patients [-5/del(5q), -7/del(7q), +8, and del(20q)] (Figure 26a-d) was observed in 1155 of the abnormal studies (70.5%; 1155 / 1639) (Figure 27). Abnormalities not typical of MDS identified by cytogenetics and confirmatory or concurrent FISH included chimerism of donor and host cells in post-transplant patients, a variety of deletions and rearrangements, and random gains and losses of chromosomes not typically associated with MDS. Each one of these were in frequencies of less than 1.5%. In the adult population, an MDS-related abnormality was detected by cytogenetic karyotyping and/or FISH in 1099 specimens as either the sole anomaly or as part of a complex karyotype with more than one anomaly present, MDS-related or not. The frequencies of each hallmark abnormality observed was determined for this population. The most frequent MDS-related abnormality observed was -5/del(5q), seen in 519 adult samples. The -7/del(7q) abnormality was the next most frequent abnormality as it was seen in 423 samples followed by +8 in 282 samples.

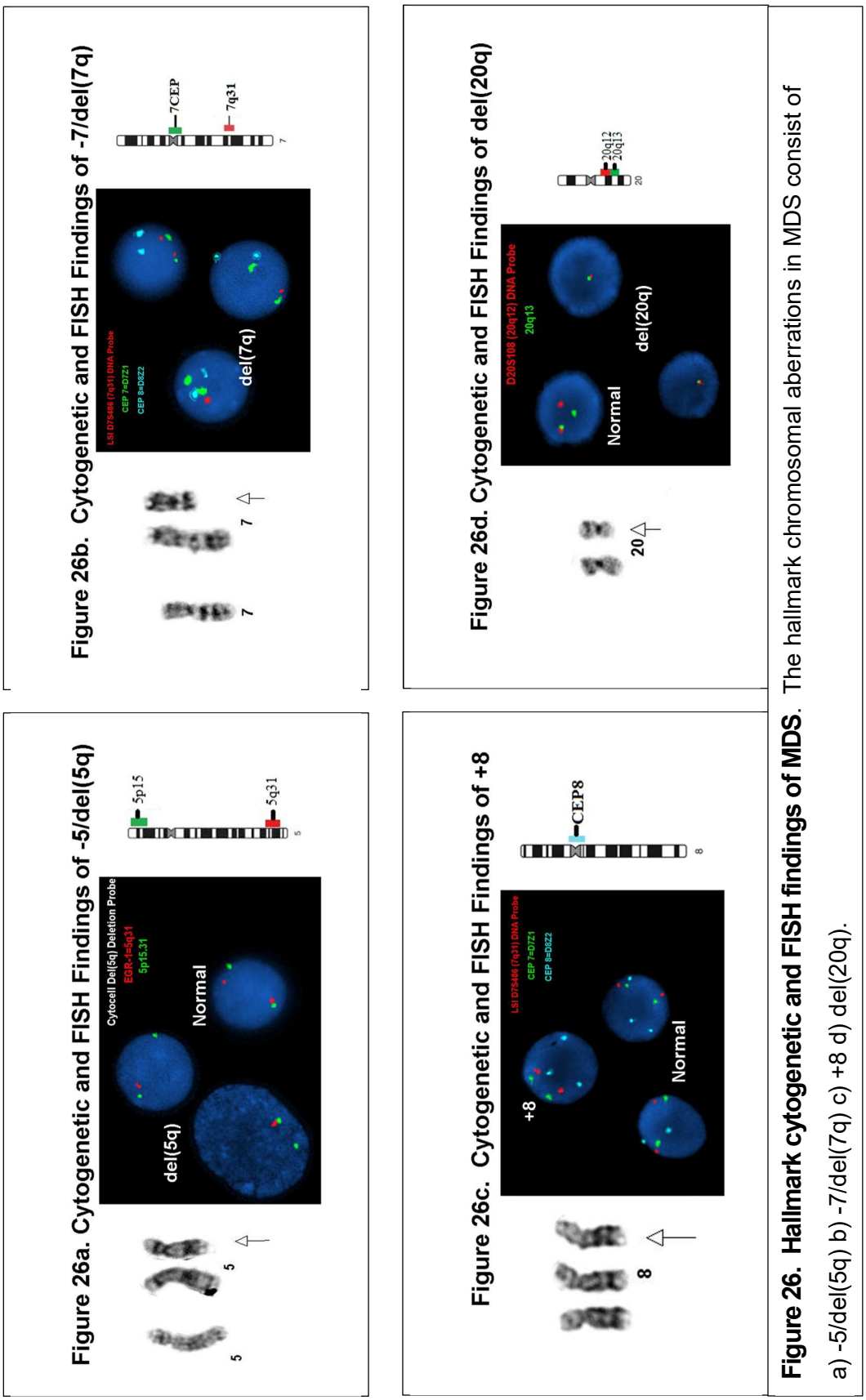



**Figure 26. Hallmark cytogenetic and FISH findings of MDS**. The hallmark chromosomal aberrations in MDS consist of a) -5/del(5q) b) -7/del(7q) c) +8 d) del(20q).

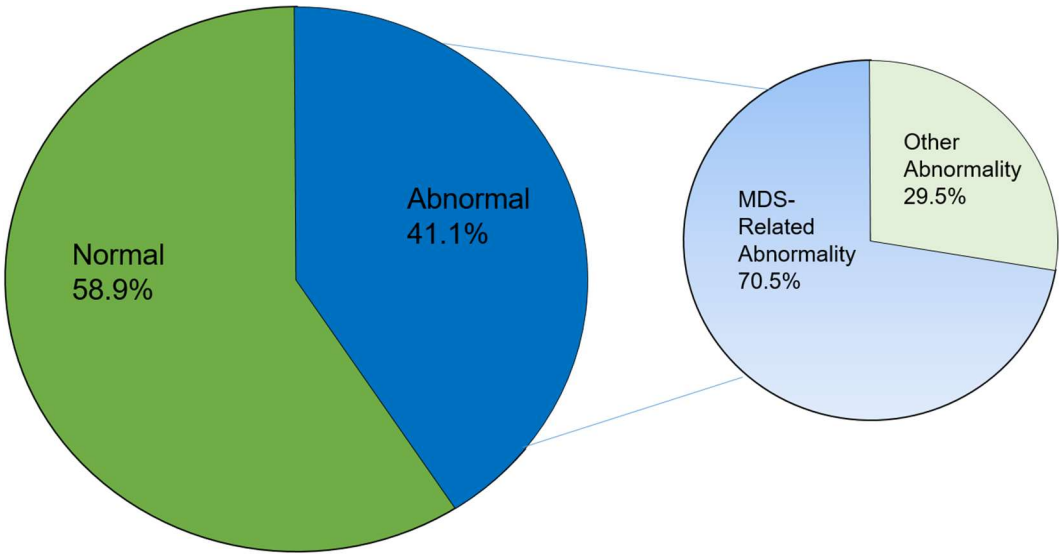



**Figure 27. Cytogenetic/FISH Findings of 2353 specimens from adult MDS (≥30 years of age) cases**. From the given time span, 2353 (58.9%) bone marrow or peripheral blood samples with a clinical diagnosis of MDS contained a normal cytogenetic karyotype and normal FISH results. An abnormal cytogenetic karyotype and/or FISH analyses was observed in 1639 (41.1%). From these samples, 1155 (70.5%) contained one of the four hallmark MDS chromosomal abnormalities of -5/del(5q), -7/del(7q), +8, or del(20q). The remaining 484 (29.5%) of the cases contained an abnormality not typical of MDS.

The least frequent MDS-related abnormality observed in the adult population, or those over the age of 30, was del(20q) in 260 specimens (Table I).

### *Conventional Cytogenetic and FISH Analyses of Pediatric Population*

MDS is a rare disease among pediatric and young adults, between the age of 0 and 29 years. A fraction of the total number of MDS specimens analyzed over the given time span included this population, 7.6% (306 / 3992). Among the 306 specimens from pediatric and young adult cases, 230 (75.2%; 230 / 306) presented normal results by conventional cytogenetics and FISH analyses. Among the 76 abnormal specimens (24.8%; 76 / 306), one or more of the hallmark MDS-related cytogenetic abnormalities was observed in 56 samples (73.7%; 56 / 76) (Figure 28). The most frequent chromosomal abnormality observed was -7/del(7q) in 28 samples. Trisomy 8 was observed in 13 samples and del(20q) in 10 samples. Unlike the adult population, among the pediatric and young adult specimens the -5/del(5q) was the least frequent abnormality observed in 8 specimens (Table II).

Further delineation between the pediatric (0-18) and young adult (19-29) population presented similar frequencies of MDS-related chromosome abnormalities thus providing the justification for expanding the pediatric analysis to include the young adults. Of the 205 pediatric only (0-18 years) specimens, 150 presented normal karyotypes and negative FISH results (73.2%; 150 / 205). Of the 55 abnormal specimens (26.8%; 55 / 205), 43 (78.2%; 43 / 55) samples contained one of the hallmark MDS-related cytogenetic abnormalities (Figure 29). Among the 101 young adult (19-29) specimens, 80 (79.2%; 80 / 101) presented normal cytogenetic and FISH findings. Of the 21 (20.8%) abnormal specimens, 13 (61.9%; 13 / 21) contained one or more of the hallmark MDS-related

| MDS-Related Abnormality | Number of Adult Specimens (≥30 years of age) |
|---|---|
| -5/del(5q) | 519 |
| -7/del(7q) | 423 |
| +8 | 282 |
| del(20q) | 260 |

**Table I. Characterization of the MDS-related chromosomal abnormalities detected by cytogenetic and FISH analyses in adult MDS**. The most frequent MDS-related abnormality observed was -5/del(5q), seen in 519 adult specimens followed by -7/del(7q) in 423 samples; and +8 in 282 samples. The least frequent MDS-related abnormality observed in the adult population was del(20q) in 260 samples.

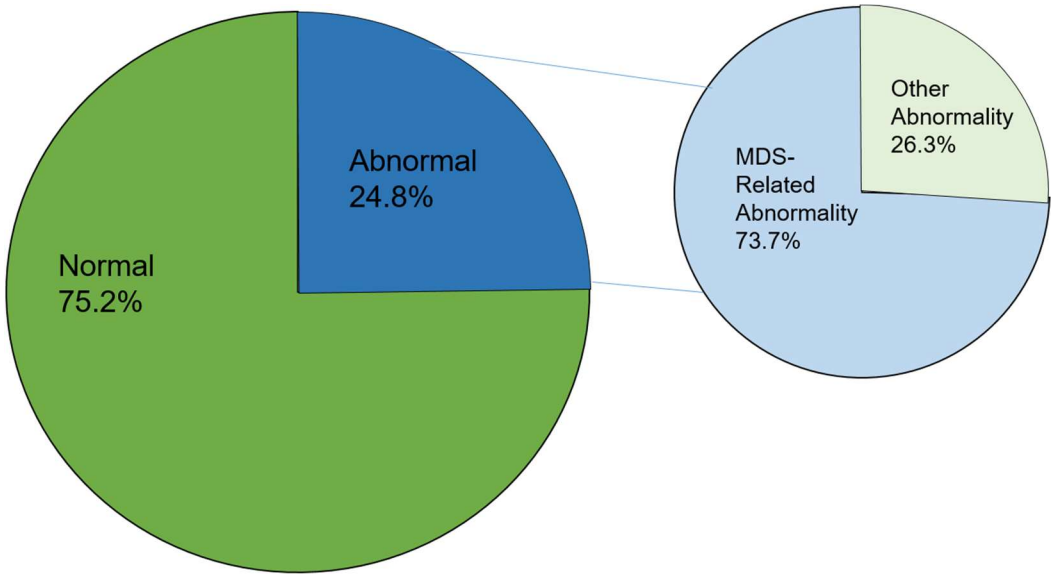



**Figure 28. Cytogenetic/FISH findings of pediatric and young adult MDS**. From the given time span of 1990-2015, 230 pediatric and young adult specimens (75.2%) presented normal results by conventional cytogenetics and FISH analyses. Among the 76 (24.8%) abnormal samples, 56 (73.7%) contained one or more of the hallmark MDS-related cytogenetic abnormalities and an abnormality not typical of MDS was noted in 20 (26.3%) samples.

| MDS-Related Abnormality | Number of Pediatric and Young Adult Specimens (0-29 Years) |
|---|---|
| -5/del(5q) | 8 |
| -7/del(7q) | 28 |
| +8 | 13 |
| del(20q) | 10 |

**Table II. Characterization of the MDS-related chromosomal abnormalities detected by cytogenetic and FISH analyses in the pediatric/young adult population**. In the pediatric and young adult population, 0-29 years, the most frequent chromosomal abnormality observed was -7/del(7q) in 28 specimens. Trisomy 8 was observed in 13 samples and del(20q) in 10 samples. The least frequent abnormality in the pediatric and young adult population was -5/del(5q) in 8 specimens.

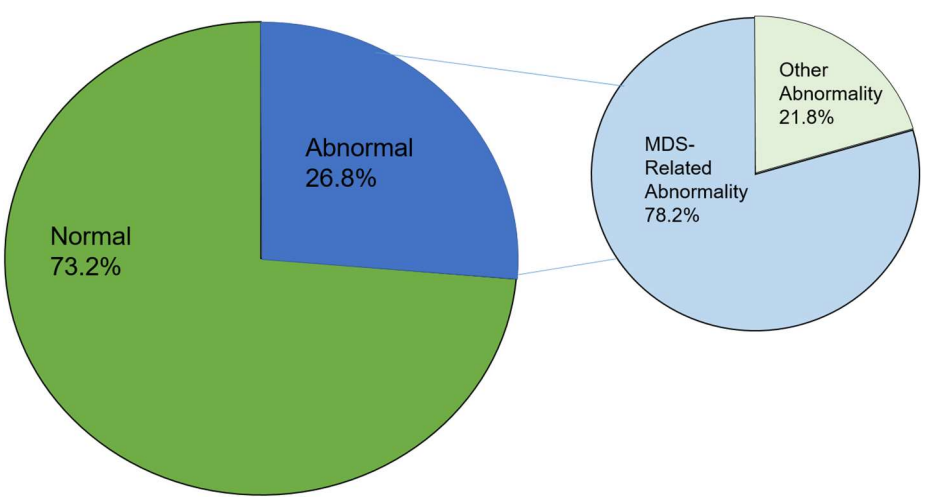



**Figure 29. Cytogenetic/FISH findings of pediatric MDS**. From 1990-2015, 205 total pediatric only (0-18 years) specimens were analyzed and 150 (73.2%) of them had a normal cytogenetic karyotype and FISH analyses. Out of the 55 specimens (26.8%) with abnormal findings, 43 specimens (78.2%) contained one of the hallmark MDS aberrations.

abnormalities (Figure 30). The most frequent abnormality of -7/del(7q) was observed in 17 pediatric samples. Trisomy 8 and del(20q) was observed in 10 samples; and -5/del(5q) was only observed in five samples among the 0-18 age group. Among the young adult population, -7/del(7q) was observed in 9 specimens and +8 and del(20q) was seen in 3 samples each. The least frequently observed abnormality of -5/del(5q) was observed in 3 specimens from the 19-29 age group (Table III).

The 20 specimens with a chromosome abnormality not typical of MDS had a range of abnormalities. The most common other abnormality detected by cytogenetics and FISH in the younger population was constitutional trisomy 21, observed in 8 samples (14.2%; 8 / 56). Other abnormalities included the following; chimerism of donor and host cells in post-transplant patients; loss of Y and 13; deletion of 6q; rearrangements of 11q, 14q, 18q, 19q; and gain of chromosome 19. Each one of these were in frequencies of 1.0% or less.

## Microarray Samples

Between January 1, 2012 to January 1, 2015, bone marrow or peripheral blood from pediatric or young adult MDS cases was saved when the original specimen was in excess after all cytogenetic and FISH studies were performed. These samples were used for extraction of DNA for high-resolution array studies. These cases were chosen based solely on their volume of original specimen and not for their cytogenetic or FISH findings. From the 93 pediatric or young adult MDS cases received in the Human Genetics Laboratory between 2012-2015, only 28 specimens contained excess whole bone marrow or blood after cytogenetic and FISH studies and were from cases with a variety of ages and cytogenetic findings (Table IV).

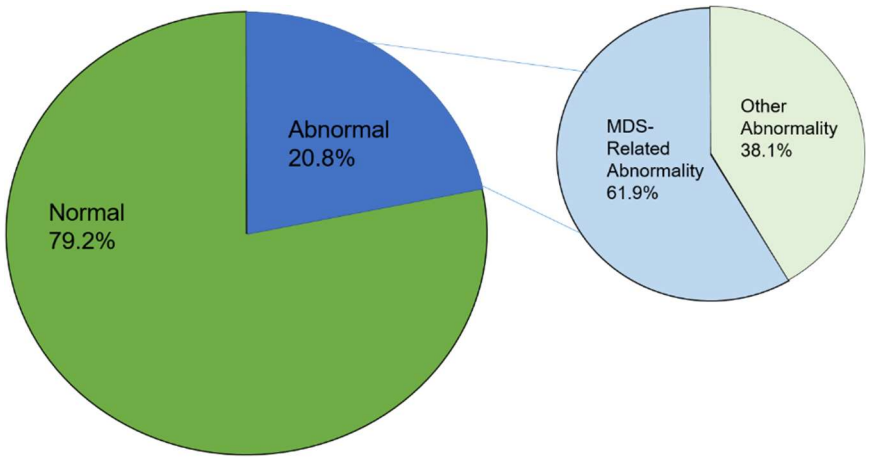



**Figure 30. Cytogenetic/FISH findings of young adult (19-29 years) MDS**. Over the given time span, 101 total specimens from young adult (19-29 years) specimens were analyzed and 80 (79.2%) presented normal cytogenetic and FISH findings. Of the 21 (20.8%) abnormal specimens, 13 (61.9%; 13/21) contained one or more of the hallmark MDS-related abnormalities.

| MDS-Related Abnormality | Number of Pediatric Specimens (0-18 years) | Number of Young Adult Specimens (19-29 years) |
|---|---|---|
| -5/del(5q) | 5 | 3 |
| -7/del(7q) | 19 | 9 |
| +8 | 10 | 3 |
| del(20q) | 10 | 3 |

**Table III. Comparison of MDS-related abnormalities observed in the pediatric and young adult MDS populations**. The observance of the hallmark abnormality of -7/del(7q) was comparable between the two groups with this being the most observed aberration. The next most frequent abnormalities included trisomy 8, del(20q), and -5/del(5q).

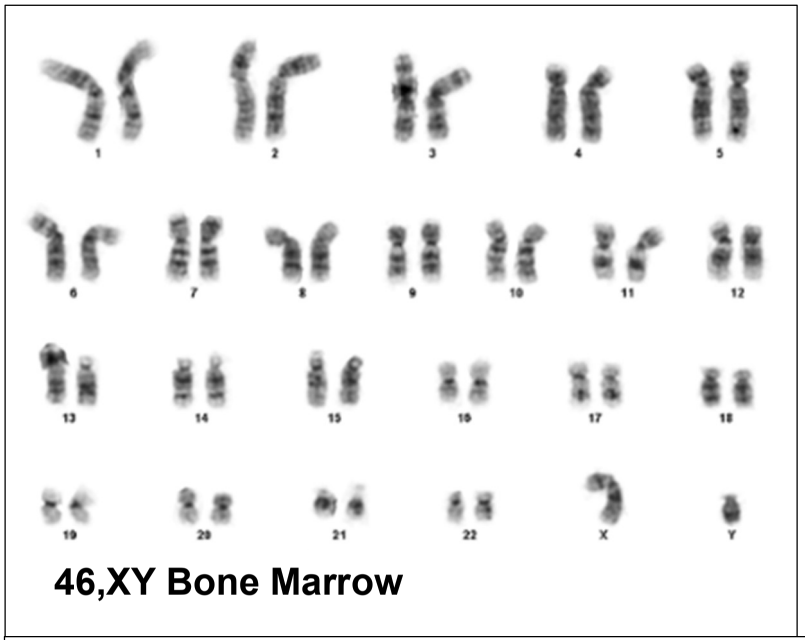
| Case ID | Age | Cytogenetic Findings | FISH Findings |
|---|---|---|---|
| 1 | 2 | 46,XX | Negative |
| 2 | 16 | 46,XY | Negative |
| 3 | 10 | 46,XY | Negative |
| 4 | 6 | 46,XY | Negative |
| 5 | 14 | 46,XY | del(20) (q12q13) [13%] |
| 6 | 3 months | 46,XX | Negative |
| 7 | 11 | 46,XX,inv(11)(q13q23)[17] | MLL (11q23) Breakapart [67%] |
| 8 | 20 | 46,XX | Null |
| 9 | 14 | 46,XY | Null |
| 10 | 14 | 46,XY | Negative |
| 11 | 7 | 45,XY,-7[7] | monosomy 7 [12%] |
| 12 | 7 months | 45,XY,-7[4] | monosomy 7 [23%] |
| 13 | 15 | 45,XX,-7[9] | monosomy 7 [28%] |
| 14 | 15 | 46,XX | Negative |
| 15 | 29 | 46,XY | Negative |
| 16 | 19 | 46,XX | Negative |
| 17 | 18 | 46,XY | Negative |
| 18 | 13 | 46,XY | Negative |
| 19 | 1 | 46,XY | Negative |
| 20 | 8 | 46,XX | Negative |
| 21 | 17 | 46,XY | Negative |
| 22 | 24 | 46,XX | Negative |
| 23 | 20 | 46,XY | Negative |
| 24 | 15 | 46,XY | del(20)(q12q13) [8%] |
| 25 | 7 | 46,XX | Negative |
| 26 | 11 months | 46,XY | Negative |
| 27 | 4 | 46,XY | Negative |
| 28 | 2 | 46,XY | Negative |

**Table IV. List of pediatric and young adult MDS specimens for microarray studies after karyotyping and FISH analyses**. The table lists the case ID, age of patient at time of intake, cytogenetic karyotyping and FISH results for the 28 cases sent for microarray testing.

Only five of these cases were from the young adult population, or those between the ages of 19 and 29. From the total cases, 21 presented normal karyotypes (Figure 31) and FISH results (Figure 32). Our justification for using cases with normal cytogenetic and FISH findings is due to the possible presence of cryptic abnormalities that can only be detected by high-resolution techniques. The six cases with abnormal cytogenetic or FISH results contained the following abnormalities;
45,XX,-7; 45,XY,-7; 45,XY,-7; 46,XX,inv(11)(q13q23); 46,XY,del(20)(q12q13); and 46,XY,del(20)(q12q13).

## Microarray Results

Out of the 28 pediatric and young adult MDS specimens available, microarray analysis was performed on 26 cases. Two cases were not utilized on array due to insufficient DNA yields. From the 26 cases, 15 (58%; 15 / 26) were found to contain genetic alterations that were determined to be pathogenic or likely pathogenic (Figure 33). From this study, 33 gene aberrations detected were determined as pathogenic or likely pathogenic and were MDS-associated genes and/or genes involved in hematopoiesis, cell cycle proliferation/regulation, apoptosis, known tumor suppressor genes, or other disease-associated genes (Table V). Of particular importance, eight of these genetic aberrations were detected in more than one case. Further analysis found that out of the five young adult specimens, three specimens had a detectable pathogenic aberration and some of these pathogenic changes were observed in the pediatric population, 0-18 age group. These results further justify our non-restrictive use of young adult specimens included into this pediatric study.



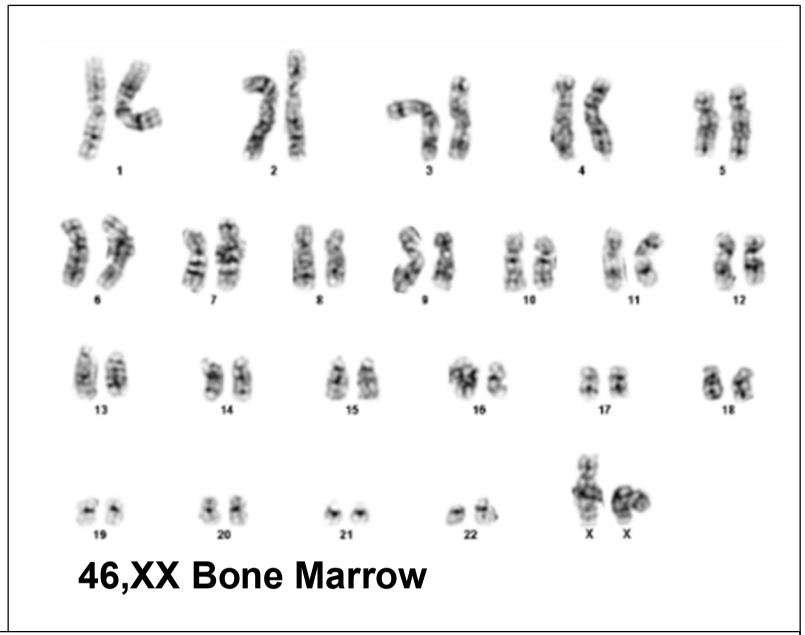




**Figure 31. Representative Images depicting normal cytogenetic karyotypes**. This image depicts: a) Case 1 showing a normal karyotype of 46,XX. b) Normal karyotype (46,XY) observed in Case 2.

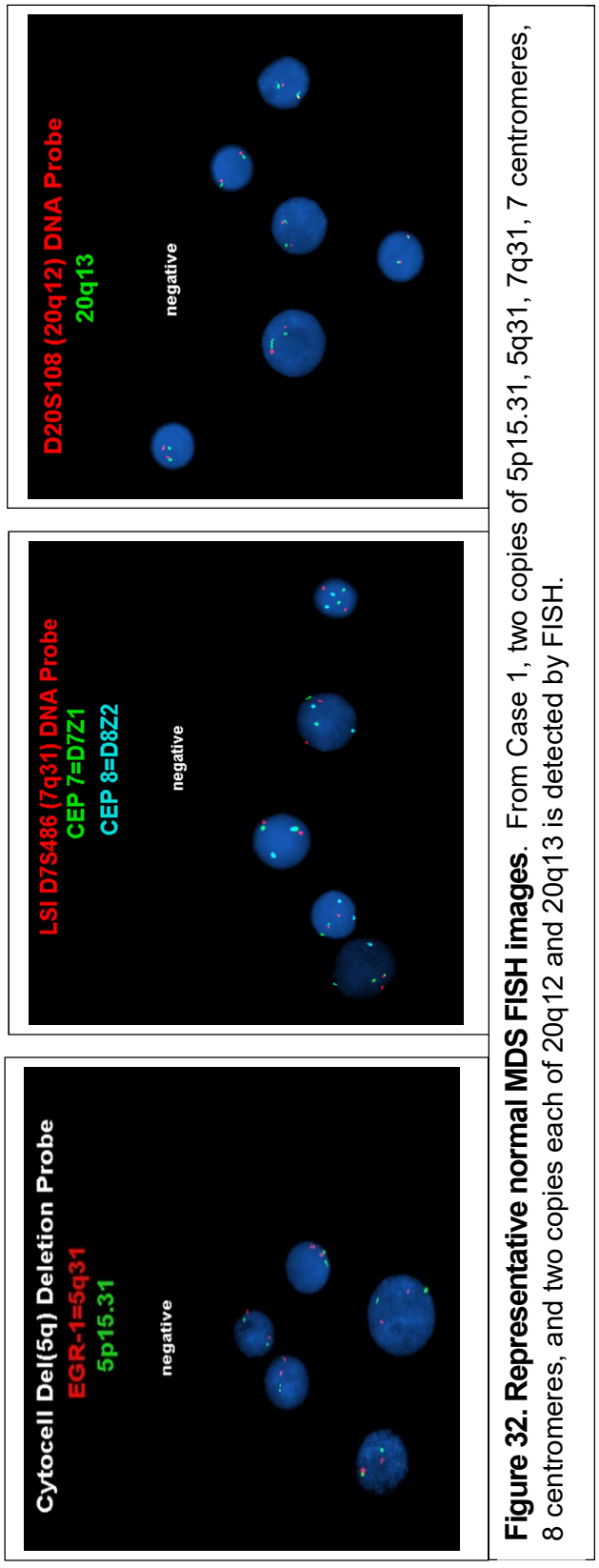



**Figure 32. Representative normal MDS FISH images**. From Case 1, two copies of 5p15.31, 5q31, 7q31, 7 centromeres, 8 centromeres, and two copies each of 20q12 and 20q13 is detected by FISH.

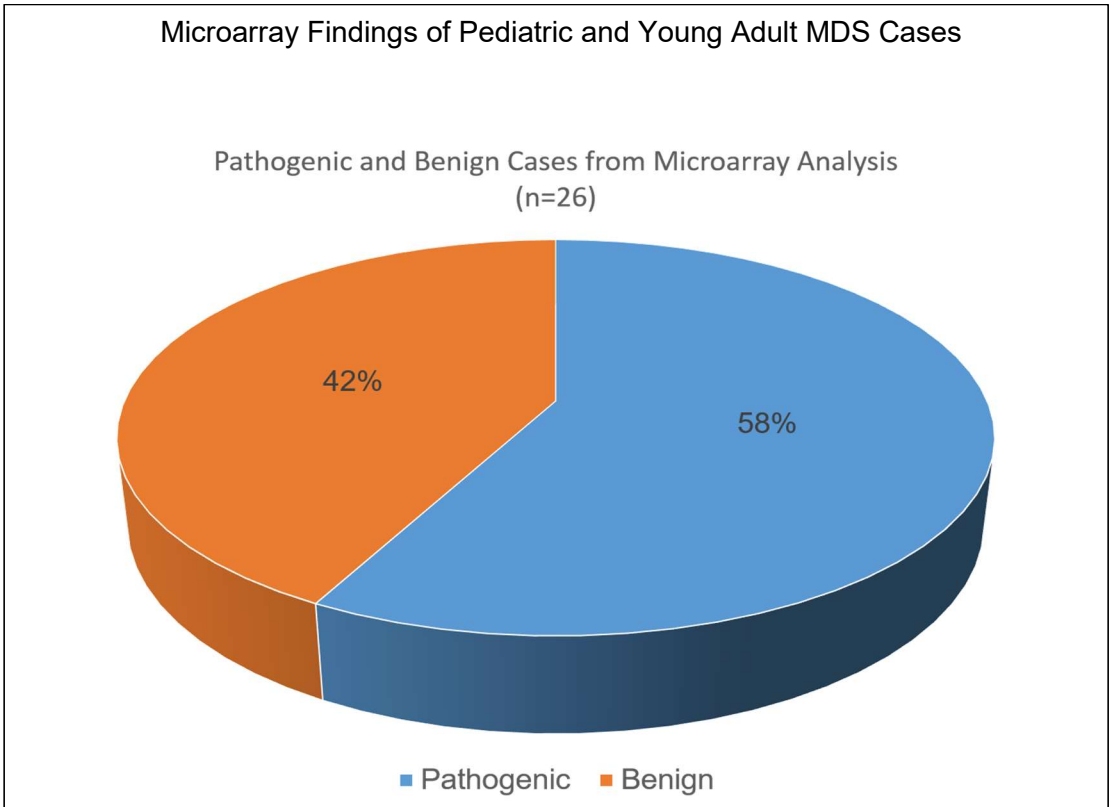



**Figure 33. Microarray findings of pediatric and young adult MDS cases**. From the 26 total pediatric and young adult MDS cases, microarray determined that over 58% (15 cases) contained a genetic alteration that was determined to be pathogenic or likely pathogenic.

| Gene | Chromosome Location | Linear Location | Copy Number State | Number of Cases |
|---|---|---|---|---|
| ***PRDM16*** | **1p36.33** | **chr1: 3,068,226 – 3,438,620** | **Gain** | **6** |
| ***IRF4*** | **6p25.3** | **chr6: 391,738 – 411,442** | **Gain/Loss** | **4** |
| ***MYH11*** | **16p13.1** | **chr16: 15,703,134 – 15,857,032** | **Gain** | **3** |
| ***ALK*** | **2p23.3** | **chr2: 29,192,773 – 29,921,610** | **Partial Loss** | **2** |
| ***CDKN2B*** | **9p21.3** | **chr9: 22,002,902 – 22,009,312** | **Loss** | **2** |
| ***PAX5*** | **9p13.2** | **chr9: 36,833,274 – 37,035,318** | **LOH** | **2** |
| ***EXT2*** | **11p11.2** | **chr11: 44,095,548 – 44,245,429** | **Partial Gain** | **2** |
| ***ERCC4*** | **16p13.12** | **chr16: 13,920,144 – 13,952,347** | **Gain** | **2** |
| *MPL* | 1p34.2 | chr1: 3,068,226 – 3,438,620 | LOH | 1 |
| *ABL2* | 1q25.2 | chr1: 179,099,326 – 179,229,692 | LOH | 1 |
| *KIF14* | 1q32.1 | chr1: 200,551,496 – 200,620,790 | Loss | 1 |
| *PIM1* | 6p21.2 | chr6: 37,170,145 – 37,175,427 | Loss | 1 |
| *RUNX2* | 6p21.2 | chr6: 45,327,799 – 45,664,031 | Partial Loss | 1 |
| *LATS1* | 6q25.1 | chr6: 149,658,152 – 149,718,255 | Loss | 1 |
| *IKZF1* | 7p12.2 | chr7: 50,303,464 – 50,405,100 | Loss | 1 |
| *TAC1* | 7q21.3 | chr7: 97,731,958 – 97,740,471 | Loss | 1 |
| *IDO1* | 8p11.21 | chr8: 39,913,808 – 39,928,789 | Loss | 1 |
| *KAT6A* | 8p11.21 | chr8: 41,929,478 – 42,051,988 | Loss | 1 |
| *CLU* | 8p21.1 | chr8: 27,596,916 – 27,615,030 | Loss | 1 |
| *FANCG* | 9p13.3 | chr9: 35,073,837 – 35,080,015 | Loss | 1 |
| *ADAMTS13* | 9q34.13 | chr9: 133,414,338 – 133,459,402 | Gain | 1 |
| *PICALM* | 11q14.2 | chr11: 85,957,170 – 86,069,880 | Loss | 1 |
| *YAP1* | 11q22.1 | chr11: 102,110,253 – 102,233,422 | Loss | 1 |
| *ETS1* | 11q24.3 | chr11: 128,458,760 – 128,587,583 | Loss | 1 |
| *PTPN11* | 12q24.13 | chr12: 112,418,731 – 112,509,917 | Partial Loss | 1 |
| *PML* | 15q24.1 | chr15: 82,536,749 – 82,540,543 | LOH | 1 |
| *RPS17* | 15q25.2 | chr15: 82,536,749 – 82,540,543 | LOH | 1 |
| *RECQL3* | 15q26.1 | chr15: 90,717,326 – 90,815,461 | LOH | 1 |
| *FANCI* | 15q26.1 | chr15: 89,243,947 – 89,317,130 | LOH | 1 |
| *CBFA2T3* | 16q24.3 | chr16: 88,874,854 – 88,977,198 | Gain | 1 |
| *FANCA* | 16q24.3 | chr16: 89,737,550 – 89,816,657 | Gain | 1 |
| *RARA* | 17q21.2 | chr17: 40,309,170 – 40,357,642 | Gain | 1 |
| *CHEK2* | 22q12.1 | chr22: 28,687,742 – 28,741,904 | Loss | 1 |

**Table V. Characterization of genetic aberrations detected by microarray**. A total of 33 genes were altered among the pediatric and young adult MDS cases by microarray. The top eight gene aberrations listed in the table were detected in more than one case. This table lists the gene name, the chromosome and linear location, the copy number state, and the number of cases that contained the aberration.

## ***Case Studies***

### *Case 1*

Case 1 was a bone marrow aspirate from a 2-year-old female with a normal cytogenetic karyotype and negative for the MDS FISH panel. High-resolution array studies using the ChAS software detected 20 aberrations in the form of gains, losses, or regions of LOH. Based upon the call parameters previously discussed, 19 of the 20 aberrations were classified as benign. A 532kbp pathogenic gain was observed by manual examination of the smooth signal on chromosome 16q24.3 located at chr16: 88,722,097 - 89,254,728, which overlaps with the *CBFA2T3* gene (16: 88,874,854 - 88,977,198) (Figure 34). This gene is associated with AML and therapy-related myeloid malignancies as determined from the Online Mendelian Inheritance in Men® (OMIM) online catalogue and the National Center for Biotechnology Information (NCBI).

### *Case 2*

Case 2 was a bone marrow aspirate from a 16-year-old male that presented a normal karyotype and was negative for the MDS FISH panel. There were 32 benign aberrations based on the classification criteria.

### *Case 3*

A bone marrow aspirate from a 10-year-old male presented a normal cytogenetic karyotype and negative FISH analyses. This case detected 32 aberrations in the form of gains, losses, or regions of LOH and all were classified as benign.

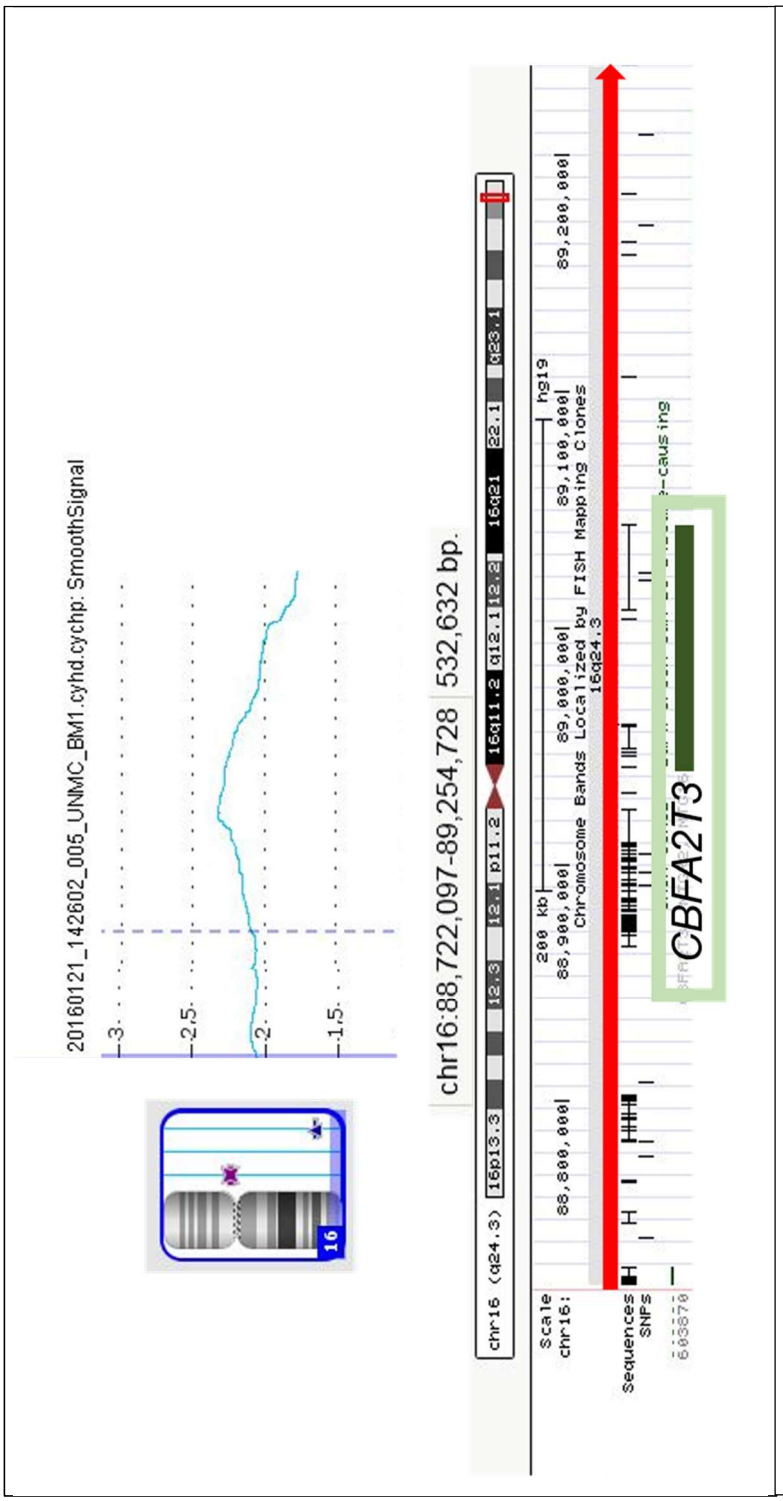



**Figure 34. Microarray results for Case 1**. A 532kbp pathogenic gain was observed on chromosome 16q24.3. This gain overlaps with the *CBFA2T3* gene and has been shown to be associated with AML and therapy-related myeloid malignancies.

***Case 4***

In Case 4, high-resolution array studies using the ChAS software detected 41 aberrations in the form of gains, losses, or regions of LOH; 39 of the 41 aberrations were classified as benign. Two aberrations were determined to overlap with pathogenic genes. A 3,558kbp mosaic gain was detected by the ChAS software on chromosome 1p36.33 located at chr1: 849,466 - 4,408,084 which overlaps with the *PRDM16* gene (1: 3,068,226 - 3,438,620), an MDS-related gene (Figure 35a). Further examination of the smooth signal in ChAS detected a 247kbp loss on chromosome 6p21.2 located at chr6: 36,988,591 - 37,236,435. This aberration overlaps with the *PIM1* gene (6: 37,170,145 - 37,175,427), a known oncogene involved in hematopoiesis (Figure 35b).

***Case 5***

In case 5, FISH analyses were positive for a deletion of 20(q12q13) in 13% of the interphase cells (Figure 36). This deletion was not detected by microarray studies due to the low-level mosaicism. However, microarray detected a 98kbp likely pathogenic deletion on chromosome 2 within the p23.3 region. The location of this deletion ranged from 29,607,409 through 29,706,398, which overlaps with a small portion of the *ALK* gene (2: 29,192,773 - 29,921,610), a known oncogene involved in various cancers as determined by OMIM and NCBI (Figure 37).

***Case 6***

This bone marrow aspirate was from a 3-month old female with a normal karyotype and negative for the MDS FISH panel. Twenty-six aberrations were classified as benign in case 6 by microarray studies. Two benign aberrations originally predicted to overlap with disease-causing genes were determined to be outside of the genes of interest

Figure 35a. Case 4 *PRDM16*

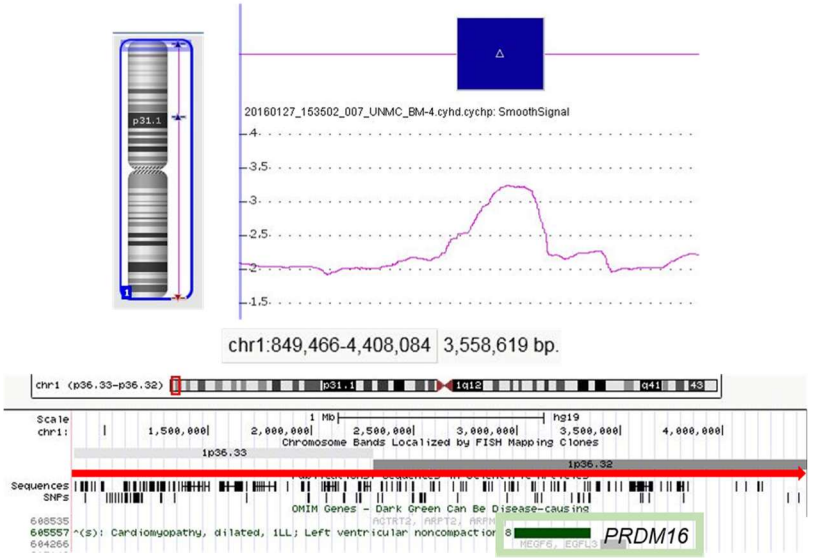



Figure 35b. Case 4 *PIM1*

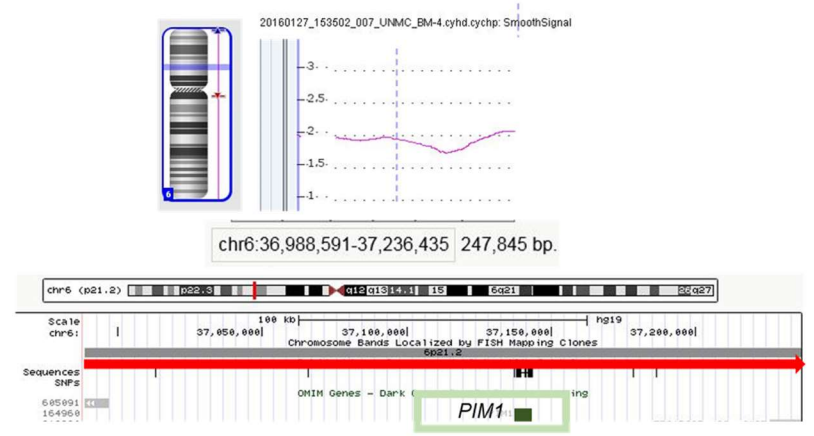



**Figure 35. Microarray results for Case 4**. Two pathogenic aberrations were detected and were determined to overlap with pathogenic genes. a) A 3,558kbp mosaic gain was detected on chromosome 1p36.33 which overlaps with the *PRDM16* gene, an MDS-related gene. b) A 247kbp loss was observed on chromosome 6p21.2 which overlaps the *PIM1* gene, a known oncogene involved in hematopoiesis.

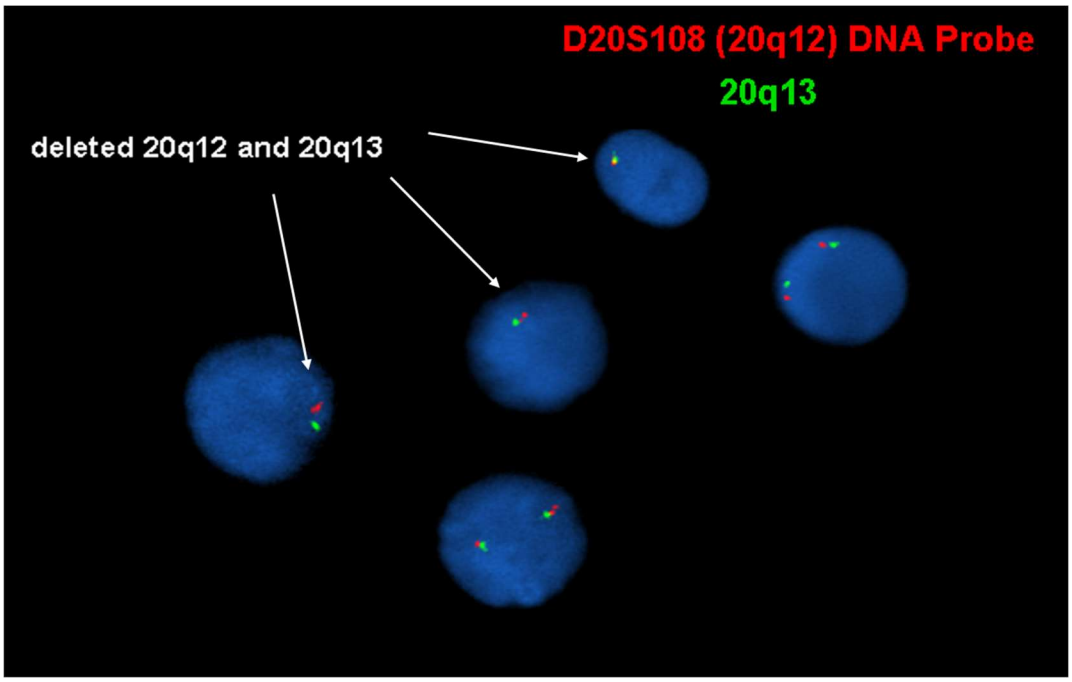



**Figure 36. FISH results for Case 5**. The FISH MDS panel detected a deletion of 20q12 and 20q13 with normal karyotypes in case 5. This image shows only one copy of each of the 20q probes.

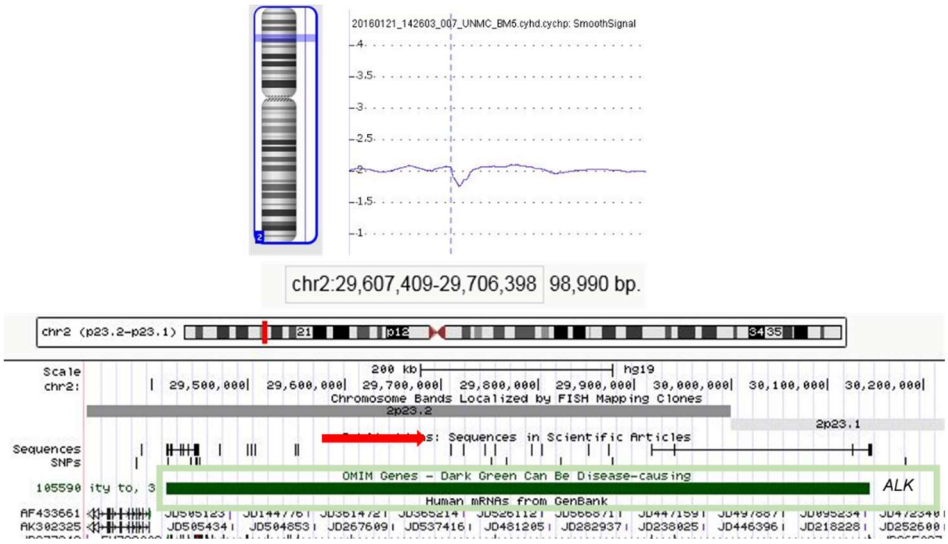



**Figure 37. Microarray results for Case 5**. One likely pathogenic aberration was detected and determined to overlap with a pathogenic gene. A 98kbp deletion was observed on chromosome 2p23.3 which overlaps a small portion of the *ALK* gene.

after further detailed examination following initial review using the ChAS software. Examination and comparisons of the aberration and gene locations determined that these aberrations did not result in the loss of the *CASP9* (1p36.13) and *FGFR1OP* (6q27) genes as previously predicted (Figure 38a-b).

### *Case 7*

Case 7 was a bone marrow aspirate from an 11-year-old female that presented an abnormal cytogenetic karyotype of 46,XX,inv(11)(q13q23) in 17 cells and was abnormal for the MLL (11q23) Breakapart FISH probe in 67% of interphase cells (Figure 39). The remaining MDS FISH panel was negative. The disruption of this gene cannot be detected by microarray studies. Balanced genomic alterations are not determined by microarray but three additional changes were determined to overlap pathogenic genes. A 774kbp gain was observed on chromosome 1p36.33 located at chr1: 2,910,012 - 3,684,184 that overlaps with the MDS-related *PRDM16* gene (1: 3,068,226 - 3,438,620). Manual examination detected a 249kbp gain on chromosome 11p11.2 located at chr11: 44,100,800 - 44,350,320 that overlaps the tumor suppressor *EXT2* gene (11: 44,095,548 - 44,245,429). Finally, a 674kbp loss on chromosome 22q12.1 located at chr22: 28,680,003 - 29,354,770 that overlaps the *CHEK2* gene (22: 28,687,742 - 28,741,904) was observed. The *CHEK2* gene has been shown to play a role in cell proliferation and tumor progression (Figure 40a-c).

### *Case 8*

A normal karyotype was presented in case 8 and FISH studies were cancelled by the requesting physician. High-resolution array studies using the ChAS software detected 32 benign aberrations.

Figure 38a. Case 6 Downstream of *CASP9*

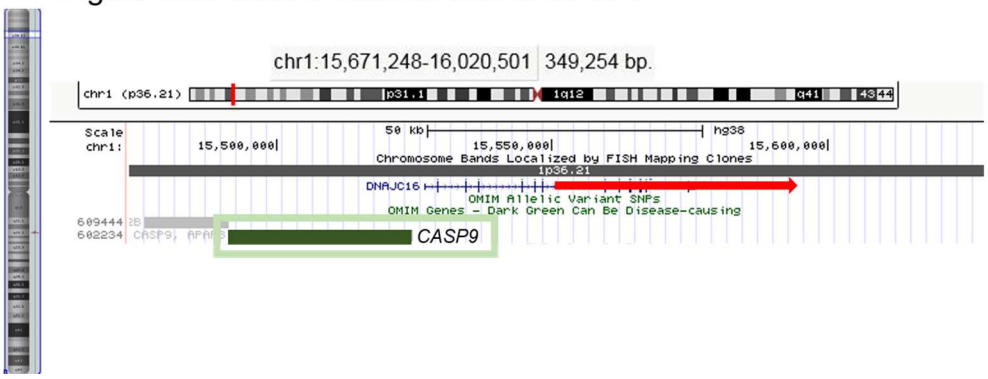



Figure 38b. Case 6 Downstream of *FGFR1OP*

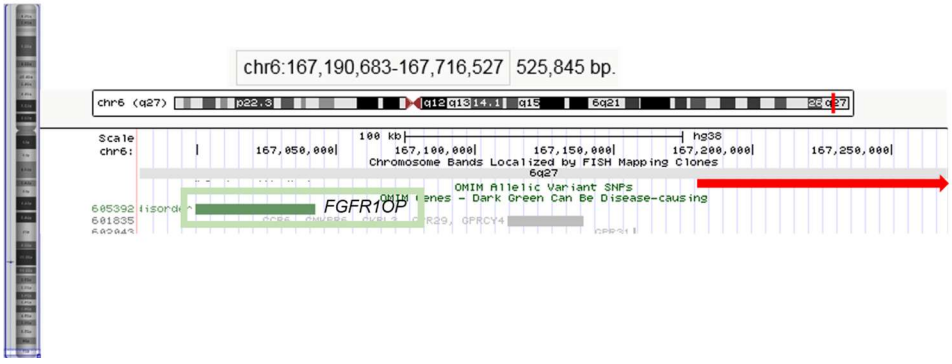
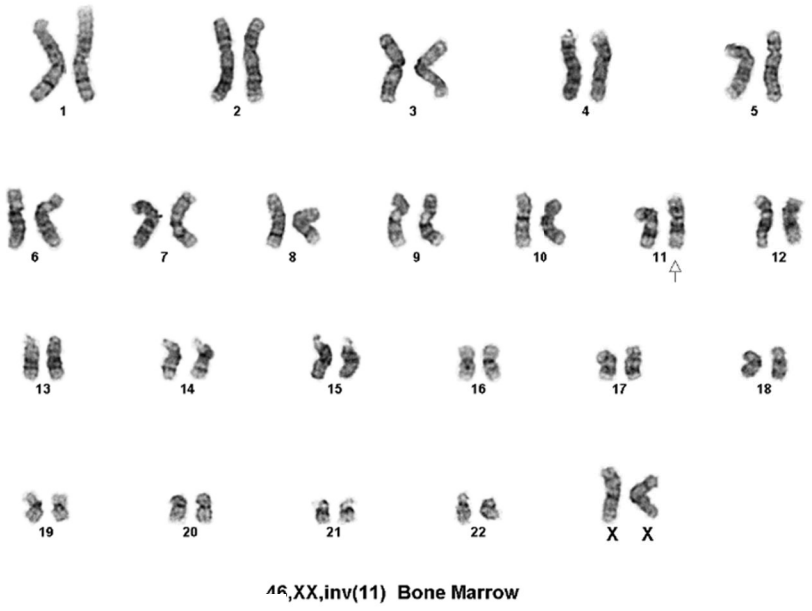



**Figure 38. Microarray results for Case 6**. ChAS originally predicted the overlap of a) 349kbp loss on chromosome 1p36.13, overlapping the apoptotic gene *CASP9* and b) 525kbp loss on chromosome 6q27, overlapping the MPD-related gene *FGFR1OP*. Further examination determined the losses as benign. The actual locations were over 144kbp and 148kbp downstream of the gene, respectively. This emphasizes the need for detailed examination following initial review using ChAS software.



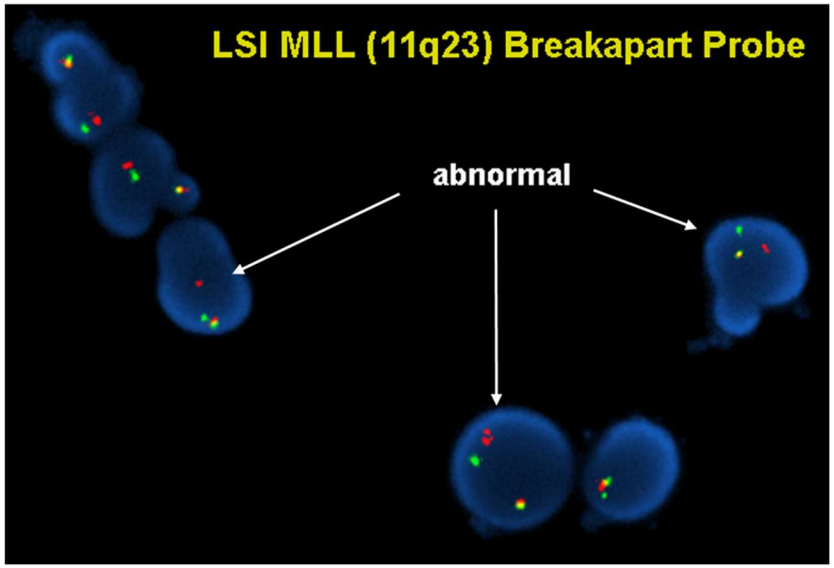




**Figure 39. Cytogenetic and FISH results for Case 7**. An abnormal cytogenetic karyotype presented 46,XX,inv(11)(q13q23). Abnormal FISH findings were positive for the MLL (11q23) Breakapart FISH probe and negative for the remaining MDS FISH panel.

Figure 40a. Case 7 *PRDM16*

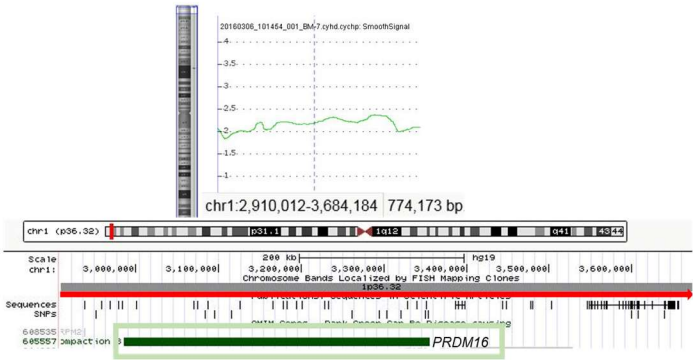



Figure 40b. Case 7 *EXT2*

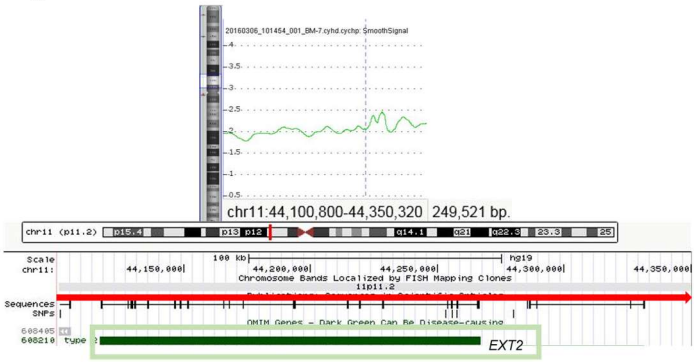



Figure 40c. Case 7 *CHEK2*

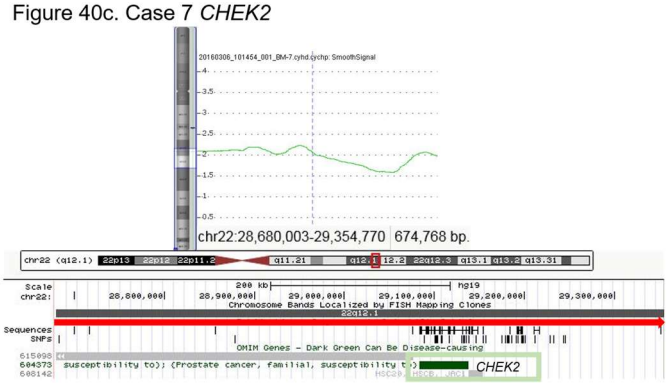



**Figure 40. Microarray results for Case 7**. Three pathogenic aberrations were detected and were determined to overlap pathogenic genes. a) A 774kbp gain was detected on chromosome 1p36.33 which overlaps the MDS-related *PRDM16* gene. b) A 249kbp gain on chromosome 11p11.2 was observed that overlaps the *EXT2* gene, known to have tumor suppressor roles. c) A 674kbp loss on chromosome 22q12.1 that overlaps the *CHEK2* gene was observed and has been shown to play a role in cell proliferation and tumor progression.

### *Case 9*

Case 9 was a bone marrow aspirate from a 14-year-old male with a normal karyotype. FISH studies were not performed since it was not ordered by the requesting physician. A total of 42 aberrations were detected and classified as benign by microarray studies.

### *Case 10*

In case 10, we utilized the OncoScan® High-resolution array studies. It is important to note that the ChAS software quality control of the MAPD, a standard deviation of the variation of the probe pair ratios, indicated that the quality of these results may have been potentially compromised. The aberrations detected included a 2.5Mbp gain on 1p36.33 (*PRDM16* gene); a 3.7Mbp LOH on 1p34.2 (*MPL* gene); a 4.0Mbp LOH on 1q25.2 (*ABL2* gene); a 39kbp loss on 2p23.2 (portion of the *ALK* gene); a 268kbp loss on 6p25.3 (*IRF4* gene); a 417kbp loss on 8p21.2 (*CLU* gene); a 47kbp loss on 9p21.3 (*CDKN2B* gene); a 6.0Mbp LOH on 9p21.1 (*PAX5* gene); a 3.8Mbp gain on 9q34.11 (*ADAMTS13* gene); and a 32.6Mkbp gain on 16p13.3 (*ERCC4* and *MYH11* gene) (Table VI). These genes have functions ranging from apoptosis, oncogenic potential, and tumor suppression. Other genes detected have known disease associations including AML, ALL, TCP, FA, NHL, MDS, and chronic myelogenous leukemia (CML).

### *Case 11*

Case 11 was a bone marrow aspirate from a 7-year-old male that presented an abnormal cytogenetic karyotype of 45,XY,-7 in seven cells and positive for monosomy 7 by FISH in 12% of interphase cells (Figure 41). Due to the low-level mosaicism of this abnormality, microarray studies were not able to detect monosomy 7. However, five

| Chromosome | Cytoband Start | Copy Number Change | Size (kbp) | Call |
|---|---|---|---|---|
| 1 | p36.33 | Gain | 2550.825 | Pathogenic *PRDM16* Gene |
| 1 | p34.2 | LOH | 3760.95 | Pathogenic *MPL* Gene |
| 2 | p23.2 | Loss | 39.566 | Likely Pathogenic Portion of *ALK* Gene |
| 6 | p25.3 | Loss | 268.34 | Pathogenic *IRF4* Gene |
| 8 | p21.2 | Loss | 417.183 | Pathogenic *CLU* Gene |
| 9 | p21.3 | Loss | 47.465 | Pathogenic *CDKN2B* Gene |
| 9 | p21.1 | LOH | 6066.542 | Pathogenic *PAX5* Gene |
| 9 | q34.11 | Gain | 3863.95 | Pathogenic *ADAMTS13* Gene |
| 16 | p13.3 | Gain | 32635.813 | Pathogenic *ERCC4* and *MYH11* Gene |

**Table VI. Microarray results for Case 10**. Numerous aberrations were detected including a 2.5Mbp gain on 1p36.33 (*PRDM16* gene); a 3.7Mbp LOH on 1p34.2 (*MPL* gene); a 4.0Mbp LOH on 1q25.2 (*ABL2* gene); a 39kbp loss on 2p23.2 (portion of the *ALK* gene); a 268kbp loss on 6p25.3 (*IRF4* gene); a 417kbp loss on 8p21.2 (*CLU* gene); a 47kbp loss on 9p21.3 (*CDKN2B* gene); a 6.0Mbp LOH on 9p21.1 (*PAX5* gene); a 3.8Mbp gain on 9q34.11 (*ADAMTS13* gene); and a 32.6Mbp gain on 16p13.3 (*ERCC4* and *MYH11* gene)

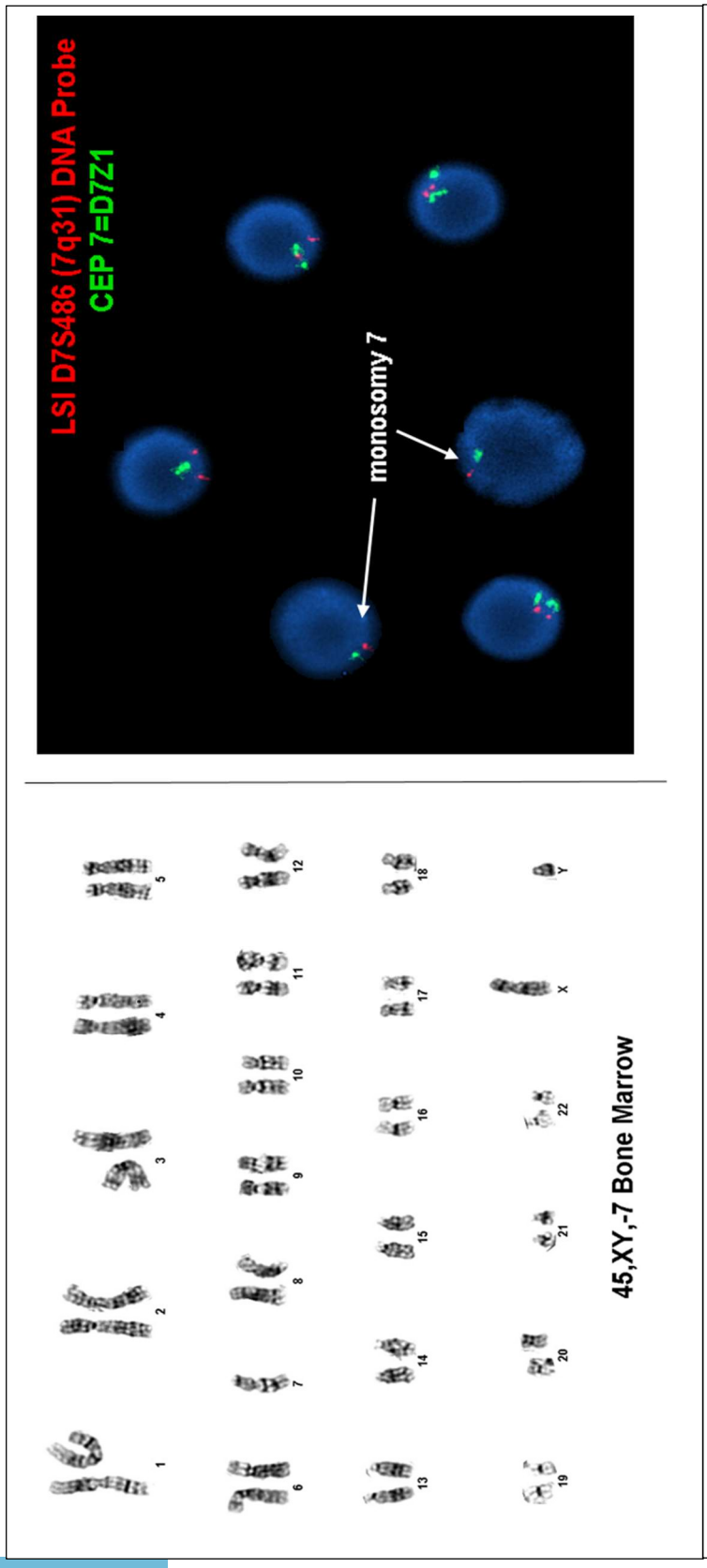



**Figure 41. Cytogenetic and FISH results for Case 11**. An abnormal cytogenetic karyotype presented 45,XY,-7 in seven cells. Abnormal FISH findings were positive for monosomy 7 in 12% of interphase cells.

pathogenic aberrations were detected utilizing OncoScan®. The aberrations detected included a 47kbp loss on 9p21.3 (*CDKN2B* gene); a 4.4Mbp LOH on 9p13.3 (*PAX5* gene); a 32.6Mbp gain on 16p13.3 (*ERCC4* and *MYH11* gene); a 1.1Mbp gain on 16q24.3 (*FANCA* gene); and a 3.5Mbp gain on 17q12 (*RARA* gene) (Table VII). These genes have been associated with tumor suppression and also have known disease associations with AML, ALL, CML, TCP, and FA.

***Case 12***

Case 12 was a peripheral blood from a 7-month old male with an abnormal cytogenetic karyotype of 45,XY,-7 in four cells and positive for monosomy 7 by FISH in 23% of interphase cells. The remaining probes in MDS FISH studies were not requested (Figure 42). Two aberrations were determined to overlap with pathogenic genes using OMIM and NCBI. A 197kbp gain was observed on chromosome 6p25.3 located at chr6: 241,540 - 439,182 which overlaps with the *IRF4* gene (6: 391,738 - 411,442), a known oncogene. A 298kbp loss on chromosome 7p12.2 located at chr7: 50,173,182 - 50,471,578 was also observed that overlaps with the ALL-related *IKZF1* gene (7: 50,303,464 - 50,405,100) (Figure 43).

***Case 13***

Case 13 was a bone marrow aspirate from a 15-year-old female presenting an abnormal cytogenetic karyotype of 45,XX,-7 in nine cells and positive for monosomy 7 in 28% of interphase cells by FISH (Figure 44). A total of 45 benign changes were observed by microarray. The level of mosaicism for this case was slightly above the validated detection rate for this assay (20-25%), yet monosomy 7 was not able to be detected by microarray.

| Chromosome | Cytoband Start | Copy Number Change | Size (kbp) | Call |
|---|---|---|---|---|
| **9** | p21.3 | Loss | 47.683 | Pathogenic *CDKN2B* Gene |
| **9** | p13.3 | LOH | 4473.806 | Pathogenic *PAX5* Gene |
| **16** | p13.3 | Gain | 32635.81 | Pathogenic *ERCC4* and *MYH11* Gene |
| **16** | q24.3 | Gain | 1110.352 | Pathogenic *FANCA* Gene |
| **17** | q12 | Gain | 3542.846 | Pathogenic *RARA* Gene |

**Table VII. Microarray results for Case 11**. The aberrations detected included a 47kbp loss on 9p21.3 (*CDKN2B* gene); a 4.4Mbp LOH on 9p13.3 (*PAX5* gene); a 32.6Mbp gain on 16p13.3 (*ERCC4* and *MYH11* gene); a 1.1Mbp gain on 16q24.3 (*FANCA* gene); and a 3.5Mbp gain on 17q12 (*RARA* gene).

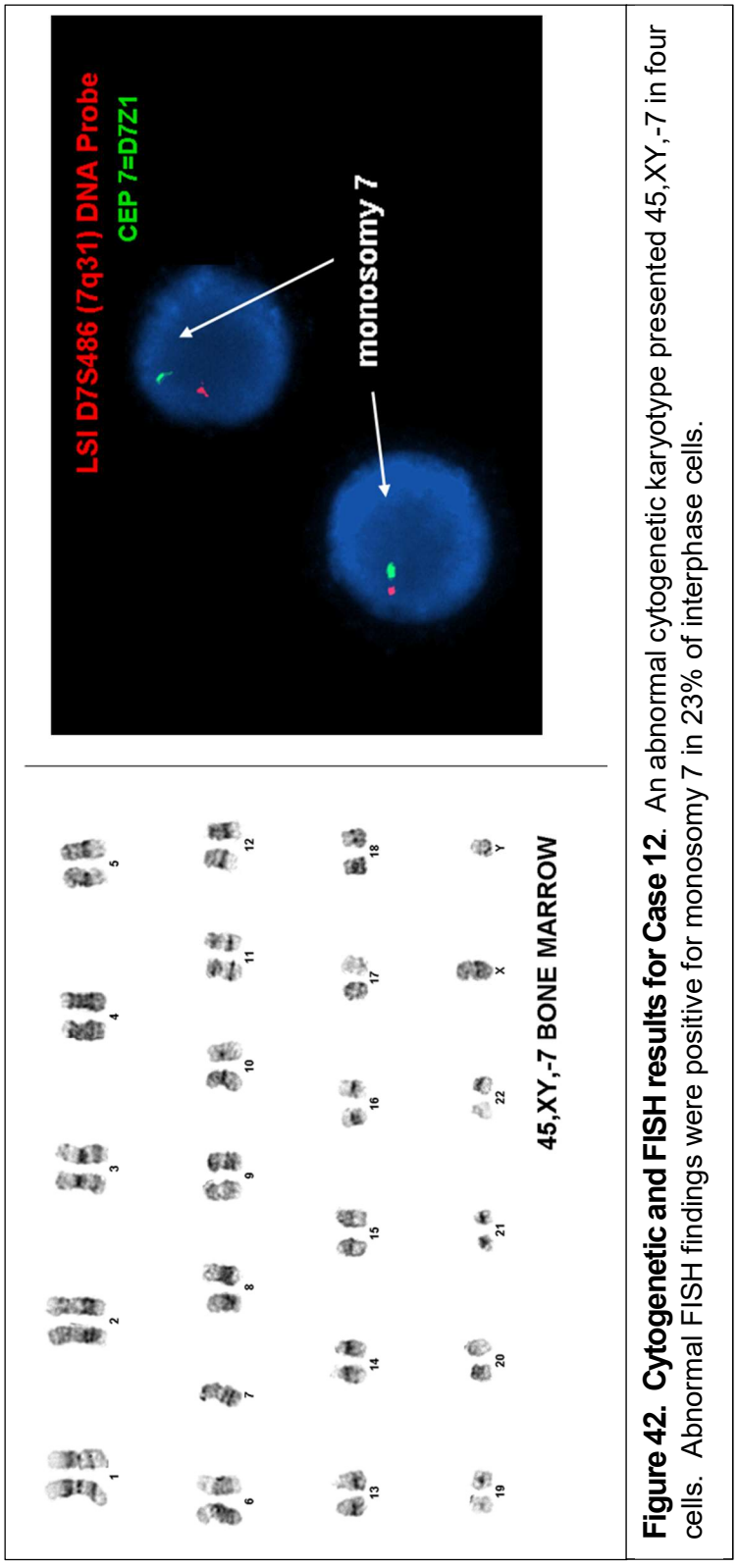



**Figure 42. Cytogenetic and FISH results for Case 12**. An abnormal cytogenetic karyotype presented 45,XY,-7 in four cells. Abnormal FISH findings were positive for monosomy 7 in 23% of interphase cells.

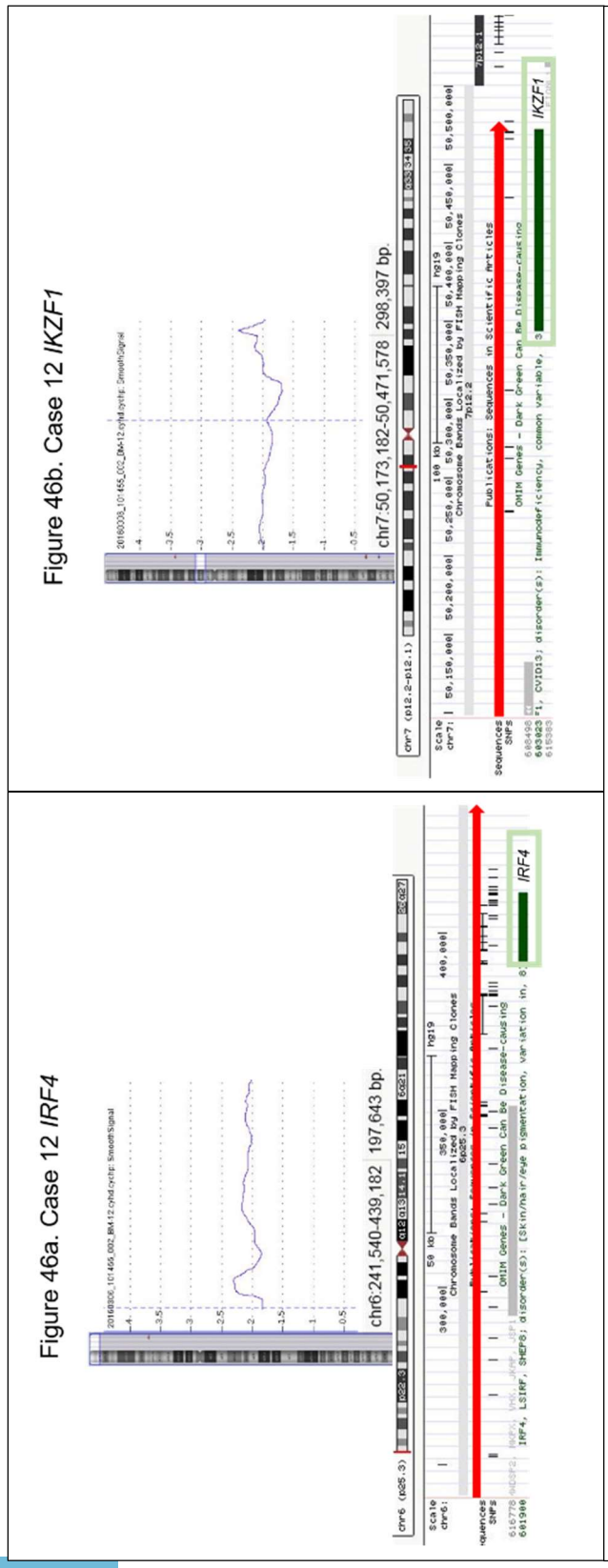
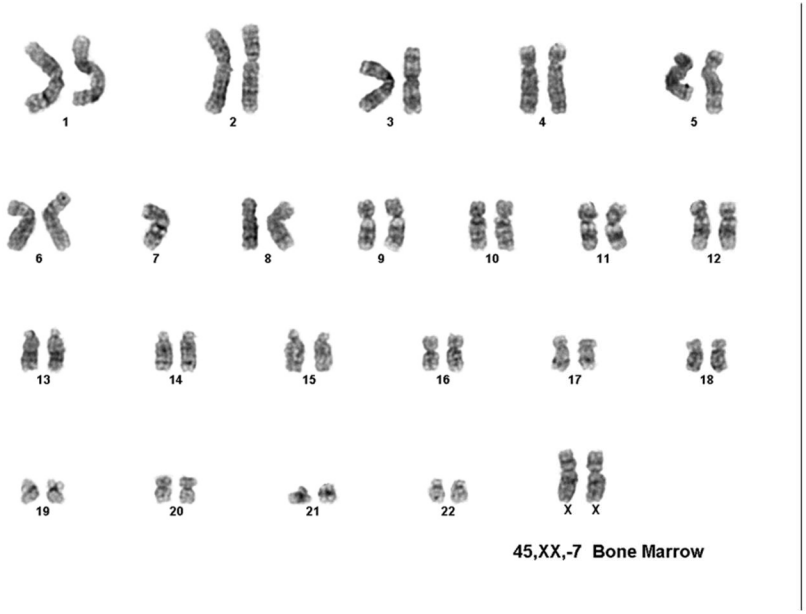



**Figure 43. Microarray results for Case 12**. Two aberrations overlapping pathogenic genes were detected. a) A 197kbp gain was detected on chromosome 6p25.3 which overlaps the *IRF4* gene, a known oncogene. b) A 298kbp loss on chromosome 7p12.2 that overlaps ALL-related *IKZF1* gene was also observed.



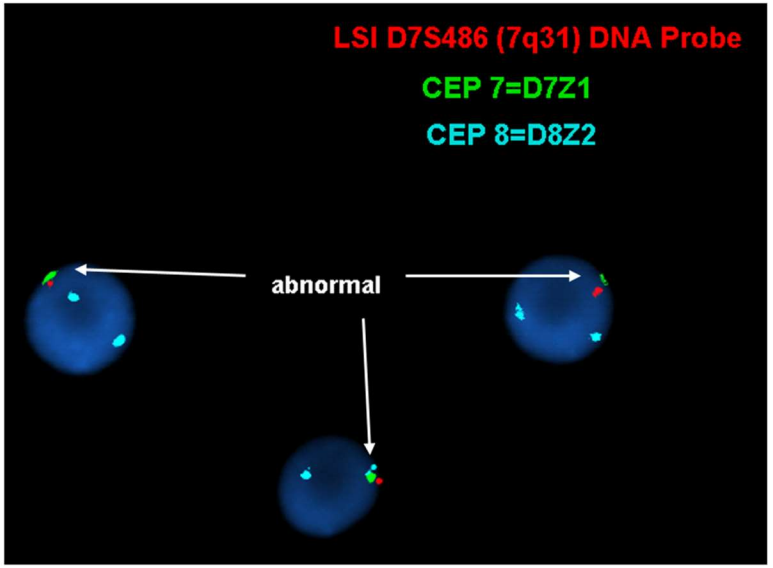




**Figure 44. Cytogenetic and FISH results for Case 13**. An abnormal cytogenetic karyotype presented 45,XX,-7. Abnormal FISH findings were positive for monosomy 7 in interphase cells. The karyotype illustrates monosomy 7. The FISH image shows representative interphase cells with one copy of centromere 7 and 7q31 region.

***Case 14***

Case 14 was a bone marrow aspirate from a 15-year-old female with a normal karyotype and FISH studies. High-resolution array studies depicting only benign aberrations was observed.

***Case 15***

In case 15, microarray detected three aberrations that overlapped pathogenic genes. A 179kbp loss was observed on chromosome 6p25.3 located at chr6: 222,619 - 402,086 which overlaps a portion of the *IRF4* gene (6: 391,738 - 411,442), a known oncogene. On chromosome 11q22.1 located at chr11: 101,752,252 - 102,239,809, a 287kbp loss was observed that overlaps the *YAP1* gene (11: 102,110,253 - 102,233,422), a gene with apoptotic functions. A 288kbp partial loss in the *PTPN11* gene (12: 112,418,731 - 112,509,917), known to be associated with MDS, was detected on 12q24.13 (chr12: 112,744,633 - 112,983,024) (Figure 45a-c).

***Case 16***

Case 16 was a bone marrow aspirate from a 19-year-old female presenting normal cytogenetic and FISH analyses and two pathogenic aberrations by microarray. A 690kbp gain overlapping the MDS-related *PRDM16* gene (1p36.33) and a 216kbp loss overlapping a portion of the *IRF4* oncogene (6p25.3) was detected (Figure 46).

***Case 17***

DNA from the excess whole bone marrow for case 17 was extracted, however, due to a poor quality of DNA (less than 5µg/mL) it could not be used for microarray studies. This case presented normal cytogenetic and FISH analyses (Figure 47).

Figure 45a. Case 15 *IRF4*

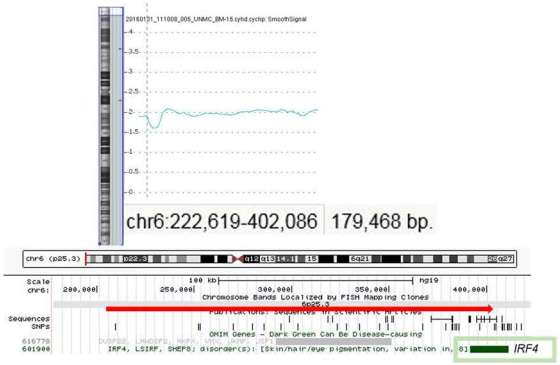



Figure 45b. Case 15 *YAP1*

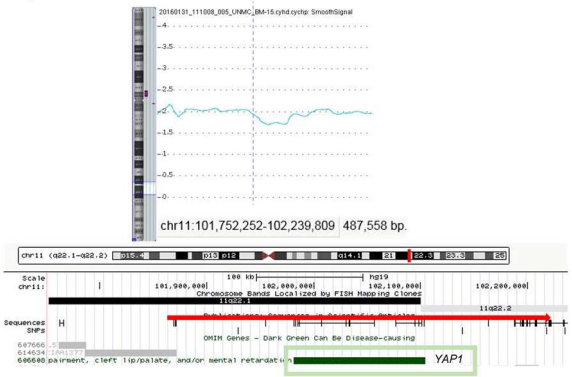



Figure 45c. Case 15 *PTPN11*

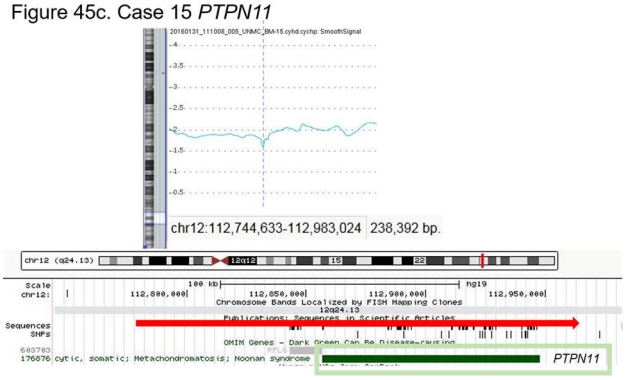



**Figure 45. Microarray results for Case 15**. Three aberrations overlapping a) a 179kbp loss on chromosome 6p25.3 which overlaps with a portion of the *IRF4* oncogene; b) a 287kbp loss on chromosome 11q22.1 overlapping the *YAP1* gene, which is involved in apoptotic functions; and c) a 288kbp loss partially involving the *PTPN11* gene located on chromosome 12q24.13 and implicated in MDS.

Figure 46a. Case 16 *PRDM16*

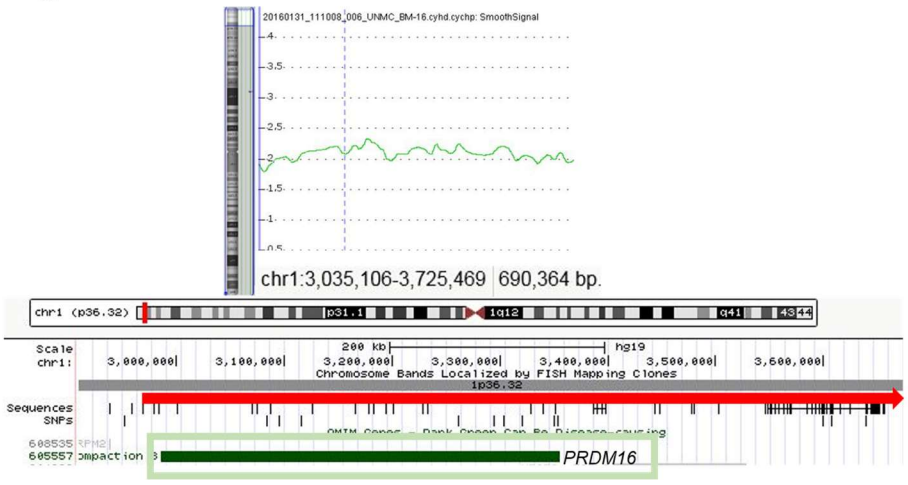



Figure 46b. Case 16 *IRF4*

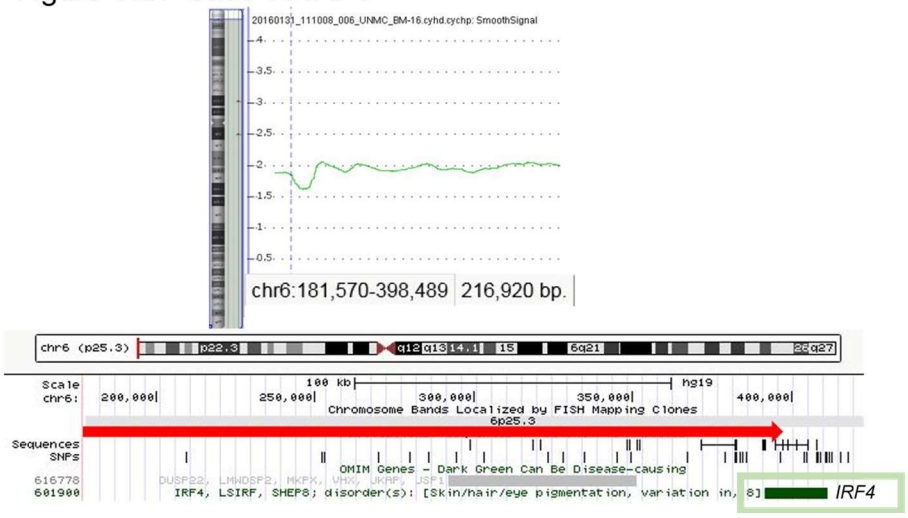



**Figure 46. Microarray results for Case 16**. Two aberrations were detected that overlap with pathogenic genes. a) A 690kbp gain was observed on chromosome 1p36.33 which overlaps the MDS-related *PRDM16* gene. b) A 216kbp loss on chromosome 6p25.3 that overlaps a portion of the oncogene *IRF4* gene was also observed.

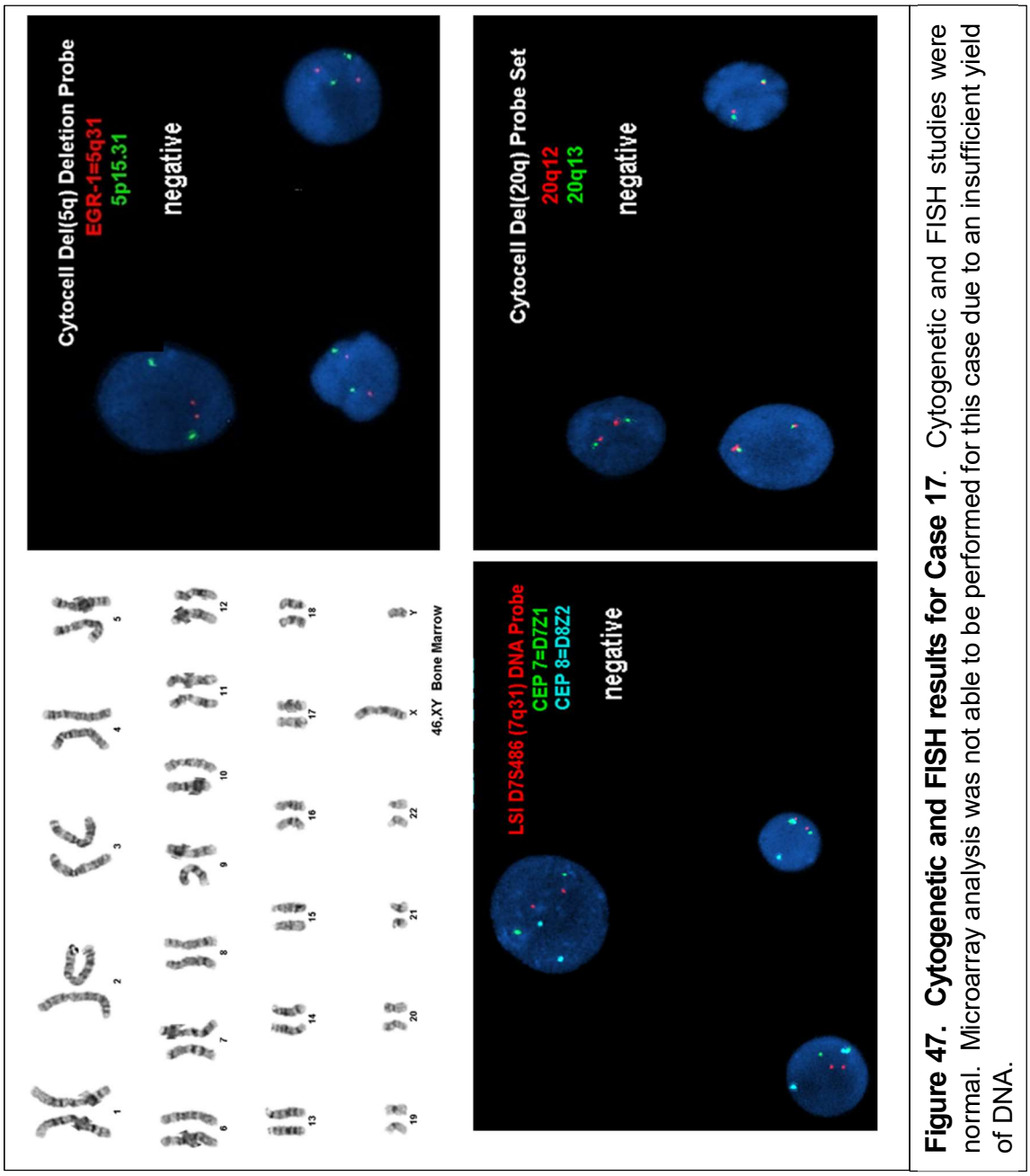



**Figure 47. Cytogenetic and FISH results for Case 17**. Cytogenetic and FISH studies were normal. Microarray analysis was not able to be performed for this case due to an insufficient yield of DNA.

***Case 18***

Case 18 was a bone marrow aspirate from a 13-year-old male with a normal karyotype, FISH, and microarray studies.

***Case 19***

Case 19 was a bone marrow aspirate from a 1-year-old male with a normal karyotype and FISH results and one pathogenic aberration by microarray. A 471kbp loss was detected on chromosome 1q32.1 located at chr1: 200,239,873 - 200,711,145 and was determined to overlap with *KIF4* gene (1: 200,551,496 - 200,620,790), a known disruptor of the cell cycle (Figure 48).

***Case 20***

Two aberrations were detected by microarray in case 20 and were determined to overlap with pathogenic genes. A 229kbp likely pathogenic loss was observed on chromosome 6p21.2 located at chr6: 45,198,969 - 45,428,856 which included a portion of the tumor suppressor *RUNX2* gene (6: 45,327,799 - 45,664,031). A 26kbp gain was detected on chromosome 16p13.11 located art ch16: 15,801,192 - 15,827,712 that overlaps a portion of the AML-associated *MYH11* gene (16: 15,703,134 - 15,857,032) (Figure 49a-b).

***Case 21***

In case 21, two aberrations overlapping with pathogenic genes were detected with normal karyotype and FISH analyses. A 4,995kbp mosaic gain on chromosome 1p36.33 including the *PRDM16* gene and a 50kbp gain on chromosome 11p11.2 involving the *EXT2* tumor suppressor gene, were detected by microarray studies (Figure 50a-b).

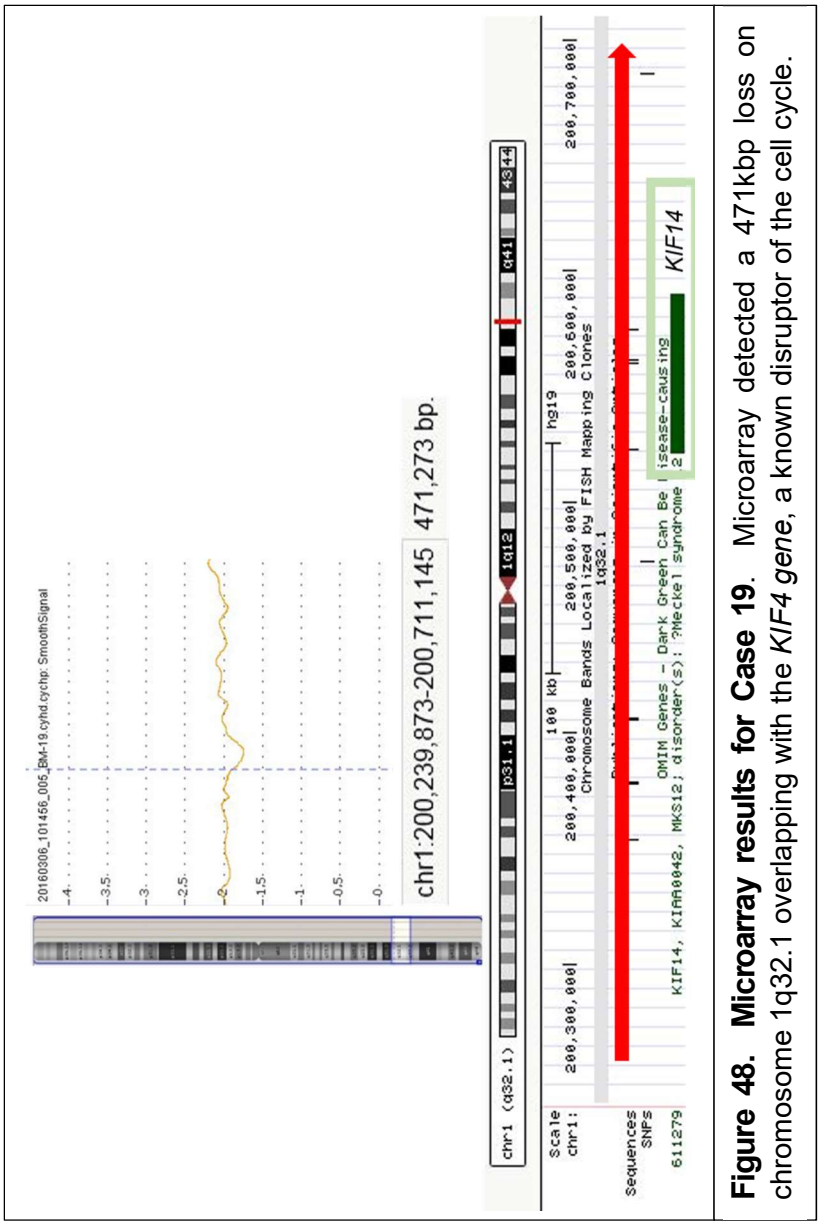



**Figure 48. Microarray results for Case 19**. Microarray detected a 471kbp loss on chromosome 1q32.1 overlapping with the *KIF4 gene*, a known disruptor of the cell cycle.

Figure 49a. Case 20 *RUNX2*

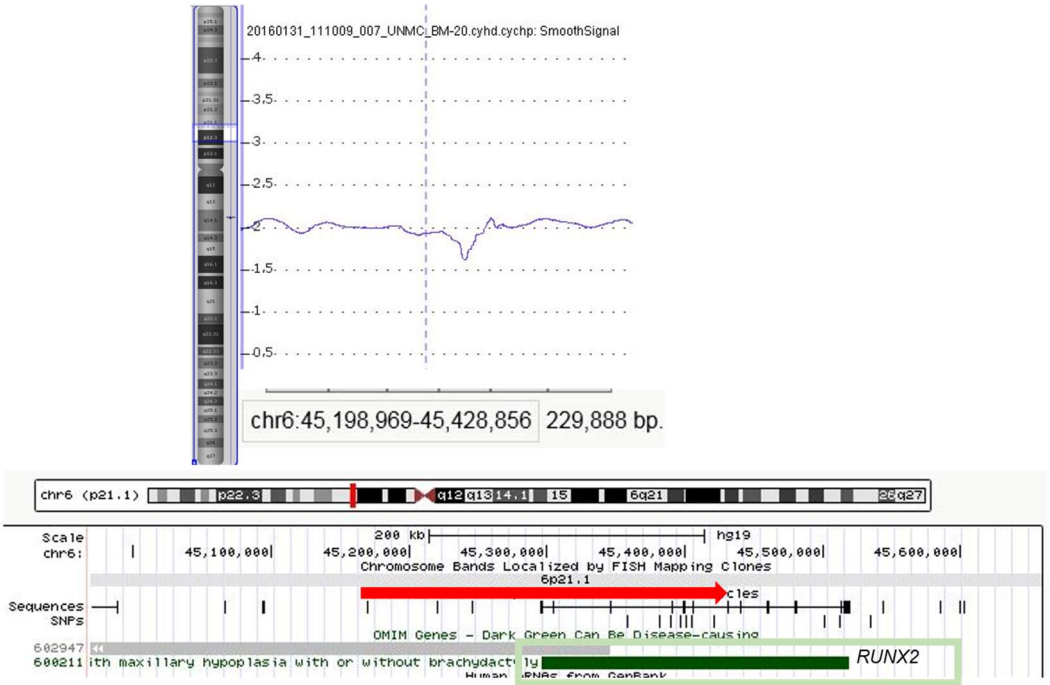



Figure 49b. Case 20 *MYH11*

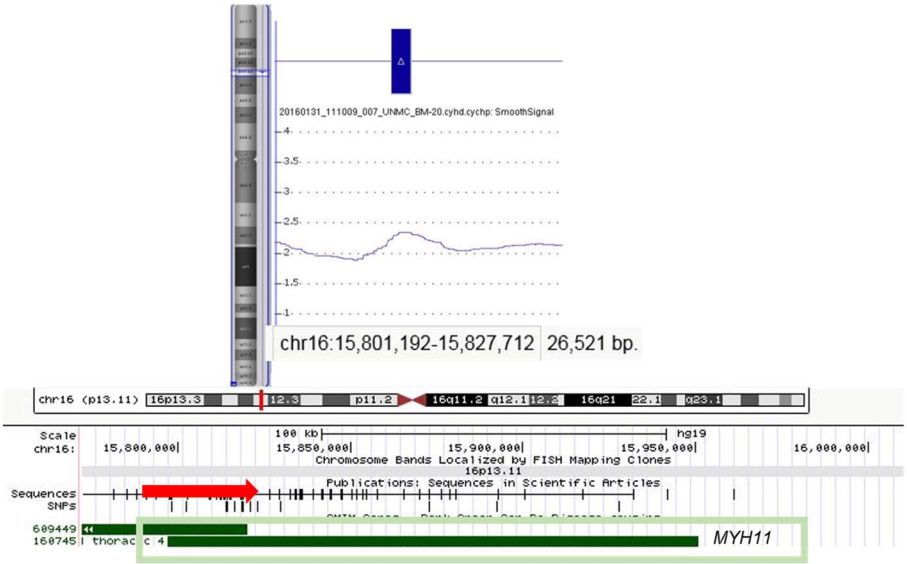



**Figure 49. Microarray results for Case 20**. Two aberrations were detected. a) A 229kbp loss was observed on chromosome 6p21.2 which partially overlaps a portion of the tumor suppressor *RUNX2* gene. b) A 26kbp gain was detected on chromosome 16p13.11 that overlaps a portion of the *MYH11* gene, a gene associated with AML.

Figure 50a. Case 21 *PRDM16*

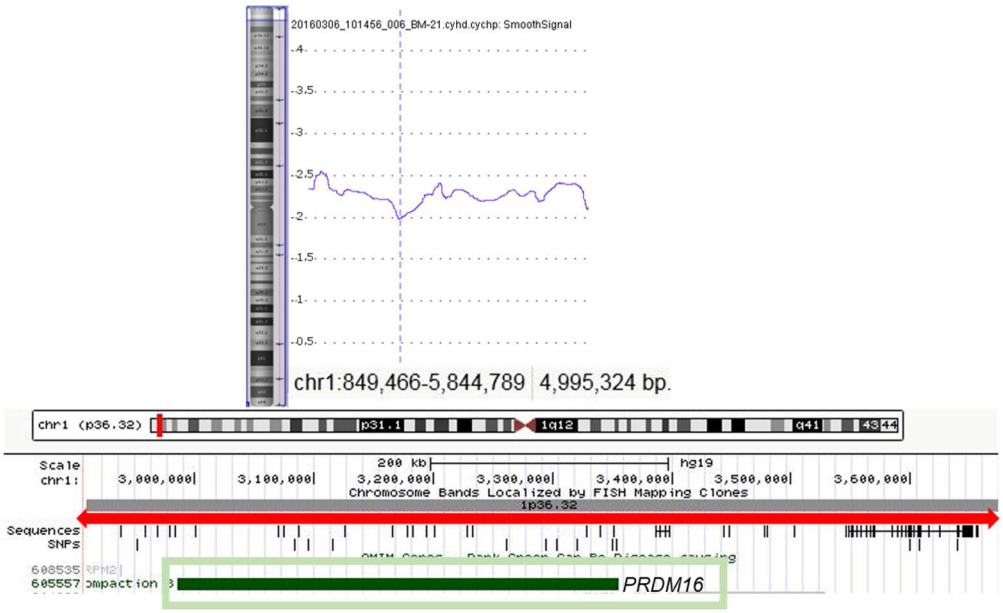



Figure 50b. Case 21 *EXT2A*

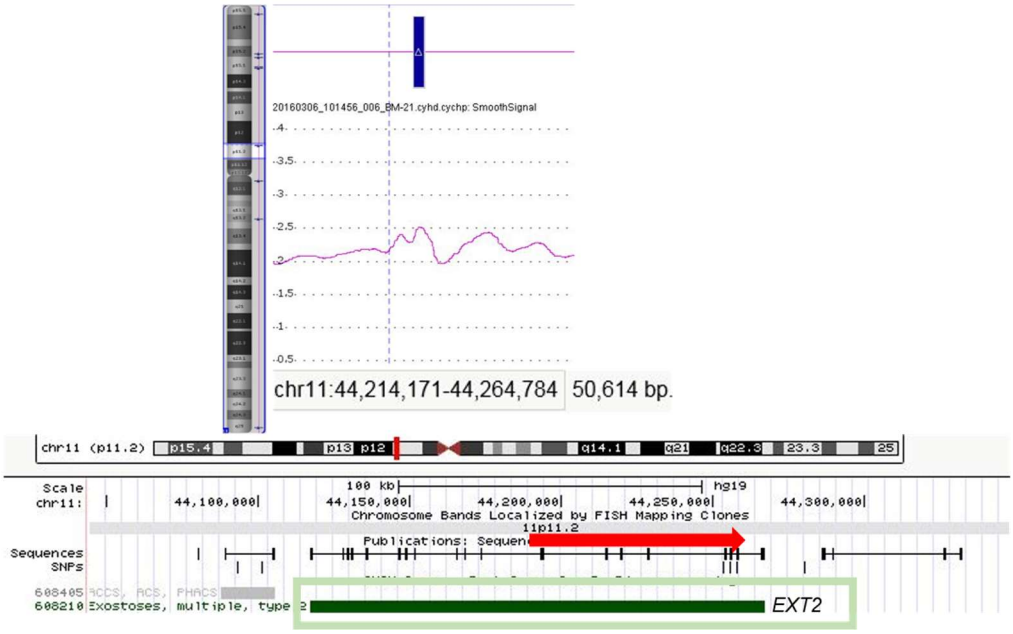



**Figure 50. Microarray results for Case 21**. Image depicts two aberrations that overlap with pathogenic genes were detected. a) A 4,995kbp mosaic gain on chromosome 1p36.33 overlapping the MDS-associated *PRDM16* gene. b) A 50kbp gain on chromosome 11p11.2 was observed that overlaps a portion of the tumor suppressor *EXT2* gene.

### *Case 22*

Case 22 was a bone marrow aspirate from a 24-year-old female with a normal karyotype with suboptimal morphology and was negative for the MDS FISH panel. Four aberrations overlapping pathogenic genes were detected. A 1.6Mbp gain on chromosome 1p36.33 overlapping the *PRDM16* gene, a known MDS-related gene; a 592kbp loss on chromosome 6q25.1 covering *LATS1*, a large tumor suppressor gene; a 454kbp loss involving *IDO1* gene with antiproliferative effects; and a large LOH region involving four additional genes were detected on chromosome 15. The genes include: *PML* (15: 73,994,672 - 74,047,818), involved in acute promyelocytic leukemia (APL); *RPS17* (15: 82,536,749 - 82,540,543), involved in Diamond-Blackfan anemia; *RECQL3* (15: 90,717,326 - 90,815,461), a known Bloom syndrome gene; and *FANCI* (15: 89,243,947 - 89,317,130), known to be involved with Fanconi's anemia (Figure 51a-d).

### *Case 23*

Case 23 was a bone marrow aspirate from a 20-year-old male with a normal karyotype, FISH, and microarray results.

### *Case 24*

Case 24 was a bone marrow aspirate from a 15-year-old male that presented a normal karyotype. FISH studies using probes for MDS revealed a deletion of 20(q12q13) in 8% of interphase cells (Figure 52). Many benign alterations were noted in microarray studies. The deletion of 20 observed by FISH was not detected by microarray due to low level mosaicism.

Figure 51a. Case 22 *PRDM16*

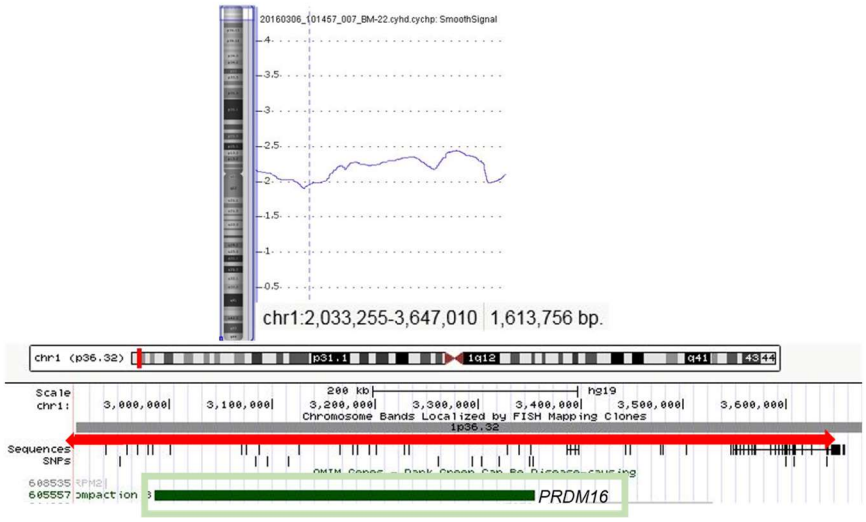



Figure 51b. Case 22 *LATS1*

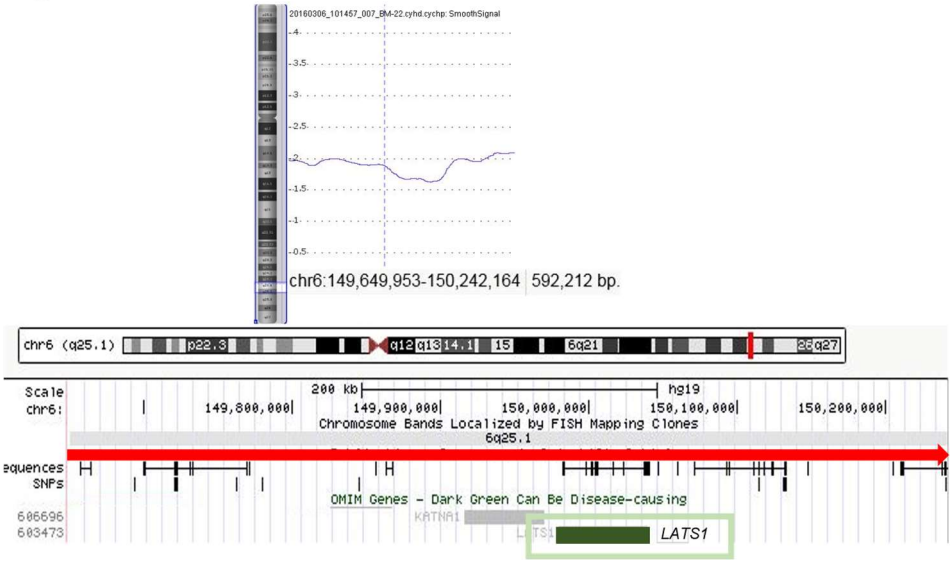



**Figure 51a-b. Microarray results for Case 22.** Four aberrations that overlap pathogenic genes were detected. a) A 1613kbp gain was detected on chromosome 1p36.33 which overlaps the *PRDM16* gene, a known MDS-related gene. b) A 592kbp loss was detected on chromosome 6q25.1 which overlaps the *LATS1* gene, a large tumor suppressor.

Figure 51c. Case 22 *IDO1*

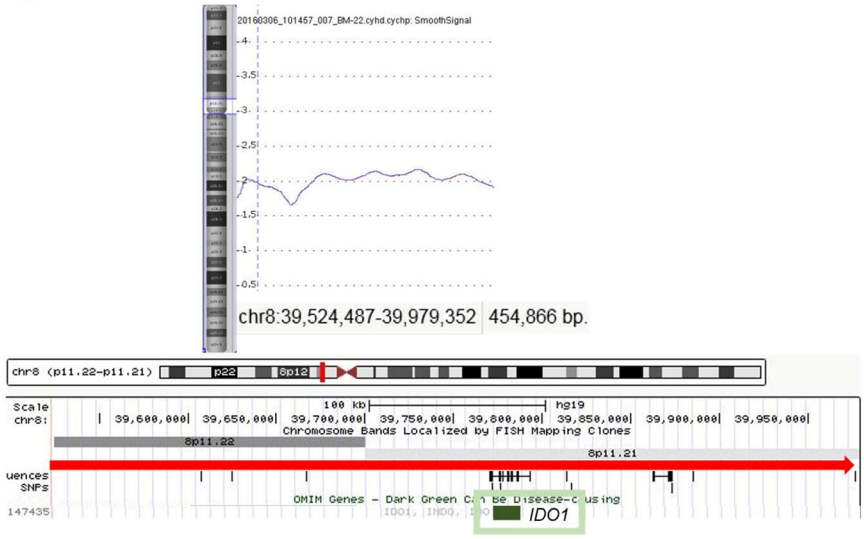



Figure 51d. Case 22 *PML, RPS17, RECQL3,* and *FANCI*


**Figure 51c-d. Microarray results for Case 22**. c) A 454kbp loss on chromosome 8p11.21 located which overlaps the *IDO1* gene, which has been shown to have antiproliferative effects on tumor. d) The image illustrates an LOH region on chromosome 15q24.-q26.1 which overlaps four genes: *PML*, involved in acute promyelocytic leukemia; *RPS17*, involved in Diamond-Blackfan anemia; *RECQL3*, a known Bloom syndrome gene; and *FANCI*, known to be involved with Fanconi's anemia.

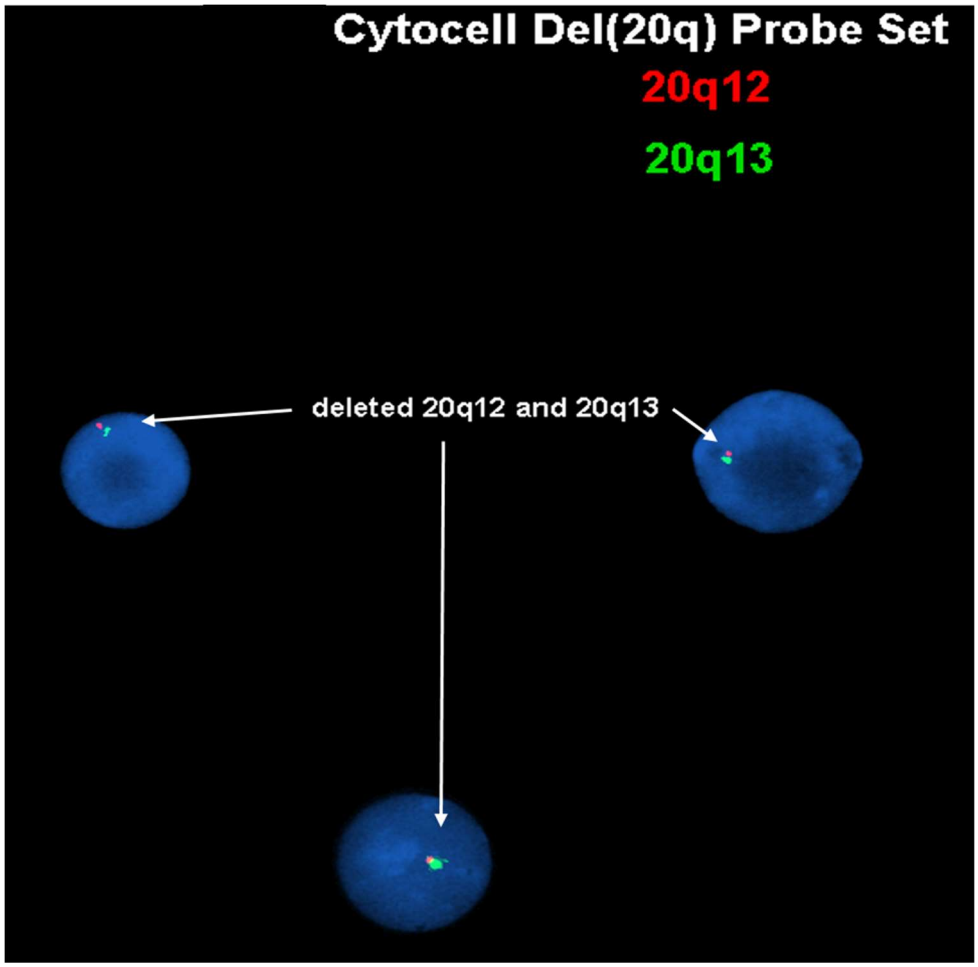



**Figure 52. FISH results for Case 24**. The FISH MDS panel detected a deletion of 20q12 and 20q13 with normal cytogenetic karyotypes. This image shows only one copy of each of the 20q probes.

***Case 25***

Case 25 was a bone marrow aspirate from a 7-year-old female with a normal karyotype and negative MDS FISH studies. A 682kbp pathogenic loss was detected on chromosome 7q21.3 located at chr7: 97,143,614 - 97,826,349. This deletion overlaps the *TAC1* gene (7: 97,731,958 - 97,740,471), a known regulator of hematopoiesis (Figure 53).

***Case 26***

In case 26, forty-two of the 45 aberrations detected by microarray were classified as benign. Three aberrations were determined to overlap with pathogenic genes. A 400kbp loss was detected on chromosome 8p11.21 located at chr8: 41,715,952 - 42,116,292 which overlaps the *KAT6A* gene (8: 41,929,478 - 42,051,988), a known AML-related gene. A 471kbp loss on chromosome 11q14.2 was detected at location chr11: 85,499,875 - 85,971,236, overlapping the AML-related gene *PICALM* (11: 85,957,170 - 86,069,880). Additionally, a 396kbp loss on chromosome 11q24.3 was detected at location chr11: 128,258,843 - 128,655,819 that overlaps with the AML-related gene *ETS1* (11: 128,458,760 - 128,587,583). (Figure 54a-c).

***Case 27***

Case 27 was a bone marrow aspirate from a 4-year-old male with a normal karyotype and normal FISH results (Figure 55). DNA from the excess whole bone marrow was extracted, however, due to a poor quality of DNA (less than 5µg/mL) it could not be used for microarray studies.

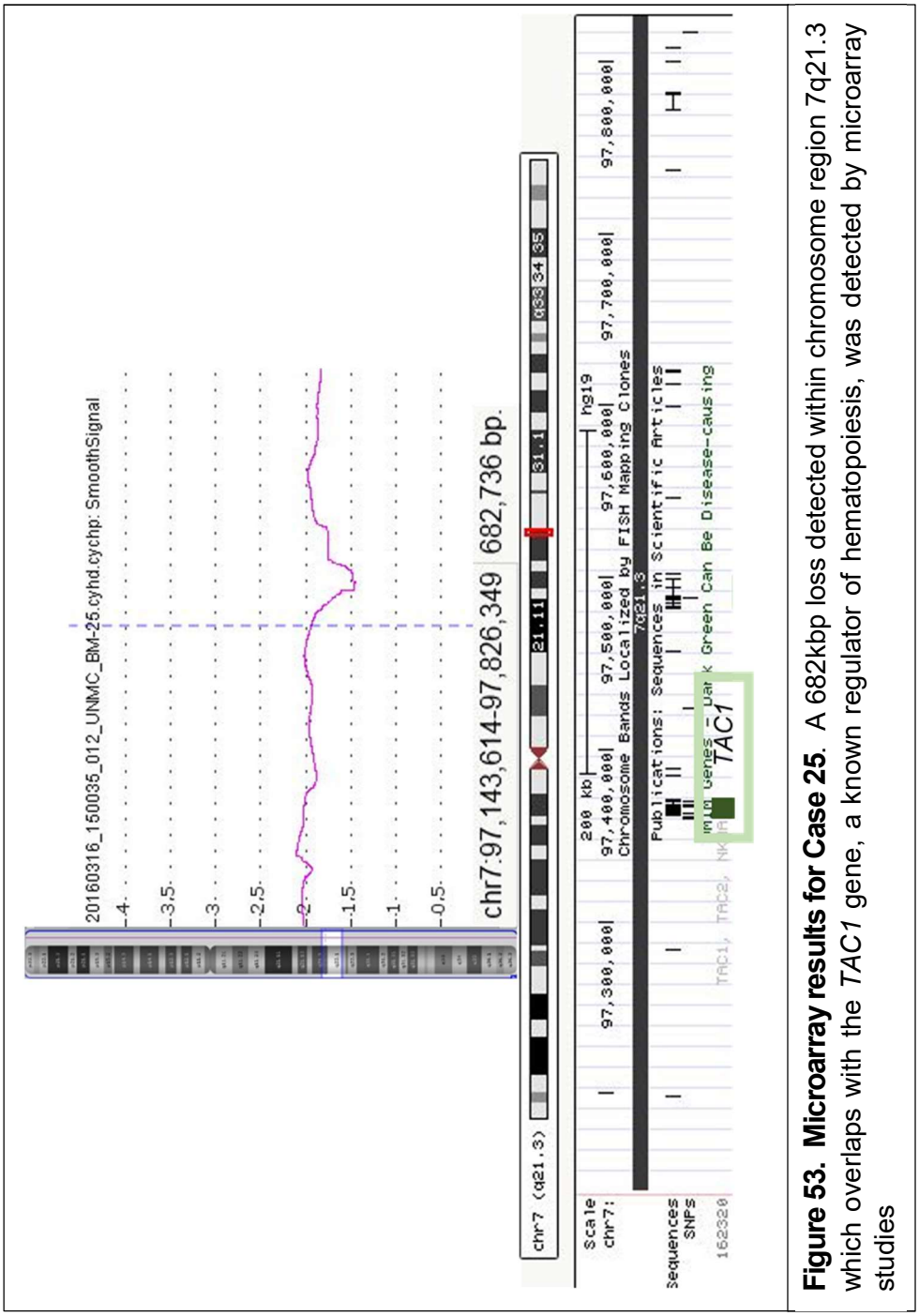



**Figure 53. Microarray results for Case 25**. A 682kbp loss detected within chromosome region 7q21.3 which overlaps with the *TAC1* gene, a known regulator of hematopoiesis, was detected by microarray studies

Figure 54a. Case 26 *KAT6A*

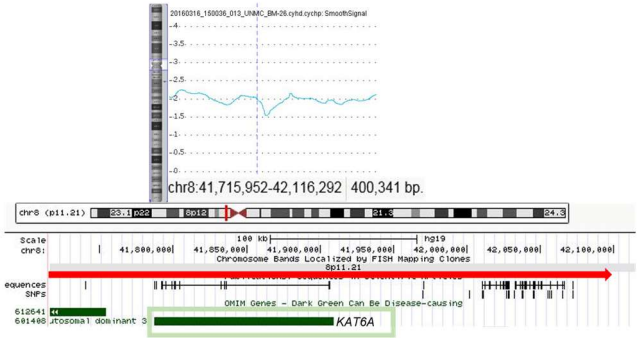



Figure 54b. Case 26 *PICALM*

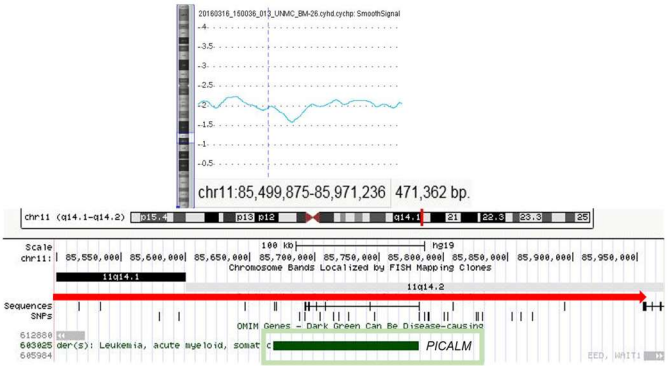



Figure 54c. Case 26 *ETS1*

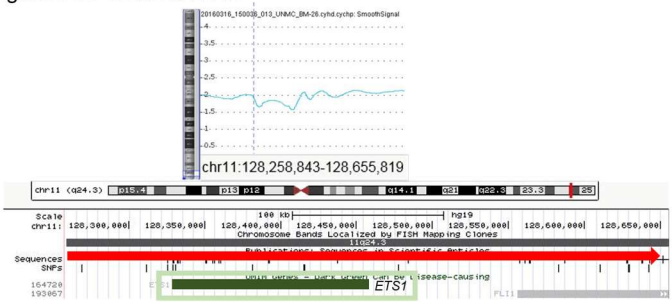



**Figure 54. Microarray results for Case 26**. Three aberrations detected were determined to involve AML-associated genes. These include a) a 400kbp loss was detected on chromosome 8p11.21 which overlaps the *KAT6A* gene; b) a 471kbp loss on chromosome 11q14.2 was detected which overlaps the *PICALM* gene; and c) a 396kbp loss on chromosome 11q24.3 was detected which overlaps the *ETS1* gene.

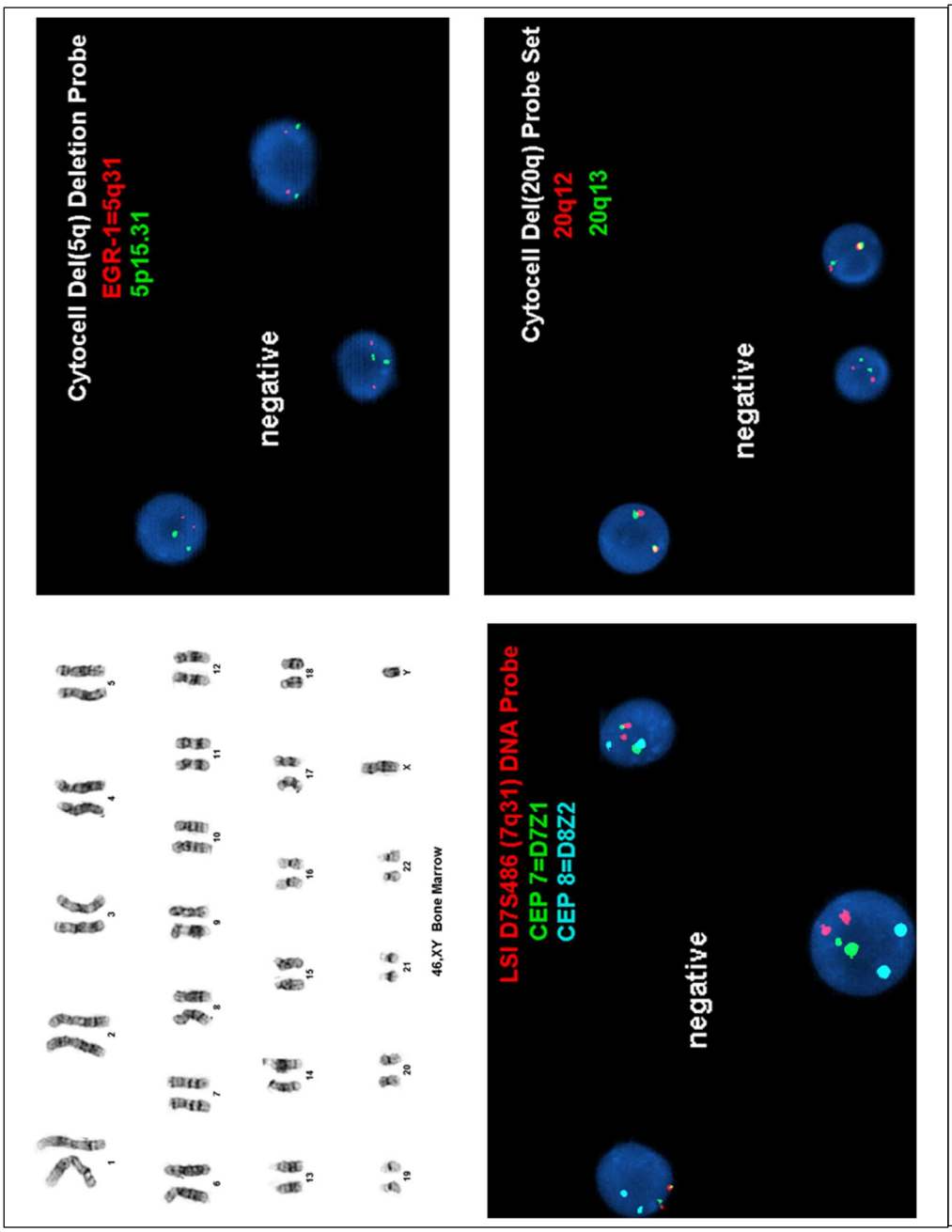



**Figure 55. Cytogenetic and FISH results for Case 27**. Cytogenetic and FISH studies were normal. Microarray analysis was not performed for this case due to insufficient yield of DNA.

***Case 28***

Case 28 was a bone marrow aspirate from a 2-year-old male that presented a normal karyotype, FISH and microarray results (Figure 56).

The utilization of high-resolution techniques allowed for the detection of novel genetic changes in this rare group of MDS. The 33 genes found to have a genomic aberration have a variety of functions not typically reported in pediatric MDS. Those of particular importance include the eight genes observed in multiple cases which may have implications in MDS based upon the findings of this study. The gain of the entire MDS-related *PRDM16* gene was detected in six cases along with a variety of other genomic aberrations equally of interest due to the novelty of these findings in the pediatric MDS population (Table VIII). The results from this study provides information useful in the characterization of the genetic landscape of this rare population of MDS.

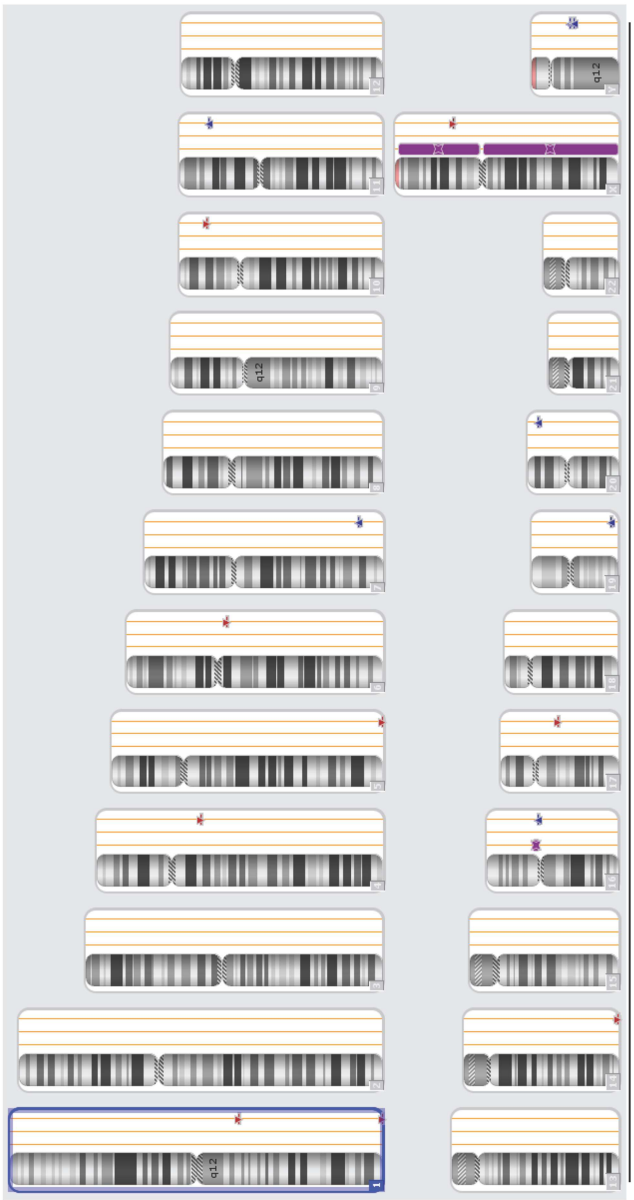


**Figure 56. Microarray results for Case 28.** Karyogram image of microarray results. All detected copy number changes were determined benign by DGV and Fullerton overlap maps.

| CASE | CYTOGENETIC AND FISH RESULTS | ADDITIONAL GENETIC ALTERATIONS AMONG CASES WITH GAIN OF *PRDM16* |
|---|---|---|
| **4** | 46,XY<br>Negative FISH | Loss of *PIM1* (6p21.2) |
| **7** | 46,XX,inv(11)(q13q23)[17]<br>MLL(11q23)<br>Breakapart[67%] | Gain of *EXT2* (11p11.2)<br>Loss of *CHEK2* (22q12.1) |
| **10** | 46,XY<br>Negative FISH | LOH of *MPL* (1p34.2)<br>LOH of *ABL2* (1q25.2)<br>Loss of *ALK* (2p23.3)<br>Loss of *IRF4* (6p25.3)<br>Loss of *CLU* (8p21.1)<br>Loss *CDKN2B* (9p21.3)<br>Loss of *FANCG* (9p13.3)<br>LOH of *PAX5* (9p13.2)<br>Gain of *ADAMTS13* (9q34.2)<br>Gain of *MYH11* (16p13.11)<br>Gain of *ERCC4* (16p13.12) |
| **16** | 46,XX<br>Negative FISH | Partial Loss of *IRF4* (6p25.3) |
| **21** | 46,XY<br>Negative FISH | Partial Gain of *EXT2* (11p11.2) |
| **22** | 46,XX<br>Negative FISH | Loss of *LATS1* (6q25.1)<br>Loss of *IDO1* (8p11.21)<br>LOH of *PML* (15q24.1)<br>LOH of *RPS17* (15q25.2)<br>LOH of *RECQL3* (15q26.1)<br>LOH of *FANCI* (15q26.1) |

**Table VIII. Characterization of the cases with the *PRDM16* gene aberration**. This table lists the six cases with the *PRDM16* gain and their respective cytogenetic and FISH results and additional genetic alterations observed by microarray analysis.

## Comprehensive Testing Results

From the retrospective study, 10.5% of abnormal samples were detected by FISH studies alone while 2.6% of abnormals were detected by cytogenetic karyotyping alone. This study presents that a total of 13.1% of abnormal samples were detected because of concurrent use of cytogenetics and FISH (Table IX).

The addition of microarray as an adjunct study improved the abnormality detection rate for a number of cases. Table X depicts comprehensive results after using all three techniques. Eleven cases had normal cytogenetics and FISH while microarray detected an abnormality. Twenty-four cases had all three tests performed, seven of these cases were normal (29%; 7 / 24) and 17 cases were abnormal (71%; 17 / 24). Of these 17 cases, there were 11 cases with pathogenic findings by microarray with normal conventional cytogenetics and FISH studies.

It is noteworthy that even though an abnormal finding was detected by microarray, in four cases microarray did not identify low level mosaic aberrations as low level mosaicism is detected by microarray above 20-25%. A loss of chromosome 7, deletion of 20, and also a balanced inversion disrupting the *MLL* gene had gone undetected by the microarray technique (Figures 38, 40, and 43; Table VII) . From this study, 60.7% (17 / 28) of cases detected an abnormality from at least one technique.

| | | |
|---|---|---|
| ***Total Normal Specimens*** | 230/306 | 75.2% |
| ***Total Abnormal Specimens*** | 76/306 | 24.8% |
| ***Abnormal FISH Alone*** | 8/76 | 10.5% |
| ***Abnormal Cytogenetics and FISH*** | 66/76 | 86.9% |
| ***Abnormal Cytogenetics Alone*** | 2/76 | 2.6% |

**Table IX. Characterization of results by cytogenetics and/or FISH analyses**. This table describes the total number and percentages of normal and abnormal pediatric/young adult MDS specimens from conventional cytogenetics and FISH studies.

| Case ID | Cytogenetic Findings | FISH Findings | Microarray Findings | Overall Comprehensive Results |
|---|---|---|---|---|
| 1 | Normal | Negative | Pathogenic | ABNORMAL |
| 2 | Normal | Negative | Benign | NORMAL |
| 3 | Normal | Negative | Benign | NORMAL |
| 4 | Normal | Negative | Pathogenic | ABNORMAL |
| 5 | Normal | Positive | Pathogenic | ABNORMAL |
| 6 | Normal | Negative | Benign | NORMAL |
| 7 | Abnormal | Positive | Pathogenic | ABNORMAL |
| 8 | Normal | Null | Benign | NORMAL |
| 9 | Normal | Null | Benign | NORMAL |
| 10 | Normal | Negative | Pathogenic | ABNORMAL |
| 11 | Abnormal | Positive | Pathogenic | ABNORMAL |
| 12 | Abnormal | Positive | Pathogenic | ABNORMAL |
| 13 | Abnormal | Positive | Benign | ABNORMAL |
| 14 | Normal | Negative | Benign | NORMAL |
| 15 | Normal | Negative | Pathogenic | ABNORMAL |
| 16 | Normal | Negative | Pathogenic | ABNORMAL |
| 17 | Normal | Negative | Null | NORMAL |
| 18 | Normal | Negative | Benign | NORMAL |
| 19 | Normal | Negative | Pathogenic | ABNORMAL |
| 20 | Normal | Negative | Pathogenic | ABNORMAL |
| 21 | Normal | Negative | Pathogenic | ABNORMAL |
| 22 | Normal | Negative | Pathogenic | ABNORMAL |
| 23 | Normal | Negative | Benign | NORMAL |
| 24 | Normal | Positive | Benign | ABNORMAL |
| 25 | Normal | Negative | Pathogenic | ABNORMAL |
| 26 | Normal | Negative | Benign | NORMAL |
| 27 | Normal | Negative | Null | NORMAL |
| 28 | Normal | Negative | Pathogenic | ABNORMAL |

**Table X. Characterization of results from comprehensive testing**. This table describes abnormalities for each of the 28 cases analyzed using cytogenetic karyotyping, FISH, and microarray techniques. From this study, 60.7% (17 / 28) of cases detected an abnormality from at least one technique.

# DISCUSSION

## Demographics

MDS has been attributed to age due to the aging of the hematopoietic system from gradual accumulation of endogenous and exogenous carcinogens over a lifetime. Studies have shown changes in the HSC system with over time including a higher frequency of stem cells, which are typically predominately quiescent, and a shift from a balanced differentiation to a more myeloid lineage than lymphoid. With age, the hematopoietic system has an up-regulation of the cell cycle that produces decreased bone marrow cellularity that leads to abnormalities of anemia and MDS or MPD (Pang, Price et al. 2011).

The occurrence of MDS before the age of 50 is rare; MDS is the most common cancer in persons over the age of 70 with incidences as high as 50 adult or elderly patients per 100,000 annually (Cui, Bueso-Ramos et al. 2010, Tefferi, Vardiman 2009, Corey, Minden et al. 2007). An overwhelming majority of specimens analyzed in our laboratory from January 1, 1990 to December 31, 2015 were from the adult and elderly populations at 92.4% (3686 / 3992) as seen in Figure 24. As depicted in Figure 23, this study supports the prevalence of MDS over the age of 70; over 53% of the MDS cases received into our laboratory were from individuals 70 years and older (1585 / 2948). In the adult and elderly MDS populations, males are more frequently diagnosed than women (Visconte, Selleri et al. 2014). Our study corroborates with the reported literature with findings of 57% males (1571 / 2771) to 43% females (1200 / 2771) from the 2771 total adult cases, age 30 and above.

Myelodysplastic syndromes among the pediatric and young adult population, or those between the ages of 0 and 29, is quite rare and represents about 5% of the hematopoietic neoplastic disorders or about 1.8 to 4 cases per million per year for this age group (Rau, Shreedhara et al. 2012, Silva, Maschietto et al. 2013, Germing, Aul et al.

2008, Chatterjee, Choudhry 2013, Mandel, Dror et al. 2002). In the past 25 years, we analyzed 306 pediatric or young adult MDS specimens, constituting 7.6% of the total MDS samples (306 / 3992), depicted in Figure 24. This study is one of the largest cohorts of MDS and supports previous documentation that is has a low prevalence in the younger population. The current report on pediatric MDS claim that this disease is seen in males and females equally (Niemeyer, Baumann 2008, Hofmann 2015a, Glaubach, Robinson et al. 2014). Our study of 119 pediatric only individuals (0-18 years) does not corroborate with these reports as depicted in Figure 25 showing a frequency more comparable to the adult populations [65 (55%) males to 54 (45%) females]. The results showed a trend that males were more often diagnosed with MDS than females in the pediatric population. Current literature notes a median age between 6.8 and 10.7 years for pediatric MDS (Hofmann 2015a, Hofmann 2015b, Glaubach, Robinson et al. 2014). Our study depicted a slightly lower median age of 6 years in the pediatric (0-18 years) population. Our study of pediatric MDS cases showed that the majority were very young, in the first three years of life. Thus, besides the ratio of males and females in the pediatric population, our present study correlates with previously published data on MDS.

## Cytogenetics and FISH

Once ineffective hematopoiesis is observed, conventional cytogenetic karyotyping and FISH are essential tools in accurate diagnosis and provide important prognostic information from certain aberrations. Nearly 40-50% of adult primary MDS studies contain a karyotypic abnormality and about 60-80% of secondary MDS samples contain an abnormality, detected by conventional techniques (Tefferi, Vardiman 2009, Haase, Germing et al. 2007, Jhanwar 2015, Haase 2008, Visconte, Selleri et al. 2014). The heterogeneic nature of MDS includes the presence of different types of chromosomal

aberrations but the most common abnormalities in the adult MDS population include one or more of the following hallmark abnormalities of -5/del(5q), -7/del(7q), +8, and del(20q) (Jhanwar 2015, Haase, Germing et al. 2007, Haase 2008, Visconte, Selleri et al. 2014).

In the adult population, these chromosome abnormalities have been well characterized; to some extent, prognosis and specific treatments have been determined based upon the presence of these anomalies. The most common MDS-associated chromosome abnormality is the loss of chromosome 5 or more frequently a deletion of the long arm of chromosome 5, observed in about 30% of the adult MDS population with a cytogenetic abnormality (Haase, Germing et al. 2007). As detailed in Table I, our studies support this observation in adult MDS samples. Nearly 33% (519 / 1563) of the total abnormal adult MDS samples analyzed in this laboratory presented -5/del(5q). Monosomy 7 or deletion of the long arm of 7 is the next most frequent cytogenetic abnormality observed in adult MDS at about 15-25% (Jhanwar 2015, Haase 2008). Our results detected -7/del(7q) at a slightly higher frequency of 27% (423 / 1563) among all abnormal adult samples. Over the given time span, 18% of the abnormal adult MDS specimens analyzed in our laboratory contained trisomy 8 (282 / 1563) which also corresponds with the current statistics of +8 typically seen in roughly 16% of adult MDS (Haase, Germing et al. 2007). Deleted 20q was observed in 16% (260 / 1563) of our subset of abnormal adult MDS samples. Several studies have reported the deletion of the long arm of chromosome 20 in 10-20% of cytogenetically abnormal MDS studies (Jhanwar 2015).

Another cytogenetic abnormality typically observed in MDS patients is the loss of the Y chromosome, but this abnormality has been associated with advanced age and not necessarily indicative of the syndrome (Vardiman, Thiele et al. 2009, Tefferi, Vardiman 2009). In our adult and elderly population of MDS, loss of Y was the most frequent

cytogenetic non MDS-related abnormality observed at 12% (190 / 1563). A variety of other abnormalities not related to MDS were observed in the adult population, however, all in frequencies less than 2%.

A limited number of reports are available on the characterization of pediatric and young adult MDS but we do know that karyotypic abnormalities are typically observed in 30 to 50% of the pediatric MDS population (Glaubach, Robinson et al. 2014). Our study showed a slightly lower frequency of cytogenetic aberrations in this young population at 24.8% (76 / 306). Of the abnormal specimens, 73.7% (56 / 76) contained one of the four hallmark MDS-related abnormalities (Table II). Current studies have documented the presence of these specific anomalies in pediatric MDS but research has shown that these entities are observed in different frequencies. Unlike the adult MDS population, -5/del(5q) is rarely observed in pediatric MDS (Hofmann 2015a, Germing, Aul et al. 2008). Our study found -5/del(5q) in 10.5% (8 / 76) of our abnormal pediatric population. This was our least frequent cytogenetic abnormality among the four hallmark aberrations. The most frequent cytogenetic abnormality in our pediatric specimens was -7/del(7q) in 36.8% (28 / 76). Current literature also shows that this abnormality is the most prevalent in the pediatric population at rates of 30% of abnormal cytogenetic studies and is observed in the adult population only 15-25% of the time (Corey, Minden et al. 2007, Glaubach, Robinson et al. 2014, Niemeyer, Baumann 2011, Hofmann 2015a, Hofmann 2015b). The next most frequent abnormality observed in the pediatric population is documented as trisomy 8, between 12-16% and in our study we witnessed +8 in 17.1% (13 / 76) (Glaubach, Robinson et al. 2014, Hofmann 2015a). This intermediate frequency is comparable to that observed in the adult population. Finally, the del(20q) abnormality has an intermediate frequency about 10-20% in both the pediatric and adult populations (Hofmann 2015a, Glaubach, Robinson et al. 2014). Our study coincides with this information with the

observation of del(20q) in 13.2% (10 / 76) of the abnormal pediatric samples. The most frequent other abnormality detected in the pediatric and young adult population is constitutional trisomy 21 in 25% (19 / 76). Trisomy 21 accounts for 20-25% of pediatric MDS and has been characterized as its own entity of ML-DS (Rau, Shreedhara et al. 2012, Glaubach, Robinson et al. 2014). The remaining pediatric and young adult samples with cytogenetic abnormalities not typical of MDS were observed in frequencies less than 1%.

This study is one of the largest retrospective studies of MDS with 3992 total MDS specimens studied over the time frame of January 1, 1990 to December 31, 2015. The goal of this study was to provide more evidence of the prevalence in the adult population and to confirm just how rare a disease MDS is in the pediatric population. Our study confirms the very infrequent occurrence of MDS in the pediatric and young adult population. This study provides confirmation of published statistics on the demographics and cytogenetic frequencies in adult MDS. This study also provides more evidence towards the frequencies of cytogenetic findings in the pediatric and young adult population and provides evidence that the most common cytogenetic abnormality of -7/del(7q) is seen in a slightly higher frequency than previously published.

Our study supports concurrent cytogenetic and FISH analyses to efficiently and accurately detect prognostically important abnormalities in MDS. Benefits of concurrent cytogenetic and FISH testing aid in the detection of cytogenetic aberrations even when mitotic index is low from compromised or limited initial specimen and when poor morphology inhibits the ability to observe higher resolution banding (Cin, Aster et al. 2010, Rigolin, Bigoni et al. 2001). Utilizing cytogenetic karyotyping will continue to detect abnormalities in very low frequencies that may be out of the abnormal range established for each FISH probe as determined during probe validation. Furthermore, this study

observed 10.5% (8 / 76) of the total abnormal pediatric/young adult samples containing one of the hallmark MDS abnormalities only detectable by FISH studies (Table IX). An accurate determination of all genetic aberrations is of the utmost importance for this rare group of MDS and is achieved with concurrent testing.

## Prognostic Implications in MDS

For MDS studies, the use of cytogenetic studies is of utmost importance in prognostic implications based upon the presence of the hallmark aberrations of -5/del(5q), -7/del(7q), +8, and del(20q). In the adult population, the prognostic scores are determined and well characterized based upon the presence of these anomalies. Limited information and studies on the pediatric MDS population has precluded the development of a pediatric specific prognostic scoring system, however, continued efforts will contribute to this cause. The information available has shown prognostically diverse cytogenetic findings between the two age groups.

The loss of chromosome 5 or deleted 5q is the most prevalent aberration in the adult population and is rarely observed in the pediatric population. Adults with -5/del(5q) are considered in the 'good' prognostic category with an overall median survival at 54 months and specific drug therapies have long been established including the use of azacitidine for MDS with 5q deletions (Tefferi, Vardiman 2009, Zou, Fink et al. 2007) This abnormality in the pediatric population presents poor overall survival and a higher propensity of progression to AML (Haase 2008).

A more favorable prognosis, median overall survival over 2 years, in the pediatric population is given when -7/del(7q) is observed cytogenetically due to being the ideal candidate for HSCT. After treatment, these pediatric patients have an overall good

prognosis with a longer-term survival. In the adult population, the median survival of an adult MDS study with this abnormality is less than 2 years (Rau, Shreedhara et al. 2012). These adult individuals are typically prone to infections and the -7/del(7q) aberration is usually part of a more complex karyotype (Greenberg, Tuechler et al. 2012).

Trisomy 8 in the pediatric population presents the most stable prognosis of the disease in the younger population while in the adult population; this abnormality correlates with an intermediate prognosis of OS at or above 23 months. In both populations, the prognostic impact of del(20q) also has an intermediate OS at or above 23 months (Greenberg 2015, Rau, Shreedhara et al. 2012). The -5/del(5q) and -7/del(7q) prognostic differences need to be the basis for the justification of characterizing the younger population as its own group. Discovering any trends in gene involvement of the pediatric population and making comparisons with the adult studies will aid in determining key differences between pediatric and adult MDS cases at the molecular level.

## Microarray

The use of high-resolution techniques including microarray is useful in the identification of any additional microdeletions or duplications that are unable to be observed by standard cytogenetic assays. In the adult population, this technique has been able to shed light on possible mutations that affect genes that regulate cellular epigenetic machinery (Shih, Abdel-Wahab et al. 2012, Bejar 2014). The specific genes involved in pediatric MDS have yet to be fully characterized and this study contributes to the ongoing research of this rare population.

Our study shows the importance of comprehensive analyses for MDS. Roughly 45% of MDS studies will present normal cytogenetic karyotypes yet a genetic imbalance cannot be ruled out (Cherian, Bagg 2006, Haase, Germing et al. 2007). The purpose of

this study was to determine genetic anomalies in the rare pediatric MDS population with a comprehensive study that included cytogenetics, FISH, and high-resolution array technologies. As described in Table XI, our findings characterized genetic aberrations in 33 genes from over 58% (15 / 26 cases) of the pediatric and young adult samples tested. The majority of these cases (11 cases) presented normal cytogenetic and FISH results at 73% as depicted in Figure 57. The ability to detect genomic loss or gain in prognostically relevant genes in pediatric MDS cases has clinical implications for the patient and higher resolution testing like microarray can achieve this detection.

This study also shows the need to continue conventional techniques of cytogenetics and FISH. Microarray analysis is typically able to detect copy number changes when present in as few as 20-25% of cells. Unfortunately, due to the mosaic nature of cancer genomes, aberrations can occur in low numbers that are not able to be detected by these higher resolution tests due to the algorithm nature of the analysis. The need for comprehensive testing is necessary in order to determine all possible genomic aberrations for the benefit of a more thorough analysis of each case.

## Current Trends in Adult MDS Array Analysis

The heterogeneous nature of MDS is seen at the genetic level in a broad spectrum of somatic or acquired gene alterations. Genetic imbalances are detected using high-resolution techniques that are increasingly becoming recognized as important diagnostic and prognostic markers that not only have clinical significance in relation to risk stratification and potential therapies but have primarily been studied and implicated in the adult population (Vardiman, Thiele et al. 2009, Kulasekararaj, Mohamedali et al. 2013, Jhanwar 2015).

| Case | Cytogenetic Results | FISH Results | Microarray Results | Reference |
|---|---|---|---|---|
| 1 | 46,XX | Negative | Loss of *CBFA2T3* | Fig. 34 |
| 4 | 46,XY | Negative | Gain of *PRDM16*<br>Loss of *PIM1* | Fig. 35 |
| 5 | 46,XY | del(20)(q12q13) [13%] | Loss of *ALK* | Figs. 37-38 |
| 7 | 46,XX,inv(11)(q13q23) | MLL (11q23) Breakapart [67%] | Gain of *PRDM16*<br>Gain of *EXT2*<br>Loss of *CHEK2* | Figs. 39-40 |
| 10 | 46,XY | Negative | Gain of *PRDM16*<br>LOH of *MPL*<br>LOH of *ABL2*<br>Loss of *ALK*<br>Loss of *IRF4*<br>Loss of *CLU*<br>Loss *CDKN2B*<br>Loss of *FANCG*<br>LOH of *PAX5*<br>Gain of *ADAMTS13*<br>Gain of *MYH11*<br>Gain of *ERCC4* | Table VI |
| 11 | 45,XY,-7 | Monosomy 7 [12%] | Loss of *CDKN2B*<br>LOH *PAX5*<br>Gain of *ADAMTS13*<br>Gain of *ERCC4*<br>Gain of *MYH11*<br>Gain of *FANCA*<br>Gain of *RARA* | Fig. 41<br>Table VII |
| 12 | 45,XY,-7 | Monosomy 7 [23%] | Gain of *IRF4*<br>Loss of *IKZF1* | Figs. 42-43 |
| 15 | 46,XY | Negative | Loss of *IRF4*<br>Loss of *YAP1*<br>Loss of *PTPN11* | Fig. 45 |
| 16 | 46,XX | Negative | Gain of *PRDM16*<br>Loss of *IRF4* | Fig. 46 |
| 19 | 46,XY | Negative | Loss of *KIF4* | Fig. 48 |
| 20 | 46,XX | Negative | Loss of *RUNX2*<br>Gain of *MYH11* | Fig. 49 |
| 21 | 46,XY | Negative | Gain of *PRDM16*<br>Gain of *EXT2* | Fig. 50 |
| 22 | 46,XX | Negative | Gain of *PRDM16*<br>Loss of *LATS1*<br>Loss of *IDO1*<br>LOH of *PML*<br>LOH of *RPS17*<br>LOH of *RECQL3*<br>LOH of *FANCI* | Fig. 51 |
| 25 | 46,XX | Negative | Loss of *TAC1* | Fig. 53 |
| 26 | 46,XY | Negative | Loss of *KAT6A*<br>Loss of *PICALM*<br>Loss of *ETS1* | Fig. 54 |

**Table XI. Details of abnormalities observed in pediatric MDS cases by microarray**. This table describes the cytogenetic and FISH findings and the specific aberrations detected by microarray fort he 26 cases that microarray was performed on. This table also lists the reference figure or table describing each case.

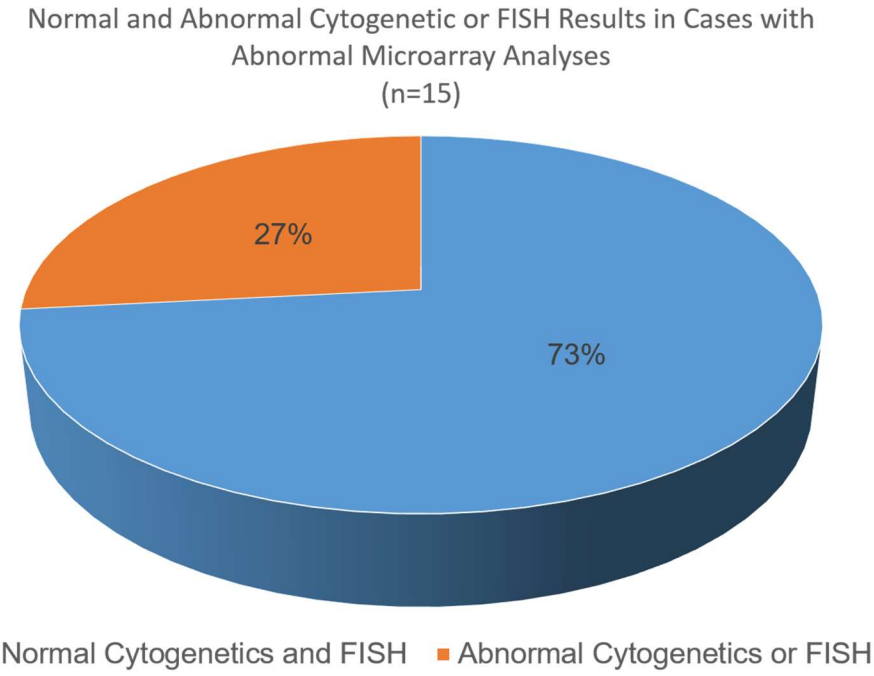



**Figure 57. Cytogenetic or FISH results in cases with abnormal microarray analyses**. From the 15 total abnormal microarray cases, 73% presented normal cytogenetic and FISH results while only 27% had an abnormal cytogenetic or FISH analysis.

Current contributions of microarray have identified over 40 key MDS-related mutations. The most frequent mutations occur in genes responsible for DNA methylation, histone modifications, transcription regulation, tumor suppression, and signal transduction in the form of gains or losses (Table XII) (Kulasekararaj, Mohamedali et al. 2013, Glaubach, Robinson et al. 2014, Bejar 2014, Shih, Abdel-Wahab et al. 2012, Papaemmanuil, Gerstung et al. 2013).

One possible mechanism for the development of MDS and other hematopoietic malignancies later in life includes the dysregulation of epigenetic genes specifically those involved in DNA methylation, which concurrently occurs more frequently as a person ages (Jhanwar 2015, Zhang, Padron et al. 2015). The most frequent mutated genes responsible for DNA methylation include *TET2* and *DNMT3A* in the adult MDS population (Santini, Melnick et al. 2013, Zhang, Padron et al. 2015, Greenberg 2015, Kulasekararaj, Mohamedali et al. 2013, Glaubach, Robinson et al. 2014). Current literature suggests that mutations in the form of copy number loss of the *TET2* gene are the most frequent aberration detected in MDS samples (Santini, Melnick et al. 2013, Zhang, Padron et al. 2015). Furthermore, MDS patients with *TET2* and loss of *DNMT3A* genetic abnormalities have been shown to respond positively to hypomethylating agents including azacitidine for treatment due to their correcting effects on epigenetic abnormalities (Hofmann 2015a, Glaubach, Robinson et al. 2014, Kulasekararaj, Mohamedali et al. 2013, Visconte, Selleri et al. 2014). According to current literature, administering these specific treatments to the pediatric population is not the ideal therapy as these mutations are rare in children and young adults.

Epigenetic histone and chromatin modifications including methylation is essential in the differentiation of stem cells to specific cell types while still maintaining a constant

| Gene | Location | Mutation | Function | Comment |
|---|---|---|---|---|
| *TET2* | 4q24 | Loss | DNA Methylation | Most prevalent gene mutation in adult MDS; responds positively to hypomethylating agents |
| *DNMT3A* | 2p23 | Loss | DNA Methylation | Responds positively to hypomethylating agents |
| *EZH2* | 7q36 | Loss | Histone/Chromatin Modifications | Mutations from del(7q) and increases myeloid progenitor formation |
| *ASXL1* | 20q11 | Loss | Histone/Chromatin Modifications | Results in excess blasts and poor prognosis |
| *RUNX1* | 21q22 | Loss | Transcription Regulation | High-risk MDS |
| *GATA2* | 3q21.3 | Loss | Transcription Regulation | Early indicator of MDS |
| *ETV6* | 12p13 | Loss | Transcription Regulation | Frequently rearranged with other known cancer genes; poor prognosis |
| *TP53* | 17p13 | Loss | Tumor Suppressor | Associated with numerous human cancers |
| *JAK2* | 9p24 | Gain | Signal Transduction | Most frequent mutation in MPN; observed with cytogenetic +8 |

**Table XII. List of the most frequent gene alterations in adult MDS**. According to current literature these genes are responsible for DNA methylation, histone modifications, transcription regulation, tumor suppression, and signal transduction in adult MDS.

stem cell population. In the adult population, certain MDS-related chromosomal abnormalities have been linked to specific methylating activities of the *EZH2* gene including deleted 7q, a frequent cytogenetic aberration in adult MDS. The loss of this gene has been shown to increase myeloid progenitor formation during hematopoiesis (Padron et al. 2015, Kulasekararaj, Mohamedali et al. 2013). Frequent mutations of *ASXL1*, a regulator of histone function, are observed in the adult MDS population in high frequency as a copy number loss and has been shown to result in excess blasts and independently results in a poor prognosis for those adults with the mutation (Visconte, Selleri et al. 2014, Greenberg 2015).

The most common genetic alterations affecting transcription regulation in the older MDS population include *RUNX1*, *GATA2*, and *ETV6*. Point mutations in *RUNX1*, also known as *AML1,* are typically associated with a high-risk MDS with a higher propensity of AML evolution (Santini, Melnick et al. 2013, Visconte, Selleri et al. 2014). The *AML1* gene acts as a regulator of myeloid differentiation and leads to a poor prognosis when mutated. Even though, loss of the *GATA2* gene has been suggested to be an early indicator of MDS initiation; it has rarely been observed in younger MDS cases (Glaubach, Robinson et al. 2014). This gene is responsible for hematopoietic cell proliferation and survival (Zhang, Padron et al. 2015). Gene aberrations of *ETV6* have adverse prognostic implications in MDS as this gene is frequently rearranged with a variety of other genes in a range of human cancers including MDS (Bejar 2014, Bejar, Stevenson et al. 2011).

Genetic alterations of *TP53* is commonly observed with complex karyotypes in the form of deletions or loss of the gene. In the adult population, loss of *TP53*, a critical tumor suppressor gene on chromosome 17p13, is associated with the isolated deleted 5q

syndrome (Greenberg 2015); this aberration is rarely observed in young populations. Mutations of this gene results in malfunctions of cell cycle regulation and DNA repair and has been observed in numerous human cancers (Zhang, Padron et al. 2015, Bejar 2014). A poor prognosis and a possible resistance to specific treatments and relapse has been observed in adult MDS cases with disruption of the *TP53* gene (Greenberg 2015).

A common gain that affects signal transduction in adult MDS cases has been documented within chromosome 9p24 resulting in a copy number gain of the *JAK2* gene and results in a better OS and lowered progression to AML (Zhang, Padron et al. 2015, Vardiman, Thiele et al. 2009). Even though rare in the pediatric population, this alteration is one of the most recognized in myeloproliferative neoplasms (MPN) including polycythemia vera (PV) and primary myelofibrosis (PMF) and has typically been observed in conjunction with trisomy 8 from karyotyping and FISH analyses (Liu, Ying et al. 2012, Kulasekararaj, Mohamedali et al. 2013). Since trisomy 8 is less frequently observed in the pediatric population, the presence of concurrent gain within 9p24 is assumed to rarely occur. A FISH probe is not included in our panel and we did not observe the gain of this region in our microarray analysis of pediatric MDS cases.

## Current Trends in Pediatric MDS Array Analysis

To date, over 40 genes have been described in the adult population and alterations of *TET2, DNMT3A, IDH1/2, EZH2, ASXL1, RUNX1, GATA2, ETV6, TP53*, and *JAK2* are some of the most frequent aberrations; however, these are rare or even non-existent in current pediatric data. Alterations in genes with DNA methylation and histone/chromatin functions have yet to be determined in the pediatric and young adult MDS population (Glaubach, Robinson et al. 2014). Limited use of array on the rare population of pediatric MDS has presented very few overlapping adult and pediatric gene involvements but

mutations of *JAK2*, *GATA2* and *RUNX1* have been seen in very low frequencies (Hofmann 2015b, Hofmann 2015a, Ismael, Shimada et al. 2012). In about 7% of primary childhood MDS, mutations of the *GATA2* gene have been observed leading to the assumption that this gene is a first hit in pediatric MDS (Kozyra, Hirabayashi et al. 2015). Currently, an increasing number of studies have been conducted on the mutations associated with juvenile myelomonocytic leukemia (JMML) and not pediatric MDS as a whole. The gene associations most frequently observed include those involved in RAS/MAPK signaling, which controls cell proliferation and apoptosis, including *NF1*, *PTPN11*, *CBL*, *NRAS*, *JAK2*, and *KRAS* (Ganapathi, Schafernak et al. 2015, Silva, Maschietto et al. 2013, Glaubach, Robinson et al. 2014, Ismael, Shimada et al. 2012). The use of high-resolution microarray is suggested to play an integral part in accurately detecting genomic alterations in JMML and delivering a more accurate diagnosis and prognosis for these young patients. More information and insight into the molecular basis of pediatric MDS is needed. Using larger pediatric and young adult MDS cohorts will aid in distinguishing novel genomic aberrations useful for a better understanding of MDS in this rare group.

## Limitations of this Study

Pediatric MDS is rare and the number of cases received in a single laboratory are scarce. To add to this, usually a small amount of whole bone marrow or cancer blood specimen is obtained from pediatric samples. Due to the nature of the disease in these young patients, the original specimen sent for cytogenetic and FISH analyses was typically at or below the recommended amount. Excess specimen for these patients is most often very limited and the elimination of a test or culture for the use of research is unethical. In our laboratory, clinical information on the patients may be limited to demographic information with limited follow up information available. The majority of pediatric MDS

specimens had limited to no follow up studies and further clinical information was unavailable.

## Gene Involvement in Pediatric MDS

In the current study, 33 genes that had a gain/loss/LOH in known pathogenic genes was observed (Table V). These genes have not been previously documented in pediatric-specific MDS cases. Disease specific genes, tumor suppressors, oncogenes and cell cycle regulators were observed and were typically aberrant in only one case with the exception of eight genes. The genes that were detected to have a copy number change in more than one case included: *PRDM16*, *IRF4, MYH11, ALK, CDKN2B, PAX5, EXT2,* and *ERCC4* (Table XIII). The most notable finding from this study is a copy number gain encompassing the entire *PRDM16* gene in six cases (21.4%; 6 / 28 cases). The genomic studies on pediatric MDS are very rare and there is a dearth of available data; therefore, the resulting effect of these genetic alterations is based upon known functions of these genes.

### *PRDM16 Gene*

The *PRDM16* gene, located on chromosome 1p36.33, belongs to the PRDM family that is involved in a wide range of biological processes including adipose development, cell fate determination, and specific diseases including cardiomyopathy, AML and more importantly MDS (Masetti, Togni et al. 2014, Duhoux, Ameye et al. 2012, Shiba, Yoshida et al. 2015, Chi, Cohen 2016, Warner, Greene et al. 2014). Leukemogenesis in humans has been attributed to the dysregulation of hematopoietic stem cell maintenance resulting

| Gene | Linear Location | Number of Cases | Copy Number State |
|---|---|---|---|
| ***PRDM16*** | chr1: 3,068,226 -3,438,620 | 6 | Gain |
| ***IRF4*** | chr6: 391,738 - 411,442 | 4 | Gain and Loss |
| ***MYH11*** | chr16: 15,703,134 -15,857,032 | 3 | Gain |
| ***ALK*** | chr2: 29,192,773 -29,921,610 | 2 | Loss |
| ***CDKN2B*** | chr9: 22,002,902 - 22,009,312 | 2 | Loss |
| ***PAX5*** | chr9: 36,833,274 - 37,035,318 | 2 | LOH |
| ***EXT2*** | chr11: 44,095,548 - 44,245,429 | 2 | Gain |
| ***ERCC4*** | chr16: 13,920,144 - 13,952,347 | 2 | Gain |

**Table XIII. List of genes that were detected in more than one case**. The table includes the gene name, the linear location, the specific case the aberration was seen in, the copy number state, size, and linear location of the aberration.

from disrupted *PRDM16*. Research has shown the involvement of *PRDM16* in AML and MDS with the t(1;3)(p36;q21) rearrangement (*MECOM*) among older patients and those presenting an overexpression of *MECOM* had a poor prognosis and less success to current therapies (Duhoux, Ameye et al. 2012). This suggests that the promotor region of *PRMD16* is important in functionality of the gene. Further studies revealed that rearrangements were not limited to *MECOM* and novel translocation partners including *ETV6* and *IKZF1* were described (Chi, Cohen 2016). These studies have led to the assumption that a differential expression of this gene is involved during dysregulation of hematopoiesis (Duhoux, Ameye et al. 2012). Functional studies for the *PRDM16* gene have been challenging due to the fact that multiple isoforms exist that have been characterized as both an oncogene (PR-lacking domain) and a tumor suppressor (PR-containing domain) (Chi, Cohen 2016, Warner, Greene et al. 2014).

The current data on *PRDM16* and MDS is not exclusive to the pediatric population and has typically been studied using the adult population. In one pediatric AML study, a novel *PRDM16* fusion transcript was detected using sequencing techniques and the investigators were able to determine that this occurrence was not only a rare finding but was observed as an overexpression of the gene (Masetti, Togni et al. 2014). Our findings in this pediatric and young adult cohort indicated a gain of the entire gene in six cases, which is assumed to lead to the overexpression of the PR-lacking domain and therefore having an oncogenic effect. In all cases with *PRDM16* alterations, conventional cytogenetics was normal. Each case presented aberrations exclusive to the case including gains, losses, and/or LOH in *PIM1, EXT2, CHEK2, MPL, ABL2, ALK, IRF4, CLU, CDKN2B, FANCG, PAX5, ADAMTS13, ERCC4, MYH11, LATS1, IDO1, FANCI, PML, RPS17*, and *RECQL3* (Table VIII). Two cases had a common loss of the *IRF4* (6p25.3) gene with gain of *PRDM16* and both cases presented normal cytogenetic karyotypes and

FISH findings (Table VI and 46). A different set of cases had a common gain of *EXT2* (11p11.2) with gain of *PRDM16* and only one of the cases presented normal conventional cytogenetic findings (Figures 40 and 50); the other case contained an abnormal inv(11)(q13q23) by cytogenetics and FISH (Figure 39). One case with the *PRDM16* gain also contained a loss of the *PIM1* gene on 6p21.2 and presented normal cytogenetic and FISH results (Figure 35). Finally, one case contained *PRDM16* aberration with multiple other aberrations within 6q, 8p, and 15q by microarray with normal cytogenetic and FISH results (Figure 51). The individual and combined roles of these various genes in cases with *PRDM16* is yet to be determined. Also, the determination of whether or not these genes are novel translocation partners within the pediatric MDS population needs to be established and can be accomplished with continued microarray analysis with a larger sample size of young MDS patients. The overall number of pediatric MDS cases is typically low and when cases are received, adequate amount of leftover specimen must be made available for DNA extraction to use in higher resolution testing. The novel findings in the *PRDM16* gene from this study has potential for being deemed a key player in the genetic landscape of the pediatric and young adult MDS population.

### *IRF4 Gene*

In four cases, an aberration of the *IRF4* gene on 6p25.3 was detected ranging from 179kbp to 268kbp gain. One case contained numerous additional copy number changes with normal cytogenetic and FISH results. One of the four cases contained a 197kbp gain of the gene along with the loss of the *IKZF1* gene. This case also presented abnormal cytogenetic and FISH results of monosomy 7 (Figures 42-43). The other two cases had a partial loss of the gene and both presented normal cytogenetic and FISH results. In addition to the *IRF4* gene, one case detected the loss of the *YAP1* and *PTPN11* genes

(Figure 45) and the other case detected gain of *PRDM16* (Figure 46). The *IRF4* gene is a known oncogene involved in T-cell lymphoma and multiple myeloma primarily and less often associated with MDS (Lohr, Stojanov et al. 2014, Kalb, Feldman 2015). The *IRF4* gene has been shown to function as a transcription factor involved in normal blood development, which is needed for key developmental stages of hematopoiesis (Adamaki, Lambrou et al. 2013). One study presented that the amount of *IRF4* gene expression in pediatric acute leukemia was twice the amount in comparison to healthy children (Adamaki, Lambrou et al. 2013). While another study determined that *IRF4* aberrations were more commonly observed in a subtype of B-cell lymphoma in the pediatric population and less commonly seen in adults (Salaverria, Philipp et al. 2011). From our study, the genomic aberration detected in this gene among the four cases is typical of current research observing gains. Even though studies have not been conducted in the MDS populations, our findings are significant for the ongoing research in determining the genomic landscape of this cohort.

### *MYH11 Gene*

A protein-coding gene associated with AML was detected and was of particular interest to this study. A gain of the *MYH11* gene on 16p13.11 was detected in three pediatric MDS cases by microarray analysis. Two of these cases had the exact same gain size and numerous additional aberrations were detected by microarray (Tables VI and VII). These two particular cases were either cytogenetically normal or presented monosomy 7 (Figure 41). One case with normal cytogenetic and FISH findings also detected a loss of the *RUNX2* gene by microarray analysis (Figure 49). *MYH11* is typically observed in AML and presents abnormal bone marrow eosinophils and is part of one of the most frequent AML-related alterations with the *CBFB* inversion of chromosome 16

usually following drug therapy (Akiyama, Yamamoto et al. 2015, Haferlach 2015). The *MYH11* inversion is frequently observed by cytogenetic and FISH analyses in acute myelomonoblastic leukemia, but a few studies have documented the presence of cryptic aberrations of *MYH11* that are only seen with higher resolution testing usually in RT-PCR (Douet-Guilbert, Chauveau et al. 2015). The evolvement of MDS to AML cannot be ruled out without further clinical data for these cases. Although microarray has not been implicated in specific studies to detect *MYH11* involvement, the higher resolution technique may have uncovered this aberration without *CBFB* in this cohort of patients for the first time.

### *ALK Gene*

In two cases analyzed, a partial loss of the *ALK* gene was detected on chromosome 2p23. *ALK* has predominately been characterized as a gene involved in a variety of tumors including anaplastic large cell lymphomas, follicular lymphoma, non-small cell lung cancer, and neuroblastoma (Tanaka, Ohwada et al. 2012, Dai, Kelly et al. 2012). Currently, there are no studies indicating an *ALK* involvement in MDS and the information available typically presents a variety of *ALK*-negative lymphomas with MDS (Tanaka, Ohwada et al. 2012). In the two cases this aberration was detected, a partial deletion was observed in each of the cases with losses of 39kbp and 98kbp. The 98kbp partial loss case presented normal cytogenetic and FISH findings. We detected numerous additional gains, losses, and regions of LOH. The other case contained a 39kbp partial loss of *ALK* as the sole aberration from microarray studies. This case presented normal cytogenetic results and abnormal FISH findings (deleted 20q in 13% of interphase cells) (Figures 37-38). *ALK*-positive gene rearrangements are in fact present in pediatric diseases but primarily in neuroblastomas (Jongmans, Loeffen et al. 2016). In 2010, one

study presented a very small population of pediatric MPD patients (6 patients) with *ALK* abnormalities only seen by cytogenetics in the form of inv(2), translocations between 2p and 2q or 4q, or a deletion of 2p. Three of these previously published case studies also contained the common monosomy 7 aberration (Röttgers, Gombert et al. 2010). In our present study, the cytogenetic findings were normal. The expression of *ALK* activates a variety of signaling pathways including hematopoietic cell proliferation and transformation (Röttgers, Gombert et al. 2010). With the limited studies available on *ALK* aberrations and MDS, we speculate that the activity of *ALK* has a variety of activities not limited to lymphomas including roles in hematopoietic cell proliferation and transformation.

### *CDKN2B, PAX5, and ERCC4 Gene*

In two cases, the same 47kbp loss on 9p21.3 and 32635kbp gain on 16p13.13 was observed. The loss of *CDKN2B* and gain of the region overlapping the *ERCC4* gene locations were the exact same in both of these cases. Both cases contained other additional aberrations. One case presented monosomy 7 by cytogenetics and FISH while normal findings were observed in the other case. These aberrations encompassed the *CDKN2B* and *ERCC4* genes, respectively. Mutations of the *CDKN2B* gene, a tumor suppressor, has been linked to a variety of cancers including melanoma, renal cell carcinoma and breast cancer but have not typically been linked to MDS (McNeal, Liu et al. 2015, Jafri, Wake et al. 2015). In a 2012 study, characterization of the methylation changes were characterized in chromosome 9p21, which includes the *CDKN2B* gene, and their alterations were considered to play a role in MDS and AML in these patients (Cechova, Lassuthova et al. 2012). This study did not define the age groups of its subset of MDS and AML patients nor a genomic copy number change. The loss of this tumor

suppressor in two cases from our study should not be disregarded due to the lack of published data and is of interest for further studies.

The *ERCC4* gene has been characterized as a key player in DNA damage repair and mutations are typically observed in HPV-positive cervical cancer, neurological abnormalities, and more note-worthy, in *ERCC4*-related Fanconi's anemia (FA) (Manandhar, Boulware et al. 2015, Ghafouri-Fard, Fardaei et al. 2016, Dong, Nebert et al. 2015). The *ERRC4* gene has enzymatic activity that encodes for important DNA repair enzymes and has been studied as a potential target for cancer treatments (Manandhar, Boulware et al. 2015). More importantly, this DNA repair gene, when mutated, causes a subtype of FA characterized by bone marrow failure and a predisposition to cancer (Bogliolo, Schuster et al. 2013). Whether this patient is in fact diagnosed with FA is not known at this time but the occurrence of this aberration can be used as a gene indicator for bone marrow disorders due to inherited disorders.

A 4.0-6.6Mbp region of LOH on chromosome 9p13.2 was detected in two cases encompassing the *PAX5* gene (Tables VI and VII). Typically, alterations of the *PAX5* have been observed in ALL cases (Anderl, König et al. 2015). A more recent study recognized a number of genes that are considered to cause familial MDS and a predisposition to MDS and AML in adult populations including the *PAX5* gene (Churpek 2014, Horwitz 2013). This finding with novel studies on *PAX5* playing a role as a hereditary factor in MDS and leukemia risk is of importance in characterizing the genetic landscape of this young population.

***EXT2 Gene***

An aberration of the tumor suppressor gene *EXT2* was observed in the form of a gain on chromosome 11p11.12 in two cases with the same additional aberration of gain of *PRDM16*. One case presented abnormal cytogenetic and FISH findings with an inversion of chromosome 11 and additional aberrations in *CHEK2* by microarray analysis (Figures 39-40). A 50kbp partial gain of *EXT2* was detected in a case that contained gain of *PRDM16* and normal cytogenetic and FISH results (Figure 50). The *EXT2* gene causes abnormal bone growths or exostoses and has been found to play a role in hereditary bone cancers (Wuyts, Van Hul et al. 1998). This gene has typically been characterized as a tumor suppressor primarily involved in osteochondromas with genetic mutations of loss or nucleotide substitutions (Stickens, Clines et al. 1996, Xia, Xu et al. 2016). This aberration has not been documented as a gain or with an involvement in MDS but it was detected in two cases with the same concurrent gains and should be one to note for future studies.

### *Protein Coding Genes of MDS, MPN, and AML: RUNX2, IKZF1, ETS1, PTPN11, PML, and CBFA2T3 Genes*

A 229kbp loss on chromosome 6p21.was detected in one patient that overlaps with the protein coding gene *RUNX2*. This patient presented normal cytogenetic and FISH results and also had a gain of *MYH11* observed by microarray analysis (Figure 49). *RUNX2* is most often associated with bone and cartilage maintenance but in knockout studies, defects in this gene have caused disruption in the hematopoietic development (Harada, Harada et al. 2004, Blyth, Cameron et al. 2005). Presently, the majority of studies focus on *RUNX1* aberrations which are found in about 20% of AML patients (Kuo, Zaidi et al. 2009). However, a recent study reported the up-regulation of *RUNX2* in AML with normal cytogenetics (Schnerch, Lausch et al. 2014). On the contrary, we observed the loss of the *RUNX2* gene but with limited information regarding *RUNX2* overall, we do

not dismiss this finding due to the role the RUNX family transcription factors have been shown to play in hematopoiesis.

A loss was detected within the protein-coding gene of *IKZF1* that was 298kbp in size in one case that also contained a gain of the *IRF4* gene by microarray (Figure 43). The *IKZF1* gene is located on 7p12.2, has more frequently been associated with ALL, and presents a poor outcome in B-ALL in the pediatric population (Kuiper, Waanders et al. 2010). In 2010, a study characterized *IKZF1* as one of the novel mutations found in MPNs in the general population so the occurrence of these mutations specifically in pediatric MPNs or even MDS has not been documented (Tefferi 2010). Defects of this gene have been shown to cause a malfunction in normal hematopoiesis and is one of the key players in regulating hematopoietic stem cell functioning (Gorzkiewicz, Walczewska 2015). The case with loss of the *IKZF1* gene in our study presented monosomy 7 cytogenetically. The defined role this gene plays in pediatric MDS specifically has yet to be established; however, the loss of chromosome 7 is more frequently observed in the young population and therefore a loss of the *IKZF1* gene is a possible important candidate gene of pediatric MDS.

A 396kbp loss on 11q24.3 was detected in the protein-coding gene *ETS1* in one case with normal cytogenetic and FISH results and additional aberrations in *KAT6A* and *PICALM* by microarray (Figure 54). This gene belongs to a family that is involved in stem cell development, apoptosis, and tumorigenesis (Dittmer 2003). More importantly, this gene has been linked to apoptosis in low-risk MDS patients as an epigenetic regulator in methylation (Del Rey, O'Hagan et al. 2013). This finding is one of particular interest since mutations of epigenetic regulators are typically more common in adult and elderly MDS and the case described in the report is from an 11-month old (Aul, Bowen et al. 1998).

More interestingly, *ETS1* has been implicated in FA in a particular subset of the population; the downregulation of this gene was observed in the Indian population presenting FA (Shyamsunder, Ganesh et al. 2013). Fanconi's anemia is typically characterized by bone marrow failure and hematological abnormalities and could play a role in this patient's diagnosis of MDS.

A 288kbp loss on chromosome 12q24.13 overlapping the *PTPN11* gene was detected in one case with additional genetic aberrations in *IRF4* and *YAP1* (Figure 45). *PTPN11* is associated with JMML, AML, and more noteworthy, pediatric MDS (Kozyra, Hirabayashi et al. 2015, Olsson, Zettermark et al. 2016, Sarper, Gelen et al. 2015). *PTPN11* helps regulate cell growth, differentiation, and transformation (Kozyra, Hirabayashi et al. 2015). This aberration has been described in current literature using gene based technologies including next generation sequencing and is frequently observed in MDS and when present, has been associated with a poor outcome including a shorter overall survival (Alpermann, Haferlach et al. 2015, Bejar 2014). This finding is significant for this study as this particular case presented normal cytogenetic and FISH results; this loss was only detected using higher resolution testing like microarray. The ability to detect genomic loss in relevant genes like *PTPN11* in pediatric MDS cases has prognostic implications for the patient and emphasizes the need for concurrent genetic testing.

The *PML* gene is often associated with the cytogenetic acute promyelocytic leukemia (APL) translocation t(15;17) with the *RARA* gene, frequently observed by karyotyping and/or FISH analyses (Rose, Haferlach et al. 2015). This aberration is rare in MDS patients and the one case that detected a region of LOH on 15q24.1, encompassing the *PML* gene, was negative for the *PML/RARA* translocation based upon cytogenetic karyotyping. In fact, cytogenetic and FISH results were normal. However,

numerous additional aberrations were detected in *PRDM16*, *LATS1*, *IDO1*, *RPS17*, *RECQL3*, and *FANCI* genes by microarray (Figure 51). The detection of this aberration provides important diagnostic and prognostic information for APL and in a few rare cases, an aberration has been detected as a cryptic anomaly not observed by conventional techniques but rearrangements with *RARA* is also observed (Gruver, Rogers et al. 2013). Genomic imbalances of the *RARA* gene were not observed in this case by microarray analyses. One study of the *PML* gene used microarray techniques to characterize loss of the gene in a variety of lymphomas and carcinomas yet LOH was not detected (Gurrieri, Capodieci et al. 2004). The LOH of *PML* in this case cannot be discredited due to the multitude of aberrations observed by microarray and should be researched further.

A 532kbp gain of the *CBFA2T3* gene was observed in one patient as the sole anomaly detected by microarray analysis (Figure 34). This gene is located on 16q24.3 and has typically been observed in young AML patients that present a poorer outcome and a higher risk of relapse (Schuback, Alonzo et al. 2014). According to current literature, an inversion of chromosome 16 encodes for a *CBFA2T3*/*GLIS2* (16p13.3) fusion protein and has been shown to lead to a more aggressive type of pediatric AML (Gruber, Gedman et al. 2012). This inversion typically is cryptic and presents a normal karyotype and cytogenetic results on this particular case did not present aberrations of the 16 chromosome and were in fact normal (Masetti, Pigazzi et al. 2013, Vogan 2013). These findings are of particular interest due to the age of the patient (2 years) with an initial clinical diagnosis of MDS. Given the published reports and facts that alteration of *CBFA2T3* was observed as a sole abnormality, it would be of interest to determine whether this genetic change confers aggressive disease with higher transformation potential from MDS to AML. The potential of AML evolvement from MDS is of concern for this particular case due to these findings.

### *Inherited Bone Marrow Failure Disorder Genes: FANCG, RPS17, RECQL3, FANCI, and FANCA Genes*

An 11.7Mbp loss on 9p13.3 was detected in one case that overlaps with the *FANCG* gene. This case contained numerous additional aberrations by microarray and presented normal cytogenetic and FISH results. The observance of the *FANCG* gene has been attributed to the onset of adult AML and is considered a founder mutation of Fanconi's anemia in different populations (Park, Chung et al. 2013, Wainstein, Kerr et al. 2013, Duan, Wang et al. 2013). The presence of bone marrow disorders, including MDS, are common from IBMF disorders like FA (Zierhut, Tryon et al. 2014).

A large region of LOH was observed in one case on chromosome 15q25.2-26.1, with numerous additional aberrations by microarray (Figure 51). This LOH region encompassed three genes of particular interest for this study. The *RPS17*, *RECQL3*, and *FANCI* gene were within this region and are especially notable due to their involvement in three different kinds of inherited bone marrow failure disorders and their associations with MDS evolvement. *RPS17* is a gene associated with Diamond-Blackfan anemia, a form of anemia that usually presents itself within the first year of life (Babushok, Bessler 2015). Diamond-Blackfan is one of the IBMF disorders that leads to abnormal blood cell formation and can lead to blood cancers and disorders, including MDS, later in life (Quarello, Garelli et al. 2012). *RECQL3* is a gene responsible for the rare genetic disorder of Bloom syndrome; typically presents patchy red skin and short statue; more importantly, patients with diagnosed Bloom syndrome have a higher risk of different cancers (Aktas, Koc et al. 2000). Bloom syndrome predisposes an individual to AML and MDS and usually presents monosomy 7 by karyotyping, a common aberration observed in the pediatric MDS population (Poppe, Van Limbergen et al. 2001). Microarray findings in this case also

showed LOH of the *FANCI* gene, one of the 17 variants that are responsible for FA that causes defective DNA repair functions, which leads to an inherited form of bone marrow disorders (Chen, Zhang et al. 2014). The three genes detected in this case are responsible for three types of IBMF disorders, which have been documented to cause a predisposition to MDS and/or AML in the pediatric population (Babushok, Perdigones et al. 2015, Babushok, Bessler 2015). Cytogenetic and FISH analyses for this case were normal.

Aberrations of the *FANCA* gene were observed in one of the MDS cases. An 1110kbp gain on 16q24.3 encompassing the *FANCA* gene was detected along with numerous additional aberrations and is considered a gene of interest for this cohort (Table VII). *FANCA* gene variants are one of the numerous genes responsible for the activation of FA (Ishiai, Kitao et al. 2008). Individuals with this genomic imbalance have an increased risk for MDS and AML from the malfunctioning repair genes responsible for this IBMF disorder (Solomon, Rajendran et al. 2015, Voso, Fabiani et al. 2015). The occurrence of this aberration is a probable cause for MDS in this subset of patients.

### *Oncogenes and Tumor Suppressors: MPL, ABL2, KIF14, PIM1, LATS1, CLU, YAP1, and CHEK2 Genes*

A 3760kbp region of LOH on 1p34.2 that encompassed the *MPL* gene was detected utilizing the OncoScan® platform on one case. This particular case contained numerous additional aberrations observed by microarray and presented normal cytogenetics and FISH results (Table VI). *MPL,* a proto-oncogene, has been associated with myelofibrosis and thrombocytopenia and one study has found a mutation of the *MPL* gene in pediatric essential thrombocythemia (ET), a very rare disease among the younger population (Ouyang, Qiao et al. 2015). Essential thrombocythemia, when present in the

pediatric population, is seen as a sporadic event or as a familial inheritance and leads to a predisposition of MDS (Teofili, Giona et al. 2007). In this same patient, a 4006kbp LOH was also observed at chromosome 1q25.2 which potentially disrupts the ALL-related gene *ABL2* (Figure 42). To date, the majority of pediatric data available correlates this gene with pediatric ALL and AML (Kuiper, van Reijmersdal et al. 2015, Raca, Gurbuxani et al. 2015).

In one case, a 471kbp loss was observed in 1q32.1 as the sole aberration detected by microarray with normal cytogenetics and FISH (Figure 48). This loss overlaps the protein-coding gene *KIF14*, identified as an oncogene in a variety of cancers including retinoblastoma, lung, breast, and ovarian cancers (Corson, Huang et al. 2005). *KIF14* functions as a regulator during the cell cycle and interacts with tumorigenic signaling pathways and mutations have been linked to cytokinesis failures when mutated (Thériault, Corson 2015). Typically, the genomic aberration of *KIF14* is in the form of gains and an association with hematopoietic disorders like MDS have yet to be described. Our results conflict with current data, as seen as a loss, yet are noteworthy for future studies as the function of *KIF14* in MDS is speculated to be fundamentally different then the previously described tissue specific activity *KIF14.*

*PIM1*, a proto-oncogene primarily expressed in B-lymphoid and myeloid cells lines has an association in hematopoietic malignancies when overexpression occurs (Ouhtit, Muzumdar et al. 2015). This is of particular interest as one case presented a 247kbp loss on 6p21.2, resulting in the loss of the *PIM1* gene (Figure 35). This case presented normal cytogenetic and FISH results and was a case that also detected an aberration in the *PRDM16* gene by microarray. The *PIM1* gene belongs to a family of kinases typically overexpressed, not inhibited, in tumor cells and the inhibition of *PIM1* is currently being

studied as a potential drug therapy for hematological cancers (Garcia, Langowski et al. 2014, Blanco-Aparicio, Carnero 2013). The presence of this aberration may have a relationship with the gain of *PRDM16* gene, the aberration also detected by microarray, yet data on this co-occurrence has not been described to date.

The tumor suppressor, *LATS1*, located on 6q25.1 was detected in one patient with a 592kbp loss along with multiple additional aberrations by microarray with normal cytogenetics and FISH (Figure 51). *LATS1* helps regulate the cell cycle and apoptosis (Xia, Qi et al. 2002). Mutations of this gene have mainly been found in non-small cell lung cancers and mesothelioma (Wan, Sun et al. 2016, Lee 2015). An association with *LATS1* and MDS is not clear but the apoptotic behavior of this gene leads to a suspicion of an involvement in hematopoietic disorders including MDS.

In one case, multiple aberrations were detected including a 417kbp loss on 8p21.1 (*CLU* gene) (Table VI). The *CLU* gene, a tumor suppressor, has been implicated in pediatric neuroblastoma and more recently as a risk gene in Alzheimer's disease (Wang, Liu et al. 2012, Yang, Li et al. 2016). The exact role *CLU* plays in human cancers is still under investigation, but roles in apoptosis, proliferation, transformation, and differentiation have been documented and more research is needed to determine its place in the genetic landscape of MDS.

A 287kbp loss was observed in 11q22.1, overlapping the *YAP1* gene in one case with other genetic aberrations by microarray (Figure 45). *YAP1* plays an oncogenic role, activates genes involved in cell proliferation, and suppresses apoptotic genes and is a gene of interest for this study as studies have shown its expression in hematopoietic stem cells and AML (Safari, Movafagh et al. 2014). Furthermore, *YAP1* has been found in cells responsible for tumor-repopulation in medulloblastoma in children (Fernandez-L,

Northcott et al. 2009). A consistent expression of *YAP1* contributes to the homeostatic balance of proliferation and apoptosis in hematopoiesis (Wang, Du et al. 2014).

A 674kbp loss was detected on 22q12.1 overlapping the *CHEK2* gene, a known tumor suppressor, in the case that presented inverted 11 by cytogenetic and FISH. This gene is commonly associated with ovarian, breast, and in rare cases pancreatic cancer but not typically implicated in MDS (Lawrenson, Iversen et al. 2015, Scelo, McKay et al. 2014). Recently, a study found an association with *CHEK2* gene alterations being association with the development of MPNs including PV (Janiszewska, Bąk et al. 2015). This study presents a new player in the molecular mechanisms behind MPNs as they have been shown to evolve into MDS and/or AML and hence our detection is noteworthy for future studies (Mascarenhas, Mesa et al. 2014).

### *Non-Specific Protein Coding Genes: TAC1, KAT6A, ADAMTS13, PICALM, IDO1, and RARA Genes*

*TAC1*, a gene located on 7q21.3, has been associated with a variety of diseases including heart disease, neurodegenerative disease, and more interestingly, has an impact on hematopoiesis (Liu, Castillo et al. 2007). One patient exhibited a 682kbp loss overlapping this gene as the only aberration; the cytogenetic and FISH analyses were normal (Figure 53). To date, cases involving pediatric MDS or hematopoietic diseases and aberrations of the *TAC1* gene are non-existent but with further testing of this young group and larger cohorts, a significant contribution may be implied into the role this gene plays in the rare pediatric population.

The protein-coding gene, *KAT6A*, is located on 8p21.1 and a 400kbp loss encompassing this gene was detected in one case along with aberrations of the *PICALM*

and *ETS1* genes by microarray (Figure 54). This gene is typically associated with AML, specifically containing the translocation with *CREBBP*, t(8;16); however, this case presented normal cytogenetic and FISH findings (Panagopoulos, Torkildsen et al. 2014). Variant gene partners of the *KAT6A* gene have been determined, some that may be cryptic, but the loss of the gene in MDS or more specifically in pediatric MDS has not been documented at this time (Saleem, Mohd. Yusoff 2015).

In one case, a gain approximately 3863kbp in size on 9q34.2 overlapping the *ADAMTS13* gene. This gene is known to be involved in thrombocytopenic purpura and was observed in addition to other aberrations (Table VII) (Krabbe, Kemna et al. 2015). Thrombocytopenia is commonly observed in MDS patients, pediatric and adult (Li, Morrone et al. 2015).

A 471kbp loss in the *PICALM* gene, located on 11q14.2 was detected in one case with normal cytogenetic and FISH findings and additional aberrations detected by microarray (Figure 54). This protein-coding gene is typically observed in Alzheimer's disease (Xu, Tan et al. 2015). Recently, studies characterized the *PICALM* gene as a regulator in normal hematopoiesis and these studies have begun using *PICALM* as a possible therapeutic target for hematopoietic disorders including PV (Ishikawa, Maeda et al. 2015). A noted loss of function in the pediatric population has yet to be characterized but with further research and larger cohorts a better understood role could be established.

A 454kbp loss was observed in one case in the protein-coding gene *IDO1* located on chromosome 8p11.21 along with a variety of other aberrations by microarray analysis (Figure 51). This gene has been implicated in a variety of roles including antimicrobial and antitumor defense and more importantly, in inflammation (Yeung, Terentis et al. 2015). The role this gene plays in pediatric MDS has not been characterized but the

dysregulation of the inflammation signaling pathways have been noted as driving forces in the MDS development and should be further studied to help define the pathogenesis of MDS.

In one of the cases, multiple aberrations were detected including a 3542kbp gain on 17q21.2 (*RARA* gene). The *RARA* gene is typically observed in patients with APL with presentation of the t(15;17) rearrangement (Rose, Haferlach et al. 2015). This rearrangement is readily observed by cytogenetic karyotyping and/or FISH analysis. At the time of specimen intake, the FISH probes specific for *RARA* were not applied due to the initial diagnosis. Cytogenetic karyotyping did not observe the classical APL rearrangements and in fact presented monosomy 7 by karyotype (Table VII). However, current studies have documented the *PML/RARA* translocation in APL as a cryptic anomaly (Gruver, Rogers et al. 2013).

The use of high-resolution microarray techniques allowed the detection of genetic changes in more than half of the cases analyzed that previously were determined normal by cytogenetics and FISH. Multiple genes with known functions are now implicated in pediatric MDS based upon this study. Those of particular importance include the gain of the MDS-related *PRDM16* gene, and aberrations in *IRF4*, *MYH11*, and *ALK* genes. The lack of genetic characterization is due to the rarity of the disease and this study contributes to the limited research available on the genetic landscape of pediatric MDS.

## Comprehensive Testing

Cytogenetic and FISH testing is considered the gold standard of care for MDS patients as it provides accurate diagnostic and prognostic information regarding the patient (Haase 2008). These techniques are essential for determining clonality of the

disease and are essential tools in therapeutic stratification (Germing, Aul et al. 2008, Costa, Valera et al. 2010). Since over 50% of MDS cases contain a cytogenetic abnormality that is able to be observed by conventional karyotyping or FISH, the continued utility of combined testing is necessary (Visconte, Selleri et al. 2014). The key for successful diagnoses and treatment is to determine the entire genetic landscape of MDS. The addition of another genetic test that increases the detection of abnormal cases will help better define this disease and genetic changes that may have prognostic impact. Multiple types of tests are needed in order to effectively observe all potential genomic aberrations. This can be accomplished with combined cytogenetic, FISH, and high-resolution microarray testing.

Conventional cytogenetic studies are used as an efficient and rapid test to observe hallmark MDS-related abnormalities of -5/del(5q), -7/del(7q), +8, and del(20q). The specificity of FISH probes can result in missed aberrations without concurrent testing of the entire genome. Hence conventional cytogenetics is useful. The use of FISH will allow the detection of aberrations even when mitotic index is low, especially where conventional karyotyping will not be effective. This is most useful when the initial specimen is limited or compromised. Fluorescence *in situ* hybridization allows for the detection of low mosaicism aberrations not observed by karyotype because more number of cells are scored. As observed in two pediatric cases studied (Figures 36 and 52), a deletion of 20q was only observed using FISH testing due to being detected in low frequencies of interphase cells [8% and 13%]. As depicted in Table IX, out of the 76 abnormal pediatric/young adult samples over the given time span, 10.5% (8 / 76) contained one of the hallmark MDS abnormalities detected by FISH studies alone. Cytogenetic karyotyping from these cases presented normal findings. Furthermore, two cases detected trisomy 21 by cytogenetic karyotyping only with normal FISH results due to the specific nature of the

probes utilized in the FISH panel. The use of concurrent cytogenetic karyotyping and FISH testing allowed for the accurate detection of genetic aberrations in 13.1% of the total number of abnormal pediatric and young adult samples studied in this laboratory from 1990 to 2015.

This study determined the impact of a more comprehensive analysis by the addition of higher-resolution microarray testing on this rare population of MDS. From the 28 cases that utilized comprehensive testing, an abnormality was detected in 60.7% (17 / 28) cases by karyotyping, FISH, and/or microarray analyses (Table X). The use of microarray was able to detect copy number gains, losses, or LOH in 33 pathogenic genes in 15 of the 28 cases (33%) (Table V). A notable finding from this study presented that the majority of the abnormal microarray cases presented normal karyotype and FISH analyses with 11 cases (73%) (Figure 57). However, among the 27% (4 cases) with abnormal cytogenetic/FISH findings, the aberrations were not detected by microarray. In case 5, the deletion of 20q was only observed in 13% of interphase cells by FISH and was below the detection rate of microarray. In three cases that presented monosomy 7 by cytogenetic karyotyping and FISH analyses, the percentage of mosaicism was fewer than the detection rate or very close to the validated rate. These findings emphasize the need for continued karyotyping and FISH testing in order to detect low frequency mosaicisms. In order to detect all prognostically relevant gene aberrations, a comprehensive study using multiple techniques is crucial.

# SUMMARY AND FUTURE DIRECTIONS

## Summary

The purpose of this study was to decipher the genetic landscape in the pediatric MDS population. Using the available, yet limited, information regarding this rare group we were able to shed light on key differences and similarities between adult and pediatric MDS. The results from the present study helps to better understand the specific genetic changes among pediatric/young adult MDS cases; clinical correlations may consequently aid in development of treatments tailored specifically for this age group of MDS cases. The results of this study included:

1) A confirmation of the rarity of MDS in the pediatric and young adult populations by presentation of a total of 306 pediatric or young adult MDS specimens, constituting 7.6% of the total MDS samples, received into our laboratory from 1990-2015.
2) The frequency of pediatric MDS has typically been reported to occur in males and females equally, however, this study found a frequency more comparable to the adult MDS data showing males have a slightly higher occurrence [65 (55%) males to 54 (45%) females].
3) The median age of pediatric/young adult MDS is 6 years according to this study which is slightly lower than the current literature documenting a median age between 6.8 and 10.7 years for pediatric MDS. This study also determined the highest occurrence of pediatric MDS during the first year of life.
4) This study supports the current literature showing the most common MDS-associated chromosome abnormality is -5/del(5q) in the adult MDS population (30%), with our findings at 33% of the cytogenetically abnormal adult MDS samples (519 / 1563). Documented studies have shown -7/del(7q) is the next most frequent cytogenetic abnormality (15-25%), followed by +8 (16%), and

del(20q) (10-20%). Our results detected -7/del(7q) at 27% (423 / 1563); +8 in 18% (282 / 1563); and del(20q) in 16% (260 / 1563) of our subset of abnormal adult MDS samples from 1990-2015.

5) These results provide evidence that the occurrence of cytogenetic aberrations observed in the young MDS population is not as high as 30-50% as previously documented, as this study found an aberration in 76 of the 306 pediatric or young adult samples (24.8%).
6) The occurrence of hallmark MDS-related abnormalities are observed in different frequencies in comparison to the elderly population. The most frequent abnormality in our study was -7/del(7q) in 36.8% of the samples (8 / 76) which is higher than the limited published information showing this aberration in 30% of pediatric and young adult MDS patients. The next most frequent abnormality observed in this study was +8 in 17.1% (13 / 76) followed by del(20q) in 13.2% (10 / 76). The -5/del(5q), the most common abnormality in the adult population, was observed at a frequency of 10.5% (8 / 76) from our cohort of young MDS specimens, thus adding and confirming to the very limited genetic information available in the young MDS cases.
7) The results from this study prove that the use of higher-resolution techniques can aid in the detection of genetic aberrations among cases with normal conventional cytogenetics and FISH analyses. Genetic aberrations in 33 genes were observed in over 58% (15 cases) of the pediatric and young adult samples tested using microarray; the majority of these cases (73%; 11 / 15 cases) presented normal cytogenetic and FISH results.
8) The use of microarray detailed 33 genes of potential interest for pediatric and young adult MDS patients. The functions of these genes include tumor

suppressors, oncogenes and cell cycle regulators as well as disease specific genes including MDS, AML, ALL, NHL, and CML. The following genes exhibited copy number changes in more than one case: *PRDM16*, *IRF4, MYH11, ALK, CDKN2B, PAX5, EXT2, and ERCC4*.

9) Most importantly, a copy number gain in the MDS-related *PRDM16* gene was detected in six cases (6 / 26; 23%) with microarray. This is a new and important finding. This is a unique and novel finding for the pediatric and young adult MDS population.

10) The results from this study prove the importance of comprehensive testing utilizing cytogenetic karyotyping, FISH, and microarray techniques in distinguishing the most accurate genetic landscape of pediatric MDS. Our study helped to detect an abnormality in 60.7% (17 / 28) of the cases by combined use of conventional cytogenetics, FISH, and/or microarray analyses and each technique proved to be beneficial in its own right.

## Future Directions

Over 50% of MDS cases present with normal karyotypes by conventional cytogenetics; mainly because abnormal clones existing in low frequency may go undetected by conventional cytogenetic studies. These cases fall into a 'Good' IPSS category. Genomic imbalances may be present, however, these aberrations can only be determined with more sensitive studies (Papaemmanuil, Gerstung et al. 2013, Bejar, Stevenson et al. 2011, Bejar 2014). Fluorescence *in situ* hybridization techniques, which is more sensitive, determines only those alterations for which the probes are used. The addition of a more sensitive microarray study may facilitate the detection of cryptic abnormalities and may be helpful in determining genomic imbalances. From the 26 cases

that were available for microarray analysis, over half contained genomic aberrations not observed by cytogenetic karyotyping or FISH. We observed multiple patterns of genetic alterations in this group of patients and in order to determine those that are prognostically important, future studies utilizing a larger cohort of pediatric MDS are warranted. The implications of aberrations in *PRDM16, IRF4, MYH11, ALK, CDKN2B, PAX5, EXT2,* and *ERCC4,* genes of particular interest since these were altered in more than two cases, have the potential to disrupt normal hematopoiesis. Our present study provides insight into the limited information available for the pediatric MDS population.

Currently, the NCCN guidelines provide information on the most up to date treatments for practical use including the risk/benefit and financial cost strategies in regards to the patient outcome, adverse events, quality of life and financial burdens (Greenberg 2015). In order to determine the entire global mutational landscape of pediatric MDS, continued efforts need to be made in determining how best to combine clinical and genetic information and apply this information in the clinical practice. A more thorough study including cytogenetic karyotyping in conjunction with FISH paired with higher resolution microarray studies could determine the most accurate genetic makeup of these patients. We observed genomic aberrations in more than half of the cases that presented normal cytogenetic and FISH findings. Furthermore, due to the inability to detect low mosaicism by microarray, the continued use of cytogenetics and FISH is crucial for the most comprehensive study for these patients. Current studies have begun using higher sensitive tests including next generation sequencing to detect mutations within select nucleotide sequences. The future of genetic testing should not be limited to conventional techniques. With more information using combined testing and more accurate data better clinical correlations can be performed which in turn will facilitate therapeutic stratification tailored for this rare group of MDS.

The results from this study will supplement the very limited information available for pediatric MDS. Further microarray testing needs to be performed on a larger subset of pediatric MDS specimens to determine the true incidences of the genomic alterations, specifically *PRDM16*. Future testing using sequencing assays will provide a more thorough analysis on the current cases and for future patients. This research has presented novel findings and has better characterized the genetic landscape in pediatric MDS.

# BIBLIOGRAPHY

ADAMAKI, M., LAMBROU, G.I., ATHANASIADOU, A., TZANOUDAKI, M., VLAHOPOULOS, S. and MOSCHOVI, M., 2013. Implication of IRF4 aberrant gene expression in the acute leukemias of childhood. *PloS one,* **8**(8), pp. e72326.

AKHTARI, M., 2011. When to treat myelodysplastic syndromes. *Oncology (Williston Park, N.Y.),* **25**(6), pp. 480-486.

AKIYAMA, H., YAMAMOTO, M., SAKASHITA, C., UMEZAWA, Y., KUROSU, T., MURAKAMI, N. and MIURA, O., 2015. Therapy-related Leukemia with Inv (16)(p13. 1q22) and Type D CBFB/MYH11 Developing after Exposure to Irinotecan-containing Chemoradiotherapy. *Internal Medicine,* **54**(6), pp. 651-655.

AKTAS, D., KOC, A., BODUROGLU, K., HICSONMEZ, G. and TUNCBILEK, E., 2000. Myelodysplastic syndrome associated with monosomy 7 in a child with Bloom syndrome. *Cancer genetics and cytogenetics,* **116**(1), pp. 44-46.

ALPERMANN, T., HAFERLACH, C., FASAN, A., SCHINDELA, S., KERN, W. and HAFERLACH, T., 2015. Prognosis of Mecom (EVI1)-rearranged MDS and AML Patients Strongly Depends on Accompanying Molecular Mutations but Not on Blast Counts. *Blood,* **126**(23), pp. 1372-1372.

ANDERL, S., KÖNIG, M., ATTARBASCHI, A. and STREHL, S., 2015. PAX5-KIAA1549L: a novel fusion gene in a case of pediatric B-cell precursor acute lymphoblastic leukemia. *Molecular cytogenetics,* **8**(1), pp. 1.

ARAI, F., HIRAO, A. and SUDA, T., 2005. Regulation of hematopoietic stem cells by the niche. *Trends in cardiovascular medicine,* **15**(2), pp. 75-79.

ARMITAGE, J.O., CARBONE, P.P., CONNORS, J.M., LEVINE, A., BENNETT, J.M. and KROLL, S., 2003. Treatment-related myelodysplasia and acute leukemia in non-Hodgkin's lymphoma patients. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **21**(5), pp. 897-906.

AUL, C., BOWEN, D.T. and YOSHIDA, Y., 1998. Pathogenesis, etiology and epidemiology of myelodysplastic syndromes. *Haematologica,* **83**(1), pp. 71-86.

BABUSHOK, D.V. and BESSLER, M., 2015. Genetic predisposition syndromes: When should they be considered in the work-up of MDS? *Best Practice & Research Clinical Haematology,* **28**(1), pp. 55-68.

BABUSHOK, D.V., PERDIGONES, N., PERIN, J.C., OLSON, T.S., YE, W., ROTH, J.J., LIND, C., CATTIER, C., LI, Y. and HARTUNG, H., 2015. Emergence of clonal

hematopoiesis in the majority of patients with acquired aplastic anemia. *Cancer genetics,* **208**(4), pp. 115-128.

BEJAR, R., 2014. Clinical and genetic predictors of prognosis in myelodysplastic syndromes. *Haematologica,* **99**(6), pp. 956-964.

BEJAR, R., STEVENSON, K., ABDEL-WAHAB, O., GALILI, N., NILSSON, B., GARCIA-MANERO, G., KANTARJIAN, H., RAZA, A., LEVINE, R.L., NEUBERG, D. and EBERT, B.L., 2011. Clinical Effect of Point Mutations in Myelodysplastic Syndromes. *N Engl J Med,* **364**(26), pp. 2496-2506.

BERNASCONI, P., CAVIGLIANO, P.M., BONI, M., CALATRONI, S., KLERSY, C., GIARDINI, I., ROCCA, B., CROSETTO, N., CARESANA, M., LAZZARINO, M. and BERNASCONI, C., 2003. Is FISH a relevant prognostic tool in myelodysplastic syndromes with a normal chromosome pattern on conventional cytogenetics? A study on 57 patients. *Leukemia,* **17**(11), pp. 2107-2112.

BLANCO-APARICIO, C. and CARNERO, A., 2013. Pim kinases in cancer: diagnostic, prognostic and treatment opportunities. *Biochemical pharmacology,* **85**(5), pp. 629-643.

BLYTH, K., CAMERON, E.R. and NEIL, J.C., 2005. The RUNX genes: gain or loss of function in cancer. *Nature Reviews Cancer,* **5**(5), pp. 376-387.

BOGLIOLO, M., SCHUSTER, B., STOEPKER, C., DERKUNT, B., SU, Y., RAAMS, A., TRUJILLO, J.P., MINGUILLÓN, J., RAMÍREZ, M.J. and PUJOL, R., 2013. Mutations in ERCC4, encoding the DNA-repair endonuclease XPF, cause Fanconi anemia. *The American Journal of Human Genetics,* **92**(5), pp. 800-806.

BRUNNING, RD. BENNETT, JM. FLANDRIN, G. MATUTES, E. HEAD, D. VARDIMAN, JW., 2001. Myelodysplastic syndromes. In: JAFFE ES, HARRIS NL, STEIN H, ed, *World Health Organization classification of tumors.* Vol 1 edn. IARC Press, Lyon, pp. 61.

CANTOR, A.B., 2015. Myeloid Proliferations Associated with Down Syndrome. *Journal of hematopathology,* **8**(3), pp. 169-176.

CANTU, C. and PROYTCHEVA, M.A., 2015. Bone marrow failure syndromes, a practical approach to diagnosis. *Journal of Hematopathology,* **8**(3), pp. 101-112.

CECHOVA, H., LASSUTHOVA, P., NOVAKOVA, L., BELICKOVA, M., STEMBERKOVA, R., JENCIK, J., STANKOVA, M., HRABAKOVA, P., PEGOVA, K., ZIZKOVA, H. and CERMAK, J., 2012. Monitoring of methylation changes in 9p21 region in patients with myelodysplastic syndromes and acute myeloid leukemia. *Neoplasma,* **59**(2), pp. 168-174.

CHATTERJEE, T. and CHOUDHRY, V.P., 2013. Childhood myelodysplastic syndrome. *Indian journal of pediatrics,* **80**(9), pp. 764-771.

CHEN, H., ZHANG, S. and WU, Z., 2014. Fanconi anemia pathway defects in inherited and sporadic cancers. *Translational Pediatrics,* **3**(4), pp. 300-304.

CHERIAN, S. and BAGG, A., 2006. The genetics of the myelodysplastic syndromes: classical cytogenetics and recent molecular insights. *Hematology (Amsterdam, Netherlands),* **11**(1), pp. 1-13.

CHI, J. and COHEN, P., 2016. The Multifaceted Roles of PRDM16: Adipose Biology and Beyond. *Trends in Endocrinology & Metabolism,* **27**(1), pp. 11-23.

CHURPEK, J.E., 2014. Inherited predisposition to myelodysplastic syndrome and acute leukemia. *Blood,* **124**(21), pp. SCI-31-SCI-31.

CIN, P., ASTER, J. and DEANGELO, D., 2010. When to Go FISHing. *American Journal of Clinical Pathology,* **133**(3), pp. 351-353.

COREY, S.J., MINDEN, M.D., BARBER, D.L., KANTARJIAN, H., WANG, J.C. and SCHIMMER, A.D., 2007. Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nature reviews.Cancer,* **7**(2), pp. 118-129.

CORSON, T.W., HUANG, A., TSAO, M. and GALLIE, B.L., 2005. KIF14 is a candidate oncogene in the 1q minimal region of genomic gain in multiple cancers. *Oncogene,* **24**(30), pp. 4741-4753.

COSTA, D., VALERA, S., CARRIO, A., ARIAS, A., MUNOZ, C., ROZMAN, M., BELKAID, M., COUTINHO, R., NOMDEDEU, B. and CAMPO, E., 2010. Do we need to do fluorescence in situ hybridization analysis in myelodysplastic syndromes as often as we do? *Leukemia research,* **34**(11), pp. 1437-1441.

CUI, W., BUESO-RAMOS, C.E., YIN, C.C., SUN, J., CHEN, S., MUDDASANI, R. and LU, G., 2010. Trisomy 14 as a sole chromosome abnormality is associated with older age, a heterogenous group of myeloid neoplasms with dysplasia, and a wide spectrum of disease progression. *Journal of biomedicine & biotechnology,* **2010**, pp. 365318.

DAI, Z., KELLY, J.C., MELONI-EHRIG, A., SLOVAK, M.L., BOLES, D., CHRISTACOS, N.C., BRYKE, C.R., SCHONBERG, S.A., OTANI-ROSA, J., PAN, Q., HO, A.K., SANDERS, H.R., ZHANG, Z.J., JONES, D. and MOWREY, P.N., 2012. Incidence and patterns of ALK FISH abnormalities seen in a large unselected series of lung carcinomas. *Molecular cytogenetics,* **5**(1), pp. 44-8166-5-44.

DAVE, B.J., HESS, M.M., PICKERING, D.L., ZALESKI, D.H., PFEIFER, A.L., WEISENBURGER, D.D., ARMITAGE, J.O. and SANGER, W.G., 1999. Rearrangements of chromosome band 1p36 in non-Hodgkin's lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **5**(6), pp. 1401-1409.

DAVE, B.J., WIGGINS, M., HIGGINS, C.M., PICKERING, D.L., PERRY, D., AOUN, P., ABROMOWICH, M., DEVETTEN, M. and SANGER, W.G., 2005. 9q34 rearrangements in BCR/ABL fusion-negative acute lymphoblastic leukemia. *Cancer genetics and cytogenetics,* **162**(1), pp. 30-37.

DAVIDS, M.S. and STEENSMA, D.P., 2010. The molecular pathogenesis of myelodysplastic syndromes. *Cancer biology & therapy,* **10**(4), pp. 309-319.

DEEG, H.J., SCOTT, B.L., FANG, M., SHULMAN, H.M., GYURKOCZA, B., MYERSON, D., PAGEL, J.M., PLATZBECKER, U., RAMAKRISHNAN, A., RADICH, J.P., SANDMAIER, B.M., SORROR, M., STIREWALT, D.L., WILSON, W.A., STORB, R., APPELBAUM, F.R. and GOOLEY, T., 2012. Five-group cytogenetic risk classification, monosomal karyotype, and outcome after hematopoietic cell transplantation for MDS or acute leukemia evolving from MDS. *Blood,* **120**(7), pp. 1398-1408.

DEL REY, M., O'HAGAN, K., DELLETT, M., AIBAR, S., COLYER, H., ALONSO, M., DIEZ-CAMPELO, M., ARMSTRONG, R., SHARPE, D. and GUTIERREZ, N., 2013. Genome-wide profiling of methylation identifies novel targets with aberrant hypermethylation and reduced expression in low-risk myelodysplastic syndromes. *Leukemia,* **27**(3), pp. 610-618.

DITTMER, J., 2003. The biology of the Ets1 proto-oncogene. *Molecular cancer,* **2**(1), pp. 1.

DONG, H., NEBERT, D.W., BRUFORD, E.A., THOMPSON, D.C., JOENJE, H. and VASILIOU, V., 2015. Update of the human and mouse Fanconi anemia genes. *Human genomics,* **9**(1), pp. 1.

DOUET-GUILBERT, N., CHAUVEAU, A., GUEGANIC, N., GUILLERM, G., TOUS, C., LE BRIS, M., BASINKO, A., MOREL, F., UGO, V. and DE BRAEKELEER, M., 2015. Acute myeloid leukaemia (FAB AML-M4Eo) with cryptic insertion of cbfb resulting in cbfb-Myh11 fusion. *Hematological oncology,* .

DUAN, X.L., WANG, Q.L., WANG, J.G., WANG, C.Y. and FAN, H., 2013. Expression of FANCG gene in acute myeloid leukemia. *Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology,* **21**(1), pp. 7-11.

DUHOUX, F.P., AMEYE, G., MONTANO-ALMENDRAS, C.P., BAHLOULA, K., MOZZICONACCI, M.J., LAIBE, S., WLODARSKA, I., MICHAUX, L., TALMANT, P., RICHEBOURG, S., LIPPERT, E., SPELEMAN, F., HERENS, C., STRUSKI, S., RAYNAUD, S., AUGER, N., NADAL, N., RACK, K., MUGNERET, F., TIGAUD, I., LAFAGE, M., TAVIAUX, S., ROCHE-LESTIENNE, C., LATINNE, D., LIBOUTON, J.M., DEMOULIN, J.B., POIREL, H.A., GROUPE FRANCOPHONE DE CYTOGENETIQUE HEMATOLOGIQUE (GFCH) and BELGIAN CYTOGENETIC GROUP FOR HAEMATOLOGY AND ONCOLOGY (BCG-HO), 2012. PRDM16 (1p36) translocations

define a distinct entity of myeloid malignancies with poor prognosis but may also occur in lymphoid malignancies. *British journal of haematology,* **156**(1), pp. 76-88.

FERNANDEZ-L, A., NORTHCOTT, P.A., DALTON, J., FRAGA, C., ELLISON, D., ANGERS, S., TAYLOR, M.D. and KENNEY, A.M., 2009. YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. *Genes & development,* **23**(23), pp. 2729-2741.

FLANDRIN, G., 2002. Classification of myelodysplastic syndromes. *Atlas of Genetics and Cytogenetics in Oncology and Haematology,* **6**(3), pp. 217-222.

FOZZA, C. and LONGINOTTI, M., 2013. The role of T-cells in the pathogenesis of myelodysplastic syndromes: passengers and drivers. *Leukemia research,* **37**(2), pp. 201-203.

FU, R.T., XUE, H.M., ZHANG, B.H., WANG, J., LIN, S.F. and CHEN, C., 2015. Correlation analysis of severe aplastic anemia immunosuppressive therapy and human leukocyte antigen alleles in pediatric patients. *Experimental and therapeutic medicine,* **10**(6), pp. 2396-2402.

GANAPATHI, K.A., SCHAFERNAK, K.T., RAO, V.K. and CALVO, K.R., 2015. Pediatric myelodysplastic/myeloproliferative neoplasms and related diseases. *Journal of Hematopathology,* **8**(3), pp. 159-167.

GARCIA, P.D., LANGOWSKI, J.L., WANG, Y., CHEN, M., CASTILLO, J., FANTON, C., ISON, M., ZAVOROTINSKAYA, T., DAI, Y., LU, J., NIU, X.H., BASHAM, S., CHAN, J., YU, J., DOYLE, M., FEUCHT, P., WARNE, R., NARBERES, J., TSANG, T., FRITSCH, C., KAUFFMANN, A., PFISTER, E., DRUECKES, P., TRAPPE, J., WILSON, C., HAN, W., LAN, J., NISHIGUCHI, G., LINDVALL, M., BELLAMACINA, C., AYCINENA, J.A., ZANG, R., HOLASH, J. and BURGER, M.T., 2014. Pan-PIM kinase inhibition provides a novel therapy for treating hematologic cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **20**(7), pp. 1834-1845.

GERMING, U., AUL, C., NIEMEYER, C.M., HAAS, R. and BENNETT, J.M., 2008. Epidemiology, classification and prognosis of adults and children with myelodysplastic syndromes. *Annals of Hematology,* **87**(9), pp. 691-699.

GERSEN, S. and KEAGLE, M., 2013. Basic Cytogenetics Laboratory Procedures. *The Principles of Clinical Cytogenetics.* 3rd edn. Springer Science+Business, pp. 53-65.

GHAFOURI-FARD, S., FARDAEI, M. and MIRYOUNESI, M., 2016. A novel 5 nucleotide deletion in XPA gene is associated with severe neurological abnormalities. *Gene,* **576**(1), pp. 379-380.

GIAGOUNIDIS, A., 2006. *Improving treatment outcomes by understanding cytogenetics in myelodysplastic syndromes.* 2. St. Johannes Hospital Duisburg, Germany: Haematologica Reports.

GINZBURG, Y. and RIVELLA, S., 2011. Beta-Thalassemia: a Model for Elucidating the Dynamic Regulation of Ineffective Erythropoiesis and Iron Metabolism. *Blood,* **118**(16), pp. 4321-4330.

GLAUBACH, T., ROBINSON, L.J. and COREY, S.J., 2014. Pediatric myelodysplastic syndromes: they do exist! *Journal of pediatric hematology/oncology,* **36**(1), pp. 1-7.

GOHRING, G., MICHALOVA, K., BEVERLOO, H.B., BETTS, D., HARBOTT, J., HAAS, O.A., KERNDRUP, G., SAINATI, L., BERGSTRAESSER, E., HASLE, H., STARY, J., TREBO, M., VAN DEN HEUVEL-EIBRINK, M.M., ZECCA, M., VAN WERING, E.R., FISCHER, A., NOELLKE, P., STRAHM, B., LOCATELLI, F., NIEMEYER, C.M. and SCHLEGELBERGER, B., 2010. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. *Blood,* **116**(19), pp. 3766-3769.

GOLDBERG, S.L., CHEN, E., CORRAL, M., GUO, A., MODY-PATEL, N., PECORA, A.L. and LAOURI, M., 2010. Incidence and clinical complications of myelodysplastic syndromes among United States Medicare beneficiaries. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **28**(17), pp. 2847-2852.

GORZKIEWICZ, A. and WALCZEWSKA, A., 2015. Functions of the Ikaros transcription factor and the role of IKZF1 gene defects in hematological malignancies. *Acta Haematologica Polonica,* **46**(1), pp. 10-19.

GREENBERG, P.L.P., 03. Myelodysplastic syndromes, version 2.2015. *Journal of the National Comprehensive Cancer Network,* **13**(3), pp. 261; 261-272; 272.

GREENBERG, P., COX, C., LEBEAU, M.M., FENAUX, P., MOREL, P., SANZ, G., SANZ, M., VALLESPI, T., HAMBLIN, T., OSCIER, D., OHYASHIKI, K., TOYAMA, K., AUL, C., MUFTI, G. and BENNETT, J., 1997. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood,* **89**(6), pp. 2079-2088.

GREENBERG, P.L., TUECHLER, H., SCHANZ, J., SANZ, G., GARCIA-MANERO, G., SOLE, F., BENNETT, J.M., BOWEN, D., FENAUX, P., DREYFUS, F., KANTARJIAN, H., KUENDGEN, A., LEVIS, A., MALCOVATI, L., CAZZOLA, M., CERMAK, J., FONATSCH, C., LE BEAU, M.M., SLOVAK, M.L., KRIEGER, O., LUEBBERT, M., MACIEJEWSKI, J., MAGALHAES, S.M., MIYAZAKI, Y., PFEILSTOCKER, M., SEKERES, M., SPERR, W.R., STAUDER, R., TAURO, S., VALENT, P., VALLESPI, T., VAN DE LOOSDRECHT, A.A., GERMING, U. and HAASE, D., 2012. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood,* **120**(12), pp. 2454-2465.

GRUBER, T.A., GEDMAN, A.L., ZHANG, J., KOSS, C.S., MARADA, S., TA, H.Q., CHEN, S., SU, X., OGDEN, S.K. and DANG, J., 2012. An Inv (16)(p13. 3q24. 3)-encoded CBFA2T3-GLIS2 fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. *Cancer cell,* **22**(5), pp. 683-697.

GRUVER, A.M., ROGERS, H.J., COOK, J.R., BALLIF, B.C., SCHULTZ, R.A., BATANIAN, J.R., FESLER, M.J. and TUBBS, R.R., 2013. Modified array-based comparative genomic hybridization detects cryptic and variant PML-RARA rearrangements in acute promyelocytic leukemia lacking classic translocations. *Diagnostic molecular pathology : the American journal of surgical pathology, part B,* **22**(1), pp. 10-21.

GURRIERI, C., CAPODIECI, P., BERNARDI, R., SCAGLIONI, P.P., NAFA, K., RUSH, L.J., VERBEL, D.A., CORDON-CARDO, C. and PANDOLFI, P.P., 2004. Loss of the tumor suppressor PML in human cancers of multiple histologic origins. *Journal of the National Cancer Institute,* **96**(4), pp. 269-279.

HAASE, D., 2008. Cytogenetic features in myelodysplastic syndromes. *Annals of Hematology,* **87**(7), pp. 515-526.

HAASE, D., GERMING, U., SCHANZ, J., PFEILSTOCKER, M., NOSSLINGER, T., HILDEBRANDT, B., KUNDGEN, A., LUBBERT, M., KUNZMANN, R., GIAGOUNIDIS, A.A., AUL, C., TRUMPER, L., KRIEGER, O., STAUDER, R., MULLER, T.H., WIMAZAL, F., VALENT, P., FONATSCH, C. and STEIDL, C., 2007. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. *Blood,* **110**(13), pp. 4385-4395.

HAFERLACH, C., 2015. Genes break barrier between MDS and AML. *Blood,* **125**(1), pp. 9-10.

HARADA, H., HARADA, Y., NIIMI, H., KYO, T., KIMURA, A. and INABA, T., 2004. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. *Blood,* **103**(6), pp. 2316-2324.

HASLE, H., NIEMEYER, C.M., CHESSELLS, J.M., BAUMANN, I., BENNETT, J.M., KERNDRUP, G. and HEAD, D.R., 2003. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia,* **17**(2), pp. 277-282.

HIGGINS, C., SOE, M. and SANGER, W., 1993. Origen GCT-CM enhances mitotic index and chromosome morphology in bone marrow from patients with hematological disorders. *Applied Cytogenetics,* **19**(4),.

HOFMANN, I., 2015a. Pediatric myelodysplastic syndromes. *Journal of Hematopathology,* **8**(3), pp. 127-141.

HOFMANN, I., 2015b. Myeloproliferative Neoplasms in Children. *Journal of hematopathology,* **8**(3), pp. 143-157.

HORWITZ, M., 2013. Insights from Familial Leukemia and Myelodysplastic Syndromes. *Blood,* **122**(21), pp. SCI-6-SCI-6.

HOWE, B., UMRIGAR, A. and TSIEN, F., 2014. Chromosome preparation from cultured cells. *Journal of visualized experiments : JoVE,* **(83):e50203. doi**(83), pp. e50203.

HOWE, R.B., PORWIT-MACDONALD, A., WANAT, R., TEHRANCHI, R. and HELLSTROM-LINDBERG, E., 2004. The WHO classification of MDS does make a difference. *Blood,* **103**(9), pp. 3265-3270.

ISHIAI, M., KITAO, H., SMOGORZEWSKA, A., TOMIDA, J., KINOMURA, A., UCHIDA, E., SABERI, A., KINOSHITA, E., KINOSHITA-KIKUTA, E. and KOIKE, T., 2008. FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway. *Nature structural & molecular biology,* **15**(11), pp. 1138-1146.

ISHIKAWA, Y., MAEDA, M., PASHAM, M., AGUET, F., TACHEVA-GRIGOROVA, S.K., MASUDA, T., YI, H., LEE, S.U., XU, J., TERUYA-FELDSTEIN, J., ERICSSON, M., MULLALLY, A., HEUSER, J., KIRCHHAUSEN, T. and MAEDA, T., 2015. Role of the clathrin adaptor PICALM in normal hematopoiesis and polycythemia vera pathophysiology. *Haematologica,* **100**(4), pp. 439-451.

ISMAEL, O., SHIMADA, A., HAMA, A., ELSHAZLEY, M., MURAMATSU, H., GOTO, A., SAKAGUCHI, H., TANAKA, M., TAKAHASHI, Y., YINYAN, X., FUKUDA, M., MIYAJIMA, Y., YAMASHITA, Y., HORIBE, K., HANADA, R., ITO, M. and KOJIMA, S., 2012. De novo childhood myelodysplastic/myeloproliferative disease with unique molecular characteristics. *British journal of haematology,* **158**(1), pp. 129-137.

JAFRI, M., WAKE, N.C., ASCHER, D.B., PIRES, D.E., GENTLE, D., MORRIS, M.R., RATTENBERRY, E., SIMPSON, M.A., TREMBATH, R.C., WEBER, A., WOODWARD, E.R., DONALDSON, A., BLUNDELL, T.L., LATIF, F. and MAHER, E.R., 2015. Germline Mutations in the CDKN2B Tumor Suppressor Gene Predispose to Renal Cell Carcinoma. *Cancer discovery,* **5**(7), pp. 723-729.

JANISZEWSKA, H., BĄK, A., HARTWIG, M., KULISZKIEWICZ-JANUS, M., CAŁBECKA, M., JAŹWIEC, B., KULICZKOWSKI, K. and HAUS, O., 2015. The germline mutations of the CHEK2 gene are associated with an increased risk of polycythaemia vera. *British journal of haematology,* .

JHANWAR, S.C., 2015. Genetic and epigenetic pathways in myelodysplastic syndromes: A brief overview. *Advances in biological regulation,* **58**, pp. 28-37.

JONGMANS, M.C., LOEFFEN, J.L., WAANDERS, E., HOOGERBRUGGE, P.M., LIGTENBERG, M.J., KUIPER, R.P. and HOOGERBRUGGE, N., 2016. Recognition of

genetic predisposition in pediatric cancer patients: An easy-to-use selection tool. *European journal of medical genetics,* **59**(3), pp. 116-125.

KALB, I. and FELDMAN, A.L., 2015. The oncogenic transcription factor IRF4 is regulated by a novel CD30/NF-κB positive feedback loop in peripheral T-cell lymphoma.

KARDOS, G., BAUMANN, I., PASSMORE, S.J., LOCATELLI, F., HASLE, H., SCHULTZ, K.R., STARY, J., SCHMITT-GRAEFF, A., FISCHER, A., HARBOTT, J., CHESSELLS, J.M., HANN, I., FENU, S., RAJNOLDI, A.C., KERNDRUP, G., VAN WERING, E., ROGGE, T., NOLLKE, P. and NIEMEYER, C.M., 2003. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. *Blood,* **102**(6), pp. 1997-2003.

KOH, Y.R., CHO, E.H., PARK, S.S., PARK, M.Y., LEE, S.M., KIM, I.S. and LEE, E.Y., 2013. A rare case of transformation of childhood myelodysplastic syndrome to acute lymphoblastic leukemia. *Annals of laboratory medicine,* **33**(2), pp. 130-135.

KONDO, M., 2010. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological reviews,* **238**(1), pp. 37-46.

KOZYRA, E.J., HIRABAYASHI, S., LOYOLA, V.B.P., PRZYCHODZEN, B., KAROW, A., CATALA, A., DE MOERLOOSE, B., DWORZAK, M., HASLE, H. and MASETTI, R., 2015. Clonal Mutational Landscape of Childhood Myelodysplastic Syndromes. *Blood,* **126**(23), pp. 1662-1662.

KRABBE, J.G., KEMNA, E.W., STRUNK, A.L., JOBSE, P.A., KRAMER, P., DIKKESCHEI, L., VAN DEN HEUVEL, L., FIJNHEER, R. and VERDONCK, L.F., 2015. Adult-onset congenital thrombotic thrombocytopenic purpura caused by a novel compound heterozygous mutation of the ADAMTS13 gene. *International journal of hematology,* **102**(4), pp. 477-481.

KUIPER, R.P., VAN REIJMERSDAL, S.V., SIMONIS, M., YU, J., SONNEVELD, E., SCHEIJEN, B., BOER, J.M., BOEREE, A., KLOUS, P. and HOOGERBRUGGE, P.M., 2015. Targeted Locus Amplification & Next Generation Sequencing for the Detection of Recurrent and Novel Gene Fusions for Improved Treatment Decisions in Pediatric Acute Lymphoblastic Leukemia. *Blood,* **126**(23), pp. 696-696.

KUIPER, R., WAANDERS, E., VAN DER VELDEN, V., VAN REIJMERSDAL, S., VENKATACHALAM, R., SCHEIJEN, B., SONNEVELD, E., VAN DONGEN, J., VEERMAN, A. and VAN LEEUWEN, F., 2010. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. *Leukemia,* **24**(7), pp. 1258-1264.

KULASEKARARAJ, A.G., MOHAMEDALI, A.M. and MUFTI, G.J., 2013. Recent advances in understanding the molecular pathogenesis of myelodysplastic syndromes. *British journal of haematology,* **162**(5), pp. 587-605.

KUO, Y.H., ZAIDI, S.K., GORNOSTAEVA, S., KOMORI, T., STEIN, G.S. and CASTILLA, L.H., 2009. Runx2 induces acute myeloid leukemia in cooperation with Cbfbeta-SMMHC in mice. *Blood,* **113**(14), pp. 3323-3332.

LAWRENSON, K., IVERSEN, E.S., TYRER, J., WEBER, R.P., CONCANNON, P., HAZELETT, D.J., LI, Q., MARKS, J.R., BERCHUCK, A., LEE, J.M., ABEN, K.K., ANTON-CULVER, H., ANTONENKOVA, N., AUSTRALIAN CANCER STUDY (OVARIAN CANCER), AUSTRALIAN OVARIAN CANCER STUDY GROUP, BANDERA, E.V., BEAN, Y., BECKMANN, M.W., BISOGNA, M., BJORGE, L., BOGDANOVA, N., BRINTON, L.A., BROOKS-WILSON, A., BRUINSMA, F., BUTZOW, R., CAMPBELL, I.G., CARTY, K., CHANG-CLAUDE, J., CHENEVIX-TRENCH, G., CHEN, A., CHEN, Z., COOK, L.S., CRAMER, D.W., CUNNINGHAM, J.M., CYBULSKI, C., PLISIECKA-HALASA, J., DENNIS, J., DICKS, E., DOHERTY, J.A., DORK, T., DU BOIS, A., ECCLES, D., EASTON, D.T., EDWARDS, R.P., EILBER, U., EKICI, A.B., FASCHING, P.A., FRIDLEY, B.L., GAO, Y.T., GENTRY-MAHARAJ, A., GILES, G.G., GLASSPOOL, R., GOODE, E.L., GOODMAN, M.T., GRONWALD, J., HARTER, P., HASMAD, H.N., HEIN, A., HEITZ, F., HILDEBRANDT, M.A., HILLEMANNS, P., HOGDALL, E., HOGDALL, C., HOSONO, S., JAKUBOWSKA, A., PAUL, J., JENSEN, A., KARLAN, B.Y., KJAER, S.K., KELEMEN, L.E., KELLAR, M., KELLEY, J.L., KIEMENEY, L.A., KRAKSTAD, C., LAMBRECHTS, D., LAMBRECHTS, S., LE, N.D., LEE, A.W., CANNIOTO, R., LEMINEN, A., LESTER, J., LEVINE, D.A., LIANG, D., LISSOWSKA, J., LU, K., LUBINSKI, J., LUNDVALL, L., MASSUGER, L.F., MATSUO, K., MCGUIRE, V., MCLAUGHLIN, J.R., NEVANLINNA, H., MCNEISH, I., MENON, U., MODUGNO, F., MOYSICH, K.B., NAROD, S.A., NEDERGAARD, L., NESS, R.B., NOOR AZMI, M.A., ODUNSI, K., OLSON, S.H., ORLOW, I., ORSULIC, S., PEARCE, C.L., PEJOVIC, T., PELTTARI, L.M., PERMUTH-WEY, J., PHELAN, C.M., PIKE, M.C., POOLE, E.M., RAMUS, S.J., RISCH, H.A., ROSEN, B., ROSSING, M.A., ROTHSTEIN, J.H., RUDOLPH, A., RUNNEBAUM, I.B., RZEPECKA, I.K., SALVESEN, H.B., BUDZILOWSKA, A., SELLERS, T.A., SHU, X.O., SHVETSOV, Y.B., SIDDIQUI, N., SIEH, W., SONG, H., SOUTHEY, M.C., SUCHESTON, L., TANGEN, I.L., TEO, S.H., TERRY, K.L., THOMPSON, P.J., TIMOREK, A., TWOROGER, S.S., VAN NIEUWENHUYSEN, E., VERGOTE, I., VIERKANT, R.A., WANG-GOHRKE, S., WALSH, C., WENTZENSEN, N., WHITTEMORE, A.S., WICKLUND, K.G., WILKENS, L.R., WOO, Y.L., WU, X., WU, A.H., YANG, H., ZHENG, W., ZIOGAS, A., COETZEE, G.A., FREEDMAN, M.L., MONTEIRO, A.N., MOES-SOSNOWSKA, J., KUPRYJANCZYK, J., PHAROAH, P.D., GAYTHER, S.A. and SCHILDKRAUT, J.M., 2015. Common variants at the CHEK2 gene locus and risk of epithelial ovarian cancer. *Carcinogenesis,* **36**(11), pp. 1341-1353.

LEE, D., 2015. Genetic Basis of Mesothelioma—More Than Asbestos Exposure. *Journal of Thoracic Oncology,* .

LI, W., MORRONE, K., KAMBHAMPATI, S., WILL, B., STEIDL, U. and VERMA, A., 2015. Thrombocytopenia in MDS: epidemiology, mechanisms, clinical consequences and novel therapeutic strategies. *Leukemia,* .

LIST, A., DEWALD, G., BENNETT, J., GIAGOUNIDIS, A., RAZA, A., FELDMAN, E., POWELL, B., GREENBERG, P., THOMAS, D., STONE, R., REEDER, C., WRIDE, K., PATIN, J., SCHMIDT, M., ZELDIS, J., KNIGHT, R. and MYELODYSPLASTIC SYNDROME-003 STUDY INVESTIGATORS, 2006. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *The New England journal of medicine,* **355**(14), pp. 1456-1465.

LIU, K., YING, C.X., CHEN, X.D., ZHOU, X.Y. and GUO, K.Y., 2012. A case report of myelodysplastic/myeloproliferative disease unclassifiable with karyotype aberration of trisomy 8 and JAK2 mutation. *Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology,* **20**(5), pp. 1139-1143.

LIU, K., CASTILLO, M.D., MURTHY, R.G., PATEL, N. and RAMESHWAR, P., 2007. Tachykinins and Hematopoiesis. *Clinica Chimica Acta,* **385**(1–2), pp. 28-34.

LOBO, N.A., SHIMONO, Y., QIAN, D. and CLARKE, M.F., 2007. The biology of cancer stem cells. *Annual Review of Cell and Developmental Biology,* **23**, pp. 675-699.

LOHR, J.G., STOJANOV, P., CARTER, S.L., CRUZ-GORDILLO, P., LAWRENCE, M.S., AUCLAIR, D., SOUGNEZ, C., KNOECHEL, B., GOULD, J. and SAKSENA, G., 2014. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. *Cancer cell,* **25**(1), pp. 91-101.

MACIEJEWSKI, J.P., TIU, R.V. and O'KEEFE, C., 2009. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. *British journal of haematology,* **146**(5), pp. 479-488.

MALLO, M., ARENILLAS, L., ESPINET, B., SALIDO, M., HERNANDEZ, J.M., LUMBRERAS, E., DEL REY, M., ARRANZ, E., RAMIRO, S., FONT, P., GONZALEZ, O., RENEDO, M., CERVERA, J., SUCH, E., SANZ, G.F., LUNO, E., SANZO, C., GONZALEZ, M., CALASANZ, M.J., MAYANS, J., GARCIA-BALLESTEROS, C., AMIGO, V., COLLADO, R., OLIVER, I., CARBONELL, F., BUREO, E., INSUNZA, A., YANEZ, L., MURUZABAL, M.J., GOMEZ-BELTRAN, E., ANDREU, R., LEON, P., GOMEZ, V., SANZ, A., CASASOLA, N., MORENO, E., ALEGRE, A., MARTIN, M.L., PEDRO, C., SERRANO, S., FLORENSA, L. and SOLE, F., 2008. Fluorescence in situ hybridization improves the detection of 5q31 deletion in myelodysplastic syndromes without cytogenetic evidence of 5q-. *Haematologica,* **93**(7), pp. 1001-1008.

MANANDHAR, M., BOULWARE, K.S. and WOOD, R.D., 2015. The ERCC1 and ERCC4 (XPF) genes and gene products. *Gene,* **569**(2), pp. 153-161.

MANDEL, K., DROR, Y., POON, A. and FREEDMAN, M.H., 2002. A practical, comprehensive classification for pediatric myelodysplastic syndromes: the CCC system. *Journal of pediatric hematology/oncology,* **24**(7), pp. 596-605.

MASCARENHAS, J., MESA, R., PRCHAL, J. and HOFFMAN, R., 2014. Optimal therapy for polycythemia vera and essential thrombocythemia can only be determined by the completion of randomized clinical trials. *Haematologica,* **99**(6), pp. 945-949.

MASETTI, R., PIGAZZI, M., TOGNI, M., ASTOLFI, A., INDIO, V., MANARA, E., CASADIO, R., PESSION, A., BASSO, G. and LOCATELLI, F., 2013. CBFA2T3-GLIS2 fusion transcript is a novel common feature in pediatric, cytogenetically normal AML, not restricted to FAB M7 subtype. *Blood,* **121**(17), pp. 3469-3472.

MASETTI, R., TOGNI, M., ASTOLFI, A., PIGAZZI, M., INDIO, V., RIVALTA, B., MANARA, E., RUTELLA, S., BASSO, G., PESSION, A. and LOCATELLI, F., 2014. Whole transcriptome sequencing of a paediatric case of de novo acute myeloid leukaemia with del(5q) reveals RUNX1-USP42 and PRDM16-SKI fusion transcripts. *British journal of haematology,* **166**(3), pp. 449-452.

MCNEAL, A.S., LIU, K., NAKHATE, V., NATALE, C.A., DUPERRET, E.K., CAPELL, B.C., DENTCHEV, T., BERGER, S.L., HERLYN, M., SEYKORA, J.T. and RIDKY, T.W., 2015. CDKN2B Loss Promotes Progression from Benign Melanocytic Nevus to Melanoma. *Cancer discovery,* **5**(10), pp. 1072-1085.

NIEMEYER, C.M. and BAUMANN, I., 2011. Classification of childhood aplastic anemia and myelodysplastic syndrome. *Hematology / the Education Program of the American Society of Hematology.American Society of Hematology.Education Program,* **2011**, pp. 84-89.

NIEMEYER, C.M. and BAUMANN, I., 2008. Myelodysplastic syndrome in children and adolescents. *Seminars in hematology,* **45**(1), pp. 60-70.

OHARA, A., KOJIMA, S., HAMAJIMA, N., TSUCHIDA, M., IMASHUKU, S., OHTA, S., SASAKI, H., OKAMURA, J., SUGITA, K., KIGASAWA, H., KIRIYAMA, Y., AKATSUKA, J. and TSUKIMOTO, I., 1997. Myelodysplastic Syndrome and Acute Myelogenous Leukemia as a Late Clonal Complication in Children With Acquired Aplastic Anemia. *Blood,* **90**(3), pp. 1009-1013.

OLSSON, L., ZETTERMARK, S., BILOGLAV, A., CASTOR, A., BEHRENDTZ, M., FORESTIER, E., PAULSSON, K. and JOHANSSON, B., 2016. The genetic landscape of paediatric de novo acute myeloid leukaemia as defined by single nucleotide polymorphism array and exon sequencing of 100 candidate genes. *British journal of haematology,* .

ORAZI, A. and GERMING, U., 2008. The myelodysplastic/myeloproliferative neoplasms: myeloproliferative diseases with dysplastic features. *Leukemia,* **22**(7), pp. 1308-1319.

ORKIN, S.H. and ZON, L.I., 2008. Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell,* **132**(4), pp. 631-644.

OUHTIT, A., MUZUMDAR, S., GUPTA, I., SHANMUGANATHAN, S. and TAMIMI, Y., 2015. Understanding the functional discrepancy of Pim-1 in cancer. *nucleus,* **5**(6), pp. 7.

OUYANG, Y., QIAO, C., WANG, J., XIAO, L. and ZHANG, S., 2015. Analysis of CALR, JAK2 and MPL gene mutations in BCR-ABL negative myeloproliferative neoplasms. *Zhonghua yi xue za zhi,* **95**(18), pp. 1369-1373.

PANAGOPOULOS, I., TORKILDSEN, S., GORUNOVA, L., TIERENS, A., TJØNNFJORD, G.E. and HEIM, S., 2014. Comparison between karyotyping-FISH-reverse transcription PCR and RNA-sequencing-fusion gene identification programs in the detection of KAT6A-CREBBP in acute myeloid leukemia. *PloS one,* **9**(5), pp. e96570.

PANG, W.W., PRICE, E.A., SAHOO, D., BEERMAN, I., MALONEY, W.J., ROSSI, D.J., SCHRIER, S.L. and WEISSMAN, I.L., 2011. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proceedings of the National Academy of Sciences,* **108**(50), pp. 20012-20017.

PAPAEMMANUIL, E., GERSTUNG, M., MALCOVATI, L., TAURO, S., GUNDEM, G., VAN LOO, P., YOON, C.J., ELLIS, P., WEDGE, D.C., PELLAGATTI, A., SHLIEN, A., GROVES, M.J., FORBES, S.A., RAINE, K., HINTON, J., MUDIE, L.J., MCLAREN, S., HARDY, C., LATIMER, C., DELLA PORTA, M.G., O'MEARA, S., AMBAGLIO, I., GALLI, A., BUTLER, A.P., WALLDIN, G., TEAGUE, J.W., QUEK, L., STERNBERG, A., GAMBACORTI-PASSERINI, C., CROSS, N.C., GREEN, A.R., BOULTWOOD, J., VYAS, P., HELLSTROM-LINDBERG, E., BOWEN, D., CAZZOLA, M., STRATTON, M.R., CAMPBELL, P.J. and CHRONIC MYELOID DISORDERS WORKING GROUP OF THE INTERNATIONAL CANCER GENOME CONSORTIUM, 2013. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood,* **122**(22), pp. 3616-27; quiz 3699.

PARK, J., CHUNG, N., CHAE, H., KIM, M., LEE, S., KIM, Y., LEE, J., CHO, B., JEONG, D. and PARK, I., 2013. FANCA and FANCG are the major Fanconi anemia genes in the Korean population. *Clinical genetics,* **84**(3), pp. 271-275.

PASSEGUE, E., WAGERS, A.J., GIURIATO, S., ANDERSON, W.C. and WEISSMAN, I.L., 2005. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *The Journal of experimental medicine,* **202**(11), pp. 1599-1611.

PITCHFORD, C., HETTINGA, A. and REICHARD, K., 2010. Fluorescence In Situ Hybridization for -5/5q, -7/7q, +8, and del(20q) in Primary Myelodysplastic Syndrome Correlates with Conventional Cytogenetics in the Setting of an Adequate Study. *American Journal of Clinical Pathology,* **133**(2), pp. 260-264.

POPPE, B., VAN LIMBERGEN, H., VAN ROY, N., VANDECRUYS, E., DE PAEPE, A., BENOIT, Y. and SPELEMAN, F., 2001. Chromosomal aberrations in Bloom syndrome patients with myeloid malignancies. *Cancer genetics and cytogenetics,* **128**(1), pp. 39-42.

QUARELLO, P., GARELLI, E., BRUSCO, A., CARANDO, A., MANCINI, C., PAPPI, P., VINTI, L., SVAHN, J., DIANZANI, I. and RAMENGHI, U., 2012. High frequency of ribosomal protein gene deletions in Italian Diamond-Blackfan anemia patients detected by multiplex ligation-dependent probe amplification assay. *Haematologica,* **97**(12), pp. 1813-1817.

RAAIJMAKERS, M.H., 2012. Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. *International journal of hematology,* **95**(1), pp. 17-25.

RACA, G., GURBUXANI, S., ZHANG, Z., LI, Z., SUKHANOVA, M., MCNEER, J. and STOCK, W., 2015. RCSD1–ABL2 fusion resulting from a complex chromosomal rearrangement in high-risk B-cell acute lymphoblastic leukemia. *Leukemia & lymphoma,* **56**(4), pp. 1145-1147.

RAU, A.T., SHREEDHARA, A.K. and KUMAR, S., 2012. Myelodysplastic syndromes in children: where are we today? *The Ochsner journal,* **12**(3), pp. 216-220.

RIGOLIN, G.M., BIGONI, R., MILANI, R., CAVAZZINI, F., ROBERTI, M.G., BARDI, A., AGOSTINI, P., DELLA PORTA, M., TIEGHI, A., PIVA, N., CUNEO, A. and CASTOLDI, G., 2001. Clinical importance of interphase cytogenetics detecting occult chromosome lesions in myelodysplastic syndromes with normal karyotype. *Leukemia,* **15**(12), pp. 1841-1847.

ROLLISON, D.E., HOWLADER, N., SMITH, M.T., STROM, S.S., MERRITT, W.D., RIES, L.A., EDWARDS, B.K. and LIST, A.F., 2008. Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the United States, 2001-2004, using data from the NAACCR and SEER programs. *Blood,* **112**(1), pp. 45-52.

ROSE, D., HAFERLACH, T., KERN, W. and HAFERLACH, C., 2015. Categorizing Molecular Mutations in MDS and AML. *Blood,* **126**(23), pp. 5222-5222.

RÖTTGERS, S., GOMBERT, M., TEIGLER-SCHLEGEL, A., BUSCH, K., GAMERDINGER, U., SLANY, R., HARBOTT, J. and BORKHARDT, A., 2010. ALK fusion genes in children with atypical myeloproliferative leukemia. *Leukemia,* **24**(6), pp. 1197-1200.

RUBIN, C., ARTHUR, D., WOODS, W., LANGE, B., NOWELL, P., ROWLEY, J., NACHMAN, J., BOSTROM, B., BAUM, E. and SUAREZ, C., 1991. Therapy-related myelodysplastic syndrome and acute myeloid leukemia in children: correlation between chromosomal abnormalities and prior therapy. *Blood,* **78**(11), pp. 2982-2988.

SAFARI, S., MOVAFAGH, A., ZARE-ADOLLAHI, D., GHADIANI, M., RIAZI-ISFAHANI, S., SAFAVI-NAINI, N. and OMRANI, M.D., 2014. MST1/2 and YAP1 gene expression in acute myeloid leukemia. *Leukemia & lymphoma,* **55**(9), pp. 2189-2191.

SALAVERRIA, I., PHILIPP, C., OSCHLIES, I., KOHLER, C.W., KREUZ, M., SZCZEPANOWSKI, M., BURKHARDT, B., TRAUTMANN, H., GESK, S., ANDRUSIEWICZ, M., BERGER, H., FEY, M., HARDER, L., HASENCLEVER, D., HUMMEL, M., LOEFFLER, M., MAHN, F., MARTIN-GUERRERO, I., PELLISSERY, S., POTT, C., PFREUNDSCHUH, M., REITER, A., RICHTER, J., ROSOLOWSKI, M., SCHWAENEN, C., STEIN, H., TRUMPER, L., WESSENDORF, S., SPANG, R., KUPPERS, R., KLAPPER, W., SIEBERT, R., MOLECULAR MECHANISMS IN MALIGNANT LYMPHOMAS NETWORK PROJECT OF THE DEUTSCHE KREBSHILFE, GERMAN HIGH-GRADE LYMPHOMA STUDY GROUP and BERLIN-FRANKFURT-MUNSTER-NHL TRIAL GROUP, 2011. Translocations activating IRF4 identify a subtype of germinal center-derived B-cell lymphoma affecting predominantly children and young adults. *Blood,* **118**(1), pp. 139-147.

SALEEM, M. and MOHD. YUSOFF, N., 2015. Fusion genes in Malignant Neoplastic Disorders of Haematopoietic System. *Hematology,* (just-accepted), pp. 1-22.

SANTINI, V., MELNICK, A., MACIEJEWSKI, J.P., DUPREZ, E., NERVI, C., COCCO, L., FORD, K.G. and MUFTI, G., 2013. Epigenetics in focus: pathogenesis of myelodysplastic syndromes and the role of hypomethylating agents. *Critical reviews in oncology/hematology,* **88**(2), pp. 231-245.

SARPER, N., GELEN, S.A., ZENGIN, E., DEMIRSOY, U. and ERCIN, C., 2015. Nonsyndromic Juvenile Myelomonocytic Leukemia With PTPN11 Mutation in a 9-Year-old Girl. *Journal of pediatric hematology/oncology,* **37**(6), pp. 486-487.

SCELO, G., MCKAY, J., HOLCATOVA, I., JANOUT, V., FORETOVA, L., FABIANOVA, E., CHABRIER, A., GABORIEAU, V. and BRENNAN, P., 2014. Uncommon CHEK2 missense variant and reduced risk of pancreatic cancer. *Cancer research,* **74**(19 Supplement), pp. 2213-2213.

SCHNERCH, D., LAUSCH, E., BECKER, H., FELTHAUS, J., PFEIFER, D., MUNDLOS, S., ENGELHARDT, M., SCHWABE, M. and WÄSCH, R., 2014. Up-regulation of RUNX2 in acute myeloid leukemia in a patient with an inherent RUNX2 haploinsufficiency and cleidocranial dysplasia. *Leukemia & lymphoma,* **55**(8), pp. 1930-1932.

SCHUBACK, H.L., ALONZO, T.A., GERBING, R.B., MILLER, K.L., KAHWASH, S., HEEREMA-MCKENNEY, A., HIRSCH, B.A., RAIMONDI, S.C., APLENC, R. and GAMIS, A.S., 2014. CBFA2T3-GLIS2 Fusion Is Prevalent in Younger Patients with Acute Myeloid Leukemia and Associated with High-Risk of Relapse and Poor Outcome: A Children's Oncology Group Report. *Blood,* **124**(21), pp. 13-13.

SEIF, A.E., 2011. Pediatric leukemia predisposition syndromes: clues to understanding leukemogenesis. *Cancer genetics,* **204**(5), pp. 227-244.

SHAFFER, L., MCGOWAN-JORDAN, J. and SCHMID, M., 2013. *An International System for Human Cytogenetic Nomenclature (2013).* Basel: S Karber.

SHIBA, N., YOSHIDA, K., SHIRAISHI, Y., HARA, Y., YAMATO, G., KABURAKI, T., SANADA, M., OKI, K., TOMIZAWA, D. and SOTOMATSU, M., 2015. Detection of Novel Pathogenic Gene Rearrangements in Pediatric Acute Myeloid Leukemia By RNA Sequencing. *Blood,* **126**(23), pp. 2575-2575.

SHIH, A.H., ABDEL-WAHAB, O., PATEL, J.P. and LEVINE, R.L., 2012. The role of mutations in epigenetic regulators in myeloid malignancies. *Nature reviews.Cancer,* **12**(9), pp. 599-612.

SHYAMSUNDER, P., GANESH, K.S., VIDYASEKAR, P., MOHAN, S. and VERMA, R.S., 2013. Identification of novel target genes involved in Indian Fanconi anemia patients using microarray. *Gene,* **531**(2), pp. 444-450.

SIEGEL, R., MA, J., ZOU, Z. and JEMAL, A., 2014. Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians,* **64**(1), pp. 9-29.

SILVA, A.G., MASCHIETTO, M., VIDAL, D.O., PELICARIO, L.M., VELLOSO, E.D., LOPES, L.F., KREPISCHI, A.C. and ROSENBERG, C., 2013. Array-CGH as an adjuvant tool in cytogenetic diagnosis of pediatric MDS and JMML. *Medical oncology (Northwood, London, England),* **30**(4), pp. 734-013-0734-1. Epub 2013 Oct 2.

SMITH, A.R., CHRISTIANSEN, E.C., WAGNER, J.E., CAO, Q., MACMILLAN, M.L., STEFANSKI, H.E., TROTZ, B.A., BURKE, M.J. and VERNERIS, M.R., 2013. Early hematopoietic stem cell transplant is associated with favorable outcomes in children with MDS. *Pediatric blood & cancer,* **60**(4), pp. 705-710.

SOLOMON, P.J., RAJENDRAN, R., RAMALINGAM, R., MENEZES, G. and SHIRLEY, A., 2015. A case report and literature review of Fanconi Anemia (FA) diagnosed by genetic testing. *Ital J Pediatr,* **41**, pp. 38.

STARY, J., BAUMANN, I., CREUTZIG, U., HARBOTT, J., MICHALOVA, K. and NIEMEYER, C., 2008. Getting the numbers straight in pediatric MDS: distribution of subtypes after exclusion of down syndrome. *Pediatric blood & cancer,* **50**(2), pp. 435-436.

STEENSMA, D.P., KOMROKJI, R.S., STONE, R.M., LIST, A.F., GARCIA-MANERO, G., HUBER, J.M., DENNISON, B. and SEKERES, M.A., 2014. Disparity in perceptions of disease characteristics, treatment effectiveness, and factors influencing treatment adherence between physicians and patients with myelodysplastic syndromes. *Cancer,* **120**(11), pp. 1670-1676.

STICKENS, D., CLINES, G., BURBEE, D., RAMOS, P., THOMAS, S., HOGUE, D., HECHT, J.T., LOVETT, M. and EVANS, G.A., 1996. The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. *Nature genetics,* **14**(1), pp. 25-32.

STONE, R.M., 2009. How I treat patients with myelodysplastic syndromes. *Blood,* **113**(25), pp. 6296-6303.

SUN, J., KONOPLEV, S.N., WANG, X., CUI, W., CHEN, S.S., MEDEIROS, L.J. and LIN, P., 2011. De novo acute myeloid leukemia with inv (3)(q21q26. 2) or t (3; 3)(q21; q26. 2): a clinicopathologic and cytogenetic study of an entity recently added to the WHO classification. *Modern Pathology,* **24**(3), pp. 384-389.

TAN, B.T., PARK, C.Y., AILLES, L.E. and WEISSMAN, I.L., 2006. The cancer stem cell hypothesis: a work in progress. *Laboratory investigation,* **86**(12), pp. 1203-1207.

TANAKA, H., OHWADA, C., HASHIMOTO, S., SAKAI, S., TAKEDA, Y., ABE, D., TAKAGI, T., OHSHIMA, K. and NAKASEKO, C., 2012. Leukemic presentation of ALK-negative anaplastic large cell lymphoma in a patient with myelodysplastic syndrome. *Internal medicine (Tokyo, Japan),* **51**(2), pp. 199-203.

TEFFERI, A., 2010. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia,* **24**(6), pp. 1128-1138.

TEFFERI, A. and VARDIMAN, J.W., 2009. Myelodysplastic Syndromes. *N Engl J Med,* **361**(19), pp. 1872-1885.

TEOFILI, L., GIONA, F., MARTINI, M., CENCI, T., GUIDI, F., TORTI, L., PALUMBO, G., AMENDOLA, A., FOA, R. and LAROCCA, L.M., 2007. Markers of myeloproliferative diseases in childhood polycythemia vera and essential thrombocythemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **25**(9), pp. 1048-1053.

THÉRIAULT, B.L. and CORSON, T.W., 2015. Kif14: A Clinically Relevant Kinesin and Potential Target for Cancer Therapy. *Kinesins and Cancer.* Springer, pp. 149-170.

TILAK, V., SOOKMANE, D.D., GUPTA, V. and SHUKLA, J., 2008. Myelodysplastic syndrome. *Indian journal of pediatrics,* **75**(7), pp. 729-732.

VALENT, P. and HORNY, H.P., 2009. Minimal diagnostic criteria for myelodysplastic syndromes and separation from ICUS and IDUS: update and open questions. *European journal of clinical investigation,* **39**(7), pp. 548-553.

VALENT, P., HORNY, H.P., BENNETT, J.M., FONATSCH, C., GERMING, U., GREENBERG, P., HAFERLACH, T., HAASE, D., KOLB, H.J., KRIEGER, O., LOKEN, M., VAN DE LOOSDRECHT, A., OGATA, K., ORFAO, A., PFEILSTOCKER, M., RUTER, B., SPERR, W.R., STAUDER, R. and WELLS, D.A., 2007. Definitions and standards in the diagnosis and treatment of the myelodysplastic syndromes: Consensus statements and report from a working conference. *Leukemia research,* **31**(6), pp. 727-736.

VARDIMAN, J.W., THIELE, J., ARBER, D.A., BRUNNING, R.D., BOROWITZ, M.J., PORWIT, A., HARRIS, N.L., LE BEAU, M.M., HELLSTRÖM-LINDBERG, E., TEFFERI, A. and BLOOMFIELD, C.D., 2009. The 2008 revision of the World Health Organization

(WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood,* **114**(5), pp. 937-951.

VISCONTE, V., SELLERI, C., MACIEJEWSKI, J.P. and TIU, R.V., 2014. Molecular pathogenesis of myelodysplastic syndromes. *Translational medicine @ UniSa,* **8**, pp. 19-30.

VOGAN, K., 2013. Recurrent fusion in pediatric AMKL. *Nature genetics,* **45**(1), pp. 11-11.

VOSO, M., FABIANI, E., ZANG, Z., FIANCHI, L., FALCONI, G., PADELLA, A., MARTINI, M., ZHANG, S.L., SANTANGELO, R. and LAROCCA, L., 2015. Fanconi anemia gene variants in therapy-related myeloid neoplasms. *Blood cancer journal,* **5**(7), pp. e323.

WAINSTEIN, T., KERR, R., MITCHELL, C.L., MADAREE, S., ESSOP, F.B., VORSTER, E., WAINWRIGHT, R., POOLE, J. and KRAUSE, A., 2013. Fanconi anaemia in black South African patients heterozygous for the FANCG c. 637-643delTACCGCC founder mutation. *SAMJ: South African Medical Journal,* **103**(12), pp. 970-973.

WAN, L., SUN, M., LIU, G.J., WEI, C.C., ZHANG, E.B., KONG, R., XU, T.P., HUANG, M.D. and WANG, Z.X., 2016. Long non-coding RNA PVT1 promotes non-small cell lung cancer cell proliferation through epigenetically regulating LATS2 expression. *Molecular cancer therapeutics,* .

WANG, H., DU, Y., ZHOU, X., LIU, H. and TANG, S., 2014. The dual functions of YAP-1 to promote and inhibit cell growth in human malignancy. *Cancer and metastasis reviews,* **33**(1), pp. 173-181.

WANG, C., LIU, Z., WOO, C.W., LI, Z., WANG, L., WEI, J.S., MARQUEZ, V.E., BATES, S.E., JIN, Q., KHAN, J., GE, K. and THIELE, C.J., 2012. EZH2 Mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR. *Cancer research,* **72**(1), pp. 315-324.

WANG, J.C. and DICK, J.E., 2005. Cancer stem cells: lessons from leukemia. *Trends in cell biology,* **15**(9), pp. 494-501.

WARNER, D.R., GREENE, R.M. and PISANO, M., 2014. PRDM16 in Development and Disease. *Human Genetics & Embryology,* **2014**.

WEST, R.R., STAFFORD, D.A., WHITE, A.D., BOWEN, D.T. and PADUA, R.A., 2000. Cytogenetic abnormalities in the myelodysplastic syndromes and occupational or environmental exposure. *Blood,* **95**(6), pp. 2093-2097.

WHICHARD, Z.L., SARKAR, C.A., KIMMEL, M. and COREY, S.J., 2010. Hematopoiesis and its disorders: a systems biology approach. *Blood,* **115**(12), pp. 2339-2347.

WILSON, A. and TRUMPP, A., 2006. Bone-marrow haematopoietic-stem-cell niches. *Nature reviews.Immunology,* **6**(2), pp. 93-106.

WILSON, A., LAURENTI, E., OSER, G., VAN DER WATH, R.C., BLANCO-BOSE, W., JAWORSKI, M., OFFNER, S., DUNANT, C.F., ESHKIND, L., BOCKAMP, E., LIÓ, P., MACDONALD, H.R. and TRUMPP, A., 2008. Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell,* **135**(6), pp. 1118-1129.

WUYTS, W., VAN HUL, W., DE BOULLE, K., HENDRICKX, J., BAKKER, E., VANHOENACKER, F., MOLLICA, F., LÜDECKE, H., SAYLI, B.S. and PAZZAGLIA, U.E., 1998. Mutations in the EXT1 and EXT2 genes in hereditary multiple exostoses. *The American Journal of Human Genetics,* **62**(2), pp. 346-354.

XIA, P., XU, H., SHI, Q. and LI, D., 2016. Identification of a novel frameshift mutation of the EXT2 gene in a family with multiple osteochondroma. *Oncology Letters,* **11**(1), pp. 105-110.

XIA, H., QI, H., LI, Y., PEI, J., BARTON, J., BLACKSTAD, M., XU, T. and TAO, W., 2002. LATS1 tumor suppressor regulates G2/M transition and apoptosis. *Oncogene,* **21**(8), pp. 1233-1241.

XU, W., TAN, L. and YU, J., 2015. The Role of PICALM in Alzheimer's Disease. *Molecular neurobiology,* **52**(1), pp. 399-413.

YANG, X., LI, J., LIU, B., LI, Y. and JIANG, T., 2016. Impact of PICALM and CLU on hippocampal degeneration. *Human brain mapping,* .

YEUNG, A.W., TERENTIS, A.C., KING, N.J. and THOMAS, S.R., 2015. Role of indoleamine 2,3-dioxygenase in health and disease. *Clinical science (London, England : 1979),* **129**(7), pp. 601-672.

ZHANG, L., PADRON, E. and LANCET, J., 2015. The molecular basis and clinical significance of genetic mutations identified in myelodysplastic syndromes. *Leukemia research,* **39**(1), pp. 6-17.

ZIERHUT, H.A., TRYON, R. and SANBORN, E.M., 2014. Genetic Counseling for Fanconi Anemia: Crosslinking Disciplines. *Journal of genetic counseling,* **23**(6), pp. 910-921.

ZOU, Y.S., FINK, S.R., STOCKERO, K.J., PATERNOSTER, S.F., SMOLEY, S.A., TUN, H.W., REEDER, C.B., TEFFERI, A. and DEWALD, G.W., 2007. Efficacy of conventional cytogenetics and FISH for EGR1 to detect deletion 5q in hematological disorders and to assess response to treatment with Lenalidomide. *Leukemia research,* **31**(9), pp. 1185-1189.

# Appendix A. Reagent names and manufacturers

| Reagent | Manufacturer |
|---|---|
| Chang Medium BMC® | Irvine Scientific, Irvine, CA |
| Colcemid® | Irvine Scientific, Irvine, CA |
| 1XTrypsin-EDTA | Irvine Scientific, Irvine, CA |
| Methyl Alcohol | Mallinckrodt Pharmaceutical |
| Glacial Acetic Acid | Mallinckrodt Pharmaceutical |
| Hanks Balanced Salt Solution | Irvine Scientific, Irvine, CA |
| Wright's Stock Solution | Sigma-Aldrich, St. Louis, MO |
| 6.8 Gurr's Buffer | BDH Laboratory, Poole, England |
| LSI® EGR-1 (5q31) DNA Probe | Abbott-Vysis, Abbott Park, IL |
| D5S23, D5S721 (5p15.2) DNA Probe | Abbott-Vysis, Abbott Park, IL |
| D7S486 (7q31) DNA Probe | Abbott-Vysis, Abbott Park, IL |
| CEP 7(D7Z1) DNA Probe | Abbott-Vysis, Abbott Park, IL |
| LSI® D20S108 (2oq12) DNA Probe | Abbott-Vysis, Abbott Park, IL |

| | |
|---|---|
| Cytocell Del(20q) Deletion Probe | Cytocell, Cambridge, UK |
| CEP 8 (D8Z1) DNA Probe | Abbott-Vysis, Abbott Park, IL |
| 0.4% Sodium Chloride and Sodium Citrate (SSC) | Sigma-Aldrich, St. Louis, MO |
| 0.3% Nonidet P-40 (NP-40) | Abbott-Vysis, Abbott Park, IL |
| 4,6-diamidino-2-phenylindole (DAPI II) in Antifade Solution | Abbott-Vysis, Abbott Park, IL |
| CytoScan® Nuclease-Free Water | Affymetrix, Santa Clara, CA |
| CytoScan® 10X Nsp I Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® 100X BSA | Affymetrix, Santa Clara, CA |
| CytoScan® Nsp I Enzyme | Affymetrix, Santa Clara, CA |
| CytoScan® 10X T4 DNA Ligase Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® 50µM Nsp I Adaptor | Affymetrix, Santa Clara, CA |
| CytoScan® T4 DNA Ligase | Affymetrix, Santa Clara, CA |
| 10X Titanium™ *Taq* PCR Buffer | Clontech Laboratories, Takara Bio Company, Mountain View, CA |
| GC-Melt Reagent | Clontech Laboratories, Takara Bio Company, Mountain View, CA |

| | |
|---|---|
| dNTP Mixture (2.5mM each) | Clontech Laboratories, Takara Bio Company, Mountain View, CA |
| PCR Primer (002) | Clontech Laboratories, Takara Bio Company, Mountain View, CA |
| 50X Titanium™ *Taq* DNA Polymerase | Clontech Laboratories, Takara Bio Company, Mountain View, CA |
| 2% TBE Precast Gel | Lonza Group LTD, Switzerland |
| 1% TBE Precast Gel | Lonza Group LTD, Switzerland |
| USB PCR Marker 50-2000bp Ladder | Affymetrix, Santa Clara, CA |
| CytoScan® Purification Beads | Affymetrix, Santa Clara, CA |
| CytoScan® Purification Wash Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® Elution Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® 10X Fragmentation Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® Fragmentation Reagent | Affymetrix, Santa Clara, CA |
| TrackIt™ 25bp DNA Ladder | Life Technologies, Carlsbad, CA |
| CytoScan® 5X TdT Buffer | Affymetrix, Santa Clara, CA |
| CytoScan® 30mM DNA Labeling Reagent | Affymetrix, Santa Clara, CA |

| | |
|---|---|
| CytoScan® TdT Enzyme | Affymetrix, Santa Clara, CA |
| CytoScan® Hyb Buffer Part 1 | Affymetrix, Santa Clara, CA |
| CytoScan® Hyb Buffer Part 2 | Affymetrix, Santa Clara, CA |
| CytoScan® Hyb Buffer Part 3 | Affymetrix, Santa Clara, CA |
| CytoScan® Hyb Buffer Part 4 | Affymetrix, Santa Clara, CA |
| CytoScan® Oligo Control Reagent 0100 | Affymetrix, Santa Clara, CA |
| GeneChip® Wash A | Affymetrix, Santa Clara, CA |
| GeneChip® Wash B | Affymetrix, Santa Clara, CA |
| GeneChip® Stain Buffer 1 | Affymetrix, Santa Clara, CA |
| GeneChip® Stain Buffer 2 | Affymetrix, Santa Clara, CA |
| GeneChip® Array Holding Buffer | Affymetrix, Santa Clara, CA |
| OncoScan® Somatic Mutation Probe Mix 1.0 | Affymetrix, Santa Clara, CA |
| OncoScan® Copy Number Probe Mix 1.0 | Affymetrix, Santa Clara, CA |
| OncoScan® Positive Control | Affymetrix, Santa Clara, CA |
| OncoScan® Negative Control | Affymetrix, Santa Clara, CA |

| | |
|---|---|
| OncoScan® Buffer A | Affymetrix, Santa Clara, CA |
| OncoScan® dNTPs (A/T) | Affymetrix, Santa Clara, CA |
| OncoScan® dNTPSs (G/C) | Affymetrix, Santa Clara, CA |
| OncoScan® SAP, Recombinant (1U/uL) | Affymetrix, Santa Clara, CA |
| OncoScan® Gap Fill Enzyme Mix | Affymetrix, Santa Clara, CA |
| OncoScan® Cleavage Buffer | Affymetrix, Santa Clara, CA |
| OncoScan® Cleavage Enzyme | Affymetrix, Santa Clara, CA |
| OncoScan® PCR Mix | Affymetrix, Santa Clara, CA |
| OncoScan® Taq Polymerase | Affymetrix, Santa Clara, CA |
| OncoScan® Buffer B | Affymetrix, Santa Clara, CA |
| OncoScan® HaeIII Enzyme | Affymetrix, Santa Clara, CA |
| OncoScan® Exonuclease I | Affymetrix, Santa Clara, CA |
| 3% TBE Precast Gel | Lonza Group LTD, Switzerland |
| NEB Low Molecular Weight Ladder | New England Biolabs, Ipswich, Massachusetts |
| OncoScan® Hybridization Mix | Affymetrix, Santa Clara, CA |

# Appendix B. Equipment product names and manufacturers

| Equipment/Supplies | Manufacturer |
|---|---|
| Thermatron Drying Chamber CDS-5® | Venturedyne, Ltd., Holland, MI |
| HYBrite™ | Abbott-Vysis, Abbott Park, IL |
| ThermoBrite™ | Abbott-Vysis, Abbott Park, IL |
| QIAcube® Automated Robot | Qiagen, Redwood City, CA |
| Qubit 3.0™ Fluorometer Instrument | ThermoFisher Scientific, Waltham, MA |
| GeneChip® Scanner 3000 7G | Affymetrix, Santa Clara, CA |
| GeneAmp™ PCR System 9700 | Applied Biosystems, Waltham, MA |
| MagnaRack Magnetic Stand | Life Technologies, Carlsbad, CA |
| NanoDrop® Spectrophotometer ND-1000 | NanoDrop Technologies, Inc, Wilmington, DE |
| 1/2" Microtube Tough-Spots | Diversified Biotech, Boston, MA |
| GeneChip® Fluidics Station 450 | Affymetrix, Santa Clara, CA |

# Appendix C. Software product name and manufacturers

| Software | Manufacturer |
|---|---|
| CytoVision® Image Analysis System | Leica Biosystems, Buffalo Grove, IL |
| Chromosome Analysis Suite (ChAS) Software | Affymetrix, Santa Clara, CA |
| GeneChip® Command Center® 3.2 | Affymetrix, Santa Clara, CA |