Clinical impact of genomic characterization of 15 patients with acute megakaryoblastic leukemia related malignancies

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Abstract

Background: Acute megakaryoblastic leukemia (AMKL) is a rare subtype of acute myeloid leukemia but is ˜500 times more likely to develop in children with Down syndrome (DS) thro

INTRODUCTION

Acute megakaryoblastic leukemia (AMKL) is a rare subtype of acute myeloid leukemia (AML), defined by the presence of at least 50% of blasts from the megakaryocytic lineage, and patients often present with thrombocytopenia or thrombocytosis. Other clinical features include dysplastic neutrophils, erythroid cells, and germ cell tumors in young men. AMKL is typically observed in children, accounting for 4-15% of AML cases, compared to <1% of adult patients. A diagnosis of AMKL can be made if the pathognomonic t(1;22)(p13.3;q13.3) translocation is observed, resulting in a *RBM15-MKL1* gene fusion, or by pathological assessment of bone marrow as described above. However, recent studies have shown that AMKL is genetically heterogeneous with distinct sub-groups based on cytogenetic and molecular alterations.1,2

Children with Down syndrome (DS) have an estimated 150- to 500-fold increased risk of AMKL compared to children without DS.3,4 In utero *GATA1* mutations drive the development of transient abnormal myelopoiesis (TAM) through impaired megakaryocytic differentiation.2,5 While most patients undergo spontaneous resolution of TAM within weeks of diagnosis, a small subset of high-risk patients presenting with life-threatening signs such as respiratory impairment, hepatic dysfunction and/or leukocytosis (WBC>100 000) may not survive.6 A further 20% of patients with TAM will go on to develop AMKL due to accumulation of additional genetic mutations and clonal expansion.7,8

Genetic analysis of non-DS AMKL has identified additional recurrent gene fusions in addition to the pathognomonic *RBM15-MKL1* fusion. Next-generation sequencing (NGS) and targeted analysis of genes commonly altered in myeloid malignancies revealed a number of recurrent genetic rearrangements that evaded cytogenetic detection.9,10 Evaluation of 89 pediatric patients with non-DS AMKL using NGS revealed an inversion on chromosome 16 associated with a *CBFA2T3-GLIS2* gene fusion in 18% of patients, rearrangements involving *KMT2A* in 17% of patients, rearrangements of the *HOX* gene cluster in 14% patients, and a t(11;12) translocation resulting in a *NUP98-KDM5A* gene fusion in 11.5% patients.9 Interestingly, the pathognomonic t(1;22)(p13.1;q13.3) translocation is observed in only 10% patients and acquired GA*TA1 mu* tations which arise in all DS-TAM are observed in 9% patients of non-DS AMKL. In addition to the stark differences in genomic profiles between DS-AMKL and non-DS AMKL, the latter is also associated with significantly worse prognosis and higher mortality rates.1,11

The recurrent genetic features carry different diagnostic, prognostic, and therapeutic implications, making comprehensive genetic testing critical for optimal patient management. We present fifteen patients with AMKL-related malignancies that illustrate various clinical scenarios and genomic strategies enabling accurate diagnosis and prognostication of AMKL.

METHODS

Patient characteristics

Patients were identified by pathological diagnosis and/or molecular findings. Comprehensive genomic evaluation using a large NGS panel for DNA and RNA has been offered at the Children's Hospital of Philadelphia since 2016. This clinical cohort was mined for patients with a pathological or molecular diagnosis of AMKL, DS-TAM or DS-AMKL. Specifically, the pathology reports of patients having undergone genomic profiling were mined for the words "AMKL", "megakaryoblastic", "CD41" or "CD61". Additionally, an internal genomic database was mined for clinical indications related to AMKL, DS-TAM or DS-AMKL, and for genetic findings associated with these malignancies, including *GATA1* mutations and the recurrent gene fusions observed in AMKL. This study was approved by the Institutional Review Board of the Children's Hospital of Philadelphia.

Cytogenetics

Chromosome analysis and fluorescence in situ hybridization (FISH) studies were performed according to standard protocols. Briefly, unstimulated bone marrow specimen were cultured and harvested after overnight incubation. G-banding metaphases were prepared using trypsin digestion followed by Giesma staining. A minimum of 20 cells were analyzed. FISH studies were performed using a panel of probes for AML and additional probes may be added based on clinical indication, where appropriate, to rule-out recurrent abnormalities.

Next-generation sequencing panel

The Comprehensive Hematological Cancer Panel includes sequence and copy number analyses of 99 (version 1) to 118 (version 2) cancer genes, and gene fusion detection involving one of 106 (version 1) to 110 (version 2) possible partner genes (exact number depends on date of service). The genes included in the current panel are listed at

(*https://apps.chop.edu/service/laboratories/olsd.cfm/division-genomic-diagnostics*). DNA and RNA extraction, sequencing and analysis was performed as previously reported.12,13 Briefly, in-house scripts are used to identify and annotate single nucleotide variants (SNVs) and small insertion-deletions (indels) detected within exonic regions +/- 10bp flanking regions and intonic regions with known mutations, and copy number variants (CNVs) are analyzed using NextGENe v2 NGS Analysis Software (Softgenetics, State College, PA). Gene fusions are detected using Archer^{TM} Analysis according to standard protocols. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Somatic mutations are classified using criteria consistent with those recommended by the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists.14 In brief, Tier 1 variants are actionable somatic variants with well-established evidence for diagnostic, prognostic or therapeutic implications. Tier 2 variants represent potentially actionable somatic variants. Tier 3 and 4 variants represent variants of unknown significance and likely benign/benign variants, respectively. Only Tier 1-2 variants are reported. Variants in *GATA1* are reported based on transcript NM_002049.3, and transcripts for remaining variants described are listed in Supplementary Table 1.

RESULTS

Non-DS AMKL

Six patients were diagnosed with AMKL unrelated to DS, of which five presented between 1 to 2 years of age, with physical symptoms including fevers, bruising, rashes, and cytopenia (Table 1). The sixth patient

presented at 11 years of age with persistent thrombocytopenia during routine follow-up 5 years post low-risk B-cell acute lymphoblastic leukemia (B-ALL) treatment. Clinical history for all patients is summarized in Table 1. Pathological evaluation was suggestive of AMKL in 4 of 6 cases, but genetic testing was required for diagnosis in patients 1 and 3 (see Discussion).

All six patients had comprehensive genomic evaluation (Fig. 1, Table 2), including cytogenetics and comprehensive NGS analyses. Cytogenetic studies revealed multiple changes in all patients, including complex, multi-clonal karyotypes in patients 1, 2 and 6 (Supplementary Table 2). Only patient 4 had a cytogenetic finding highly suggestive of an AMKL-related translocation, a pericentric inversion associated with *CBFA2T3-GLIS2.* In contrast, in all six patients a gene fusion of diagnostic significance was detected using the NGS panel, including one patient with *NUP98-KDM5A* (Fig. 2), one patient with *KMT2A -MLLT6* , and four patients with *CBFA2T3-GLIS2* (Fig. 3). Interestingly, only two patients had clinically significant somatic mutations, both of which were in *SETD2* , a methyltransferase commonly altered in leukemias, typically associated with poor prognosis and may confer chemoresistance (Table 2).15 Overall, the genomes of these leukemias are characterized by large structural changes resulting in often cryptic, recurrent gene fusions with a relatively low mutational burden (Fig. 1). All five patients with follow-up information available underwent bone marrow transplant due to the high-risk of relapse.

Down syndrome related transient abnormal myelopoiesis (DS-TAM)

Four patients in our cohort were diagnosed with DS-TAM, and presented between 2 days to 6 weeks of life (Table 1). As expected, a *GATA1* SNV was observed in each patient, and no fusion events were observed (Table 2, Fig. 4). The *GATA1* mutations were present at high variant allele fraction (VAF), ranging from 42 – 95%.

Despite similar genetic profiles, significantly different clinical outcomes were observed (clinical details not available for patient 7). Patients 8 and 9 presented with high-risk features including leukocytosis and respiratory distress and were thus treated with low-dose cytarabine according to standard protocols.6 Patient 8 developed significant co-morbidities including ascites, hepatic dysfunction and disseminated intravascular coagulation, and ultimately passed away at 3 months of age due to multi-organ failure secondary to his complete atrioventricular canal. Patients 9 and 10 remain leukemia-free at 20 months and 1 year of age, respectively.

Down syndrome-related AMKL (DS-AMKL)

In contrast to the patients with DS-TAM, the five patients diagnosed with DS-AMKL presented as toddlers (14 months to 25 months), and only patient 12 had a known prior history of DS-TAM. Patients were treated according to the Children's Oncology Group (COG) AAML1531 protocol, and all patients remain disease-free (follow-up time 8.5-30 months).

The genetic profiles in these patients were distinct from patients with DS-TAM or AMKL (Fig. 1). Although all patients had *GATA1* mutations, they also had additional molecular and/or cytogenetic changes (range 2 – 4 additional changes; Table 2). Two patients had trisomy 8, patient 12 had other cytogenetic changes, and all five patients had sequence mutations in pathways known to be involved in DS-AMKL, including two with frameshift mutations in *CTCF* . The other mutations involve genes in the RAS pathway (*JAK1, JAK2, NRAS, PTPN11*), cohesin complex (*RAD21),* epigenetic regulators (*SUZ12*), and spliceosome components (*SRSF2)* .

DISCUSSION

In our cohort, the three groups of patients displayed distinct genomic profiles (Fig. 1). Non-DS AMKL is characterized by large cytogenetic changes, often with complex genomes, recurrent gene fusions, and a paucity of sequencing mutations. DS-TAM and DS-AMKL are characterized by trisomy 21 and *GATA1* frameshift mutations with additional cytogenetic and sequence mutations observed in DS-AMKL exclusively.

Genomic-guided management in non-DS AMKL

While a diagnosis of non-DS-AMKL has been historically associated with poor prognosis, these patients are clinically heterogeneous and recent studies identified recurrent genomic changes which delineate patients into distinct prognostic groups. Inaba and colleagues defined 3 risk groups based on karyotypes, with high-risk patients having normal karyotypes, –7, 9p abnormalities, including t(9;11)(p22;q23), –13/13q–, or –15 (16). Low-risk patients have 7p abnormalities while the remaining patients are considered intermediate risk. More recently, recurrent gene fusions have been used to define prognosis with *NUP98-KDM5A* ,*CBFA2T3-GLIS2* , and *KMT2A* rearrangements being associated with poor prognosis, *HOX* rearrangements and *RBM15-MKL1* are associated with intermediate prognosis, and *GATA1* mutations are associated with good prognosis.9,11 Current risk stratification in COG is based on specific cytogenetic/molecular features with allogenic HSCT during the first remission for patients with high-risk AML.

The AMKL-associated gene fusions have been shown to be important drivers of leukemogenesis associated with distinct transcriptional programs and clinical outcomes.9 For instance, both *CBFA2T3* and *GLIS2*are transcriptional regulators, and the *CBFA2T3-GLIS2* fusion results in increased hedgehog, JAK-STAT and growth factor signaling pathways leading to enhanced self-renewal of hematopoietic stem cells.10,17 Interestingly, we identified this fusion in 4 of 6 patients with non-DS AMKL, indicating it may be more common than previously thought in pediatric patients, although our study is limited in size (see Study Limitations below). Our panel may have a higher detection rate for this fusion due to the open-ended PCR technology used and increased sensitivity of using a targeted fusion panel as opposed to whole transcriptome sequencing which may be less efficient for fusions with lower expression.

In some cases a diagnosis of AMKL cannot be achieved based on morphological or immunophenotypic information alone, and comprehensive genomic evaluation can reveal recurrent AMKL-associated mutations allowing for an integrated diagnosis of AMKL. Patients 1 and 3 did not receive a definitive diagnosis until genetic testing revealed*NUP98-KDM5A* and *CBFA2T3-GLI3* gene fusions, respectively. Patient 1 was initially diagnosed with undifferentiated AML and upon identification of the fusion gene, his diagnosis was changed to AMKL. Additionally, the complex cytogenetic aberrations observed further confirmed a poor prognosis for this patient. For patient 3, while flow cytometry revealed megakaryocytic differentiation, he presented with a temporal sarcoma which complicated diagnosis. Evaluation of bone marrow and correlation with genetic features confirmed a diagnosis of AMKL. Because children with AML and *NUP98* or *CBFA2T3* fusions treated with chemotherapy alone have poor relapse-free survival 11, both patients underwent a matched unrelated donor bone marrow transplant.

Significance of genomic evaluation in patients with DS

Even within DS-related myeloid malignancy, the clinical heterogeneity is striking. Despite the fact that all patients with DS-TAM acquire*GATA1* mutations, up to two thirds remain asymptomatic (*i.e.* silent TAM), while the remaining third (˜10% of newborns with DS) develop clinical TAM.18,19 In most patients, TAM resolves spontaneously, however in 20-30% of patients, TAM transforms to MDS or AML, most commonly AMKL, 1 to 3 years later. One theory postulates that the initiating *GATA1* clone occurs in tissues of fetal hematopoeisis, which could explain the spontaneous remission observed in most patients with DS-TAM within the first year of life, when bone marrow hematopoeisis takes over.2 If additional driver mutations arise in these clones prior to extinction of fetal hematopoeisis stem cells, there is risk of AMKL. In our cohort, only one of the five patients with DS-AMKL was known to have TAM prior to their AMKL diagnosis, suggesting the remaining four had silent TAM and accumulated additional driver mutations prior to extinction of fetal hematopoeisis stem cells. Interestingly, eight of the nine patients with DS-TAM or DS-AMKL in this cohort are male, consistent with previous studies which noted a trend towards sex bias in development of DS-TAM/AMKL.20,21

The acquired *GATA1* mutations in TAM/AMKL are predominantly frameshift mutations in exon 2, resulting in expression of a shorter isoform due to an alternative translation initiation codon at residue 84 thereby skipping the frameshift mutations and the boundary between exon 2 and intron 2. These mutations may be embryonic lethal as they have rarely been reported as germline changes.22 (22). In contrast, pathogenic germline variants in *GATA1* are mostly missense mutations in exon 4 encoding the N-f protein domain, and

are associated with X-linked anemia, neutropenia, thrombocytopenia, and platelet abnormalities, but not leukemias (Fig. 4).(23). 23

All DS-AMKL harboured additional somatic mutations, consistent with a prior exome sequencing study showing a higher mutation burden in individuals with DS-AMKL compared to DS-TAM.8 All patients with DS-AMKL had at least one additional driver mutation with an enrichment in cohesion genes, epigenetic regulators, *CTCF* , and other critical signaling pathway genes.2,7 Here, we identify the second AMKL patients with somatic mutations in *SUZ12* and *SRSF2* , respectively.8 Of note, two *GATA1* mutations were observed in patient 14 (DS-AMKL), which likely arose independently in distinct clonal populations; indeed visualization in IGV confirmed that these two variants were mutually exclusive. This finding is consistent with the theory that cells with trisomy 21 have a "mutator phenotype" specific to certain loci such as *GATA1* .19 In two published individuals with more than one *GATA1* mutation and genetic profiling at the time of DS-TAM and AMKL diagnoses, the minor TAM subclone expanded and acquired additional mutations to become the major AMKL clone.7,8

Identifying patients with DS-TAM at greatest risk of AMKL is not evident a priori as these patients have indistinguishable pathology at the time of initial diagnosis. The natural history study by the COG evaluated multiple clinical risk factors and only time to TAM resolution reached border-line significance with leukemia-free survival.6 Of note, neither *GATA1* mutation type nor diagnostic blast count is associated with progression to AMKL.2,6 An outstanding question is whether serial, high-depth sequencing of genes involved in TAM to AMKL progression may help in early detection of DS-AMKL transformation.

Importance of comprehensive evaluation

Accurate and timely diagnosis and prognostication require multiple molecular and cytogenetic techniques, combining fast turnaround times with thorough evaluation. At the moment, clinical laboratories utilize various techniques to offer such comprehensive testing; however RNA studies which are required to detect most AMKL-related gene fusions are not considered standard of care.

Detection of *GATA1* mutations is important for diagnosis of TAM, especially "silent" TAM where infants have a small fraction of clones with *GATA1* mutations but no clinical phenotype.19 These infants are at increased risk of developing DS-AMKL. Pending studies will determine whether periodic mutation screening can improve outcomes for these infants with "silent" TAM and thus the clinical utility of screening for *GATA1* mutations in all patients with DS.2,19

Cytogenetic evaluation can rapidly identify canonical chromosomal rearrangements associated with non-DS AMKL as well as identify prognostic structural changes. For example, a pericentric inversion of chromosome 16 was detected with cytogenetics in the bone marrow of patient 4, strongly suggesting a *CBFA2T3-GLIS2* gene fusion which was subsequently confirmed with the NGS RNA panel. However, many of the recurrent rearrangements associated with AMKL are cytogenetically cryptic and were detected using the RNA sequencing only. Indeed, despite four patients harboring the recurrent *CBFA2T3-GLIS2* with standard breakpoints, only one displayed the typical cytogenetic mechanism, a pericentric inversion 16 (patient 4, Fig. 3). In the remaining two remaining patients with this fusion with karyotype analysis, one was caused by a balanced translocation between the two chromosome 16 homologs, and the other likely involved a complex rearrangement in which only a balanced translocation between chromosomes 16 and 19 could be observed (patient 5).

In summary, high-throughput DNA and RNA profiling is required for optimal management of AMKL-related malignancies, and cytogenetic studies can provide diagnostic results in a short timeframe and may help to reveal underlying mechanism of genomic alterations.

Study limitations

One caveat to this study is the possibility of missing fusion genes involving genes of the *HOX* gene cluster which are not included in the current RNA panel. However, some partner genes that have been observed in *HOX* fusions are included, such as *EWSR1* . Based on prior studies using unbiased transcriptomic profiling,

it is expected that ˜15% of patients with non-DS AMKL carry *HOX* rearrangements, which are associated with intermediate prognosis. Alternatively, our cohort may be biased towards patients with aggressive disease referred to the Children's Hospital of Philadelphia. This is supported by the lack of patients with genetic features associated with good (*GATA1* somatic mutations) or intermediate prognosis (*HOX* -rearrangements and *RBM15-MKL1* fusion). Indeed, electronic medical record search for pathological features of AMKL did not reveal any additional patients.

CONCLUSION

AMKL-related malignancies display both clinical and genomic heterogeneity. Recurrent molecular and cytogenetic changes can predict prognosis and guide management decisions. Genomic diagnostic strategies must include integrated genomic evaluation to identify these critical genetic changes and inform personalized patient care.

CONFLICT OF INTERESTS

MML is on the SAB of Roche Sequencing Solutions. The rest of the authors disclose no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Genomic profile of ten patients with acute megakaryocytic leukemia (AMKL). Patients with AMKL are defined by recurrent gene fusions, while patients with Down Syndrome (DS) transient myeloproliferative disease (TAM) have *GATA1* mutations with high variant allele fraction (VAF), and patients with DS-AMKL have additional somatic variants in signaling pathway genes. Only variants classified as Tier 1-2 are listed. See Table 2 for complete genomic profile of each individual and Supplementary Table 1 for detailed variant information.

Figure 2. Complex cytogenetics observed in patient 1. The stemline is shown on the right, which was observed in 11 of 21 cells (see Supplementary Table 2 for complete nomenclature). FISH studies demonstrate the ring chromosome identified by karyotype originates from chromosome 3 and contains at least the 3q26 locus (EVI1 gene). The EVI1 probe also localized to a derivative chromosome 21 which was involved in a three-way translocation with chromosomes 3 and 19. This was also observed with FISH for RUNX1 (21q22.12)

which localized both to the derivative chromosome 21 and a derivative chromosome 19. Finally, a terminal deletion of 20q was confirmed in 144/200 interphase cells.

Figure 3. Diverse cytogenetic mechanisms resulting in *CBFA2T3-GLIS2* gene fusions. A) Representative cytogenetic and FISH images used to identify chromosome 16 abnormalities in patients 2, 4 and 5. See Supplementary Table 2 for complete nomenclature. Cytogenetics are not available for patient 3. B) Representative screenshot from the Archer software demonstrating a *CBFA2T3-GLIS2* gene fusion between exons 11 and 3 of *CBFA2T3* and *GLIS2* , respectively (i.e. C11G3). The same breakpoint was observed in all four patients.

Figure 4. *GATA1* variant spectrum in germline and somatic setting. A) *GATA1* variants listed in ClinVar as Pathogenic or Likely Pathogenic or in HGMD as "disease-associated" for hereditary conditions. B) *GATA1* sequence variants listed in COSMIC identified in hematopoietic or lymphoid tissues. Intronic variants which may affect splicing are not included. Numbering based on RefSeq transcript NM_002049.3.

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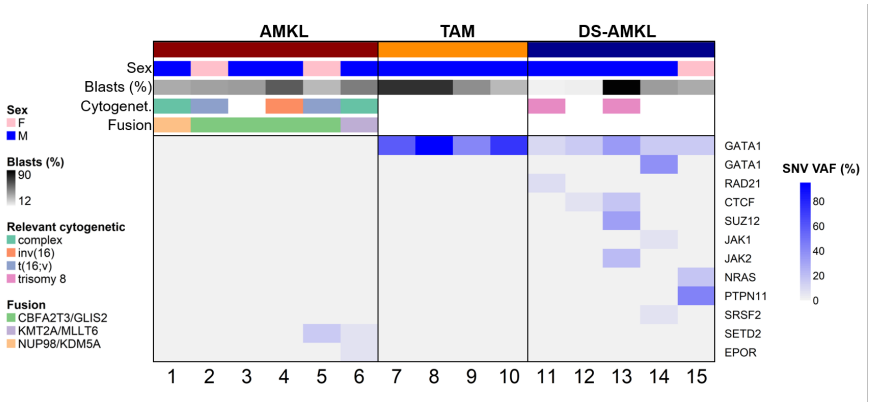

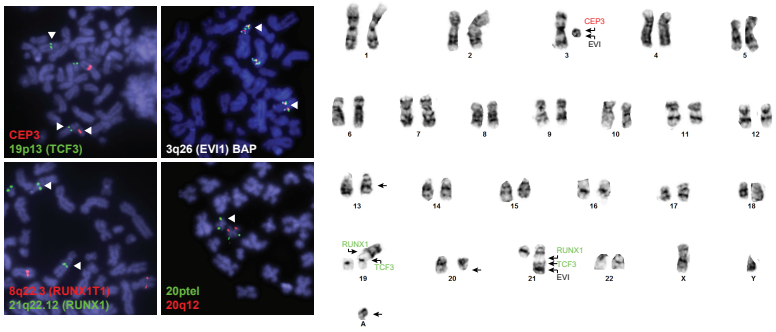

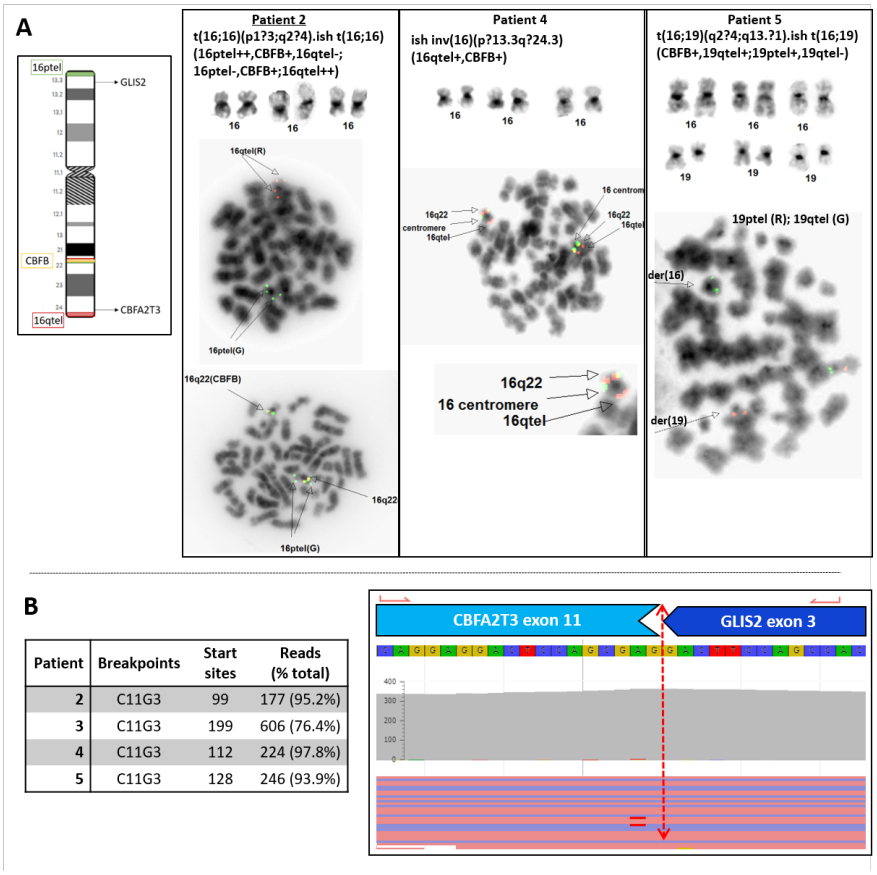

Patient	Breakpoints	Start sites	Reads (% total)
2	C11G3	99	177 (95.2%)
3	C11G3	199	606 (76.4%)
4	C11G3	112	224 (97.8%)
5	C11G3	128	246 (93.9%)