F．研究発表

1. 論文発表

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2. 学会発表

なし

G．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患等克服研究事業（難治性疾患克服研究事業））
分担研究報告書

遺伝性貧血の病態解明と診断法の確立に関する研究

Fanconi 貧血の診断・診断ガイドラインの作成

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研究要旨：Fanconi 貧血（FA）は種々の身体異常と小児期から進行する骨髄不全、白血化や高発がんを特徴とする遺伝性疾患である。染色体脆弱検査と骨髄不全症や白血化、固形腫瘍の発症等の臨床症状および FANCD2 のモノユビキチン化や、multiplex Ligation-dependent Probe Amplification（MLPA）法と京都大学放射線生物研究センターの高田穣研究室における FA 遺伝子のゲノムシーケンス等により FA 診断を試みた。次世代シーケンスの導入により異常遺伝子の解析の精度は飛躍的に向上したが、片アレルのみの検出例や変異が確認出来ない症例もみられ、染色体脆弱性と臨床症状を照合した診断が重要である。

A．研究目的

Fanconi 貧血（FA）の身体症状、臨床症状は一様ではなく、原因遺伝子も現在では 16 という多数の遺伝子が同定されている。FA の造血不全の唯一の治療法は造血細胞移植であり、アルキル化剤を中心とした移植前処置に用いられる薬剤に過敏性があるため、適切な移植方法を選択するためにも確実な診断が必要である。染色体断裂試験、FANCD2 モノユビキチン化試験、MLPA 法および FA 遺伝子解析に臨床所見を加えて診断の検討を行い、適切な診断ガイドラインの作成することを目的とした。

B．研究方法

身体異常および骨髄不全、固形腫瘍の発症等の臨床所見、染色体脆弱試験、FANCD2 のモノユビキチン化、FANCA の MLPA 法に京都大学放射線生物研究センター高田穣研究室にて解析された FA 遺伝子の変異を合わせて検討した。

（倫理面への配慮）

「ヒトゲノム・遺伝子解析研究に関する倫理指針」と「臨床研究に関する倫理指針」を順守し、インフォームドコンセントに基づいた科学的にも倫理的にも妥当な研究の計画と実施している。説明同意書には検体の使用および保存中止請求書類も加え、遺伝子カウンセリングの体制も整えている。また、平易な文面で記載された小児用の説明書も作成し、家族だけではなく患児の理解や同意を得る努力を行っている。

C．研究結果

骨髄異形成症候群や急性骨髄性白血病への進行は 37 例にみられ、移植後も含めて 14 例に固形腫瘍の発症がみられた。うち、2 例は骨髄不全の発症はみられず、乳幼児期に固形腫瘍を発症した。ほか、舌がん、食道がんなどの頭頸部がんが多く、いずれも 20 代から 40 代前半の若年発症であった。また、成人の 2 例においては骨髄不全の程度は極めて軽度であり、免疫異常が疑われ、診断には注意を要する。MLPA 法を用いた 61 症例の検討では、36 例が FANCA シーケンスで A 群と断定され、そのうちの 24 例が A 群 MLPA 法にて片アレルまたは両アレルの検出が可能であった（66.3%）。MLPA 法での検出はリンパ球、骨髄細胞などの造血細胞だけでなく、皮膚・骨髄線維芽細胞でも同等に検出が可能であった。東海大学における総計 80 例の日本人 FA の遺伝子解析では、FA 遺伝子のゲノムシーケンスより 35 例の FANCA と 20 例の FANCG 遺伝子の変異が京都大学放射線生物研究センターの高田穣研究室にて同定された。欧米諸国に比較的多くみられる FANCC は 1 例も検出されず、FANCD1, FANCE, FANCP も各 1 例確認された。既知遺伝子が全く検

出されなかった症例は6例（7.5%）認められた。

D．考察

MLPA法での検出はリンパ球、骨髄細胞などの造血細胞だけでなく、皮膚・骨髄線維芽細胞でも同等に検出が可能であり、A群のMLPA法はDNAを抽出すれば既知の変異であれば、約2/3の症例においては同定が可能であり、迅速な診断が期待される。次世代シーケンス導入により、異常遺伝子の解析の精度は飛躍的に向上したが、片アレルのみの検出例や変異の異常が確認出来ない症例もみられ、染色体脆弱性と臨床症状を照合した診断が重要である。

E．結論

臨床像のみあるいは遺伝子解析でのみで本疾患を確定診断するのは困難である。小児期に発症した再生不良性貧血患者や若年発症の頭頸部、婦人科領域の固形がんの患者には、全例にDNA架橋剤添加による染色体断裂試験を行い、FAを除外することが望まれる。乳幼児期における固形腫瘍の発症で身体異常を有する症例や、骨髄不全が軽度で免疫異常が疑われ身体異常を伴う場合には、FAの可能性があり注意を要する。

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G. 知的財産権の出願・登録状況

1. 特許取得
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2. 実用新案登録
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分担研究報告書

遺伝性貧血の病態解明と診断法の確立に関する研究

ゼブラフィッシュを用いたDBAの遺伝子解析

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研究要旨：リボソームの生合成に関与する因子の異常と遺伝性貧血との関連が注目されている。ダイアモンド・ブラックファン貧血の患者で新たに同定されたリボソームタンパク質遺伝子 *RPL27* の変異と疾患発症との関連を調べるために、ゼブラフィッシュで *RPL27* のスプライシングを阻害した。その結果、成長不良や尾部の屈曲などの形態異常および造血の著しい低下が示され、*RPL27* の変異が疾患発症に大きく関わっている可能性が明らかになった。

A．研究目的

リボソームの生合成には200種類以上もの因子が関与し、これらをコードする遺伝子に変異が確認された疾患はリボソーム病と呼ばれる。そのひとつであるダイアモンド・ブラックファン貧血（DBA）の患者では、リボソームタンパク質（RP）S19遺伝子の変異が最も多くみられ、その割合は約25%を占める。さらに、10種類のRP遺伝子の変異も報告されているが、未だに4割の患者では責任遺伝子が同定されていない。本研究では、DBA患者のエキソーム解析で新たに発見されたDBA候補遺伝子 *RPL27* について、ゼブラフィッシュを用いた機能解析を行うことで疾患発症との関連を明らかにすることを目的とした。

B．研究方法

1.　*rpl27* 発現抑制胚の作製

*RPL27*遺伝子の第1イントロンのアクセプター部位に変異を持つ患者では、開始コドンを含む第2エキソンが欠損したmRNAが発現していた。そこで、遺伝子構造が同じであるゼブラフィッシュにおいて造血との関係を調べるために、オーソログである *rpl27* の第1イントロンのアクセプター部位を標的とし、スプライシングを阻害するアンチセンスオリゴ（MO）を設計した。これを濃度5.0 μg/μlで受精卵に注入し、24時間後に全RNAを回収して逆転写PCRを行った。

2.　RT-PCRによる転写産物の解析

rpl27 のスプライシングを調べるために、受精後25時間の正常胚とMO注入胚から全RNAを回収して、逆転写PCRとシーケンシングで *rpl27* の転写産物を解析した。

3.　血球数の観察

造血への影響を観察するためにヘモグロビン染色を行った。49時間胚を染色液（0.6 mg/ml *o*-dianisidine、0.01 M 酢酸ナトリウム pH4.5、0.63%過酸化水素、40%エタノール）で7～10分間静置後、PBSで5分間3回リンスし、1%メチルセルロース中で実体顕微鏡を用いて心臓部周辺にみられる顆粒状の赤茶色の血球を観察した。

C．研究結果

1.　*RPL27*遺伝子の構造解析

エキソーム解析で新たに発見された *RPL27* の変異を持つ患者では、開始コドンを含む第2エキソンが欠損していた。そこで、*RPL27* と造血との関連をゼブラフィッシュで解析するために、ヒトとゼブラフィッシュの *RPL27*遺伝子の構造解析を行った。その結果、これらのエキソン・イントロン構造は同じで、特にエキソンの翻訳領域の長さは411 bpで同一であった。また、翻訳領域とアミノ酸配列のアラインメントを行った結果、それぞれの相同性は84%と96%で、非常によく保存されていた。これらの結果から、ゼブラフィッシュにスプライシングを阻害するMOを注入することで患者と同様の異常を再現出来ることが考えられた。

2. ノックダウン胚における形態形成の観察

rpl27 MO の注入によるスプライシング阻害がゼブラフィッシュの形態形成にどのような影響を与えるのかを観察した。その結果、受精後 25 時間では、体長の短縮、不完全な卵黄伸長部の形成、腹側に屈曲した尾部が見られた。このような表現型は、*in vitro* 転写で合成した *rpl27* mRNA を同時に注入することで回復することを確認した。

3. 赤血球形成における影響

造血への影響を観察するために、受精後 49 時間でヘモグロビン染色を行った。野生型胚の心臓と卵黄嚢の表面は血球が高密度に存在していた。これに対し、MO を注入した胚ではほとんど血球を確認することが出来なかった。しかし、MO と *rpl27* mRNA の混合液を注入すると約 7 割の胚で、血球数の回復が見られた。このことから、ゼブラフィッシュにおいて *rpl27* は、赤血球の形成に必要であることが示唆された。

D．考察

rpl27 ノックダウン胚で得られた尾部の屈曲や赤血球の減少などの表現型は、DBA の主症状とされる貧血や骨格異常などの特徴に類似している。また、DBA の代表的な原因遺伝子である *rps19* をノックダウンしたゼブラフィッシュで見られる表現型にも類似していた。これらのことから、*rpl27* ノックダウン胚は、DBA の疾患モデルになり得ることが考えられる。しかし、*rpl27* がどのように造血や骨格形成に関与するのかはこれから検証していく必要がある。

E．結論

ゼブラフィッシュを用いた解析により、DBA 患者で新たに同定された *RPL27* 遺伝子の変異は、DBA 発症の原因であると推測された。

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G．知的財産権の出願・登録状況

1. 特許取得
該当なし

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該当なし

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Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

◎は、本研究によることが明記されている論文
○は、本研究に関連する論文

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Ⅳ．研究成果の刊行物・別冊

nature genetics

The landscape of somatic mutations in Down syndrome–related myeloid disorders

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Transient abnormal myelopoiesis (TAM) is a myeloid proliferation resembling acute megakaryoblastic leukemia (AMKL), mostly affecting perinatal infants with Down syndrome. Although self-limiting in a majority of cases, TAM may evolve as non-self-limiting AMKL after spontaneous remission (DS-AMKL). Pathogenesis of these Down syndrome–related myeloid disorders is poorly understood, except for *GATA1* mutations found in most cases. Here we report genomic profiling of 41 TAM, 49 DS-AMKL and 19 non-DS-AMKL samples, including whole-genome and/or whole-exome sequencing of 15 TAM and 14 DS-AMKL samples. TAM appears to be caused by a single *GATA1* mutation and constitutive trisomy 21. Subsequent AMKL evolves from a pre-existing TAM clone through the acquisition of additional mutations, with major mutational targets including multiple cohesin components (53%), *CTCF* (20%), and *EZH2*, *KANSL1* and other epigenetic regulators (45%), as well as common signaling pathways, such as the JAK family kinases, *MPL*, *SH2B3* (*LNK*) and multiple RAS pathway genes (47%).

TAM represents a transient proliferation of immature megakaryoblasts that occurs in 5–10% of perinatal infants with Down syndrome[1,2]. Although morphologically indistinguishable from AMKL, TAM is self-limiting in the majority of cases and usually terminates spontaneously within 3–4 months of birth[1]. Hepatic infiltration of myeloid cells is a common finding and can be severe enough to be fatal, owing to hepatic failure, with liver fibrosis occurring in 5–16% of cases[2–4]. Moreover, even when spontaneous remission is achieved, approximately 20–30% of surviving infants develop DS-AMKL years after remission, although some DS-AMKL cases have no documented history of TAM[4]. In contrast to non–Down syndrome–related AMKL (non-DS-AMKL), which generally shows poor prognosis, individuals with DS-AMKL typically have a favorable prognosis. In molecular pathogenesis of these Down syndrome–related myeloid disorders, *GATA1* mutations are detected in virtually all affected infants, suggesting their central role in Down syndrome–related myeloid proliferation[5,6]. However, it is still open to question whether a *GATA1* mutation is sufficient for the development of TAM in individuals with Down syndrome, what is the cellular origin of the subsequent AMKL, whether additional gene mutations are required for progression to AMKL, and, if so, what are their gene targets, although several genes have been reported to be mutated in occasional cases with DS-AMKL, including *JAK1*, *JAK2* and *JAK3* (refs. 7–10), *TP53* (refs. 10,11), *FLT3* (ref. 8) and *MPL*[12]. We reasoned that identifying a comprehensive registry of gene mutations and tracking them at a clonal level using massively parallel sequencing would provide vital information for addressing these questions.

RESULTS

Genomic landscape of Down syndrome–related myeloid neoplasms

We performed whole-genome sequencing of 4 trios consisting of samples from TAM, AMKL and complete remission phases (**Supplementary Figs. 1** and **2** and **Supplementary Table 1**). In total,

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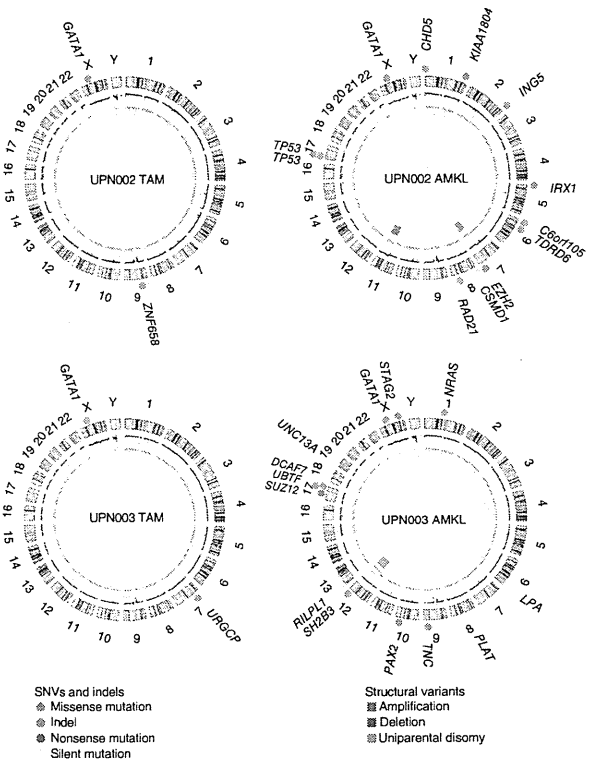

Figure 1 Representative Circos plots of paired TAM and DS-AMKL cases. Locations of somatic mutations, including of missense, frameshift, nonsense and silent mutations (colored circles), are indicated. Total (black) and allele-specific (red and green for alleles showing relatively larger and smaller copy numbers, respectively) genomic copy numbers, as well as somatic structural variants (colored bars), are indicated in the inner circle. Sample IDs are shown within each plot; plots were created with Circus[53].

we confirmed 411 single-nucleotide variants (SNVs) and 17 small nucleotide insertions and deletions (indels) by Sanger sequencing and/or deep resequencing (**Supplementary Fig. 1** and **Supplementary Table 2**). We detected only a few structural variants, including deletion, amplification and uniparental disomy, in the TAM and DS-AMKL genomes (**Fig. 1** and **Supplementary Fig. 3**). The mean number of validated somatic mutations in DS-AMKL samples (71 or 0.023 mutations/Mb) was twice the number observed in TAM samples (36 or 0.012 mutations/Mb) (**Supplementary Fig. 1a**). Mutation numbers in samples from both phases were substantially lower than in most other cancers (**Supplementary Fig. 4**), although differences in mutation rates could partly be affected by different definitions and algorithms for mutation calling. The spectrum of mutations was over-represented by C-to-T and G-to-A transitions in both TAM and DS-AMKL samples, resembling the mutational spectra in gastric and colorectal cancers[13] and in other blood cancers (**Supplementary Fig. 1b**)[14,15]. We unmasked the details of clonal evolution and expansion leading to AMKL through the use of deep sequencing of individual mutations detected by combined whole-genome and whole-exome sequencing (**Fig. 2** and **Supplementary Table 2**). Intratumoral heterogeneity was evident at initial diagnosis with TAM and in the AMKL phase in all cases (**Supplementary Fig. 5**). In UPN001, UPN002 and UPN004, AMKL evolved from one of the major subclones in the TAM phase with a shared *GATA1* mutation, as reported previously in relapsed acute myeloid leukemia (AML) in adults (**Fig. 2a,b,d**)[15]. In contrast, UPN003 showed a unique pattern of clonal evolution, in which AMKL originated from a minor subclone in the TAM phase that was totally unrelated to the predominant clone in terms of somatic mutations, with no mutation shared by both phases, and carried an independent *GATA1* mutation (**Fig. 2c**). In both scenarios, progression to AMKL seemed to be accompanied by many additional mutations, including common driver mutations that were absent in the original TAM population, indicating a multistep process of leukemogenesis.

Exome sequencing

We further investigated non-silent mutations by whole-exome sequencing of additional samples to generate a full registry of driver mutations that are relevant to the development of TAM and subsequent progression to AMKL (**Supplementary Fig. 6** and **Supplementary Table 1**). We detected *GATA1* mutations in all TAM and DS-AMKL cases, indicating sufficient sensitivity in our whole-exome analysis. In total, we confirmed 26 and 81 non-silent somatic mutations identified in the exome analysis of 15 TAM and 14 DS-AMKL samples, respectively, with 3 *GATA1* mutations common to both phases (**Supplementary Table 3**). The mean number of non-silent mutations was significantly higher in DS-AMKL samples (5.8; range of 1–11) than in TAM samples (1.7; range of 1–5) ($P = 0.0002$) (**Fig. 3a**). Of the 107 mutations, 84 were single-nucleotide substitutions that were mostly within coding sequences, except for 4 splice-site mutations. We also observed predominantly C-to-T and G-to-A transitions for non-silent substitutions (**Supplementary Fig. 7**). The remaining mutations were frameshift ($n = 21$) or non-frameshift ($n = 2$) indels, most frequently involving *GATA1* ($n = 13$). One individual with DS-AMKL (UPN004) had no SNVs or indels (**Fig. 3a**), but copy number analysis identified a large deletion at 16q involving the *CTCF* locus (**Supplementary Fig. 3**), suggesting that the alteration of *CTCF* could be a driver event in this case. Therefore, at least one additional genetic lesion other than *GATA1* mutation was detected in our whole-exome sequencing, despite the low frequency of leukemic cells appearing to show the morphology of immature megakaryoblasts (blast percentage) in many cases, which is a known characteristic of DS-AMKL samples[16,17]. Whole-exome sequencing results suggested the presence of intratumoral heterogeneity in the majority of DS-AMKL cases (**Fig. 3b**).

Spectrum of recurrent mutations in DS-AMKL

Recurrently affected genes are of primary interest in identifying driver mutations. Whereas *GATA1* was the only recurrent mutational target in TAM samples, an additional eight genes were recurrently mutated in the DS-AMKL samples, including *RAD21*, *STAG2*, *NRAS*, *CTCF*, *DCAF7*, *EZH2*, *KANSL1* and *TP53* (**Table 1**). These genes are expressed in a wide variety of hematopoietic compartments, including in both myeloid and lymphoid cells, except for *EZH2*, whose expression is largely confined to $CD34^+$ cells[18] (**Supplementary Fig. 8**). We also found that these genes were expressed in DS-AMKL cells at similar levels to common hematopoietic genes[19], although we did not observe significant difference in their expression levels in DS-AMKL and non-DS-AMKL cells (**Supplementary Fig. 9**).

We then performed targeted deep sequencing of these 8 genes in an extended set of 109 samples (including 29 samples in 25 discovery cases) consisting of 41 TAM, 49 DS-AMKL and 19 non-DS-AMKL samples (**Supplementary Tables 1** and **4**). We also included additional genes in targeted sequencing that were either functionally related to the above eight genes or were mutated only in single cases but had been previously reported to be mutated in DS-AMKL (*JAK3*) or other myeloid neoplasms (*SH2B3*, *SUZ12*, *SRSF2* and *WT1*), together with other common mutational targets in adult myeloid malignancies

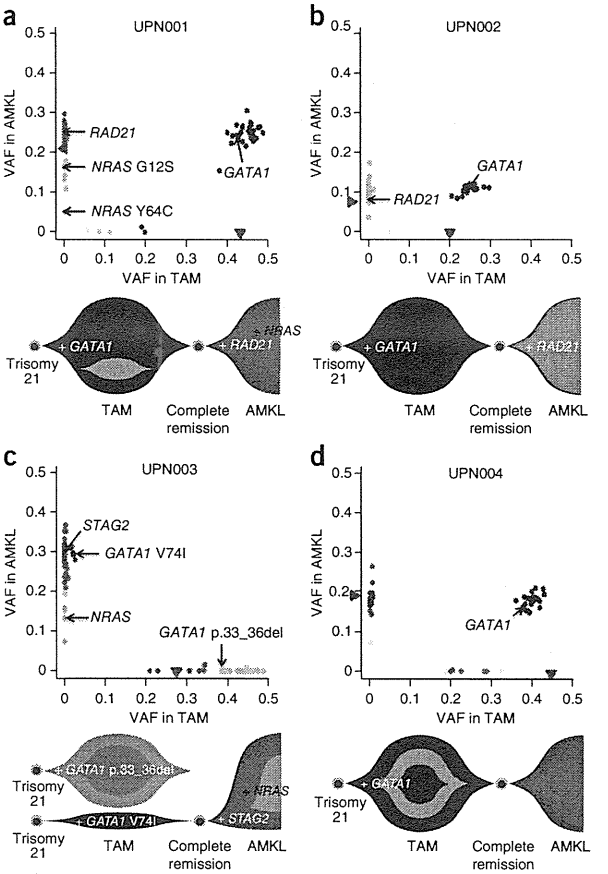

Figure 2 Clonal evolution of Down syndrome–related myeloid disorders. (**a–d**) Observed VAFs of validated mutations listed in **Supplementary Table 2** in both TAM and AMKL phases are shown in diagonal plots (top) for UPN001 (**a**), UPN002 (**b**), UPN003 (**c**) and UPN004 (**d**), where VAFs of genes on the X chromosome in male cases or in regions of uniparental disomy were halved. Half the value of the blast percentage, which corresponds to the allele frequency of a heterozygous mutation distributed in all tumor cells, is also shown by a red arrowhead, except for UPN003 AMKL, for which clinical data were not available. Driver mutations including in *GATA1*, *STAG2*, *RAD21* and *NRAS* are indicated by black arrows. Predicted chronological behaviors of different leukemia subclones are depicted below each diagonal plot. Distinct mutation clusters are indicated by color. In UPN001, UPN002 and UPN004, founding clones of TAM shown in blue became dominant in the AMKL samples, in which some subsequent subclones evolved through the serial acquisition of SNVs. In contrast, in UPN003, a subclone in the TAM phase (blue) and not the founding clone of TAM (aqua) became dominant in the AMKL sample. VAFs of some mutations were higher than for *GATA1* but seem to be actually equivalent to it given the error range of PCR-based deep sequencing.

(**Supplementary Fig. 10** and **Supplementary Tables 5** and **6**). We also analyzed by RT-PCR two recurrent fusion genes previously reported in non-DS-AMKL cases, *RBM15-MKL1* (*OTT-MAL*)[20,21] and *CBFA2T3-GLIS2* (refs. 22,23).

Mutations of cohesin and associated molecules

Major components of the cohesin complex, including *RAD21* and *STAG2*, were frequent targets of gene mutations in DS-AMKL (**Table 1**). Including an additional mutation in *NIPBL*, 8 of the 14 discovery DS-AMKL cases (57%) had a mutated cohesin or associated component (**Supplementary Table 3**). Cohesin is a multiprotein complex consisting of 4 core components, including the SMC1, SMC3, RAD21 and STAG proteins[24,25]. In concert with several functionally associated proteins, such as the NIPBL and ESCO proteins, cohesin is engaged in the cohesion of newly replicated sister chromatids by forming a ring-like structure[25], preventing their premature separation before late anaphase. Cohesin has also been implicated in post-replicative DNA repair and long-range regulation of gene expression[26–30]. Targeted deep sequencing confirmed recurrent mutations and deletions in all core cohesin components (*STAG2*, *RAD21*, *SMC3* and *SMC1A*) and in *NIPBL* in 26 of 49 DS-AMKL cases (53%) but in none of the 41 TAM cases, although 2 non-DS-AMKL cases (11%) had *STAG2* mutations (**Fig. 4a,b** and **Supplementary Tables 7** and **8**). Strikingly, all mutations and deletions in different cohesin components were completely mutually exclusive, suggesting that cohesin function was the common target of these mutations. All but one *STAG2* mutation (encoding a p.Arg370Gln substitution) was either a nonsense, frameshift or splice-site change (**Fig. 4a,b**, **Supplementary Figs. 11** and **12a**, and **Supplementary Table 7**). Similarly, 6 of 9 *RAD21* mutations were heterozygous nonsense or frameshift alterations. Four of the five mutations in *NIPBL*, *SMC1A* and *SMC3* were also nonsense or splice-site changes causing abnormal exon skipping (**Fig. 4a** and **Supplementary Table 7**). Thus, most of these mutations were thought to result in premature truncation, leading to loss of cohesin function. The leukemogenic mechanism of mutated cohesin components is still elusive; some studies have implicated aneuploidy caused by cohesin dysfunction in oncogenic actions[31]. However, DS-AMKL cases have been characterized by a largely normal karyotype[32]. We found no significant difference in the frequency of aneuploidy between cases with mutated and wild-type cohesin in the current DS-AMKL cohort. Many cases with mutated cohesin had completely normal karyotypes, except for constitutive trisomy 21, arguing against the hypothesis that aneuploidy has a major role in the pathogenesis of cohesin-mutated DS-AMKL (**Fig. 5a**).

CTCF mutations

Given the high frequency of cohesin mutations, new recurrent *CTCF* mutations were of particular interest because the functional interaction of cohesin and CTCF proteins has been of emerging interest in the long-range regulation of gene expression[26,30,33,34]. CTCF is a zinc-finger protein implicated in diverse regulatory functions, including transcriptional activation and/or repression, insulation, formation of chromatin barrier, imprinting and X-chromosome inactivation[35]. CTCF binds to target sequence elements and blocks the interaction of enhancers and promoters through DNA loop formation (insulator activity)[36], and several lines of evidence suggest that cohesin occupies CTCF-binding sites to contribute to the long-range regulation of gene expression by participating in the formation and stabilization of a repressive loop[26,37]. *CTCF* was mutated or deleted in ten DS-AMKL cases (20%), one TAM case (2%) and four non-DS-AMKL cases (21%), with seven mutations representing nonsense, frameshift or splice-site changes and an additional six alterations representing deletions resulting in the loss of protein function (**Fig. 4a,b**, **Supplementary Figs. 11** and **12b**, and **Supplementary Tables 7** and **8**). To our knowledge, this is the first report of frequent recurrent *CTCF* mutations in cancer, although rare mutations (occurring in approximately 2% of cases) have recently been reported in breast cancer sequencing[38].

Mutations in epigenetic regulators

EZH2, which encodes a catalytic subunit of the Polycomb repressive complex 2 (PRC2) that is responsible for di- and trimethylation of histone H3 lysine 27 (H3K27)[39], is another recurrent mutational target in DS-AMKL (**Table 1**). Inactivating mutations in *EZH2* have

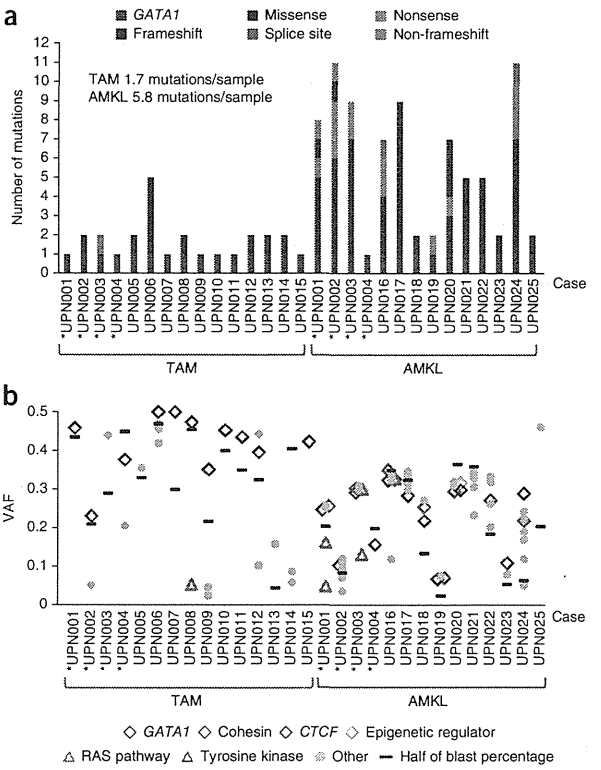

Figure 3 Somatic mutations detected by whole-exome sequencing of Down syndrome–related myeloid disorders. (**a**) Number of validated somatic mutations in 25 individuals with TAM and DS-AMKL identified by whole-exome sequencing. Paired samples are indicated by asterisks. The mutation rates per phase are given. (**b**) VAFs of individual mutations determined by deep sequencing, with VAFs adjusted for genomic copy numbers. Long indels of >3 bp were excluded from the analysis because their VAFs were difficult to accurately estimate. The VAF for each sample estimated on the basis of blast percentage is indicated by a purple horizontal bar.

been reported in up to 13% of myelodysplastic syndromes and related chronic myeloid neoplasms[40]. Although rarely mutated in adult AML[41], *EZH2* represents one of the most frequently mutated and deleted genes in childhood AMKL, as we identified mutations or deletions in 16 of 49 DS-AMKL cases (33%) and in 3 of 19 non-DS-AMKL cases (16%) (**Fig. 4a,b**, **Supplementary Fig. 12c** and **Supplementary Tables 7** and **8**). No other PRC2 components were mutated, except for *SUZ12*, which was mutated in a single DS-AMKL case (**Fig. 4a** and **Supplementary Table 7**). Although frequent mutations in other epigenetic regulators, including in *TET2*, *IDH1* or *IDH2*, *DNMT3A* and *ASXL1*, are cardinal features of myeloid neoplasms in adults, we rarely found these mutations in DS-AMKL and non-DS-AMKL cases, only identifying occasional *DNMT3A* ($n = 1$), *ASXL1* ($n = 1$) and *BCOR* ($n = 2$) mutations in DS-AMKL (**Fig. 4a**).

KANSL1 (encoding KAT8 regulatory NSL complex subunit 1; also known as MSL1V1 or NSL1) represents a new recurrent mutational target in human cancer (**Table 1**), although haploinsufficiency of *KANSL1* through germline deletions or mutations has been implicated in a congenital disease known as 17q21.31 microdeletion syndrome (MIM 610443)[42,43]. We found heterozygous mutations in *KANSL1* in three DS-AMKL and three non-DS-AMKL cases, and most of these mutations were nonsense or frameshifts, leading to loss of protein function (**Fig. 4a** and **Supplementary Table 7**). KANSL1 protein is necessary and sufficient for the activity of the KAT8 (MOF) histone acetyltransferase complex, which is engaged in the acetylation of histone H4 lysine 16 (H4K16), leading to transcriptional activation. Loss of acetylation of H4K16 has been reported to be a common hallmark of human cancer, and other histone acetyltransferases for H4K16 have been reported to form recurrent fusion partners in leukemia, including MOZ and MORF[44], suggesting a role for compromised H4K16 acetylation by *KANSL1* mutations in leukemogenesis. Of interest, KANSL1 is also responsible for the acetylation of the TP53 tumor suppressor that is important for TP53-dependent transcriptional activation[45]. KAT8 also interacts with a histone H3 lysine 4 (H3K4) methyltransferase, MLL, and the interaction of MLL and KAT8 complexes facilitates the cooperative recruitment of both complexes to gene promoters and enhances transcription initiation at target genes[45]. Thus, impaired TP53 function and/or deregulated expression of MLL gene targets could also contribute to leukemogenesis by *KANSL1* mutations.

Table 1 Recurrently mutated genes other than *GATA1* in DS-AMKL samples in whole-exome sequencing

Gene	Mutation type	RefSeq	Amino acid change	Nucleotide change	Sample (UPN) number
CTCF	Splice site	NM_006565	p.Gly318_splice	c.953–2A>G	016
CTCF	Frameshift	NM_006565	p.Asn314fs	c.940_941insAC	020
DCAF7	Missense	NM_005828	p.Leu340Phe	c.1018C>T	001
DCAF7	Missense	NM_005828	p.Leu340Phe	c.1018C>T	003
EZH2	Frameshift	NM_004456	p.710_716del	c.2129_2148delATCACAGGATAGGTATTTTT	001
EZH2	Missense	NM_004456	p.Arg25Gln	c.74G>A	002
KANSL1	Frameshift	NM_001193466	p.Arg720fs	c.2159_2160insCG	020
KANSL1	Nonsense	NM_001193466	p.Arg462*	c.1384C>T	024
NRAS	Missense	NM_002524	p.Gly12Ser	c.34G>A	001
NRAS	Missense	NM_002524	p.Tyr64Cys	c.191A>G	001
NRAS	Missense	NM_002524	p.Gly12Ala	c.35G>C	003
RAD21	Nonsense	NM_006265	p.Arg139*	c.415A>T	001
RAD21	Frameshift	NM_006265	p.374_375del	c.1120_1124delTCTTT	002
RAD21	Missense	NM_006265	p.Leu611Arg	c.1832T>G	018
RAD21	Nonsense	NM_006265	p.Arg65*	c.193C>T	024
STAG2	Nonsense	NM_001042750	p.Arg604*	c.1810C>T	003
STAG2	Nonsense	NM_001042750	p.Arg216*	c.646C>T	019
STAG2	Frameshift	NM_001042750	p.Asn863fs	c.2588_2589insT	020
TP53	Nonsense	NM_000546	p.Glu68*	c.202G>T	002
TP53	Non-frameshift	NM_000546	p.157_162del	c.469_486delGTCCGCGCCATGGCCATC	002

Other mutations in DS-AMKL

RAS pathway mutations are common in hematopoietic malignancies and other human cancers but have not to our knowledge been described in DS-AMKL. In the current cohort, we identified RAS pathway