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A 15q24 microdeletion in transient myeloproliferative disease (TMD) and acute megakaryoblastic leukaemia (AMKL) implicates PML and SUMO3 in the leukaemogenesis of TMD/AMKL

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Summary

Transient myeloproliferative disorder (TMD) of the newborn and acute megakaryoblastic leukaemia (AMKL) in children with Down syndrome (DS) represent paradigmatic models of leukaemogenesis. Chromosome 21 gene dosage effects and truncating mutations of the X-chromosomal transcription factor GATA1 synergize to trigger TMD and AMKL in most patients. Here, we report the occurrence of TMD, which spontaneously remitted and later progressed to AMKL in a patient without DS but with a distinct dysmorphic syndrome. Genetic analysis of the leukaemic clone revealed somatic trisomy 21 and a truncating *GATA1* mutation. The analysis of the patient's normal blood cell DNA on a genomic single nucleotide polymorphism (SNP) array revealed a *de novo* germ line 2·58 Mb 15q24 microdeletion including 41 known genes encompassing the tumour suppressor *PML*. Genomic context analysis of proteins encoded by genes that are included in the microdeletion, chromosome 21-encoded proteins and GATA1 suggests that the microdeletion may trigger leukaemogenesis by disturbing the balance of a hypothetical regulatory network of normal megakaryopoiesis involving PML, SUMO3 and GATA1. The 15q24 microdeletion may thus represent the first genetic hit to initiate leukaemogenesis and implicates PML and SUMO3 as novel components of the leukaemogenic network in TMD/AMKL.

Keywords: transient myeloproliferative disorder, GATA1, microdeletion 15q24 syndrome, trisomy 21, leukaemogenesis.

Children with Down syndrome (DS) are known to carry a 10- to 20-fold risk for developing acute lymphoblastic leukaemia (ALL) and a 400-fold risk for acute myeloid leukaemia (AML) (Zipursky *et al*, 1987, 1992; Avet-Loiseau *et al*, 1995). The predominant type of AML in DS is acute megakaryoblastic leukaemia (AMKL). Five to ten percent of newborns with DS are estimated to develop a transient myeloproliferative disorder (TMD), which is characterized by a proliferation of megakaryoblasts (Zipursky *et al*, 1999).

TMD is usually discovered within the first 3 weeks of life. Children with TMD can be asymptomatic or severely ill. In addition to symptoms of acute leukaemia, TMD may be complicated by liver fibrosis and cardiopulmonary disease. TMD usually remits spontaneously within the first 3 months

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of life, but 20–30% of patients develop AMKL before the age of 4 years (Zipursky *et al*, 1992).

N-terminally truncating mutations of the X-chromosomal transcription factor GATA1, whose normal function is essential for normal megakaryocytic and erythroid differentiation, are characteristic of both TMD and AMKL (Pevny *et al*, 1995; Shivdasani *et al*, 1997; Wechsler *et al*, 2002; Mundschau *et al*, 2003). These mutations are short insertions, deletions and point mutations of exon 2, which result in the introduction of a premature termination codon (Hitzler *et al*, 2003). These mutations do not appear to induce RNA degradation by nonsense-mediated decay, probably because translation is re-initiated at a downstream ATG translation initiation codon (Holbrook *et al*, 2004; Bhuvanagiri *et al*, 2010; Neu-Yilik *et al*, 2011), thus giving rise to an N-terminally truncated GATA1 variant (GATA1s) (Li *et al*, 2005).

The cooperation of GATA1s and trisomy 21 in inducing TMD and AMKL is one of the important enigmas of clinical oncology. It has been hypothesized that trisomy 21 provides a proliferative advantage of megakaryocytic precursors and that second hit somatic *GATA1* mutations result in TMD and leukaemia (Malinge *et al*, 2009). Curiously, however, in some patients with TMD, both trisomy 21 and GATA1s are somatic, suggesting that there may be independent TMD-initiating mutations (Wechsler *et al*, 2002; Cushing *et al*, 2006).

The patient reported here features a germ line 15q24 microdeletion including the tumour suppressor gene *PML*. His TMD and AMKL cells showed somatic trisomy 21 and GATA1s. This finding thus suggests that deletion of genes that are included in the microdeletion in general and possibly PML in particular are candidates for alternative first hit mutations in TMD and AMKL.

Patient details and methods

Clinical definition of the syndrome

The patient reported here is a boy who was born at 34 weeks of gestation by caesarian section because of premature placental separation. He had multiple congenital anomalies: congenital heart disorder (tetralogy of Fallot), unilateral iris coloboma, bilateral microtia and dysplastic ears, costovertebral anomalies, toe anomalies, hypospadia and cryptorchidism (Fig 1). The boy showed bilateral mixed deafness, hypotonia and severe psychomotor retardation. Cranial magnetic resonance imaging revealed non-obstructive hydrocephalus and hypoplasia of the cerebellar hemispheres. He developed significant behavioural problems with self-injurious behaviour, head-banging, trichotillomania and a sleeping disorder. On examination at the age of 6 years, his height and weight were below the 3rd centile, he was macrocephalic (occipitofrontal circumference > 97th centile), had a long, triangular face with a high forehead and a long, pointed chin, hypertelorism, down-slanting palpebral fissures, and microtia with dysplastic ears bilaterally. He was severely mentally retarded and had no speech. Diagnostic tests for deletion 22q11·2 and subtelomeric screening revealed regular results. Clinically, CHARGE syndrome (Coloboma of the eye, Heart defects, Atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities and deafness) was considered but discarded because cranial computer tomography revealed regular semicircular canals of the inner ear.

The patient was born with the diagnostic features of TMD. The blood count on the first day of life showed leukocytosis ($91{\cdot}1 \times 10^9$/l) without anaemia (Hb 154 g/l) or thrombocytopenia (platelet count 227×10^9/l). The differential count revealed 72% blast cells and bone marrow cytology on day 5 showed 50% blasts (Fig 2A). Immunophenotyping showed strong expression of the progenitor cell markers CD34 and CD117, the myeloid marker CD33, the megakaryocytic marker CD61, and glycophorin A. In summary, these blasts had the morphological and immunological characteristics of megakaryoblasts. The TMD remitted spontaneously within 70 d.

When aged 22 months the child developed thrombocytopenia (31×10^9/l) without anaemia (Hb 117 g/l) or leucocytosis/leucopenia (10×10^9/l). The peripheral blood smear included 16% blasts. Bone marrow cytology and immunophenotyping revealed the typical appearance and antigen profile of AMKL (Fig 2B). The patient received dose-reduced polychemotherapy according to the Berlin-Frankfurt-Münster

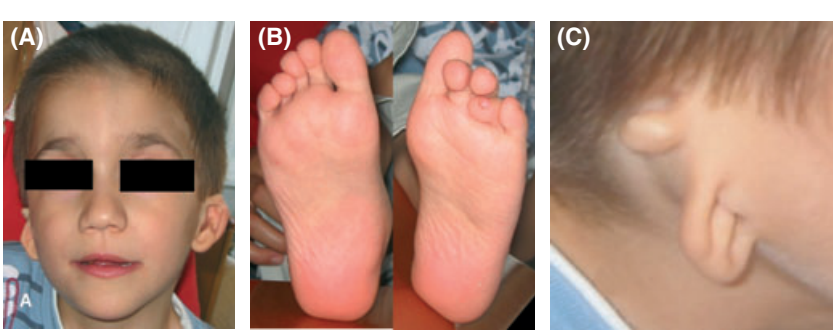

Fig 1. Syndromal stigmata of the patient with the novel 15q24 microdeletion. Panel A: facial stigmata including high anterior hair line, down slanting palpebral fissures, broad eyebrows, broad nasal bridge, dysmorphic outer ears. Panel B: Toe anomalies of the left foot. Panel C: dysmorphic outer right ear.

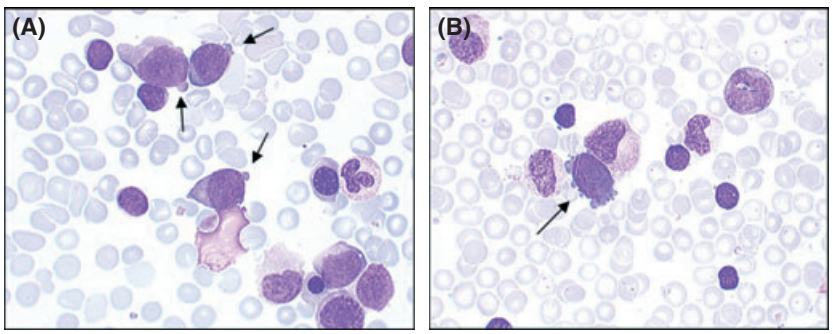

Fig 2. Bone marrow cytology during the TMD (panel A) and AMKL (panel B) phases revealing abnormal megakaryoblasts with round nuclei, non-granulated cytoplasm and cytoplasmatic blebbing (indicated by arrows). Original magnification ×50.

AML protocol (AML-BFM 2004) for DS children (www.kinderkrebsinfo.de). Remission was achieved after the first chemotherapy cycle. Currently the boy is in complete continuous remission 8 years after the diagnosis of AMKL was made.

Methods

Blood and bone marrow samples and the clinical photographs were obtained with informed consent of both parents. The haematological analyses were performed by standard methods.

GATA1 sequencing. Genomic DNA was isolated from peripheral blood leucocytes or bone marrow cells using routine procedures. DNA was amplified using primers GATA Ex2F (5′-AAAGGAGGGAAGAGGAGCAG-3′) and GATA Ex2R (5′-GACCTAGCCAAGGATCTCCA-3′). The polymerase chain reaction product was purified by NucleoSpin® Extract II Kit. Sequencing was done by GATC biotech (Konstanz, Germany).

Single nucleotide polymorphism (SNP) array. SNP array analysis was performed as part of the 'German Mental Retardation Network' (MRNET), which is funded by the German Federal Ministry of Education and Research (BMBF) as a part of the National Genome Research Network.

Genomic DNA was isolated from peripheral blood leucocytes using routine procedures and DNA quality was assessed by NanoDrop spectrophotometer (ND-1000) optical density metrics and gel electrophoresis. Samples were processed in accordance with the manufacturer's instructions. Genotypes were called with Affymetrix Genotyping Console (GTC) Software v3 using the Birdseed algorithm with default calling threshold of 0·5 and a prior size of 10 000 bases in a simultaneous analysis of the patient-parents trio. Samples were required to have a minimum Quality Control SNP call rate of 97% and were analysed with Copy Number Analyzer for GeneChip (CNAG), and the Affymetrix CN4 algorithms within GTC v3. The predicted copy numbers as well as the start and end of each copy number variation segment were determined using the Hidden Markov Model (HMM) incorporated in each package. Interpretation was based on Human Genome Build 36.

Genomic context analysis. Genomic context analysis of interactions between *GATA1*, chromosome 21 and genes affected by microdeletion 15q24 was performed with the STRING 8.1 software (Szklarczyk *et al*, 2011).

Results

Cytogenetic and molecular genetic analysis

Cytogenetic analysis of peripheral blood and bone marrow in the first week of life during the TMD phase showed trisomy 21 mosaicism affecting 52% (peripheral blood, 16/31 metaphases) and 100% (bone marrow, 3/3 interpretable metaphases) of examined cells (47,XY + 21; 46,XY). During remission, no trisomy 21 was detected in the bone marrow, whereas cytogenetic analysis of the AMKL cells revealed the presence of trisomy 21 mosaicism and trisomy 8. Cytogenetic analysis of fibroblasts in remission after AMKL revealed a normal karyotype in 100 cells thus not supporting the diag-

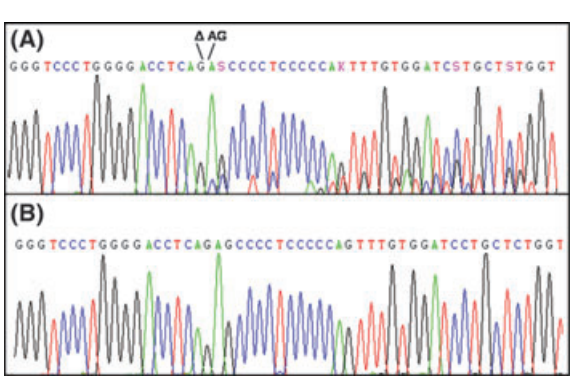

Fig 3. Identification of an AG-dinucleotide deletion at position 150/151 in exon 2 of the *GATA1* gene. Panel A: The indicated AG dinucleotide deletion is identified in the bone marrow DNA against a background of normal sequence. Panel B: only normal *GATA1* sequence was found in the remission bone marrow.

nosis of germ line trisomy 21 mosaicism. Molecular genetic analysis of bone marrow DNA during the TMD and AMKL phases showed the presence of a previously described truncating AG dinucleotide deletion of the *GATA1* gene in exon 2 at position 150/151 (Hitzler *et al*, 2003) (Fig 3A). The *GATA1* mutation was absent in blood and bone marrow cells obtained during remission of TMD and AMKL (Fig 3B). In summary, the diagnosis of TMD in a child with a malformation syndrome that is distinct from DS was made.

We next performed an analysis of a genomic single nucleotide polymorphism (SNP) array as part of the MRNET study (German Mental Retardation Network). DNA was extracted from peripheral blood cells taken 51 months after remission had been achieved. The analysis revealed a 2·58 Mb *de novo* microdeletion on chromosome 15, chromosomal band 15q24.1–15q24.2, position 70750326–73331875, covering 2·582 Mb and 1367 markers (start: CN_721025, end: CN_699268) (UCSC genome browser on Human Mar.2006 Assembly (hg18); Fig 4D). This deletion includes 41 known genes and open reading frames (Table I) and causes a rare contiguous gene deletion syndrome (Online Mendelian Inheritance in Man database number #613406). The deletion was not detected in the parents' genomes.

Similar microdeletions of chromosome region 15q24 have previously been described in 15 patients with mild to moderate developmental delay, muscular hypotonia, joint laxity, short stature, digital abnormalities, genital abnormalities and characteristic facial features (high forehead and anterior hair line, typical eyebrow, epicanthus, broad nasal base, long smooth philtrum, full lower lip, dysplastic ear lobes; Fig 5) (Sharp *et al*, 2007; Klopocki *et al*, 2008; Andrieux *et al*, 2009; El-Hattab *et al*, 2009; Masurel-Paulet *et al*, 2009; Van Esch *et al*, 2009). Because the syndromal features of this patient are consistent with this known 15q24 microdeletion syndrome, we interpret these findings to indicate that the deletion is probably of germ line origin. 15q24 microdeletions have not been identified in an extended cohort of 450 individuals drawn from populations with European, Asian and African ancestry whose genomes have been analysed by tiling oligonucleotide arrays (Conrad *et al*, 2010). Neither could it be detected in the 1000 Genomes Project pilot phase (Mills *et al*, 2011). We next investigated whether the 15q24/*PML* deletion might be found in other patients with TMD and trisomy 21 mosaicism. However, SNP-array analysis of three further patients did not identify this deletion, suggesting that the 15q24 microdeletion is not a common genetic anomaly in trisomy 21 independent TMD (Fig 4).

Next, we performed a genomic context analysis using the STRING 8.1 software of the deleted genes in 15q24 and of genes located on chromosome 21 (Szklarczyk *et al*, 2011). Interestingly, this analysis revealed that the known tumour suppressor gene *PML* (inducer of promyelocytic leukaemia), which is deleted by the 15q24 microdeletion, interacts with *GATA2* and probably also with the highly homologous *GATA1* on the X-chromosome (Tsuzuki *et al*, 2000). Furthermore, PML is known to be inactivated by SUMO3 (small ubiquitin-related modifier 3) which is encoded on chromosome 21 and is expressed in a gene dosage-dependent fashion (Fu *et al*, 2005; Weisshaar *et al*, 2008).

Discussion

The functional molecular network underlying the clinical association of trisomy 21 and GATA1s mutations with spontaneously regressing TMD and progression to AMKL represents one of the important enigmas in clinical haematology and oncology. It has been suggested previously that elevated chromosome 21 gene dosage results in increased proliferation of megakaryocytic progenitors (Izraeli, 2006; Chou *et al*, 2008). It is unknown which gene products encoded by chromosome 21 are functionally important but the transcription factors RUNX1 and BACH1 are candidates, because they are known to play a role in megakaryopoiesis (Ichikawa *et al*, 2004; Toki *et al*, 2005). Furthermore, chromosome 21-encoded miRNAs may also play a role in DS leukaemogenesis (Malinge *et al*, 2009). While trisomy 21 is considered to be a common first hit in TMD, GATA1s mutations are thought to be necessary second, albeit early, hits (Look,

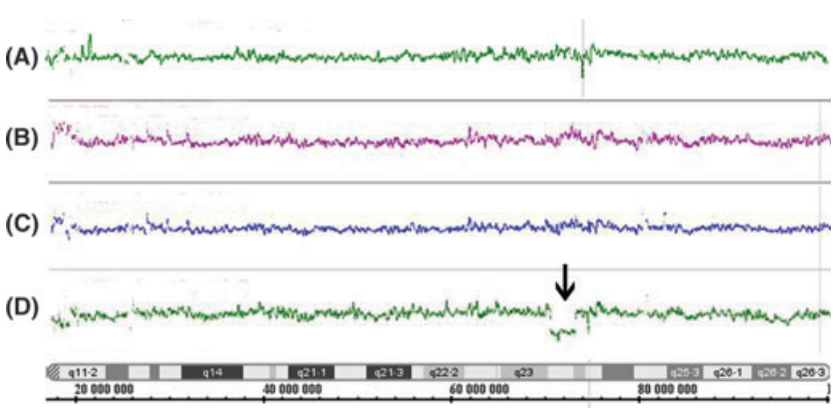

Fig 4. SNP array of chromosome 15 in patients with TMD and trisomy 21 mosaicism. Patient D is the patient presented in this report (arrow indicates deletion in 15q24.1–15q24.2).

Table I. Genes and open reading frames affected by the 15q24microdeletion. The PML gene is highlighted.

HEXA	*ADPGK*	*TBC1D21*	*ISLR*	*CLK3*	*SCAMP2*
C15orf34	*NEO1*	*LOXL1*	*STRA6*	*EDC3*	*MPI*
TMEM202	*HCN4*	*STOML1*	*CCDC33*	*CYP1A2*	*C15orf17*
ARIH1	*C15orf60*	***PML***	*CYP11A1*	*CSK*	*COX5A*
GOLGA6B	*NPTN*	*GOLGA6A*	*SEMA7A*	*LMAN1L*	*RPP25*
HIGD2B	*CD276*	*LOC283731*	*UBL7*	*CPLX3*	*SCAMP5*
BBS4	*C15orf59*	*ISLR2*	*ARID3B*	*ULK3*	

2002; Hitzler *et al*, 2003). Functionally, GATA1s has been reported to downregulate *RUNX1* and to upregulate *BACH1* (Bourquin *et al*, 2006), which may contribute to the block of megakaryocytic development. In some cases, further leukaemogenic hits can be identified at the transition of TMD to AMKL, which is exemplified by the trisomy 8 found in the AMKL cells of the patient reported here.

Patients with somatic trisomy 21 that is only found in the TMD/AMKL cells are rare but may provide important clues to leukaemogenesis, because other first hits are likely to operate in these cases. The important question arising here is whether the 15q24 microdeletion represents such an alternative first hit or whether the combination of the germ line 15q24 microdeletion with the somatic trisomy 21 and the GATA1s mutation in the TMD and the AMKL is mere coincidence. A potential oncogenic role of the 15q24 microdeletion is suggested by the occurrence of ALL in one out of 15 patients with the 15q24 microdeletion syndrome (Fig 5) (El-Hattab *et al*, 2009). Although co-incidence cannot, of course, be ruled out, functionally neutral microdeletions are usually <1 Mb in size and 15q24 microdeletions have not been identified in several genome wide analyses (Conrad *et al*, 2010; Mills *et al*, 2011). Assuming that the 15q24 microdeletion may be functionally active in the process of leukaemogenesis, the analysis of interactions between the gene products of this locus and GATA1 and chromosome 21-encoded genes is important. The *PML* gene product that was identified by our genomic context analysis represents an interesting candidate. PML is essential for the formation of specific nuclear bodies (PML-NB). These subnuclear structures have multiple functions, such as transcriptional regulation, post-translational modification, release of proteins and promotion of nuclear events in response to cellular stress (Van Damme *et al*, 2010). Various mechanisms of the tumour suppressive function of PML could be identified: It participates in the regulation of the TP53 response to oncogenic signals, such as RAS (Pearson *et al*, 2000), and contributes to the interaction with diverse co-repressors, such as histone deacetylase-1, which is important for MAD (Mothers against decapentaplegic)-mediated transcriptional repression (Khan *et al*, 2001). Furthermore, PML inactivates the phosphorylated serine-threonine kinase Akt, a key component of the tumourigenic PI3K (phosphatidylinositol-3 kinase)/Akt pathway (Trotman *et al*, 2006). PML can inhibit neoangiogenesis in neoplastic and ischaemic conditions (Bernardi *et al*, 2006) and its deficiency can induce neoplasias of the

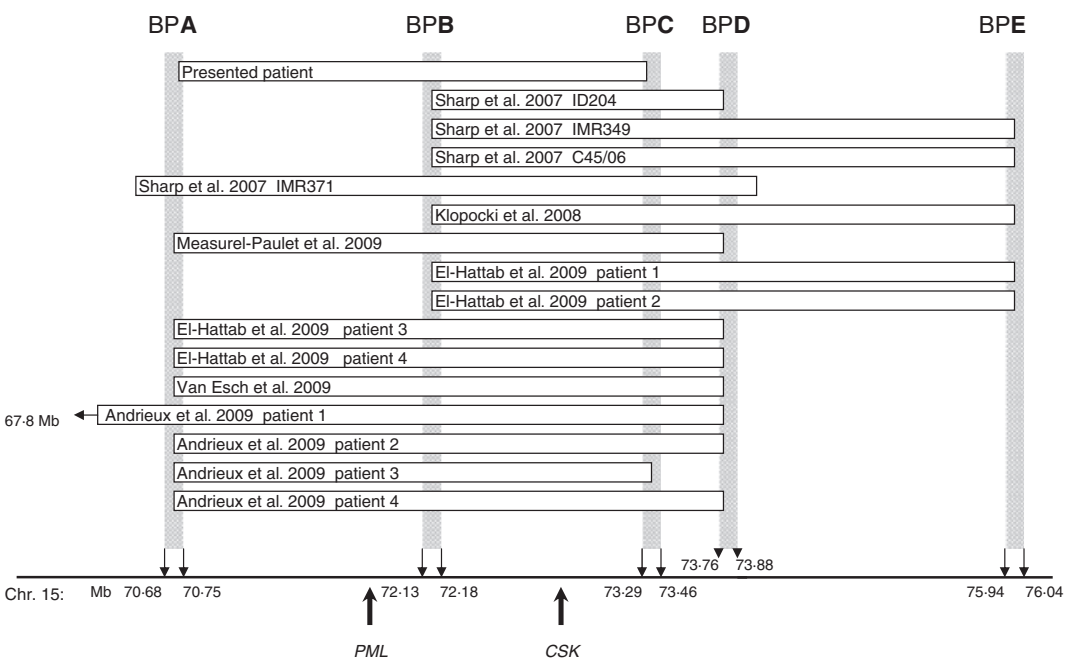

Fig 5. Schematic overview of the genomic localization of 15q24 microdeletions of the patient presented here and in 15 previously reported patients. BP A–E represent the five breakpoints as described by El-Hattab *et al* (2009). The exact genomic position on chromosome 15 is indicated in Mb. Patient 1, reported by El-Hattab *et al* (2009), developed acute lymphoblastic leukaemia. Localizations of the *PML* and *CSK* genes are indicated by arrows.

prostate (Trotman *et al*, 2006). More specifically, PML has been reported to be downregulated in AML and AMKL (Bourquin *et al*, 2006) and is known to stimulate the normal activity of the GATA transcription factors, which are known to induce megakaryocytic differentiation (Tsuzuki *et al*, 2000). GATA2 is also known to stimulate megakaryocytic development in cells with a GATA1s mutation (Huang *et al*, 2009). Hypothetically, deletion of *PML* may thus reduce GATA activity, which may result in disturbed megakaryocytic development.

A potential functional relationship between the genetic abnormalities found in this patient might thus be explained by the following hypothetical model (Fig 6): The heterozygous 15q24 microdeletion in the germ line of the patient reduces the expression of *PML*. The reduced PML activity downregulates normal GATA1 and GATA2 function, which inhibits normal megakaryocytic differentiation. This negative effect on GATA activity is confounded by the somatic GATA1s mutation that inactivates normal GATA1 function and by the overexpression of *SUMO3* from the triplicated chromosome 21 (Ait Yahya-Graison *et al*, 2007), probably resulting in an increase of PML degradation (Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008; Weisshaar *et al*, 2008). Therefore, elevated activity of SUMO3 in cells with the somatic trisomy 21 may aggravate the reduced expression of *PML* in 15q24 microdeleted cells and thus contribute to the reduced GATA1 function. Furthermore, sumoylation has been reported to directly repress transcriptional activity of *GATA1* (Collavin *et al*, 2004) thus suggesting an additional role of the *SUMO3* triplication in inducing TMD and AMKL by disturbing this regulatory network. The identification of the 15q24 microdeletion thus implicates PML and SUMO3 as novel components of the leukaemogenic network in TMD/AMKL.

Although we did not identify any known interactions of the products of the other genes that are affected by the microdeletion with GATA1 or chromosome 21 gene products, the effect of the 15q24 microdeletion may, of course, go beyond the deletion of *PML*. Specifically, the microdeletion includes the *CSK* gene, whose product downregulates the tyrosine kinase activity of the Src proto-oncogene by phosphorylation. Several oncogenic mechanisms that are important for cell transformation by Src oncoprotein interfere with this phosphorylation. Although we have not directly tested whether *CSK* is downregulated in the TMD/AMKL of this patient, *CSK* might function as a tumour suppressor in this context (Armstrong *et al*, 1992).

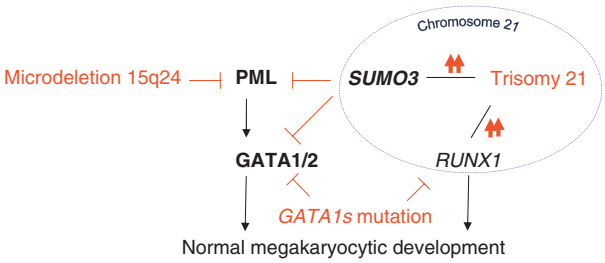

Fig 6. Working model of a hypothetical leukaemogenic network of protein interactions including microdeletion 15q24/PML, GATA1s, and trisomy 21/SUMO3: normal megakaryocytic development depends on normal RUNX1 and GATA1 function. GATA1s does not support normal megakaryopoiesis and inhibits RUNX1. In case of the microdeletion 15q24, we hypothesize that the PML haploinsufficiency may downregulate normal GATA1 function and that this effect may be confounded by the overexpression of the chromosome 21-encoded SUMO3, which is known to inactivate *PML*.

In conclusion, we suggest that the 15q24 microdeletion may be an important component of a hypothetical leukaemogenic network that includes deletion of *PML*, potential trisomy 21-related *SUMO3* gene dosage effects, and a truncating *GATA1* mutation. It will now be important to search for this deletion in other non-DS patients with TMD/AMKL and to analyse the role of *PML* and *SUMO3* in experimental systems of TMD/AMKL.

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Author contributions

JWJ, AJ, TD, JOK and PB analysed patient material and data. UM, CRB, SHe, WB and KAK gave expertise advice. UG, MB and OR performed analysis with SNP array. DR contributed patients' material. SHa and AEK coordinated research and analysis and wrote the paper.

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