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New insights into the genetic basis of TAR (thrombocytopenia-absent radii) syndrome

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Thrombocytopenia with absent radii (TAR) syndrome is a rare disorder combining specific skeletal abnormalities with a reduced platelet count. Rare proximal microdeletions of 1q21.1 are found in the majority of patients but are also found in unaffected parents. Recently it was shown that TAR syndrome is caused by the compound inheritance of a low-frequency noncoding SNP and a rare null allele in *RBM8A*, a gene encoding the exon-junction complex subunit member Y14 located in the deleted region. This finding provides new insight into the complex inheritance pattern and new clues to the molecular mechanisms underlying TAR syndrome. We discuss TAR syndrome in the context of abnormal phenotypes associated with proximal and distal 1q21.1 microdeletion and microduplications with incomplete penetrance and variable expressivity.

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Clinical features

Thrombocytopenia with absent radii (TAR) syndrome is characterized by a reduction in the number of platelets (the cells that make the blood clot) (generally below $50 \times 10^9\,L^{-1}$, normal range $150–350 \times 10^9\,L^{-1}$) and the absence of one of the bones in the forearm (the radius) but with preservation of the thumb.

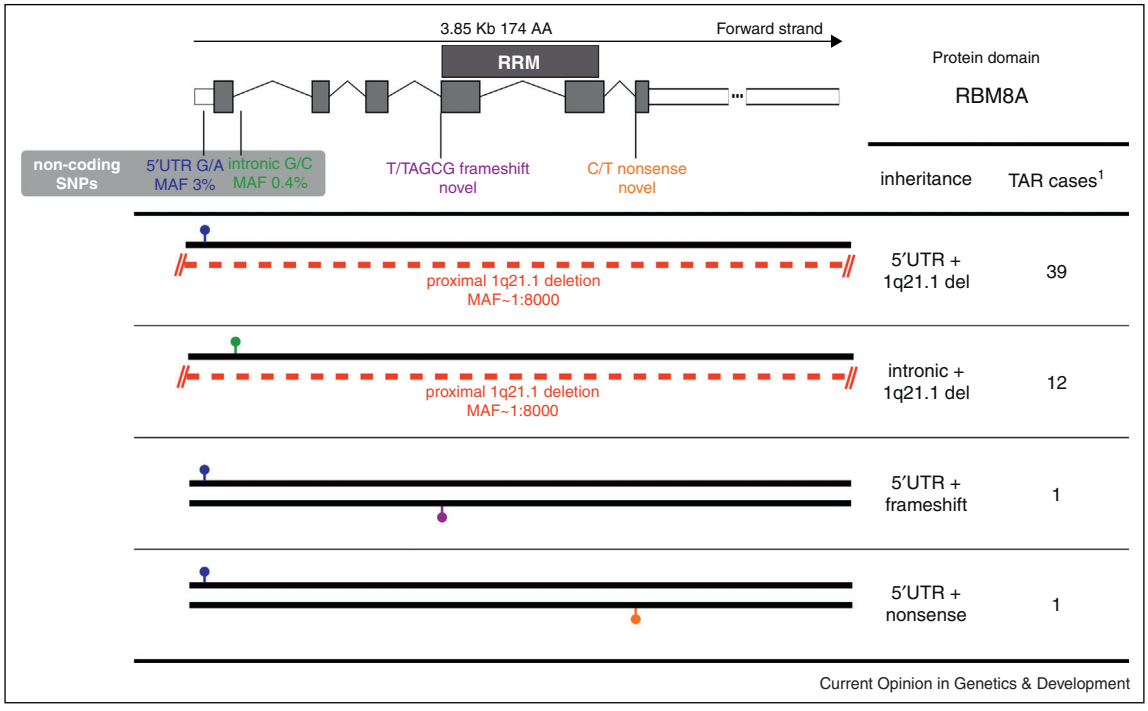
TAR syndrome was first described by Gross *et al.* [1] and Shaw and Oliver in 1959 [2], but Judith Hall was the first to define it as a syndrome in 1969, presenting clinical findings in a cohort of 40 patients [3]. The presence of the thumbs distinguishes TAR from other syndromes that combine blood abnormalities with absence of the radius, such as Fanconi anemia [3–5]. The severity of skeletal abnormalities varies from absence of radii to virtual absence of upper limbs (phocomelia) with or without lower limb defects, such as malformations of the hip and knee [3,5]. TAR cases have low numbers of megakaryocytes, the platelet precursor cells that reside in the bone marrow, and cases frequently present with bleeding episodes in the first year of life [3,5]. A remarkable feature of TAR syndrome is that the platelet count can improve with age and bleeding diminishes [5]. Other symptoms have been described in a series of 34 TAR patients [6], with renal anomalies and cardiac anomalies in respectively 23% and 15% of patients, and 47% suffering from intolerance to cow's milk.

Genetic mechanism

TAR syndrome has an incidence of approximately 1 in 240 000 births [7] and was thought to be inherited as an autosomal recessive disease [8] based on finding affected siblings. There is however no clear evidence of increased incidence in consanguineous families with only one case reported [9]. On the other hand, vertical parent-to-child transmission has been reported [10], as well as the case of a male patient and maternal uncle [11]. This unusual inheritance pattern has complicated the application of classic linkage analysis methods and homozygosity mapping approaches. Initial attempts to resolve the genetic basis of TAR syndrome have therefore consisted mainly of candidate gene studies [12,13] and identification of abnormal karyotypes [6]. Stripploi *et al.* [13] failed to identify mutations in c-mpl, the receptor for thrombopoietin, the principal cytokine regulating platelet production. No mutations were identified either in HoxA10, HoxA11, and Hox12 [12] even though HoxA11 has been associated with amegakaryocytic thrombocytopenia [14].

In 2007 Klopocki *et al.* [15••] identified proximal microdeletions of 1q21.1 in all of 30 TAR patients tested. The deletion was inherited paternally in 5 cases and maternally in 12 cases and occurred *de novo* in a further 5 cases [15••]. The deletion is rare but segregates in the population: it was observed twice in a set of 8329 unaffected adult controls [16]. The parents of TAR patients who carried the microdeletion were unaffected. The authors therefore suggested that the deletion was required but

Figure 1



Compound inheritance of a low-frequency noncoding SNP and a rare null mutation in *RBM8A* causes TAR syndrome. The gray box shows the two noncoding SNPs in respectively the 5′UTR (chr1: 145 507 646 G/A, hg19) and the first intron (chr1: 145 507 765 G/C, hg19) of *RBM8A* that were identified in a series of 55 TAR cases analyzed by Albers *et al.* [17••]. The minor allele frequency (MAF) of each SNP is indicated below each variant. One of these two noncoding SNPs was present with either a rare proximal 1q21.1 deletion (see also Figure 3), or a novel loss-of-function mutation (frameshift insertion or nonsense mutation) in RBM8A in 53 TAR cases. The four inheritance patterns and the corresponding number of cases that were observed in Ref. [17••] are shown below the gene.

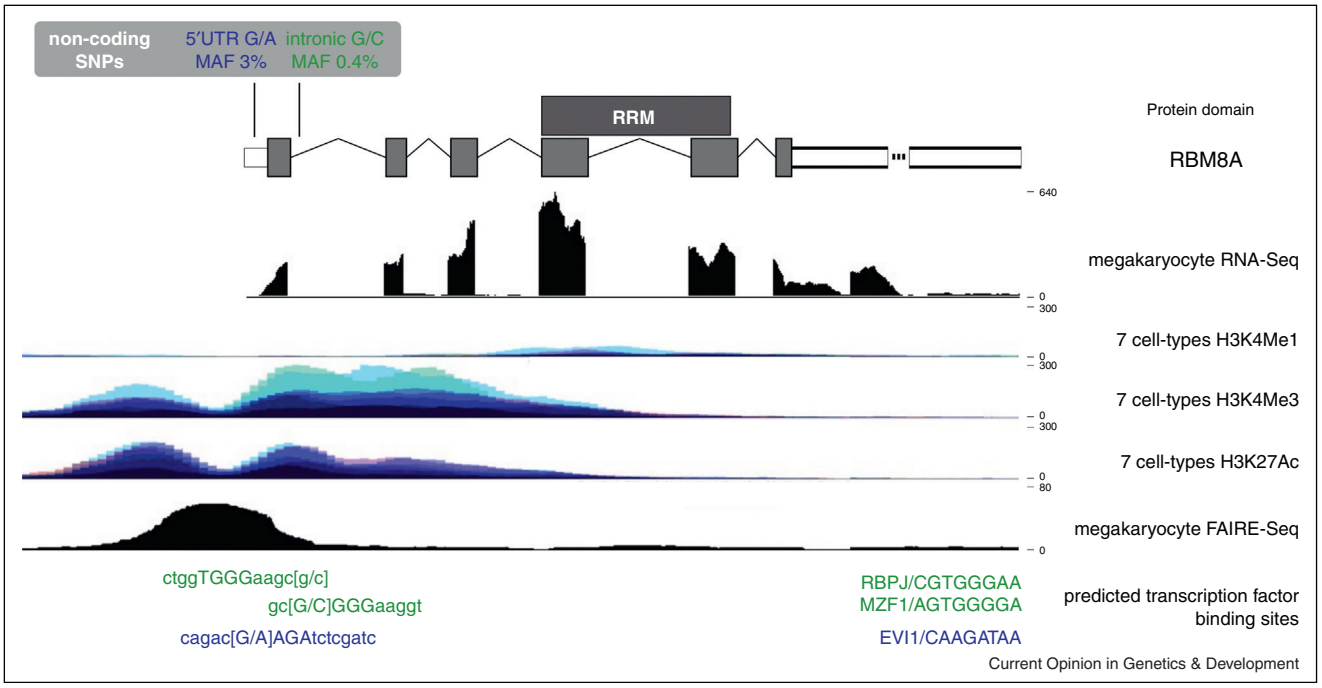
not sufficient to explain TAR and that a second causative allele (sometimes described as a modifier) must exist. They sequenced the protein coding sequence of 10 genes in the ~200 kb region that was deleted in all 30 patients, but no mutations were identified.

Low-frequency noncoding SNPs in *RBM8A*

In order to identify the second causative allele, we used high-throughput sequencing of DNA enriched for protein-coding genes (exome-sequencing) in five unrelated TAR cases with a 1q21.1 deletion [17••]. Assuming autosomal recessive inheritance, we hypothesized that the second causative allele would most likely be located in the 200 kb minimal deleted region identified by Klopocki *et al.* However, we also could not identify any rare deleterious protein-coding variants in the same gene in all five cases. We then considered all low-frequency variants (<5%) in the minimal deleted region, regardless of their predicted consequences, as potentially causative. This allowed us to identify a low-frequency SNP (allele frequency 3%) in the 5′UTR region of the gene *RBM8A* in four of the TAR cases sequenced and a low-frequency SNP (allele frequency 0.4%) in the first intron of the same gene in the last case (Figure 1). The frequency of the TAR deletion (1/8329, Ref. [16]) and the frequency of two noncoding SNPs are roughly consistent with the incidence of 1:240 000 reported in Ref. [7]. In principle, the technique of exome-sequencing is focused on enriching for exonic regions. However, due to partial overlaps with the hybridization probes and capture design to enable detection of intronic splice site mutations, it is often possible to call sequence variants within 50 bp of the targeted regions. This allowed us to identify both the 5′UTR SNP and the intronic SNP from the targeted resequencing of exons.

The findings were confirmed by Sanger sequencing in a further 48 individuals with TAR and a 1q21.1 deletion, with co-inheritance of the 5′UTR SNP in 35 cases and the intronic SNP in a further 11. Crucially, an additional two patients who did not have the deletion but carried the 5′UTR SNP were found to carry a loss of function mutation on the other *RBM8A* allele (frame shift insertion and premature termination codon). From these data we concluded that it is the compound (bi-allelic) inheritance

Figure 2



The noncoding SNPs found in TAR cases are located in regulatory elements in megakaryocytes, the precursor cells of platelets. Sequencing of RNA from cord-blood-derived megakaryocytes showing that *RBM8A* is expressed in megakaryocytes. Histone modifications from the ENCODE Project [59] in seven cell lines (GM12878, H1-hESC, HSMM, HUVEC, K562, NHEK, and NHLF) indicate the presence of regulatory elements at the promoter and first intron of *RBM8A*. H3K4Me1 is often found near regulatory elements, H3K4Me3 is often found near promoters and H3K27Ac is often found near active regulatory elements (UCSC Genome Browser). FAIRE marking regions of open chromatin showing that the 5′UTR SNP and first intron SNP are accessible to DNA binding proteins in megakaryocytes. Computational modeling predicted that the 5′UTR SNP minor allele creates a binding site for the transcription factor Evi1, a transcriptional repressor. It was shown that *in vitro* the minor allele of the 5′UTR shows increased binding of Evi1 [17••]. Thus, epigenetic annotation facilitates interpretation of sequence variants in nonprotein-coding sequence.

of a noncoding SNP together with a null mutation in *RBM8A* that causes TAR syndrome.

A number of unaffected parents were found to be homozygous for the 5′UTR SNP, demonstrating that being homozygous for one of the two regulatory variants is not sufficient to cause TAR syndrome. The two noncoding TAR SNPs are present at low frequency in European population, but were not detected in African populations in Phase 1 of the 1000 Genomes Project [18]. There have been reports of TAR in the Nigerian population [19], and it would therefore be interesting to see if the mechanism of inheritance and sequence variants in *RBM8A* described above explain TAR in that population as well.

Reduced expression of Y14, the protein encoded by *RBM8A*, in TAR patients

The two noncoding variants are located in regulatory elements in megakaryocytes, the precursor cell of platelets (Figure 2) [17••]. The level of Y14, the protein encoded by *RBM8A*, was found to be significantly lower in the platelets of TAR patients [17••]. This strongly suggests that the mechanism by which the compound inheritance of the noncoding variant and the rare null allele causes TAR syndrome is by reducing the expression of Y14 below a critical threshold [17••]. How this happens exactly is not clear, and the molecular mechanism may be different for the 5′UTR SNP and the intronic SNP. In reporter assays the minor 5′UTR allele and the intronic allele led to decreased transcription in megakaryocytic cell lines, but not in a vascular endothelial cell line [17••]. Together with the noncoding nature of the two SNPs, this strongly suggests tissue-dependent and possibly developmental stage-dependent effects of the two noncoding SNPs on *RBM8A* expression. The minor allele of the 5′UTR SNP was furthermore shown to result in increased binding of the transcription factor EVI1 *in vitro* [17••]. However, it is not clear at this stage if EVI affects transcription by binding to the DNA (by acting as a transcriptional repressor in competition with transcription factors binding to the normal allele), or by inhibiting translation by binding to the RNA. For the intronic SNP, reduced protein binding to the mutant DNA sequence was demonstrated *in vitro*, but

we could not confirm definitively which specific transcription factor binds to this particular regulatory region of the *RBM8A* gene [17••].

RBM8A/Y14 is a member of the exon-junction complex

Y14 is a small 174 aa protein with an RNA-binding domain (Figure 1). Y14 is one of the four components of the core exon-junction complex (EJC), which is involved in basic cellular functions such as nuclear export and subcellular localization of specific transcripts [20,21], translational enhancement [22] and nonsense-mediated RNA decay (NMD) [21,23,24]. The EJC is also associated with splicing. It is deposited 24 nt upstream of exon–exon junctions following pre-mRNA splicing [25]; *in vivo* the majority of exon junctions carry an EJC [26]. It was suggested that EJCs form 'super-complexes' with other EJCs to promote mRNA packaging and compaction [26]. Knockdown of the Y14 ortholog *tsu* in *Drosophila melanogaster* results in major defects in abdomen formation [27], and is lethal in *Danio rerio* [17••], highlighting the critical importance of Y14 and the EJC during embryonic development [28].

Biological mechanisms implicated in hematological features of TAR syndrome

What is not clear at this stage is how a deficiency in Y14 exerts its effect at a cellular level and in particular how it affects the production of megakaryocytes and platelets. Several studies have focused on the characterization of the nature of the thrombocytopenia in TAR patients. There are clearly a low number of megakaryocyte progenitors in the bone marrow in TAR patients [5,29,30] and this also translates *in vitro* where megakaryocyte colony output is virtually absent from patients' bone marrow progenitors [29–31]. In contrast, erythroid and myeloid colony growth from the TAR infants marrow cells was preserved, which strongly suggests a lineage specific maturation defect or a differentiation blockage [31]. Several studies have therefore focused on potential signaling defects in TAR patients as an explanation for this observation; in particular downstream of the main cytokine that controls megakaryocyte differentiation (thrombopoietin, TPO) [32–34]. The most recent study showed defects in thrombopoietin signal transduction in the platelets of 12/13 pediatric patients [34]. In particular these authors showed a correlation between the lack of phosphorylation of the Jak2 kinase (directly downstream of the thrombopoietin receptor) and the platelet count. Interestingly this defect corrected with age with 10/11 adult samples showing normal Jak2 phosphorylation in response to TPO [34]. At this stage, there is no clear evidence of how a deficiency in the EJC affects megakaryocyte maturation and how it would have an influence on the defective cell signaling described above.

Microdeletions and microduplications in the 1q21.1 region are associated with a variety of phenotypes

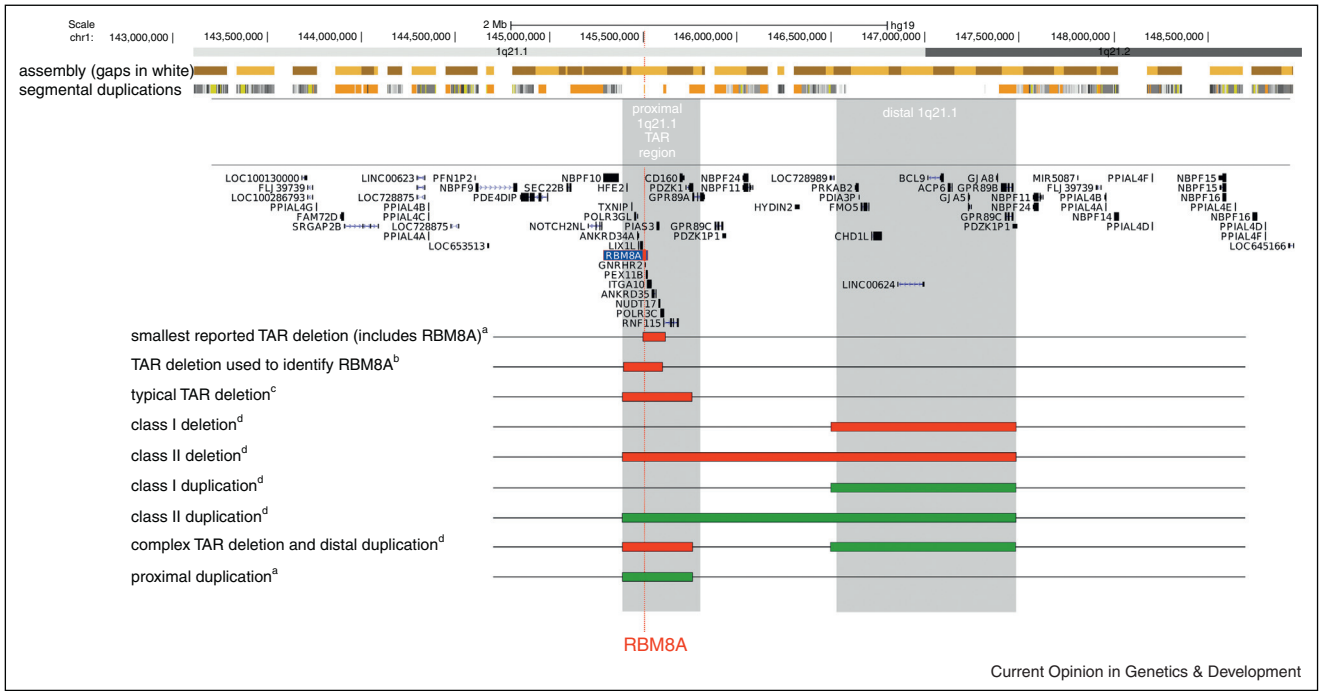
Chromosomal region 1q21.1 is structurally complex: it contains many segmental duplications (SDs) and the region still contains several assembly gaps (Figure 3) [15••,35–43]. Studies of microdeletions and microduplications of 1q21.1 showed that the break points of these structural variants tended to co-occur with these SDs [16,40•,42•], suggesting that the cause of many *de novo* microdeletions and microduplications in this region is nonallelic homologous recombination [36,42•,44,45]. As an illustration of the likely impact of these repetitive regions in 1q21.1 on the size of the deletion, the majority (28/30) of the TAR patients studied by Klopocki *et al.* carried a 500 kb deletion, and only one patient carried a substantially smaller deletion (the 'minimal deletion' used to identify the noncoding TAR mutations in Ref. [17••]). Given the qPCR probes used only variations in the distal end but not in the proximal coordinates of the deletion were identified in that study [15••].

Microdeletions and microduplications of 1q21.1 are associated with a wide range of phenotypes. The deletions associated with TAR syndrome are located proximally (Figure 3). Distal 1q21.1 deletions and duplications are associated with microcephaly or macrocephaly [40•,41], schizophrenia [38,39], and a spectrum of developmental delay, neuropsychiatric abnormalities, and dysmorphic features and congenital anomalies [16,35,37,40•,42•] but are not associated with a specific syndrome [42•]. Patients with a deletion or duplication spanning both the TAR region and the distal region have been reported ([42•]; the 'class II' deletions and 'class II' duplications in Ref. [40•]), as well as patients with a deletion in the TAR region and a duplication in the distal region [40•]. Weak evidence for proximal 1q21.1 duplications in the absence of distal duplications being deleterious has been reported at $P = 0.03$ [46•] and $P = 0.051$ [16]. In a study of 15 767 children with intellectual disability and various congenital defects, distal deletions were found to be most strongly associated with disease of all 1q21.1 rearrangements [16]. Both the proximal and distal deletions and duplications have been observed in healthy control cohorts, so all rearrangements of 1q21.1 exhibit incomplete penetrance, although undiagnosed more subtle phenotypes may be present.

Source of phenotypic variability

TAR syndrome provides an illustration of the challenge of interpreting rare and large copy number variants. The genetic heterogeneity underlying TAR syndrome appears to be limited, yet in addition to the three essential features of TAR, a wide range of additional phenotypes can be observed. This begs the question of what accounts for the phenotypic variability observed in TAR syndrome. One possibility is that it is simply variation in

Figure 3



Microdeletions and duplications of 1q21.1 are associated with a wide range of phenotypes. TAR syndrome is associated with proximal deletions of 1q21.1 (see also Figure 1). Distal 1q21.1 deletions and duplications are associated with microcephaly or macrocephaly [40•,41], schizophrenia [38,39], and a spectrum of developmental delay, neuropsychiatric abnormalities, and dysmorphic features and congenital anomalies [16,35,37,40•,42•] but are not associated with a specific syndrome [42•]. The approximate breakpoints of the proximal and distal deletions and duplications are indicated by the gray backgrounds. Segmental duplications (SDs) longer than 1 kb are shown, with SDs with more than 98% sequence similarity shown in orange. The region contains many SDs and sequence gaps. Note that what was previously known (in build hg18) as the distal 1q21.1 deletion/duplication region partially overlaps 1q21.2 in the UCSC browser of build hg19. **(a)** The smallest deletion observed in a TAR patient [46•] includes *RBM8A*. **(b)** This atypical 200 kb TAR deletion was identified in a 2007 study of 30 TAR patients [15••] and was used to identify *RBM8A* as the gene underlying TAR syndrome in 2012 [17••]. **(c)** Typical TAR deletion observed in 28 of 30 TAR patients in the 2007 study [15••]. **(d)** A range of deletions and duplications was observed in two 2008 studies of the 1q21.1 region [40•,42•]. The 'class I' and 'class II' terminology is taken from Ref. [40•]. **(e)** Recently, proximal duplications in the TAR region in the absence of distal rearrangements were found to be weakly associated with the development of abnormal phenotypes [16,46•].

gene expression, which may be further modified by environmental factors and statistical chance [47] that accounts for the variability in phenotypes associated with TAR. Subtle variations in activity of an essential gene of which a complete knockout is incompatible with development may result in a range of malformations. Alternatively, it is possible that further modifier alleles on the nondeleted chromosome account for the variability, including epigenetic alleles. For instance, the cow-milk allergy and cardiac anomalies frequently observed in TAR patients have also been observed in individuals referred for cytogenetic testing found to carry a proximal 1q21.1 deletion but without TAR syndrome [46•]; this could be a consequence of incomplete penetrance of the TAR mutations (noncoding variant combined with a null allele) or of the existence of additional modifier alleles in the proximal 1q21.1 region in genes other than *RBM8A*. Interestingly, a sex-bias has been frequently reported for TAR with an increased incidence in females (ratios vary from 1:1.5 to 1:3.8, see Ref. [48] for an overview). A detailed study of how the severity of the TAR phenotype (skeletal abnormalities and thrombocytopenia) and the range of additional phenotypes in TAR correlate with the genotype of each individual patient would be of interest. TAR shows that even relatively high-frequency variants can have strongly deleterious effects when combined with a rare deletion. It cannot be excluded that similar effects can be identified for other genes in 1q21.1.

Although precedent for a noncoding functional SNP modifying a deletion phenotype had been reported for Sotos syndrome and factor XII deficiency [49], modifier alleles and two locus models, distinct from the Knudson second hit somatic event model [50], have recently attracted increasing attention [51–53]. Coding variants in the COMT gene on the nondeleted allele of individuals carrying a 22q11.2 allele can affect cognitive function [54,55]. Girirajan *et al.* demonstrated that a second large

CNV at a distinct genomic locus can contribute to phenotypic variability in patients with developmental disorders [56]. At the cystic fibrosis locus, an upstream di-nucleotide repeat can modulate exon 9-skipping of the CFTR gene, but only when activated by the T5 allele of the polymorphic polythymidine tract in the 3′ splice site of exon 9 [57]. This explains the incomplete penetrance of the T5 polymorphism [58], analogous to noncoding SNPs explaining the incomplete penetrance of the 1q21.1 deletion in TAR syndrome.

Whole-genome high-throughput sequencing can simultaneously detect copy number variation and noncoding/regulatory small variants that act as modifiers. Although this will require large sample sizes, it may prove a way forward to dissect the phenotypic variability associated with copy number variation in rare disorders. With annotation of noncoding regions [59] becoming increasingly richer through large collaborative efforts such as the ENCODE Project [59], and in particular the BLUEPRINT Project [60], which focuses on creating a highly detailed epigenetic annotation of hematological cell types, interpretation of additional causative alleles that do not affect protein-coding sequence but instead affect gene expression has become feasible. The annotation of gene expression patterns in different cell types and developmental stages should provide insight into possible developmental aspects associated with the noncoding mutations involved in TAR syndrome. Finally, integration with the data from large genome-wide association studies of platelet parameters [61] may provide further insights into downstream effects of Y14 deficiency on platelet function.

Conclusion

TAR syndrome is caused by the compound (bi-allelic) inheritance of one of two noncoding single-nucleotide variants and a rare null allele in *RBM8A*. The two noncoding variants, located in the 5′UTR and first intron, explain the incomplete penetrance of the proximal 1q21.1 deletion and this new insight into the inheritance pattern will benefit diagnostic testing as well as counseling. The complex inheritance pattern ultimately results in reduced expression of Y14, the protein encoded by *RBM8A* and a core member of the exon-junction complex (EJC), in platelets. Further research is needed to explain how Y14 insufficiency, and presumably subsequent defect of the EJC, explains the unique skeletal, hematological and additional features of TAR syndrome.

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