<5% and platelet counts are not elevated. By strictly adhering to the 2008 WHO criteria, we provide important molecular information in a relatively homogenous and well-defined group of patients. Morphological diagnoses are often subjective, with inter-observer variability, and inaccurate classification of myeloid neoplasms can result in erroneous estimation of associated molecular abnormalities. Specifically, it is important to note that BM ring sideroblasts are sometimes seen in both PMF and ET, and it is possible to misdiagnose some of these cases as RARS-T or RARS.[10,11]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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OPEN

The germline sequence variant rs2736100_C in TERT associates with myeloproliferative neoplasms

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Myeloproliferative neoplasms (MPN) constitute a group of clonal hematological disorders characterized by an expansion and accumulation of one or more mature cell types of the myeloid lineages. There are four main groups of MPNs that can be classified by the presence or absence of the Philadelphia (Ph) chromosome.[1] Chronic myeloid leukemia is accompanied by the Ph chromosome, whereas polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are not.[1] In all three Ph-negative MPN subtypes (hereafter called MPN), a recurrent somatic mutation is frequently found in the pseudokinase region of Janus kinase 2 ($JAK2^{V617F}$).[2] The mutation is observed in about 80–95% of PV cases, 50% of ET cases and 60% of PMF cases.[2] Owing to the activating nature of the mutation, it leads to constant stimulation of myeloid proliferation.[2] Family studies have shown that germline variants may predispose to the disease as the risk of MPN among first-degree relatives of MPN patients in Sweden is five to sevenfold greater than that in the general population.[2] Furthermore, common germline single-nucleotide polymorphisms (SNPs) at the *JAK2* locus have been associated with MPN.[3–5]

The aim of the current study was to search for germline sequence variants that associate with risk of MPNs by performing genome-wide association analysis. For the association analysis, we used sequence variants identified by whole genome sequencing (WGS), to an average depth of 22 ×, of DNA isolated from white blood cells of 2230 Icelanders (Supplementary Material). Using imputation assisted by long-range haplotype phasing, the genotype probabilities of all the 34.2 million sequence variants identified were determined for Icelanders genotyped with Illumina (San Diego, CA, USA) SNP chips. Furthermore, using Icelandic genealogical information, genotype probabilities were calculated for individuals who are close relatives of chip-typed individuals (familial imputation) (Supplementary Material). We then tested the identified variants for association with MPN, using 237 Icelanders diagnosed with MPN, including PV ($n = 98$), ET ($n = 40$), PMF ($n = 26$) and 34 128 Icelandic controls with imputed genotypes (Table 1 and Supplementary Table S1). It is worth stressing that somatic MPN mutations do not affect the association analysis as the variants identified through WGS and subsequently imputed into MPN cases are almost exclusively from individuals not diagnosed with MPN (one MPN case, diagnosed 9 years after blood draw, among the 2230 sequenced individuals).

Assuming a multiplicative model, we observed a genome-wide significant association between MPN in the Icelandic population

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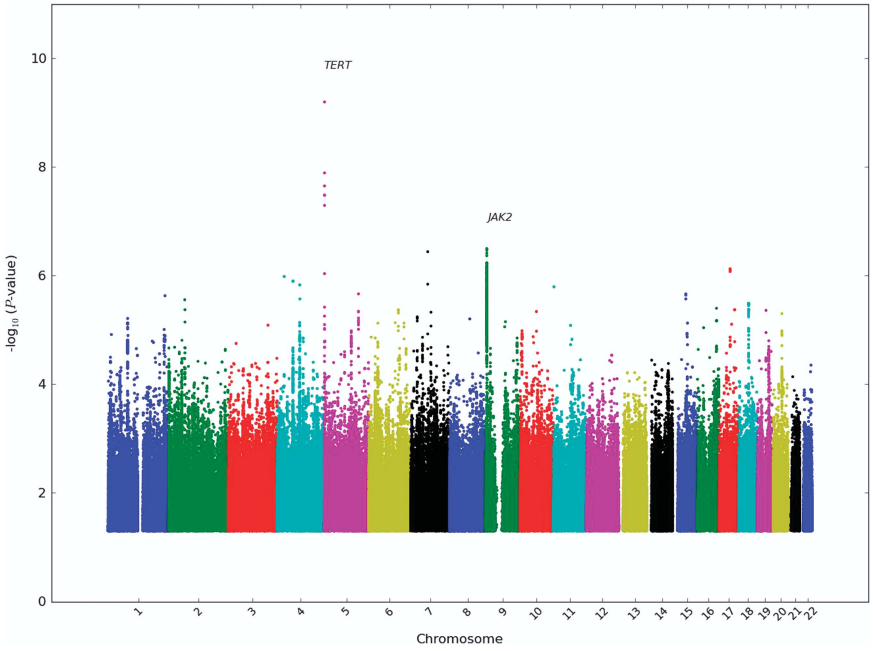

Figure 1. Genome-wide association results for MPN case ($N = 237$) and control ($N = 34\,128$) analysis in Iceland. The $-\log10$ of the allelic *P*-values <0.05 for the 34.2 million sequence variants tested is presented. The chromosomal distribution is shown as a Manhattan plot.

Table 1. Association of the *TERT* variant rs2736100_C and the *JAK2* variant rs1034072_A with MPN in Icelandic samples

Phenotype		*TERT rs2736100_C*		*JAK2 rs1034072_A*[a]	
	N	P-*value*	*OR*	P-*value*	*OR*
MPN (Ph-negative)	237	6.39×10^{-10}	2.09	3.19×10^{-7}	1.85
Polycythemia vera	98	1.10×10^{-5}	2.32	8.03×10^{-8}	2.76
Essential thrombocythemia	40	2.87×10^{-3}	2.25	0.25	1.39
Primary myelofibrosis	26	2.46×10^{-2}	2.42	0.27	1.56

Abbreviations: ET, essential thrombocythemia; JAK, janus kinase; MPN, myeloproliferative neoplasm; OR, odds ratio; PMF, primary myelofibrosis. Of the 237 MPN cases, 74 were diagnosed as MPN unclassifiable and 1 MPN case had two sub-phenotypes assigned. Risk allele frequency for TERT rs2736100 is 49.32%; risk allele frequency for JAK2 rs1034072 is 28.21%. [a]rs1034072 is the strongest signal at the JAK2 locus in our data and it is highly correlated ($r^2 = 0.91$) with the previously reported JAK2 germline variant rs10974944.

and a common variant rs2736100_C located in the second intron of the *TERT* gene at the *TERT-CLPTM1L* locus (allele frequency (AF) = 49.3%, odds ratio (OR) = 2.09, *P*-value $= 6.39 \times 10^{-10}$) (Figure 1 and Table 1). In addition, we replicate the association of rs10974944_G in *JAK2* previously reported to associate with MPN[5] (AF = 28.7%, OR = 1.78, *P*-value $= 1.90 \times 10^{-6}$; Supplementary Table S2) and detect an even stronger association between MPN and a second variant, rs1034072_A, in *JAK2* (AF = 28.2%, OR = 1.85, *P*-value $= 3.19 \times 10^{-7}$; Table 1). This variant is highly correlated with rs10974944_G ($r^2 = 0.91$; Supplementary Table S2). As reported for rs10974944_G, the association of the *JAK2* variant rs1034072_A with PV (OR = 2.76) is stronger than that with the other MPN subphenotypes (ET, OR = 1.39 and PMF, OR = 1.56)[5] (Table 1). In contrast, the effect of the risk allele of rs2736100_C at the TERT-CLPTM1L locus is similar for the three MPN subphenotypes, with OR 2.32 (PV), 2.25 (ET) and 2.42 (PMF) (Table 1).

We tested all variants at the *TERT-CLPTM1L* locus that associate with MPN with a *P*-value $< 1 \times 10^{-5}$ and those that have been associated with cancer and idiopathic pulmonary fibrosis,[6,7] conditioning on rs2736100. None of these SNPs remained significantly associated with MPN after conditioning the association on rs2736100 (Supplementary Table S3). This raises the possibility that rs2736100 is the causative variant at this locus as the association was based on WGS data and therefore, all simple variants at this locus were tested.

In a recent publication, rs2736100_C is one of the eight variants that were shown to associate with long telomeres in white blood cells,[8] suggesting that this SNP acts on the *TERT* gene encoding the reverse transcriptase of the telomerase complex essential for maintaining the telomere length. To our knowledge, functional effect of rs2736100 has not been evaluated. However, for rs7705526, an SNP located 542 bp telomeric to rs2736100 and the variant at the *TERT-CLPTM1L* locus that shows the highest correlation with rs2736100 ($r^2 = 0.52$; Supplementary Table S3), an increased enhancer activity has been described for the allele correlated with rs2736100_C.[9] Therefore, rs2736100_C might increase transcription of *TERT* through increased enhancer activity and this enhanced expression could mediate the MPN risk.

It is well documented that MPN cells have shorter telomeres than normal cells[10] as do cells in many types of neoplasms.[11] Short telomeres have been proposed to promote tumor formation by inducing genomic instability. The association of *TERT* rs2736100_C with increased risk for MPN and with longer telomeres thus appears to be a paradox. However, the biology of the *TERT* gene and its link to cancer (including MPN) is complicated and could

even be mediated through functions other than telomerase-mediated extensions of telomeres such as its effect on cell DNA repair, cycle regulators or on cell signaling.[12] This hypothesis is further supported by a lack of association with MPN of rs10936599_C in the *TERC* gene (template-containing telomerase RNA that is part of the telomerase complex; Supplementary Table S4) that shows the strongest reported genome-wide association with increased telomere length.[8]

An association of *TERT* rs2736100_C was observed in Iceland with an increase in red blood cell counts similar to a previous report[13] (effect (s.d.) = 0.019, *P*-value = 9.07×10^{-6}, $N = 76\,739$ individuals; Supplementary Table S5). In addition, we observed an association with elevated platelet counts (effect (s.d.) = 0.025, *P*-value = 5.26×10^{-8}, $N = 103441$ individuals) and white blood cell counts (effect (s.d.) = 0.016, *P*-value = 4.10×10^{-4}, $N = 126\,853$ individuals). The association with white blood cell counts is limited to cells of the myeloid linage (Supplementary Table S5) and the effect on blood cell counts remained unchanged after excluding MPN cases. In addition to the association of rs2736100_C with the three subphenotypes of MPN, these results for the blood cell counts indicate that the *TERT* variant asserts its effect on a common myeloid progenitor. As this effect on cell counts is seen in individuals without MPN it could mean that the risk of MPN conferred by the variant is mediated through an effect on cell numbers. In line with no effect on lymphocyte counts, rs2736100_C did not associate with lymphoproliferative malignancies (Supplementary Table S6). In contrast to the *TERT* variant, the germline *JAK2* risk variant rs1034072_A is not associated with red blood cell count (*P*-value = 0.91, effect (s.d.) = 0.001, $N = 76\,739$ individuals), but associates with decrease in platelet count (*P*-value = 2.50×10^{-4}, OR = −0.019, $N = 103\,441$ individuals) (Supplementary Table S5). This might indicate that *TERT* and *JAK2* predispose to MPN through different mechanisms.

The recurrent somatic mutation $JAK2^{V617F}$ is present in a large fraction of MPN cases.[2] We analyzed available blood samples from Icelandic MPN cases ($N = 103$) for the $JAK2^{V617F}$ somatic mutation using a real-time quantitative-PCR assay (Supplementary Material). We excluded blood samples drawn more than 2 years before MPN diagnosis ($N = 43$). In the remaining samples ($N = 62$) we observed positive $JAK2^{V617F}$ somatic mutation status in 69.4% of MPN cases and 80.6% of PV cases ($N = 31$) (Supplementary Table S7), in line with previous studies.[2] The germline MPN risk allele in *JAK2*, rs10974944_G, has been shown to associate with increased allelic burden of the $JAK2^{V617F}$ somatic mutation.[14] We tested the counts of the MPN risk alleles rs1034072_A in *JAK2* and rs2736100_C in *TERT* for correlation with $JAK2^{V617F}$ allele burden. We observed a significant correlation between the number of germline risk alleles and allelic burden of $JAK2^{V617F}$ at the *JAK2* locus ($P = 0.02$, effect = 10.28% of allelic burden per germline risk allele) but not at the *TERT* locus ($P = 0.77$) (Supplementary Table S8). However, owing to our small MPN sample set, a weak effect of the germline *TERT* variant on $JAK2^{V617F}$ allele burden cannot be excluded. Recurrent somatic mutations in the *TERT* promoter region, C228T and C250T, shown to increase TERT expression, have recently been reported in several cancer types.[15] We screened for these mutations by Sanger sequencing in the MPN cases used for screening the $JAK2^{V617F}$ mutation ($N = 62$) and observed only a single occurrence of the the C228T *TERT* mutation.

In conclusion, we report a common germline variant in *TERT* that associates with MPN. The risk mediated by the variant rs2736100_C is large (OR = 2.09) and in our data even larger than the reported *JAK2* germline signal rs1034072_A (OR = 1.85). As the *TERT* variant is more common than the *JAK2* variant (49.3% vs 28.2%) its population attributable risk is larger (57.7% vs 34.9%). Unlike the *JAK2* variant, the *TERT* variant exerts similar risk on the three MPN subtypes PV, ET and PMF. Our data also suggest that the germline *TERT* variant does not associate with the recurrent somatic $JAK2^{V617F}$ mutation. Association of the *TERT* variant with increased counts of myeloid white blood cells, red blood cells and platelets but not lymphoid cells, in the general population, together with similar effects on all three subpopulations of MPN suggests that the variant exerts its effect on hematopoiesis by increasing proliferation of cells derived from common myeloid progenitor.

CONFLICT OF INTEREST

For the authors who are affiliated with deCODE genetics/AMGEN, we declare competing financial interests as employees. The remaining authors declare no conflict of interest.

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Low rate of calreticulin mutations in refractory anaemia with ring sideroblasts and marked thrombocytosis

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Refractory anaemia with ring sideroblasts (RARS) and marked thrombocytosis (RARS-T) was proposed in the World Health Organisation 2001 classification of tumours of haematopoietic and lymphoid tissues and retained as a provisional entity in the 2008 version.[1] RARS-T is characterised by a high rate of *JAK2*^{V617F} mutations[2–4] or the presence of mutations in exon 10 of *MPL* (myeloproliferative leukaemia).[5,6] The existence of RARS-T as an entity independent from RARS and essential thrombocythemia (ET) was a matter of debate, as certain specialists favoured the hypothesis that RARS-T was a form of ET with $>15\%$ of ring sideroblasts in the bone marrow, whereas others thought that RARS-T is derived from RARS with secondary thrombocytosis developing through the acquisition of the *JAK2*^{V617F} mutation.

We recently demonstrated that RARS-T differed from RARS and ET from a clinical, biological and prognostic point of view.[4] The presence of high rates of splicing factor 3B subunit 1 (*SF3B1*) mutations in RARS-T and the absence of these mutations in ET strengthened the hypothesis that RARS-T was a distinct entity.[7–9]

However, the myeloproliferative features of RARS-T are not totally explained by *JAK2*^{V617F} and *MPL*^{W515L} mutations, as these account for only 50% and 1% of RARS-T, respectively. The question remains about other causative mutations responsible for the myeloproliferative part of RARS-T. The challenge is the same in myeloproliferative neoplasms (MPN), as 50–60% of ET and primary myelofibrosis (PMF) do not present with any *JAK2* or *MPL* mutations.

The recent discovery of a high rate of calreticulin (*CALR*) mutations in *JAK2*-non-mutated MPNs was a great step towards better understanding of the molecular pattern of these diseases. Mutations in exon 9 of *CALR* have been reported as mutually exclusive of *JAK2* and *MPL* mutations, and found in 67–71% of ET and 56–88% of PMF with wild-type *JAK2* or *MPL*.[10,11] These mutations are insertions or deletions leading to a frameshift responsible for modifying the C-terminal part of the protein. Following these modifications, the C-terminal part of the protein becomes positively charged and the reticulum targeting KDEL sequence is abrogated, which disturbs its cellular localisation.

Up to now, *CALR* mutations have only been explored in small RARS-T cohorts, with a mutation frequency ranging from 0 to 12.5%.[10,11] The purpose of our study was to analyse the frequency of *CALR* mutations in a large RARS-T cohort in order to determine whether *CALR* mutations may also be responsible for thrombocytosis in this disease. We therefore analysed a large cohort of 95 RARS-T patients, with 29 RARS as the control.

According to the WHO 2008 classification, patients are diagnosed with RARS-T if they fulfil the following criteria: i) anaemia (haemoglobin level <125 g/l for females and <135 g/l for males) with erythroid dysplasia and $>15\%$ ring sideroblasts; ii) thrombocytosis with $>450\times10^9$ platelets/l; iii) $<5\%$ blast cells in the bone marrow; iv) presence of large atypical megakaryocytes similar to those observed in *BCR-ABL1*-negative MPN; v) no secondary cause of ring sideroblasts; and vi) no karyotype abnormalities such as del(5q), t(3;3)(q21;q26) or inv(3)(q21q26).[1] One hundred and twenty-four samples including 95 RARS-T and 29 RARS from seven European centres in three European countries were collected and tested. This cohort has been published previously.[4,9]

For each patient, demographic (gender and age at diagnosis) and biological data (blood cell count, bone marrow exploration, ring sideroblasts, karyotype and molecular explorations) were collected.

The *SF3B1* mutations were analysed with a sensitive next-generation amplicon deep-sequencing assay (454 Life Sciences, Branford, CT, USA) with a median coverage of 500 reads. This approach was able to detect mutations with a sensitivity below 1%.[9]

The *JAK2*^{V617F} mutation was analysed by allele-specific real-time PCR to estimate the allele burden according to the method published by Lippert *et al.*[12] with a sensitivity of 1%. *JAK2* exon 12 analysis was performed according to the method by Schnittger *et al.*[13] and the *MPL*^{W515} mutations were analysed by high-resolution melting curve analyses followed by Sanger sequencing if positive, as previously published by Schnittger *et al.*[14]

The *CALR* exon 9 mutations were screened by fragment analysis and Sanger sequencing according to the method by Klampfl *et al.*[10]

Approval for the study was obtained from the ethics committee of each institution and the procedures were carried out in accordance with the Helsinki Declaration of 1975, as revised in 2000.

A total of 124 cases with 95 RARS-T and 29 RARS (including 62 males and 62 females) were recorded in the study, which is, to our best knowledge, the largest series of myelodysplastic syndromes with ring sideroblasts (MDS-RS) studied for *CALR* mutations. The median age at diagnosis was 74 years and 73 years for the RARS-T and RARS cohort, respectively (Table 1). A karyotype was available in 112 cases (87 RARS-T and 25 RARS). Seventy-five (86.2%) RARS-T

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