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The *CCND1* 870G>A polymorphism is a risk factor for t(11;14) (q13;q32) multiple myeloma

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URLs

The R suite can be found at <http://www.r-project.org/>

Detailed information on the tag SNP panel can be found at <http://www.illumina.com/dbSNP>: <http://www.ncbi.nlm.nih.gov/projects/SNP/>

HapMap: <http://www.hapmap.org/>

1000Genomes: <http://www.1000genomes.org/>

KASPAR allele specific PCR: <http://www.lgcgenomics.com/kasp-genotyping-reagents>

IMPUTE: <https://mathgen.stats.ox.ac.uk/impute/impute.html>

SNPTEST: <http://www.stats.ox.ac.uk/~marchini/software/gwas/snpstest.html>

EIGENSTRAT: <http://genepath.med.harvard.edu/~reich/Software.htm>

Wellcome Trust Case Control Consortium: www.wtccc.org.uk

METAL: www.sph.umich.edu/csg/abecasis/metal

Mendelian Inheritance In Man: <http://www.ncbi.nlm.nih.gov/omim>

Medical Research Council (MRC) Myeloma-IX trial: <http://public.ukcrn.org.uk>

Author Contributions

KH, HG, GJM, and RSH designed the study. RSH and GM obtained financial support in the UK and KH and HG in Germany. RSH and KH drafted the manuscript with contributions from GJM, DCJ and NW. DC, FH, BC, DCJ, YPM, SED and NW performed statistical and bioinformatics analyses; PB coordinated UK laboratory analyses. DCJ managed and prepared Myeloma IX and Myeloma XI Case Study DNA samples and performed expression analyses. HG, KN, NW and DH coordinated and managed German DNA samples. MWH and NW managed and prepared mantle cell lymphoma samples. LE managed the HNR samples. PH, TWM and MMN performed and coordinated genotyping of the German controls. KH and AF coordinated genotyping, performed by PH and MMN. DCJ, MFK, NLL and BAW performed UK expression analyses. FMR performed UK and AJ German FISH analyses. GJM, FED, WAG, and GHJ performed ascertainment and collection of UK case samples. All authors contributed to the final paper.

Competing Interests Statement

The authors declare no competing financial interests.

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Abstract

A number of specific chromosomal abnormalities define the subgroups of multiple myeloma. In a meta-analysis of two genomewide association studies of multiple myeloma totaling 1,661 patients we investigated risk for developing a specific tumor karyotype. The t(11;14)(q13;q32) translocation in which *CCND1* is placed under the control of the immunoglobulin heavy chain enhancer was strongly associated with the *CCND1* 870G>A polymorphism ($P=7.96 \times 10^{-11}$). These results provide for a model in which a constitutional genetic factor is associated with risk of a specific chromosomal translocation.

Multiple myeloma is a malignancy of the plasma cells that primarily localize to the bone marrow^{1,2}. Multiple myeloma can be broadly divided into hyperdiploid (HD) and non-hyperdiploid subtypes with further subdivision based on the presence of *IGH* translocation, most common of which are t(11;14)(q13;q32) and t(4;14)(p16;q32)³. These translocations cause transcriptional activation of *FGFR3/MMSET* and *CCND1* which contribute to MM biology³.

To explore the possibility of an influence of constitutional genotype on multiple myeloma karyotype we analyzed data from an expanded genomewide association study (GWAS) of multiple myeloma previously reported⁴. Briefly, cases of multiple myeloma from the UK and Germany were genotyped using Illumina OmniExpress BeadChips. The UK GWAS comprised 1,321 cases recruited from the UK Medical Research Council (MRC) Myeloma-IX trial. Genotype frequencies were compared with publicly accessible genotype data generated by the UK Wellcome Trust Case Control Consortium 2 (WTCCC2) study of 2,698 individuals from the 1958 British Birth Cohort (known as 58C)⁵ and 2,501 individuals from the UK Blood Service (UKBS) collections that had been genotyped using Illumina Human 1.2M-Duo Custom_v1 Array BeadChips (see Online Methods). The German GWAS comprised the original 384 multiple myeloma cases recruited from the Heidelberg University Clinic plus an additional 698 patients recruited through the same source. Genotype frequencies were compared with genotype data generated by the Heinz-Nixdorf Recall (HNR) study of 2,132 individuals⁶ from the German population who had been genotyped using Illumina Human Omni1-Quad BeadChips (Online Methods).

Genotype data were filtered on the basis of pre-specified quality control measures. SNPs showing deviation from Hardy-Weinberg equilibrium with $P < 1.0 \times 10^{-6}$ in controls, having a call rate $< 95\%$ or a minor allele frequency $< 1\%$ were excluded. Samples were removed if closely related or if they had a non-Northern and Western European descent (CEU) ancestry (Online Methods; Supplementary Figures 1 and 2). After filtering, 414,804 autosomal SNPs common to both case-control series remained (Supplementary Figure 1).

To explore the relationship between SNP genotype and translocations involving the *IGH*-locus (t(4;14), t(11;14) and other translocations), as well as HD status, we restricted our analysis to the 1,660 multiple myeloma patients with fluorescence *in situ* hybridization (FISH) and/or karyotype data (Supplementary Figure 1). Translocation data were available on 1,655 multiple myeloma patients, 12% with t(4;14) and 17% with t(11;14) and ploidy status was available for 1,535 patients, 55% of which had HD (Supplementary Table 1).

Prior to undertaking the meta-analysis of the two GWAS by subtype, we searched for potential errors and biases in the data sets. Quantile-quantile plots of χ^2 values for all cases against controls showed that there was limited inflation of the test statistics (genomic control inflation factor λ before and after Eigenstrat adjustment = 1.03, 1.01, and 1.17, 1.03, for UK and German studies respectively; Supplementary Figure 3). Using data from UK and German GWASs, we derived the combined OR and confidence interval (CI) for each SNP under a fixed-effects model along with the associated P -value for each multiple myeloma subtype (comparing case subtypes with all controls). Heterogeneity between different tumor subtypes was assessed by case-only analyses with the tumour characteristic as the outcome variable.

Only one SNP, rs603965, showed a statistically significant association with multiple myeloma risk (*i.e.* $P < 5.0 \times 10^{-8}$) that was subtype specific. All other associations were generic. The rs603965 genotype was associated with an OR of 1.82 for t(11;14) multiple myeloma ($P = 7.96 \times 10^{-11}$; Table 1, Supplementary Table 2). Furthermore, case-only analysis provided supportive evidence that the rs603965 association being specific to t(11;14) multiple myeloma ($P = 2.07 \times 10^{-11}$). Since multiple myeloma is the result of the transformation of plasma cells localized with the bone marrow and genotyping was performed on lymphocyte DNA obtained prior to chemotherapy in $> 80\%$ of cases, it is highly unlikely that somatic changes are influencing study findings. To confirm the fidelity of Illumina Bead Chip genotypes for rs603965 we retyped all t(11;14) cases and a subset of the controls from both studies using orthogonal technologies (Supplementary Table 3).

Associations for all other *IGH* translocations and HD multiple myeloma were either non-significant (*i.e.* $P > 0.05$) or were generic, being nonsignificant in a case-only analysis. Since multiple myeloma subgroups were not mutually exclusive and a common control series was used we performed a meta-analysis, using ASSET7, exploring all possible subsets of “non-null” studies (*i.e.* testing whether some associations in subsets are negatively, positively, or not associated). This method identifies the subset with the strongest association signal and accounts for the multiple tests required by the subset search (see Online Methods). This analysis provided further strong support for the rs603965 association with t(11;14) multiple myeloma ($P = 6.20 \times 10^{-9}$; Supplementary Figure 4).

rs603965 maps to a 62.4kb region of linkage disequilibrium (LD) at 11q13.3 (69,462,910bps) and is responsible for the 870G>A polymorphism in cyclin D1 (*CCND1*; MIM 168461). Additional support for the 11q13.3 association was provided by rs649392 ($P= 5.47 \times 10^{-9}$) and rs1352075 ($P= 1.48 \times 10^{-8}$) which are in LD with rs603965 ($r^2=0.47$ and 0.66 respectively; Supplementary Table 2). Cyclin D1 is a component of the core cell cycle machinery and increased levels are detected in many human cancers including multiple myeloma. In addition to activating cyclin-dependent kinases CDK4 and CDK6, cyclin D1 has kinase independent functions in DNA repair notably directly binding RAD51, a recombinase that drives homologous recombination^{8,9}. Overproduction of a D group cyclin is a common feature of multiple myeloma and in the t(11;14) subtype it is *CCND1* which is upregulated through close proximity to the powerful E μ enhancer of the *IGH* locus as a result of reciprocal translocation.

To further examine the relationship between 11q13.3 genotype and the risk of t(11;14) multiple myeloma, we imputed unobserved genotypes in the UK and German GWAS cases and controls using data from the 1000 Genomes Project (see Online Methods). This analysis did not provide evidence for a stronger association than that provided by rs603965 (Figure 1 and Supplementary Table 4). Conditional analysis provided for a model in which rs603965 genotype was sufficient to capture the association at 11q13.3.

In multiple myeloma the immunoglobulin gene translocations are generated by abnormal class switch recombination (CSR) events occurring during the germinal center stage of B cell development. Such abnormal CSR events are usually present in 100% of clonal cells and are also detectable in monoclonal gammopathy of unknown significance (MGUS), a premalignant phase of multiple myeloma, consistent with their early development in multiple myeloma^{3,10}. Normal class switch recombination involves a recognition step and double strand (DSB) DNA cleavage steps which is driven by activation induced deaminase. These DSBs are then repaired using translocation prone pathways not involving classical non-homologous end joining (NHEJ)¹¹ that can result in the formation of reciprocal translocation to other DSBs located elsewhere in the genome. We therefore examined the relationship between the rs603965 genotype and the risk of MGUS in 155 German patients, 40 of which were t(11;14) positive. As with multiple myeloma the frequency of the G allele was significantly higher in MGUS patients with t(11;14) than in those without the translocation (0.69 and 0.55 respectively, $P=0.03$). This observation is, therefore, entirely consistent with the impact of rs603965 on t(11;14) multiple myeloma risk arising early in the evolution of the disease. However, the absence of an association between rs603965 and the risk of translocations other than t(11;14) suggests the impact of genotype is not entirely through the facilitation of errors in class switch recombination.

t(11;14) is also a feature of other B-cell malignancies, notably mantle cell lymphoma (MCL) in which the translocation is mediated via abnormal VDJ recombination at the *IGH* locus. However, the breakpoints in MCL are to some extent clustered whereas the breakpoints in t(11;14) multiple myeloma are heterogeneous spanning several hundred kb in the 5' region of *CCND1*¹². To explore the possibility that the rs603965 genotype might influence the advent of a t(11;14) translocation in MCL we studied 45 German MCL patients and compared the allele frequencies with the 2,124 controls. Allele frequencies were not

different between the cases and controls (frequency of G allele in cases 0.54, controls 0.53; $P=0.85$), a finding consistent with data from a recently reported analysis of 121 MCL cases and 1,780 controls which also showed no evidence of a relationship between rs603965 genotype and MCL risk ($P=0.95$)¹³. The absence of an association is consistent with a different etiological basis for t(11;14) multiple myeloma and MCL 14,11.

Deregulation of a D group cyclin is a key feature of multiple myeloma and constitutes a major component of the TC (translocation cyclin D) classification of this disorder. *CCND1* is highly over-expressed in t(11;14) multiple myeloma as a consequence of its rearrangement in close proximity to the Ig enhancer region and in addition it is overexpressed in the majority of HD myeloma via an alternate mechanism. Normal plasma cells are non-cycling and as a consequence do not express cyclin D1, however, multiple myeloma results from the transformation of a plasmablast during class switch recombination. The level of expression of cyclin D1 at this stage of plasma cell development is unknown. We found no association between rs603965 genotype and the overall level of *CCND1* mRNA expression in CD138-selected primary multiple myeloma or lymphoblastoid cell lines (Supplementary Table 5). rs603965 has, however been shown to differentially influence the alternative splicing of *CCND1* mRNA, the 870G allele creating an optimal splice donor site at the exon 4/intron4 boundary resulting in the cyclin D1a transcript⁹. By contrast, 870A hinders splicing allowing for read-through into intron 4 and production of the variant cyclin D1b transcript⁹. Although 870A is preferentially associated with D1b production the A allele is not fully penetrant⁹. In CD138-selected plasma cells we found a significant allele-specific correlation between 870G genotype and D1a/D1b transcript ratio, in all multiple myeloma, HD and t(11;14) restricted samples (Supplementary Table 5).

Over the last decade the *CCND1* 870G>A polymorphism has been variously reported to be risk for a large number of cancers with the majority of studies linking the 870A to cancer risk^{9,15}. The molecular basis for the association between 870G and an almost doubling of the risk of developing t(11;14) multiple myeloma remains to be established. The two isoforms of *CCND1* differ in their fundamental biological and biochemical properties¹⁶. Cyclin D1b conveys greater transforming capacity¹⁶ but lacks the DNA damage response associated with cyclin D1a¹⁷. Although Cyclin D1b appears to have no cell regulatory properties in B lymphocytes¹⁸ both isoforms are detectable in multiple myeloma cell lines and primary patient myeloma cells but their respective role in myelomagenesis remains unresolved¹⁹. One potential model is that 870G facilitates clonal expansion post-translocation and over-expression of *CCND1*, but evidence supporting a differential oncogenic potential of D1a as compared with D1b is lacking. Moreover, in this respect, we found no evidence for an association with HD multiple myeloma. The lack of an association of *CCND1* genotype with other subgroups of multiple myeloma, which are also characterized by IG translocations but in this case deregulating other genes, argues against the genotype facilitating the development of translocation through a general mechanism. Although speculative, collectively our data are compatible with the 870G genotype impacting on the early evolution of t(11;14) multiple myeloma.

In conclusion our findings demonstrate a relationship between *CCND1* 870G>A and risk of t(11;14) multiple myeloma and provide the first evidence for genetic variation being a

determinant of the risk of acquiring a specific chromosomal translocation. This association was not found in the original GWAS due to the low frequency of this subtype and thus our findings underscore the value of conducting subtype specific analyses for cancer.

Online Methods

Ethics

Collection of samples and clinico-pathological information from subjects was undertaken with informed consent and relevant ethical review board approval in accordance with the tenets of the Declaration of Helsinki.

Genomewide association studies

The UK-GWAS has been previously described. Briefly, the study was based on multiple myeloma cases (ICD-10 C90.0; 469 male; mean age at diagnosis 63.9 years, SD 9.9) ascertained through the UK Medical Research Council (MRC) Myeloma-IX trial²⁰. All cases were UK residents. For controls, we used publicly accessible data generated by the Wellcome Trust Case Control Consortium from the 1958 Birth Cohort (58C; also known as the National Child Development Study)⁵ and National Blood Service (NBS). Genotyping of both sets of cases was conducted using Illumina Human 1-2M-Duo Custom_v1 Array chips.

The German-GWAS comprised 384 multiple myeloma cases which were the subject of a previous publication and an additional series of 698 multiple myeloma cases (388 males and 310 females, mean age at diagnosis of 59 years; SD 9.3) recruited at the University Clinic, Heidelberg. As before the additional cases were genotyped using Illumina Human OmniExpress-12 v1.0 arrays according to the manufacturer's protocols (Illumina, San Diego, USA). DNA samples with GenCall scores <0.25 at any locus were considered "no calls". A SNP was deemed to have failed if <95% of DNA samples generated a genotype at the locus. For controls, we used genotype data on 2,132 healthy individuals enrolled into the Heinz Nixdorf Recall (HNR) study⁶.

Mantle cell lymphoma cases

The rs603965 genotype was determined in 45 German patients with mantle cell lymphoma recruited from the University Clinic, Heidelberg (37 male, mean age at diagnosis 60.3 years; SD 8.1).

Monoclonal gammopathy of unknown significance (MGUS) cases

The analysis was based on 155 MGUS cases ascertained through the University Clinic, Heidelberg (80 male, mean age at diagnosis 59.5 years; SD 12.0).

Validation of SNP genotypes

Confirmation of the accuracy of Illumina BeadChip rs603965 genotypes in cases and controls, we: (1) Sequenced all the German t(11;14) cases using ABI310 technology (Applied Biosystems, Foster City, CA); (2) Genotyped all German t(11;14) cases and a subset of controls, all UK t(11;14) cases and a subset of controls using Kaspar Allele

specific PCR (LCG Genomics) (Supplementary Table 3). PCR primer sequences and conditions are available upon request.

Statistical and bioinformatic analysis

The same quality control metrics on the new German GWAS data were applied as in our previous multiple myeloma study⁴. Briefly, samples were excluded if <95% of SNPs were successfully genotyped (10 sample) or if identity-by-state (IBS) probabilities for pairs of samples were <0.80 (13 samples). To identify individuals of divergent ancestry we conducted a principal component analysis using smartPCA from the EIGENSTRAT21 package and a pruned SNP set²². We excluded 70 individuals from analyses. SNPs having a minor allele frequency <1% or a call rate <95% were excluded. SNPs showing departure from Hardy-Weinberg equilibrium in controls at $P < 1 \times 10^{-6}$ were also excluded.

Main analyses were undertaken using R (v2.6), Stata v.10 (State College, Texas, US), PLINK (v1.06) and EIGENSTRAT software. The association between each SNP and cytogenetic subgroup of multiple myeloma was assessed by the Cochran-Armitage trend test. The possibility of differential genotyping of cytogenetic subgroups and controls were evaluated using quantile-quantile (Q-Q) plots of test statistics. Odds ratios (ORs) and associated 95% confidence intervals (CIs) were calculated by unconditional logistic regression. To fully examine the effects of population substructure on our findings, we performed principal-components analysis using the EIGENSTRAT21 software. Meta-analysis was firstly conducted using standard methods²³. Cochran's Q statistic was calculated to test for heterogeneity²³ and the I^2 statistic to quantify the proportion of the total variation due to heterogeneity calculated; I^2 values >75% are considered characteristic of large heterogeneity²⁴.

Meta-analysis was also performed using the R statistical package ASSET (ASsociation analysis based on subSETs) which explores all possible subsets of "non-null" studies to identify the strongest association signal and then evaluates the significance of the signal while accounting for multiple tests required by the subset search⁷.

Prediction of the ungenotyped SNPs was carried out using IMPUTEv2 based on the 1000 genomes phase 1 integrated variant set (b37) from March 2012. Imputed data were analyzed using SNPTEST v2 to account for uncertainties in SNP prediction. LD metrics were calculated in plink using 1000 genomes data and plotted using SNAP. LD blocks were defined according to HapMap recombination rate (centimorgans per megabase), as determined using the Oxford recombination hotspots²⁵ and the previously set distribution of confidence intervals²⁶.

Fluorescence *in situ* hybridization (FISH)

FISH and ploidy classification of UK samples was conducted using the methodology described by Chiecchio *et al*²⁷. FISH and ploidy classification of German samples was performed as previously described²⁸. The XL IGH Break Apart probe (MetaSystems, Altussheim Germany) was used to detect any IgH translocation in German samples.

Relationship between SNP genotype and mRNA expression

To examine for a relationship between rs603965 genotype and *CCND1* expression in multiple myeloma (CD138 selected plasma cells), we made use of Affymetrix Human Genome U133 Plus 2.0 array data we previously generated on the plasma cells from 192 multiple myeloma patients from the MRC Myeloma IX trial²⁹. To examine for a relationship between SNP genotype and expression levels in lymphocytes we made use of publicly available expression data generated on lymphoblastoid cell lines from Geneva GenCord individuals³⁰ MuTHER resource³¹ and HapMap³³² using Sentrix Human-6 Expression BeadChips (Illumina, San Diego, USA)^{33,34}.

To quantitatively evaluate the expression of *CCND1a* and *CCND1b* transcripts in CD138 selected plasma cells, total RNA was extracted using RNA/DNA mini kit or Allprep kit, Qiagen and cDNA was then synthesised using SuperScript II reverse transcriptase with Oligo (dT)_{12–18} primer (Invitrogen) according to the manufacturer's instructions. Custom PCR primers were designed using Primer 3 to Exon4-Intron4 (short isoform -CCND1b), together with previously described primers to 3UTR (long isoform – CCND1a)³⁵(Supplementary table 6). CDNA was amplified using a 7500 real-time PCR cyclor with SYBR® Green Reagents according to the manufacturer's protocol. The relative quantification of *CCND1* isomers was determined by Comparative C_T (C_T) method using 7500 Software version v2.0.6 (Applied Biosystems™, Foster city, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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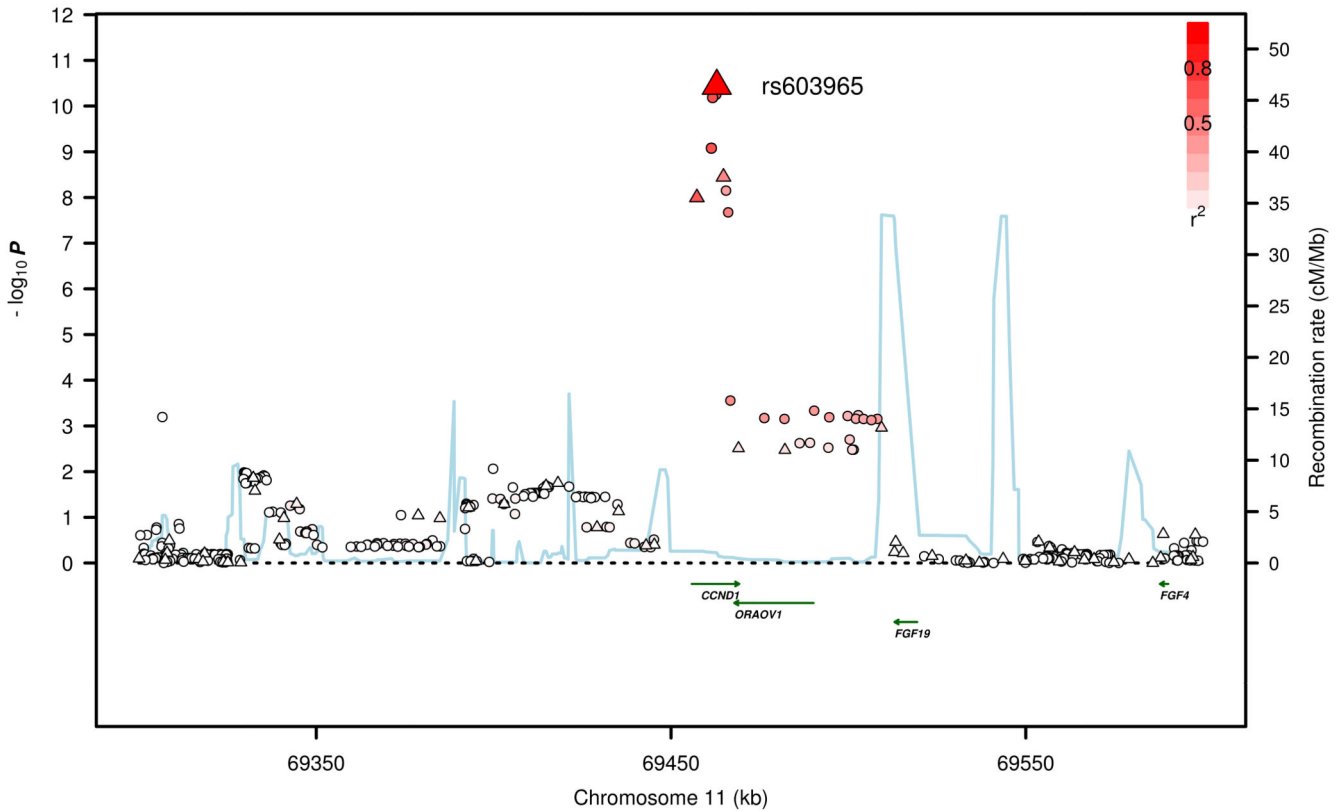


Figure 1. Regional plot of association results and recombination rates around the *CCND1* locus at chromosome 11q13.

Triangles denote directly typed SNPs and circles imputed SNPs. $-\log_{10} P$ values (y axis) of the SNPs are shown according to their chromosomal positions (x axis). The color intensity of each symbol reflects the extent of LD with rs603965, from white ($r^2 = 0$) to dark red ($r^2 = 1.0$). Genetic recombination rates, estimated using HapMap CEU samples, are shown with a light blue line. Physical positions are based on NCBI build 37 of the human genome. Genes have been redrawn to show their relative positions; therefore, maps are not to physical scale.

Table 1
Summary results for the rs603965 association with t(11;14) multiple myeloma risk.

(a) Case-control analysis (b) case-only analysis

(a) Case-control												
	<u>t(11;14) cases</u>				<u>Controls</u>				OR^b	95% CI^c	P	P
	RAF^a	GG	GA	AA	RAF	GG	GA	AA				
UK GWAS	0.72	57	41	10	0.56	1615	2561	1023	2.02	1.53-2.72	4.17x10 ⁻⁶	*
German GWAS	0.67	76	86	17	0.53	610	1031	465	1.72	1.37-2.16	3.08x10 ⁻⁶	7.67x10 ⁻⁶
Combined									1.82	1.52-2.19	7.96x10⁻¹¹	2.92x10⁻¹⁰
									<i>P_{het}=0.40 I²=0.00</i>			
(b) Case-only												
	<u>t(11;14) cases</u>				<u>Non t(11;14) cases</u>				OR^b	95% CI^c	P	P
	RAF^a	GG	GA	AA	RAF	GG	GA	AA				
UK GWAS	0.72	57	41	10	0.55	197	313	135	2.06	1.50-2.82	7.55x10 ⁻⁶	*
German GWAS	0.67	76	86	17	0.52	186	360	163	1.89	1.48-2.43	5.66x10 ⁻⁷	*
Combined									1.95	1.61-2.38	2.07x10⁻¹¹	*
									<i>P_{het}=0.67 I²=0.00</i>			

^aRisk allele frequency (RAF).

^bOdds ratio.

^c95% Confidence Interval.

^dEigenstrat adjusted *P*-values. Uncalled genotypes are not shown.

* no adjustment necessary