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## Molecular dissection of NRG1-ERBB4 signaling implicates *PTPRZ1* as a potential schizophrenia susceptibility gene

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### Abstract

Neuregulin and the neuregulin receptor ERBB4 have been genetically and functionally implicated in schizophrenia. In this study, we used the yeast two-hybrid system to identify proteins that interact with ERBB4, to identify genes and pathways that might contribute to schizophrenia susceptibility. We identified the MAGI scaffolding proteins as ERBB4-binding proteins. After validating the interaction of MAGI proteins with ERBB4 in mammalian cells, we demonstrated that ERBB4 expression, alone or in combination with ERBB2 or ERBB3, led to the tyrosine phosphorylation of MAGI proteins, and that this could be further enhanced with receptor activation by neuregulin. As MAGI proteins were previously shown to interact with receptor phosphotyrosine phosphatase  $\beta/\zeta$  (RPTP $\beta$ ), we postulated that simultaneous binding of MAGI proteins to RPTP $\beta$  and ERBB4 forms a phosphotyrosine kinase/phosphotyrosine phosphatase complex. Studies in cultured cells confirmed both a spatial and functional association between ERBB4, MAGI and RPTP $\beta$ . Given the evidence for this functional association, we examined the genes coding for MAGI and RPTP $\beta$  for genetic association with schizophrenia in a Caucasian United Kingdom case-control cohort ( $n \sim 1400$ ). *PTPRZ1*, which codes for RPTP $\beta$ , showed significant, gene-wide and hypothesis-wide association with schizophrenia in our study (best individual single-nucleotide polymorphism allelic  $P = 0.0003$ ; gene-wide  $P = 0.0064$ ; hypothesiswide  $P = 0.026$ ). The data provide evidence for a role of *PTPRZ1*, and for RPTP $\beta$  signaling abnormalities, in the etiology of schizophrenia. Furthermore, the data indicate a role for

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RPTP $\beta$  in the modulation of ERBB4 signaling that may in turn provide further support for an important role of neuregulin/ERBB4 signaling in the molecular basis of schizophrenia.

### Keywords

case-control studies; association; protein-tyrosine-phosphatase; receptor protein tyrosine kinases; neuregulins; ERBB4

### Introduction

There is now a large body of evidence that the neuregulin 1 (*NRG1*) gene influences risk for schizophrenia, with an initial linkage and association study in the Icelandic population followed by more than 10 case-control studies and more than five family-based association studies providing positive evidence for association.<sup>1-4</sup> *NRG1* spans a 1.4-megabase region of chromosome 8p13, and is comprised of at least 20 exons. The gene undergoes complex alternative splicing, generating at least 15 protein isoforms that fall into six families (identified as I-VI).<sup>5</sup> These gene products include soluble isoforms, as well as isoforms with one or two transmembrane domains. Gene products of *NRG1* include heregulin, neu differentiating factor, acetylcholine receptor-inducing activity, glial growth factor 2 and sensory and motor neuron-derived factor. Much of the positive association for *NRG1* in schizophrenia centers around the first two exons of *NRG1* (E187 and E1006, using the nomenclature of Steinhorsdottir *et al.*<sup>5</sup>), around the region of the Icelandic ‘core at-risk haplotype’ first demonstrated to be associated with the disorder. These two alternatively spliced exons are involved in specifying the type IV and type II isoforms of neuregulin 1.<sup>5</sup>

Expression studies of neuregulin 1 in postmortem samples from subjects with schizophrenia provide support for neuregulin 1 abnormalities in the disorder. Expression of type I-containing isoforms were elevated in schizophrenia.<sup>6,7</sup> Moreover, a single-nucleotide polymorphism (SNP; SNP8NRG243177) and a four-marker haplotype (both derived from the Icelandic haplotype) were significantly associated with the expression of type IV neuregulin isoforms in the brain, with the risk allele or haplotype associated with increased expression of these isoforms.<sup>7</sup>

*NRG1* gene products can activate intracellular signaling pathways through interactions with members of the ERB family of receptor tyrosine kinases (ERBB2, ERBB3 and ERBB4).<sup>8-11</sup> Of the three ERBB receptors that are involved in *NRG1* signaling, ERBB2 lacks an active ligand-binding domain and ERBB3 lacks an active catalytic domain, so both of these receptors must form heterodimers for signal transduction.<sup>8-11</sup> In contrast, ERBB4 can form functional homodimers as well as heterodimers. Recently, it has been shown that ERBB4 is a substrate for  $\gamma$ -secretase cleavage, which results in the liberation of the ERBB4 intracellular/cytoplasmic domain (EICD), which may then be involved in cytoplasmic and/or nuclear signaling.<sup>8-11</sup> ERBB4 plays a critical role in the development of the brain, and has been shown to directly influence the proliferation, organization and migration of cells in the rostral migratory stream.<sup>12-14</sup>

There is now emerging evidence that the gene for *ERBB4* also influences risk for schizophrenia,<sup>15,16</sup> especially when considered together with *NRG1*.<sup>16</sup> Moreover, there is evidence from postmortem mRNA studies for alterations in the levels of *ERBB4*<sup>15</sup> (as well as the *ERBB3* coreceptor<sup>17–21</sup>) in schizophrenia. A recent study that investigated neuregulin stimulation of *ERBB4* in slices of prefrontal cortex from control and schizophrenic subjects demonstrated enhanced activation of *ERBB4* by neuregulin in schizophrenia.<sup>22</sup> Finally, mouse models with disruptions in *NRG1-ERBB4* signaling appear to capture some of the behavioral phenotypes associated with schizophrenia, and in certain cases these altered behaviors can be ameliorated by antipsychotics.<sup>1,23–25</sup>

Altogether, the data support a role for abnormal *NRG1-ERB* signaling in schizophrenia. Dissecting the pathways of *NRG1-ERB* signaling in the central nervous system may therefore contribute to the elucidation of pathways of schizophrenia susceptibility and may also reveal targets for interventions. The functional and genetic evidence implicating *ERBB4* in schizophrenia suggests that this is a particularly interesting protein for further study.

In the current study we carried out a yeast two-hybrid analysis,<sup>26</sup> with the cytoplasmic domain of *ERBB4* as bait. We identified two proteins that interacted with *ERBB4*, one of which was the scaffolding protein *MAGI-2*.<sup>27</sup> *MAGI* proteins have been shown to bind to receptor phosphotyrosine phosphatase  $\beta/\zeta$  (*RPTP\beta*),<sup>28,29</sup> indicating the possible existence of a kinase–phosphatase complex brought together by a *MAGI* scaffold. In support of this, tyrosine phosphorylation of *MAGI* proteins was increased in the presence of *ERBB4* and decreased in the presence of *RPTP\beta*. In light of these data, we examined *PTPRZ1*, the gene coding for *RPTP\beta*, and *MAGI-1-3*, for evidence of association with schizophrenia. We obtained hypothesis-wide evidence for genetic association between *PTPRZ1* and schizophrenia, consistent with the general hypothesis of an etiological role for *NRG1-ERBB4* signaling in schizophrenia.

## Materials and methods

### Yeast two-hybrid screening

The cytoplasmic domain of the cyt1 isoform of *ERBB4* (i.e., the isoform which includes the cytoplasmic exon containing a docking site for phosphatidylinositol 3-kinase (*PI3K*)<sup>30</sup>) was cloned into the DUAL-hybrid bait vector in frame with the LexA DNA-binding domain, and the resultant construct confirmed by sequencing. After validating that the 96 kDa fusion protein was expressed in yeast and that it did not, by itself, activate reporter gene expression,  $1.3 \times 10^7$  colonies from an adult mouse brain cDNA library (complexity  $1 \times 10^6$ ; Clontech, Mountain View, CA, USA) were screened. Seventy clones were identified and subsequently reassayed, and 12 of these clones remained positive after an X-Gal filter test. These 12 clones were analyzed by retransformation with either the *ERBB4* bait or with the control bait. All 12 clones showed bait dependency and were considered specific interactors in the yeast assay. Restriction analysis and sequencing of all clones indicated the existence of four unique clones among the 12. Yeast two-hybrid screening was carried out together with Dualsystems Biotech (Zurich, Switzerland).

### cdNA constructs

Full-length MAGI-1 and MAGI-2 were cloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA), with the addition of an hemagglutinin (HA) tag at the N terminal. In addition, the ERBB4 EICD, with an initiator methionine and a Kozak<sup>31</sup> consensus sequence, was cloned into pcDNA3. Similarly, full-length receptor RPTP $\beta$  (short form) was cloned into pcDNA3. Expression vectors for the JMa splice variants of ERBB4 has been described previously.<sup>32</sup> All ERBB4 constructs were of the cyt1<sup>30</sup> splice variant.

### Transfection of mammalian cells

H4 human neuroglioma cells, maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>, were transfected with the indicated plasmids, using Lipofectamine (Invitrogen), following the manufacturer's protocol.

### Co-immunoprecipitation

For co-immunoprecipitation from cell lysates, cells were harvested in lysis buffer containing 1 × phosphate-buffered saline (PBS)/0.5% Triton X-100/protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The lysate was sonicated using a Misonix microson sonicator (Misonix Inc., Farmingdale, NY, USA) and centrifuged at 16 000 *g* for 1 h to clear cellular debris. Cleared lysate (5  $\mu$ g) was immunoprecipitated using antibodies as indicated, followed by the addition of 20  $\mu$ l of protein A agarose (Invitrogen). The immunoprecipitate was washed three times in lysis buffer and eluted by boiling in sodium dodecyl sulfate (SDS) sample loading buffer containing 1M Tris-HCl (pH6.8)/10% SDS/50% glycerol/200mM  $\beta$ -mercaptoethanol/bromophenol blue. Samples were run on a 10% NOVEX SDS-polyacrylamide gel (Invitrogen) and transferred to membranes. Immunoblotting was carried out using primary antibodies as indicated, followed by secondary horseradish peroxidase-labeled anti-mouse antibody, and detection using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rochford, IL, USA).

### Colocalization

Cells were fixed in 4% formaldehyde in PBS prewarmed to 37°C for 15 min and permeabilized in 0.1% Triton X-100 for 2 min. Cells were incubated with PBS/2% BSA for 1 h at room temperature, and then with antibodies as indicated. After three washes with 1 × PBS/0.2% BSA, cells were incubated with secondary antibody. Coverslips with adherent cells were mounted onto slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were captured using a Leica TCSSP (UV) confocal microscope in the Mount Sinai Microscopy Shared Research Facility.

### Tyrosine phosphorylation

To characterize tyrosine phosphorylation, MAGI-1 or MAGI-2 were transfected into 293 cells, together with ERBB2/3/4 constructs and/or RPTP $\beta$ . Twenty-four hours after transfection, cells were split into smaller wells, and allowed to adhere for an additional 8h. For neuregulin treatment, cells were then washed in serum-free medium and serum-starved for 12 h, followed by treatment for 15min with either 100 ng/ml of recombinant human neuregulin (NRG1-beta EGF domain, R&D systems, Minneapolis, MN, USA) or vehicle.

Cells were subsequently lysed in phosphorylation lysis buffer (50mM Hepes (pH 7.5), 100mM NaCl, 10% glycerol, 1mM EGTA, 1.5mM MgCl<sub>2</sub>, 100mM NaF, 0.6mM Na<sub>3</sub>VO<sub>4</sub> (activated) and Complete Protease EDTA-free Inhibitor Tablets (Roche)), with 2% SDS. After boiling for 2 min, lysates were spun to remove residual debris, and an equal volume of phosphorylation lysis buffer, with 10% Triton X-100, was added. MAGI proteins were then recovered by immunoprecipitation using anti-HA antibody. After electrophoresis and transfer to nitrocellulose, total MAGI was determined by immunoblotting with the anti-HA antibody, whereas tyrosine-phosphorylated MAGI was determined by immunoblotting with an anti-phosphotyrosine antibody (4G10, Upstate, Boston, MA, USA).

### Genetic association analysis

The case sample consisted of 673 unrelated subjects with schizophrenia (455 males). All were white and born in the UK or Ireland, and had a consensus diagnosis of schizophrenia according to DSM-IV criteria made by two independent raters following a semistructured interview by trained psychiatrists or psychologists. Full details concerning diagnostic practices and demographics are reported elsewhere.<sup>33</sup> The controls for the core sample were 716 individuals (482 males) matched to cases for age, sex and ethnicity, chosen from more than 1400 blood donors recruited from the National Blood Transfusion Service. They were not specifically screened for psychiatric illness but unless there is a major ascertainment bias towards patient inclusion, the use of unscreened controls does not affect power for disorder with the population prevalence of schizophrenia.<sup>34</sup> Multicentre and Local Research Ethics Committee approval were obtained, and all subjects, cases and controls, gave written informed consent to participate. Markers that were significantly associated in the primary sample were genotyped in an additional set of controls ( $n = 617$ ; 233 males) that was drawn from the same source. Although matched for ethnicity, they were not matched for gender. For none of the markers in which this additional sample was typed did the allele frequencies in controls vary by gender ( $P_{\min} = 0.12$  for marker rs10278079) or between the two sets of controls ( $P_{\min} = 0.34$  for marker rs1147502).

The SNaPshot (Applied Biosystems, Foster City, CA, USA) protocol for pooled DNA genotyping has been described in detail.<sup>35</sup> Positive data in the pooled analyses were followed up by individual genotyping, thereby excluding false positives arising from measurement error. Because pooled analysis usually overestimates evidence for association, and because we genotyped a large number of markers in each gene, to strike a balance between sensitivity and follow-up genotyping burden, individual genotyping was undertaken where the estimated  $P$ -value for one marker was  $< 0.01$ , or where more than one marker gave an estimated  $P$ -value from pooled analysis of  $< 0.05$ . Individual genotyping was performed with the Sequenom MassARRAY using HME and iPLEX chemistries (Sequenom, San Diego, CA, USA, <http://www.sequenom.com>) according to the recommendations of the manufacturers. All assays were optimized in 30 reference CEU trios (Utah residents with ancestry from Northern and Western Europe) from HapMap (<http://www.hapmap.org>). All plates contained a mixture of cases, controls, blanks and CEU samples. Genotypes were called in duplicate by a rater blind to sample identity and blind to the other rater. Genotypes of CEU samples were compared to those available on the HapMap to provide a measure of genotyping accuracy. Genotyping assays were only considered suitable for analysis if our

own data from CEU individuals were the same as those in the HapMap during optimization and in sample plates.

For statistical analyses, pooled data were tested for association using contingency tables created by multiplying double the number of individuals represented in each pool by the estimated allele frequencies. Contingency tables were also used for single-marker case-control analysis. Haplotypes were analyzed using UNPHASED.<sup>36</sup> Genotypes were tested for Hardy-Weinberg equilibrium using a  $\chi^2$  goodness-of-fit test. Analyses of linkage disequilibrium (LD) between markers ( $r^2$  and  $D'$ ) were performed using Haploview (<http://www.broad.mit.edu/mpg/haploview/index.php>). The number of effective independent SNPs assayed was estimated by the spectral decomposition method of Nyholt.<sup>37</sup> The truncated product of  $P$ -values to derive overall gene-wide significance was calculated by multiplying all  $P$ -values reaching a threshold of  $P < 0.05$  to achieve a product  $P$ -value.<sup>38</sup> The significance of this product was established by permuting case-control status for all genotyped markers over 100 000 replicates and counting the number of times this product was exceeded.

## Results

### Identification of MAGIs as ERBB4-interacting proteins

The cytoplasmic domain of ERBB4 was used to screen a mouse brain library for interacting proteins. We identified two classes of interacting clones, one including domains of the MAGI-2 scaffolding protein, and one including domains of the transcription factor PLZF. MAGI proteins include three highly related proteins (MAGI-1, MAGI-2 and MAGI-3), which are comprised of six PDZ domains (blue spheres, Figure 1), a guanylate kinase domain (oval) and WW domains (orange spheres). In the yeast two-hybrid screen, the MAGI-2 clones included the two WW domains and the downstream PDZ domain (PDZ1), corresponding to exons 5–9 of the gene (underlined in Figure 1). Glutathione S-transferase-pulldown experiments using recombinant proteins corresponding to the various domains of MAGI-2 confirmed that PDZ1 was able to bind the C terminus of ERBB4 (data not shown).

After demonstrating specificity of the MAGI-2/ERBB4 interaction in yeast (data not shown), we validated the interactions between MAGI and ERB4 in mammalian cells (Figure 2). We transfected human neuroglioma H4 cells with either control plasmid (vector alone) or MAGI-2, in the presence or absence of either full-length ERBB4 or EICD. Immunoprecipitation of MAGI-2 with anti-HA antibodies resulted in the co-precipitation of full-length ERBB4, the cytoplasmic fragment (CTF) of ERBB4 resulting from normal processing, and the EICD fragment (Figure 2).

Expression of exogenous ERBB4 demonstrated primarily perinuclear localization, consistent with a localization of ERBB4 to membrane compartments (Figure 3a). Expression of exogenous MAGI-2 alone resulted in a more diffuse staining of the cytoplasm, whereas expression of EICD alone resulted in diffuse cytoplasmic and nuclear localization (data not shown). When we co-transfected MAGI-2 with either EICD or ERBB4, we observed colocalization of MAGI-2 with the other proteins, particularly in cytoplasmic compartments (Figure 3a). Interestingly, ERBB4 showed a more pronounced reticular staining in the

presence of MAGI-2, as compared to in its absence (Figure 3a), indicating that MAGI-2 altered the steady-state distribution of ERBB4.

We then used purified rat oligodendrocytes, in which we had confirmed that ERBB4 and MAGI (predominantly MAGI-1) were expressed, to demonstrate colocalization of endogenous proteins (Figure 3b). Double labeling for ERBB4 and MAGI demonstrated colocalization of the two proteins.

### Modulation of MAGI phosphorylation on tyrosine

ERBB4 is a receptor tyrosine kinase, found either as a homodimer, or, more typically, as a heterodimer with ERBB2 or ERBB3. As MAGI proteins interact with ERBB4, we investigated whether MAGI proteins are substrates for tyrosine phosphorylation in the presence of ERBB4. When MAGI-1 (Figure 4a) or MAGI-2 (not shown) were co-transfected with ERBB4 (in the absence or presence of ERBB2 or ERBB3), we observed increased tyrosine phosphorylation of the MAGI proteins, when compared to cells transfected with MAGI proteins alone. We then transfected cells with ERBB4, ERBB2 and MAGI-1, treated them with vehicle or with neuregulin 1, and examined tyrosine phosphorylation of MAGI proteins. In the presence of neuregulin 1, we observed an approximately twofold increase in phosphorylation of MAGI-1 on tyrosine (Figure 4b). Similar results were observed when cells were transfected with ERBB3 and ERBB4 in place of ERBB2 and ERBB4 (data not shown).

MAGI proteins, including MAGI-1 and MAGI-3, have previously been shown to bind to RPTP $\beta$  (through PDZ4, SH, unpublished).<sup>28,29</sup> Furthermore, RPTP $\beta$  has been shown to dephosphorylate tyrosinephosphorylated MAGI-1.<sup>29,39</sup> As our data indicated that MAGI proteins could bind and be phosphorylated by a tyrosine kinase (ERBB4), we sought to determine whether MAGI proteins could represent a functional (and possibly physical) link between a phosphotyrosine kinase and a phosphatase activity. We therefore transfected cells with ERBB2, ERBB4 and MAGI-1, in the presence or absence of RPTP $\beta$ , and assayed the tyrosine phosphorylation of MAGI-1. Addition of RPTP $\beta$  resulted in a significant decrease in the phosphorylation of MAGI-1 (Figure 4b), supporting a functional relationship between ERBB receptors, MAGI proteins and RPTP $\beta$ . Similar results were observed with MAGI-2 (data not shown). Colocalization of ERBB4, MAGI and RPTP $\beta$  in oligodendrocytes indicated that such a complex could form endogenously (Figure 3b).

### Genetic association analysis of PTPRZ1 with schizophrenia

Our interest in dissecting ERBB4 signaling was to contribute to the understanding of the etiology of schizophrenia. As RPTP $\beta$  appears to modulate ERBB4 (and ERBB2/ERBB4, ERBB3/ERBB4) signaling, we next asked whether sequence variants in *PTPRZ1*, which encodes RPTP $\beta$ , or any of the genes encoding MAGI proteins, were genetically associated with schizophrenia. We carried out association analysis using a two-staged approach. In the first stage, we looked for evidence of association by genotyping pools of DNA of cases and controls, followed by individual genotyping, if indicated. We required gene-wide significance to go on to the second stage, where we genotyped additional SNPs within the gene.

In stage 1 of the analysis of *PTPRZ1*, we genotyped in pools 19 polymorphic SNPs selected from dbSNP and HapMap ([www.hapmap.org](http://www.hapmap.org)) with a focus on exonic sequences based on reference sequence NM\_002851. Most of the exons were within 2 kb of a polymorphic SNP, the exceptions being exons 10 (3 kb from the nearest SNP), exon 16 (3.6 kb), exon 17 (3.8 kb), exon 28 (3.3 kb), exon 29 (4.3 kb) and exon 30 (5.6 kb) where the nearest markers were not polymorphic. The positions of the markers are indicated in Figure 5 and the results of the pooled analyses are presented in Table 1. Four markers (rs1196513, rs13241278, rs1196471 and rs1147502) yielded nominally significant evidence ( $P = 0.05$ ) for association. Assays were designed for three of the markers (we dropped rs1196513 as this was highly correlated ( $r^2 = 0.91$ ) with rs1196471 in the CEU trios). However, the designed assays did not yield reliable genotypes at rs1196471 (but see below). Both rs13241278 and rs1147502 yielded nominally significant evidence for allelic association ( $P = 0.016$  and  $P = 0.05$ , respectively) in the case–control sample. To determine if this evidence for association was due to sampling variation in the controls, we typed approximately 600 additional blood donor controls for the two markers. The combined results are given in Table 2. The evidence for rs1147502 remained effectively unchanged ( $P = 0.02$ ), whereas that for rs13241278 improved somewhat ( $P = 0.003$ ). To correct for multiple testing, we estimated the effective number of independent SNPs using the method of Nyholt<sup>37</sup> based on the Caucasian European Utah Resident (CEU) dataset in HapMap. The number of independent SNPs (*MeffLi*) was estimated at eight. However, five of the 19 SNPs were not included in HapMap and therefore these were added to give a total of 13 independent SNPs, a conservative estimate because it assumes no LD between any of the additional five markers and any other marker. Thus, for rs13241278, the phase 1 genewide corrected  $P$ -value is 0.039. All genotypes were in Hardy–Weinberg equilibrium for cases and controls.

Because the pooled analysis was biased towards exons (Figure 5) and also inaccuracies in pooling may result in type II error,<sup>34</sup> to follow up our findings, we undertook a more thorough and effectively independent analytic approach based on HapMap. We selected markers using the program Tagger<sup>40</sup> based on all markers available at the time in HapMap (Hapmap Data Ref # 19/phase II, Oct 05, on NCBI B34 assembly dbSNP b124) to derive a set of tagSNPs such that each common allele (minor allele frequency (MAF) 0.1) was captured with  $r^2 > 0.8$  by a single marker. This suggested 27 tagSNP markers (Figure 5), which we genotyped in our core sample, in addition to rs1196471, which we had been unsuccessful in genotyping in phase 1. The results from the individual genotyping of the tagSNPs are presented in Table 3. Five of the markers gave nominally significant evidence for association ( $P = 0.05$ ). These were rs6466808 ( $P = 0.004$ ), rs2693657 ( $P = 0.009$ ), rs10278079 ( $P = 0.011$ ), rs1147497 ( $P = 0.032$ ) and rs1206381 ( $P = 0.042$ ). Additionally, rs1196471, a phase 1 marker, yielded nominally significant evidence for association ( $P = 0.01$ ) but this was not as strong as estimated by the pooled analysis (Table 1). As before, we typed approximately 600 additional blood donor controls for the markers showing strongest evidence: rs6466808, rs2693657, rs1196471 and rs10278079. The combined results are given in Table 3. The evidence for rs2693657 remained unchanged ( $P = 0.01$ ), whereas that for rs6466808 and rs10278079 became stronger ( $P = 0.0002$  and  $0.001$ , respectively). Again, we could not obtain satisfactory genotypes with rs1196471 so these were excluded. All genotypes were in Hardy–Weinberg equilibrium for cases and controls.



To evaluate the evidence for overall association in the context of testing multiple markers, we applied the truncated product of  $P$ -values method.<sup>38</sup> This is a gene-wide test which produces a single significance level for evidence of overall association at a gene, allowing for independent association information from multiple SNPs, multiple testing, and the degree to which the markers are in LD.<sup>38</sup> We multiplied together all  $P$ -values reaching a threshold of  $P = 0.05$  to achieve a product  $P$ -value. The significance of this product was established by permuting case-control status over 100 000 replicates and counting the number of times this product was exceeded. This yielded a gene-wide significance of  $P = 0.0080$ , which is stronger to that obtained in phase 1. We also determined the significance, in the context of multiple testing, of the single best marker rs6466808 by running 100 000 permutations of case-control status with all genotyped markers. This gave a similar gene-wide level of significance to that of the other method ( $P = 0.0064$ ).

We also performed sliding window haplotype analyses based on all the markers we had genotyped. Although several haplotypes yielded nominally significant evidence for association ( $P_{\min} = 0.006$ ), none was more significantly associated than the individual markers.

We analyzed in the DNA pools 18 polymorphic SNPs in *MAGI-1*, 24 SNPs in *MAGI-2* and 21 SNPs in *MAGI-3*. Several markers yielded nominally significant association in the pools and were individually genotyped (Supplementary Tables). Nominally significant signals were confirmed by individual genotyping for markers in *MAGI-1* (rs9880851,  $P = 0.033$ ), *MAGI-2* (rs2868865,  $P = 0.011$ ) and *MAGI-3* (rs1230661,  $P = 0.028$ ), but none reached gene-wide significance following correction for multiple testing, so these genes were not further pursued using the more thorough HapMap-based approach.

## Discussion

NRG1-ERBB4 signaling has been genetically and functionally implicated in schizophrenia, and downstream molecules involved in this pathway and molecules that modulate NRG1-ERBB4 signaling might also play important roles in schizophrenia pathogenesis. Using the cytoplasmic domain of ERBB4, we have identified MAGI proteins as ERBB4-interacting proteins. We further showed that MAGI proteins are tyrosine phosphorylated when ERBB4 is present, either alone or in combination with ERBB2 or ERBB3. MAGI proteins serve as scaffolding proteins underlying the cell membrane, recruiting signaling molecules (such as phosphatase and tensin homolog (PTEN)), channels, receptors and cell adhesion molecules to specific domains in the cell membrane.<sup>27,41</sup> Some of these interactions can be modulated by threonine-serine phosphorylation of MAGI.<sup>42</sup> It would be of interest to determine whether tyrosine phosphorylation of MAGI proteins might also modulate their interactions with other molecules, modulating the activating different signaling components. Nonetheless, our results indicate that MAGI proteins are apparent downstream molecules in NRG1-ERBB4 signaling that could serve as a site to integrate or modulate signaling events in the cells.

MAGI proteins have previously been shown to interact with RPTP $\beta$ .<sup>28,29,39</sup> We and others,<sup>29,39</sup> have observed that RPTP $\beta$  can dephosphorylate MAGI proteins. In addition, we

found that RPTP $\beta$ , ERBB4 and MAGI proteins can colocalize in cells. Our data suggest that MAGIs can bring ERBB4 tyrosine kinase and RPTP $\beta$  tyrosine phosphatase to domains in the cell membrane, modulating the balance among signaling pathways downstream of these molecules. Given that NRG1-ERBB4 signaling has been etiologically linked with schizophrenia, we carried out a genetic association analysis of *PTPRZ1*. Several SNPs yielded significant evidence for association, and the evidence in support of association remained significant when corrected for multiple testing at the genewide level ( $P = 0.0064$ ), and also at the experimentwide level (correcting for all genes examined,  $n=4$ ,  $P = 0.026$ ). As the associated SNPs are in introns, it will require further studies to determine how this pattern of association might affect *PTPRZ1* gene function. However, if it can be demonstrated that RPTP $\beta$  antagonizes NRG1-ERBB4 signaling *in vivo*, then we would predict that reduced functioning of RPTP $\beta$  would result in a phenotype like that of enhanced NRG1-ERBB4 signaling.<sup>22</sup> This would provide a potential mechanism by which *PTPRZ1*/RPTP $\beta$  could be involved in the molecular basis of schizophrenia.

*PTPRZ1* gene products are expressed mainly in the nervous system.<sup>43</sup> There are four splicing isoforms of *PTPRZ1*, two of which are transmembrane receptor forms (RPTP $\beta$ , short and long) and the other two are soluble forms that lack both a transmembrane region and tyrosine phosphatase activity (phosphacan, short and long).<sup>44–50</sup> The extracellular region of *PTPRZ1* consists of several modular structural domains, including a carbonic anhydrase domain, a fibronectin type III repeat, a spacer domain (all of which are present in all four isoforms), and a region containing ~860 amino acids (that is missing in the RPTP $\beta$  and phosphacan short forms) where several glycosaminoglycan chains can be attached.<sup>44</sup> The carbonic anhydrase domain interacts with the cell recognition molecule contactin,<sup>51</sup> whereas the spacer region can interact with several other cell adhesion molecules including NrCAM, L1CAM, NCAM, contactin-1 and contactin-2/TAG-1.<sup>52–56</sup>

*PTPRZ1* gene products are expressed from early developmental to adulthood in glial cells<sup>47,57–59</sup> as well as in certain population of neurons<sup>60–63</sup> and are implicated in neuron–glial cell interactions that involve on bi-directional signaling, including myelination and node of Ranvier formation, through interactions with neuronal cell adhesion molecules.<sup>55,56</sup> Interestingly, *in situ* hybridization analysis has shown that *PTPRZ1* is expressed in the subventricular zone of the lateral ventricles in early embryos and in adults, in the subgranular zone in the dentate gyrus of the hippocampus of adults,<sup>57,64</sup> and in adult glial progenitor cells,<sup>65</sup> suggesting that *PTPRZ1* also has a function in adult stem/progenitor cells. In addition, it has been shown that inhibition of *PTPRZ1* expression or inhibition of RPTP $\beta$  activity in adult glial progenitors leads to their differentiation into mature oligodendrocytes,<sup>65</sup> supporting a role for RPTP $\beta$  activity in the maintenance of glial progenitors in an undifferentiated state. Using experimental allergic encephalomyelitis (EAE), an inducible mouse model for multiple sclerosis (MS), *PTPRZ1*-deficient mice could not repair EAE lesions, although *PTPRZ1* expression is increased in human remyelinating oligodendrocytes of MS biopsies.<sup>66</sup> Taken together, these data suggest that *PTPRZ1* could be implicated in adult cell renewal, repair of the nervous system and/or oligodendrocyte development. It should be noted that NRG1-ERBB4 signaling has been shown to play crucial roles in these processes, supporting the possibility that *PTPRZ1* might function partly through modulation of the NRG1-ERBB4 signaling pathway.

A role for *PTPRZ1* in oligodendrocyte development is intriguing in light of evidence for altered oligodendrocyte functioning in schizophrenia. Several postmortem studies in schizophrenia have demonstrated reduced expression of oligodendrocyte-related genes,<sup>17–21,67–72</sup> some of which may be susceptibility genes for schizophrenia.<sup>33,72–75</sup> Moreover, several studies have evidence for deficits in oligodendrocytes in the disorder.<sup>76–79</sup> These oligodendrocyte abnormalities may in turn contribute to the loss of coherence of axon tracts, and reduced connectivity, in schizophrenia.<sup>80,81</sup> Alterations in *NRG1-ERBB4* and/or *PTPRZ1* signaling may cause these oligodendrocyte abnormalities, in addition to potential neuronal effects.

In summary, using proteomic and cell–biological approaches, we identified a signaling complex comprised of *ERBB4*, *MAGI* and *RPTPβ*. Moreover, genetic evidence suggests that *PTPRZ1* might influence susceptibility for schizophrenia. Further studies delineating the role of *PTPRZ1* in the brain will elucidate the role of this gene in *ERBB4* signaling, and may contribute to a better understanding of schizophrenia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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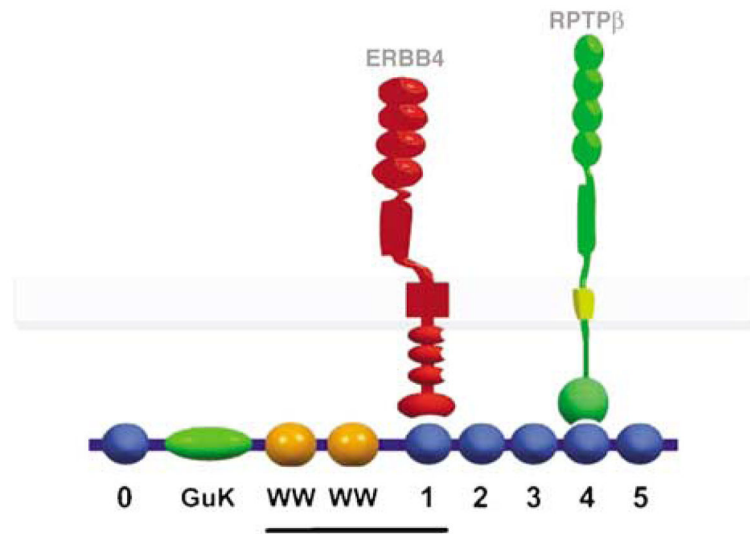
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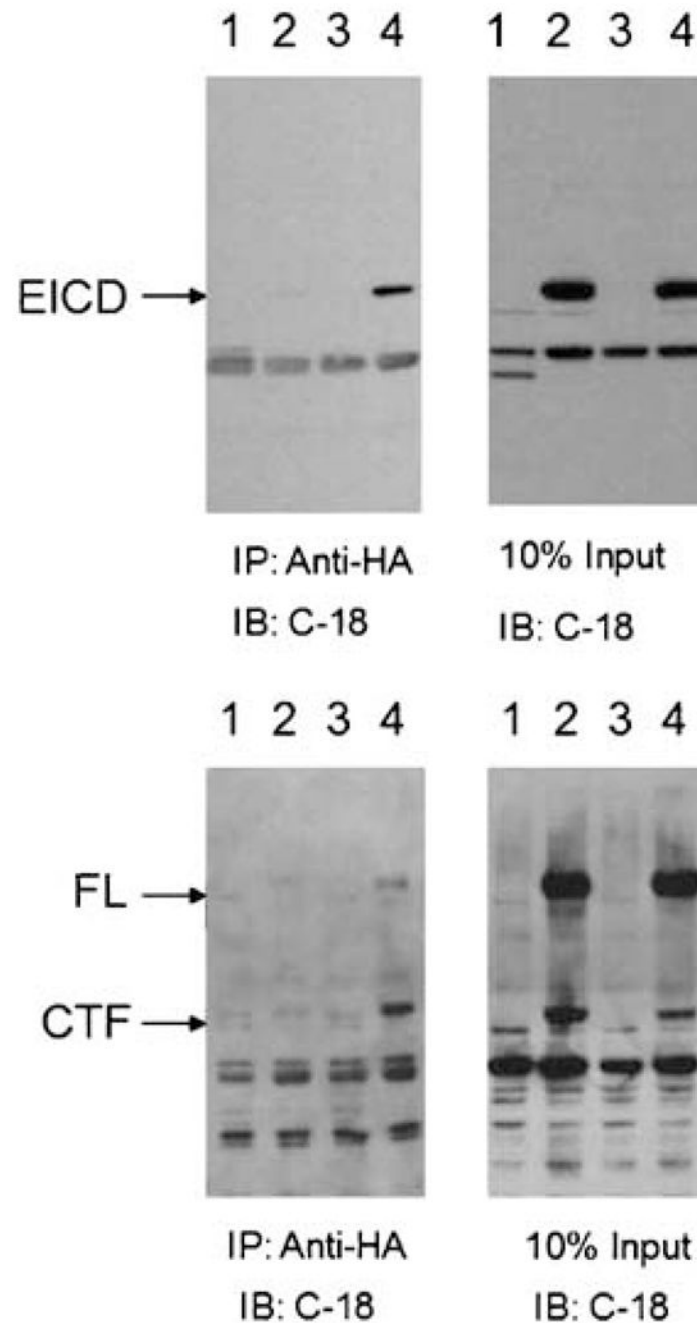
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**Figure 1.** Scheme of ERBB4-MAGI-RPTP $\beta$  interactions. PDZ domains of MAGI are numbered 0–5. ERBB4 binds to PDZ1, whereas RPTP $\beta$  binds to PDZ4. The line indicates the MAGI-2 sequence recovered in the yeast two-hybrid screen.





**Figure 2.**

Co-precipitation of MAGI-2 and ERBB4. Cells were transfected with: (1) MAGI-2; (2) EICD (top panels) or ERBB4 (bottom panels); (3) vector; or (4) MAGI-2 and either EICD (top panels) or ERBB4 (bottom panels). Cells were lysed and subjected to immunoprecipitation (IP) with anti-HA antibody (to precipitate HA-tagged MAGI-2), and the amounts of EICD, ERBB4, or cleaved, C-terminal fragment of ERBB4 (CTF), were determined by immunoblotting (IB) with an antibody (C-18) against the C terminal of ERBB4 (left panels). In parallel, an aliquot of cell lysate from transfected cells (10% of the

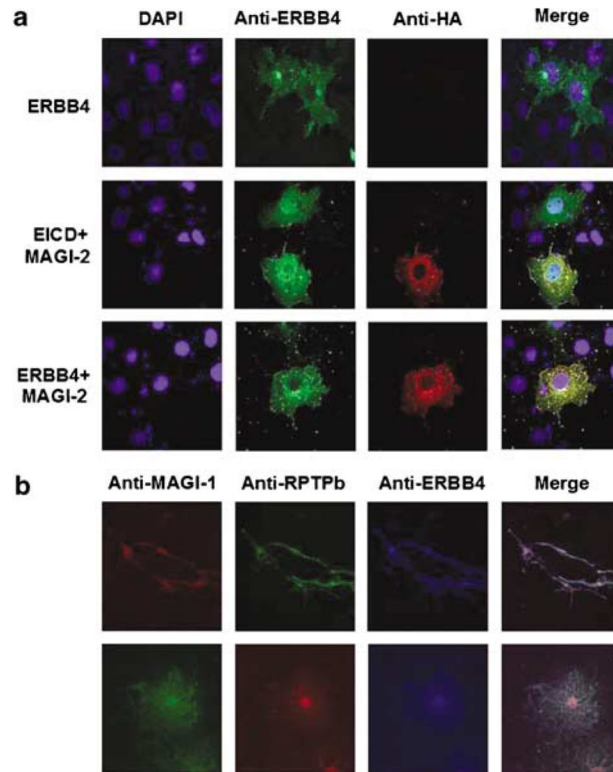
amounts used for the immunoprecipitation) was immunoblotted to confirm expression of transfected proteins (right panels).

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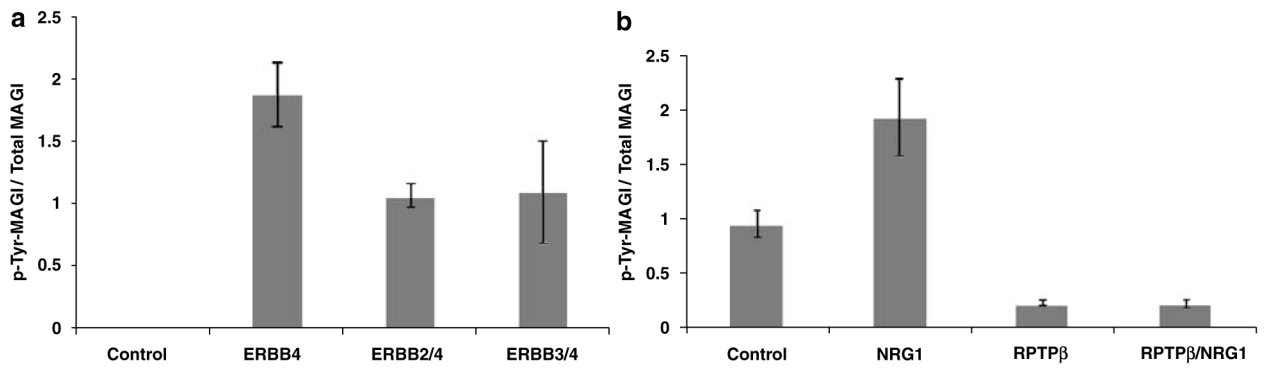
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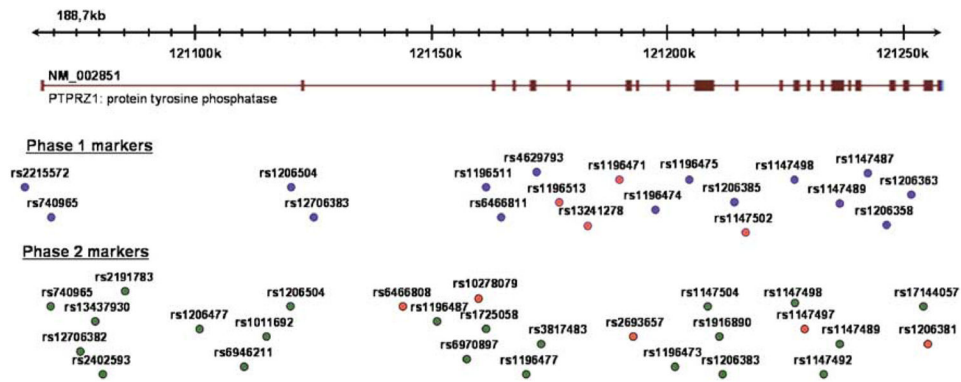
**Figure 3.**

Co-localization of MAGI with ERBB4 and RPTP $\beta$ . (a) Colocalization of MAGI-2 with ERBB4. H4 cells were transfected with ERBB4, ERBB4 and HA-tagged MAGI-2, or ERBB4 EICD and HA-tagged MAGI-2 and the proteins visualized by immunocytochemistry with an antibody directed against the C-terminus of ERBB4 and with an anti-HA antibody. (b) Colocalization of endogenous MAGI-1 with ERBB4 and RPTP $\beta$ . ERBB4, MAGI and RPTP $\beta$  were visualized in rat oligodendrocyte progenitor cells (top) and premyelinating oligodendrocytes (bottom) using anti-ERBB4, anti-MAGI-1 and/or anti-RPTP $\beta$  antibodies.



**Figure 4.**

Tyrosine-phosphorylation of MAGI proteins. **(a)** Phosphorylation of MAGI-1 by ERB-receptor kinases. Cells were transfected with MAGI-1, in the presence or absence of ERBB2, ERBB3 and ERBB4 and tyrosine phosphorylation of MAGI-1 determined. **(b)** Phosphorylation/dephosphorylation of MAGI-1. Cells were transfected with MAGI-1, ERBB2 and ERBB4, in the presence or absence of RPTP $\beta$ . Transfected cells were treated with vehicle or NRG1, and tyrosine phosphorylation of MAGI-1 determined. Similar results were observed with MAGI-2 (data not shown).



**Figure 5.** Position of the markers analyzed across the *PTPRZ1* gene. Markers analyzed in phase 1 and phase 2 are shown, with markers with a nominal  $P > 0.05$  indicated in red.

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**Table 1**

Summarized pooled genotyping for PTPRZ1

No.	SNP ID	Position	Distance to next SNP (bases)	Nucleotide change <sup>b</sup>	Minor allele frequency <sup>a</sup>		Difference	$\chi^2$	P
					Cases	Controls			
1	rs2215572	121064040		G/A	0.466	0.485	-0.019	0.84	0.36
2	rs740965	121068064	4024	G/T	0.165	0.143	0.022	2.53	0.11
3	rs1206504	121121853	53789	G/A	0.446	0.425	0.021	1.92	0.17
4	rs12706383	121124481	2628	C/T	0.089	0.099	-0.010	1.34	0.25
5	rs1196511	121161514	37033	T/C	0.359	0.337	0.021	1.36	0.24
6	rs6466811	121165045	3531	A/G	0.129	0.125	0.003	0.07	0.80
7	rs4629793	121170753	5708	A/G	0.383	0.357	0.026	1.99	0.16
8	rs1196513	121176314	5561	C/T	0.399	0.449	-0.050	6.97	0.008
9	rs13241278	121181151	4837	C/T	0.478	0.510	-0.041	4.60	0.03
10	rs1196471	121189416	8265	C/T	0.328	0.392	-0.064	13.46	0.0002
11	rs1196474	121196863	7447	C/T	0.117	0.120	-0.003	0.00	0.97
12	rs1196475	121203726	6863	T/C	0.364	0.393	-0.029	2.17	0.14
13	rs1206385	121213098	9372	A/G	0.317	0.292	0.025	1.93	0.17
14	rs1147502	121215433	2335	T/G	0.463	0.501	-0.038	3.84	0.05
15	rs1147498	121225025	9592	C/A	0.167	0.183	-0.016	2.68	0.10
16	rs1147489	121233636	8611	G/A	0.250	0.274	-0.023	1.91	0.17
17	rs1147487	121239221	5585	C/T	0.400	0.424	-0.024	1.58	0.21
18	rs1206358	121244523	5302	T/A	0.422	0.396	0.026	1.90	0.17
19	rs1206363	121250046	5523	A/G	0.349	0.329	0.020	1.59	0.21

Abbreviation: SNP, single-nucleotide polymorphism.

<sup>a</sup>Minor allele frequencies in pools of 648 subjects with schizophrenia and 712 controls.

<sup>b</sup>Minor allele presented first.

Table 2

Individual genotyping of significant phase 1 markers

No.	SNP ID	Location	Nucleotide change <sup>a</sup>	Number of cases	Number of controls	Minor allele frequency		Allelic P-value	$\chi^2$	Genotypic P-value
						Cases	Controls			
1	rs13241278	121181151	C/T	594	1267	0.478	0.531	0.003	9.43	0.009
2	rs1147502	121215433	T/G	661	1317	0.463	0.502	0.02	6.27	0.04

Abbreviation: SNP, single-nucleotide polymorphism.

<sup>a</sup>Minor allele presented first.

Table 3

## Individual genotyping of phase 2 tagSNPs

No.	SNP ID	Location	Distance to next SNP (bases)	Nucleotide change <sup>a</sup>	Number of cases	Number of controls	Minor allele frequency <sup>a</sup>		$\chi^2$	Allelic P-value	$\chi^2$	Genotypic P-value
							Cases	Controls				
1	rs740965 <sup>b</sup>	121068064		G/T	660	707	0.165	0.147	1.69	0.19	2.11	0.35
2	rs12706382	121074224	6160	T/C	659	698	0.118	0.125	0.31	0.58	0.40	0.82
3	rs13437930	121076328	2104	C/G	650	706	0.099	0.105	0.29	0.59	0.45	0.80
4	rs2402593	121079103	2775	G/A	665	709	0.232	0.236	0.08	0.77	3.28	0.19
5	rs2191783	121079987	884	T/C	665	710	0.165	0.151	1.01	0.32	2.23	0.33
6	rs1206477	121101096	21109	C/G	663	707	0.452	0.455	0.01	0.91	0.03	0.99
7	rs6946211	121109294	8198	T/C	662	710	0.157	0.138	1.99	0.16	3.32	0.19
8	rs1011692	121114514	5220	C/T	650	709	0.143	0.126	1.80	0.18	2.90	0.23
9	rs1206504 <sup>b</sup>	121121853	7339	G/A	662	708	0.446	0.424	1.25	0.26	1.43	0.49
10	rs6466808	121142483	20630	A/G	662	1121	0.323	0.380	13.72	0.0002	15.77	0.0004
11	rs1196487	121146390	3907	C/G	662	705	0.477	0.443	3.19	0.074	3.60	0.17
12	rs6970897	121156410	10020	G/A	652	705	0.102	0.084	2.71	0.10	5.02	0.081
13	rs10278079	121159221	2811	G/A	657	1287	0.302	0.355	10.45	0.001	12.00	0.002
14	rs1725058	121159315	94	A/G	663	705	0.167	0.158	0.66	0.42	0.72	0.70
15	rs1196477	121168118	8803	A/G	654	700	0.386	0.396	0.30	0.58	0.41	0.81
16	rs3817483	121171520	3402	G/A	665	710	0.116	0.133	1.89	0.17	2.32	0.31
17	rs13241278 <sup>c</sup>	121181151	9631	C/T	594	1267	0.478	0.531	8.85	0.003	9.43	0.009
18	rs1196471 <sup>c</sup>	121189416	8265	C/T	594	673	0.328	0.375	6.65	0.01	6.72	0.03
19	rs2693657	121191768	2352	A/G	596	1281	0.327	0.369	6.93	0.01	6.84	0.03
20	rs1196473	121201610	9842	G/A	665	709	0.130	0.133	0.04	0.85	0.07	0.97
21	rs1147504	121207901	6291	A/G	665	710	0.464	0.493	2.32	0.13	2.45	0.29
22	rs1916890	121210293	2392	G/A	656	703	0.111	0.127	1.68	0.19	1.79	0.41
23	rs1206383	121210312	19	A/G	652	702	0.003	0.001	0.83	0.36	-	-
24	rs1147502 <sup>c</sup>	121215433	5121	T/G	661	1317	0.463	0.502	5.45	0.02	6.27	0.04
25	rs1147498 <sup>b</sup>	121225025	9592	C/A	666	708	0.167	0.18	0.86	0.35	1.28	0.53
26	rs1147497	121225630	605	G/T	649	703	0.351	0.391	4.61	0.03	5.57	0.06



No.	SNP ID	Location	Distance to next SNP (bases)	Nucleotide change <sup>a</sup>	Number of cases	Number of controls	Minor allele frequency <sup>a</sup>		$\chi^2$	Allelic P-value	$\chi^2$	Genotypic P-value
							Cases	Controls				
27	rs1147492	121231427	5797	T/C	643	701	0.488	0.458	2.37	0.12	2.56	0.28
28	rs1147489 <sup>b</sup>	121233636	2209	G/A	643	701	0.239	0.269	3.12	0.08	4.92	0.09
29	rs17144057	121253030	19394	G/T	662	709	0.119	0.126	0.31	0.58	0.70	0.71
30	rs1206381	121253330	300	C/A	653	702	0.374	0.413	4.14	0.04	5.96	0.05

Abbreviation: SNP, single-nucleotide polymorphism.

<sup>a</sup>Minor allele presented first. Its frequencies in cases and controls are presented in columns 8 and 9. All markers were in Hardy–Weinberg equilibrium (HWE) in cases and controls.

<sup>b</sup>Overlapping markers from pooling and tagSNPs analysis.

<sup>c</sup>Markers significant from pooling experiment.