Published in final edited form as:
Hum Genet. 2009 November ; 126(5): 655–666. doi:10.1007/s00439-009-0714-x.

The *ERAP2* gene is associated with preeclampsia in Australian and Norwegian populations

Matthew P. Johnson[1,5,6], **Linda T. Roten**[2,5], **Thomas D. Dyer**[1], **Christine E. East**[3], **Siri Forsmo**[4], **John Blangero**[1], **Shaun P. Brennecke**[3], **Rigmor Austgulen**[2], and **Eric K. Moses**[1]

[1]Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX, 78227 U.S.A.

[2]Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, 7006 Norway.

[3]Department of Perinatal Medicine, Royal Women's Hospital & Department of Obstetrics & Gynaecology, University of Melbourne, Parkville, 3052 Australia.

[4]Department of Public Health and General Practice, Norwegian University of Science and Technology, Trondheim, 7006 Norway.

Abstract

Preeclampsia is a heritable pregnancy disorder that presents new onset hypertension and proteinuria. We have previously reported genetic linkage to preeclampsia on chromosomes 2q, 5q and 13q in an Australian/New Zealand (Aust/NZ) familial cohort. This current study centered on identifying]the susceptibility gene(s) at the 5q locus. We firstly prioritized candidate genes using a bioinformatic tool designed for this purpose. We then selected a panel of known SNPs within 10 prioritized genes and genotyped them in an extended set of the Aust/NZ families and in a very large, independent Norwegian case/control cohort (1,139 cases, 2,269 controls). In the Aust/NZ cohort we identified evidence of a genetic association for the endoplasmic reticulum aminopeptidase 1 (*ERAP1*) gene (rs3734016; p_{uncorr}=0.009) and for the endoplasmic reticulum aminopeptidase 2 (*ERAP2*) gene (rs2549782; p_{uncorr}=0.004). In the Norwegian cohort we identified evidence of a genetic association for *ERAP1* (rs34750; p_{uncorr}=0.011) and for *ERAP2* (rs17408150; p_{uncorr}=0.009). The *ERAP2* SNPs in both cohorts remained statistically significant (rs2549782; p_{corr}=0.018; rs17408150; p_{corr}=0.039) after corrections at an experiment-wide level. The *ERAP1* and *ERAP2* genes encode enzymes that are reported to play a role in blood pressure regulation and essential hypertension in addition to innate immune and inflammatory responses. Perturbations within vascular, immunological and inflammatory pathways constitute important physiological mechanisms in preeclampsia pathogenesis. We herein report a novel preeclampsia risk locus, *ERAP2*, in a region of known genetic linkage to this pregnancy specific disorder.

Keywords

Preeclampsia; Chromosome 5q linkage; ERAP2; Genetic association; LRAP

6To whom correspondence should be addressed; mjohnson@sfbrgenetics.org, P +1 210 258 9465, F +1 210 258 9595.
5These authors contributed equally to this work

Introduction

Preeclampsia is a serious pregnancy specific disorder with an incidence of 2–5% reported in most population-based studies (Brown et al. 2000). The clinical features used in diagnosis are new onset hypertension and proteinuria during pregnancy (Brown et al. 2000). Preeclampsia is associated with substantial maternal and fetal morbidity and mortality. Expeditious delivery of both fetus and placenta is often the only effective means of halting the progression of the disease and alleviating the maternal symptoms. If pre-term intervention is required there can be both short- and long-term complications from prematurity for the neonate. Women who have suffered a preeclamptic pregnancy are also at an elevated risk of later life cardiovascular disease (Chesley et al. 1976; Funai et al. 2005; Irgens et al. 2001).

There have been numerous etiological hypotheses proposed for preeclampsia involving immunological, vascular, ischemic and inflammatory disturbances (Ilekis et al. 2007). While there continues to be active debate on the origins of this disease, it is now widely acknowledged that preeclampsia is a complex disorder involving both genetic and environmental risk factors (Ilekis et al. 2007). The evidence for genetic involvement is strong with large epidemiological studies demonstrating that there are both maternal and paternal genetic contributions to preeclampsia susceptibility (Esplin et al. 2001; Lie et al. 1998; Skjaerven et al. 2005). However, like the majority of other common complex diseases the mode of inheritance is unclear and positional cloning efforts have been initiated in attempts to identify the genetic risk factors. Medium density genome-wide linkage scans for maternal genes have uncovered evidence for locus heterogeneity amongst Icelandic (Arngrímsson et al. 1990), Australian/New Zealand (Fitzpatrick et al. 2004; Johnson et al. 2007; Moses et al. 2006; Moses et al. 2000), Dutch (Lachmeijer et al. 2001; Oudejans et al. 2004) and Finnish (Laivuori et al. 2003) familial cohorts.

We have recently re-analyzed the Australian/New Zealand family-based genome-wide linkage scan data (Fitzpatrick et al. 2004; Moses et al. 2000) under the hypothesis that the underlying liability of preeclampsia susceptibility is inherently quantitative. Under this assumption, any preeclampsia susceptibility gene will represent a quantitative trait locus (QTL), thus allowing a more refined variance components-based procedure utilizing a biological threshold model for preeclampsia in the statistical analysis routines. The application of this efficient genetic linkage analysis method resolved and strengthened the chromosome 2 linkage signal to 2q22 (Moses et al. 2006). This analysis approach has also identified two novel preeclampsia susceptibility QTLs to chromosomes 5q and 13q (Johnson et al. 2007).

We have recently reported on our efforts to identify the susceptibility gene(s) at the 2q22 QTL using an extended cohort of Australian families and an independently ascertained Norwegian case/control cohort. Evidence for association with polymorphisms in the activin A receptor, type IIA (*ACVR2A* [MIM 102581]) gene was found (Fitzpatrick et al. 2009; Roten et al. 2009). We now report on our efforts to identify the susceptibility gene(s) at the 5q QTL.

Subjects and Methods

Australian Familial Cohort

The Australian/New Zealand (Aust/NZ) familial cohort consists of the original set of 34 (26 Australian, 8 New Zealand) families that we have previously used to localize the 2q, 5q and 13q preeclampsia susceptibility QTLs (Fitzpatrick et al. 2004; Johnson et al. 2007; Moses et al. 2006; Moses et al. 2000) and an additional 40 (Australian) families that we have subsequently ascertained and recently described (Fitzpatrick et al. 2009). The original 34 families are hereafter called “The 34 Family Cohort” and the entire familial sample is hereafter called “The 74 Family Cohort”. All family members are of Caucasian origin.

Norwegian Case/Control Cohort

All Norwegian patient samples used in this current study were retrospectively identified from the Nord-Trøndelag County in Norway as part of a large multipurpose health survey conducted from 1995 to 1997 (the HUNT2 study) (Holmen et al. 2003). More than 65,000 inhabitants participated. The people living in Nord-Trøndelag County are considered representative of the Norwegian population, and are well suited for genetic studies because of ethnic homogeneity (<3% non-Caucasians) (Holmen et al. 2004; Holmen et al. 2003). This study population is described in detail elsewhere (Moses et al. 2008).

Preeclampsia diagnosis

Preeclampsia diagnosis in the Australian and Norwegian cohorts was based on the development of hypertension and an elevation of protein levels in the urine during pregnancy. Multiple blood pressure readings and a quantitative (24 hr urine sample) or qualitative (random proteinuria dipstick reading) test were performed.

Australia—Preeclampsia in the Australian cohort was diagnosed by qualified clinicians in accordance with guidelines from the Australasian Society for the Study of Hypertension in Pregnancy (Brown et al. 1993; Brown et al. 2000) and has been described in detail elsewhere (Fitzpatrick et al. 2009; Johnson et al. 2007; Moses et al. 2006; Moses et al. 2000). Women with pre-existing hypertension or other medical conditions known to predispose for preeclampsia (e.g. renal disease, diabetes, twin pregnancies or fetal chromosomal abnormalities) were excluded. The 74 Family Cohort (n=480) therefore included 140 women coded as affected (20 with eclampsia, 120 with preeclampsia) and 90 women coded as unaffected (normotensive and non-proteinuric). Women who were eclamptic experienced convulsions or unconsciousness in their perinatal period in addition to pregnancy induced hypertension and proteinuria.

Norway—Preeclampsia in the Norwegian cohort was diagnosed in accordance with the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy 2000) and has been described previously (Moses et al. 2008; Roten et al. 2009). Preeclamptic women and women who had non-preeclamptic pregnancies in the HUNT cohort were identified by linking the HUNT database with the Medical Birth Registry of Norway (MBRN) database as previously described (Moses et al. 2008). Since 1967 clinical information pertaining to all deliveries in Norway has been sent to the MBRN. We identified 1,139 women registered with preeclampsia (cases) and 2,269 women with a history of non-preeclamptic pregnancies (controls) with blood samples available at the HUNT biobank.

Positional Candidate Gene Prioritization using GeneSniffer

The objective prioritization of positional candidate genes residing within the bounds of the 5q QTL was performed using the computer program GeneSniffer (http://www.genesniffer.org/) as previously described (Johnson et al. 2007; Moses et al. 2006). A set of disease-specific keywords are assigned a weighting score of 10, 5 or 1 (10 being the highest) and are inputted into GeneSniffer to assist in prioritizing positional candidate genes. The keywords assigned a score of 10 were pre-eclampsia, preeclampsia, eclampsia, pregnancy-induced hypertension, pregnancy induced hypertension, pregnancy hypertension, gestational hypertension, PIH, toxaemic pregnancy and decidua. The keywords assigned a score of five were toxaemia, pregnancy, placenta, spiral artery, glomerular endotheliosis, implantation, trophoblasts, cytotrophoblasts, proteinuria and placental bed. The keywords assigned a score of one were uterus, uterine, amnion, chorion, NK cell, hypertension, gestation, gestational, oedema, edema,

maternal, endothelial, endothelium, haemostasis, coagulation, coagulopathy, thrombophelia, ischemia, hypoxia and allograft.

Positional Candidate SNP Selection

Publicly available sequence SNPs were selected from the NCBI SNP database (*Homo sapiens* dbSNP build 125).

Australian Genotyping

Extraction of genomic DNA from peripheral blood samples has been previously described (Moses et al. 2000). SNP typing in The 74 Family Cohort was performed with Illumina's GoldenGate® SNP Genotyping Assay (Illumina Inc., San Diego, U.S.A.). The design of two allele specific oligos and one locus specific oligo in conjunction with a universal set of amplification primers applied to a bead array substrate makes the GoldenGate assay highly robust and specific in a large multiplex reaction. A custom SNP pool was genotyped on an Illumina 8×12 Sentrix Array Matrix (SAM) and each SAM was imaged on the Illumina BeadStation 500GX BeadArray Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). SNP genotype clustering and individual sample genotype calls were interrogated using the Illumina BeadStudio software, Genotyping Module (version 2.3.41).

Norwegian Genotyping

SNPlex genotyping—DNA for genotyping was extracted from peripheral blood samples stored in the HUNT biobank as previously described (Moses et al. 2008; Roten et al. 2009). The Applied Biosystems SNPlex™ Genotyping System (Applied Biosystems, Foster City, U.S.A.) was employed for SNP genotyping. All samples were electrophoretically separated on a 3730 DNA Genetic Analyzer (Applied Biosystems), and automated allele calls and genotype clustering was performed using Applied Biosystems' GeneMapper® Software (version 4.0) as previously described (Moses et al. 2008; Roten et al. 2009).

iPLEX Gold genotyping—Replicated SNP genotyping was performed using the iPLEX Gold assay on the MassARRAY® system (Sequenom Inc., San Diego, U.S.A.) at the Centre for Integrative Genomics (CIGENE), hosted by the Norwegian University of Life Sciences at campus Aas. The Sequenom MassARRAY system utilizes a Matrix-Assisted Laser Desorption Ionization – Time Of Flight (MALDI-TOF) mass spectrometer for genotyping. The technology is based on an allele-specific primer extension reaction where short primers are extended according to the base composition in the template sequence, and then separated by mass (Storm et al. 2003). Differences in mass are automatically translated by the MassARRAY Typer software into specific genotype calls. The iPLEX Gold genotyping assay allows multiplexing of up to 40 SNPs simultaneously.

Statistical Analysis

Genotype error checking—Genotypes pertaining to the Australian cohort not conforming to Mendelian inheritance laws were identified and assessed using SimWalk2 (Sobel and Lange 1996). Mendelian discrepancies and spurious recombinations were removed by blanking those genotypes identified by SimWalk2 as having a high probability of being in error. Genotype concordance in the Norwegian cohort was assessed for the regenotyped SNPs across the SNPlex and iPLEX Gold assays.

SNP allele frequency estimation—Maximum likelihood techniques that account for pedigree structure were used to estimate allelic frequencies and their standard errors using

SOLAR (Almasy and Blangero 1998). Tests for deviations from Hardy-Weinberg expectations were also performed in SOLAR.

Intra- and inter-genic SNP linkage disequilibrium estimates—Estimates of pairwise linkage disequilibria parameters amongst intra- and inter-genic SNPs were used in a basic correlation method to assess all disequilibria jointly in SOLAR. In this approach, SNP genotypes are scored as −1, 0 and 1 (for the AA, AB and BB genotypes, respectively) and the correlations among these data vectors are calculated to give an unbiased estimate of the squared linkage disequilibrium (LD) correlation, rho (ρ).

SNP association analysis—SNP association analyses were conducted with SOLAR's QTLD procedure (Blangero et al. 2005). This procedure performs a test for population stratification and two commonly used association tests: the quantitative transmission disequilibrium test (QTDT) (Abecasis et al. 2000) and the measured genotype (Boerwinkle et al. 1986). The QTDT procedure scores allele transmissions throughout the entire pedigree structure for quantitative or qualitative traits (Abecasis et al. 2000) and it has been modified in SOLAR to work with discrete traits using a threshold model (Duggirala et al. 1997). The measured genotype test uses a standard threshold model assuming an underlying normal distribution of liability. The threshold model and its assumptions are near identical to those used in standard logistic regression but benefits from the ease of interpretation with regard to genetic effects. The measured genotype test of association can assess the extent of genotypic mean differences (or the liability or risk scale) between case and control singletons assuming a model of additive gene action (Boerwinkle et al. 1986). Due to the non-familial structure of the Norwegian case/control cohort we can only present the measured genotype test statistic for this cohort.

Experiment-wide corrections—Simulation and permutation analyses (n=10,000) were performed in SOLAR to obtain experiment-wide significance values for SNP associations in the Australian and Norwegian cohorts, respectively.

P-value interpretation—In this study we denote all observed p-values as uncorrected (p_{uncorr}). Results subsequently corrected at an experiment-wide level are presented as not significant (NS) ($p_{corr}>0.1$), borderline significant ($0.05\leq p_{corr}<0.1$) or significant ($p_{corr}<0.05$).

Ethics

Australia—Ethical approval for the recruitment of Aust/NZ preeclampsia families was granted by the Royal Women's Hospital Research and Ethics Committees, Melbourne, Australia. Written informed consent was obtained from study participants prior to them being phlebotomized. Ethical approval for the quantitative genetic analysis of The 34 Family Cohort plus molecular genetic interrogation across the 5q QTL in The 74 Family Cohort was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

Norway—Prior approval to use the Norwegian case/control cohort for genetic studies was obtained by the Regional Committee for Medical Research Ethics, Norway and approved by the National Data Inspectorate and The Directorate of Health and Social Welfare. Ethical approval for genotyping and statistical analysis of the Norwegian cohort was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

Results

Positional Candidate Gene Prioritization

We used the GeneSniffer computer program to interrogate the 3-LOD drop (99% confidence) interval at the Australian 5q preeclampsia QTL to prioritize positional candidate genes *in silico*. GeneSniffer interrogated 299 genomic regions in the 5q critical region from D5S424 (90.87 cM) to D5S2115 (136.75 cM) using NCBI Human Build 35.1. These 299 genomic regions included 222 confirmed genes, 30 expressed sequence tags, 19 predicted genes, 8 interim locus IDs and 20 conflicting gene models. The top 20 ranked gene hits are presented in Table 1.

We used this data along with knowledge on current and emerging concepts of the pathophysiology of preeclampsia (Gammill and Roberts 2007) to select 10 genes for further analysis. These genes included 5 of the top 6 GeneSniffer ranked genes (*LNPEP*, *IL4*, *CSF2*, *ERAP1* and *CRHBP*), an additional 3 genes within the top 20 (*ERAP2*, FLJ90650 [*LVRN*] and *IL13*) and another 2 genes within the top 50 (*IL3* and *IL5*) (Table 1). The *IL3* and *IL5* genes form part of the 5q cytokine gene cluster with *IL4*, *CSF2* and *IL13*, and have previously been implicated in inflammatory mediated disorders (Marsh et al. 1994; Postma et al. 1995).

Candidate SNP selection

For the 10 prioritized genes (highlighted in Table 1) we selected 55 validated SNPs from NCBI's SNP database (*Homo sapiens* dbSNP build 125) for further genetic analyses (Table 2). In selecting these SNPs our initial focus was on likely functional variants (coding sequence SNPs) and those in known gene regulatory regions (proximal promoter, 5'UTR and 3'UTR). In order to effectively cover known SNP variation within the *CRHBP* and *IL13* genes we also selected validated intronic SNPs at ~4.0 and ~0.3 kb intervals, respectively.

SNP genotyping and association analysis in the Australian cohort

In the Australian cohort one SNP failed the Illumina GoldenGate assay design and 10 SNPs were non-polymorphic (Table 2). In order to effectively analyze SNP genotype data an additional four SNPs were excluded due to observing fewer than 5 copies of the minor allele (Table 2). The remaining 40 SNPs genotyped in the Australian cohort exhibited a very high individual sample genotype success rate of ≥98.1% and all SNPs conformed to Hardy-Weinberg expectations ($p>0.05$).

Based on a prior hypothesis that the underlying liability of preeclampsia is inherently quantitative and therefore any preeclampsia gene will represent a QTL (Johnson et al. 2007; Moses et al. 2006) we have independently analyzed the 10 prioritized 5q QTL positional candidate genes. Evidence for association ($p_{uncorr}<0.10$) was found for three genes (*CRHBP*, *ERAP1* and *ERAP2*) with the QTDT and measured genotype test statistics (Table 2). None of the tested SNPs within the remaining seven genes (*LNPEP*, FLJ90650, [*LVRN*], *IL3*, *CSF2*, *IL5*, *IL13* and *IL4*) were associated with preeclampsia in the Australian cohort ($p_{uncorr}\geq0.10$) (Table 2).

To correct our observed QTDT and measured genotype p-values we used SOLAR to simulate a quantitative trait with a heritability of 0.51 (Moses et al. 2006) and assigned affection status to a fixed number of affected women (n=140) with the highest simulated trait values. The QTLD analysis procedure (Blangero et al. 2005) was repeated 10,000 times to our simulated trait and an experiment-wide p-value for each SNP was determined at an alpha threshold of 0.05. Allowing for the total experiment we present a borderline association for *ERAP1* and a significant association for *ERAP2* with preeclampsia susceptibility in the Australian cohort with the QTDT statistic (Table 2).

SNP genotyping and association analysis in the Norwegian cohort

In the Norwegian cohort two SNPs failed the SNPlex genotyping assay and 13 SNPs were non-polymorphic (Table 2). To promote valid use of our statistical analysis an additional SNP was excluded due to a very poor individual genotype success rate of 14.8% (Table 2). Successfully genotyped SNPs using Applied Biosystems' SNPlex assay resulted in a less than favorable individual sample genotype success rate of ≤82.0%. In order to minimize the loss of statistical power due to loss of individual genotypes we attempted to replicate the seven SNPs showing a borderline (uncorrected) association ($p_{uncorr}<0.10$) on an independent genotyping platform (Table 2). Replicated genotyping was performed on Sequenom's MassARRAY using the iPLEX Gold genotyping assay. One of the seven SNPs selected for re-genotyping failed to be designed into the iPLEX Gold SNP pool (Table 2). The use of this independent platform resulted in a far superior individual sample genotyping success rate of ≥98.5%. The genotypes for the remaining six, successfully replicated SNPs in the Norwegian cohort replaced the original SNPlex generated genotypes prior to statistical analysis. All SNPs conformed to Hardy-Weinberg expectations ($p>0.05$) with the exception of rs10050860, rs2287987, rs3734016, rs17408150 and rs2069744. Evidence for association ($p_{uncorr}<0.10$) was found for three genes (*CRHBP*, *ERAP1* and *ERAP2*) with the measured genotype test (Table 2). None of the tested SNPs within the remaining seven genes (*LNPEP*, FLJ90650 *[LVRN]*, *IL3*, *CSF2*, *IL5*, *IL13* and *IL4*) were associated with preeclampsia in the Norwegian cohort ($p_{uncorr}\geq0.10$) (Table 2).

To correct our observed measured genotype p-values we ran a permutation analysis procedure in SOLAR. This procedure held the individual genotype constant whilst randomizing the disease status 10,000 times ($\alpha<0.05$). Allowing for the total experiment we present a borderline association for *CRHBP* and *ERAP1*, and a significant association for *ERAP2* with preeclampsia susceptibility in the Norwegian cohort with the measured genotype test statistic (Table 2).

Independent SNP association in the Australian and Norwegian cohorts

In the Australian and the Norwegian cohorts we present a borderline association for the *ERAP1* SNPs rs3734016 and rs34750, respectively. The *ERAP2* association result in both the Australian (rs2549782) and Norwegian (rs17408150) cohorts corroborates an experiment-wide significant association for *ERAP2* with preeclampsia genetic susceptibility, albeit at different SNP loci.

SNP linkage disequilibrium

All patterns of LD are measured by the squared value of the pairwise correlation among intra- or inter-genic genotypes (ρ^2) and we shall herein refer to observed patterns of LD as a set of isocorrelated redundant variants (IRVs) (Blangero et al. 2005). We performed both intra- and inter-genic genotype pairwise correlations for *ERAP1* and *ERAP2* due to their contiguous physical location on 5q15 and their suggested derivation from a common ancestral gene (Tanioka et al. 2003).

For *ERAP1* we observe a very similar pattern of IRVs in both the Australian and Norwegian cohorts (Figures 1a and 1b, respectively). The difference between the Australian and Norwegian *ERAP1* IRV sets is a result of a different SNP in each cohort failing to be genotyped (see Table 2). Irrespective of this, the *ERAP1* SNPs in the Australian (rs3734016) and Norwegian (rs37450) cohorts shown to have a borderline association with preeclampsia are within independent sets of IRVs.

For *ERAP2* we observe an identical pattern of IRVs in both the Australian and Norwegian cohorts (Figures 1a and 1b, respectively). The *ERAP2* SNPs in the Australian (rs2549782) and

Norwegian (rs17408150) cohorts shown to be significantly associated with preeclampsia genetic susceptibility reside within independent sets of IRVs.

There is no evidence of inter-genic isocorrelated redundant SNP variants between *ERAP1* and *ERAP2* in either of the Australian or Norwegian cohorts (Figures 1a and 1b, respectively).

Bioinformatic analysis of the ERAP1 and ERAP2 SNPs

The *ERAP1* and *ERAP2* SNPs reported in both cohorts reside within regions most likely to affect the three-dimensional (3-D) structure and function of their respective proteins. We therefore predicted the importance of these SNPs and how they may affect the ERAP1 and ERAP2 protein folding kinetics using PolyPhen (Sunyaev et al. 2001). The rs3734016 *ERAP1* SNP in the Australian cohort was predicted to be benign. The intronic rs34750 *ERAP1* SNP in the Norwegian cohort is highly correlated with the missense rs26653 SNP (see Figure 1b), which was also predicted to have a benign affect on the ERAP1 protein.

The rs2549782 (Australia) and rs17408150 (Norway) SNPs were predicted to possibly and probably damage the 3-D ERAP2 protein structure and function, respectively. The missense rs2549782 SNP also resides within and juxtaposes the second Glutamic acid residue (**E**) in the highly conserved zinc-binding $HEXXH(X)_{18}$**E** motif. This motif is a distinguishing characteristic of the zinc-dependent aminopeptidases and is essential in their enzymatic activity (Hooper 1994).

Discussion

The genetic basis of preeclampsia has been the subject of widespread speculation for many decades with epidemiological data strongly supporting both maternal and paternal contributions to susceptibility (Esplin et al. 2001; Lie et al. 1998; Skjaerven et al. 2005). Underlying essential hypertension, immune maladaptation to the fetal ‘allograft’ and an exaggerated inflammatory response to pregnancy are most widely hypothesized to play a role in pathogenesis (Ilekis et al. 2007). However, there have been no convincing genetic data to support these theories. In our on-going family-based studies we have initially focused on identifying the maternal genetic contributions to preeclampsia susceptibility. In this current study we now report evidence of a genetic association between *ERAP2* and preeclampsia susceptibility.

The associated *ERAP2* SNPs are missense, population specific, independent of each other and could possibly be playing a functional role, although it is not obvious what effect these amino acid changes have on the activity of the ERAP2 enzyme nor if these SNPs can in some way affect the transcription of the *ERAP2* gene. These seemingly independent associations may arise due to a number of possibilities. We may have uncovered evidence for allelic heterogeneity within the *ERAP2* locus and surrounding genomic sequence to preeclampsia genetic susceptibility. Examples of this phenomenon for other complex disorders include Crohn’s disease (Hugot et al. 2001) and rheumatoid arthritis (Plenge et al. 2007). Each SNP may differ in their genetic effect size in each population which may render our Australian cohort insufficiently powered to detect the rarer, and possibly older rs17408150 SNP. Or we may have observed a false positive association(s). However, since *ERAP2* is clustered within a relatively narrow genomic interval (~270 kb) with the related genes *ERAP1* and *LNPEP* (Tanioka et al. 2003), we cannot rule out that there are as yet unidentified functional variants at this locus that are independent of, or in LD with the *ERAP2* SNPs showing significant association. Notwithstanding this, on review of the known biology of ERAP1, ERAP2 and LNPEP, we suggest that all three genes represent compelling positional candidate susceptibility genes for preeclampsia.

On the basis of sequence similarity (43–49% at the amino acid level) the ERAP1, ERAP2 and LNPEP enzymes have been assigned to an oxytocinase subfamily of the M1 zinc-dependent aminopeptidase gene family (Tanioka et al. 2003). This family of enzymes cleave the N-terminus of numerous proteins thereby enabling them to play an important role in a plethora of biological functions including protein maturation and protein stability, cellular maintenance, growth and development, and defense mechanisms (Taylor 1993). Of major relevance to preeclampsia are their roles in blood pressure regulation, immune recognition and the inflammatory response during pregnancy.

LNPEP, also commonly known as PLAP (placental leucine aminopeptidase), has been identified as an important placental peptidase involved in regulating the peptide hormones oxytocin, vasopressin and angiotensins (Mizutani and Tomoda 1996). Throughout a normal healthy pregnancy there is a steady increase of LNPEP levels in the maternal serum with a subsequent decline after parturition (Yamahara et al. 2000). There however appears to be a significant bi-directional change in maternal serum levels of LNPEP activity in pregnancies complicated with preeclampsia. This enzyme's activity significantly rises in the early stages of a preeclamptic pregnancy only to then dramatically fall below normal levels as the pregnancy progresses and worsens (Mizutani and Tomoda 1996). In light of this knowledge it has been proposed that a marked reduction in serum protease levels in preeclampsia leads to inadequate degradation of vasopressin and angiotensin II and thereby increases placental vascular resistance (Mizutani and Tomoda 1996).

ERAP1 and *ERAP2* also play a key role in blood pressure regulation via their involvement in the renin-angiotensin system. *In vitro* studies using Chinese Hamster Ovary cells characterized the ERAP1 enzyme to effectively cleave and inactive angiotensin II in addition to converting kallidin to bradykinin (Hattori et al. 2000). In the same system, the ERAP2 enzyme was demonstrated to have the ability to cleave angiotensin III to angiotensin IV and to convert kallidin to bradykinin with no hydrolytic activity against oxytocin, vasopressin or angiotensin II (Tanioka et al. 2003). A subsequent genetic study investigating known and novel *ERAP1* SNPs in a Japanese case/control cohort identified a significant association of the missense rs30187 SNP with essential hypertension (Yamamoto et al. 2002). This SNP was shown to reduce by 60% the efficiency of ERAP1 to cleave angiotensin II to angiotensin III and to cause a 70% reduction in the enzymes ability to convert kallidin into bradykinin (Goto et al. 2006).

The immune maladaptation theory for preeclampsia proposes that there is an abnormal maternal immune response to antigenic challenge by the fetal allograft, analogous to the rejection seen in tissue transplant recipients (Dekker and Sibai 1999). The recent reported association between the *ERAP1* gene and the HLA class I mediated autoimmune disease ankylosing spondylitis adds another plausible role for these enzymes in the etiology of preeclampsia (Wellcome Trust Case Control Consortium and The Australo-Anglo-American Spondylitis Consortium 2007). *ERAP1* and *ERAP2* are co-localized within the endoplasmic reticulum (ER), and exhibit a complementary role in trimming NH_2-extended precursor peptides (Saveanu et al. 2005). The additional peptide processing within the ER enables the 8–10 residue mature epitopes to be "loaded" into the peptide-binding groove of MHC class I molecules for transport to the cell surface and presentation to the immune system (Rock et al. 2004). During early human pregnancy MHC class I HLA molecules expressed by invading trophoblast cells at the site of placentation are involved in a maternal-fetal immunological interaction with decidual (natural killer) immune cells via the killer immunoglobulin-like receptors (KIR). This is thought to be crucial for the establishment of immune tolerance to the fetal allograft and successful placentation. Much attention has been focused on this interaction in preeclamptic pregnancies, arguing that unfavorable genotype combinations of both the HLA and KIR molecules may be at play (Moffett and Hiby 2007). The possible involvement of genetic variation in *ERAP1* and *ERAP2* in this interaction is now of great interest.

A recently popularized theory is that preeclampsia is caused by a systemic inflammatory response involving both leucocytes and the endothelium (Sargent et al. 2003). This inflammatory response is also present in a normal pregnancy, albeit in milder form. The inflammatory stimulus was proposed to come from the placenta, via syncytiotrophoblast apoptotic debris, which is shed in normal pregnancy and in increased amounts in preeclampsia. From a functional perspective the ERAP enzymes represent plausible biological candidates having a role in an exaggerated inflammatory response to pregnancy. ERAP1 cleaves cell surface receptors for the pro-inflammatory cytokines IL-1 (IL-1Ra), IL-6 (IL-6Ra) and TNF (TNFR1), thereby down regulating their signaling. Genetic variants of *ERAP1* could therefore have pro-inflammatory effects through this mechanism in light of the increased circulating IL-6 levels during pregnancy (Sargent et al. 2006).

Although we did not find significant associations between *LNPEP* or *ERAP1* (including the rs30187 SNP) and preeclampsia it would be premature to exclude these genes as risk factors for preeclampsia. On the basis of its physical proximity to *ERAP2* it is possible that there is other functional genetic variation in *ERAP1* that is in linkage disequilibrium with the significant *ERAP2* SNP associations, but not with the other *ERAP1* SNPs tested. The same argument applies to LNPEP and deep re-sequencing of all three genes in the Australian families followed by further association analyses in the Australian and Norwegian populations will be required to resolve this. To our knowledge, there are currently no genetic association data with *ERAP2* and hypertension but given our finding of association with preeclampsia it would now be timely for such studies.

Note in support: During the review of this paper a study was published that shows that the expression of the *ERAP2* gene is reduced in first trimester placental tissue from preeclamptic pregnancies (Founds et al. 2009).

Acknowledgments

This work was supported by National Institutes of Health grants HD049847 (to E.K.M., S.P.B. and J.B.) and MH059490 (to J.B.); a grant from the Semp Russ Foundation of the San Antonio Area Foundation (to M.P.J.); The Norwegian University of Science and Technology (NTNU) (R.A.); the Central Norway Regional Health Authority; and the Medical Faculty and Office of International Relations at NTNU (to L.T.R., Ph.D. scholar). M.P.J. is the recipient of a Cowles Postdoctoral Fellowship. This investigation was conducted in facilities constructed with support from Research Facilities Improvement Program grants RR13556 and RR017515 from the National Center for Research Resources, National Institutes of Health. The Nord-Trøndelag Health Study (the HUNT study) is a collaboration between the HUNT Research Centre, the Faculty of Medicine, NTNU, the Norwegian Institute of Public Health and the Nord-Trøndelag County Council. We thank Dr. Jeremy Jowett for bioinformatic analyses and Mr. Anthony Borg for technical assistance. We thank all the preeclampsia families and unrelated preeclamptic women whose participation made this work possible and the support of all the clinicians and research midwives who contributed to this study.

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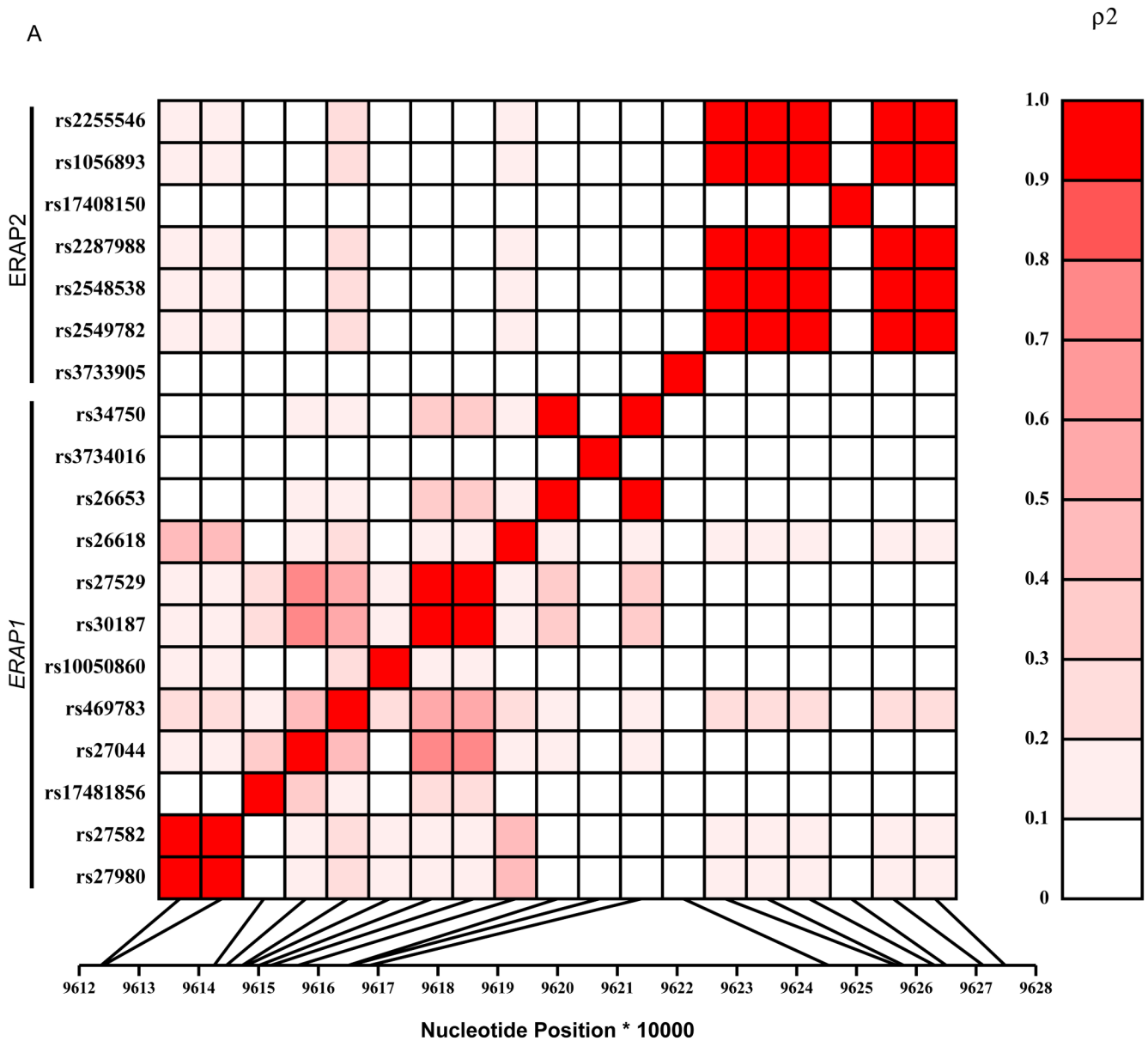

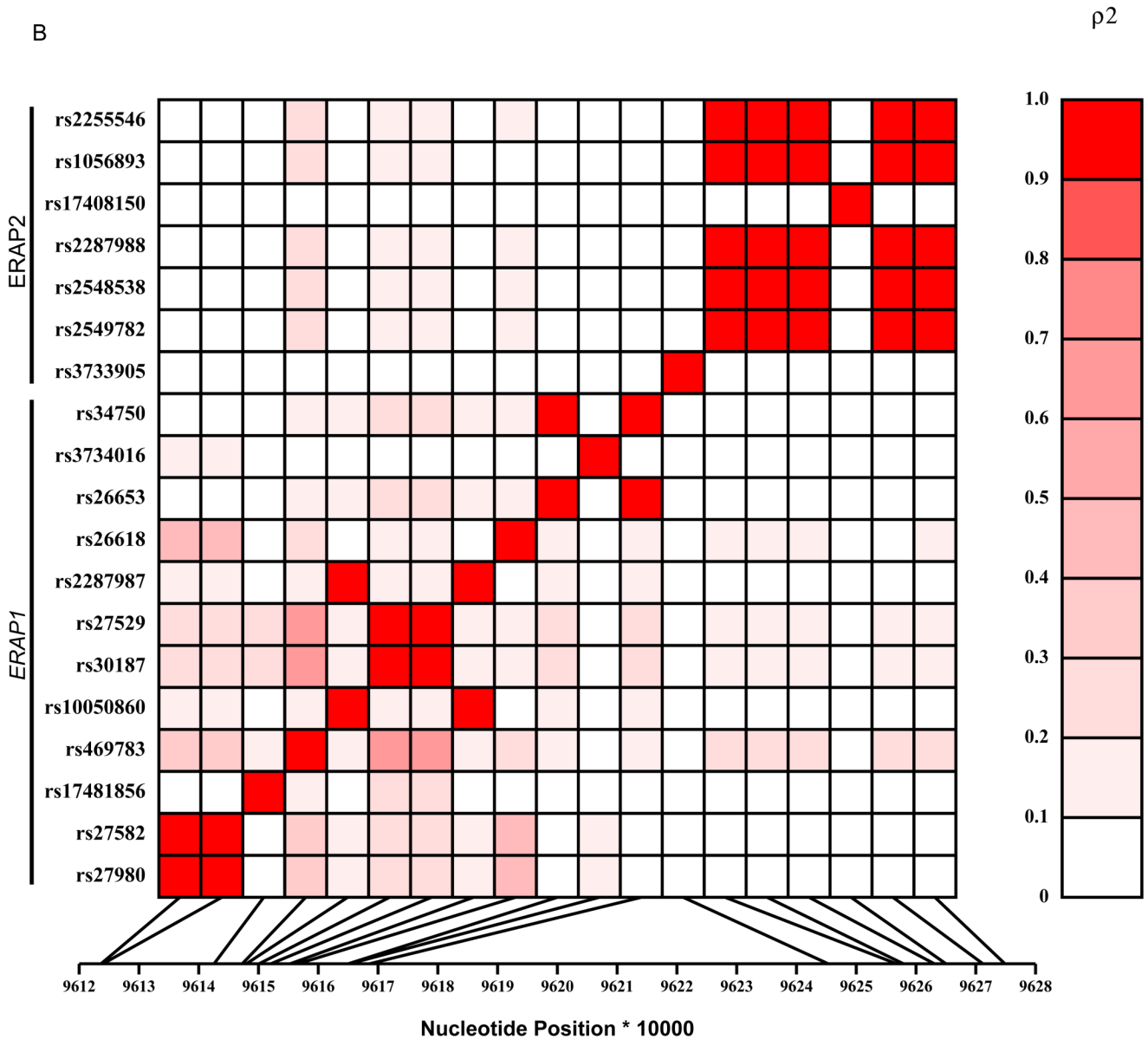

Figure 1.

The pattern of *ERAP1* and *ERAP2* IRVs in (a) the Aust/NZ familial cohort and (b) the Norwegian case/control cohort. The metric to the right of the plot (ρ^2) indicates the strength of the correlation amongst genotypes at each SNP locus with 0.0 indicating no correlation (white) and 1.0 indicating a perfect correlation (red).

Table 1

The GeneSniffer top 20 ranked 5q QTL positional candidate genes.[a,b]

Rank	Hit Score	Symbol[c]	Symbol[d]	OMIM	Gene ID	Mb
1	3050	FLJ39485[e]			285603	96.30
2	**1764**	***LNPEP***	***LNPEP***	**151300**	**4012**	**96.30**
3	**978**	***IL4***	***IL4***	**147780**	**3565**	**132.04**
4	**830**	***CSF2***	***CSF2***	**138960**	**1437**	**131.44**
5	723	*SIAT8D*	*ST8SIA4*	602547	7903	100.17
6	**692**	***ARTS-1***	***ERAP1***	**606832**	**51752**	**96.12**
7	**617**	***CRHBP***	***CRHBP***	**122559**	**1393**	**76.28**
8	495	*GLRX*	*GLRX*	600443	2745	95.18
9	450	*EDIL3*	*EDIL3*	606018	10085	83.27
10	438	*SLC22A5*	*SLC22A5*	603377	6584	131.73
11	415	*NR2F1*	*NR2F1*	132890	7025	92.95
12	408	LOC391827	KRT18P16		391827	123.00
13	**397**	***LRAP***	***ERAP2***	**609497**	**64167**	**96.24**
14	**384**	**FLJ90650**	***LVRN***	**610046**	**206338**	**115.33**
15	381	*DHFR*	*DHFR*	126060	1719	79.96
16	332	*RASA1*	*RASA1*	139150	5921	86.60
17	**320**	***IL13***	***IL13***	**147683**	**3596**	**132.02**
18	307	*SEMA6A*	*SEMA6A*	605885	57556	115.81
19	284	*RIOK2*	*RIOK2*		55781	96.52
20	284	LOC391819	KRT18P42		391819	108.77
29	**179**	***IL3***	***IL3***	**147740**	**3562**	**131.42**
47	**141**	***IL5***	***IL5***	**117850**	**3567**	**131.91**

[a] The 9th and 10th positional candidate genes selected for genotyping were ranked 29th and 47th, respectively.

[b] Bold data highlights the 10 genes prioritized for further analysis.

[c] NCBI Human Genome Build 35.1.

[d] NCBI Human Genome Build 36.2 (gene identifiers used in this article).

[e] This record has been replaced with Gene ID 4012 (*LNPEP*).

Table 2

Prioritized chromosome 5q positional candidate SNPs tested in the Australian and Norwegian preeclampsia cohorts.

Gen	SNP	Function	Aust/NZ cohort						Norwegian cohort							
			GoldenGate genotyping						SNPlex genotyping			iPLEX Gold genotyping				
			Allele (frequency)		MG P_{uncorr}	MG P_{corr}	QTDT P_{uncorr}	QTDT P_{corr}	Allele (frequency)		MG P_{uncorr}	Allele (frequency)		MG P_{uncorr}	MG P_{corr}	
CRHBP	rs32897	Intronic	A (0.849)	G (0.151)	0.914		0.993		A (0.818)	G (0.182)	0.947					
	rs6453267	Intronic	Excluded[a]						Excluded[b]							
	rs7721519	Intronic	C (0.618)	T (0.382)	0.117	NS	0.075	NS	C (0.620)	T (0.380)	0.016	C (0.627)	T (0.373)	0.038	0.083	
	rs1875999	3'UTR	A (0.665)	G (0.335)	0.062	NS	0.065	NS	A (0.648)	G (0.352)	0.052	A (0.650)	G (0.350)	0.030	0.065	
ERAP1	rs27980	3'UTR	A (0.690)	C (0.310)	1.000		0.378		A (0.660)	C (0.340)	0.540					
	rs27582	3'UTR	G (0.697)	A (0.303)	0.700		0.149		G (0.660)	A (0.340)	0.514					
	rs17481856	Synonymous	G (0.874)	A (0.126)	0.374		0.365		G (0.869)	A (0.131)	0.676					
	rs27044	Missense	C (0.709)	G (0.291)	0.252		0.188		Failed genotyping							
	rs469783	Synonymous	T (0.559)	C (0.441)	0.477		0.094		T (0.563)	C (0.437)	0.762					
	rs10050860	Missense	C (0.781)	T (0.219)	0.951		0.720		C (0.804)	T (0.196)	0.088	C (0.800)	T (0.200)	0.066	NS	
	rs30187	Missense	C (0.680)	T (0.320)	0.533		0.377		C (0.661)	T (0.339)	0.558					
	rs27529	Synonymous	G (0.678)	A (0.322)	0.418		0.571		G (0.660)	A (0.340)	0.499					
	rs2287987	Missense	Failed assay design						T (0.794)	C (0.206)	0.075	T (0.801)	C (0.199)	0.063	NS	
	rs26618	Missense	T (0.777)	C (0.223)	0.933		0.352		T (0.773)	C (0.227)	0.752					
	rs26653	Missense	G (0.735)	C (0.265)	0.628		0.953		G (0.710)	C (0.290)	0.062	Failed assay design				
	rs3734016	Missense	G (0.960)	A (0.040)	0.078	NS	0.009	0.074	G (0.944)	A (0.056)	0.196					
	rs34750	Intronic	C (0.736)	G (0.264)	0.595		0.881		C (0.709)	G (0.291)	0.071	C (0.709)	G (0.291)	0.011	0.082	
ERAP2	rs3733905	Missense	C (0.995)	T (0.005)	0.633		1.000		C (0.997)	T (0.003)	0.368					
	rs2549782	Missense	T (0.572)	G (0.428)	0.015	NS	0.004	0.018	T (0.494)	G (0.506)	0.480					
	rs2548538	Synonymous	T (0.572)	A (0.428)	0.015	NS	0.004	0.018	T (0.493)	A (0.507)	0.372					
	rs2287988	Synonymous	A (0.572)	G (0.428)	0.015	NS	0.004	0.018	A (0.493)	G (0.507)	0.514					
	rs17408150	Missense	T (0.963)	A (0.037)	0.614		0.675		T (0.961)	A (0.039)	0.044	T (0.958)	A (0.042)	0.009	0.039	
	rs1056893	Synonymous	T (0.572)	C (0.428)	0.014	NS	0.004	0.018	T (0.491)	C (0.509)	0.624					
	rs2255546	Synonymous	A (0.573)	G (0.427)	0.015	NS	0.004	0.018	A (0.492)	G (0.508)	0.513					
LNPEP	rs3797799	Missense	NP						NP							
	rs12520455	Missense	NP						NP							
	rs2303138	Missense	G (0.918)	A (0.089)	NS		NS		G (0.922)	A (0.078)	NS					
	rs17087233	Missense	NP						NP							
	rs11746232	Missense	A (0.909)	G (0.091)	NS		NS		A (0.903)	G (0.097)	NS					
	rs2287927	Synonymous	NP						NP							
LVRN	rs10062297	Synonymous	T (0.820)	C (0.180)	NS		NS		T (0.830)	C (0.170)	NS					
	rs12520255	Missense	C (0.820)	T (0.180)	NS		NS		C (0.832)	T (0.168)	NS					
	rs1445708	Synonymous	C (0.597)	T (0.403)	NS		NS		C (0.573)	T (0.427)	NS					
	rs17482536	Synonymous	C (0.981)	T (0.019)	NS		NS		C (0.977)	T (0.023)	NS					
	rs7724126	Missense	Excluded[a]						NP							
	rs7712021	Synonymous	T (0.656)	C (0.344)	NS		NS		T (0.652)	C (0.348)	NS					
	rs1560079	Missense	NP						NP							
	rs17138632	Missense	G (0.971)	T (0.029)	NS		NS		G (0.959)	T (0.041)	NS					
	rs17138681	Missense	NP						NP							
	rs1508886	3'UTR	A (0.506)	G (0.494)	NS		NS		A (0.540)	G (0.460)	NS					
IL3	rs40401	Missense	C (0.790)	T (0.210)	NS		NS		C (0.772)	T (0.228)	NS					
CSF2	rs2069625	Synonymous	Excluded[a]						NP							
	rs25882	Missense	T (0.806)	C (0.194)	NS		NS		T (0.791)	C (0.209)	NS					
IL5	rs2069818	Synonymous	NP						NP							
	rs1800474	Synonymous	NP						NP							
IL13	rs1295687	Intronic	C (0.941)	G (0.059)	NS		NS		C (0.915)	G (0.085)	NS					
	rs3212145	Intronic	NP						NP							

Gen	SNP	Function	Aust/NZ cohort						Norwegian cohort					
			GoldenGate genotyping						SNPlex genotyping			iPLEX Gold genotyping		
			Allele (frequency)		MG P_{uncorr}	MG P_{corr}	QTDT P_{uncorr}	QTDT P_{corr}	Allele (frequency)		MG P_{uncorr}	Allele (frequency)	MG P_{uncorr}	MG P_{corr}
	rs2069744	Intronic	C (0.991)	T (0.009)	NS		NS		C (0.995)	T (0.005)	NS			
	rs2069747	Intronic	NP						NP					
	rs1295686	Intronic	G (0.826)	A (0.174)	NS		NS		G (0.793)	A (0.207)	NS			
	rs20541	Missense	C (0.823)	T (0.177)	NS		NS		C (0.794)	T (0.206)	NS			
	rs848	3’UTR	G (0.826)	T (0.174)	NS		NS		G (0.795)	T (0.205)	NS			
IL4	rs17772853	5’UTR	C (0.995)	T (0.005)	NS		NS		Failed genotyping					
	rs2070874	5’UTR	C (0.858)	T (0.142)	NS		NS		C (0.844)	T (0.156)	NS			
	rs4986964	Missense	Excluded[a]						NP					

Abbreviations: QTDT, quantitative transmission disequilibrium test; MG, measured genotype test; P_{uncorr}, uncorrected p-value; P_{corr}, corrected p-value; NP, non-polymorphic; NS, not significant.

[a] NP excluded from statistical analysis (observed minor allele count < 5).

[b] NP excluded from statistical analysis (individual genotype success rate = 14.8%).