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Chorionic Gonadotropin Beta gene variants are associated with recurrent miscarriage in two European populations

Short title: *HCG beta* genes and recurrent miscarriage

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Abstract

Context: The incidence of recurrent miscarriage (RM; ≥3 consecutive pregnancy losses) is estimated as 1%-2% in fertile couples. Familial clustering of RM has suggested the contribution of a genetic component.

Objective: Low level of HCG in maternal serum during the first trimester of the pregnancy is a clinically accepted risk factor for miscarriage. We sought to study whether variation in *Chorionic Gonadotropin beta* subunit genes (*CGB*) expressed in placenta may contribute to the risk of RM.

Design: Resequencing of *CGB5* and *CGB8,* the two most actively transcribed loci of the four *HCG beta* duplicate genes.

Setting: A case-control study involving two sample sets, from Estonia (n=194) and from Finland (n=185).

Patients: RM patients (n=184) and fertile controls (n=195).

Results: From 71 identified variants in *CGB5* and *CGB8*, 48 SNPs were novel. Significant protective effect was associated with two SNPs located at identical positions in intron 2 in both *CGB5* (p=0.007, OR=0.53) and *CGB8* (p=0.042, OR=0.15); and with four *CGB5* promoter variants (p<0.03; OR=0.54-0.58). The carriers of minor alleles had reduced risk of RM. The haplotype structure of the *CGB8* promoter was consistent with balancing selection; a rare mutation in *CGB8* initiator element was detected only among patients (n=3). In addition, three rare non-synonymous substitutions were identified among RM cases as possible variants increasing the risk of recurrent pregnancy loss.

Conclusions: The findings encourage studying the functional effect of the identified variants on *CGB* expression and HCG hormone activity to further elucidate the role of *CGB* variation in RM.

Introduction

Recurrent miscarriage (RM) or habitual abortion is defined as three or more consecutive pregnancy losses before 22 gestational weeks or the spontaneous abortion of an embryo/fetus weighing less than 500g. The occurrence of RM is estimated as 1%-2% of fertile couples (1, 2). Although the patients with RM undergo multiple diagnostic tests to detect parental chromosomal anomalies, maternal thrombophilic, endocrine, or immunological disorders, over 50% of the RM cases are classified as idiopathic (3). An increased prevalence of miscarriage among first-degree relatives of the women suffering from RM (4) suggests genetic contribution in recurrent pregnancy loss. Possible candidates include genes regulating the development of maternal immunotolerance and inflammatory response, coagulation, angiogenesis, vascular tone, and apoptosis. Prime candidates of the molecular causes of RM have been various trombophilic gene mutations (5-7). Convincing data has also been reported on the association between the miscarriage rates and the polymorphisms in *HLA-G* gene expressed on the surface of the invading cytotrophoblasts (8).

So far, major interest has focused on the physiological response of the mother to the pregnancy. Less attention has been paid to the placental proteins coded by the fetal genome with contribution from both maternal and paternal genes and their variants. One of the first proteins produced by the conceptus is human chorionic gonadotropin (HCG), also known as “the pregnancy hormone” due to its essential role in human reproduction. The main function of HCG is to delay the apoptosis of the *corpus luteum* during the first trimester of pregnancy. HCG has several paracrine effects in the process of implantation (9), angiogenesis and placentation (10, 11), and development of maternal immunotolerance (12). Low level and non-exponential increase of HCG in maternal serum during the first trimester of the pregnancy is a clinically accepted risk factor for miscarriage (13-15).

The hormone-specific HCG beta-subunit is expressed by syncytiotrophoblasts of placenta and is encoded by four *Chorionic Gonadotropin Beta* genes (*CGB, CGB5, CGB7* and *CGB8*) located within the *LHB/CGB* gene cluster at 19q13.3 (Fig. 1B). Among the four *HCG beta* duplicate genes *CGB8* and *CGB5* are the most actively transcribed and contribute together 62-82% to the total pool of beta-subunit mRNA transcripts (Fig.1A, 16-18). Our previous data on the *HCG beta* genes showed that (i) their diversity level is one of the highest reported for human genes; (ii) there is high interindividual and intergenic difference in expression and (iii) mRNA transcription level is significantly lower in cases of RM compared to normal first trimester pregnancies (18-20). Now we have addressed the question whether particular variants in these genes may contribute to pregnancy failure. High genetic variation in the *LHB/CGB* region and the aim to capture both rare and common variation prompted us to choose resequencing instead of traditional genotyping. We analyzed *CGB5* and *CGB8* in Estonian and Finnish RM cases (n=184) and fertile women (n=195) by comparing variation and haplotype patterns between the two groups. Consistent with hypothesis of the study, we identified genetic variants in *HCG beta* genes either significantly increasing or reducing a subject’s risk to experience recurrent pregnancy loss.

Subjects and Methods

Study subjects

The study was approved by the Ethics Committees of the University of Tartu, Estonia (protocols no 117/9, 16.06.03, 126/14, 26.04.2004) and the Department of Obstetrics and Gynecology, Helsinki University Central Hospital Outpatient Clinic for women with recurrent miscarriage (protocol no 298/E2/2000). Subjects were recruited and blood samples for the DNA extraction were collected at the Women’s Clinic of Tartu University Hospital and Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Tallinn, Estonia in 2003-2007; and in the Department of Gynaecology and Obstetrics of the Helsinki University Hospital in Finland during 2001–2004. Written informed consent was obtained from every study participant. In both participating centers patients with at least ≥3 abortions during the first trimester of pregnancy were recruited (n=184; age 18-40 yrs). As maternally and paternally derived gene variants contribute equally to the function of a fetal genome, the patient group included both, the women and their partners, who had experienced recurrent pregnancy losses. In Estonian sample collection the patient group consisted of 32 couples and 29 females with RM, and additional 3 couples with ≥3 unsuccessful *in vitro* fertilization procedures. In Finnish sample collection the RM group consisted of 40 couples and 5 females with RM (detailed description in 21, 22). The control group (n=195) consisted of age-matched fertile women with no history of miscarriage and either at least one normal pregnancy (the Finnish subjects, n=100) or more stringently, ≥3 successful deliveries (the Estonian subjects, n= 95). The control group was designed under the assumption that fertile women with no history of spontaneous abortions are carrying gene variants supporting successful pregnancies. Their male partners were not recruited into the control group as detailed reliable information on their past reproductive history was unavailable.

All patients had a normal karyotype tested from peripheral blood lymphocyte cultures. Female patients having uterine anomalies were excluded by ultrasonography or hysterosonogram.

Amplification and resequencing of CGB5 and CGB8

DNA was extracted from peripheral blood using a protocol based on the salting-out method for DNA extraction. The *CGB5* (~ 1.7 kb fragment) and *CGB8* (long-range PCR ~ 8.3 kb; nested PCR ~2.5 kb fragment) genomic regions (Fig. 1C) were amplified and resequenced using previously described primers and conditions (19). The resequenced region involving *CGB8* covered 2050 bp including the entire *CGB8* (1474 bp), 400 bp of 5' upstream region. The resequenced region for *CGB5* (1468 bp) covered the full genic region and part of 3'downstream region (Fig. 1C). Additional primers were designed for the analysis of the 5'upstream region of the *CGB5* gene (450 bp) using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Specificity of the PCR products was verified in three steps: (1) design of unique primer pairs for specific amplification of only one of the seven duplicated genes; (2) verification of monomorphic status of gene-specific positions used as markers for each individual gene (Supplementary Fig. S1); (3) test for Hardy-Weinberg Equilibrium (HWE) for each identified SNP. Primer sequences for PCR and resequencing are listed in Supplementary Table S1. The sequences were resolved using either ABI 3730 X1 or ABI 3730 XL DNA Analyzer (Applied Biosystems) and assembled into a contig as described (19). Polymorphisms were identified using the PolyPhred program (Version 6.02) (http://www.phrap.org/phredphrapconsed.html) (23) and confirmed by manual checking. A genetic variant was verified only if it was observed in both forward and reverse orientations. In case of indel heterozygosity the genotype of the subject was confirmed using two independent forward and two reverse primers. The nomenclature of the identified polymorphisms was based on the GenBank reference sequences: NM_033043.1 GI:15451747 for *CGB5*, NM_033183.2 GI:146229337 for *CGB8*.

Data analysis

Allele frequencies were estimated and conformance to HWE was calculated (α = 0.05). In total 8 rare SNPs in 5'upstream region of *CGB5* were found to be deviating from HWE, as one individual was homozygous for minor allele of all these SNPs.

Haplotypes were inferred from unphased genotype data using the Bayesian statistical method in the program PHASE 2.1.1 (http://www.stat.washington.edu/stephens/; 24), applying the model allowing recombination. The running parameters were: number of iterations = 1000, thinning interval = 1, burn-in = 100; the –X10 parameter was used for increasing the number of iterations of the final run of the algorithm.

Sequence diversity parameters and neutrality tests were calculated using DnaSP (ver. 4.0; http://www.ub.es/dnasp/; 25) with the most probable phased haplotypes as an input sequence. The direct estimate of per-site heterozygosity (π) was derived from the average pairwise sequence differences, while Watterson's θ represents an estimate of the expected per-site heterozygosity based on the number of segregating sites (S). The basis of the Tajima's D statistic (26) is the difference between the π and θ estimates: under neutral conditions $\pi = \theta$ and $D^T = 0$. The Ewens-Watterson homozygosity test implemented in Arlequin 2.000 software (http://cmpg.unibe.ch/software/arlequin3/; 27) was used to test the hypothesis that haplotypes are selectively neutral. An excess of rare variants (= homozygosity excess) indicates directional selection, while an excess of intermediate frequency variants (= homozygosity deficiency) indicates balancing selection. The relationship between inferred haplotypes was analyzed with NETWORK 4.201 software (http://www.fluxus-technology.com) using the Median-Joining network algorithm (28). Haplotype networks of *CGB5* and *CGB8* were calculated using (i) SNPs located in genic region from the transcription initiation site until the end of the mRNA and (ii) promoter SNPs located 5'upstream of the genic region. Singleton polymorphisms were excluded from network calculations (cannot be reliably phased) performed with default parameters. The descriptive statistics of linkage disequilibrium (LD), r^2 was calculated for pairs of markers and summarized by Haploview software (29).

The significance of the association between the identified SNPs in *CGB5* and *CGB8* genes and occurrence of RM was tested using Cochran-Armitage test for trend implemented in statistical analysis package JMP® 6.0.3 with Genomics module 2.0.6 (http://www.jmp.com/software/genomics/). The same test was applied to address the interpopulation (Estonians, Finns) differentiation. Odds ratio (OR) with 95% confidence intervals (CI) were calculated to show the strength and direction of the association. In all tests, $p<0.05$ was considered statistically significant.

Results

Resequencing of CGB5 and CGB8

We sequenced the entire genic and 5'upstream regions of *CGB5* and *CGB8* genes in a sample collection consisting of Finnish and Estonian patients with recurrent miscarriage (RM) (n=184; n=85 Finns, n=99 Estonians) and fertile controls (n=195; n=100 Finns, n=95 Estonians). For every subject the entire sequenced region covered 4.3 kbp (Fig. 1B-C). In total 71 variants were identified: 29 and 19 SNPs in the genic part of *CGB5* and *CGB8*, respectively; 18 and 3 SNPs in the 5' upstream regions of *CGB5* and *CGB8*, respectively; and 2 SNPs 3'downstream of *CGB5* (Table 1). Among the 71 detected SNPs 48 (68%) were novel variants, previously not described in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and literature. Neither *CGB5* nor *CGB8* has been covered by the most recent version of HAPMAP (http://www.hapmap.org/; release March 2008). The diversity parameter π that describes the mean nucleotide diversity per bp differed in the genic and 5' upstream regions (Table 2). Among fertile women the diversity of *CGB5* (π=2.71x10^{-3}) and *CGB8* (~π=2.01x10^{-3}) 5' upstream regions was approximately twofold higher compared to the genic regions of *CGB5* (~π=1.69x10^{-3}) and *CGB8* (~π=9x10^{-4}) (Table 2).

Two thirds of the identified variants (n=41; 58%) were shared by Estonian and Finnish sample collections. In both sample collections there were 15 population-specific SNPs represented as single or low frequency variants (<2%). Majority of the shared SNPs showed no differences (p>0.05) among the two study populations. Significant difference in allele frequencies was detected for 8 out of 71 SNPs, most being rare variants (Table 1). Linkage disequilibrium (LD) between the identified SNPs in the resequenced region was nearly absent in both population samples (Fig. 2).

Four SNPs represented non-synonymous amino acid changes: *CGB5* p.Val76Leu in a single Finnish RM patient, *CGB8* p.Arg28Trp and *CGB8* p.Pro93Arg in single Estonian patients, and *CGB8* p.Val49Ile in one Finnish and two Estonian patients, and also seven Estonian fertile women (Table 1). Further experimental studies have to be conducted before drawing any conclusions about their effect on the hormone function.

CGB5 and CGB8 variants lowering the risk for recurrent pregnancy loss

A case-control study targeting the association of identified *CGB5* and *CGB8* genetic variants with RM was carried out separately for the Estonian (RM cases n=99; fertile women defined as controls n=95) and the Finnish subjects (cases n=85; controls n=100) as well as for the joint dataset. The comparison of single marker and haplotype distribution in the two sample sets revealed low population stratification (Table 1, Supplementary Fig. S2) facilitating the joint analysis in order to increase the statistical power of the study.

In the full case-control sample set a significant association with RM was detected for the *CGB5* 5'upstream polymorphisms (c5EF-155, c5EF-147, c5EF-144, c5EF-142) (p<0.03; OR=0.58 [95% CI 0.35-0.93]; Cochran-Armitage trend test) (Table 3). Analysis of the Estonian (p=0.083; OR=0.54 [95% CI 0.27-1.1]) and the Finnish (p≤0.131; OR=0.58 [95% CI 0.29-1.19]) subsamples supported trend for association in both study populations independently, but the p-values did not reach statistical significance (p>0.05) due to reduced samples sizes. The significant association with all four *CGB5* promoter polymorphisms results from higher minor allele frequency (MAF) in fertile women (12.05%-13.08%) compared to RM group (7.10%-7.92%). This difference between the control group and RM cases was consistent in both study populations: 13.16% compared to 8.08 % and 11-13% compared to 5.95-7.74%, in Estonians and Finns, respectively (Table 3). On the haplotype network of *CGB5* upstream region the promoter variants carrying the minor alleles of the four polymorphisms form a remote clade (H1-H2, H10-H11; Fig. 3A).

Among the *CGB5* genic SNPs a strong protective effect was detected for the minor allele of intron 2 c5EF1038 (p<0.007; OR=0.53 [95% CI 0.32-0.85]), represented with the frequency 14.36% in fertile women compared to 8.15% in the RM group (Table 3). This effect reached statistical significance in the separate analysis of the Finnish subjects (p=0.036; OR=0.48 [95% CI 0.24-0.97]) and showed a trend for association in the Estonian (p=0.079; OR=0.57 [95% CI 0.30-1.08]) subsample. No increase in protection towards RM was detected for the combination of the minor alleles of the *CGB5* 5'upstream and the intronic SNP (data not shown).

Notably, the association of four *CGB5* SNPs with the protective effect towards pregnancy loss was sufficiently robust to remain significant even when only the female RM patients (n=109) were considered as cases (Table 4). A separate analysis of male RM patients revealed similar trends for association and protective effect sizes as compared to female RM cases, although the p-values were non-significant possibly due to smaller sample size (n=75) that reduced the statistical power. However, the inclusion of both sexes gave a stronger effect than gender-specific analysis in all but one SNP (c5EF1038; Table 3, 4) further supporting the contribution of both, maternal and paternal genes in the reproductive success.

Population-specific associations were detected in the Finnish sample collection with two rare SNPs (MAF <10%) in *CGB8*: c8EF301 (p=0.034) and c8EF1045 (p=0.025) (Table 3). Interestingly, the protective variant in the intron 2 of *CGB8* (c8EF1045) is located at the same position within the gene as the *CGB5* intronic variant (c5EF1038).

Rare CGB8 promoter variants increase the susceptibility to recurrent miscarriages

The resequenced *CGB8* 5'upstream region stands out with only 3 SNPs (two common and one rare) compared to the respective region for *CGB5* with 18 SNPs (Table 1). The rare allele A of SNP c8EF-4 was solely represented in patients, one from Finland and two from Estonia (Cochran-Armitage trend test, p=0.071). This polymorphism is located within the AP1-like sequence overlapping the *HCG beta* initiator element critical for basal transcription and downstream of the Ets-2 binding site acting as a major enhancer of *HCG beta* gene expression (30).

We applied two neutrality tests to explore observed versus expected distribution of SNPs and haplotypes in the 5'upstream region of *CGB8*. Both, the Tajima's D statistic (D^T=2.29, p<0.05; Table 2) as well as Ewens-Watterson homozygosity test (p=0.007) indicated a possible scenario of balancing selection driving the three apparently most efficient *CGB8* promoter variants (H1, H3, H4) to high frequency in both populations (Fig. 3B; Supplementary Table S2: Supplementary Fig. S2). The rare variant H2 carried the minor allele of c8EF-4, identified solely in patients. Notably, the haplotype combining the minor alleles of c8EF-287 (C; MAF=25.2%) and c8EF-186 (T; MAF=39.7%) is expected to be present with the frequency of 10%, but was not observed in the current study (Fig. 3B; Supplementary Table S2).

Discussion

Here we report the first case-control study targeting the variation in *HCG beta* genes in association with recurrent miscarriages (RM). Most association studies on RM have so far focused on susceptibility variants of maternal genes involved in physiological adaptation to pregnancy, such as development of immunotolerance at feto-maternal interface or alterations in fibrinolytic and coagulation pathways. As these genes also contribute to complex diseases, the role of their variants in susceptibility to RM may not be specific (5, 8, 22, 31). *HCG beta* genes are expressed in blastocysts shortly after fertilization (20, 32) and are essential for successful implantation. Thus, a genetic variant of these genes is more likely to have an effect on pregnancy outcome. Our study focused on *CGB8* and *CGB5* that provide the major fraction of HCG *beta* mRNA transcripts and the resequencing method was chosen instead of genotyping.

The human *CGB8* and *CGB5* genes are located among the seven duplicate genes within the *LHB/CGB* gene cluster. Major complications in targeting duplicated genes in association studies are high sequence similarity (>92%), high diversity, large number of population-specific variants and low LD due to high gene conversion activity (19, 33). These characteristics make it technically challenging to select reliable tag-SNPs and establish genotyping methods capable of targeting unique SNPs in duplicated genes. In the 379 subjects we identified only 14 out of 30 (47%) SNPs in *CGB5* present in a public SNP database NCBI dbSNP and 9 out of 44 (20%) in *CGB8*. Several of the variants not observed in our study have been predicted *in silico* or by using high-throughput methods and may actually be multisite or paralogous gene variants (34). Alternatively, some of these SNPs could indeed represent variants specific to other than Estonian or Finnish populations. For example, an amino acid substitution Val79Met (nomenclature based on mature protein; from ATG p.Val99Met) in *CGB5* exon 3 has been reported at carrier frequency 4.2% in a random population from the Midwest of the United States (35) but it was absent in a 580 DNA samples originating from five European populations (36). In the current study, in relatively large samples sets drawn from two neighboring populations, one third of the identified variants (MAF<2%) were found in only one population, although the sample size was sufficient to identify all common variants (MAF>5%) originally described in a large mutation screening of *LHB/CGB* genes (Table 1; 19). Full resequencing data collected in this study enabled to identify several rare non-synonymous and promoter variants and to conduct haplotype analysis.

Consistent with the hypothesis of the study, we identified genetic variants in *HCG beta* genes significantly increasing or reducing the risk of RM. A protective effect was detected for the minor alleles of two SNPs (c5EF1038 and c8EF1045) located at the identical positions in intron 2 in both *CGB5* and *CGB8* and for four *CGB5* promoter variants (c5EF-155; c5EF-147; c5EF-144; c5EF-142). The carrier status of the minor alleles of these six SNPs reduced the risk of RM 1.7-fold in comparison to the wildtype carriers. Interestingly, the "protective" alleles of the *CGB5* promoter SNPs form a motif (C-del-C-A; H2 on Fig. 3A) identical to the promoter sequence of *CGB8* (Fig. 1D), which has been shown to be most actively transcribed *HCG beta* gene (18). The actual contribution of these sequence variants to mRNA transcription and splicing efficiency is still to be explored.

The current data suggest the *CGB8* and especially its promoter region to be under stronger functional constraint compared to *CGB5* in spite of high DNA sequence similarity (98-99%) between the two genes (Supplementary Fig. S1). Firstly, we detected > 2 times less polymorphisms in *CGB8* genomic region (n=22) compared to *CGB5* (n=49*)*. Secondly, three rare *CGB8* variants that may exhibit an effect on hormone action were present exclusively in RM patients (p.Arg28Trp, p.Pro93Arg and a c8EF-4 within proximal promoter) compared to only one such SNP in *CGB5 (*p.Val76Leu). Thirdly, the applied neutrality tests indicated a balancing selection in the promoter region of *CGB8*, but not of *CGB5*. Additionally, we identified only three of the four predicted major *CGB8* promoter haplotypes (Fig. 3B). The haplotype combining the minor alleles of c8EF-287 (MAF 25.2 %) and c8EF-186 (MAF 39.7%) was absent in the current dataset in spite of the relatively high minor allele frequency. The discrepancy between observed (0%) and expected (10%) frequency may be explained by the localization of these SNPs within Sp1/AP-2 binding sites (37) residing in the critical region for the trophoblast-specific expression as well as cAMP-responsiveness of the *HCG beta* gene transcription (38, 39). Functional studies should reveal whether these sequence variants indeed possess a combinatory effect influencing the binding of the AP-2 and Sp1 transcription factors to the promoter of *HCG beta* and alter the transcription of genes.

One of the key factors in obtaining reliable results in a case-control study is a clearly defined study group and replication of the results in an independent dataset. We applied parallel analysis of case-control sample sets collected from two neighboring countries in order to confirm the robustness of the association across populations. As the stratification was low between these populations we also conducted a joint analysis of the two sample sets in order to raise the statistical power. Although there were minor differences in subject recruitment, the obtained results were concordant in two populations and the strength of detected associations increased in the analysis of the pooled dataset. In addition we identified two gene variants lowering the risk of RM in the Finnish dataset only possibly owing to the specific demographic history of the Finnish population (40).

In conclusion, these data from two populations provide the first evidence for the role of the variation in *HCG beta* genes in contributing to the susceptibility of RM. The findings encourage further studies addressing the functional effect of the identified promoter, intronic and rare protein-altering variants on *HCG beta* gene expression and HCG hormone activity. The diagnostic application of our findings may facilitate the improvement of early and preventive treatment of RM.

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References

1. **Berry CW, Brambati B, Eskes TK, Exalto N, Fox H, Geraedts JP, Gerhard I, Gonzales Gomes F, Grudzinskas JG, Hustin J, Jouppila P, Lindblom BKA, Mantoni N, Montenegro N, Nogales Fernandes F, O'Rahilly R, Pedersen JF, Peters PWJ, Regan L, Rushton DI, van Straaten HWM, Tarlatzis BC, Wells M** 1995 The Euro-Team Early Pregnancy (ETEP) protocol for recurrent miscarriage. Hum Reprod 10:1516–1520
2. **Jauniaux E, Farquharson RG, Christiansen OB, Exalto N** 2006 Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. Hum Reprod 21: 2216-2222
3. **Li TC, Makris M, Tomsu M, Tuckerman E, Laird S** 2002 Recurrent miscarriage: aetiology, management and prognosis. Hum Reprod Update 8:463-481
4. **Christiansen OB** 1996 A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects. Hum Reprod Update 2:271-293
5. **Goodman CS, Coulam CB, Jeyendran RS, Acosta VA, Roussev R** 2006 Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss? Am J Reprod Immunol 56:230-236
6. **Rey E, Kahn SR, David M, Shrier I** 2003 Thrombophilic disorders and fetal loss: a meta-analysis. Lancet 361:901-908
7. **Robertson L, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GDO, Walker ID, Greaves M, Brenkel I, Regan L, Greer IA** 2006 Thrombophilia in pregnancy: a systematic review. Br J Haematol 132:171–196
8. **Hviid TV** 2006 HLA-G in human reproduction: aspects of genetics, function and pregnancy complications. Hum Reprod Update 12:209-232
9. **Licht P, Fluhr H, Neuwinger J, Wallwiener D, Wildt L** 2007 Is human chorionic gonadotropin directly involved in the regulation of human implantation? Mol Cell Endocrinol 269:85-92
10. **Toth P, Lukacs H, Gimes G, Sebestyen A, Pasztor N, Paulin F, Rao CV** 2001 Clinical importance of vascular LH/hCG receptors - a review. Reprod Biol 1:5-11
11. **Herr F, Baal N, Reisinger K, Lorenz A, McKinnon T, Preissner KT, Zygmunt M** 2007 HCG in the regulation of placental angiogenesis. Results of an in vitro study. Placenta S:85-93
12. **Kayisli UA, Selam B, Guzeloglu-Kayislim O, Demir R, Arici A** 2003 Human chorionic gonadotropin contributes to maternal immunotolerance and endometrial apoptosis by regulating Fas-Fas ligand system. J Immunol 171:2305-2313
13. **Buyalos RP, Glassman LM, Rifka SM, Falk RJ, Macarthy PO, Tyson VJ, DiMattina M** 1992 Serum beta-human chorionic gonadotropin, estradiol and progesterone as early predictors of pathologic pregnancy. J Reprod Med 37:261-266
14. **Dumps P, Meisser A, Pons D, Morales MA, Anguenot JL, Campana A, Bischof P** 2002 Accuracy of single measurements of pregnancy-associated plasma protein-A, human chorionic gonadotropin and progesterone in the diagnosis of early pregnancy failure. Eur J Obstet Gynecol Reprod Biol 100:174-180
15. **Tong S, Wallace EM, Rombauts L** 2006 Association between low day 16 hCG and miscarriage after proven cardiac activity. Obstet Gynecol 107:300-304
16. **Bo M, Boime I** 1992 Identification of the transcriptionally active genes of the chorionic gonadotropin beta gene cluster in vivo. J Biol Chem 267:3179-3184
17. **Miller-Lindholm AK, LaBenz CJ, Ramey J, Bedows E, Ruddon RW** 1997 Human chorionic gonadotropin-beta gene expression in first trimester placenta. Endocrinology 138:5459-5465
18. **Rull K, Laan M** 2005 Expression of beta-subunit of human chorionic gonadotropin genes during the normal and failed pregnancy. Hum Reprod 20:3360-3368
19. **Hallast P, Nagirnaja L, Margus T, Laan M** 2005 Segmental Duplications and Gene Conversion: Human Luteinizing Hormone/ Chorionic Gonadotropin Beta Gene Cluster. Genome Res 15:1535-1546
20. **Rull K, Hallast P, Uusküla L, Jackson J, Punab M, Salumets A, Campbell RK, Laan M** 2008 Fine-scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. Mol Hum Reprod 14:23-31

21. **Ulander VM** 2007 Venous thromboembolism during pregnancy and the impact of thrombophilia in pregnancy complications. Ph.D.thesis, University of Helsinki, Faculty of Medicine, Institute of Clinical Medicine (http://urn.fi/URN:ISBN:978-952-10-3671-2).
22. **Kaare M, Ulander VM, Painter JN, Ahvenainen T, Kaaja R, Aittomäki K** 2007 Variations in the thrombomodulin and endothelial protein C receptor genes in couples with recurrent miscarriage. Hum Reprod 22:864-868
23. **Bhangale TR, Stephens M, Nickerson DA** 2006 Automating resequencing-based detection of insertion-deletion polymorphisms. Nat Genet 38:1457-1462
24. **Stephens M, Smith N, Donnelly P** 2001 A new statistical method for haplotype reconstruction from population data. Am J Hum Gen 68:978-989
25. **Rozas J, Rozas R** 1999 DnaSP version 3: an integrated program for molecular population and molecular evolution analysis. Bioinformatics 15:174-175
26. **Tajima F** 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595
27. **Schneider S, Roessli D, Excoffier L** 2000 Arlequin ver. 2.000: A software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland
28. **Bandelt HJ, Forster P, Röhl A** 1999 Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37-48
29. Barrett JC, Fry B, Maller J, Daly MJ 2005 Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263-265
30. **Ghosh D, Ezashi T, Ostrowski MC, Roberts RM** 2003 A central role for Ets-2 in the transcriptional regulation and cyclic adenosine 5'-monophosphate responsiveness of the human chorionic gonadotropin-beta subunit gene. Mol Endocrinol 17:11-26
31. **Dosiou C, Giudice LC** 2005 Natural Killer Cells in Pregnancy and Recurrent Pregnancy Loss: Endocrine and Immunologic Perspectives. Endocr Rev 26: 44-62
32. **Jurisicova A, Antenos M, Kapasi K, Meriano J, Casper RF** 1999 Variability in the expression of trophectodermal markers beta-human chorionic gonadotrophin, human leukocyte antigen-G and pregnancy specific beta-1 glycoprotein by the human blastocyst. Hum Reprod 14:1852-1858
33. **Sedman L, Padhukasahasram B, Kelgo P, Laan M** 2008 Complex signatures of locus-specific selective pressures and gene conversion on human growth hormone/chorionic somatomammotropin genes. Hum Mutat 0: 1-13, DOI 10.1002/humu.20767 [Epub ahead of print, May 12]
34. **Fredman D, White SJ, Potter S, Eichler EE Den Dunnen JT, Brookes AJ** 2004 Complex SNP-related sequence variation in segmental genome duplications. Nat Genet 36:861-866
35. **Miller-Lindholm AK, Bedows E, Bartels CF, Ramey J, Maclin V, Ruddon RW** 1999 A naturally occurring genetic variant in the human chorionic gonadotropin-beta gene 5 is assembly inefficient. Endocrinology 140:3496-3506
36. **Jiang M, Savontaus ML, Simonsen H, Williamson C, Müllenbach R, Gromoll J, Terwort N, Alevizaki M, Huhtaniemi I.** 2004 Absence of the genetic variant Val79Met in human chorionic gonadotropin-beta gene 5 in five European populations. Mol Hum Reprod 10:763-766
37. **Johnson W, Jameson JL** 1999 AP-2 (activating protein 2) and Sp1 (selective promoter factor 1) regulatory elements play distinct roles in the control of basal activity and cyclic adenosine 3',5'-monophosphate responsiveness of the human chorionic gonadotropin-beta promoter. Mol Endocrinol 13:1963-1975
38. **Albanese C, Kay TW, Troccoli NM, Jameson JL** 1991 Novel cyclic adenosine 3',5'-monophosphate response element in the human chorionic gonadotropin beta-subunit gene. Mol Endocrinol 5:693-702
39. **Steger DJ, Hecht JH, Mellon PL** 1994 GATA-binding proteins regulate the human gonadotropin alpha-subunit gene in the placenta and pituitary gland. Mol Cell Biol 14: 5592-5602
40. **Norio R, Nevanlinna HR, Perheentupa J** 1973 Hereditary diseases in Finland; rare flora in rare soul. Ann Clin Res:109–141

Legends to Figures

Figure 1. Genomic and expressional context for the design of the association study targeting *HCG beta* genes. (A) The contribution of each individual gene into the total mRNA transcript pool of all six *CGB* genes (18). (B) Schematic presentation of the *LHB/CGB* gene cluster with genes marked as black wide arrows in the direction of transcription on sense strand. (C) The position of long-range PCR primers (black arrows) and extent of resequenced *CGB5* and *CGB8* regions (short black bars). Gene exons are depicted with grey boxes. Capital letters correspond to the primer sequences listed in Supplementary Table S1. (D) The aligned consensus sequences of the 5'upstream element of *LHB/CGB* genes. The nucleotide positions distinctive for each *HCG beta* and *LHB* gene co-localizing with *CGB5* SNPs c5EF-155, c5EF-147, c5EF-144 and c5EF-142 (Table 1) are highlighted.
All positions are given relative to mRNA transcription start site.

Figure 2. LD structure of the resequenced *CGB5-CGB8* genomic region in Estonian and Finnish sample sets. The plot has been drawn based on r^2 statistic and polymorphisms are ordered as located on the sense strand of genomic DNA sequence. Singleton variants have been excluded for reliable estimation of LD pattern. The direction of transcription of *CGB5* and *CGB8* genes is depicted with arrows. Polymorphisms with a significant association to recurrent miscarriages (Table 3) are given above the LD plot.

Figure 3. Median-Joining (MJ) networks of predicted haplotypes in the 5' upstream region of *CGB5* (A) and *CGB8* (B). Singleton polymorphisms were excluded from the analysis, because of unreliable phasing. The size of each node is proportional to the haplotype frequency in the total dataset. The relative distribution of each haplotype among the recurrent miscarriage (RM) cases (black) and fertile controls (white) is indicated. Haplotype nomenclature is shown in Supplementary Table S2. (A) The carrier status of haplotypes H1-H2 and H10-H11 lowered 1.7-fold the risk of RM. (B) Haplotype H2 defined by the minor allele of a proximal promoter mutation c8EF-4 was exclusively identified in RM patients in both study populations, Estonians and Finns.
MJ networks of predicted haplotypes in the genic regions of *CGB5* and *CGB8* are shown in Supplementary Fig. S3.

TABLE 1. Characteristic of SNPs identified in *CGB5* and *CGB8* in Estonian and Finnish sample sets.

SNP code[a]	Position relative to ATG	Location	Allele[b] major/minor Aminoacid change[c]	Minor allele frequency in a subsample (%)		population difference p-value[d]	rs number[e]
				Estonian (n=194)	Finnish (n = 185)		
			Variants in *CGB5* genomic region				
c5F-447	-812	5’ upstream	T/G	0	S (Co)	0.304	
c5F-399	-764		T/C	0	0.82	0.167	
c5EF-322	-687		T/C	0.52	0.82	0.667	
c5EF-315	-680		T/G	S (Pa)	1.9	0.071	
c5EF-314	-679		C/A	S (Pa)	1.09	0.234	
c5EF-309	-674		C/T	S (Pa)	1.09	0.234	
c5EF-306	-671		T/G/C	S (Pa)	2.45	0.037	
c5EF-291	-656		C/T	21.65	17.66	0.162	rs4801789
c5F-204	-569		A/G	0	1.36	0.051	
c5F-191	-557		T/C	0	1.36	0.051	
c5EF-155	-520		G/C	10.57	9.24	0.543	
c5EF-147	-512		G/del	10.57	8.7	0.373	
c5EF-144	-509		T/C	10.57	10.6	0.989	
c5EF-142	-507		T/A	10.57	10.6	0.989	
c5EF-82	-447		G/A	1.8	1.63	0.853	
c5EF-30	-395		G/C	0.77	1.36	0.429	
c5E-28	-393		C/T	1.55	0	0.016	
c5EF-1	-366		A/G	S (Co)	S (Co)	0.973	
c5E101	-265	5'UTR	C/T	0.52	0	0.166	
c5EF138	-228		A/G	12.11	6.76	0.011	**rs710899**
c5E157	-209		C/T	0.52(Pa)	0	0.166	
c5F206	-160		C/T	0	S (Co)	0.305	
c5F324	-42		G/A	0	0.54 (Co)	0.146	
c5EF345	-21		G/C	22.16	16.76	0.055	**rs12610392**
c5F354	-12		G/del	0	1.08	0.040	
c5E519	154	intron1	G/T	0.52	0	0.166	
c5E525	160		A/G	0.52	0	0.166	**rs35621293**
c5E527	162		G/A	0.52	0	0.166	
c5E529	164		G/A	S (Pa)	0	0.328	
c5EF544	179		T/G	33.25	25.68	0.024	**rs3956245**
c5E551	186		C/T	0.77	0	0.089	**rs4002422**
c5F553	188		T/C	0	0.54 (Co)	0.146	
c5EF580	215		G/A	21.65	17.03	0.104	**rs34524624**
c5F660	295		A/C	0	S (Pa)	0.305	
c5EF666	301		C/T	32.73	25.41	0.027	**rs35871536**

c5EF789	424	exon2	G/A p.Pro24Pro	21.13	17.03	0.148	**rs35133942**
c5E912	547	intron2	G/C	0.77	0	0.089	
c5E918	553		C/G	0.52	0	0.166	**rs33933429**
c5EF1038	673		C/T	11.86	10.81	0.651	**rs34335161**
c5EF1069	704		A/G	1.55	2.43	0.376	**rs33976607**
c5F1111	746		A/T	0	0.54 (Co)	0.146	
c5EF1115	750		C/T	3.35	0.54	0.005	**rs34935416**
c5F1178	813	exon3	G/C p.Val76Leu	0	S (Pa)	0.305	
c5EF1258	893		C/T p.Tyr102Tyr	4.64	0.81 (Co)	0.001	**rs35756580**
c5E1390	1025		C/A p.Pro146Pro	S (Co)	0	0.328	
c5EF1402	1037		T/C p.Ser150Ser	S (Co)	S (Pa)	0.973	
c5EF1426	1061		G/A p.Ser158Ser	S (Co)	S (Pa)	0.973	
c5E1501	1136	3’ down-stream	T/C	S (Pa)	0	0.328	
c5EF1660	1295		A/T	1.29	1.08	0.502	

Variants in *CGB8* genomic region

c8EF-287	-659	5' up-stream	T/C	26.49	23.85	0.417	**rs4801790**
c8EF-186	-558		G/T	40.21	39.08	0.754	**rs8102901**
c8EF-4	-376		T/A	0.52 (Pa)	S (Pa)	0.627	
c8EF105	-268	5'UTR	G/C	4.12	0.57 (Co)	0.003	**rs34212754**
c8EF108	-265		C/T	39.95	39.08	0.808	**rs13345685**
c8EF301	-72		T/A	5.41	6.32	0.597	**rs35930240**
c8EF432	60	intron1	A/C	0.52	0.57 (Co)	0.913	
c8F461	89		T/C	0	S (Pa)	0.290	
c8EF523	151		G/T	S (Co)	0.86 (Pa)	0.264	
c8F526	154		T/G	0	S (Co)	0.290	rs2387591
c8EF541	169		G/C	39.69	39.37	0.928	**rs13345575**
c8F551	179		G/T	0	S (Co)	0.290	
c8F558	186		T/C	0	S (Co)	0.290	
c8EF673	301		T/C	S (Co)	S (Co)	0.938	
c8E806	434	exon2	C/T p.Arg28Trp	S (Pa)	0	0.343	
c8EF869	497		G/A p.Val49Ile	2.32	S (Pa)	0.017	
c8EF925	553	intron2	G/C	2.06	3.16	0.341	rs2303050
c8EF1045	673		C/T	0.52	1.72 (Co)	0.112	**rs33943298**
c8EF1076	704		G/A	1.8	2.01	0.836	
c8EF1122	750		T/C	1.8	2.01	0.836	

c8E1237	865	exon3	C/G p.Pro93Arg	S (Pa)	0	0.343
c8E1418	1046		A/T pArg153Arg	S (Pa)	0	0.343

[a] a SNP code includes gene and sample name (e.g.c5=*CGB5*; E=Estonians, F=Finns), location relative to mRNA start site; GenBank references: NM_033043.1 GI:15451747 for *CGB5*, NM_033183.2 GI:146229337 for *CGB8*; non-synonymous changes detected only in patients are underlined; [b]alleles at the coding strand; [c]coding from ATG including signal protein; [d]Cochran-Armitage test for trend; [e]Variants originally described by Hallast et *al.,* 2005 (19) are highlighted in **bold.** All variants identified in the current study have been submitted to dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), accession numbers ss105106983 – ss105107053.

S - singleton SNP; Co - only among fertile women with no miscarriages; Pa - only among RM patients

TABLE 2. Diversity parameters and neutrality tests of the *CGB5* and *CGB8* genes in fertile women and patients of recurrent miscarriages (RM).

	CGB5			***CGB8***		
	Full region[a]	5'upstream region[b]	Genic region[c]	Full region[a]	5'upstream region[b]	Genic region[c]
No of SNPs	46	17	29	23	3	20
Fertile women						
Diversity (π)[d]	0.00193	0.00271	0.00169	0.00119	0.00201	0.00095
Tajima D[e]	-1.12980	-1.16944	-0.88132	-0.35277	**2.29389***	-0.96141
P-value of Ewens-Watterson *F* statistic[f]	ns	ns	ns	ns	**0.007**	ns
RM Patients						
Diversity (π)[d]	0.00172	0.00195	0.0016	0.00115	0.00199	0.0009
Tajima D[e]	-1.23744	-1.23550	-0.91052	-0.56306	1.26879	-1.06361
P-value of Ewens-Watterson *F* statistic[f]	ns	ns	ns	ns	ns	ns

[a]SNPs in 5'upstream and genic regions; [b]SNPs located in the region of –435 bp up to –1 bp from the start site of mRNA sequence; [c]SNPs located in mRNA seqence: +1 bp up to +1082 bp from the start site of mRNA sequence; [d]Estimate of nucleotide diversity (π) per basepair from average pairwise differences among individuals; [e]The basis of the Tajima's D statistics ($D^{T)}$ is the difference between observed (π) and expected (θ) diversity estimates: under neutral conditions $\pi = \theta$ and $D^T = 0$; [f]This statistic tests the observed allele frequency spectrum with the expected allele frequency spectrum under the neutral model (Hardy-Weinberg Equilibrium).

*p=0.0169; ns - non-significant (p>0.05)

TABLE 3. Variants in *CGB5* and *CGB8* genes significantly associated with recurrent miscarriage (RM). Association P-values and odds ratio (OR) with 95% confidence interval (CI) was calculated by Cochran-Armitage test for trend.

	Estonians (n=194)				Finns (n=185)				All individuals (n=379)			
	MAF (%)		p-value	OR (95%CI)	MAF (%)		p-value	OR (95%CI)	MAF (%)		p-value	OR (95%CI)
SNP	Fertile women n=95	RM patients n=99			Fertile women n=100	RM patients n=85			Fertile women n=195	RM patients n=184		
c5EF-155	13.16	8.08	0.083	0.54 (0.27-1.1)	11.50	6.55	0.129	0.58 (0.29-1.19)	12.31	7.38	0.024	0.57 (0.35-0.93)
c5EF-147	13.16	8.08	0.083	0.54 (0.27-1.1)	11.00	5.95	0.094	0.52 (0.24-1.13)	12.05	7.10	0.018	0.54 (0.32-0.91)
c5EF-144	13.16	8.08	0.083	0.54 (0.27-1.1)	13.00	7.74	0.131	0.61 (0.31-1.17)	13.08	7.92	0.023	0.58 (0.36-0.93)
c5EF-142	13.16	8.08	0.083	0.54 (0.27-1.1)	13.00	7.74	0.131	0.61 (0.31-1.17)	13.08	7.92	0.023	0.58 (0.36-0.93)
c5EF1038	14.47	9.09	0.079	0.57 (0.30-1.08)	14.00	7.06	0.036	0.48 (0.24-0.97)	14.36	8.15	0.007	0.53 (0.32-0.85)
c8EF301*	5.79	5.05	0.740	0.86 (0.35-2.12)	8.85	3.21	0.034	0.35 (0.12-0.96)	7.33	4.24	0.072	0.55 (0.29-1.06)
c8EF1045*	0.53	0.51	0.977	0.97 (0.06-15.6)	3.13	0	0.025	na	1.83	0.28	0.042	0.15 (0.02-1.03)

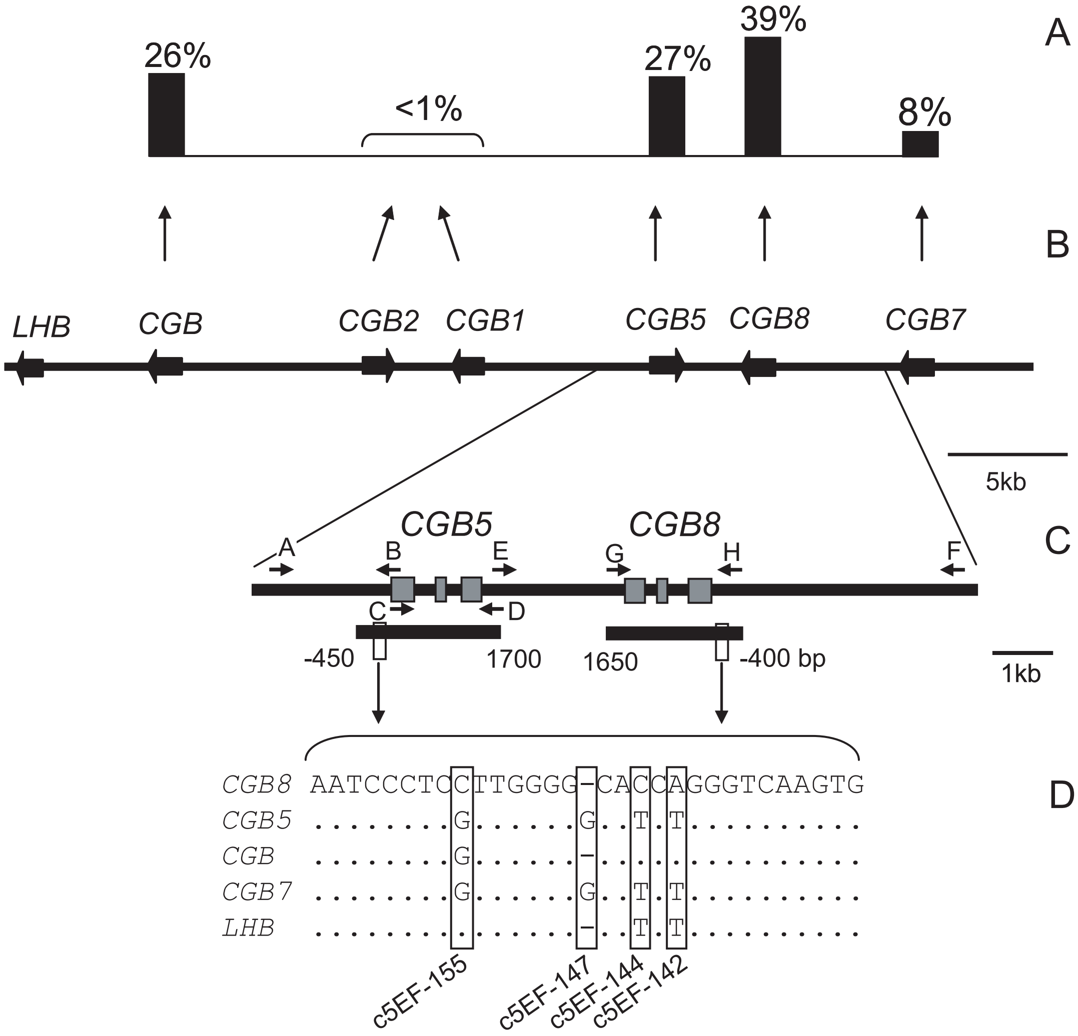
*significant association (p<0.05) only in one population

MAF - minor allele frequency; na - not applicable as monomorphic among fertile women

TABLE 4. Case-control analysis after subdividing recurrent miscarriage (RM) patients by gender. Cochran-Armitage test for trend was performed for the variants in *CGB5* and *CGB8* genes showing significant association with RM in the full sample set (Table 3).

	Fertile women n=195	Female RM patients n=109			Male RM patients n=75		
SNP	MAF %	MAF %	p-value	OR (95%CI)	MAF %	p-value	OR (95%CI)
c5EF-155	12.31	7.34	0.058	0.59 (0.32-1.03)	7.43	0.105	0.57 (0.26-1.13)
c5EF-147	12.05	6.88	0.039	0.53 (0.28-0.98)	7.43	0.115	0.57 (0.28-1.15)
c5EF-144	13.08	7.34	0.031	0.53 (0.29-0.95)	8.78	0.171	0.64 (0.34-1.22)
c5EF-142	13.08	7.34	0.031	0.53 (0.29-0.95)	8.78	0.171	0.64 (0.34-1.22)
c5EF1038	14.36	6.88	0.006	0.45 (0.25-0.81)	10	0.182	0.66 (0.36-1.22)

MAF - minor allele frequency; OR - odds ratio; CI - confidence interval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B
LHB
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CGB ....G......–................
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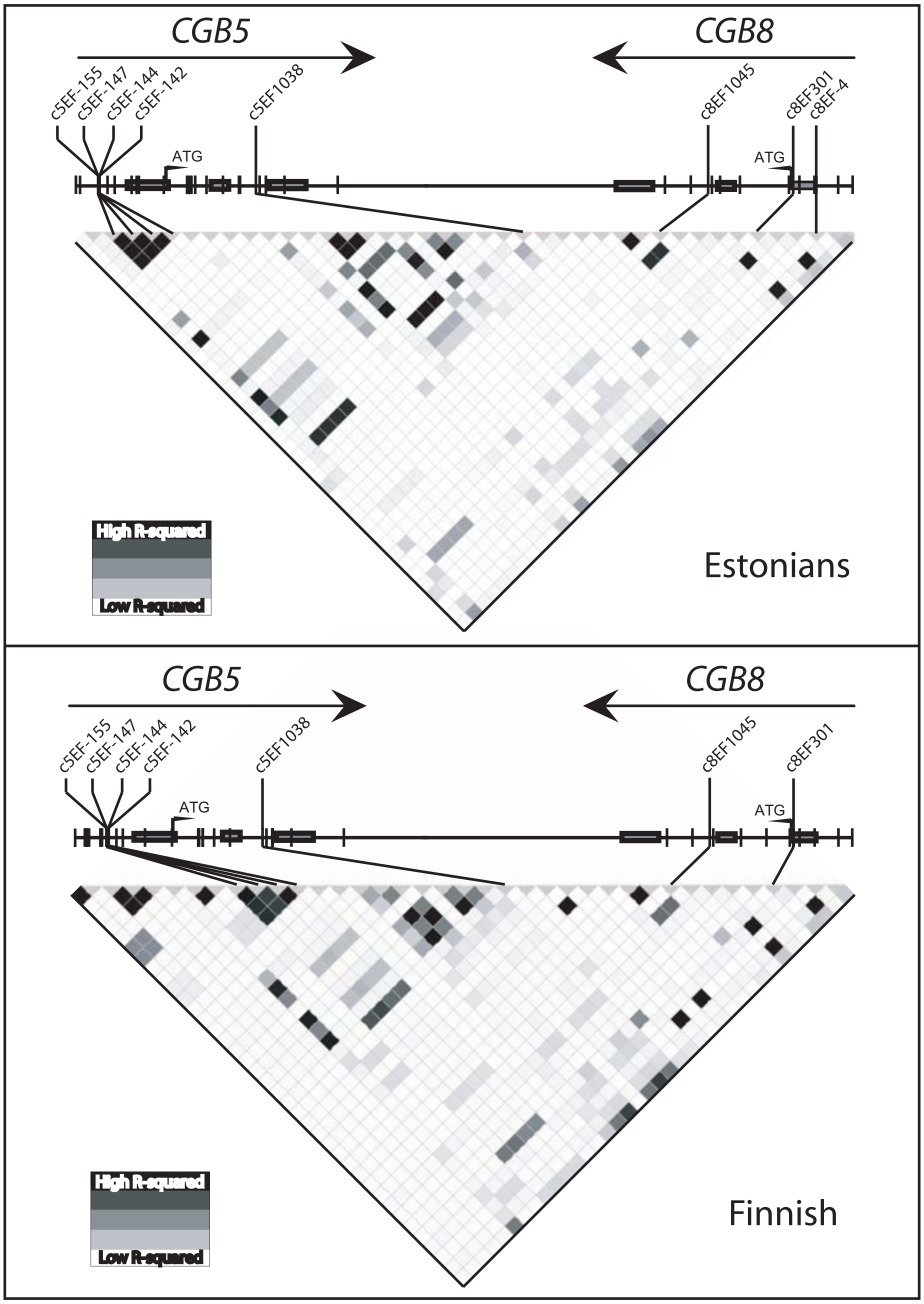
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ATG
ATG
High R-squared
Low R-squared
Estonians
CGB5
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c5EF-155
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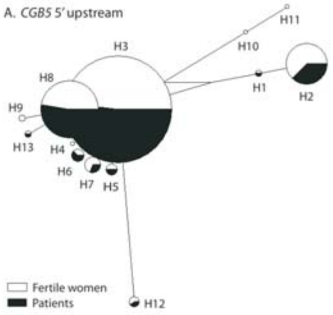
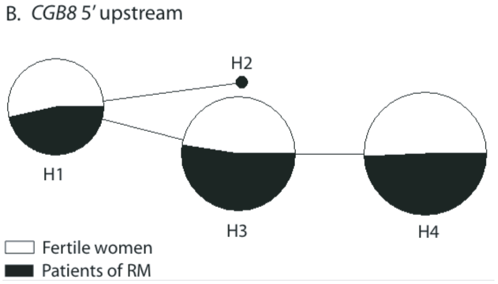


A. *CGB5* 5′ upstream



B. *CGB8* 5′ upstream