The haplotype of the growth-differentiation factor 15 gene is associated with left ventricular hypertrophy in human essential hypertension

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ABSTRACT

GDF15 (growth-differentiation factor 15) is a novel antihypertrophic factor which is induced in the heart in response to pressure overload and plays an important regulatory role in the process of hypertrophy. In the present study, we have investigated the relationship between GDF15 gene variants and left ventricular hypertrophy in human essential hypertension. A community-based hypertensive population sample of 1527 individuals (506 men and 1021 women) was genotyped for three GDF15 genetic variants, including one tag variant \(-3148C>G\) (rs4808793) and two exonic variants \(+157A>T\) (rs1059369) and \(+2438C>G\) (rs1058587). The effects of those variants on gene expression were studied by use of luciferase reporter assays and the determination of plasma GDF15 levels. Only the tag variant \(-3148G\) was significantly associated with a lower risk of left ventricular hypertrophy \(\text{odds ratio} = 0.75\) (95% confidence interval, 0.63–0.89; \(P = 0.0009\)). Multiple regression analyses confirmed that \(-3148G\) predicted the decrease in left ventricular end-diastolic diameter \((\beta = -0.10, P = 0.0001)\), end-systolic diameter \((\beta = -0.09, P = 0.0007)\), mass \((\beta = -0.11, P < 0.0001)\) and indexed mass \((\beta = -0.12, P < 0.0001)\). These effects were independent of conventional factors, including gender, age, body surface area, blood pressure, diabetes, cigarette smoking and alcohol consumption. The transcription activity of the \(-3148G\)-containing construct was increased 1.45-fold \((P = 0.015)\) at baseline and 1.73-fold \((P = 0.008)\) after stimulation with phenylephrine when compared with the \(-3148C\) construct. The \(-3148G\) allele was also associated with a significant increase in the plasma GDF15 level in hypertensive subjects \((P = 0.04)\). In conclusion, the results show that a promoter haplotype containing the \(-3148G\) variant increases GDF15 transcription activity and is associated with favourable left ventricular remodelling in human essential hypertension.

Key words: blood pressure, cardiac remodelling, growth-differentiation factor 15 (GDF15), hypertension, hypertrophy, pressure overload.

Abbreviations: ACS, acute coronary syndrome; ANF, atrial natriuretic factor; BP, blood pressure; BSA, body surface area; C/EBPγ, CCAAT/enhancer-binding protein; CHB, Chinese Han in Beijing; CI, confidence interval; DBP, diastolic BP; DMEM, Dulbecco’s modified Eagle’s medium; GDF15, growth-differentiation factor 15; IVST, interventricular septal thickness; LD, linkage disequilibrium; LV, left ventricular; LVH, LV hypertrophy; LVIDD, LV internal dimension at end-diastole; LVISD, LV internal dimension at end-systole; LVM, LV mass; LVM_{BSA}, LV indexed to BSA; NCS, newborn calf serum; OR, odds ratio; PE, phenylephrine; PWT, posterior wall thicknesses; RWT, relative wall thickness; SBP, systolic BP; TGFβ, transforming growth factor β.

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INTRODUCTION

LVH [LV (left ventricular) hypertrophy] is a strong independent risk factor for cardiovascular morbidity and all-cause mortality [1,2]. The development of LVH is dependent on genetic predisposition and the accumulation of risk factors, including BP (blood pressure), age, gender, lifestyle and diabetes [3]. BP can only account for approx. 50% of the increase in LVM (LV mass) in hypertensive patients [4]. Much of the BP-independent variation in cardiac mass may be attributable to inherited genetic causes [5,6].

GDF15 (growth-differentiation factor 15) is a distant member of the TGFβ (transforming growth factor β) superfamily. GDF15 was originally found to be involved in tumour progression and invasiveness [7], but growing evidence has shown that GDF15 is also a protective factor in response to cardiovascular injury [8,9]. Although baseline expression of GDF15 is negligible in the heart, it is up-regulated rapidly in the myocardium following the pathological or environmental stress [8,9]. After being secreted as a disulfide-linked dimeric protein, GDF15 suppresses the development of maladaptive hypertrophy and limits myocardial ischaemia/reperfusion injury [8,9]. Furthermore, the elevated concentration of GDF15 in plasma has been associated with an increase in the risk of developing future cardiovascular events in women [10], and an increase in the risk of death in patients with chronic heart failure and non-ST-elevation ACS (acute coronary syndrome) [11–13]. The involvement of GDF15 in cardiac regulation makes it a new target factor in response to cardiovascular injury [8,9]. Although evidence has shown that GDF15 is also a protective factor in tumour progression and invasiveness [7], but growing evidence has shown that GDF15 is also a protective factor in response to cardiovascular injury [8,9].

Given the rapid change in GDF15 expression levels in response to pressure overload and the important role of GDF15 in the regulation of cardiac remodelling, we hypothesized that genetic variants which influence the expression and function of GDF15 may confer the susceptibility to LVH and cardiac size in human hypertension. To test the hypothesis, we investigated the potential relationship of genetic variants of GDF15 with LVH and LVM in a community-based hypertensive population.

MATERIALS AND METHODS

Study population

The study subjects were recruited from a community-based cross-sectional study, which investigated the prevalence and risk factors of LVH in a Chinese hypertensive population. The present study was conducted in Xinyang County, a central region in China from March 2005 to August 2005. The study was approved by the Institutional Review Boards of the participating hospitals and the Ministry of Science and Technology of China. All subjects who participated in the study provided written informed consent.

BP was measured by trained professionals with a standardized mercury sphygmomanometer. Two readings were recorded at least 30 s apart, after a minimum of 5 min of rest, in the sitting position on the subject’s right upper arm. The average was used for analysis. Hypertension was defined as an SBP (systolic BP) ≥140 mmHg or a DBP (diastolic BP) ≥90 mmHg, or the use of antihypertensive medication. In total, 15835 subjects of both sexes were invited to participate in the study from 60 rural communities; of those, 13444 subjects (5270 men and 8174 women) completed the survey, and 5440 patients had essential hypertension (40.5%). Among the hypertensive patients, 4869 underwent echocardiography (89.5%).

A total of 25 communities were randomly selected from the 60 communities for this association study. All hypertensive patients with detailed echocardiography determination were enrolled. Patients were excluded if they took antihypertensive drugs, or had valvular heart disease, pulmonary hypertension or coronary heart disease. Eventually, 1527 subjects were recruited, 506 men and 1021 women.

Echocardiographic measurement

M-dimensional and bi-dimensional echocardiography was performed using commercially available instruments [HP 5500 (Phillips Medical System); or HDI 3000 (ATL)]. Three physician-echocardiographers who were blinded to the patients’ genotypes supervised the examination. Measurements included LVIDD and LVISD (LV internal dimension at end-diastole and end-systole respectively), IVST (interventricular septal thickness), and PWT (posterior wall thicknesses) in diastole up to three cardiac cycles, in accordance with methods outlined by the American Society of Echocardiography [14]. Two cardiologists analysed each recording and the mean values used were the LVM. LVM was calculated using the equation:

\[
LVM(g) = 0.8 \times [1.04 \times (LVIDD + IVST + PWT) - LVIDD)^3] + 0.6 [15].
\]

Intra-and inter-observer coefficients of variation for LVM were 11–13% respectively. LVM was indexed to BSA (body surface area) to obtain LVM_{BSA}. LVH was defined using the partition values of LVM_{BSA} > 115 g/m^2 for men and > 96 g/m^2 for women [16]. RWT (relative wall thickness) was calculated as 2 × the PWT/LVIDD ratio [17].

Clinical data collection

All the participants completed a questionnaire on current medication and lifestyle. A blood sample was taken from all participants after overnight fasting. Plasma and urine biochemical variables were determined by using...
standard methods on an automatic analyser (Hitachi 7060). Diabetes mellitus was diagnosed when the subject had a fasting glucose > 7.0 mmol/l or was taking medication for diabetes [18]. Smoking was defined as a history of smoking > 2 pack-years and/or smoking in the last year. Alcohol consumption was defined as continuously drinking for 1 year with the frequency of at least once per week.

Selection of the GDF15 variants
The genetic architecture of the GDF15 gene and its flanking regions (9.0 kb in size) were analysed by using the pairwise method of the Haploview version of the Tagger program in the HapMap CHB (Chinese Han in Beijing) database [19]. There is a strong LD (linkage disequilibrium) block in the promoter region, including five variants: rs12459566 (located at position −3690T > C, relative to the transcription start site), rs4808793 (−3148C > G), rs8101804 (−862C > T) and rs12459782 (−474C > T) in the promoter region, and rs1059519 (+56C > G) in exon 1 (Figure 1). The natural haplotype block of the promoter region was confirmed by sequencing the 3.9-kb 5′ upstream promoter region and 100 bp of exon 1 in 30 randomly selected subjects. All five variants were in strong LD (D′ > 0.9 and r² > 0.9). Therefore any one of the five variants could reflect the natural haplotype block of GDF15 (T-C-C-C-C and C-G-T-T-G). The variant −3148C > G (rs4808793) was selected for the following genotyping assay because there is a natural BsrI digestion site within the sequence.

Two other potential functional genetic variants, including the exonic variants rs1059369 (+157A > T, in exon 1) and rs1058587 (+2438C > G, in exon 2), were
also selected for genotyping. These variations result in non-synonymous amino acid changes at residue 48T > S in the precursor protein and 6H > D in the mature protein of GDF15 respectively. The variant rs1059369 was in complete LD with variant rs1804826 ($D' = 1, r^2 = 1$) according to the HapMap CHB data.

**Genotyping**
Genomic DNA was extracted from cellular buffy coat. The three selected polymorphisms, $-3148C > G$ (rs4808793), +157A > T (rs1059369) and +2438C > G (rs1058587), were genotyped in all 1527 subjects by PCR-RFLP (restriction-fragment-length polymorphism). The conditions for the PCR are shown in Supplementary Table S1 (available at http://www.ClinSci.org/cs/118/cs1180137add.htm). Reproducibility of genotyping was confirmed by bidirectional sequencing in 100 randomly selected samples and the reproducibility was 100%.

**Luciferase reporter constructions**
The 1138 bp fragments (from −1061 to +77) were amplified from genomic DNA with the forward primer 5′-CCGCCCTCGAGCATGTAATCCCACCACCA-AG-3′ (containing a XhoI restriction site underlined) and the reverse primer 5′-CCGCAAGCTTAGCAGGACACCAGC-AAACCAGG-3′ (containing a HindIII restriction site underlined). The resultant PCR products were double-digested with XhoI/HindIII and cloned into the multiple cloning sites of pGL3-basic vector (Promega). The vector containing the genotype C-C-C (rs8101804-rs12459782-rs1059519) was designated as pGL3-GDF15-wild and the vector containing the genotype T-T-G (rs4808793-rs1058587-rs1059369) was designated as pGL3-GDF15-mutant (Figure 1). The GDF15 promoter sequences in both vectors were confirmed by direct sequencing.

**DNA transfection and luciferase assay**
Primary cultures of cardiac myocytes were prepared from ventricles of 1–2-day-old neonatal Sprague–Dawley rat hearts by a method reported previously [20]. After separation from non-myocyte fibroblasts through pre-plating, myocytes were replated at a density of 0.5 × 10^4 in 24-well plates. Following 48 h of culture in DMEM (Dulbecco’s modified Eagle’s medium; Life Technologies) containing 10% (v/v) FBS (fetal calf serum; Hyclone), cardiomyocytes were grown in DMEM (Dulbecco’s modified Eagle’s medium; Life Technologies) containing 10% (v/v) NCS (newborn calf serum) for 24 h. Luciferase activity was determined and normalized to the pRL-null luciferase activity using the Dual Luciferase assay kit (Promega). Three independent experiments were performed in triplicate.

**Determination of genotype-dependent plasma levels of GDF15**
The plasma level of GDF15 antigen was determined in 136 hypertensive patients (43 males) randomly selected from the recruited subjects. To minimize any potential confounding effects, the subjects were recruited only when they met the following criteria: (i) 40–65 years of age; (ii) SBP ≥160 mmHg and/or DBP ≥100 mmHg; (iii) using neither antihypertensive medication nor NSAIDs (non-steroidal anti-inflammatory drugs); and (iv) no history of diabetes mellitus. The variant $-3148C > G$ (rs4808793) was genotyped in all patients. Of the patients, 60 were homozygous for the C allele; 64 were heterozygous for the G allele and 12 were homozygous for the G allele. The GDF15 concentrations were determined twice by using an ELISA kit (R&D Systems) as described previously [21].

**Statistical analysis**
Results are presented as percentages, medians (25–75th percentiles) or means (S.E.M.), as indicated. A χ2 test was applied to compare qualitative variables and the Hardy–Weinberg equilibrium. Differences in GDF15 plasma levels were analysed by the non-parametric Mann–Whitney test between two groups, or the Kruskal–Wallis test among distinct groups. Other quantitative variables were tested with a Student’s t test or one-way ANOVA. The associations of the quantitative echocardiography variables with genetic variants were detected by multiple regression analyses adjusted for age, gender, BSA, SBP, DBP, cigarette smoking, alcohol consumption and diabetes status. The ‘gene–dosage effect’ of the positively associated variants was tested by linear regression, based on the number of polymorphic alleles (0, 1 or 2). Stepwise multiple linear regression analysis was used to examine whether the number of variant alleles carried by each subject had statistical influence on LV diameters and LVM, independent of the conventional covariates mentioned above. The α level for entry and removal of terms at each forward step was 0.10. Multiple testing was adjusted by using the Bonferroni correction. Two-tailed values of $P < 0.05$ were considered statistically significant. Analyses were performed with SPSS 13.0 for Windows.

**RESULTS**
The genotype frequencies for all the three GDF15 variants were in accordance with the Hardy–Weinberg equilibrium. No LD was found between any pairwise analysis among the three selected variants. As shown in Table 1, only $-3148C > G$ was found to be
Table 1 Association of the three variants of GDF15 studied with LVH in the study population

ORs were calculated with $2 \times 3$ or $2 \times 2$ cross-tabulation.

(A) $-3148C > G$ (rs4808793)

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype (n)</th>
<th>Allele (n)</th>
<th>P value</th>
<th>C</th>
<th>G</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>With LVH</td>
<td>318 (58.9%)</td>
<td>189 (35.0%)</td>
<td>33 (6.1%)</td>
<td>0.005</td>
<td>835 (76.6%)</td>
<td>255 (23.4%)</td>
<td>0.75 (0.63–0.89)</td>
</tr>
<tr>
<td>Without LVH</td>
<td>497 (50.4%)</td>
<td>408 (41.3%)</td>
<td>82 (8.3%)</td>
<td></td>
<td>1402 (71.0%)</td>
<td>572 (29.0%)</td>
<td></td>
</tr>
</tbody>
</table>

(B) $+157A > T$ (rs1059369)

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype (n)</th>
<th>Allele (n)</th>
<th>P value</th>
<th>A</th>
<th>T</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>With LVH</td>
<td>253 (46.9%)</td>
<td>227 (42.0%)</td>
<td>60 (11.1%)</td>
<td>0.109</td>
<td>733 (67.9%)</td>
<td>347 (32.1%)</td>
<td>1.113 (0.95–1.30)</td>
</tr>
<tr>
<td>Without LVH</td>
<td>412 (41.7%)</td>
<td>469 (47.5%)</td>
<td>106 (10.7%)</td>
<td></td>
<td>1293 (65.5%)</td>
<td>681 (34.5%)</td>
<td></td>
</tr>
</tbody>
</table>

(C) $+2438C > G$ (rs1058587)

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype (n)</th>
<th>Allele (n)</th>
<th>P value</th>
<th>C</th>
<th>G</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>With LVH</td>
<td>286 (53.0%)</td>
<td>214 (39.6%)</td>
<td>40 (7.4%)</td>
<td>0.203</td>
<td>786 (72.8%)</td>
<td>294 (27.2%)</td>
<td>1.162 (0.99–1.37)</td>
</tr>
<tr>
<td>Without LVH</td>
<td>482 (48.8%)</td>
<td>412 (41.7%)</td>
<td>93 (9.4%)</td>
<td></td>
<td>1376 (69.7%)</td>
<td>598 (30.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Significantly associated with LVH ($P = 0.005$). The C allele of the $-3148$ locus was more frequently identified in individuals with LVH, whereas the G allele was more often found in individuals without LVH ($OR (odds ratio), 0.75 [95% CI (confidence interval) 0.63–0.89]; P = 0.0009$). The difference was still statistically significant after the Bonferroni correction ($P < 0.017$; i.e. $0.05/3$), suggesting the minor allele $-3148G$ may be a ‘protective’ allele.

Table 2 shows the clinical characteristics according to the three genotypes of $-3148C > G$. Among the anthropometric and biochemical variables, DBP decreased significantly with an increase in G alleles ($P = 0.015$). With respect to the echocardiographic parameters, the $-3148C > G$ genotype was associated with LVIDD, LVIDS, LVM and LVMBSA in the whole sample. The association remained, revealing remarkably low $P$ values, after correction for other conventional factors that might influence cardiac size, such as age, gender, BSA, SBP, DBP, cigarette smoking, alcohol consumption and diabetes status (Table 3). The coefficients of linear regression of $-3148$ and each cardiac variable on the number of G alleles were all statistically significant ($P < 0.0001$), suggesting that $-3148G$ had a ‘gene–dosage’ or ‘addictive’ effect on these variables.

Stepwise multiple regression analyses showed that, of all of the variables that might influence cardiac size, $-3148G$ was one of the two (the other is BSA) most important independent predictors of LV diameters, non-indexed and indexed LVM (Table 3).

As the G allele of $-3148$ reflects the C-G-T-T-G ($-3690/-3148/-862/-474/+56$) haplotype of the GDF15 gene, C-G-T-G might be a protective haplotype against cardiac remodelling in hypertension. To test whether the variants affected the transcriptional activity of the GDF15 minimal promoter ($-1061$ to $+77$, relative to the transcript start site), the mutant haplotype (T-T-G) and the wild-type haplotype (C-C-C) were transfected into neonatal rat cardiomyocytes. As shown in Figure 1, the promoter activity of mutant promoter was 1.45-fold higher ($P = 0.015$) than that of the wild-type construct at baseline, and 1.73-fold higher ($P = 0.008$) after the stimulation with PE. Moreover, PE significantly increased luciferase activity of the GDF15 mutant promoter in transfected cardiomyocytes ($P = 0.02$), but failed to increase the activity of wild-type promoter. These results suggest that the mutant haplotype promoter was more sensitive to the hypertrophic stimulation and had higher transcription activity than the wild-type haplotype promoter.

To investigate further the expression change induced by the variant in the promoter, the plasma concentrations of GDF15 were determined in hypertensive patients (Figure 2). The $-3148G$ allele carriers had a significant increase in GDF15 levels ($P = 0.015$) that of the wild-type construct at baseline, and 0.73-fold higher ($P = 0.008$) after the stimulation with PE. Moreover, PE significantly increased luciferase activity of the GDF15 mutant promoter in transfected cardiomyocytes ($P = 0.02$), but failed to increase the activity of wild-type promoter. These results suggest that the mutant haplotype promoter was more sensitive to the hypertrophic stimulation and had higher transcription activity than the wild-type haplotype promoter.
consistent with those from the luciferase activity study.

DISCUSSION

The results of the present study show that a GDF15 haplotype, which significantly increases the promoter activity and consequently the plasma level of GDF15, was associated with a decrease in LVIDD, LVISD, LVM and LVMBSA in hypertensive patients. The effects were independent of BP levels, age, gender and other clinical factors that influence cardiac size. To the best of our knowledge, this is the first demonstration that genetic variants of GDF15 are associated with favourable cardiac hypertrophy in human essential hypertension.

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GDF15 has been identified as an antihypertrophic endocrine factor [9]. It is barely detectable in the heart at baseline, but is up-regulated rapidly following pressure overload. The increase in GDF15 levels might be an intrinsic protective mechanism that counter-regulates the hypertrophic stimuli. In our present study, we found that rs4808793 (−3148C>G) was associated with an increase in GDF15 promoter activity and circulating GDF15 protein levels. Furthermore, −3148G was negatively associated with LVM in both quantitative and dichotomized phenotypes. It is noteworthy that the significant decrease in LVM and LVM_BSA with the number of −3148G alleles are contributed to by decreased ventricular diameters, rather than differences in wall thickness. This is consistent with the putative role of GDF15 in modifying cardiac mass in animal models. It has been shown that Gdf15-overexpressing mice with a low- or medium-expression level have normal LV thickness, but significantly a smaller LV chamber compared with the wild-type mouse. Conversely, Gdf15 gene-targeted mice have larger L V dimensions after being normalized to body weight [9]. Therefore one can speculate that, in response to hypertension, the haplotype of the GDF15 transcription regulatory region might promote the GDF15 antihypertrophic effects by increasing the protein level.

It has been reported that increased levels of circulating GDF15 are associated with an increased risk of death in patients with non-ST-elevation ACS and chronic heart failure [11,12]. However, the high GDF15 level is unlikely to be a cause of these cardiovascular events, because overexpression of GDF15 by injection of recombinant GDF15 protein has been shown to attenuate heart failure in mlp (muscle lim protein) gene-targeted mice [9]. As pathological cardiac hypertrophy and heart failure are continuously progressive, the protective effect of the GDF15 haplotype on LVH might only function within a given stage in hypertensive patients. At an early stage, the GDF15 plasma level is elevated primarily due to promoter activation and plays an antihypertrophic role; however, if the haemodynamic stress persists, this counter-regulatory role would be limited and unable to provide protection. Indeed, the protective role played by GDF15 in the heart is reminiscent of ANF (atrial natriuretic factor). Although the ANF level is strongly correlated with the degree of ventricular dysfunction and eventual mortality, hypertensive patients carrying the ANF gene promoter variant, which is significantly associated with lower plasma pro-ANF, have an increased LVM [22].

The promoter haplotype is located in the main 5’ region of GDF15 gene. This region contains the binding sites for the important transcriptional factors that regulate the expression of GDF15, such as p53, Sp1 and Sp3 [23–26]. Indeed, the variants −862 and −474 are both located immediately adjacent to the potential binding sites for p53, which is essential for the induction of GDF15 expression in tumours [24,25]. Moreover, analysis of these sequences, using the MAPPER database (http://bio.chip.org/mapper/), predicts that the variant −862T introduces a novel consensus eukaryotic transcription factor C/EBPγ (CCAAT/enhancer-binding protein γ)-binding site [27]. C/EBPγ has been reported to function as an activator and modulate the expression of several genes [28,29]. Therefore it is possible that any of the five variants in the LD block could regulate the expression of GDF15 in the response to hypertension via altering the interactive effects of the transcription factors. These need to be elucidated in future studies.

Neither exonic variants +157A>T nor +2438C>G were associated with LVM or the concentration of GDF15 (results not shown), consistent with a previous study which reports that variant H6D (now called +2438C>G) is not associated with the subsequent risk of cardiovascular events and GDF15 plasma level in women [10]. It is more likely that the cardiac protective role of GDF15 is mainly restricted to the promoter variants which regulate gene expression, rather than those which alter the protein structure or function.

The strengths of our present study include that the clinical relevance of the GDF15 promoter haplotype was explored in a community-based hypertensive population, and the functional change was confirmed in vitro and in vivo. As our hypertensive patients reside in the same area and are of the same nationality, the possibility of population stratification is low [30]. Nevertheless, our findings should be confirmed in other independent hypertensive cohorts and explored in the general population. As only the promoter region was sequenced, the information of additional GDF15 genetic
variants is insufficient. Moreover, the plasma GDF15 level should be measured in the general population and the triangular relationship between BP, the GDF15 level and LVH should be assessed. Considering the powerful ability of LVH to predict morbidity and mortality, it is necessary to perform a longitudinal follow-up study in the future to elucidate whether there are protective effects of GDF15 polymorphisms on heart failure.

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Table S1 Description of the GDF15 variants, primers and restriction enzymes

<table>
<thead>
<tr>
<th>Variant</th>
<th>Primer</th>
<th>PCR product (bp)</th>
<th>Tm (°C)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3148C &gt; G forward, 5'-AGTGAGTCCTTGTGTCTCTTAC-3'</td>
<td>251</td>
<td>58</td>
<td>BsrI</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Reverse, 5'-GCAGGCTGGTGTAGAGTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+157A &gt; T forward, 5'-GGCTGCTGGGGGTGGGAG-3'</td>
<td>209</td>
<td>61</td>
<td>BsrGI</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Reverse, 5'-GCAAGTTTCTCGGGACCCTCAGAGTTGTAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+2438C &gt; G forward, 5'-GCAGAATCTTCGTCCGCAC-3'</td>
<td>165</td>
<td>62</td>
<td>AvaII</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Reverse, 5'-CCAGCCCAGGTCTTCCAG-3'</td>
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</table>

¹ These two authors contributed equally to the work.

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