Author's response to reviews

Title: Protective effect of a rare genetic variant in the hypoxia inducible factor-1 alpha gene on type 1 and type 2 diabetes

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Author's response to reviews: see over
Dear Dr Erik Alexandersson,

Please find enclosed the revised version of MS: 1519522404259209 manuscript (original title: Protective effect of a rare genetic variant in the hypoxia inducible factor-1 alpha gene on type 1 and type 2 diabetes)

The original title has been changed as a consequence of the reviewer’s opinion. The new title is: “Association of hypoxia inducible factor-1 alpha gene polymorphism with both type 1 and type 2 diabetes in a Caucasian (Hungarian) sample”.

We express our appreciation to our Reviewers for their suggestions. We agree with all of the major and minor comments, they have been considered and incorporated into the revised manuscript.

A Competing interests section has been included between the Conclusions and Authors contributions section.


Maria Sasvari-Szekely
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Answer to Reviewer #1 (George N Goulielmos):
We thank the reviewer for finding our manuscript interesting, clear and suitable for publication as well as the appreciation of our double genotyping methodology. We have been using this approach from the very beginning of our molecular genetic studies, as we have seen many errors in published genotyping protocols resulting in an overestimation of one of the alleles and/or deviations from the Hardy Weinberg equilibrium. Therefore we believe that a strong control on the genotyping protocols has a vital importance in reliable association studies.

The revised manuscript incorporated both minor comments of the reviewer, as follows:

“1) A positive point of the article is that it presents some assumptions about the putative role of the gene polymorphism analyzed in the pathogenesis of diabetes. However, the inability of Hlatky et al (2007) and Nagy et al (this paper) to replicate former results presented by Yamada et al (2005) dealing with the HIF-1 alpha transcriptional activity make me a bit concerned as regards with the real functional significance of the polymorphism under study. Thus, I would avoid characterizing as “protective” the effect of this polymorphism; I would prefer the term “non-predisposing” rather than protective, given that this is still in fact one issue of debate in the literature, in case that alleles are found significantly more prevalent in controls than in patients.”

Answer:
We agree that results are contradictory on the functional role of rs11549465 SNP in HIF-1α gene, therefore the title of the manuscript needs revision.
The original title was: "Protective effect of a rare genetic variant in the hypoxia inducible factor-1 alpha gene on type 1 and type 2 diabetes"
The revised title is: "Association of hypoxia inducible factor-1 alpha gene polymorphism with both type 1 and type 2 diabetes in a Caucasian (Hungarian) sample."

Up till now only the rs11549465 SNP has been investigated in association studies between the HIF-1α gene and diabetes, as this is as exon polymorphisms hypothetically effecting the function of the protein. There are however, numerous other SNPs in strong linkage with the rs11549465 which also might play an important role in this effect. Therefore, in the light of the reviewer’s suggestion we omitted the expression of “protective effect” from the title, as well as the name of the SNP, emphasizing the association between the gene and the disease. Here we studied only the rs11549465 SNP of HIF-1α gene as our primary goal was the replication of previous findings in a Caucasian sample. Our data show a clear replication of association between the HIF-1α gene variants and diabetes, described previously by Yamada et al (2005) in a Japanese population. In addition, we demonstrated that the HIF-1α gene polymorphism is associated not only to T2DM but also to T1DM.
Replication of association results in independent populations is of vital importance. Therefore our new title shows that this work was done on a Caucasian (Hungarian) sample. We believe that the revised title reflects the main content of the manuscript more precisely.

“2) I recommend a first approach to be done regarding the plausible role of the mutation analyzed in the conformation of the protein. This may give some explanations dealing with the functional significance of this SNP.”

Answer:
We agree with the reviewer that the approach of a gene polymorphism from the view of protein conformational is a very important issue. Unfortunately, our knowledge on the functional effects of the studied HIF-1α SNP is contradictory in the literature at the moment.
Nevertheless, we tried to give a more detailed review of the molecular-structural background in both the “Background” and the “Discussion” sections of the revised manuscript.

As the HIF1 protein has been extensively investigated thus its amino acid sequence, regulatory domains, amino acid hydroxylation sites, etc., are well documented in the literature, we inserted a more detailed description of this knowledge into the “Background” section of the revised manuscript:

“The HIF-1α protein contains five functional domains. The basic helix–loop–helix (bHLH) domain is specifically required for the binding of DNA [17], the Per/Arnt/Sim (PAS) domain is involved in dimerisation of the α and β subunits. Transcriptional activation and interaction with coactivators are mediated by two transactivation domains in the C terminal half of HIF-1α, termed as N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains [18]. Negative regulation of HIF-1α under normoxic conditions occurs via the oxygen-dependent degradation (ODD) domain, which partly overlaps with N-TAD.[19, 20] Under normoxic conditions, HIF-1α is hydroxylated on proline residues (P402, P564) by a family of oxygen-dependant prolyl hydroxylases which mediate high affinity binding to the von-Hippel-Landau (VHL) protein, a component of the E3 ubiquitin-protein ligase complex that ubiquitinates HIF-1α, thereby targeting it for degradation.[21].”

The HIF-1α polymorphism we have studied is located within exon 12 of the gene, and causes the proline in the 582 amino acid position to change to serine. The P582 is located in a trans activation domain of the HIF protein near to the domain of oxygen dependent degradation, however, it is not a known hydroxylation site. Therefore there are no evidences for a direct role of P582S in changing the degradation of the HIF-1α protein - although, one might consider a conformational change in the HIF protein influencing indirectly the hydroxylation of the prolines. Another possibility was suggested by Tanimoto and colleagues claiming that the conformational change of the protein might enhance the recruitment of transcriptional cofactors. To our best knowledge none of these possibilities have any direct experimental support.

Nevertheless we agree with the reviewer that it is important to discuss the possible mechanism behind the studied genetic variation of HIF-1α protein, as it might generate further investigations to solve the problem. Therefore, we inserted the following paragraph into the “Discussion” section of the revised manuscript.

„The polymorphism investigated in our study causes a proline to serine change in the 582 position which is within the N-TAD near the ODD domain of the HIF-1α protein [37]. Proline 582 has not been proven to be a HIF-1α hydroxylation site, and it is not known whether it mediates VHL binding. Moreover, the serine-proline substitution in this position does not appear to alter VHL binding in vitro to a fragment of HIF-1α after hydroxylation at proline 564 [25].”

„Tanimoto and colleagues suggested that the conformational changes caused by the amino acid substitution either might alter protein stability, or could enhance recruitment of transcriptional cofactors that interact with HIF-1α. Since, the authors could not detect any differences in degradation, the altered transactivational properties was taken into consideration as a possible molecular effect of Pro582Ser change [38]. Further investigations in the field of cancer research demonstrated the rs11549465 variant to have enhanced transcription activities in in-vitro studies under both normoxic and hypoxic conditions [38, 39] associated with increased tumor microvessel density in head and neck cancer, and in prostate cancer. In conclusion, changes in the transactivational properties of the studied genetic variants could be hypothesized, however, their effect probably depends on the specific coactivators of various cell types.”
Reviewer #2 (Yukio Horikawa):

We thank the reviewers for his highly relevant suggestions. Most of them required insertion of further experimental and theoretical details which we fulfilled with pleasure. One figure (Fig 1) was also inserted according to the reviewer’s suggestion.

Note: The title of the paper has been changed according to the other reviewer’s suggestion. The original title was: „Protective effect of a rare genetic variant in the hypoxia inducible factor-1 alpha gene on type 1 and type 2 diabetes” The revised title is: “Association of hypoxia inducible factor-1 alpha gene polymorphism with both type 1 and type 2 diabetes in a Caucasian (Hungarian) sample”.

Major revisions

“1. In Methods section, the authors should show the power estimation for this case-control study of rs 11549465, which has a relatively small sample number. Judging from the genotyping data, the total number of this study is 890, not 900.”

Answer:
- This section has been added to the revised manuscript:
  “G*Power 3.1.0 (Faul et. al, 2007) has been used for computing a post hoc test for achieved power. Input parameters included the population effect size=0.1 (as a conventionally small effect size ), alpha=0.05, df=1 for our 2x2 and df=2 for our 2x3 analyses. Power estimates were 85% for the 2x2 contingency tables and 77% for the 2x3 contingency tables, which are generally considered acceptable (Cohen, 1988).”
  - The total number of participants in the abstract has been corrected from 900 to 890. Sorry for the mistake and thank you for noticing it.
  “2. In Table 2, the authors should show p-values of HWD for genotyping of case and control, respectively. They also should perform logistic regression analyses with adjustment for at least age, sex, and BMI, and show odds ratio values with 95% confidence intervals”.

Answer:
- P-values of the HWD have been added to the “Methods” section of the revised manuscript:
  “Obtained genotype frequencies were compared to the calculated frequencies based on the Hardy-Weinberg equilibrium and no significant differences were observed (P=0.740 for all participants, P=0.444 for the control group, P=0.853 for the total patient group. Within the patient group P=1.000 for the DM1 and P=0.808 for the DM2 groups).”
  - The suggested analyses were performed, methods and results were added to the manuscript:
  “Risks were examined by odds ratios (ORs) with 95% confidence intervals (CIs), using unconditional logistic regression models adjusted for age (years) and sex.”
  „The presented data demonstrated an under representation of the T allele in all patient groups suggesting a protective effect of the T allele against diabetes. It is worth mentioning that – although the numbers are rather small – TT homozygotes are extremely rare among patients (0.6%), while their incidence is more than 4 fold higher in the control group (2.5%). With other words, the C allele is a risk factor for both type 1 or type 2 diabetes (OR=1.56, CI: 1.41-1.71). The above risk estimate was based on unconditional logistic regression adjusted for age (years) and sex using data from a total of 879 participants with all data available (535 patients and 344 controls).”
  - The BMI index was not included in the regression model, due to the lack of sufficient number of data in the control group. However, possible significant effect of the BMI index has been assessed, and added to the results:
„Possible association of the HIF-1α genotypes and age or BMI index has also been tested by using the T-present vs. T-absent genotypes as independent groups. Analyses were performed independently in the control, DM1 and DM2 groups, (data on BMI index of controls was available for only 50 subjects). No significant associations were found, mean age and mean BMI of the tested T-present and T-absent genotype groups were similar (data not shown).“

“3. The authors should show the functional analyses data for the mutant of HIF-1α under either normoxic or hypoxic condition, even though it did not reach statistically significant differences.”

**Answer:**
Thank you for suggesting the detailed description of functional analysis of mutant of HIF-1α which we inserted into the revised manuscript into the “Methods”, “Results” and into the “Discussion” sections (see below). Data inserted (see Fig 1) show the expression assay of HIF-1α allelic variants under normoxia and hypoxia, as well. In the “Discussion” we explain that our negative result might originate from the relatively high endogenous HIF-1α activity in our cell line. We also conclude that a possible source of the contradictory results in the literature on the functional analysis of Pro582Ser lies in the variability of cell lines tested.

**Inserted paragraphs in the “Methods” section:**

**Plasmid constructs**
The pGL3-Control luciferase reporter vector (Promega, Madison, WI) was used as a control. The pHRE vector – which is a modified pGL3-Control plasmid (figure 1A) – containing five contiguous hypoxia responsive elements (5’-GATCTGAGACAGCACGTCAGTGGGC-3’) in front of the luciferase reporter gene, was a generous gift from Dr. M. Geiszt (Institute of Physiology, Semmelweis University, Budapest, Hungary). The wild type and p.P582S mutant HIF-1α expression vectors were a kind gift of Dr. Yukio Horikawa, Department of Diabetes and Endocrinology, Gifu University School of Medicine, Gifu, Japan.

**Cell cultures, hypoxic treatment, transient transfection**
SK-N-FI (neuroblastoma) cell line was grown in Dulbecco’s modified Eagle’s medium, high Glucose (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Normoxic cultures were kept in 21% O2, 74% N2 and 5% CO2 in humidified atmosphere. Hypoxic samples were incubated in a humidified atmosphere of 1% O2, 94% N2 and 5% CO2 in a modular incubator chamber (Billups-Rothenberg, USA). A mixture of 0.1 μg pHRE reporter construct, 0.1 μg HIF-1α expression vector and 0.1 μg pCMV-β-gal and 6 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfect 1.2 × 10⁶ SK-N-FI cells plated 24 hours before transfection in six-well plates. Luciferase and β-galactosidase activities were detected using the Luciferase Assay System kit (Promega, Madison, WI) and by ONPG (O-nitrophenyl-β-D-galactopyranoside) cleavage rate, respectively. Three parallels were used in all transfections and all experiments were performed in triplicates.”

**Inserted paragraphs in the “Results” section:**
“In order to gain more evidence for the functional role of P582S amino acid change, transcriptional activity of the allelic variants were compared in an in vitro reporter gene system (see Figure 1). Five contiguous hypoxia responsive elements (HRE) were inserted into the pGL3-Control plasmid (pGL3-C) for the assay of HIF-1α transcriptional activity (see Figure 1. A). Two allelic forms (wt = wild type, p.P582S= rare variant) of Hif1 α expression vector were cotransfected with pHRE in separate experiments using SK-N-FI (neuroblastoma) cell line. Relative Hif1 α activity was measured in cell extracts as a ratio of
luciferase activity and the β-galactosidase activity applied as transfection control. The obtained results were normalized for the low activity of “empty” vector (pGL3-C) under normoxia, and labeled on Figure 1 as relative luciferase activity. As expected, a 2-3 fold Hif1α transcriptional activity was measured under hypoxia (filled columns) compared to normoxia (open columns), except in case of “empty” vector. No significant differences were found between allelic variants (pHRE + wt and pHRE + p.P582S) either under normoxia or under hypoxia in our conditions. It should be noted, however, that the endogenous Hif-1α activity was relatively high in our system, as measured by pHRE without cotransfection of any of the allelic variants.”

New section: LEGENDS TO FIGURES

“Figure 1. The effect of the p.P582S mutation on the binding affinity of the HIF-1α to hypoxia responsive element (HRE) in SK-N-FI cells. A. Schematic description of pHRE vector, which is a modified pGL3-Control plasmid, containing five HIF-1α binding sites (HRE) in front of the SV40 promoter and the luciferase reporter gene. B. No significant difference could be detected between the transcriptional activities of the pHRE constructs co-transfected with either wild type or p.P582S mutant HIF-1α, neither in normoxic nor under hypoxic conditions. Luciferase activity was normalized to the β-galactosidase activity. Data are presented as fold increments over the normoxic pGL3-Control activity and shown as mean ± SD. Results of a representative experiment are shown as measured in triplicates. Similar data were obtained from three independent transfection experiments.”

Figure 1.

Inserted paragraphs in the “Discussion” section:

“We also attempted to demonstrate the functional importance of HIF-1α variants, however, we did not find any significant differences in the transcriptional activity of the HIF-
1α variants using a luciferase reporter system (see Figure 1). One possible reason of this contradiction might be a relatively high endogenous HIF-1α activity in our cell line as measured in the presence of the luciferase vector with hypoxia responsive elements (Figure 1, pHRE) in the absence of any HIF-1α expression vector.

Tanimoto and colleagues suggested that the conformational changes caused by the amino acid substitution either might alter protein stability, or could enhance recruitment of transcriptional cofactors that interact with HIF-1α. Since, the authors could not detect any differences in degradation, the altered transactivational properties was taken into consideration as a possible molecular effect of Pro582Ser change [38]. Further investigations in the field of cancer research demonstrated the rs11549465 variant to have enhanced transcription activities in in-vitro studies under both normoxic and hypoxic conditions [38, 39] associated with increased tumor microvessel density in head and neck cancer, and in prostate cancer. In conclusion, changes in the transactivational properties of the studied genetic variants could be hypothesized, however, their effect probably depends on the specific coactivators of various cell types.”

Minor revisions

1. In Methods section, rather than Abstract, the authors should mention the name of the enzyme used for genotyping.”

Answer:

We omitted the name of the enzyme from the abstract. The “Method” section included the name of the enzyme in the original form therefore it remained unchanged in the revised version.

2. The authors should mention whether any polymorphisms other than that of the HIF-1α gene is reported to be associated with both type 1 and type 2 diabetes.

Answer:

Thank you for pointing out the lack of details in discussing all available data on common genetic factors of type 1 and type 2 diabetes. Actually, there are not too many studies on this field, and according to our best knowledge all the previous findings have been shortly referred to in the conclusions part of the first version. We fully agree, however, that this is a very important issue in this paper. Therefore, we inserted a more accurate list of the common gene variants, as well as a more detailed discussion part to emphasize these previous findings, as follows:

„For example, the common variant of the peroxisome proliferator activated receptor γ gene isoform 2 (PPARγ2) Pro12Ala that has been consistently reported to associate with T2DM was recently shown to be associated with T1DM, as well [35]. Moreover, Galanakis and coworkers just recently have shown that the intron 4 a/b polymorphism of the endothelial nitric oxide synthase gene (eNOS) is associated with both type 1 and type 2 diabetes [36].”