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Delphine Fradin, Clémence Mille, Nadia Naoui, Chris Groves, Mark Mccarthy, Samy Hadjadj, François Paeou, Pierre Bougnères

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Allele Dependent DNA Methylation of the Human Insulin Gene Promoter in T2D patients and Controls.

Introduction

Numerous SNPs located at various gene loci related to insulin secretion have been identified by GWAS of T2D or of insulin-related traits (review in Bonnefond et al. 2010). Genetics however seems to explain yet a limited part of these phenotypes (Wheeler et al. 2011). Non genetic factors are certainly of utmost importance, and possibly include epigenetic factors at the interface of genetics and environment (Chen et al. 2011).

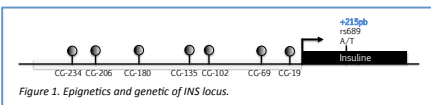
Genotype dependent variations of neighboring epigenotype have been reported at several other loci across the genome and called ASM (Allele Specific DNA Methylation). Shoemaker et al. showed that 23% to 37% of heterozygous SNPs are associated with epigenetic variation in several cell line (Shoemaker et al. 2010) while Zhang et al. found that ASM is likely to affect 10% of all human genes (Zhang et al. 2009). The finding of a link between genetic variants and epigenomic marks may have major consequences for a better understanding of the "missing heritability".

Several studies have established a significant association between the class III VNTR, in complete linkage disequilibrium with the allele T of rs689, and T2D (On et al. 1999, Permutt et al. 1990).

Previous studies have found that *INS* gene expression is regulated by epigenetic mechanisms (Chakrabarti et al. 2003, Mutskov et al. 2007 et 2009) such as DNA methylation (Kuorda et al. 2009, Yang et al. 2010). We also found an association between DNA methylation of *INS* promoter and Type 1 Diabetes (Fradin et al. 2012).

The *INS* proximal promoter encompasses 7 CpGs located at position -234, -206, -180, -135, -69 and -19bp relative to the TSS, too sparse to be considered a CpG island, thus the *INS* gene promoter is considered as a low CpG promoter. Three CpG residues are important to the binding of regulatory transcription factors: the -234 CpG site is part of the E2 box, -206 and -180 CpG sites are parts of cAMP responsive element (CRE) 1 and 2 respectively (Figure 1).

We performed genetic and epigenetic analysis of the *INS* promoter in whole blood cell (WBC) from two T2D case-control cohorts to study the relationship between DNA methylation levels and rs689 genotypes.



Materials and Methods

Patients. Non-immortalized WBC samples were taken from participants of the Type 2 diabetes cohorts from Poitiers (T2D cases) and from participants of the MONA LISA Lille study (controls). A sample of 132 non obese T2D patients aged less than 65 years, studied in mean 7 yrs after T2D diagnosis, as well as 186 age- and BMI-matched controls was provided by M. McCarthy.

Tissues. Human liver (n=14), peritoneum (n=26), skin (n=4), intestine (n=2) and islets of Langerhans (n=4) were provided by F. Pattou. Each tissue comes from a different person.

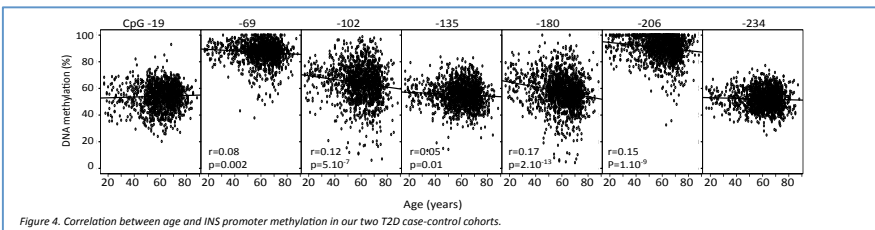
Isolation of genomic DNA and bisulfite genomic conversion and pyrosequencing. Nucleic acids were extracted from WBC or tissue using phenol/chloroform procedure. Genomic DNA was treated with EZ-96 DNA Methylation-Gold Kit, according to manufacturer's protocol (Zymo Research Corporation). We PCR-amplified the bisulfite treated genomic DNA using unbiased nested primers (sequences on request) and performed quantitative pyrosequencing (PyroMark Q96 MD, Qiagen). The percentage of methylation for each CpG site was determined for each subject using the Q-CpG methylation software (Qiagen) according to the standards in Tost and Gut (2001).

Rs689 genotyping. Rs689 was genotyped by restriction fragment length polymorphism as previously described (Le Stunff et al. 2001).

Statistical analysis. Differences in DNA methylation of the insulin promoter between rs689 genotypes were analyzed using non-parametric Kruskal-Wallis test. Correlations were calculated as adjusted R square that measures the proportion of the variation in the dependent variable accounted for by the explanatory variables. Differences in DNA methylation of the insulin promoter between T2D patients and non-diabetic controls were analyzed using non-parametric Wilcoxon rank sum test. All statistical analysis were conducted using R 2.10.1.

Relationship of methylation with age.

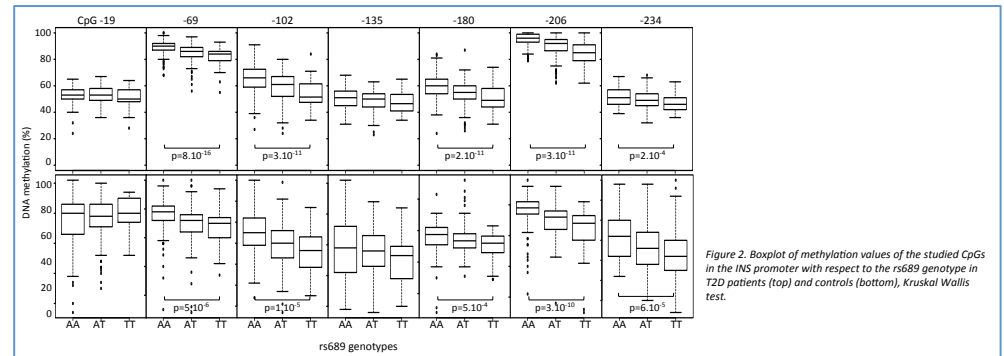
Age related changes in methylation were not uniform across the insulin gene promoter. Methylation of CpGs -135, -180 and -206 decreased slightly but significantly with age in the two French and UK cohorts (Figure 4) by losing 1.4, 1.8 and 1% methylation per 10 years. Other CpGs showed weaker but consistent changes with age, except for CpG -19 and -234.



Results

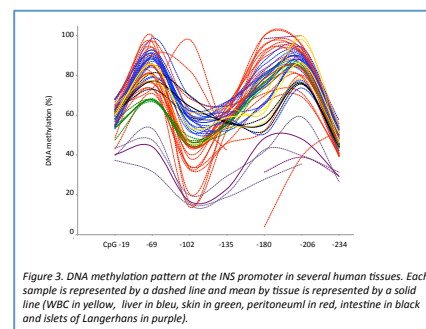
Influence of cis genotype upon methylation.

DNA methylation at CpG -69, -102, -180, -206 and -234 were found to be associated with rs689 genotypes in T2D patients (N=491) and controls (N=361) from France (Figure 2). We replicated this finding in a second T2D case-control cohort from the UK where methylation at CpGs -69, -180 and -206 showed a comparable association with rs689 genotypes.



Methylation has a comparable pattern in whole blood cells and other tissues

Comparable methylation levels and pattern were found in WBC, liver, skin, peritoneum and intestine (Figure 3). In contrast, islets of Langerhans, while following a comparable pattern of methylation had lower levels of methylation than WBC at all studied CpGs.



Intergroup variation of methylation.

We found no T2D-related global directional change in methylation level that would affect all CpGs, nor did neighbouring CpGs adopt systematic changes in methylation in T2D cases or controls.

Consistently, CpG -19 showed a decreased DNA methylation in T2D cases compared with non-diabetic controls ($p=2.10^{-16}$) in French and UK cohorts (Table 1).

	French cohort		p-value
	T2D cases	Controls	
n	491	361	
CpG -19	54 ± 6	58 ± 7	2.10 ⁻¹⁶
CpG -69	86 ± 7	86 ± 7	0.6
CpG -102	61 ± 1	62 ± 1	0.51
CpG -135	50 ± 8	57 ± 8	2.10 ⁻¹⁶
CpG -180	56 ± 9	55 ± 10	0.24
CpG -206	91 ± 7	86 ± 6	2.10 ⁻¹⁶
CpG -234	50 ± 7	50 ± 6	0.67

	UK cohort		p-value
	T2D cases	Controls	
n	348	337	
CpG -19	48 ± 12	55 ± 10	1.10 ⁻¹⁰
CpG -69	89 ± 10	88 ± 7	0.004
CpG -102	67 ± 18	66 ± 13	0.05
CpG -135	59 ± 10	56 ± 9	0.0007
CpG -180	58 ± 18	62 ± 12	0.012
CpG -206	91 ± 11	93 ± 7	0.84
CpG -234	57 ± 9	52 ± 7	1.10 ⁻¹³

Table 1. CpG methylation in the *INS* promoter in our two case-control cohorts. Results are expressed as mean ± sd. P-values are calculated by Wilcoxon rank sum test.

Conclusion

Our study reports the characterization of the DNA methylation pattern of the *INS* gene promoter in human WBC. DNA methylation at CpG -19 of the *INS* promoter was consistently decreased in T2D cases compared to controls in our French and UK cohorts. We identified an influence of the rs689 genotype upon DNA methylation in certain CpGs of the *INS* promoter. These observed methylation differences emphasize the importance to consider both genetic and epigenetic variation when studying genotype-phenotype associations.