Circadian Gene Variants and Susceptibility to Type 2 Diabetes: A Pilot Study

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Abstract

Background: Disruption of endogenous circadian rhythms has been shown to increase the risk of developing type 2 diabetes, suggesting that circadian genes might play a role in determining disease susceptibility. We present the results of a pilot study investigating the association between type 2 diabetes and selected single nucleotide polymorphisms (SNPs) in near nine circadian genes. The variants were chosen based on their previously reported association with prostate cancer, a disease that has been suggested to have a genetic link with type 2 diabetes through a number of shared inherited risk determinants.

Methodology/Principal Findings: The pilot study was performed using two genetically homogeneous Punjabi cohorts, one resident in the United Kingdom and one indigenous to Pakistan. Subjects with (N = 1732) and without (N = 1780) type 2 diabetes were genotyped for thirteen circadian variants using a competitive allele-specific polymerase chain reaction method. Associations between the SNPs and type 2 diabetes were investigated using logistic regression. The results were also combined with in silico data from other South Asian datasets (SAT2D consortium) and white European cohorts (DIAGRAM+) using meta-analysis. The rs7602358G allele near PER2 was negatively associated with type 2 diabetes in our Punjabi cohorts (combined odds ratio [OR] = 0.75 [0.66–0.86], p = 3.18 × 10⁻⁵), while the BMAL1 rs1102277ST allele was associated with an increased risk of the disease (combined OR = 1.22 [1.07–1.39], p = 0.003). Neither of these associations was replicated in the SAT2D or DIAGRAM+ datasets, however. Meta-analysis of all the cohorts identified disease associations with two variants, rs2292912 in CRY2 and rs12315175 near CRY1, although statistical significance was nominal (combined OR = 1.05 [1.01–1.08], p = 0.008 and OR = 0.95 [0.91–0.99], p = 0.015 respectively).

Conclusions/significance: None of the selected circadian gene variants was associated with type 2 diabetes with study-wide significance after meta-analysis. The nominal association observed with the CRY2 SNP, however, complements previous findings and confirms a role for this locus in disease susceptibility.

Introduction

Accumulating evidence suggests that dysregulation of the circadian clock plays an important role in glucose homeostasis and the development of type 2 diabetes. Cross-sectional and prospective studies have shown that voluntary sleep curtailment to 6 hours or less per day is associated with increased fasting glucose levels, hyperinsulinaemia and reduced insulin sensitivity, leading to an increased risk of diabetes [1,2]. Laboratory-imposed circadian desynchrony, resulting in misalignment between behavioural cycles (such as sleep/wake and fasting/feeding) and endogenous circadian rhythm, has also been shown to result in
increased postprandial glucose and insulin levels, increased mean arterial blood pressure and decreased leptin levels [3]. These metabolic disturbances could explain the increased risk of diabetes, obesity and cardiovascular disease observed in shift-workers [4,5].

Circadian rhythms are controlled and maintained by transcription-translation-based networks of positive and negative feedback loops that oscillate with 24-hour rhythmicity [6]. A recent study in mice showed that mutations in the genes encoding the CLOCK and BMAL1 transcription factors resulted in impaired glucose tolerance, reduced insulin secretion and defects in islet development, while ablation of the endogenous pancreatic clock resulted in the development of diabetes [7]. Variants of circadian genes, such as MTNR1B, CRY2, PER3, PER2 and BMAL1, have recently been implicated as determinants of fasting glucose levels and/or diabetes risk in humans [8–12], while single nucleotide polymorphisms (SNPs) in the CLOCK and PER2 genes were reported to be associated with measures of obesity [13,14]. Further investigations of circadian genes as risk markers for metabolic disease are therefore warranted.

We aimed to investigate the role of circadian gene variants as susceptibility determinants for type 2 diabetes. Before embarking on a comprehensive tag SNP analysis, however, we carried out a pilot study of selected variants chosen on the basis of their previously reported association with prostate cancer, another condition where risk has been shown to be modified by circadian misalignment [15]. The relationship between type 2 diabetes and prostate cancer is controversial. Although a number of studies have suggested that diabetic men appear to be less prone to prostate cancer than non-diabetic men (relative risk = 0.84 from a recent meta-analysis [16]), this negative correlation between the diseases has not been observed in all populations. Despite this epidemiological inconsistency, there is compelling evidence of a genetic link between the two diseases. Variants in HNF1B, UCP2, SLC2A2, IGF2BP2, TCF7L2 and CAPN11 have been shown to predispose to type 2 diabetes and protect against prostate cancer or vice versa [17,18]. A recent study also reported an inverse association between the risk of prostate cancer and a genetic risk score for type 2 diabetes [19], although a subsequent study of a subset of 17 diabetes-associated SNPs in a multiethnic cohort found no evidence of an impact on prostate cancer risk [20]. The JAZF1 gene has been implicated in both disorders, although the cancer-protective and diabetes-predisposing effects are mediated by different SNPs [17]. Similarly, independent association signals for the two diseases have been identified in the THIADA1 gene by genome-wide association studies (GWAS) [21,22]. A recent report by Zhu et al [23] suggested that twelve variants in, or close to, nine circadian-related genes were associated with overall prostate cancer risk, or risk of more or less aggressive disease. The aim of our pilot study was to determine whether these SNPs also influence the risk of type 2 diabetes.

Materials and Methods

Ethics Statement

Informed written consent was obtained from all participants and the study was approved by the Birmingham East, North and Solihull Research Ethics Committee (for participants resident in the United Kingdom) and the Baqai Institute of Diabetology and Endocrinology Institutional Review Board (for participants resident in Pakistan).

Study Participants

The study was performed using two populations of South Asian origin. UK-resident subjects (892 with type 2 diabetes, 471 normoglycaemic individuals) were recruited from Birmingham and Coventry as part of the United Kingdom Asian Diabetes Study (UKADS) [24] (UKADS registered clinical trial number: ISRCTN30297969). Pakistan-based subjects (840 with type 2 diabetes, 1309 normoglycaemic individuals) were recruited from the Mirpur region of Azad Kashmir (Diabetes Genetics in Pakistan study, DGP). All individuals were of Punjabi ancestry and originated predominantly from Mirpur. Diagnosis of type 2 diabetes was established using World Health Organisation criteria [25]. Normoglycaemia was defined as random blood glucose ≤7.0mmol/l in the UKADS control subjects and fasting whole blood glucose ≤5.6mmol/l in the DGP control subjects. Details of the study subjects have been published previously [26].

Genotyping

The subjects (N = 3512) were genotyped for selected circadian gene variants using the KASPar method (KBiosciences, Hoddesdon, UK). The twelve SNPs previously associated with prostate cancer were investigated (CLOCK, rs11133373; BMAL1, rs7950226; PER1, rs885747 and rs2289591; PER2, rs7602358; PER3, rs1012477; CRY1, rs12315175; CRY2, rs2292912; CSX1E, rs1534491; NPSR2, rs1369481; rs895521 and rs17024926) [23]. In addition we also genotyped for BMAL1 rs11022775, as this has previously been shown to be part of a susceptibility haplotype for type 2 diabetes along with rs7950226 [12]. For all SNPs, genotyping success rates were >96% and error rates in 384 duplicate samples were <0.6%.

Statistical Analysis

Statistical analysis was performed using STATA IC (version 10.1/StatCorp Corporation, College Station, TX, USA). Genotype frequencies for each SNP were checked for deviation from Hardy-Weinberg equilibrium in the normoglycaemic subjects using an exact test. The association between SNPs and type 2 diabetes was tested using logistic regression, adjusting for age and sex. The indigenous and migrant populations were analysed separately in the first instance; odds ratio (OR) values were then combined using inverse variance weighted meta-analysis, implemented in METAN. A study-wide significance threshold of p<0.0039 was applied to the analysis of the 13 SNPs in these datasets. To improve sample size and get a truer picture of the impact of the SNPs on disease risk, meta-analysis was used to combine data from the pilot study with summary statistics from the GWA phases of the expanded DIAbetes Genetics Replication And Meta-analysis (DIAGRAM+) study [27] and the South Asian Type 2 Diabetes (SAT2D) study [28]. The DIAGRAM+ cohort comprised 8,130 cases with type 2 diabetes and 38,987 control subjects of white European ancestry, while the SAT2D dataset consisted of 5,561 South Asian individuals with type 2 diabetes and 14,458 ethnically-matched control subjects. Heterogeneity of OR values between the UKADS and DGP study populations, and between the combined UKADS/DGP dataset, SAT2D and DIAGRAM+ cohorts, was assessed using Cochran’s Q statistic. Haplotype analysis for the BMAL1 locus was performed using a logistic regression framework implemented in PLINK [29].

Results

The clinical characteristics of the UKADS/DGP study subjects are shown in Table S1. None of the studied SNPs deviated significantly from Hardy-Weinberg equilibrium in the control groups from these cohorts after correction for the number of tests performed. Table 1 shows the OR values (with 95% confidence intervals) and significance values for the association of type 2
Table 1. Odds ratios and p values for the association of circadian gene variants with type 2 diabetes.

| Gene region | SNP   | Allele (minor/common) | UKADS/DGP | SAT2D | DIAGRAM+ | ALL DATASETS |
|-------------|-------|------------------------|-----------|-------|----------|-------------|
|             |       |                        | MAF       | OR (95% CI) | p       | OR (95% CI) | p       | OR (95% CI) | p       | OR (95% CI) | p       | phet     |
| PER3        | rs1012477 | C/G                   | 0.05      | 1.11 (0.89–1.39) | 0.345 | 0.95 (0.85–1.06) | 0.345 | 0.98 (0.93–1.04) | 0.510 | 0.98 (0.94–1.03) | 0.438 | 0.447     |
| BMAL1       | rs11022775 | T/C                  | 0.16      | 1.22 (1.07–1.39) | **0.003** | 0.98 (0.93–1.04) | 0.519 | 0.91 (0.83–0.99) | **0.044** | 0.99 (0.94–1.04) | 0.633 | 0.001     |
| CLOCK       | rs11133373 | G/C               | 0.38      | 0.93 (0.84–1.03) | 0.150 | 0.98 (0.92–1.04) | 0.449 | 0.99 (0.95–1.04) | 0.796 | 0.98 (0.95–1.01) | 0.267 | 0.476     |
| CRY1        | rs12315175 | C/T                | 0.07      | 0.94 (0.78–1.12) | 0.471 | 0.92 (0.84–1.01) | 0.089 | 0.96 (0.91–1.01) | 0.085 | 0.95 (0.91–0.99) | **0.015** | 0.794     |
| NPAS2       | rs1369481 | T/C               | 0.24      | 0.94 (0.84–1.05) | 0.264 | 1.10 (1.04–1.16) | **3.78x10^-6** | 0.99 (0.95–1.04) | 0.780 | 1.03 (0.99–1.06) | 0.102 | 0.003     |
| CSHN1E      | rs1534891 | T/C                | 0.19      | 1.03 (0.91–1.18) | 0.613 | 0.98 (0.92–1.03) | 0.389 | 1.00 (0.95–1.06) | 0.908 | 0.99 (0.96–1.03) | 0.728 | 0.639     |
| NPAS2       | rs17024926 | C/T                | 0.32      | 1.06 (0.96–1.18) | 0.268 | 0.93 (0.89–0.98) | **0.007** | 1.01 (0.97–1.05) | 0.595 | 0.99 (0.96–1.02) | 0.336 | 0.018     |
| PER1        | rs2289591 | A/C               | 0.14      | 0.96 (0.85–1.11) | 0.582 | 1.00 (0.93–1.09) | 0.908 | 0.96 (0.92–1.01) | 0.101 | 0.97 (0.93–1.01) | 0.150 | 0.624     |
| CRY2        | rs2292912 | C/G               | 0.27      | 1.02 (0.91–1.13) | 0.752 | 1.05 (1.00–1.12) | 0.057 | 1.05 (1.00–1.10) | 0.056 | 1.05 (1.01–1.08) | **0.008** | 0.846     |
| PER2        | rs7602358 | G/T               | 0.16      | 0.75 (0.66–0.86) | **3.18x10^-5** | 0.99 (0.93–1.06) | 0.832 | 1.03 (0.98–1.08) | 0.269 | 0.99 (0.96–1.03) | 0.648 | 1.01x10^-4 |
| BMAL1       | rs7950226 | A/G               | 0.46      | 1.04 (0.95–1.15) | 0.406 | 1.02 (0.97–1.07) | 0.471 | 0.99 (0.92–1.07) | 0.785 | 1.01 (0.98–1.05) | 0.451 | 0.700     |
| PER1        | rs885747  | C/G               | 0.29      | 0.96 (0.86–1.07) | 0.492 | 1.03 (0.96–1.10) | 0.456 | NA            | NA    | 1.01 (0.95–1.07) | 0.813 | 0.328     |
| NPAS2       | rs895521  | C/G               | 0.15      | 0.91 (0.80–1.04) | 0.184 | 1.00 (0.94–1.08) | 0.875 | 0.97 (0.92–1.03) | 0.327 | 0.98 (0.94–1.02) | 0.294 | 0.439     |

MAF – minor allele frequency in UKADS/DGP normoglycaemic control subjects, OR (95% CI) – allelic odds ratio with 95% confidence interval, p – significance level for disease association (p values less than 0.05 are shown in bold), phet – significance level of heterogeneity of odds ratios between datasets, NA – data not available, SNP failed QC in meta-analysis.

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### Figure 1. Association of 13 circadian gene SNPs with type 2 diabetes

- Forest plot showing the association of 13 circadian gene SNPs with type 2 diabetes in South Asian (UKADS/DGP and SAT2D) and white European (DIAGRAM+) cohorts.

**Circadian Genes and Risk of Type 2 Diabetes**

| Gene region | SNP     | Study          | Odds Ratio (95% CI) |
|-------------|---------|----------------|--------------------|
| PER3        | rs1012477 | DIAGRAM+ UKADS/DGP | 0.98 (0.93, 1.04)  |
|             |         | SAT2D          | 0.96 (0.85, 1.06)  |
|             |         | Subtotal       | 1.11 (0.89, 1.39)  |
|             |         | I-squared 0.0%, p = 0.447 | 0.98 (0.94, 1.03) |
| BMAL1       | rs11022775 | DIAGRAM+ UKADS/DGP | 0.91 (0.83, 1.00)  |
|             |         | SAT2D          | 0.98 (0.93, 1.04)  |
|             |         | Subtotal       | 1.22 (1.07, 1.39)  |
|             |         | I-squared 84.7%, p = 0.001 | 0.99 (0.94, 1.04) |
| CLOCK       | rs11133373 | DIAGRAM+ UKADS/DGP | 0.99 (0.95, 1.04)  |
|             |         | SAT2D          | 0.98 (0.92, 1.04)  |
|             |         | Subtotal       | 1.03 (0.84, 1.03)  |
|             |         | I-squared 0.0%, p = 0.475 | 0.98 (0.95, 1.01) |
| CRY1        | rs12315175 | DIAGRAM+ UKADS/DGP | 0.96 (0.91, 1.01)  |
|             |         | SAT2D          | 0.92 (0.84, 1.01)  |
|             |         | Subtotal       | 0.94 (0.86, 1.04)  |
|             |         | I-squared 0.0%, p = 0.794 | 0.95 (0.91, 0.99) |
| NPAS2       | rs1369481  | DIAGRAM+ UKADS/DGP | 0.99 (0.95, 1.04)  |
|             |         | SAT2D          | 1.10 (1.04, 1.16)  |
|             |         | Subtotal       | 0.94 (0.84, 1.05)  |
|             |         | I-squared 82.4%, p = 0.033 | 1.03 (0.99, 1.06) |
| CSNK1E      | rs1534891  | DIAGRAM+ UKADS/DGP | 1.00 (0.95, 1.06)  |
|             |         | SAT2D          | 0.98 (0.92, 1.03)  |
|             |         | Subtotal       | 1.03 (0.91, 1.17)  |
|             |         | I-squared 0.0%, p = 0.639 | 0.99 (0.96, 1.03) |
| NPAS2       | rs17024926 | DIAGRAM+ UKADS/DGP | 1.01 (0.97, 1.05)  |
|             |         | SAT2D          | 0.93 (0.89, 0.98)  |
|             |         | Subtotal       | 1.06 (0.96, 1.18)  |
|             |         | I-squared 75.2%, p = 0.018 | 0.99 (0.96, 1.02) |
| PER1        | rs2289591  | DIAGRAM+ UKADS/DGP | 0.96 (0.92, 1.01)  |
|             |         | SAT2D          | 1.00 (0.93, 1.09)  |
|             |         | Subtotal       | 0.96 (0.83, 1.11)  |
|             |         | I-squared 0.0%, p = 0.624 | 0.97 (0.93, 1.01) |
| CRY2        | rs2292912  | DIAGRAM+ UKADS/DGP | 1.05 (1.00, 1.10)  |
|             |         | SAT2D          | 1.05 (1.00, 1.13)  |
|             |         | Subtotal       | 1.05 (1.00, 1.12)  |
|             |         | I-squared 0.0%, p = 0.845 | 1.05 (1.01, 1.08) |
| PER2        | rs7602358  | DIAGRAM+ UKADS/DGP | 1.03 (0.98, 1.08)  |
|             |         | SAT2D          | 0.99 (0.93, 1.06)  |
|             |         | Subtotal       | 0.75 (0.66, 0.86)  |
|             |         | I-squared 89.1%, p = 0.000 | 0.99 (0.95, 1.03) |
| BMAL1       | rs7950226  | DIAGRAM+ UKADS/DGP | 0.99 (0.92, 1.07)  |
|             |         | SAT2D          | 1.02 (0.97, 1.07)  |
|             |         | Subtotal       | 1.04 (0.95, 1.15)  |
|             |         | I-squared 0.0%, p = 0.700 | 1.01 (0.98, 1.05) |
| PER1        | rs885747   | SAT2D          | 1.03 (0.96, 1.10)  |
|             |         | UKADS/DGP      | 0.96 (0.86, 1.07)  |
|             |         | Subtotal       | 1.01 (0.95, 1.07)  |
|             |         | I-squared 0.0%, p = 0.328 | 0.97 (0.92, 1.03) |
| NPAS2       | rs895521   | DIAGRAM+ UKADS/DGP | 0.97 (0.92, 1.03)  |
|             |         | SAT2D          | 1.00 (0.94, 1.08)  |
|             |         | Subtotal       | 0.91 (0.83, 1.04)  |
|             |         | I-squared 0.0%, p = 0.439 | 0.98 (0.94, 1.02) |

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**Figure 1.** Association of 13 circadian gene SNPs with type 2 diabetes. Forest plot showing the association of 13 circadian gene SNPs with type 2 diabetes in South Asian (UKADS/DGP and SAT2D) and white European (DIAGRAM+) cohorts.
diabetes with each of the 13 studied SNPs in our South Asian populations, along with the DIAGRAM+ and SAT2D cohorts. OR values did not differ significantly between the indigenous and migrant Punjabi populations in our study; therefore results are presented for the combined UKADS/DGP dataset. Sex-specific analysis of this cohort showed no significant differences in OR values between males and females (Table S2).

Two of the variants were associated with type 2 diabetes in the UKADS/DGP cohort at a study-wide significant level (Table 1). The minor allele (T) of BMAL1 rs11022775 was associated with susceptibility to the disease (p = 0.003). The rs11022775T/rs11022775A BMAL1 haplotype was also nominally associated with an increased risk of diabetes in this cohort before correction for multiple testing (p = 0.007); this effect appeared to be driven by the rs11022775 SNP. In contrast, the minor allele (G) of rs7602358 near the PER2 locus appeared to confer protection against type 2 diabetes in both the UKADS (p = 0.003) and DGP (p = 0.004) datasets (combined cohort, p = 3.18 × 10⁻⁴). Neither of these observations was confirmed in the SAT2D or DIAGRAM+ datasets, however. The BMAL1 rs11022775 SNP was nominally associated with disease in the DIAGRAM+ cohort, but disease risk appeared to be conferred by the C allele, not T as seen in UKADS/DGP. No evidence of an association with rs7602358 was seen in the DIAGRAM+ or SAT2D datasets. In the latter cohort, two NPAS2 SNPs (rs1369481 and rs17024926) were associated with type 2 diabetes, the former with a p value of 3.78 × 10⁻⁴, but this was not replicated in the UKADS/DGP or DIAGRAM+ datasets (Table 1).

Meta-analysis of all the datasets failed to confirm the disease associations with the BMAL1 and PER2 variants. In contrast, type 2 diabetes was associated with rs12315175, close to the CRY2 gene, and rs2292912, located in the NPAS2 gene, with type 2 diabetes, the former with a p value of 3.78 × 10⁻⁴. As for the SNPs described above, however, this was not replicated in either of the other datasets and no consistent direction of effect was observed.

The analysis of the SAT2D data showed two potentially interesting associations with the NPAS2 locus, with the rs369461 variant achieving a reasonable level of significance (p = 3.78 × 10⁻⁴). As for the SNPs described above, however, this was not replicated in either of the other datasets and no consistent direction of effect was observed.

Although meta-analysis of the datasets did not confirm disease associations with the PER2 and BMAL1 variants, it did provide nominal evidence of associations with CRY2 rs2292912 and CRY1 rs12315175. The former SNP is located ~4.6 kb from rs11605924, which was reported to be associated with type 2 diabetes in the MAGIC study (p = 1.7 × 10⁻⁴) [9], with a similar effect size (OR = 1.04) to that seen for rs2292912 in the current meta-analysis (OR = 1.05). These two variants are not in strong linkage disequilibrium (LD) (r² = 0.3 in HapMap Data Release 27; www.hapmap.ncbi.nlm.nih.gov), suggesting that there might be two independent association signals for type 2 diabetes at this locus. The association with rs12315175 near CRY1 is a novel finding. Interestingly recent studies in mice have suggested a role for Cry1 in glucose homeostasis; hepatic overexpression of the protein was shown to lower blood glucose concentrations and improve insulin sensitivity in insulin-resistant db/db mice [30], while transgenic mice expressing a mutant form of Cry1 developed hyperglycaemia associated with an early-onset insulin-secretory defect [31]. The role of CRY1 in human diabetes may therefore warrant further investigation.

It is interesting to note that the variants displaying significant disease associations in individual populations in our study appear to have opposite directions of effect on type 2 diabetes and prostate cancer; that is the minor alleles of the CRY1 and NPAS2 variants that appear to increase the risk of diabetes in one or more of our datasets are associated with a decreased risk of prostate cancer in the study of Zhu et al [23], while the putative diabetes-protective alleles near PER2 and CRY1 appear to confer an increased risk of prostate cancer. These findings are consistent with the inverse disease relationship reported by meta-analysis of epidemiological data [16]. It should be noted, however, that none of the prostate cancer associations described by Zhu et al [23] achieved genome-wide significance and the variants are yet to be confirmed as genuine risk determinants for the disease.

As our pilot study investigated only selected circadian variants, we cannot exclude the possibility that other SNPs at these loci have an influence on disease susceptibility. It is unlikely that such an influence would be major, however, as we would expect this to have been picked up by the genome-wide association analysis of the DIAGRAM+ and SAT2D datasets [27,29]. Nevertheless it is possible that variants of these genes have a more modest effect, which did not reach the threshold for follow-up in these studies.

In conclusion our study has confirmed the association between type 2 diabetes and variants of the CRY2 gene and suggested a potential role for the CRY1 gene in disease development. Together with previous reports of associations between fasting glucose/diabetes and the MTNR1B and CRY2 loci [9,9], our data support a
role for the circadian clock in the regulation of glucose homeostasis.

Supporting Information

Table S1 Clinical characteristics of subjects stratified by study population, disease status and sex. (DOC)

Table S2 Sex-specific analysis of circadian gene variants in UKADS/DGP cohort. (DOC)

Table S3 Minor allele frequencies of circadian SNPs in UKADS/DGP, DIAGRAM + and SAT2D datasets. (DOC)

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Author Contributions

Conceived and designed the experiments: MAK ST. Performed the experiments: SDR. Analyzed the data: SDR. Contributed reagents/materials/analysis tools: MZH ASS JB JPO SK ST AB. Wrote the paper: MAK. Contributed to discussion and reviewed/edited the manuscript: SDR MZH ASS JB JPO SK ST AB.

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