The search for circadian clock components in humans: new perspectives for association studies

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Individual circadian clocks entrain differently to environmental cycles (*zeitgebers*, e.g., light and darkness), earlier or later within the day, leading to different chronotypes. In human populations, the distribution of chronotypes forms a bell-shaped curve, with the extreme early and late types - larks and owls, respectively - at its ends. Human chronotype, which can be assessed by the timing of an individual's sleep-wake cycle, is partly influenced by genetic factors - known from animal experimentation. Here, we review population genetic studies which have used a questionnaire probing individual daily timing preference for associations with polymorphisms in clock genes. We discuss their inherent limitations and suggest an alternative approach combining a short questionnaire (Munich ChronoType Questionnaire, MCTQ), which assesses chronotype in a quantitative manner, with a genome-wide analysis (GWA). The advantages of these methods in comparison to assessing time-of-day preferences and single nucleotide polymorphism genotyping are discussed. In the future, global studies of chronotype using the MCTQ and GWA may also contribute to understanding the influence of seasons, latitude (e.g., different photoperiods), and climate on allele frequencies and chronotype distribution in different populations.

Key words: Human clock genes; Chronotype; Morningness/eveningness; Association studies; Latitudinal hypothesis

Introduction

When circadian rhythms are investigated under constant conditions, their endogenous periods often deviate from 24 h. Under natural conditions, circadian clocks are normally entrained by environmental signals (*zeitgebers*), predominantly by light. The underlying molecular mechanism generating the internal day is thought to be based upon a transcriptional regulatory loop of so-called clock genes and their products (1).

Circadian rhythms have been demonstrated in all phyla, from cyanobacteria to mammals (2) and species-specific dedicated clock genes have been identified in most model systems by reverse genetics (3-10). In humans, the as-

sumptions about the mechanisms of the molecular clock remain hypothetical, as identification of human clock genes are predominantly based on their sequence similarity with those in other animals. Although many of the mammalian clock genes have been shown to oscillate in cultured human fibroblasts (11), experiments do not prove that these genes and their products are involved in the molecular rhythm generation in humans.

To characterize the relevance of mammalian clock genes in humans, association studies of a circadian phenotype with naturally occurring genetic variation have been conducted. So far, these efforts are based primarily on a phenotyping instrument that queries time-of-day preferences, resulting in a score (Horne-Østberg Morningness-

Eveningness Questionnaire - MEQ) (12), rather than in actual timing (phase of entrainment, chronotype). Genetic variability in humans indeed reflects individual time-of-day preferences; a list of positive associations described over the last decade for 6 clock genes is given in Table 1.

Here, we review these studies and discuss inherent problems possibly responsible for inconsistencies of some results. We suggest alternative strategies based on new phenotyping and high-throughput genotyping methodologies. In addition, we propose studies investigating latitudinal clines in genetic variations related to circadian clock adaptation.

Family-based and association studies

The inter-individual differences of human time-of-day preferences (MEQ) or chronotype (Munich ChronoType Questionnaire, MCTQ) can be manifest in extreme cases as a rare familial syndrome. Thus, extreme morningness can result in the familial advanced sleep phase syndrome (ASPS) and extreme eveningness in the delayed sleep phase syndrome (DSPS). The association of several clock gene variants with morningness-eveningness scores but also with its extreme forms, ASPS or DSPS, have been investigated in various studies, however, with partly conflicting results. The monogenic autosomal dominant familial ASPS (13) has, for example, been associated with a mutation in the genes period 2 (*Per2;* Table 1). However, results are inconsistent across different pedigrees. Satoh et al. (14) characterized pedigrees with familial ASPS that were not carriers of the earlier described *Per2* mutation,

whereas a mutation at the casein kinase one delta (*CKIδ*) gene was later found to be leading to the same phenotype (Table 1).

Besides finding ASPS to be associated in isolated families with (rare) single nucleotide mutations, the same phenotype has also been associated in the general population with a length polymorphism in the period 3 (*Per3*) gene, in form of variable number of tandem repeats (VNTR; Table 1) (15,16). A long VNTR form (5-repeats) was associated with morningness, whereas a short form (4-repeats) was associated with eveningness. A replication of these findings in a Brazilian population found the long VNTR allele associated with eveningness rather than with morningness (17) (Table 1). The authors speculated that the discrepant findings could be due to the influence of different latitudes, as has been previously shown for clock genes in flies (18). However, the hypothesis of a latitudinal cline in distinct allele frequencies of this *Per3* polymorphism could not be sustained (19). Another apparent conflict (not taking possible ethnic influences into account) was found for a rare haplotype of *Per3* (G647, P864, 4-repeat, T1037, R1158). Whereas it associates with DSPS in Japanese patients (16) (Table 1), one of its alleles (G647) associates with morningness in Europeans (20).

Again, unequivocal associations have been found for a single nucleotide polymorphism (SNP) of the *CLOCK* gene 5'-UTR region (T3111C). It was found to associate with eveningness both in Europeans (21) (Table 1) and Japanese (22) (Table 1), but this association was not reproducible in other studies (23-25). Two gene variants, one of them actually responsible for the phenotype and the other

Table 1. Reports on variants of human clock genes associated with individual time-of-day preference (listed in chronological order).

Gene	Position	Population	Syndrome/sleep preference	Literature
hCLOCK	T3111C-5'-UTR	European	E	21
HPer2	Missense mutation in the CKIε binding region; S662G	Pedigree	ASPS	51
HPer3	H4 haplotypes [G647, P864, 4-repeat, T1037, R1158]	Japanese	DSPS	16
hCLOCK	T3111C-5'-UTR	Japanese	E	22
hPer3	G647	Swedish/Finish/ Austrian/German	M	20
hPer3	4 or 5 repeats - 54 bp in exon 18	British	Short allele - DSPS Long allele - M	15
hCK1ε	S408N	Japanese	Protect against DSPS	52
hPer3	4 or 5 repeats - 54 bp in exon 18	Brazilian	Long allele - DSPS	17
hPer2	C111G - 5'-UTR	British	M	53
hCKIδ	T44A	Pedigree	ASPS	54
hPer1	T2434C	British	M	55

Ethnic origins of the populations listed are: Caucasian for European; Asian for Japanese; European Caucasian and mixed ethnic origins for the Brazilian population. ASPS = advanced sleep phase syndrome; DSPS = delayed sleep phase syndrome; M/E = morningness/eveningness preferences; UTR = untranslated region.

being the one that is investigated, can often co-segregate (when they are in linkage disequilibrium, LD). LD between the investigated and the actual phenotype-causing variant could be one of the reasons for such a spurious association since LD levels vary among alleles in different populations. Thus, an association found in one population may not be reproducible in another. To investigate this hypothesis for the case of the T3111C polymorphism, Pedrazzoli et al. (25) investigated an exonic *CLOCK* variant (T257G), which showed almost complete LD with the T3111C SNP but found no association of either variant with time-of-day preferences or DSPS in the Brazilian population, indicating that neither was responsible for the phenotype.

The discrepant results of studies associating time-of-day preferences or sleep phase syndromes with clock gene mutations, SNPs, or VNTRs could have at least three reasons: i) population-specific LD levels between the investigated and the causal variant, as well as, population stratification. The first assumption will not hold for every investigated population because allele frequencies vary among different populations influencing the LD levels between loci. Therefore, it is essential to be aware of the ethnic composition of the populations for which findings are reported (Table 1). However, this information is not always clearly specified by authors. Admixture (miscegenation between populations) can dynamically generate LD among loci, because allele frequencies of ancestor populations will take several generations of recombination to achieve equilibrium. As a consequence, the confounding effect of stratification may take a long time to disappear. The results of association studies may be biased by heterogeneities; these can be generated either by mixing samples from different origins, or by samples from the same origin, but taken from an admixed population. ii) Insufficiently accurate phenotyping. Here, we discuss alternative strategies to reduce the ambiguity in phene-gene associations concerning the circadian clock in humans. iii) Gene-gene and gene-environment interactions. Chronotype is a multi-factorial trait; thus, simple straightforward associations between variants in a single gene cannot necessarily be used to predict the phenotype.

Population specificity and population stratification

Allele frequencies and LD levels between gene variants can vary drastically among subjects of distinct ancestry (26), so that association with a certain variant in LD with the causal variant for one population may not be found in other populations or may be undetectable or ambiguous in ethnically heterogeneous populations. To attenuate replication failures due to population-specific allele frequencies and LD levels, many more SNPs can be assessed with new high-throughput genotyping methodologies. The HapMap (dense maps of LD for a large number of SNPs; http://www.hapmap.org) allows the selection of SNPs that tag haplotype blocks from populations either of African, Asian or European ancestry. This approach allows covering large regions of the genome with a relatively low number of SNPs, which are used as markers for other co-segregating variants. Designing association studies based on the HapMap drastically increases the chances of "tagging" the causal variant with an LD-based strategy, thus, preventing inconsistencies that may result in single SNP association studies. However, admixture can still affect the reliability of the LD mapping strategy. Population stratification can bias associations in studies based on a single SNP or on thousands of SNPs, consequently resulting in possible replication failures. Decreasing heterogeneity of the sample can minimize stratification problems. For this purpose, molecular markers for ethnicity can be used, a way of controlling, or rather correcting for admixture. This procedure is a routine strategy in genome-wide association studies; panels of ethnic markers are already published, revealing clear ethnic distinctions in particular allele frequencies even within European populations (27).

Improving phenotyping

Beyond the methodological improvements described above, the best prerequisite for unequivocal results in phene-gene association studies is optimal phenotyping. The MEQ, used in most circadian association studies so far, is an excellent psychological instrument that has been used extensively in research beyond trying to find human clock genes (28-32). However, the questions of the MEQ are mostly subjective, assessing time-of-day preferences based on a personal "feeling best rhythm" or on hypothetical situations (e.g., "approximately what time would you get up if you were entirely free to plan your day?") (33), or in relationship to the habits of others (e.g., "I get up later than most people.") (34). The MEQ yields plausible results, but does not explicitly assess actual times (e.g., of sleep or activity) nor does it distinguish between free and work days. In addition, age- and sex-specific changes in time-of-day preferences are not sufficiently quantified and are, thus, not easily incorporated into the phenotyping procedure, which would be essential for accurate phenotyping.

We have developed the MCTQ, which allows a quantitative assessment of entrained phase (chronotype) by taking all the considerations mentioned above into account (35-39). The MCTQ shows good correlation with the

MEQ score (40). However, the best correlation exists between the MEQ score and a single question of the original version of the MCTQ (r = -0.80), essentially asking ("what chronotype are you - extreme, moderate, slightly early or late?") (39). Sleep-timing on free days, which is the initial basis for chronotyping in the MCTQ, correlates less with the MEQ score (r = -0.74) and correlation decreases with every correction procedure, necessary for determining a quantitative value for an individual chronotype (see below).

The initial MCTQ determination of chronotype is based on the mid-sleep time on free days (MSF). Since many subjects accumulate a considerable amount of sleep-debt on workdays, which they compensate for on free days, MSF has to be corrected (MSF$_{sc}$; correlation with the MEQ score: r = -0.66). Moreover, chronotype depends on sex and age (39). The large number of entries in our database (currently 65,000) allows us the determination of extremely accurate correction factors for the dependencies of MSF$_{sc}$ on age and sex (MSF$_{sasc}$; correlation with the MEQ score: r = -0.59). These adjustments are a crucial prerequisite for epidemiological and genetic association studies profiting from a more accurate measure of chronotype (39).

Circadian gene networks

The current model of the clock has outgrown the single feedback-loop hypothesis presuming a network of molecular oscillator (41). In addition, chronotype will be influenced by more than just the molecular oscillator components. Phase of entrainment of the oscillator depends on input signals and when phase of entrainment is determined by an output variable of the circadian clock (e.g., timing of sleep or the melatonin profile), it additionally depends on how the output is coupled to the oscillator. Thus, chronotype is definitely a polygenic trait involving genes concerning input(s), oscillator(s) and output(s). In gene networks, the specific effect of a SNP can only be unambiguous when it is compared in an otherwise homogeneous genome. If, however, individuals differ in more than one single nucleotide or other polymorphisms, interpretations become difficult. The theory of entrainment (42) predicts, for example, that if one polymorphism results in a shorter free-running period (still being longer than 24 h) and another in reducing the sensitivity to the *zeitgeber*, chronotype may not change, although it would with either polymorphism alone (albeit in opposed directions). A polymorphism resulting in an increased *zeitgeber* transduction would result in a later chronotype, if the free-running period of the individual is shorter than 24 h and in an earlier chronotype if the period is longer. These are only two examples of many others highlighting the complications that arise when investigating a single polymorphism association with a complex phenotype. These complications can theoretically be dealt with in genome-wide association studies when using computations that involve the interactions of more than one polymorphism in determining chronotype.

Gene-environment interactions: expanding research on human clock genetics beyond finding clock genes

Once we can optimize the designs and methods of association studies, we can start to ask more complex biological questions, such as genetic adaptation to different latitudes and climates, relationships between light exposure, state of industrialization and genetic chronotype, or even whether food influences genetic chronotype dispositions.

Among these, the latitudinal question is probably the most amenable. Light plays a crucial role within the complexity of the circadian clock. Day length (photoperiod) depends on time of year and the amplitude of its seasonal changes on latitude. These geographical specificities of light could be relevant in adaptation processes strengthening or weakening the impact of components within the circadian network that should be detectable in the allele frequencies of populations living at different latitudes. We have recently shown that the human clock entrains to sun times (predominantly to dawn) even for different longitudes within a single time zone (36). Latitudinal clines related to human reproduction (43,44), human mortality (45) or genetic geographic variations between photoperiodic diapauses and the circadian eclosion rhythm in *Drosophila* (46-49) have been observed. In addition, synchronization of circadian clocks to natural light-dark cycles is challenged at very high latitudes due to the extreme photoperiods (50). Therefore, a relationship between an adaptive circadian system and fitness is likely. Comparing the genomes between indigenous and more recently immigrated populations might reveal clock genes that are important for adaptation to photoperiod.

Conclusions

Genome-wide studies of the genetic variability underlying chronotype and the circadian system in general will not only help us to identify those genes that are relevant for circadian regulation in humans (we may even find new genes which then can be looked for in other animals) but also how the circadian system has evolved. Understand-

ing the mechanisms underlying chronotype will open new possibilities for identifying extreme chronotypes and how to help them to adjust better to social time. It will also open avenues to improve individual treatment in many diseases which are not caused by defects in the circadian system but the symptoms of which are strongly modulated by the clock and will pave the way to optimizing chrono-pharmacological interventions. The characterization of new genes and polymorphisms influencing chronotype will also strengthen *in vitro* functional studies investigating the mo-

lecular mechanisms controlling the period of free running rhythms in humans. Finally, the insights into the genetics behind chronotype may increase the appreciation of this phenomenon and may consequently lead to political changes facilitating more appropriate, flexible, or individualized social schedules (e.g., in school and work).

Acknowledgments

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