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LETTER TO THE EDITOR

Germline polymorphisms of circadian genes and gastric cancer predisposition

Dear Editor,

Gastric cancer represents a remarkable disease burden worldwide, ranking among the first five tumor types in incidence and mortality [1]. Germline DNA variation has been extensively investigated in terms of predisposition to sporadic gastric cancer, which represents more than 90% of all cases [2]. Currently available evidence shows that the fraction of disease burden that can be attributable to known risk polymorphisms is small (< 20%) [2].

Single germline variations of circadian genes (also called clock genes) have been associated with the predisposition of different tumor types [3]. The circadian clock is a time-tracking rhythmic biological system with a periodicity of about 24 hours that enables organisms to anticipate environmental changes and allow them to modify their behavior and physiological functions in the most efficient way. Circadian rhythms are controlled by proteins encoded by circadian genes, which have been discovered in all studied species. Remarkably, the disruption of these rhythms has been linked with risk of different diseases including cancer. In regards to the latter, a growing wealth of evidence supports the potential tumor suppressor role of the biological clock [3, 4].

As the role of circadian gene germline variants has never been explored in the field of gastric cancer susceptibility, with the present work, we intended to test the hypothesis that specific single nucleotide polymorphisms (SNPs) of the circadian genes, such as *CLOCK*, *NPAS2*, *PER1*, *PER2*, *RORA*, and *TIMELESS*, could significantly increase or decrease the predisposition to develop gastric cancer. We considered the 10 SNPs of the above listed 6 circadian genes that are known to be functional or associated with cancer risk or prognosis. The main features of the SNPs are described in our previous study [5].

We conducted a retrospective study based on a total of 1065 subjects comprising of 455 cases of gastric cancer and 610 healthy controls. All of them were of European ancestry. The median age of onset for gastric cancer was 67 years (range, 27-90 years). Among these gastric cancer patients, 249 (54.7%) were males and 206 (45.3%) were females. The median survival was 30.0 months, ranging from 1.0 to 293.0 months. These datasets were already employed in our previous studies [5, 6] and the detailed characteristics of the subjects are summarized in Table 1 and Supplementary Table 1.

Genotyping was performed by real-time PCR. Multivariate logistic regression analysis was performed to assess the associations employing four models of inheritance: allelic, recessive, dominant, and co-dominant. The detailed methods are available in Supplementary information. All the preselected SNPs were successfully genotyped, and no departures from Hardy-Weinberg equilibrium were observed (Supplementary Table 2). The average genotyping success rate of selected SNPs in all participants was 98.9% (range, 96.0%-100%). The mean statistical power for this analysis was 61%. Detailed statistical power for each SNP is reported in Supplementary Table 3.

Associations between the selected circadian genes genetic variations and gastric cancer predisposition were tested assuming 4 models of inheritance. The results are summarized in Table 2. We used odds ratios (ORs) and their corresponding 95% confidence intervals (CI) to measure the strength of association between each polymorphism and gastric cancer susceptibility. Overall, the genetic variants significantly associated with gastric cancer predisposition were: *NPAS2* rs895520, *PER1* rs3027178, *PER2* rs934945, *RORA* rs339972. In particular, the present analysis suggested that *NPAS2* rs895520 minor allele (A) was associated with an increased susceptibility to gastric cancer of 24% under an additive (per allele OR, 1.24; 95% CI, 1.01-1.52; $P = 0.036$), recessive (OR, 1.56; 95% CI, 1.09-2.24; $P = 0.016$) and co-dominant (OR, 1.62; 95% CI, 1.07-2.44; $P = 0.022$) model of inheritance. *PER1* rs3027178, a genetic variant with a synonymous functional effect was associated with a reduced predisposition (per allele OR, 0.80; 95% CI, 0.64-0.99; $P = 0.037$). *PER2* rs934945 (C > T) is located on the last exon of *PER2*

Abbreviations: CI, confidence interval; Ctrl, control; N/A, not applicable; OR, odds ratio; SNP, single nucleotide polymorphism.

TABLE 1 Characteristics of 455 gastric cancer patients and 610 healthy controls retrospectively included in the present study

Characteristic	Gastric cancer patients *n* (%)	Healthy controls *n* (%)
Median age (range, years)	67 (27-90)	48 (14-92)
Gender		
Male	249 (54.7)	336 (55.2)
Female	206 (45.3)	274 (44.8)
Source of controls		
Hospital	N/A	340 (55.7)
Population	N/A	270 (44.3)
Patient status		
Alive	150 (33.0)	N/A
Dead	305 (67.0)	N/A
Median survival (range, months)	30.0 (1.0-293.0)	N/A
Tumor stage		
I	131 (28.8)	N/A
II	84 (18.5)	N/A
III	109 (24.0)	N/A
IV	131 (28.8)	N/A

Abbreviation: N/A, not applicable.

locus and has a missense functional effect, leading to the substitution of Glycine-Glutamic acid. Carriers of at least one copy of the minor allele had a decreased predisposition to develop gastric cancer (28%) employing a dominant genetic model (OR, 0.72; 95% CI, 0.53-0.98; $P = 0.037$). Employing a co-dominant model heterozygotes had a 31% risk reduction as compared to homozygotes for the common allele (C) (OR 0.69; 95% CI 0.50-0.94; $P = 0.019$). *RORA* rs339972 C allele was associated with a decreased predisposition to develop gastric cancer assuming an additive (per allele OR, 0.78; 95% CI, 0.63-0.98; $P = 0.032$) or dominant (OR, 0.75; 95% CI, 0.56-1.00; $P = 0.049$) genetic model.

To the best of our knowledge, this is the first scientific work investigating the relations between circadian genes DNA genetic variations and the susceptibility to gastric cancer. Therefore, we could not know a priori the genotype-phenotype relation of these SNPs; as a consequence, we tested 4 genetic models of inheritance: allelic, recessive, dominant and co-dominant. When testing the allelic/recessive/dominant models, for those polymorphisms which were significantly associated with the phenotype in more than one model, the best fitting model was considered the one with the lower *P* value. Our results indicated that *NPAS2* rs895520 best-fitted model for the association with gastric cancer was the recessive model of inheritance, while *RORA* rs339972 was the allelic model. Interestingly, we found similar results regarding *NPAS2* rs895520 in our previous work on associations of circadian genes polymorphisms with soft tissue sarcoma susceptibility [5], while there was no difference in terms of *P* value for *RORA* rs339972 comparing the allelic and the dominant model, nevertheless, both were associated with sarcoma susceptibility as it was for gastric cancer. Since the maximum power was reached when the 'true' mode of inheritance of the disease susceptibility loci and the genetic model used in the analysis were concordant [7], it is worth determining the genotype-phenotype relation for each SNP.

We tested the co-dominant model as well, for two reasons: its robust method [7] and its application in testing the circadian genes SNPs associations with different neoplasms [8, 9]. Employing the co-dominant model *PER2* rs934945 heterozygotes had a decreased predisposition compared to homozygotes for the common allele (C) of 31%. Karantanos et al. [9] found no association of *PER2* rs934945 with colorectal cancer neither with the allelic nor with the co-dominant model. Dai and colleagues [8] found no association of *PER2* rs934945 with breast cancer in overall analysis while found a significant association in subgroup analysis. Homozygotes for the minor allele (T) had an increased risk of developing breast cancer only in a specific *CLOCK* rs3805151 background (homozygosis for the common allele C). This was in line with the shared idea that genetic variations have different effects in different neoplasms. In particular, this was recently highlighted for prognosis in an interesting work performed by Chang and Lai [4]. They performed a comprehensive study of circadian genes in 21 cancer types that considered genomic, transcriptomic and phenotypic (clinical prognosis) data and they found that circadian genes were substantially altered by somatically acquired deletions and amplifications. Core circadian genes, *PERs, CRY2, CLOCK, NR1D2, RORA* and *RORB* exhibited global patterns of somatic loss and downregulation across multiple tumor types and that loss-of-function of these genes resulted in increased death risks in patients. However, tumor suppressive qualities appeared to be cancer type-specific. Opposite trend was obtained for bladder and stomach cancers as their "low" loss-of-function of putative tumor-suppressive circadian genes were found to be associated with adverse survival outcomes [4]. In our previous study concerning the associations of gastric cancer prognosis and germline variation of circadian genes [6] we had a similar approach. We found that germline polymorphisms in the circadian pathway were associated with the survival of patients with gastric cancer, independently of established prognostic factors such as disease stage and patient age at diagnosis. In particular, combined information deriving from two SNPs (rs3749474 and rs1801260, two variants of the *CLOCK* gene 3'-UTR) allowed us to classify patients into a high or low *CLOCK* transcription, with the latter showing a significantly worse prognosis (about 70% increased risk of death). This apparent discrepancy highlights that gastric cancer prognosis and circadian genes relations need further in-depth analysis.

TABLE 2 Multivariate logistic regression analysis of circadian gene genotypes and gastric cancer predisposition under 4 models of inheritance

					Models of inheritance							
					Co-dominant		Additive		Recessive		Dominant	
Gene	SNP	Genotype	No. of healthy controls	No. of gastric cancer patients	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
CLOCK	rs1801260	TT	323	236	Ref		0.92 (0.74-1.15)	0.464	0.65 (0.39-1.09)	0.100	1.00 (0.75-1.33)	0.979
		TC	228	183	1.09 (0.80-1.47)	0.582						
		CC	56	36	0.67 (0.39-1.14)	0.136						
		Undetermined	3	0	N/A	N/A						
	rs3749474	CC	259	173	Ref		1.07 (0.87-1.32)	0.522	1.05 (0.69-1.60)	0.810	1.12 (0.83-1.50)	0.464
		CT	266	206	1.12 (0.82-1.53)	0.483						
		TT	83	57	1.12 (0.71-1.75)	0.627						
		Undetermined	2	19	N/A	N/A						
NPAS2	rs895520	GG	211	138	Ref		1.24 (1.01-1.52)	0.036	1.56 (1.09-2.24)	0.016	1.19 (0.88-1.62)	0.257
		GA	294	199	1.06 (0.76-1.47)	0.735						
		AA	103	107	1.62 (1.07-2.44)	0.022						
		Undetermined	2	11	N/A	N/A						
	rs2305160	GG	283	211	Ref		1.03 (0.83-1.28)	0.807	1.18 (0.73-1.91)	0.490	0.99 (0.74-1.31)	0.926
		GA	264	190	0.95 (0.70-1.28)	0.738						
		AA	59	48	1.15 (0.70-1.90)	0.586						
		Undetermined	4	6	N/A	N/A						
PER1	rs3027178	TT	281	226	Ref		0.80 (0.64-0.99)	0.037	0.70 (0.44-1.11)	0.132	0.76 (0.57-1.01)	0.061
		TG	253	185	0.80 (0.59-1.08)	0.141						
		GG	76	44	0.63 (0.39-1.02)	0.062						
PER2	rs934945	CC	386	314	Ref		0.79 (0.60-1.04)	0.087	1.34 (0.54-3.34)	0.530	0.72 (0.53-0.98)	0.037
		CT	206	129	0.69 (0.50-0.94)	0.019						
		TT	17	12	1.20 (0.48-3.00)	0.704						
		Undetermined	1	0	N/A	N/A						
	rs7602358	TT	358	230	Ref		1.17 (0.92-1.48)	0.210	0.86 (0.46-1.62)	0.640	1.30 (0.97-1.74)	0.082
		TG	213	184	1.35 (1.00-1.83)	0.053						
		GG	38	24	0.98 (0.51-1.86)	0.940						
		Undetermined	1	17	N/A	N/A						
RORA	rs339972	TT	312	238	Ref		0.78 (0.63-0.98)	0.032	0.69 (0.42-1.14)	0.146	0.75 (0.56-1.00)	0.049
		TC	233	168	0.78 (0.58-1.06)	0.118						
		CC	62	35	0.62 (0.37-1.04)	0.073						
		Undetermined	3	14	N/A	N/A						

(Continues)

TABLE 2 (Continued)

Gene	SNP	Genotype	No. of healthy controls	No. of gastric cancer patients	Models of inheritance							
					Co-dominant		Additive		Recessive		Dominant	
					OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
	rs10519097	CC	422	333	Ref		0.82 (0.61-1.10)	0.186	0.66 (0.23-1.92)	0.444	0.82 (0.59-1.13)	0.217
		CT	173	101	0.83 (0.60-1.16)	0.282						
		TT	14	7	0.63 (0.22-1.84)	0.399						
		Undetermined	1	14	N/A	N/A						
TIMELESS	rs7302060	TT	181	141	Ref		1.00 (0.82-1.23)	0.986	0.88 (0.61-1.27)	0.505	1.10 (0.81-1.50)	0.549
		TC	304	228	1.16 (0.83-1.62)	0.376						
		CC	121	80	0.95 (0.63-1.45)	0.814						
		Undetermined	4	6	N/A	N/A						

Note: In the co-dominant model the genotype is considered as a categorical variable and the common allele genotype is the reference; in the additive model the genotype is considered as a continuous variable; in the recessive model the genotype is considered as a categorical variable with 2 categories (homozygous for the common allele + heterozygous, homozygous for the variant allele); in the dominant model the genotype is considered as a categorical variable with 2 categories (homozygous for the variant allele + heterozygous, homozygous for the common allele). Bold values indicate significant associations (*P* value < 0.05).

Abbreviations: OR, odds ratio; CI, confidence interval; SNP, single nucleotide polymorphism; N/A, not applicable.

Moreover, we could not replicate the data reported by Qu and colleagues [10] on the association between *PER* variants and prognosis. Different ethnicity (European *vs*. Asian), sample size (the Asian series was more than two-fold larger) and disease stage composition (only our study included patients with advanced and metastatic gastric cancer) might partly explain this discrepancy. Nevertheless, differences were found by 2 groups studying *PER2* expression as a prognostic factor for gastric cancer in patients with Asian ethnicity. Zhao and colleagues [11] found that PER2 expression was downregulated in most gastric cancer tissues, while Hu and colleagues [12] found that it was upregulated.

To our knowledge, this is the first analysis investigating the hypothesis of an association between germline genetic variations of the circadian pathway with gastric cancer susceptibility. The power of our study is not optimal, and the present study should be considered as a pilot work that warrants further validation in different datasets. Nevertheless, our results showed that the 4 circadian clock variants were clinically and statistically associated with gastric cancer predisposition.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Padova University Hospital (identifier: prot#448). Written informed consent was obtained from all patients.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article and its additional files.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

C.B. and S.M.: analyzed data and co-wrote the manuscript. S.R.: performed experiments. A.M.: managed clinico-pathological data. D.N.: provided critical revision of the manuscript. All authors read and approved the final manuscript.

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Senthilkumar Rajendran[1,2*]
Clara Benna[1,2*]
Alberto Marchet[3]
Donato Nitti[1,2]
Simone Mocellin[1,4]

[1]*Department of Surgery Oncology and Gastroenterology, University of Padova, Padua 35128, Italy*
[2]*First Surgical Clinic, Padova University Hospital, Padua 35128, Italy*
[3]*Multidisciplinary Day-surgery Unit, Padova University Hospital, Padua 35128, Italy*
[4]*Surgical Oncology Unit, Veneto Institute of Oncology IOV-IRCCS, Padua 35128, Italy*

Correspondence
Clara Benna, Department of Surgery Oncology and Gastroenterology, University of Padova, Padua 35128, Italy and First Surgical Clinic, Padova University Hospital, Padua 35128, Italy.
Email: clara.benna@unipd.it

[*]These authors contributed equally to this work.

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SUPPORTING INFORMATION

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