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EPIDEMIOLOGY

The circadian gene *NPAS2* is a novel prognostic biomarker for breast cancer

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Abstract Mounting evidence suggests that neuronal PAS domain protein 2 (*NPAS2*) and other circadian genes are involved in tumorigenesis and tumor growth, possibly through their control of cancer-related biologic pathways. A missense polymorphism in *NPAS2* (Ala394Thr) has been shown to be associated with risk of human tumors including breast cancer. The current study further examined the prognostic significance of *NPAS2* in breast cancer by genotyping the Ala394Thr polymorphism and measuring *NPAS2* expression. DNA extracted from 348 breast cancer tissue samples was analyzed for *NPAS2* genotype using the TaqMan allelic discrimination assay. Of these, 287 also had total RNA available for use in real-time PCR assays to determine *NPAS2* expression. *NPAS2* genotypes and expression levels were analyzed for associations with prognostic outcomes, as well as correlations with clinical characteristics. A high level of *NPAS2* expression was strongly associated with improved disease free survival (AHR = 0.43, 95% CI: 0.21–0.86, *P* trend = 0.022) and overall survival (AHR = 0.42, 95% CI: 0.19–0.96, *P* trend = 0.036). In addition, there was a borderline, but nonsignificant association between the *NPAS2* genotype corresponding to Thr394Thr and disease free survival (AHR = 1.82, 95% CI: 0.96–3.46). The Ala/Ala, Ala/Thr, and Thr/Thr genotypes were also differentially distributed by tumor severity, as measured by TNM classification (χ^2 (6df, $N = 344$) = 14.96, $P = 0.020$). These findings provide the first evidence suggesting prognostic significance of the circadian gene *NPAS2* in breast cancer.

Keywords Circadian gene · *NPAS2* · Breast caner · Survival

Introduction

Circadian rhythm is the 24 h oscillation of many biologic and physiologic processes, such as cell division, proliferation, and metabolism [1, 2]. Circadian oscillations are maintained by transcriptional/posttranslational feedback loops among the core circadian genes [3]. Up to now, nine mammalian core circadian genes have been identified, which are expressed in the SCN as well as peripheral tissues [4]. *NPAS2* is the largest circadian gene (176.68 kb) and is located on chromosome 2 at 2q11.2 [5]. It is a member of the basic helix-loop-helix (bHLH)-PAS family of transcription factors that is expressed in the mammalian forebrain and several peripheral tissues [5, 6]. NPAS2, an essential component of the feedback loop, dimerizes with ARNTL, another core circadian protein, and binds to DNA sequences to activate gene transcription [7, 8]. NPAS2/ARNTL heterodimer controls the transcription of two other circadian genes *PER* and *CRY*, which are required for maintaining biologic rhythms in many organisms [7–10].

Emerging data have demonstrated that *NPAS2* has a substantial impact on tumor related biologic pathways, possibly through regulation of cancer-related genes, such as those involved in cell cycle checkpoint and DNA repair.

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In mice, the NPAS2/ARNTL heterodimer has been shown to bind to the promoter of the oncogene *Myc* and suppress its transcription [11]. Our functional assays have also demonstrated that *NPAS2* affects cell cycle arrest and DNA repair, and an expression assay has further indicated that *NPAS2* regulates the expression of critical genes in those processes [12].

NPAS2 is a conserved gene and has only one nonsynonymous polymorphism in its entire coding sequences, according to the NCBI dbSNP database. This SNP, an A to G coding polymorphism (dbSNP ID: rs2305160), results in a substitution at amino acid position 394 from alanine (Ala) to threonine (Thr). The Thr variants (Ala/Thr and Thr/Thr genotypes) have been previously shown to be associated with reduced risk of non-Hodgkin's Lymphoma, especially B-cell lymphoma [13], while the Ala/Thr genotype has been associated with decreased risk of breast cancer [14], as well as reduced risk of prostate cancer [15].

As a potential tumor suppressor, *NPAS2* expression could be deregulated in tumorigenesis. Expression of *PER1* and *PER2*, two *NPAS2* target genes, has been shown to be down-regulated in breast cancer compared to normal breast tissue [16, 17]. However, the expression of *NPAS2* in cancer has yet to be determined. In addition, little is known about the genetic and expression variations of *NPAS2* in relation to clinical characteristics of breast cancer. No association between circadian genes and cancer survival has been reported previously, apart from a very recent report showing that the combination of low *CRY1* and low *ARNTL* expression was a prognostic factor in ovarian cancer, but either *CRY1* or *ARNTL* alone was not associated with ovarian cancer survival [18]. To evaluate whether *NPAS2* could be a potential marker to predict breast cancer prognosis, we analyzed *NPAS2* genotype and phenotype (mRNA expression) using tissue samples from breast cancer patients in the current study.

Materials and methods

Breast cancer sample collection

Three hundred and forty eight patients who underwent surgery for primary breast cancer in the Department of Gynecologic Oncology at University of Turin between January 1998 and July 1999 were recruited into a clinical study of breast cancer. The study was approved by the university's ethical review committee. Fresh tumor samples were collected from the patients during surgery. The tissue specimens were snap-frozen in liquid nitrogen immediately after resection and then stored at −80°C until analysis. Clinical and pathological information collected for the study includes age at surgery, tumor grade, tumor size, number of lymph nodes that tested positive for cancer, histologic type, postoperative treatment, and treatment response. Tumors were staged according to the TNM (Tumor classification, lymph Node status, and Metastases) classification system [19]. Patients were followed from surgery through Feb 2007. Follow-up was scheduled every 4 months for the first 2 years, every 6 months from year 3 to year 5, and once a year thereafter. The median follow-up time among the patients was 86.2 months, ranging from 8 to 108 months. During the follow-up, information on relapse and death was collected; 81 patients experienced relapse, and 60 died during the course of follow-up. The average age at surgery was 57 years, and the range was 23–83 years. REMARK (REporting recommendations for tumor MARKer prognostic studies) is a set of guidelines which establishes standards for reporting relevant details of tumor marker studies, including prespecified hypotheses, patient and specimen characteristics, assay methods, study design, data analysis, data presentation, and interpretation of the study findings [20]. The research design and data presentation of this study is adherent to REMARK guidelines.

Genotyping

Frozen tumor samples were pulverized manually in liquid nitrogen. Genomic DNA was extracted from approximately 100 mg of tissue powder following a standard phenol–chloroform protocol. The TaqMan assay was used to determine *NPAS2* genotypes (C_15976652_10 for SNP rs2305160, Applied Biosystems, Inc., Foster City, CA). The assay was performed using PCR conditions of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Plates were then read by the ABI7500 instrument and data were analyzed by the allelic discrimination function of the Fast 7500 software (Applied Biosystems, Inc.). Determination of genotypes was performed independently by observers blinded to clinical data.

Real-time PCR

Pulverized tissue powders were also used for total RNA extraction following a standard phenol–chloroform protocol. The RNA samples were treated with DNase to remove genomic DNA contamination and were concentrated using the RNeasy MinElute Cleanup kit (Qiagen Inc., Valencia, CA). One microgram of total RNA from each sample was processed for conversion to cDNA using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA).

cDNA samples from 287 patients were available to be used in the real-time PCR assay. Primers for *NPAS2* (forward: TCTGGATCACAGAGCACCTC, reverse: CAG GAGCTCCAGGTCATCA) and hypoxanthine-guanine

phosphoribosyltransferase (*HPRT1*) (forward: GGATATAAGCCAGACTTTGTTGG, reverse: CAAACATGATTCAAATCCCTGA), were designed in-house and chemically synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Real-time PCR was performed in duplicate using the Power SYBR green master mix (Applied Biosystems, Inc.) on an ABI7500 Fast real-time PCR instrument (Applied Biosystems, Inc.). The PCR conditions included incubation at 95°C for 10 min to activate Taq polymerase, and 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 30 s. In each sample, the expression of *NPAS2* was normalized to the expression of the housekeeping gene *HPRT1*, and *NPAS2* expression was quantified as $1{,}000 \times 2^{-\Delta Ct}$. Calculation of *NPAS2* expression levels from the real-time PCR data was independently performed by observers blinded to clinical data.

Statistical analysis

All statistical analyses were performed using the SAS statistical software, version 9.1 (SAS Institute Inc., Cary, NC). Associations of *NPAS2* genotypes and expression with clinical characteristics, including age at surgery, histologic subtype, tumor size, lymph node involvement, tumor grade, and estrogen and progesterone receptor status were examined using the χ^2 test (for genotype) or analysis of variance (ANOVA, for expression). Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using Cox proportional hazards regression analysis to examine the association of *NPAS2* genotypes and expression levels with patient survival. An adjusted hazard ratio (AHR) with 95% CI was calculated from a Cox proportional hazards model that included age at diagnosis (continuous), TNM stage (4 categories), grade (3 categories), histologic subtype (2 categories), estrogen receptor (continuous) and progesterone receptor status (continuous). No variables used in the modeling violated the proportional hazards assumption, and no notable interactions were detected. *P* values <0.05 were considered statistically significant. Kaplan–Meier survival analysis was used to plot the proportion of the population that were alive (overall survival) or cancer-free (cancer-free survival) by the length of follow-up, in months.

Results

NPAS2 genotypes and expression were associated with clinical characteristics

There was no significant difference in *NPAS2* expression by genotype at Ala394Thr ($P = 0.704$), and therefore *NPAS2* expression was considered independent from its genotype at this particular locus. Distributions of the *NPAS2* genotypes and mRNA expression by various clinical and pathological variables are shown in Table 1. A significant association was noted with tumor severity, as measured by TNM classification, with genotypes unequally distributed across the four TNM stages (χ^2 (6df, $N = 344$) $= 14.96$, $P = 0.020$). The vast majority of the patients were in PT1c and PT2 TNM stages (93%). The Ala/Thr

Table 1 Distributions of *NPAS2* genotypes and expression levels with clinical characteristics

Variable	Genotype			Expression
	Ala/Ala	Ala/Thr	Thr/Thr	Median[a]
Age (years)				
<50	60 (55%)	35 (32%)	14 (13%)	726.2
50–65	63 (46%)	56 (41%)	17 (13%)	861.8
65+	45 (45%)	43 (43%)	11 (11%)	1,172.0
P value[b]		0.493		0.171
Histologic type				
Ductal	108 (50%)	82 (38%)	26 (12%)	744.2
Lobular	27 (49%)	23 (42%)	5 (9%)	1,230.7
Mix	15 (43%)	13 (37%)	7 (20%)	1,400.0
Others	17 (46%)	16 (43%)	4 (11%)	822.5
P value[b]		0.810		0.145
Lymph node				
Negative	84 (47%)	70 (39%)	24 (13%)	900.5
Positive	81 (51%)	63 (39%)	16 (10%)	726.2
P value[b]		0.586		0.079
Tumor grade				
G1/2	89 (46%)	78 (40%)	28 (14%)	1,059.4
G3	76 (53%)	55 (38%)	13 (9%)	751.6
P value[b]		0.237		**0.021**
TNM classification				
PT1a, PT1b	10 (32%)	18 (58%)	3 (10%)	730.5
PT1c	76 (46%)	63 (38%)	28 (17%)	1,047.3
PT2	69 (58%)	40 (34%)	10 (8%)	580.1
PT3, PT4	11 (44%)	13 (52%)	1 (4%)	1,240.0
P value[b]		**0.020**		**0.011**
Estrogen receptor status				
Negative	59 (50%)	48 (40%)	12 (10%)	812.4
Positive	104 (47%)	86 (39%)	30 (14%)	893.8
P value[b]		0.637		0.170
Progesterone receptor status				
Negative	87 (54%)	58 (36%)	16 (10%)	870.0
Positive	75 (42%)	76 (43%)	26 (15%)	820.7
P value[b]		0.085		0.966

[a] *NPAS2* expression was normalized to the expression of *HPRT1* and quantified as $1{,}000 \times 2^{-\Delta Ct}$

[b] *P* values are for the χ^2 test (genotyping) or for analysis of variance (expression), and significant results ($P < 0.05$) are shown in bold

and homozygous Thr/Thr genotypes were differentially distributed by tumor severity, as measured by TNM Classification of Malignant Tumors stage. The expression of *NPAS2* was higher in lobular than ductal carcinomas. It was also higher in low grade tumors compared to high grade tumors ($P = 0.021$), and slightly higher in lymph node negative cases compared to lymph node positive cases ($P = 0.079$).

NPAS2 genotype Thr/Thr was associated with poor survival

The adjusted survival analysis showed that there was a borderline, but not statistically significant association between the homozygous Thr/Thr genotype and poor disease-free survival compared to the Ala/Ala and Ala/Thr genotypes (AHR = 1.82, 95% CI: 0.96–3.46) (Table 2). The Thr/Thr genotype also appeared to be associated with poor overall survival compared to the Ala/Ala and Ala/Thr genotypes, although again, this association did not reach statistical significance (AHR = 1.78, 95% CI: 0.81–3.91). We further stratified the analysis by adjuvant treatments including adjuvant chemotherapy, adjuvant hormonal therapy, and both. However, no significant results were detected (data not shown). Kaplan–Meier survival analysis was also performed in order to generate survival curves (Fig. 1). There were no significant associations detected between *NPAS2* genotypes and overall survival ($P = 0.854$) or disease-free survival ($P = 0.679$).

High NPAS2 expression was associated with improved survival

Cox proportional hazards regression analysis was also performed for *NPAS2* expression while adjusting for patient age at surgery, disease stage, tumor grade, histologic subtype, lymph node involvement, estrogen status, and progesterone status in the analysis. *NPAS2* expression levels were grouped into three categories: low, medium, and high, based on the tertile distribution of *NPAS2* expression among the control subjects. As shown in Table 2, high levels of *NPAS2* expression were associated with better disease-free survival in both unadjusted and adjusted survival analyses (HR = 0.38, 95% CI: 0.19–0.75, P trend <0.001; and AHR = 0.43, 95% CI: 0.21–0.86, P trend = 0.022). A similar significant association between high *NPAS2* expression and better overall survival was also observed (HR = 0.38, 95% CI: 0.17–0.86, P trend = 0.017; and AHR = 0.42, 95% CI: 0.19–0.96, P trend = 0.036). No significant results were detected (data not shown) if analyses were performed by stratifying the sample according to adjuvant treatments including adjuvant chemotherapy, adjuvant hormonal therapy, and both. Kaplan–Meier survival analysis was also performed, and survival curves are presented in Fig. 2. We found a borderline, but not statistically significant association between the level of *NPAS2* expression and overall survival ($P = 0.060$), and a significant association with disease-free survival ($P = 0.017$).

Discussion

The genetic variant in *NPAS2* (Ala394Thr) studied here, has previously been shown to be a susceptibility biomarker for human cancers in our earlier case–control studies [13–15]. The distribution of *NPAS2* genotypes detected in the current group of breast cancer samples is similar to those previously reported [14]. Our previous findings suggest that the variant Thr allele might have a protective role in cancer

Table 2 *NPAS2* genotype/expression and breast cancer survival from Cox proportional hazards analysis

NPAS2	Disease-free survival		Overall survival[b]	
	HR	AHR[a]	HR	AHR[a]
NPAS2 genotype				
Ala/Ala	1	1	1	1
Ala/Thr	0.83 (0.51–1.34)	0.89 (0.55–1.46)	0.86 (0.48–1.53)	0.88 (0.49–1.59)
Thr/Thr	1.06 (0.55–2.01)	1.73 (0.88–3.40)	1.02 (0.46–2.23)	1.69 (0.74–3.84)
P for trend	0.857	0.384	0.858	0.553
Any Ala	1	1	1	1
Thr/Thr	1.15 (0.62–2.12)	1.82 (0.96–3.46)	1.09 (0.51–2.30)	1.78 (0.81–3.91)
NPAS2 expression				
Low	1	1	1	1
Medium	0.92 (0.54–1.57)	0.97 (0.56–1.69)	0.66 (0.34–1.30)	0.72 (0.36–1.45)
High	**0.38 (0.19–0.75)**	**0.43 (0.21–0.86)**	**0.38 (0.17–0.86)**	**0.42 (0.19–0.96)**
P for trend	**0.007**	**0.022**	**0.017**	**0.036**

Significant results ($P < 0.05$) are shown in bold

[a] Adjusted Hazard Ratio, adjusting for age, TNM stage, grade, histologic type, ER and PR

[b] Breast cancer-specific death

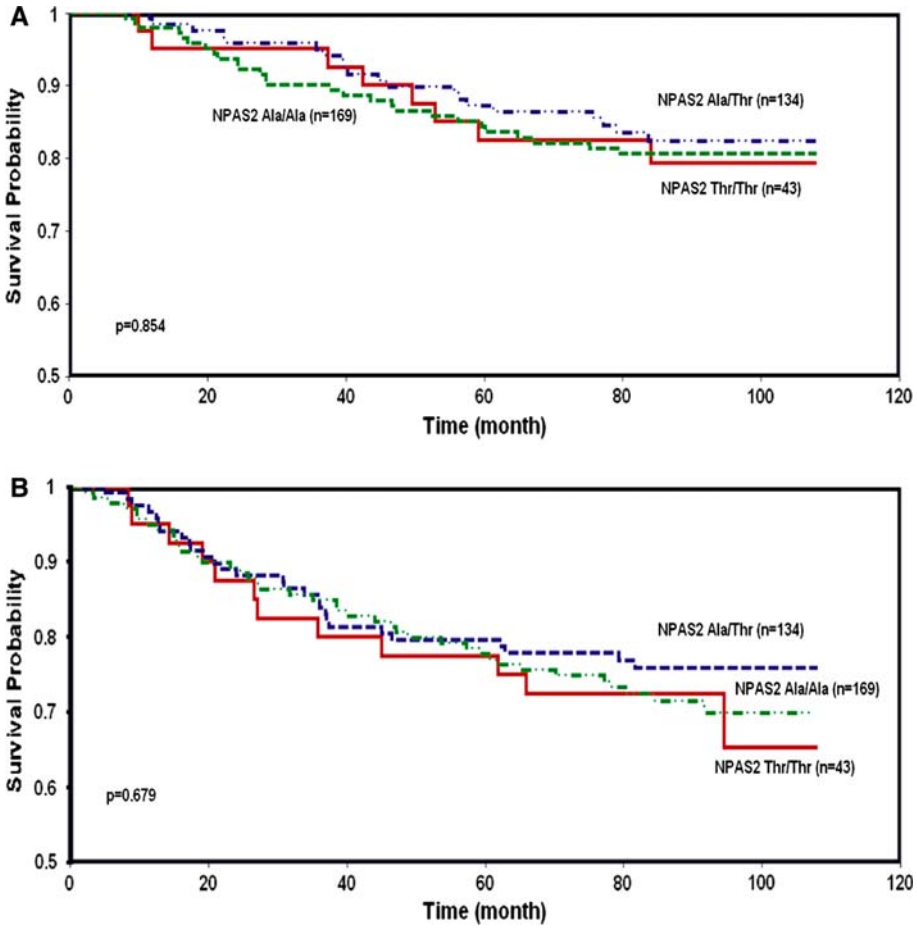

Fig. 1 Kaplan–Meier survival analysis; **a** overall survival and **b** disease-free survival, by *NPAS2* genotypes. The figure demonstrates the effect of the *NPAS2* genotypes on survival (months) among breast cancer patients. The genotypes are Ala/Ala (homozygous wild type, $n = 169$); Ala/Thr (heterozygous, $n = 134$); and Thr/Thr (homozygous variant, $n = 43$). There were no significant associations detected between *NPAS2* genotypes and overall survival ($P = 0.854$) or disease-free survival ($P = 0.679$)

development. However, the current results show that the homozygous Thr394Thr genotype is marginally associated with poorer disease-free survival in breast cancer patients. These findings, suggesting a protective role in tumor development, but a deleterious effect in terms of survival, could be due to the potential tumor suppressor role of the *NPAS2* gene in cell cycle checkpoint and DNA damage response [7]. A pathway-based expression analysis of genes important for DNA damage signaling demonstrated that knockdown of *NPAS2* significantly represses the expression of several cell cycle and DNA repair genes [12]. The Ala394Thr polymorphism is located in the PAS domain of NPAS2, which is a signal sensor domain and may affect protein sensory capacity or impede NPAS2/ARNTL heterodimerization [14]. Since the Thr allele is associated with protection from tumor initiation, this variant may enhance the tumor suppressor capacity of *NPAS2* and facilitate DNA repair capacity. After tumor formation, however, tumor cells with high DNA repair capability are hard to destroy in tumor treatments and may cause poor survivorship. Our observations are also congruent with previous findings that genetic polymorphisms associated with increased cancer risk tend to be related to disease-free survival of cancer patients [21].

In addition to *NPAS2* genotypes, our results demonstrated that high levels of *NPAS2* expression are significantly associated with tumor grade, overall survival, and disease-free survival in breast cancer patients. These results, especially those relating high *NPAS2* expression to small size, low grade, and lymph node negative tumors, suggest that high *NPAS2* expression may have a protective role in breast cancer progression. Tumors with small size, low tumor grade, and negative lymph node involvement usually predict better survival, which could explain the observed correlation between high *NPAS2* expression and better survival. Subgroup analyses were also done to explore the effects of adjuvant treatment on the relationship between *NPAS2* and breast cancer survival and no significant associations were detected in any of the treatment subgroups. Since the sample size was quite small, subgroup analyses may not have sufficient power to detect any significant associations.

Although the molecular mechanisms for the role of *NPAS2* in tumor progression are not clear, as a transcriptional regulator, *NPAS2* may influence the expression of other cancer-relevant genes. NPAS2 binds to E-Box motifs of its target genes and may directly or indirectly affect circadian regulation and other cancer related pathways

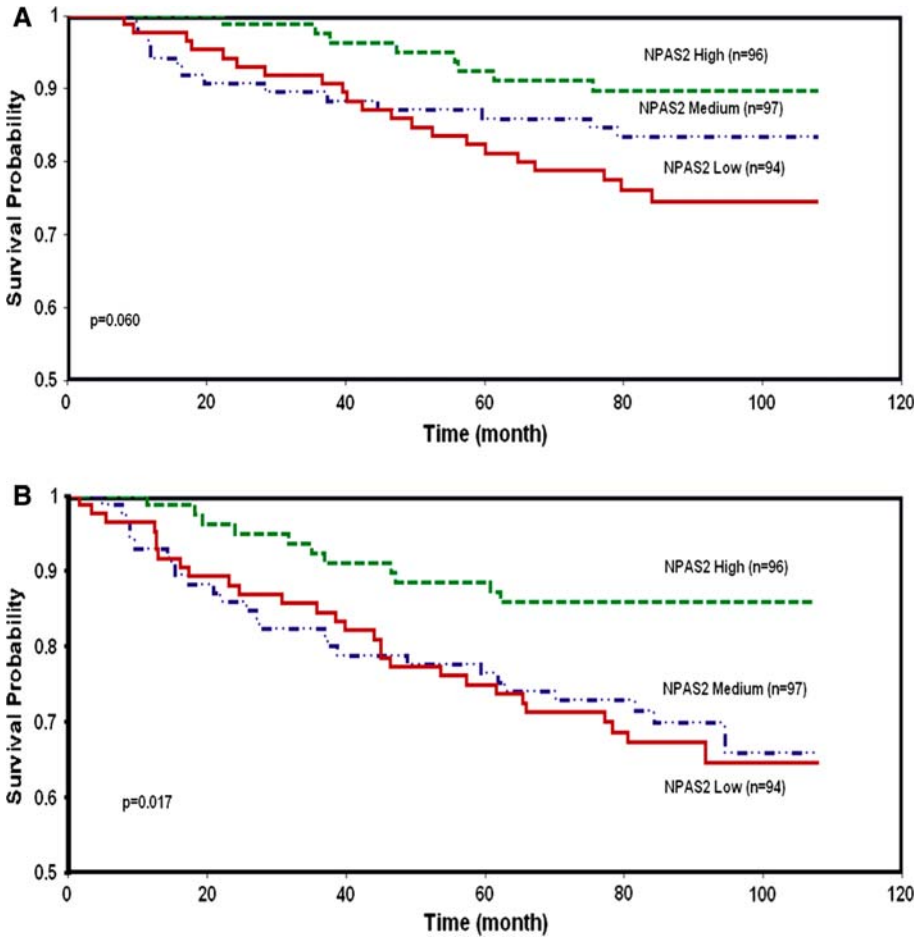

Fig. 2 Kaplan–Meier survival analysis; **a** overall survival and **b** disease-free survival, by levels of *NPAS2* expression. The figure demonstrates the effect of the levels of *NPAS2* expression on survival (months) among breast cancer patients. *NPAS2* expression was grouped into three categories: low ($n = 94$), medium ($n = 97$), and high ($n = 96$), based on the tertile distribution of *NPAS2* expression among the control subjects. The level of *NPAS2* expression showed a borderline, but nonsignificant association with overall survival ($P = 0.06$), and was significantly associated with disease-free survival ($P = 0.017$)

[22]. For example, the heterodimer of transactivators CLOCK or NPAS2 with ARNTL regulate the transcription of *PER1*, *PER2*, *CRY1*, and *CRY2* [7]. Two of these circadian regulators, *PER1* and *PER2*, have been linked to cell cycle and DNA damage response pathways [11]. Overexpression of either *PER1* or *PER2* in cancer cells inhibits their neoplastic growth and increases their rate of apoptosis. Loss or dysregulation of *PER1* and *PER2* expression has been found in many types of human cancer, and their expression levels are decreased in breast tumors relative to normal breast tissue [17]. Recently, it has also been shown that *PER1*, *PER2*, *CRY2*, *CLOCK*, and *CSNK1E* were all significantly down-regulated in ovarian cancer [18]. Decreased expression of circadian genes may result in disturbance of cell cycle regulation, and is correlated with tumor size [23]. On the other hand, overexpression of *PER1* and *PER2* may decrease cancer cell growth both in vitro and in vivo [24, 25], and induces apoptosis [26]. Given that *NPAS2* positively controls *PER1* and *PER2* expression, high levels of *NPAS2* expression may increase *PER1* and *PER2* expression and consequently decrease the growth of cancer cells. This could be one molecular explanation for the observed association between high levels of *NPAS2* expression and better survival among breast cancer patients.

One potential limitation of this study is that a single sample, taken at one time point, was used to represent *NPAS2* expression. The expression of circadian genes, including *NPAS2*, usually undergoes a daily oscillation. This raises a concern that this may not be representative of the *NPAS2* expression levels, which are present at different stages of the circadian phase. However, cancer tissues are composed of heterogeneous populations of tumor cells that are not circadian synchronized. Recently, Tokunaga et al. showed that there was no relationship between tumor removal time and the expression level of circadian genes in ovarian tumor [18]. Furthermore, it has been determined that circulating breast tumor cells have no circadian rhythm [27]. As such, adjacent individual tumor cells may be in various stages of the circadian phase, and it is therefore appropriate to compare expression levels between patients, as any sample of tumor cells should represent the average expression for a given individual over all stages.

In summary, the findings from the current study indicate that the expression of *NPAS2* may serve as a strong prognostic biomarker, and the genotype of *NPAS2* may be

a marginal prognostic biomarker, in addition to its association with breast cancer risk. This is one of the first reports relating expression levels of a circadian gene and cancer survival, and further examination of *NPAS2* and other circadian genes as prognostic biomarkers in additional cancer types is warranted.

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