ONCOLOGY

Expression of Circadian *Per1* and *Per2* Genes in the Liver and Breast Tumor Tissues of HER2/neu Transgenic Mice of Different Age

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The expression of *Per1*, *Per2*, and *Cry1* circadian genes in the liver and breast tumors were studied by real-time PCR in FVB/N mice of different age transfected with *HER-2/neu* gene. The expression of *Per1* and *Per2* genes in breast tumor tissue decreased in comparison with their expression in the lever. The expression of these genes decreased with age in both the liver and tumor tissue.

Key Words: *circadian genes; liver; breast tumor; aging*

Alternation of the day/night circadian cycle is one of the regulators of physiological rhythms in humans and animals. It is the key factor in adaptation of an organism to 24-h changes in environmental conditions (temperature, illumination, food availability, *etc.*) [1,11]. Significant shifts in circadian rhythms caused by genetic defects or exposure to environmental factors can lead to serious disorders in physiological processes [8]. Disturbances of circadian rhythms can be a risk factor for the development of some tumors, *e.g.* breast cancer [2,9,13]. In addition, genetic variety of circadian genes can be responsible for different conditions of physiological processes regulated by these genes and hence, result in different risk of various pathologies and drug sensitivity. Molecular mechanisms regulating the involvement of these genes in carcinogenesis processes in aging mammals remain little studied.

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Here we studied the role of circadian rhythm genes in the development of breast carcinoma (BC) in aging transgenic HER-2/neu mice.

MATERIALS AND METHODS

The study was carried out on archive specimens of tissues embedded in paraffin, which were collected from FVB/N homozygotic transgenic mice aged 8-15 months (as the tumors are detected in these mice from the age of 5-7 months) carrying excessive copies of *HER-2/neu* oncogene [3,4]. Twenty-three BC specimens and 11 specimens of normal liver tissue were examined. Paraffin sections (5-6 μ) were prepared using Thermo Scientific histological station.

The sections were placed into microtubes and RNA was isolated [10]. The volume and quality of total RNA were evaluated by electrophoresis and spectrophotometry. cDNA first chain was synthesized using a reverse transcription kit (Silex). Total RNA (1 μg), 0.5 μg/μl random hexaprimers, and sterile water (to a volume of 20 μl) were mixed in a 1.5 ml micro-

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tube. The mixture was incubated in a thermostat (5 min, 70°C), after which the tube was cooled. Ten-fold reaction buffer (2.5 μl), 4 μl 1.5 mM mixture of deoxynucleotide triphosphate (dNTP), and 0.5 μl (200 U) reverse transcriptase M-MLV were added to the tube. The mixture was incubated 10 min at ambient temperature and then 60 min at 37°C. The reaction was stopped by 10-min heating at 70°C. The quality and volume of the resultant cDNA were evaluated by spectrophotometry. Gene expression was evaluated by real time PCR.

SYBR Green intercalating stain served as a fluorophore for detection of the products. Amplification was carried out in an iCycler with an iQ5 optical attachment (Bio-Rad) using amplification kits (Sintol). The PCR mixture (25 μl) contained: 50 ng cDNA, 12.5 μl 2xSYBR Green RT-PCR Reaction Mix, 0.8 μl reverse and 0.8 μl forward primers (10 μM), 9.5 μl nuclease-free water. Specific primers for genes amplification: *Per1* f: AGGAGGCCCCGGAAGTAGT; *Per1* r: AGCCTGAAAGTGCATCCTGATT; *Per2* f: GACGGGTCGAGCAAAGGA; *Per2* r: GGGAAAAGTCCACATATCCATTCA; *Cry1* f: GAAGAGGACGCACAGAGTGTTG; *Cry1* r: TCTGGAAAAATGTGTCC; *Gapdh* f: CCATCAACGACCCCTTCATT; *Gapdh* r: TCTCGTGGTTCACACCCCATC. The PCR protocol: PCR cycles: 10 sec at 95°C, 30 sec at 60°C (40 cycles). Specificity of amplification products was verified by melting of PCR fragments: 1 min at 95°C, 1 min at 62°C, and 10 sec at 62°C (70 cycles, the temperature elevated by 0.5°C in each cycle).

Gene expression was evaluated by the ratio of target/reference gene signal intensities.

The significance of differences between the control and tumor specimens was evaluated using the Mann—Whitney test.

RESULTS

The level of *Per1* gene expression was significantly ($p<0.05$) lower in BC tissue of mice aged 8-15 months than in normal liver tissue, except the age of 9 months, when no significant differences were detected (Table 1). Our data are in line with the results of studies demonstrating decreased expression of circadian genes in BC patients [5,12]. On the other hand, *Per1* hyperexpression in HCT116 rectal carcinoma cells stimulated their sensitivity to apoptosis induced by infrared exposure [7]. It can be hypothesized that *Per1* gene plays a certain role in BC growth inhibition. Since *Per* gene protein product is a repressor of *Clock* and *Bmal1* genes, this result suggested disorders in the circadian rhythm in BC of mice carrying *HER-2/neu* oncogene.

It was shown on the model of mice with mutant *Per2* gene that inactivation of that gene promoted the development of tumor in inbred mice and stimulated their sensitivity to γ-radiation [6]. The level of *Per2* gene expression in the liver tissue was in most cases

TABLE 1. Relative Expression of Circadian Genes in the Liver and Breast Adenocarcinomas in HER-2/neu Transgenic Mice (*M*±*m*; *n*=2-4).

Organ	Age, months	Expression of circadian genes, relative expression		
		Per1	*Per2*	*Cry1*
Liver	8	4.68±0.68	0.03±0.01	0.17±0.14
	8.5	1.19±0.4^{oo}	1.244±0.25^{oo}	0.33±0.2
	9	0.35±0.33^{oo}	0.044±0.01^{++}	0.12±0.05
	11	0.74±0.03^{oo}	0.153±0.03^{oo++}	0.56±0.33
	15	0.057±0.026$^{oo+\#\#}$	0.773±0.19^{oo}	0.152±0.06
Mammary gland	8	0.327±0.195**	0.02±0.006	1.48±0.7
	8.5	0.17±0.19*	1.4±0.2^{oo}	0.41±0.17
	9	0.595±0.174	0.04±0.01^{++}	0.22±0.04
	11	0.035±0.006**^{x}	0.069±0.39^{+}	0.38±0.02^{xx}
	15	0.071±0.04^{x}	0.447±0.08^{oo++xx}	0.205±0.05$^{\#}$

Note. Each value represents the data of 3 independent experiments. *$p<0.05$, **$p<0.001$ compared to the liver. Differences in the expression of a gene in a group: compared to the parameter at the age of 8 months: $^{oo}p<0.001$; compared to the parameter at the age of 8.5 months: $^{+}p<0.01$, $^{++}p<0.001$; compared to the parameter at the age of 9 months: $^{x}p<0.01$, $^{xx}p<0.001$; compared to the parameter at the age of 11 months: $^{\#}p<0.01$, $^{\#\#}p<0.001$.

higher than in breast tumors. The difference was significant ($p<0.05$) in mice aged 11 months.

The *Cry1* gene protein product is a repressor of *Clock* and *Bmal1* genes expression, and hence we compared the expression of this gene in normal liver and BC tissue of animals. The expression of *Cry1* gene in these tissues virtually did not differ in mice aged 8.5-15 months. However, at the age of 8 months the expression of this gene in tumor tissue was 1.4-fold higher than in the liver ($p<0.05$).

These data attest to reducted expression of *Per1* and *Per2* circadian rhythm genes in breast tumors of transgenic HER-2/neu mice. Presumably, the regulation of circadian genes is disturbed during carcinogenesis and the disorders manifest primarily in reduced expression these genes. Changed expression of circadian genes, in turn, could modulate activation of signal pathways regulating the cell cycle and apoptosis, thus potentiating oncogenesis.

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