Deletion of the *hPER3* gene on chromosome 1p36 in recurrent ER-positive breast cancer.

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We thank. YH Fu and LJ Ptáček for providing *Per3* knockout mice and Z Werb for providing FVB-MMTV-*neu* mice. We also thank MD To for helpful discussion of the manuscript. These studies were supported by grants from National Cancer Institute (U01 CA84244) to A. Balmain, from Spanish Ministry of Education and Culture (EX-2005-1059) and Department of Defense (BC063443) to J. Climent, from "Ramon y Cajal" Program, Fondo de Investigaciones Sanitarias (PI070057), "Junta de Castilla y León" and Sandra Ibarra Foundation to J. Perez-Losada and from California Breast Cancer Research Program (I5FB-0099) to KY. Jen. A. Balmain acknowledges support from the Barbara Bass Bakar Chair of Cancer Genetics.

The authors declare that they have no competing financial interest.

ABSTRACT

The PER3 gene is a member of a conserved family of genes linked to control of the circadian cycle in flies, mice and humans. We show that deletion of the *PER3* gene located on human chromosome 1p36 is directly related to tumor recurrence in patients with estrogen receptor (ER) positive breast cancers treated with Tamoxifen. Low expression of *PER3* mRNA is associated with poor prognosis, particularly in a subset of tumors that are ER-positive, and either luminal-A type or ERBB2-positive tumors. Mice deficient in *Per3* showed increased susceptibility to breast cancer induced by carcinogen treatment or by over-expression of *Erbb2*. Epidemiological evidence suggests that disruption of sleep patterns plays a significant role in susceptibility to breast cancer, and inherited genetic variants in PER3 have previously been associated with both phenotypes. Disruption of PER3 function could provide a link between deregulation of sleep homeostasis and breast tumorigenesis, and may serve as an indicator of probability of tumor recurrence in patients with ER-positive tumors.

INTRODUCTION

Chromosomal region 1p36 is among the most commonly deleted regions in human cancers. Deletion of 1p36 is especially frequent in breast tumors and is associated with progression and lymph node metastasis[1], poor prognosis[2] higher rate of recurrence[3], larger tumor size and DNA aneuploidy[4]. However, no direct relationship between breast carcinogenesis or prognosis and any specific tumor suppressor gene on 1p36 has been established. Recent elegant studies have identified CHD5[5] and more recently KIF1B[6] as candidate tumor suppressor genes in this region, but no specific roles for these genes in breast cancer development have been demonstrated.

The human *PER3* gene is located within 1.5Mb of CHD5, and the mouse homologue is a member of the *Period* gene family that controls circadian rhythms[7,8]. Members of the *Period* family of circadian rhythm genes (*Per1* and *Per2*) have been implicated in cell cycle control, DNA damage responses and tumor progression[9-13]. Although inactivation of *mPer3* in the mouse germline has only subtle effects on circadian clock function[14], it has been shown that m*Per3* transcripts exhibit a clear circadian rhythm both in the suprachiasmatic nucleus (SCN)[7] and in mouse peripheral tissues[15]. Similar data have been shown in human peripheral blood cells, where circadian oscillations were more robust for *PER3* expression than for other clock genes including *PER1* and *PER2*[16,17]. The possible functions of *PER3* in tumor development have not been explored, but links to breast cancer are supported by biochemical studies demonstrating the existence of complexes including proteins of the PER family together with the estrogen receptor[18,19], and by reports of association between a polymorphism in the human *PER3* gene and breast cancer susceptibility[20].

The location of the PER3 gene within a region that is commonly deleted in breast cancers suggested a possible link to epidemiological studies showing an association between disrupted sleep cycles and higher risk higher risk of developing breast cancer[21,22]. We used a combination of human breast tumor analysis and mouse models to show that disruption of *PER3* may serve as a prognostic biomarker of tumor recurrence in patients with ER+, Luminal A and/or ERBB2+ tumors.

RESULTS

Deletion of 1p36 and loss of *PER3* genetic variants in breast cancers.

We previously reported genome-wide array CGH profiles of 185 lymph node negative breast cancers from a Spanish cohort[23], of whom 85 received anthracycline chemotherapy (Chemo group), and 95 received no chemotherapy (non-Chemo group). To search for genetic events related to resistance to hormonal (Tamoxifen) therapy, we divided the non-Chemo group into two subgroups based on whether they had received hormonal treatment. Of the 95 patients in the non-Chemo group, 59 patients with ER and/or PgR positive tumors received Tamoxifen, whereas 36 did not receive any treatment. Analysis of CGH profiles for these patients revealed that deletion of chromosome 1p was associated with recurrence in this subgroup of ER+ Tamoxifen treated patients ($p < 0.05$ after multiple testing correction using method of Benjamini & Hoffberg) (Supplementary Fig. 1).

The chromosome 1p36 locus is frequently deleted in many human tumors, but the region of deletion is large, and separate, non-overlapping chromosome fragments have been implicated[24-26]. This suggests that multiple tumor suppressor genes are involved. We considered *PER3* to be a good candidate for involvement in breast cancer because of its location within one of the minimal deletion regions on 1p36.2 (Refs. 5,6), as well as the epidemiological[20] and mechanistic[18] data linking circadian rhythm genes to hormone status and breast cancer. We therefore examined the copy number status of *PER3* by quantitative TaqMan analysis in DNA samples from 180 breast cancer patients. The relationship between the frequency of deletion or copy number gain and clinico-pathological characteristics of the patients is shown in Supplementary Table 1. The number of copies of *PER3* showed a significant gene dosage association with recurrence-free survival at 10 years (Fig. 1a, $p= 0.01$). The proportion of disease free surviving patients after 10 years was lowest in patients with single copy *PER3* deletion (56% ± 8.6; red line) , compared to those with two (75% ± 4.0; blue line) or more (89% ± 5.6; green line) copies of the *PER3* gene (Fig. 1a). Further analysis showed that the effect of PER3 deletion was most pronounced in the Tamoxifen treated group, with no significant association in the non-treated or chemotherapy-treated

groups (Figs.1b-d). Among the 59 patients who only received Tamoxifen treatment (Fig. 1d), patients with single copy *PER3* deletions had a significantly lower disease-free survival rate at 10 years (47% ±12) than those with normal *PER3* (84%±6) or copy number gains (100% survival) (p=0.007). To look for potential inactivating mutations in PER3 in breast cancers, we initially sequenced the complete coding region of *PER3* in a panel of 35 breast cancer cell lines. No clear pathogenic (nonsense or missense) mutation was identified. However many known[27] and some other unknown polymorphisms and alternative splicing isoforms were found (see online supplementary data for full detailed description). One of the polymorphic variants identified by sequencing had been associated in other studies with breast cancer susceptibility[20] and also with disruption of sleep homeostasis[28-30]

Low expression of *PER3* is associated with reduced survival

We next examined *PER3* gene expression in 413 breast tumor expression arrays taken from two publicly available data sets (Van de Vijver[31] 2002, n=295 and Chin[32] 2007, n=118). A full description of the stratification of the patients into different subgroups according to *PER3* expression together with disease-free survival curves for all patients in each sub-group is shown in Figures 2 and 3. Patients with lower *PER3* expression ("*PER3* low", n=122) were significantly more likely to recur than those with normal or higher expression ("*PER3* normal/high", n=291) (Fig. 2a; p=0.013). Disease-free survival analysis showed that *PER3* low patients had significantly worse survival rates than *PER3* normal/high patients (p<0.001). ER status is an important predictor of recurrence and greatly influences treatment regimes[33,34]. If low expression of *PER3* segregates with ER status, any effect of low *PER3* expression could be confounded with the effect of ER status. We therefore performed a subset analysis of *PER3* in ER+ and ER- tumors. Low *PER3* levels were significantly associated with recurrence (p= 0.01) and shorter disease-free survival times (p<0.001) in patients with ER+, but not ER- tumors (Fig. 2b). We conclude that the association between low *PER3* expression and recurrence in the complete patient sample set was driven by the ER+ tumors, with no effect being detected in the ER- tumors. These data are

in agreement with the independent association between deletion of *PER3* and recurrence specifically in the Tamoxifen-treated (ER positive) patients in Figure 1d.

We next asked whether stratifying tumors according to their molecular subtype[35,36] could reveal additional information. The tumors were labeled using a nearest centroid classifier and a label was only assigned if correlation with a target class was above 0.1 (Refs. 31,32). This resulted in samples labeled Luminal A (n=90), Luminal B (n=68), ERBB2 (n=56), Normal-like (n=17), Basal (n=73), or Unclassified (n=109) (Fig. 3 and supplementary Fig 4). Of these groups, low *PER3* expression had significant association with recurrence only in Luminal A-type (p=0.007) or ERBB2-type tumors (p=0.03) (Fig. 3b). Disease-free survival analysis for Luminal A and ERBB2-type tumors indicated that *PER3* low patients had lower disease free survival rates at 10 years than those patients with *PER3* normal/high (28%± 10 vs 84%±4) for Luminal A ($p<0.001$) and (30%± 8 vs 68%±8) for ERBB2-type ($p= 0.004$). There was also a striking effect on overall survival rate at 10 years in all the patients and in the subgroups of ER positive, Luminal A and ERBB2 patients (Fig. 4): The ten year overall survival rate for ER+ patients with low *PER3* was 55% ± 6 vs. 79% ± 3 for normal/high patients ($p < 0.001$) (Fig. 4b). The overall survival rate was 25% ± 8 for ERBB2 patients with low *PER3*, vs. 70% ± 7 for ERBB2 patients with normal/high *PER3* ($p<0.001$) (Fig. 4f). The overall Survival rate at 10 years in Luminal-A patients with low *PER3* was 34% ± 11 vs. 83% ± 3 for patients with normal/high *PER3* ($p<0.001$) (Fig. 4g). Importantly, multivariate analysis showed that *PER3* expression is significant independently from all the prognostic factors tested both for Disease Free Survival ($p<0.001$) and Overall survival ($p=0.001$) (Table 1).

We next evaluated possible links between expression levels and probability of tumor recurrence for all 54 annotated genes in the 1p36.31-1p36.22 (chr1:6,084,440-9,512,808 (3.5 Mb in size)) region. Gene expression was discretized as described for *PER3* and log rank *p* values were calculated using the *survival* library for R. This analysis showed that *PER3* was the only gene with an uncorrected $p < 0.05$ in all data sets analyzed. Although chromosome engineering studies have previously identified *CHD5* as a candidate tumor suppressor gene within the minimal deletion region on 1p36.2 (Ref. 5), no association of *CHD5* expression levels with recurrence or survival was found in any of the subgroups of breast cancer

patients analyzed (Supplementary Figs. 5 and 6). These data do not exclude the possibility that *CHD5* plays an important role as a tumor suppressor in other tumor types.

Inactivation of *Per3* increases breast tumor susceptibility in mouse models.

In order to investigate a possible causal association between loss of *Per3* function and breast tumor development, we performed two studies involving mouse models of breast cancer. A total of 86 mice carrying normal or inactivated alleles of the *Per3* gene (17 wild-type $Per3^{+/+}$, 35 heterozygous $Per3^{+/-}$ and 34 null $Per3^{-/-}$) were treated by oral gavage with 7, 12-dimethylbenz[a]anthracene (DMBA), a protocol known to induce breast cancer in sensitive strains of mice[37]. Eight mice (two heterozygous and six null) were found dead before the end point and no tissues were collected from them. The median follow-up of the remaining 78 mice included in the study was 8.3 months (range 3.8 – 15.0). All of the mice treated with DMBA developed tumors of various kinds including lymphoma and solid tumors of the lung, ovary, and skin (Supplementary table 5). However, development of breast tumors was specifically associated with *Per3* deficiency. Thirty-six percent of $Per3^{-/-}$ mice treated with DMBA developed breast tumors, while 12% of the $Per3^{+/-}$ mice developed breast tumors. In striking contrast, none of the control $Per3^{+/+}$ mice developed a breast tumor ($p= 0.005$) (Fig. 3a). A group of 65 mice (19 wild-type, 25 heterozygous, and 21 null) were used as controls with no DMBA gavage treatment. Two of the $Per3^{-/-}$ control mice developed sporadic breast tumors, but none of the remaining mice were found sick or developed any other class of tumor during the time course of this experiment (24 months).

The second mouse model was based on the observation that low levels of *Per3* expression were strongly associated with recurrence in ERBB2-type human breast cancers. MMTV-Neu mice overexpress *ErbB2* in the mammary gland, and spontaneously develop breast tumors[38]. We generated a total of 79 MMTV-Neu positive mice of which 30 (38%) were $Per3^{+/+}$, 35 (44%) were $Per3^{+/-}$, and 14 (18%) were $Per3^{-/-}$. The median follow-up of all mice was 14.9 months (range 6.3 – 25.8). All $Per3^{-/-}$ mice developed breast tumors, whereas 25 (71%) of the $Per3^{+/-}$ and 14 (47%) of the $Per3^{+/+}$ mice developed breast tumors**.** The proportion of $Per3^{-/-}$ null mice free of tumors at 15 months (21% ± 8) was significantly lower than the

proportion in the heterozygous and the wild-type mice (63% ±6 in both *Per3*$^{+/-}$ and *Per3*$^{+/+}$, p = 0.003). Histological analysis of tumors from both models of breast cancer showed that loss of *Per3* did not affect the tumor class or morphology, since both DMBA-induced and MMTV-Neu-induced tumors in *Per3*-/- mice resembled equivalent tumors from *Per3* wild type animals (data not shown). We also evaluated the possible loss of the wild type *Per3* allele in tumors from the *Per3* heterozygous mice. No loss was observed suggesting that homozygous loss is not essential in this mouse model.

DISCUSSION

Our data indicate that deletion and/or reduced expression of the *PER3* gene on human chromosome 1p36 is associated with breast cancer recurrence, particularly in ER+ patients treated with Tamoxifen who did not receive chemotherapy. No effect of deletion was seen in patients with basal type ER- breast tumors. Within the ER+ category, the effect was primarily in tumors classified as Luminal A or ERBB2, but not in the Luminal B type which share some expression features with basal tumors[35,36]. Direct evidence for a causal role for loss of *PER3*, rather than an alternative gene in this commonly deleted region of the genome[5,6], comes from analysis of two different mouse models of breast cancer. Both chemically-induced and *Neu(ErbB2)*-induced breast cancers are increased in frequency and/or reduced in latency in mice carrying inactivated *Per3* alleles. Although these data do not prove that *Per3* is the only functional tumor suppressor gene in this chromosome interval, they indicate that *Per3* is a *bona fide* tumor suppressor in these mouse models, with a key role in breast tissue.

While disruption of the mouse *Period* gene family members *Per1* and *Per2* by gene targeting induces biological clock phenotypes[39], loss of *Per3* function induces only subtle effects on circadian rhythm[14,40] . Nevertheless, evidence in favor of *PER3* involvement both in sleep disruption and in breast cancer comes from studies of a human structural polymorphism in the *PER3* coding sequence that has been associated with delayed sleep phase syndrome, diurnal preference and waking performance[28,41,42], but also with increased breast cancer risk[20], particularly in premenopausal women.

Although the specific molecular mechanisms remain to be elucidated, increasing evidence points to a role for circadian rhythm genes in cell cycle control and DNA damage responses[11,43] as well as in hormonal control of gene expression[18,19]. PER2 has been identified as an estrogen-inducible ER co-repressor that forms heterodimers with PER3 to enter the nucleus. Deletion of *PER3* prevents nuclear import, and instead promotes accumulation of PER2 in the cytoplasm[44]. Whether coordinated functional deregulation of all *PERIOD* family genes occurs in breast cancers remains to be determined. Elucidation of the relationship between control of sleep homeostasis and circadian rhythms, *PER* gene expression and DNA damage responses may help in understanding the epidemiological data linking sleep disruption to breast cancer susceptibility[18,21,22], but further detailed studies will be required to elucidate the exact mechanisms involved.

METHODS

SAMPLE SELECTION

We used three previously published breast cancer data sets that included clinical, gene expression and/or array Comparative Genomic Hybridization (CGH) data[31,32]. Data on disease-free survival (defined as the time to a first event) and overall survival were available for all the patients in the three data sets except one patient in the Chin *et al.*[32] samples.

COPY NUMBER ANALYSIS OF *PER3*

All tumor DNA samples were obtained from frozen breast tumors with >50% tumor cells[23]. The genomic sequence of *PER3* (GenBank accession NM_016831.1) was used to design a set of primers and probe specific to the *PER3* gene (Primer Express software version 1.0 (Applied Biosystems)). The primers for *PER3* were 5'- GGAGTGAGAAACCGGTGTCTGT-3' (forward) and 5'-GCCCGCAGCCTGCTT -3' (reverse). The probe for *PER3* was 5'-(6-FAM) -CTGACTGCAAAGTGAG-(TAMRA)-3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. The primers and probe for RNase P used as an endogenous control gene were obtained from Applied Biosystems. The RNase P probe was labeled at 5' end with VIC (Applied Biosystems) instead of FAM. *PER3* copy number was determined by relative quantification using the ΔΔCt method normalized to the RNase P copy number of two[45]**.** To analyze the results from the copy number experiment we used the TaqMan® Gene Copy Number Assays Macro File (Applied Biosystems).

ISOLATION and SEQUENCING OF *PER3* cDNA.

We analyzed the sequence of PER3 cDNA in 35 breast cancer cell lines (see supplementary Tables 2 and 3, and Supplementary Fig. 2). No evidence for the presence of any non-conservative tumor-

specific structural changes was detected, although several known polymorphisms were found in this analysis.

PER3 GENE EXPRESSION ANALYSIS

We examined *PER3* expression in 413 breast tumor expression arrays taken from Van de Vijver[31] 2002 (n=295) and Chin[32] 2007 (n=118). In each dataset a sample s_i in the set *S* was labeled as "*PER3* Low", "*PER3* normal", or "*PER3* high" using the rule:

If $s_i \leq$ (*mean[S] - ½ *standard deviation[S]*), assign LOW

If $s_i \geq$ *(mean[S] + ½ *standard deviation[S]),* assign HIGH

Otherwise, assign NORMAL.

This method allowed us to compare relative *PER3* expression levels across both data sets fused as a single group of patients.

STATISTICAL ANALYSIS

The association between *PER3* deletion or *PER3* expression and clinical-pathological parameters was analyzed using Fisher's Exact test. All reported *P* values were two tailed. Significant differences in disease-free and overall survival time were calculated using the Cox proportional hazard (log-rank) test. Multivariate Cox Regression Analysis was used to prove statistical independence of *PER3* from other known prognostic factors. Statistical analysis was performed using SPSS version 12.0.

MICE AND TUMOR INDUCTION

Wild-type (*Per3*$^{+/+}$) and *Per3* knockout (*Per3*$^{-/-}$) 129/sv mice (provided by Drs. YH Fu and LJ Ptáček, UCSF) were bred and treated according to Laboratory Animal Resource Center (LARC) regulations. 7-week-old female mice from the F_2 intercross population (*Per3*$^{+/+}$, *Per3*$^{+/-}$ and *Per3*$^{-/-}$) were treated with 6 doses of 1 mg of 7, 12-dimethylbenz[a]anthracene (DMBA) diluted in corn oil by weekly oral gavage. A second group of mice was treated only with corn oil as a group control. In a second

experiment, male *Per3*$^{-/-}$ mice were crossed with female FVB mice expressing the *Neu (ErbB2)* protooncogene under control of the MMTV 3'-LTR promoter[38] (provided by Dr. Z Werb, UCSF) to generate F_1 transgenic mice heterozygous for *Per3* (*Neu/Per3*$^{+/-}$). F_1 males and females were intercrossed to produce the F_2 generation consisting of *Neu/ Per3*$^{+/+}$, *Neu/ Per3*$^{+/-}$ and *Neu/ Per3-/-* animals. Identification of animal genotypes is described in the Supplementary Data.

In the DMBA gavage experiment female mice were examined every three days for sickness or symptoms of tumor development for up to 19.7 months. MMTVneu/*Per3* transgenic female mice were examined weekly for mammary tumor development by palpation for up to 25.8 months. Mice that showed significant weight loss, morbidity or excessive tumor burden were sacrificed by cervical dislocation after being anesthetized according to the UCSF Animal Care and Use (IACUC) protocol. Tumors and tissues were fixed in 4% neutral buffered paraformaldehyde for histological examination. Mice found dead were censored from the study.

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Figure. 1.- **Association between Per3 deletion and disease-free survival in breast cancer patients. (a)** TaqMan copy number analysis of *PER3* in 180 lymph node negative breast cancer tumors (top left panel), showing decreased survival of patients with *PER3* deletions. Patients who received no treatment (36 patients, **(b)**) or were treated with anthracycline chemotherapy (85 patients, **(c)**) showed no effect of *PER3* deletion. **(d)** A subset of 59 patients that were ER and/or PGR positive and were treated only with tamoxifen showed strong association between survival and low *PER3* copy number.

Figure. 2.- **Association between PER3 gene expression and survival of breast cancer patients. (a)** *PER3* low expression (red) was found in 122 (30%) patients from both data sets. Kaplan-Meier analysis for all patients indicates that those patients with tumors with low expression of *PER3* (red) have lower disease free survival rates at 10 years than those patients with normal/high expression of *PER3 (*blue*)*.
(b) Comparison of *PER3* expression with Estrogen Receptor (ER) status. Low expression of *PER3* was less common in ER+ tumors, however those patients with ER+ tumors and low *PER3* expression show a higher risk of recurrence (lower left panel). No effect was seen in patients with ER- tumors. (right panel)

Figure. 3.- **Effect of *PER3* expression levels on survival according to molecular subtypes.** Kaplan–Meier estimates of Disease-Free Survival among the 413 patients, according to the Per3 expression. Patients were stratified using the Sorlie et al.[33,36] tumor classification. **(a)** In the Basal Tumors, the low expression of *PER3* gene had no effect in patient recurrence however in the Non Basal tumors those patients whose tumors had low expression of *PER3* showed a significant increase of recurrence. **(b)** The increase in recurrence was observed mainly in the Luminal A and ERBB2+ subgroup of tumors whereas no significant difference was observed in the Luminal B subgroup. P values were obtained using the log-rank test**.**

Figure. 4.- Kaplan-Meier Estimates of Overall Survival.
The different expression levels of Per3 were evaluated in all the patients **(a)** and the different subgroups of patients based on **(b)** ER positive **(c)** ER negative, and based on the different molecular subtypes using Sorlie et al[35,36] classification, **(d)** Basal, **(e)** Non Basal, **(f)** ERBB2+, **(g)** Luminal A and **(h)** Luminal B tumors. P values were obtained using the log-rank test**.**

Figure. 5.- Effect of loss of Per3 on tumor susceptibility in two different mouse models. (a) Breast cancer incidence in a group of mice treated with 7,12-dimethyl-benz[a]anthracene (DMBA) based in the different genotypes (WT +/+, HET +/-, Null -/-) **(b)** Kaplan-Meier estimates of probability of Tumor Free Survival in the group of MMTVneu-PER3 mice. P values were obtained using the log-rank test.

a

Variable	Disease Free Survival		Overall Survival	
	Hazard ratio (95% IC)	*P-value*	*Hazard ratio (95% IC)*	*P-value*
PER3	2.13 (1.40 - 3.24)	<0.001	2.04 (1.34 - 3.10)	0.001
Tumor Size	1.72 (1.13 - 2.63)	0.012	2.02 (1.31 - 3.12)	0.002
Age (< 40 years)	0.49 (0.32 - 0.74)	0.001	0.54 (0.35 - 0.83)	0.005
ER	0.75 (0.49 - 1.15)	0.19	0.53 (0.35 - 0.80)	0.003
Lymph Node	1.36 (0.90 - 2.06)	0.14	1.85 (1.18 - 2.77)	0.007
Tumor Grade				
good	0.93 (0.55 - 1.60)	0.8	1.05 (0.61 - 1.80)	0.87
intermediate	1.18 (0.74 - 1.89)	0.48	1.38 (0.87 - 2.20)	0.17

b

Variable	Disease Free Survival		Overall Survival	
	Hazard ratio (95% IC)	*P-value*	*Hazard ratio (95% IC)*	*P-value*
PER3	2.92 (1.71 – 4.97)	<0.001	2.63 (1.49 – 4.63)	0.001
Tumor Size	1.62 (0.96 - 2.63)	0.072	1.87 (1.05 – 3.32)	0.03
Age (< 40 years)	0.58 (0.33 - 0.99)	0.047	0.57 (0.32 – 1.04)	0.06
ER	All tumors are ER positive		All tumors are ER positive	
Lymph Node	1.40 (0.83 - 2.39)	0.21	2.07 (1.18 - 2.77)	0.02
Tumor Grade				
good	1.14 (0.59 – 2.23)	0.69	1.09 (0.54 – 2.24)	0.8
intermediate	1.34 (0.73 – 2.46)	0.34	1.32 (0.70 – 2.49)	0.38

Table 1.

a.- **Cox proportional hazard ratio multivariate analysis**. Risk of distant recurrence or death among patients with breast cancer. The analysis included the 413 patients from two different data bases [31,32]

b.- **Cox proportional hazard ratio multivariate analysis for ER positive samples**. Risk of distant recurrence or death among patients with breast cancer. The analysis included the 302 patients with ER positive breast tumors from two different data bases [31,32]