Genotyping Per3 KO.

Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR. Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. Mol Cell Biol. 2000 Sep; 20(17):6269-75.

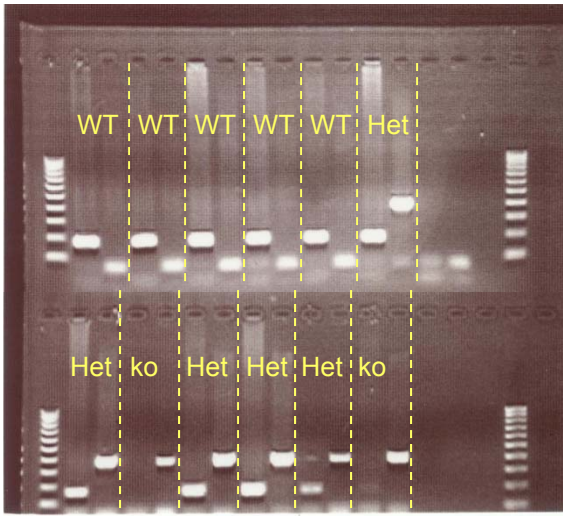
Genotypes were determined by PCR analysis of tail biopsy DNA).

The PCR method was done using three different primers,

a *forward primer in intron 3* (3-43; 5' TCTGTGAGTTCTTCCGTGTCTGTTll) (present only in the wild-type [WT] allele),
a *primer located in the NEO cassette* (Neo6-2; 5'TGCCCCAAAGGCCTACCCGCTTCC), and
a common reverse primer in exon 4 (3-41; 5' GTCTTGAGGGGCAAGCAGGTCGAC).
The presence of the WT allele led to the amplification of a ca*. 200-bp band* from primers 3-43 and 3-41, while the presence of the targeted allele was detected by amplification of a ca*. 400-bp band* with primers Neo6-2 and 3-41

The PCR protocol consisted of 3 min at 95°C, 30 cycles of amplification (each consisting of
30 s at 94°C,
30 s at 60°C, and
90 s at 72°C), and
a final extension phase (10 min at 72°C).

Products were separated on 1.5% agarose gels and viewed by UV transillumination with ethidium bromide.



MMTV-Neu Mice Genotyping.

Li B, Rosen JM, McMenamin-Balano J, Muller WJ, Perkins AS. neu/ERBB2 cooperates with p53-172H during mammary tumorigenesis in transgenic mice. Mol Cell Biol. 1997 Jun;17(6):3155-63

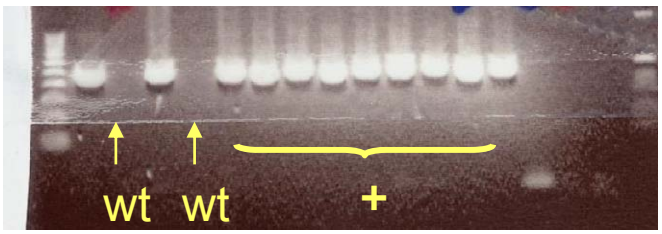
Primers
Neu1: GGAAGTACCCGGATGAGGAGGGCATATG
Neu2: CCGGGCAGCCAGGTCCCTGTGTACAAGCCG

PCR reacction:

Hotstart PCR buffer	5 µl
Neu1 primer 10 µM	1 µl
Neu2 primer 10 µM	1 µl
10 mM dNTP	1 µl
Hotstart Taq polymerase	0. 5 µl
DNA	2 µl
H2O	39. 5 µl

Program: MMTV-Neu
94°C 15 min;
35 cycles of: 94°C 30 sec, 60°C 1 min, 72°C 1 min;
72°C 2 min; 20°C (o/n)

Expectation: MMTV-Neu 660 bp band, wt: no band



		Pe3 copy number						
		Deleted		Normal		Gain		
	n	n	(%)	n	(%)	n	(%)	p-value
All patients	180	33	**18%**	117	**65%**	30	**17%**	
Estrogen Receptor								
ER positive	100	17	**17%**	68	**68%**	15	**15%**	0.89
ER negative	58	12	**21%**	34	**58%**	12	**21%**	
Stage								
I	67	11	**16%**	47	**70%**	9	**14%**	0.15
II	106	20	**19%**	67	**63%**	19	**18%**	
Age at diagnosis (years)								
< 40	19	2	**11%**	12	**63%**	5	**26%**	0.02
40-50	36	1	**3%**	29	**80%**	6	**17%**	
>= 50	125	30	**24%**	76	**61%**	19	**15%**	
Histological Grade								
Well differenciated	26	3	**12%**	16	**62%**	7	**27%**	0.65
Intermediate	55	12	**22%**	37	**67%**	6	**11%**	
Poorly differenciated	10	2	**20%**	6	**60%**	2	**20%**	
Hormonal Status								
Premenopausic	49	5	**10%**	35	**71%**	9	**19%**	0.16
Postmenopausic	114	26	**23%**	71	**63%**	17	**15%**	
Treatment								
Chemo	85	11	**13%**	58	**68%**	16	**19%**	0.53
Non Chemo	95	22	**23%**	59	**62%**	14	**15%**	
Only Hormonal	59	16	**27%**	37	**63%**	6	**10%**	
No Treatment	36	6	**17%**	22	**61%**	8	**22%**	

TABLE 1. Frequency of copy number of *PER3* related with the clinical data of 180 lymph node negative breast cancer patients from Climent et al 2007.
The median follow up of all the patients is 82 months, range (1.5 – 219 months)

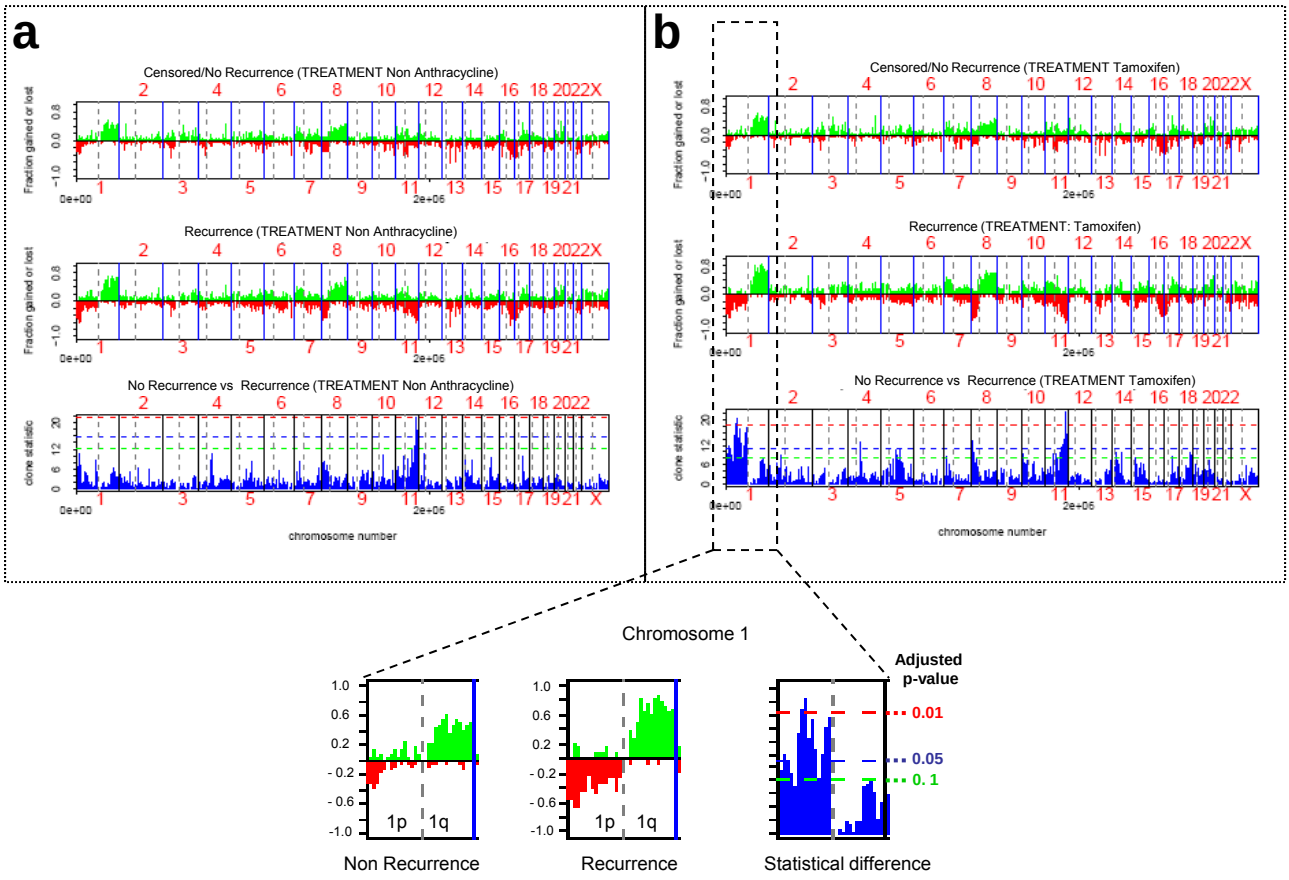


Figure 1.- **Copy number analysis by array-CGH .**

(A) In 95 lymph node negative breast cancer patients who did not received systemic chemotherapy, BAC clones showing deletion and corresponding to chromosomal region 11q21-q25, were strongly associated with patient relapse (data previously published in Climent et al 2007).

(B) In 59 patients from the previous group (A) who were ER and/or PGR positive and were treated only with tamoxifen, additional clones showing deletion and corresponding to chromosomal region 1p were strongly associated with patient recurrence.

Genome-wide analysis of DNA-copy number changes of tumor samples was performed using array CGH on a microchip with ~2.460 BAC and P1 clones printed in triplicate (UCSF Hum Array 2.0) with a resolution of 1.4 Mb across the genome. Methods and analytical procedures have been described previously in detail.

Climent J, Dimitrow P, Fridlyand J, et al. Deletion of chromosome 11q predicts response to anthracycline-based chemotherapy in early breast cancer. Cancer Res. 2007 ;67(2):818-26

Snijders AM, Nowak N, Segraves R, et al. Assembly of microarrays for genome-wide measurement of DNA copy number. *Nat Genet* 2001;**29**(3)**:**263-4.

COPY NUMBER ANALYSIS OF *PER3*

All tumor DNA samples were obtained from frozen breast tumors with >50% tumor cells. 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 RNaseP used as an endogenous control gene were obtained from Applied Biosystems. The RNaseP 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 RNaseP copy number of two[25]**.** 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 y Tables 2 and 3, and Figure 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 sequencing in Breast Cancer Cell Lines

We performed a mutation screening covering whole coding region of PER3 by direct Sanger sequencing in 35 breast cancer cell lines. We synthesized cDNA from 35 breast cancer cell lines and did RT-PCR using designed 7 forward and reverse primers (**Table 2**) for PER3 coding region. PCR reactions were carried out in a volume of 25 ul containing 100 ng cDNA, 10 pmol of each primer, 250 mM each dNTP, 0.5 U of Taq polymerase and the reaction buffer provided by the supplier (Qiagen, Hilden, Germany). Whole PER3 coding regions were sequenced using the Taq dideoxy terminator cycle sequencing kit and an ABI 3730 DNA sequencer (Applied Biosystems).

We could identify several single nucleotide polymorphisms and silent mutations (V419M, S445S, I606I, V639G, L697L, T725T, P745P, L827P, P856A, S864S, T1010T, M1028T, and H1149R). No clear pathogenic mutations like nonsense and missense mutations were identified **(Table 3)**

Table 2. Primer sequences of PER3 mutation screening

Fragment	Forward primer	Sequence	Reverse primer	Sequence	Size (bp)
1	PER3_RT1F	gaaaagctcctcggagatga	PER3_RT1R	tcatgtcttgaggtgcaagc	704
2	PER3_RT2F	aacaggctgctttgatcctg	PER3_RT2R	gtgggctcgttcgaacttta	696
3	PER3_RT3F	cagttggtccagctttgtga	PER3_RT3R	tcatctgccttgtggttctg	681
4	PER3_RT4F	ggatttgaggaacgatgagc	PER3_RT4R	gtgttcgagctgctgctgt	696
5	PER3_RT5F	gcaagaaagcaggagcaaag	PER3_RT5R	tggagattcagagggtctgg	700
6	PER3_RT6F	gtcgtcagcaatgagtccaa	PER3_RT6R	gagaatgcgctcaggtgtct	700
7	PER3_RT7F	aaaatgggcagcaatctcag	PER3_RT7R	ggtttggggctcattctagc	702

Table 3. Polymorphisms and silent mutations of PER3 in 35 breast cancer cell lines

Name	Fragment	Codon	Nucleotide change	Aminoacid change
V419M	3	419	GTG-->ATG	Val-->Met
S445S	3	445	AGT -->AGC	Ser-->Ser
I606I	4	606	ATA-->ATT	Ile-->Ile
V639G	4	639	GTC-->GGC	Val-->Gly
L697L	4	697	AAG-->AAA	Lys-->Lys
T725T	4	725	ACT-->ACA	Thr-->Thr
P745P	4	745	CCG-->CCA	Pro-->Pro
L827P	5	827	CTG-->CCG	Leu-->Pro
P856A	5	856	CCT-->GCT	Pro-->Ala
S864S	5	864	TCG-->TCA	Ser-->Ser
M1028T	6	1028	ATG-->ACG	Met-->Thr
T1010T	6	1010	ACA-->ACG	Thr-->Thr
H1149R	7	1149	CAT-->CGT	His-->Arg

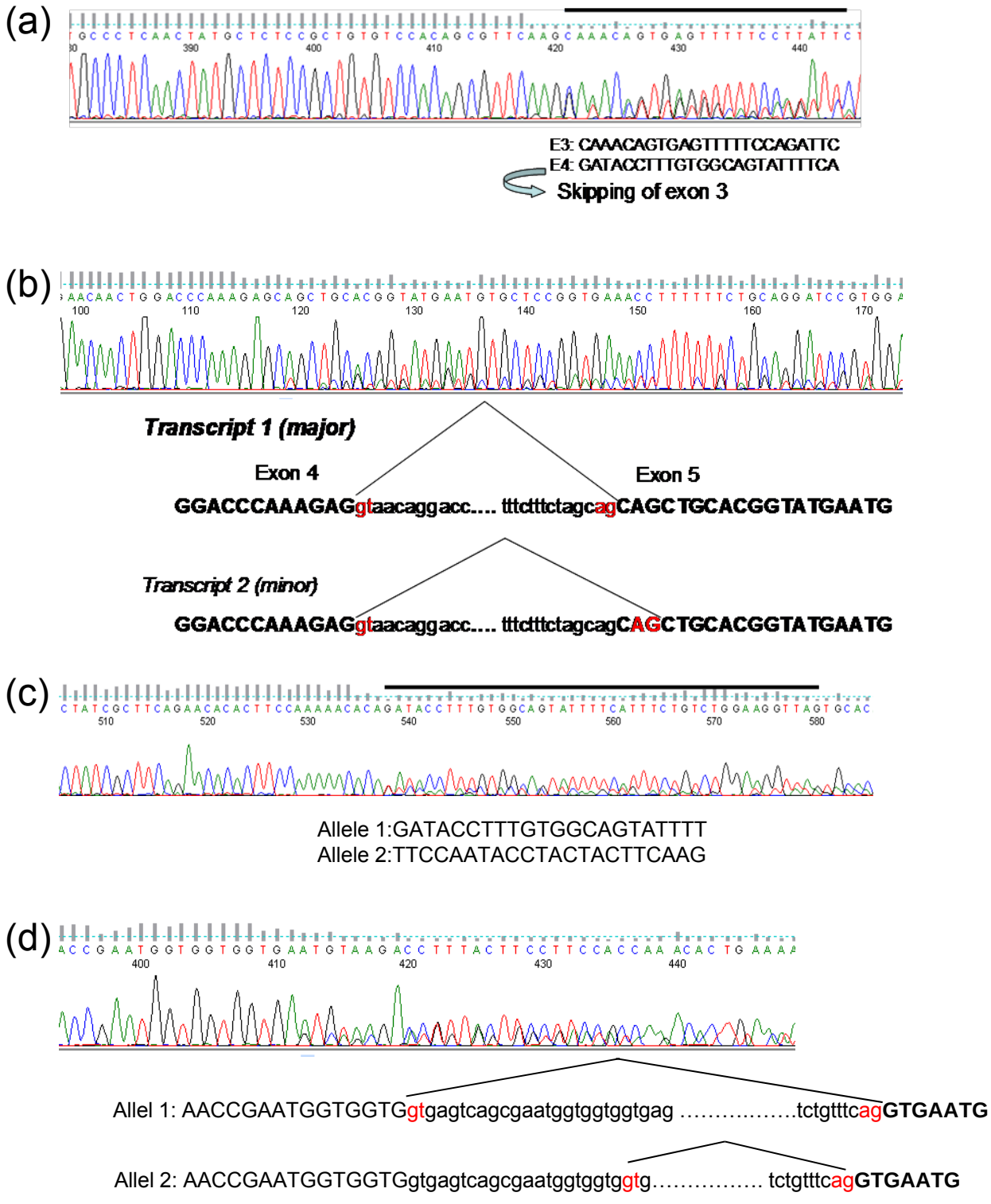


Figure 2

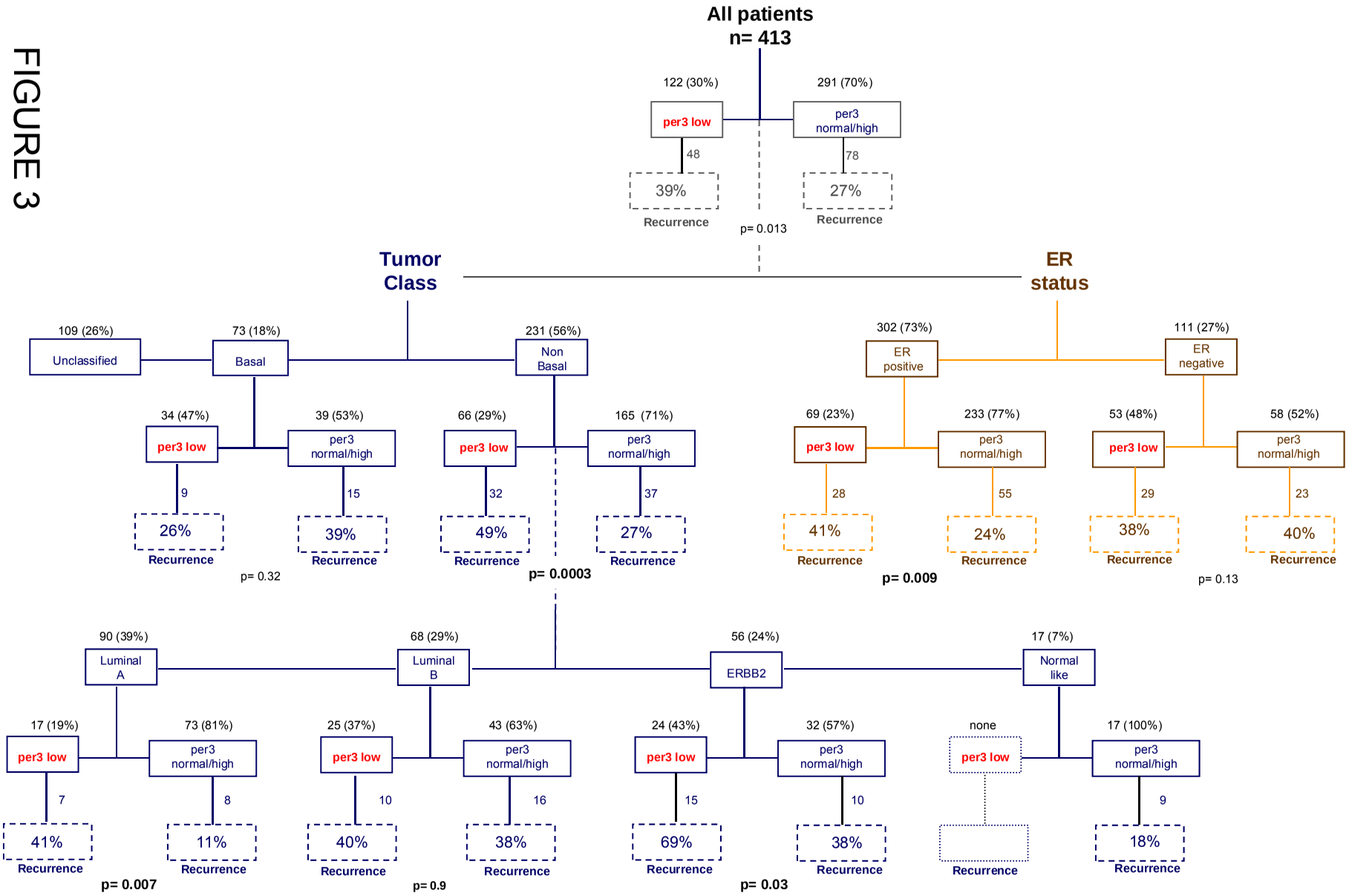
Four alternative splicing isoforms were identified. (a) exon 3 skipping isoform was found in three breast cell lines (b) Differentially expressed two isoforms were identified in intron 4. The major isoform contains one more amino acid (Alanine, GCA) than minor form in the beginning of exon 5. All 35 breast cancer cell lines showed higher expression of the major isoform (containing one more Alanine) allele by RT-PCR and sequencing analysis.

(c-d) Additional two alternative splicing were found in fragment 1 and 3, respectively

		Per3 low		Per3 normal/high		
	n	n	(%)	n	(%)	p-value
All patients	412	122	30%	291	70%	
Estrogen Receptor						
ER positive	301	69	23%	233	77%	<0.001
ER negative	111	53	48%	58	52%	
Tumor size						
<= 20mm	202	48	24%	154	76%	0.02
>20mm	209	72	34%	137	66%	
Lymph Node Status						
Negative	202	56	28%	146	72%	0.45
Positive	211	66	41%	145	69%	
Age at diagnosis (years)						
< 40	79	30	62%	49	38%	
40 - 44	102	26	75%	76	25%	
45 - 49	115	39	34%	76	66%	
>= 50	115	27	23%	89	77%	
Histological Grade						
Well differenciated	78	24	30%	55	70%	0.99
Intermediate	109	32	29%	77	71%	
Poorly differenciated	138	42	30%	96	70%	
Tumor Class						
Basal	73	34	47%	39	53%	0.001
ERBB2	56	24	43%	32	57%	
Luminal A	90	17	19%	73	81%	
Luminal B	67	25	37%	43	63%	
Normal-like	17	0	0%	17	100%	
Unclassified	110	22	80%	87	80%	
Treatment						
Chemo	171	49	29%	123	71%	0.4
Non Chemo	239	72	30%	167	70%	
Hormonal	105	12	25%	36	75%	0.3
Non Hormonal	305	60	31%	145	69%	
Only Chemo	114	35	31%	79	69%	0.21
Chemo + Hormonal	55	14	26%	41	76%	
Only Hormonal	48	12	25%	36	75%	
No Treatment	191	60	31%	131	69%	

TABLE 4. Relationship between *Per3* expression levels and clinical-pathological data of the 413 patients from Van de Vijver et al 2002, and Chin et al. 2006

FIGURE 3


All patients
n= 413
122 (30%)
per3 low
48
39%
Recurrence
291 (70%)
per3 normal/high
78
27%
Recurrence
p= 0.013
Tumor Class
ER status
109 (26%)
Unclassified
73 (18%)
Basal
231 (56%)
Non Basal
34 (47%)
per3 low
9
26%
Recurrence
39 (53%)
per3 normal/high
15
39%
Recurrence
p= 0.32
66 (29%)
per3 low
32
49%
Recurrence
165 (71%)
per3 normal/high
37
27%
Recurrence
p= 0.0003
302 (73%)
ER positive
69 (23%)
per3 low
28
41%
Recurrence
233 (77%)
per3 normal/high
55
24%
Recurrence
p= 0.009
111 (27%)
ER negative
53 (48%)
per3 low
29
38%
Recurrence
58 (52%)
per3 normal/high
23
40%
Recurrence
p= 0.13
90 (39%)
Luminal A
17 (19%)
per3 low
7
41%
Recurrence
73 (81%)
per3 normal/high
8
11%
Recurrence
p= 0.007
68 (29%)
Luminal B
25 (37%)
per3 low
10
40%
Recurrence
43 (63%)
per3 normal/high
16
38%
Recurrence
p= 0.9
56 (24%)
ERBB2
24 (43%)
per3 low
15
69%
Recurrence
32 (57%)
per3 normal/high
10
38%
Recurrence
p= 0.03
17 (7%)
Normal like
none
per3 low
Recurrence
17 (100%)
per3 normal/high
9
18%
Recurrence

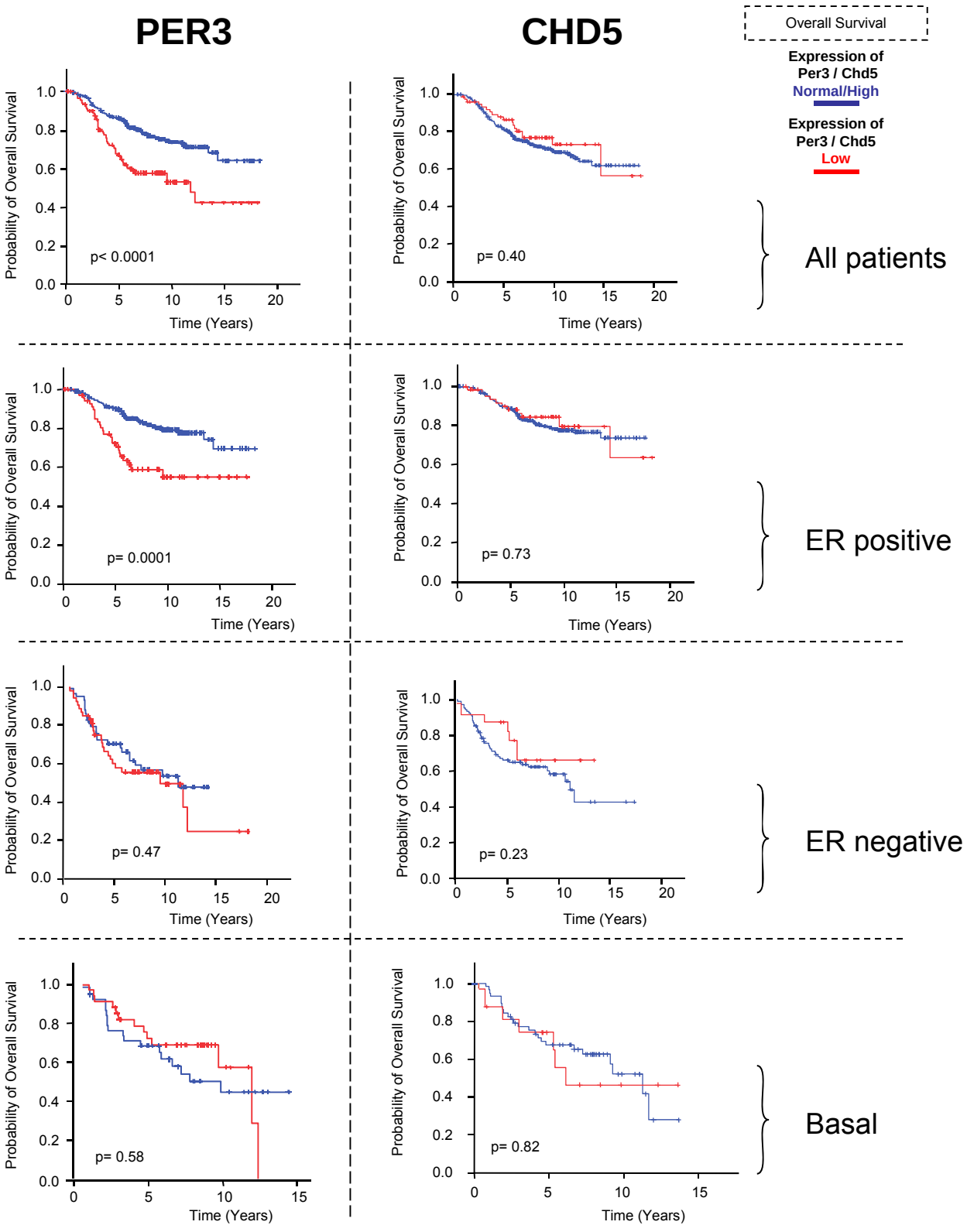


FIGURE 4.- Differences between Kaplan Meier Estimates for Overall Survival according the expression levels of Per3 (left column) and Chd5 (right column) in all patients (top) and three different subgroups of patients based on ER positive, ER negative and basal type tumors. P-values were calculated using log-rank test.

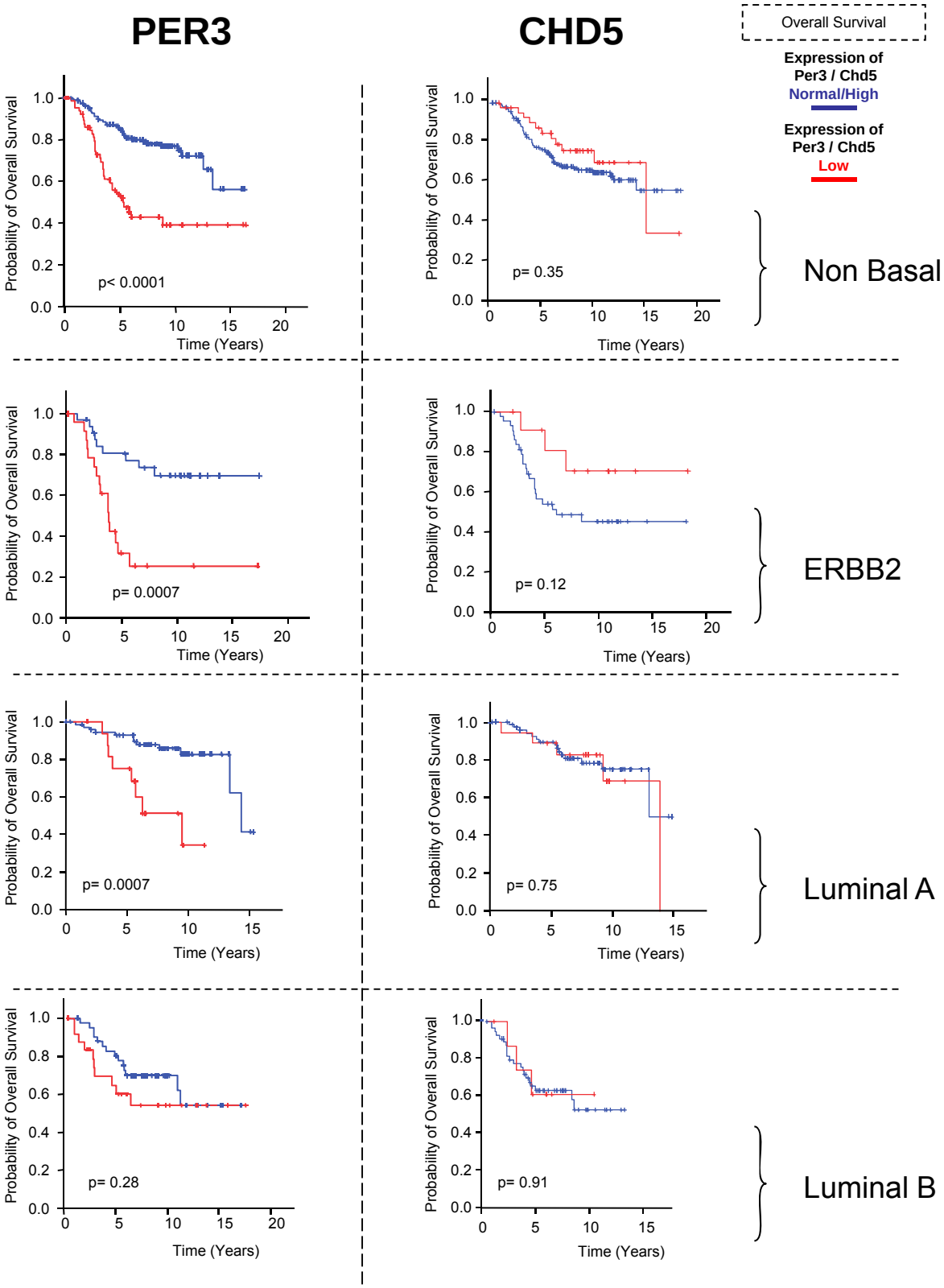


FIGURE 5.- Differences between Kaplan Meier Estimates for Overall Survival according the expression levels of Per3 (left column) and Chd5 (right column) in 4 different subgroups of patients based on Non basal, ERBB2, Luminal A and Luminal B tumor subtypes. P-values were calculated using log-rank test.

	Genotype						
	WT		HET		NULL		Total
Number of PER3 mice	**17**		**33**		**28**		**78**
Tumor type	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	n
Lymphoma or leukemia	7	41	18	55	9	32	34
Lung	7	41	12	36	10	36	29
Ovary	5	29	4	12	7	25	16
Breast hyperplasia	1	6	1	3	2	7	4
Breast	**0**	**0**	**4**	**12**	**10**	**36**	14
Skin Ca	3	18	3	9	1	4	7
Liver /kidney	0	0	0	0	3	11	3
Ca in rectal prolapsus	0	0	1	3	1	4	2
Utherus	1	6	0	0	0	0	1
Subcutaneous sarcoma	0	0	2	6	0	0	2

TABLE 5. Number of tumors generated by the treatment with DMBA (7, 12-dimethylbenz[a]anthracene) by oral gavage in PER3 mice.