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Expression of checkpoint kinase 2 in breast carcinomas: correlation with key regulators of tumor cell proliferation, angiogenesis, and survival

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Summary. Checkpoint kinase 2 (Chk2) is a cell-cycle-checkpoint kinase that may act as a tumor suppressor gene due to its important role in DNA damage signaling and cell cycle regulation. The role of Chk2 expression in mammary tumorigenesis, however, is still poorly understood. This study was designed to assess the relationship between the expression of Chk2 and well-established prognostic factors, including disease-free-survival and overall survival; and several regulators of cell proliferation and invasiveness in breast carcinomas, including oncogenes, tumor suppressor genes, apoptosis-related proteins, and angiogenesis-related markers. Immunohistochemistry with 27 primary antibodies was performed in 100 formalin-fixed paraffin-embedded samples of not otherwise specified invasive ductal carcinomas. Clinical data were retrieved from medical files. In normal mammary parenchyma adjacent to the tumors Chk2 stained the nuclei of epithelial cells. Downexpression of Chk2 protein was observed in 23 carcinomas and correlated with advanced disease. Among the regulators of tumor cell proliferation and invasiveness analyzed, the downexpression of Chk2 correlated only with reduced expression of p27 and telomerase. There was no difference between the overall survival and disease-free survival rates according to Chk2 status. In conclusion, Chk2 correlated with reduced expression of h-TERT and p27, but not with angiogenic factors. Chk2 expression also did not interfere in the outcome of the patients.

Key words: Checkpoint kinase 2, Breast, Carcinoma, Immunohistochemistry, Prognosis

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Introduction

The majority of human tumors, including breast carcinomas, develop as a result of the accumulation of genetic and epigenetic alterations that may translate into a wide range of alterations in cell morphology, structure and function (Daidone et al., 2004). Several types of genetic damage that occur in cancer cells give them an abnormal growth advantage, including activation of protooncogenes into oncogenes, inactivation of tumor suppressor genes that would normally slow or stop abnormal cell growth, impairment of the apoptotic pathways, and the ability to override genes that regulate cell senescence (Rieger, 2004). The end result of accumulated genetic errors is cells that can reproduce without restriction, invade local tissues, and ultimately, establish distant metastases.

Checkpoint kinase 2 (Chk2) is a cell-cycle-checkpoint kinase that phosphorylates p53 and BRCA1 in response to DNA damage (Vahteristo et al., 2002). The relationship of Chk2 to human cancer studies is developing rapidly with increasing evidence that Chk2 plays a role in tumor suppression (Ahn et al., 2004). In mammary carcinomas, however, the role of Chk2 and its relationship with the aforementioned mechanisms of tumorigenesis is far from understood. This study aims to evaluate the relationships between the expression of Chk2 and several key regulators of cell proliferation in invasive breast carcinomas, including oncogenes (c-erbB-2, c-erbB-3, epithelial growth factor receptor or EGFR, cyclin D1, and telomerase or hTERT), tumor suppressor genes (BRCA1, p53, p16, p21, and p27), and apoptosis-related proteins (BAG-1, bax, bcl-2, caspase 8, and survivin). The expression of angiogenesis-related proteins (extracellular matrix metalloproteinase inducer or EMMPRIN, matrix metalloproteinase 1 or MMP1, matrix metalloproteinase 2 or MMP2, plasminogen activator inhibitor or PAI, tissue inhibitor of matrix metalloproteinase 1 or TIMP1, tissue inhibitor of matrix

metalloproteinase 2 or TIMP2, and vascular endothelial growth factor or VEGF) was also analyzed according to Chk2 expression. In addition, the following well-established prognostic factors were evaluated: age, menstrual status, tumor size, tumor grade, nodal status, staging, overall survival, disease-free survival, and the expression of hormonal receptors and Ki67.

Materials and methods

Subject

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Ethics Committee. One-hundred cases of sporadic (non-familial) breast carcinomas diagnosed between 1992 and 1995 were retrieved from the files of the Department of Pathology of the Ribeirão Preto School of Medicine. The criterion for selection was based on the histopathologic diagnosis. The cases were selected to represent the three histologic grades of not otherwise specified (NOS) invasive ductal carcinomas according to the Scarff-Bloom & Richardson grading system modified by Elston & Ellis (Bloom and Richardson, 1957; Elston and Ellis, 1991; Fitzgibbons et al., 2000). The histologic types selected were: grade I invasive ductal carcinoma (n=32), grade II invasive ductal carcinoma (n=39), and grade III invasive ductal carcinoma (n=29). None of patients had received any treatment before the biopsy procedure. The clinical data were collected from the medical files. Conventional clinical features were evaluated, including age, menstrual status, pathological grading, tumor size, lymph node metastasis, and outcome. All patients were females. After the biopsy, all patients underwent surgical excision of the tumor (lumpectomy or mastectomy) followed by postoperative adjuvant systemic chemotherapy that included 5-FU (600 mg/m^2), methotrexate (40 mg/m^2), and cyclophosphamide (600 mg/m^2) every three weeks for 6 cycles. Patients who received antiestrogen therapy alone without chemotherapy were excluded from the study.

Immunohistochemistry

All tissue samples had been routinely fixed in 4% neutral formalin and embedded in paraffin. Briefly, 3-µm-thick sections were cut from paraffin blocks containing representative tumor samples. Paraffin sections were de-waxed in xylene, rehydrated through a series of graded alcohols, placed in 10 mM citrate buffer and submitted to heat retrieval using a vapor lock for 40 minutes. After heating, the slides were allowed to cool to room temperature and briefly washed with Tris-buffered saline. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 minutes. Normal serum (Novostain Super ABC kit, Novocastra, Newcastle upon Tyne, UK) was used for 30 minutes in order to block non-specific immunoassaying. Immunohistochemical staining was performed using an avidin-biotin peroxidase system (Novostain Super ABC kit, Novocastra). The primary antibodies were incubated overnight at room temperature. The source and the dilution of the primary antibodies are shown in table 1. Following washes in PBS, biotinylated universal secondary antibody (Novostain Super ABC kit, Novocastra) was applied for 30 minutes. The sections were incubated with the avidin-biotin complex reagent (Novostain Super ABC kit, Novocastra) for 30 minutes and developed with 3.3'-diaminobenzidine tetrahydrochloride (DAB) in phosphate-buffered saline, pH 7.5, containing 0.036% hydrogen peroxide for 5 minutes. Light Mayer's hematoxylin was applied as a counterstain. The slides were then dehydrated in a series of ethanols and mounted with Permount (Fischer, Fairlawn, NJ).

Samples of small bowel and normal kidney were used as positive controls for Chk2 and c-erbB-3, respectively. Cases of invasive ductal carcinoma previously known to be positive for c-erbB-2, cyclin D1, estrogen receptor, p16, p21, p53, p27, PAI, progesterone receptor, and VEGF were used as positive controls. Normal skin was used as the positive control for EGFR. Normal mammary tissue was used as the positive control for BRCA1, and a normal tonsil was used as the positive control for BAG-1, bax, bcl-2, Ki67, and hTERT. Samples of normal large bowel were used as positive controls for TIMP1. Samples from normal testis were used as control for caspase 8 and survivin. Samples of colonic adenocarcinoma were used as positive control for EMMPRIN, MMP1, MMP2, and TIMP2. Negative controls for immunostaining were prepared by omission of the primary antibody.

Chk2 staining was graded as positive (at least 60% of tumor cells showing moderate to intense nuclear immunoreactivity) or negative (fewer than 60% of tumor cells showing weak or no immunoreactivity) (Kilpivaara et al., 2005). According to the literature, the immunohistochemistry results were evaluated as follows (Umekita et al., 2002; Barnes et al., 2003; Ikeda et al., 2003; Lebeau et al., 2003; Abd El-Rehim et al., 2004; Singh et al., 2004; Sirvent et al., 2004). Expression was binary graded for BAG-1 (positive: at least 10% of neoplastic cells with nuclear or cytoplasmic expression), bax (positive: at least 10% of neoplastic cells with cytoplasmic expression), bcl-2 (positive: at least 10% of neoplastic cells with cytoplasmic expression), caspase-8 (positive: at least 10% of neoplastic cells with nuclear or cytoplasmic expression), c-erbB-3 (positive: at least 5% of neoplastic cells with cytoplasmic staining), cyclin D1 (any nuclear staining), EGFR (positive: at least 5% of neoplastic cells with cytoplasmic or membrane staining), hTERT (positive: at least 10% of neoplastic cells with nuclear expression), p16 (positive: at least 5% of neoplastic cells with nuclear or cytoplasmic expression), p21 (positive: at least 10% of neoplastic cells with nuclear expression), p27 (positive: at least 50% of neoplastic cells with nuclear expression), survivin

(positive: at least 10% of neoplastic cells with nuclear or cytoplasmic expression), and VEGF (positive: at least 1% of neoplastic cells with cytoplasmic staining).

Assessment of labeling for BRCA1 was quantitative, and the percentage of neoplastic cells stained was obtained for each tumor. The cases were then assigned to three categories: 0 to 25%, 26 to 50%, and more than 51% of neoplastic cells stained for BRCA1 (Nieto et al., 2003). Either nuclear or cytoplasmatic staining was considered for BRCA1 interpretation. The cases were interpreted as ER, PR or p53 positive if more than 10% of the neoplastic cells showed nuclear staining (Ribeiro-Silva et al., 2003). Ki67 labeling rate was obtained by the percentage of neoplastic cells displaying nuclear staining. The tumors were then grouped into three categories: less than 10%, between 10 and 50% and more than 50% of neoplastic cells positive for Ki67 (Milde-Langosch et al., 2000).

C-erbB-2 expression was scored according to the degree and the proportion of membrane staining (Rhodes et al., 2002). C-erbB-2 expression was negative for a score of 0 or 1+. A score of 0 was defined as no staining or membrane staining in less than 10% of tumor cells. A score 1+ comprised faint or partial staining of the membrane in more of 10% of tumor tissue. Overexpression of c-erbB-2 was scored as 2+ when a weak to moderate complete membrane staining was present in more than 10% of tumor cells. A score of 3+ was interpreted as a strong, complete membrane staining in more than 10% of tumor.

Statistical analysis

The chi-square test was used to compare Chk2 expression with clinicopathologic features and immunohistochemical results. The strength of the association of Chk2 with other variables was tested with Spearman rank correlation. Estimation of overall survival and disease-free survival was performed using the Kaplan-Meier method, and differences between survival curves were assessed with the log-rank test. Multivariate analyses were performed using the Cox proportional hazards model. All tests were 2-tailed, and a p-value of <0.05 was considered to be significant. Statistical analysis was performed using the Graph Pad Prism 4 software (San Diego, USA) and StatPlus 2005 3.5.0 RC (AnalystSoft).

Results

Clinical findings

The median age of patients (n=100) included in this study was 54.2 years (range 25-85 years). Thirty-four patients were premenopausal, 64 were post-menopausal, and 2 were hysterectomized. The hysterectomized women aged 65 and 71 year-old, so, for statistical analyses, they were considered post-menopausal. The

Table 1. Source and dilution of the primary antibodies.

ANTIBODY	CLONE	SOURCE	DILUTION
BAG-1	5C5	Novocastra, Newcastle upon Tyne, UK	1:50
Bax	Polyclonal	Dako, Carpinteria, CA	1:100
bcl-2	Bcl-2/100/D5	Novocastra, Newcastle upon Tyne, UK	1:50
BRCA1	MS13	Serotec, Oxford, UK	1:100
Caspase 8	11B6	Novocastra, Newcastle upon Tyne, UK	1:50
c-erbB-2	CB11	Novocastra, Newcastle upon Tyne, UK	1:300
c-erbB-3	RTJ1	Novocastra, Newcastle upon Tyne, UK	1:100
Checkpoint kinase 2 (Chk2)	DCS 270.1	Novocastra, Newcastle upon Tyne, UK	1:50
Cyclin D1	P2D11F11	Novocastra, Newcastle upon Tyne, UK	1:50
Epidermal growth factor receptor (EGFR)	EGFR.113	Novocastra, Newcastle upon Tyne, UK	1:50
Extracellular matrix metalloproteinase inducer (EMMPRIN)	8D6	Santa Cruz, Palo Alto, CA	1:50
Estrogen receptor (ER)	6F11	Novocastra, Newcastle upon Tyne, UK	1:100
Human telomerase reverse transcriptase (hTERT)	44F12	Novocastra, Newcastle upon Tyne, UK	1:100
Ki67	MM1	Novocastra, Newcastle upon Tyne, UK	1:150
Matrix metalloproteinase 1 (MMP1)	3B6	Novocastra, Newcastle upon Tyne, UK	1:50
Matrix metalloproteinase 2 (MMP2)	8B4	Santa Cruz, Palo Alto, CA	1:50
p16	6H12	Novocastra, Newcastle upon Tyne, UK	1:50
p21	4D10	Novocastra, Newcastle upon Tyne, UK	1:50
p27	1B4	Novocastra, Newcastle upon Tyne, UK	1:50
p53	DO-7	Novocastra, Newcastle upon Tyne, UK	1:100
p63	4A4	Santa Cruz, Palo Alto, CA	1:100
Plasminogen activator inhibitor (PAI)	TJA6	Novocastra, Newcastle upon Tyne, UK	1:50
Progesterone receptor	16	Novocastra, Newcastle upon Tyne, UK	1:150
Survivin	FL-142	Santa Cruz, Palo Alto, CA	1:100
Tissue inhibitor of matrix metalloproteinase 1 (TIMP1)	6F6a	Novocastra, Newcastle upon Tyne, UK	1:100
Tissue inhibitor of matrix metalloproteinase 2 (TIMP2)	3A4	Novocastra, Newcastle upon Tyne, UK	1:100
Vascular endothelial growth factor (VEGF)	A-20	Santa Cruz, Palo Alto, CA	1:200

median size of tumors was 4.1 cm (range 0.5-14 cm). Thirty-three patients were lymph node negative. Twenty-eight patients had 1 to 3 positive nodes, while 39 had more than 3 nodal metastasis. Table 2 summarizes the clinical findings. The series is heterogeneous because the samples were randomly selected. Chk2 did not correlate with age, menstrual status, tumor size, tumor grade, and nodal status (Table 3). Eight patients were stage I, 42 were stage II, 42 were stage III, and 8 were stage IV. Chk2 negativity correlated with advanced disease (stages III and IV) (p=0.0004).

Immunohistochemical findings

In normal mammary parenchyma adjacent to the tumors Chk2 stained the nuclei of epithelial cells. In invasive breast carcinomas, 77 of 100 cases showed the same pattern as verified in normal glands (Fig. 1). Chk2 protein expression was reduced in 23 carcinomas.

The positivity rate for the other markers used in this study was: BAG-1 (55% of carcinomas), Bcl-2 (45%), c-erbB-3 (60%), cyclin D1 (41%), EGFR (31%), ER (53%), h-TERT (49%), p16 (44%), p21 (25%), p27 (38%), p53 (35%), PR (47%), and survivin (69%). Twenty-six carcinomas expressed BRCA1 in less than 25% of neoplastic cells. In 26 carcinomas, 25 to 50% of neoplastic cells stained for BRCA1, while in 48 more than 50% of neoplastic cells stained for BRCA1. Fifty-three carcinomas exhibited less than 10% of neoplastic cells stained for Ki67. In 30 carcinomas, 10 to 50% of neoplastic cells were positive for Ki67, while 17 carcinomas expressed Ki67 in more than 50% of the neoplastic cells. Twenty-seven percent of carcinomas were positive for c-erbB-2 (scores 2 and 3).

In the univariate analysis Chk2 correlated only with hTERT (p=0.0039) and p27 (p=0.0269). The multivariate analysis showed that this findings are significant (p<0.001). The relationship between chk2 expression and the immunohistochemical markers is specified in Tables 3 to 5. The correlation rank between the variables is specified in Table 6.

Clinical outcomes

Median follow-up was 6.5 years (range 1-13 years). Thirty of 100 patients died due to the tumor. Thirty-three

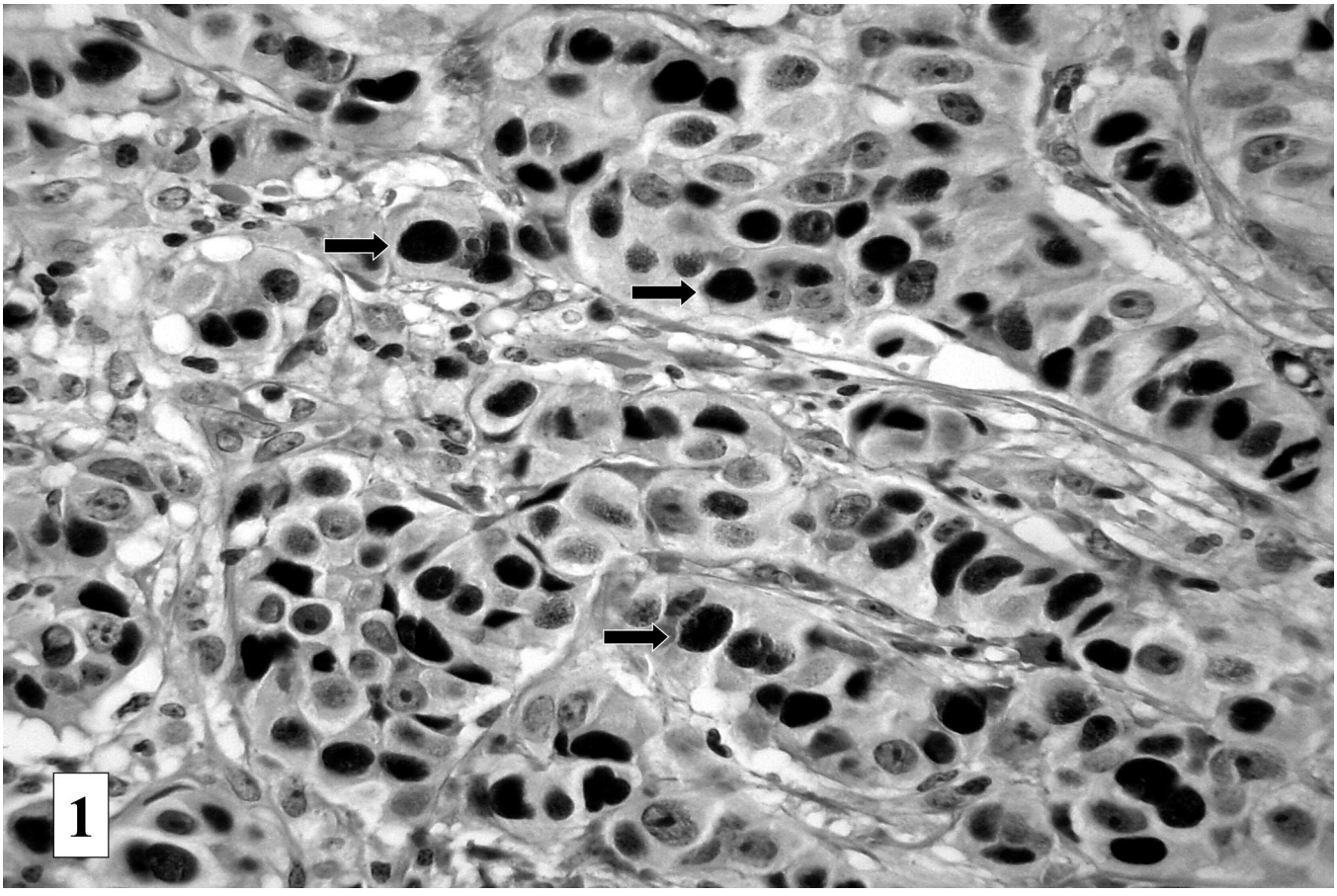

Fig. 1. Malignant mammary cells positive for Chk2 (dark nuclear staining, arrows) (immunohistochemistry, original magnification x400).

patients remained alive after 5 years of follow-up. Among them 25 were alive 10 years or more after the initial diagnosis. Thirty-seven patients were excluded from this analysis because they did not have a follow-up of five years or because the cause of death was unrelated to the tumor. The average overall survival of patients whose tumors were negative and positive for CHK2 was 7.7 and 5.7 years, respectively. This difference was not statistically significant (p=0.6102) (Fig. 2).

Forty-five of 100 patients had recurrence or metastases. Twenty-six patients remained disease free 5 years after treatment. Among them, 19 did not have recurrence or metastasis 10 years or more after the initial diagnosis. Twenty-nine patients were excluded from this analysis because they did not have a follow-up of five years. The average disease-free survival of patients whose tumors were negative and positive for CHK2 was 7.6 and 4.7 years, respectively, with the difference being statistically significant in the univariate analysis (p=0.0059) (Fig. 2). The Cox multivariate analysis, however, showed that Chk2 is not an independent variable (p=0.5659, RR=1.5398).

Discussion

The mammalian cell cycle is regulated by a complex system of inhibitory and stimulatory factors. The aberrant expression of these regulators has been implicated in the pathogenesis of the human cancers. Regulation of the cell cycle depends on the complex association between cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. According to their effect, these regulators may act as promoters (cyclin D1) or inhibitors ($p16^{INK4b}$, $p21^{WAF-1/CIP1}$ or $p27^{KIP-1}$). When DNA is damaged or replicated defectively, the cell cycle is arrested at a checkpoint in the interphase by inhibiting the activity of Cdc25 phosphatase. In arrested cells, Cdc25 is phosphorylated at Ser-216 by Chk1 or Chk2 kinase to lose its activity (Walworth et al., 1993). So, Chk2 is an important kinase which inhibits the cell cycle at checkpoints, especially in terms of DNA damage at the S phase or G2 phase. To be activated, Chk2 also requires a checkpoint protein kinase named ataxia telangiectasia mutated (ATM), which phosphorylates and activates Chk2 (Matsuoka et al., 1998, 2000; Hiral et al.,

Table 2. Clinicopathologic features.

FEATURE	N
Total	100
Age (years)	
<30	3
30-50	31
50-70	47
>70	19
Menstrual status	
Premenopausal	34
Post-menopausal	64
Hysterectomized	2
Tumor size (cm)	
<2	17
2-5	57
>5	26
Tumor grade Bloom & Richardson	
I	32
II	39
III	29
Nodal status	
Negative	33
1-3	28
>3	39
Staging	
I	8
II	42
III	42
IV	8

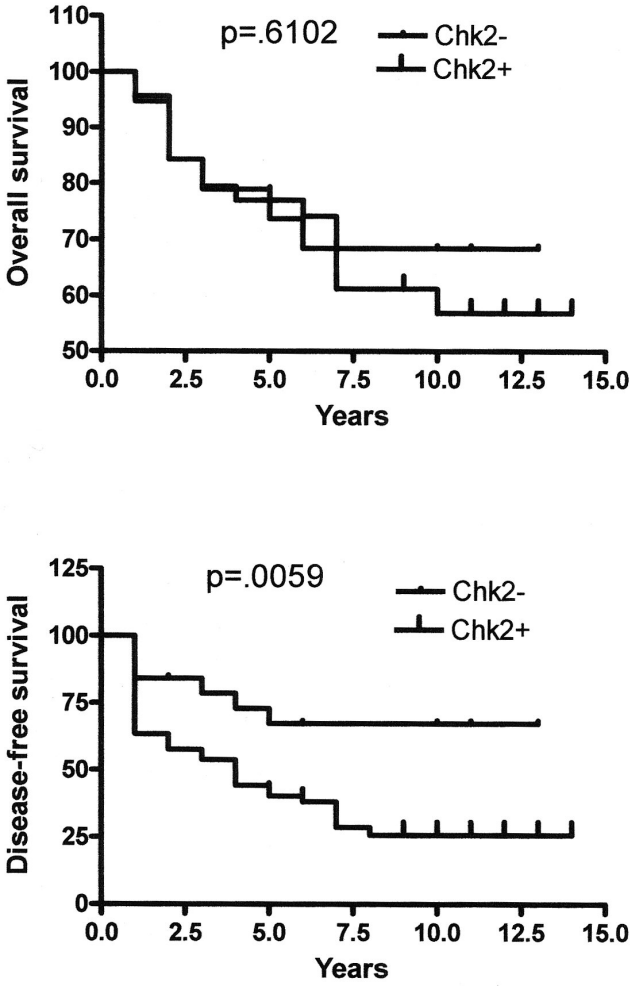

Fig. 2. Overall survival and disease-free survival according to Chk2 status.

2000).

These data indicate that Chk2 kinase may act as a tumor suppressor due to its important role in DNA damage signaling, cell cycle regulation and DNA damage-induced apoptosis (Zhang et al., 2004). Indeed, both germline and somatic loss-of-function Chk2 mutations occur in human tumors, the former linked to the Li-Fraumeni syndrome, and the latter found in diverse types of sporadic malignancies, including non-Hodgkin lymphomas, urinary bladder carcinoma, Hodgkin's lymphoma, and non-small cell lung cancer (Tort et al., 2002; Bartkova et al., 2004; Kato et al., 2004; Staalesen et al., 2004; Zhang et al., 2004).

In familial breast carcinomas, several patterns of mutations in the Chk2 gene have been identified (Allinen et al., 2001; Staalesen et al., 2004). A protein-truncating variant of Chk2 named 1100delC is associated with a moderate increase in breast cancer risk. However, the frequency of this allele in multiple-case breast cancer families is low (0.6 to 5.5% of familial breast cancer not associated with either BRCA1 or BRCA2 mutations) (Meijers-Heijboer et al., 2002; Vahteristo et al., 2002; Syrjakoski et al., 2004; Jekimovs et al., 2005). Chk2

Table 3. Relationship between Chk2 expression and clinicopathologic variables of prognostic significance.

FEATURE	CHK2 -	CHK2 +	P-VALUE
Total	23	77	
Age (years)			0.5286
<30	0	3	
30-50	8	23	
50-70	9	38	
>70	6	13	
Menstrual status			0.8030
Premenopausal	7	27	
Post-menopausal	16	50	
Tumor size (cm)			0.4104
<2	6	11	
2-5	12	45	
>5	5	21	
Tumor grade			0.2701
Bloom & Richardson			
I	10	22	
II	9	30	
III	4	25	
Nodal status			0.1121
Negative	11	22	
1-3	3	25	
>3	9	30	
Staging			0.0004
I	4	4	
II	6	36	
III	7	35	
IV	6	2	
Estrogen Receptor			0.8131
Negative	10	37	
Positive	13	40	
Progesterone Receptor			0.1566
Negative	9	44	
Positive	14	33	
P53			0.4553
Negative	17	48	
Positive	6	29	
c-erbB-2			0.2973
0	16	40	
1+	3	14	
2+	0	8	
3+	4	15	
Ki67			0.3320
<10%	15	38	
11-50%	6	24	
>50%	2	15	

Table 4. Relationship between Chk2 expression and key regulators of the cell cycle, apoptosis-related proteins, and oncogenes.

FEATURE	CHK2-	CHK2+	P-VALUE
Total	23	77	
BAG-1			0.4731
Negative	8	35	
Positive	15	42	
Bax			0.1202
Negative	5	6	
Positive	18	71	
bcl-2			0.2380
Negative	10	45	
Positive	13	32	
BRCA1			0.3516
<25%	5	21	
25-50%	4	22	
>50%	14	34	
Caspase 8			1
Negative	11	38	
Positive	12	39	
c-erbB-3			0.0963
Negative	13	28	
Positive	10	49	
Cyclin D1			0.6301
Negative	15	44	
Positive	8	33	
EGFR			0.7977
Negative	15	54	
Positive	8	23	
hTERT			0.0039
Negative	18	33	
Positive	5	44	
p16			0.6392
Negative	14	42	
Positive	9	35	
p21			0.5789
Negative	19	57	
Positive	4	20	
p27			0.0269
Negative	19	43	
Positive	4	34	
Survivin			
Negative	9	22	0.4412
Positive	14	55	

mutations in non-familial carcinomas are rare; however, loss of function in Chk2 via down-regulation of its expression occurs in a proportion of sporadic cases probably due to epigenetic changes (Ingvarsson et al., 2002; Miller et al., 2002; Sullivan et al., 2002). The biological significance of this finding is still poorly understood.

Since it permits to locate proteins in single cells or tissues, immunohistochemistry has become a standard technique for the study of the expression of several genes. In our study 77% of carcinomas exhibited a normal staining pattern. These data agree with the results of Kilpivaara et al. (2005), who found a normal staining pattern in 78.9% of carcinomas in a larger series (440 tumors) (Kilpivaara et al., 2005). Although the reduced expression of Chk2 correlated with advanced disease (p=0.0004), there was no correlation between Chk2 expression and several other indicators of aggressiveness, including age, tumor size, tumor grade, nodal status, and the expression of hormonal receptors, p53, c-erbB-2 and Ki67. Besides, there was also no statistical difference in overall survival according to Chk2 status. In the univariate analysis Chk2 correlated with disease-free survival. However, the multivariate analysis showed that Chk2 is not an independent variable.

Among the several regulators of tumor cell proliferation analyzed, including apoptosis-related proteins, the reduced expression of Chk2 correlated only with p27 and human telomerase reverse transcriptase (hTERT) expression.

Human telomeres are long, repetitive sequences of TTAGGG that protect the chromosomes from end-to-end fusion, recombination, and degradation, all events that can lead to cell death. At cell replication, telomeres cannot be completely replicated. They are gradually shortened, and when the telomeres reach a critical threshold, cell replication is arrested in what is called "replicative senescence." (Wai, 2004). The mechanism for maintaining telomere integrity is controlled by telomerase, a ribonucleoprotein enzyme that specifically restores telomere sequences lost during replication by using an intrinsic RNA component as a template for polymerization (Baykal et al., 2004). Telomerase has two core functional components required for its activity:

Table 5. Expression of VEGF, metalloproteinases and their inhibitors according to Chk2 status.

FEATURE	CHK2-	CHK2+	P-VALUE
Total	23	77	
EMMPRIN			1
0	21	69	
<20%	2	6	
20-50%	0	2	
>50%	0	0	
MMP1			0.1654
0	17	37	
<20%	5	30	
20-50%	1	7	
>50%	0	3	
MMP2			0.4015
0	17	65	
<20%	5	9	
20-50%	1	1	
>50%	0	2	
PAI			0.8839
0	20	65	
<20%	2	6	
20-50%	1	4	
>50%	0	2	
TIMP1			0.0936
0	8	15	
<20%	5	9	
20-50%	1	17	
>50%	9	36	
TIMP2			0.1082
0	14	27	
<20%	1	12	
20-50%	2	16	
>50%	6	22	
VEGF			0.6343
Negative	12	34	
Positive	11	43	

Table 6. Correlation between Chk2 expression and other variables.

FEATURE	SPEARMAN R	P-VALUE
Age (years)	0.1301	0.1967
Menstrual status	0.1900	0.0581
Tumor size (cm)	0.2030	0.0427
Tumor grade	0.3981	0.0000
Nodal status	0.1338	0.1842
Staging	0.0563	0.5779
Estrogen Receptor	-0.0119	1.0935
Progesterone Receptor	-0.0739	1.5356
P53	0.0173	0.8637
c-erbB-2	0.1953	0.0514
Ki67	0.1727	0.0856
BAG-1	0.1450	0.1500
bcl-2	-0.0240	1.1875
BRCA1	-0.2131	1.9667
c-erbB-3	-0.0403	1.3095
Cyclin D1	0.0322	0.7502
EGFR	-0.0347	1.2688
hTERT	0.2464	0.0134
p16	0.0310	0.7594
p21	0.1731	0.0849
p27	-0.0238	1.1858
Survivin	0.0718	0.4776
EMMPRIN	0.1073	0.2876
MMP1	0.2338	0.0191
MMP2	0.0447	0.6580
PAI	-0.0177	1.1390
TIMP1	0.1511	0.1334
TIMP2	0.1907	0.0572
VEGF	0.2081	0.0377

the catalytic subunit of human telomerase reverse transcriptase (hTERT) and a telomerase RNA template (hTR) (Kirkpatrick et al., 2003). Telomerase activity is almost absent in somatic cells, but is detected in embryonic stem cells and in the vast majority of tumor cells. Tumor cells, in fact, may contain stable telomeres that confer immortality to the cancer cells, which are thus able to replicate indefinitely. In breast carcinomas hTERT is frequently activated in early carcinogenesis and its activity is associated with cell proliferation, aggressiveness, nodal metastases and a poor clinical outcome (Mokbel et al., 1999; Poremba et al., 2002; Yano et al., 2002; Kirkpatrick et al., 2004; Liu et al., 2004). Downexpression of Chk2 correlated with decreased expression of hTERT.

According to Tauchi et al. (2003) the inhibition of telomerase activity induces the activation of ATM and Chk2, and subsequently increased the expression of p21(CIP1) and p27(KIP1) (Tauchi et al., 2003). These data are very interesting because in the present study we verified a statistically significant relationship between the expression of Chk2 with both telomerase and p27. P27 is a member of the KIP/CIP family of cyclin-dependent kinase inhibitors which acts as a negative cell-cycle regulator and is thought to play a role in tumor suppression (Barnes et al., 2003). The progressive reduction in p27 protein immunohistochemical staining with increasing histological grading is a well-established finding occurring in breast cancer (De Paola et al., 2002; Barnes et al., 2003; Troncone et al., 2004). In the present study, downexpression of chk2 correlated with reduced expression of p27.

Angiogenesis, the process of new blood vessel formation, plays a central role in both local tumor growth and distant metastasis in breast cancer. Extensive laboratory data suggest that angiogenesis plays an essential role in breast cancer development, invasion, and metastasis (Schneider and Miller, 2005). Recent years have seen the discovery of many regulators of the angiogenic process. Foremost among these is vascular endothelial growth factor (VEGF), the ligand for a family of specific transmembrane receptors which regulate the angiogenic process (Sledge, 2002). Angiogenesis is also related to the ability of the tumor to degrade the proteins in the extracellular matrix. For this purpose, there is a family of zinc-dependent endopeptidases named matrix metalloproteinases (MMPs) that degrades the basement membrane and extracellular matrix. MMP expression could be attributed to tumor stromal cells and is partially regulated by tumor-stroma interactions via tumor cell-associated extracellular matrix metalloproteinase inducer (EMMPRIN) (Tang et al., 2005). MMPs are also associated with a family of endogenous inhibitors named tissue inhibitors of metalloproteinases (TIMPs). (Duffy et al., 2000). Under normal physiologic conditions, the MMPs and TIMPs exist in an exquisite balance. This balance is disrupted during active angiogenesis (Schneider and Miller, 2005). Plasminogen activator inhibitor (PAI) is an important component of the plasminogen/plasmin system since it is the main inhibitor of the tissue-type and urokinase-type plasminogen activator (Gils and Declerck, 2004). Consequently, PAI also plays an important role in angiogenesis. In the present study none of these markers correlated with Chk2 status.

In conclusion, Chk2 correlated with reduced expression of h-TERT and p27, but not with angiogenic factors. Chk2 expression also did not interfere in the outcome of the patients.

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