The E2F-Regulated Gene *Chk1* Is Highly Expressed in Triple-Negative Estrogen Receptor–/Progesterone Receptor–/HER-2– Breast Carcinomas

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Abstract

We previously showed that checkpoint kinase 1 (*Chk1*) and *Clasp*, two DNA-damage checkpoint proteins, were down-regulated by 1,25-dihydroxyvitamin D₃, a known inhibitor of cell proliferation. In the present study, we aimed to investigate the transcriptional regulation of *Chk1* and *Clasp* and to study their expression levels in human breast cancer tissue. Transient transfection experiments in MCF-7 breast cancer cells showed that promoter activities of *Chk1* and *Clasp* were regulated by the E2F family of transcription factors. Subsequently, transcript levels of *Chk1*, *Clasp*, and *E2F1* were determined by quantitative reverse transcriptase-PCR analysis in 103 primary invasive breast carcinomas and were compared with several clinicopathologic variables in breast cancer. A strong correlation was found between *Chk1* and *Clasp* transcript levels. Transcript levels of *Chk1*, *Clasp*, and *E2F1* were highest in histologic grade 3 tumors and in tumors in which the expression of estrogen receptor (ER) and progesterone receptor (PR) was lost. Moreover, *Chk1* expression was significantly elevated in grade 3 breast carcinomas showing a triple-negative ER–/PR–/HER-2– phenotype compared with other grade 3 tumors. Further research is warranted to validate the use of *Chk1* inhibitors in triple-negative breast carcinomas for which treatment strategies are limited at present. [Cancer Res 2007;67(14):6574–81]

Introduction

Eukaryotic cells have developed complex checkpoint pathways that monitor DNA for damage or incomplete replication. Checkpoint pathways are amplified upon detection of aberrant DNA structures and lead to a delay in cell cycle progression during which damage can be repaired or replication be completed. Alternatively, in case of heavily damaged or seriously deregulated cells, checkpoint activation can result in apoptosis. As such, checkpoint mechanisms are essential for the maintenance of genomic integrity (1). When vertebrate cells experience replication

arrest or undergo DNA damage by UV irradiation, the ATR kinase [ataxia telangiectasia mutated (ATM)- and Rad3-related kinase] phosphorylates and activates the Chk1 protein kinase. Activated Chk1 inhibits Cdc25 phosphatases, which control inhibitory phosphorylation sites on cyclin-dependent kinases, the latter being critical regulators of cell cycle transitions (2). Full activation of Chk1 by ATR requires Clasp, which may act as a scaffolding protein that brings together ATR and Chk1 (3). Apart from being a signal mediator in checkpoint control, Clasp was suggested to act as a sensor of DNA replication (4). Moreover, Clasp is shown to be involved in cell proliferation as overproduction of Clasp stimulates cell growth, suggesting an oncogenic role for Clasp (5).

The common occurrence of checkpoint defects distinguishes cancer cells from normal cells, thereby providing a potential target for therapeutic intervention. As a possible strategy, checkpoint signaling can be inhibited to enhance the effect of concomitant treatment with DNA-damaging drugs or radiation. Targeting of ATM/ATR as well as Chk1 has already provided encouraging results (1, 6–8). Indeed, when the G₂ or S checkpoint is abrogated by inhibition of Chk1, checkpoint-deficient cancer cells will undergo mitotic catastrophe and eventually apoptosis. However, also, Clasp may be a candidate to target as depletion of Clasp by small interference RNA increases the sensitivity of cultured human cells to genotoxic stress and promotes cell death (9).

We previously identified *Chk1* and *Clasp* in a cluster of genes that were quickly down-regulated in mouse MC3T3-E1 and in mouse mammary carcinoma cells by treatment with 1,25-dihydroxyvitamin D₃, which is a known inhibitor of cell proliferation (10). Approximately 30% of the genes in this down-regulated cluster were known to be transcriptional targets of the E2F family of transcription factors. *In silico* promoter analysis further showed an additional 20% of genes to contain E2F-binding sites in their promoter. *Clasp* was found to be present among those newly identified E2F target genes.

In the present study, we aimed to investigate *Chk1* and *Clasp* expression in human breast cancer tissue. Because promoter analysis of human *Clasp* (during the course of our study; ref. 11) and *Chk1* (12) indicated that these genes were transcriptional targets of the E2F family of transcription factors, we sought to correlate *Chk1* and *Clasp* transcript levels with those of *E2F1*. *Chk1*, *Clasp*, and *E2F1* transcript levels were further compared with those of the cell proliferation marker *Ki-67* and with several clinicopathologic variables in breast cancer such as tumor size and grade, estrogen receptor (ER) and progesterone receptor (PR) expression, HER-2 status, and the presence of lymph node metastases.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell culture—proliferation assay and RNA extraction. MC3T3-E1 cells (Riken Cell Bank) were maintained in α -MEM with 2 mmol/L glutamax-I containing 10% heat-inactivated fetal bovine serum (FBS, Biocrom KG), 100 units/mL penicillin, and 100 μ g/mL streptomycin. MCF-7 cells (American Type Culture Collection) were maintained in DMEM with 2 mmol/L glutamax-I containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin. For serum starvation experiments, MC3T3-E1 cells were seeded in α -MEM without FBS either in 25-cm² flasks (200,000 per flask) for cell cycle analysis or in six-well dishes (65,000 per well) for RNA extraction. After 48 h, culture medium was replaced by medium with 10% FBS. For analysis of cell cycle distribution, cells (10⁶) were fixed in ice-cold 75% ethanol. Fixed cells were washed with PBS supplemented with 0.05% Tween 20 and resuspended in PBS with 0.5 mg/mL propidium iodide and 1 mg/mL RNase A (Sigma). Samples were analyzed with a FACSort flow cytometer (Becton Dickinson, BD Biosciences). Total RNA was isolated using the RNeasy kit (Qiagen).

Plasmids. Promoter fragments of murine *Chk1* (−223/+43), human *Chk1* (−572/+34), murine *Claspin* (−250/+70), and human *Claspin* (−246/+52) were obtained through PCR amplification and cloned into the pGL3-Basic reporter vector (Promega). Site-directed mutagenesis to mutate the E2F-binding sites in the promoter regions of murine *Chk1* and *Claspin* was done by use of the QuickChange II Site-Directed Mutagenesis kit (Stratagene) according to the instructions of the manufacturer. For murine *Chk1*, the E2F-binding site TTTGGCGGGAAA (at position +9 to +20 relative to transcription start site) was mutated to TGTGACGTGACA. For murine *Claspin*, the E2F-binding site GCGCGAAA (at position −24 to position −16 relative to transcription start site) was mutated to GCGTGACA. Expression plasmids pcDNA-HA-E2F1, E2F2, E2F3, and E2F4 were kind gifts of Dr. J. Nevins (Duke University Medical Center, Durham, NC). The CMV-HA-E2F5 expression plasmid was a gift of Dr. J. Magae (Institute of Research and Innovation, Chiba, Japan). Mutant *E2F1* (mutated at position 132) was a gift of Dr. K. Helin (Copenhagen, Denmark). The β -galactosidase expression vector pcDNA3.1(−)/Myc-His/*lacZ* and the pcDNA3.1/Myc-His vector were obtained from Invitrogen.

Transfection assays. Exponentially growing MC3T3-E1 cells were transfected with Eugene 6 (Roche Diagnostics) in 24-well dishes (20,000 per well) with 100 ng of luciferase reporter vector (or representative control vector), 50 ng of the different E2F constructs (or the empty pcDNA3.1/Myc-His), and 10 ng of pcDNA3.1(−)/Myc-His/*lacZ*. Exponentially growing MCF-

7 cells were seeded in 24-well dishes (50,000 per well) and transfected with Eugene 6 with 500 ng of luciferase reporter (or representative control vector), 250 ng of the different E2F constructs (or empty pcDNA3.1/Myc-His), and 25 ng of pcDNA3.1(−)/Myc-His/*lacZ*. Cells were lysed 48 h after transfection (with Reporter Lysis Buffer, Roche Diagnostics), and luciferase activity was measured with the Luciferase Assay System (Promega) and normalized to β -galactosidase activity, which was measured by means of the Galacto-Light Plus System (Applied Biosystems).

Case selection. Primary invasive breast carcinomas from 103 postmenopausal women (range 50–89 years) were retrieved from the files of the Department of Pathology of the University Hospital of the Katholieke Universiteit Leuven, Belgium. All patients underwent mastectomy or local wide excision with axillary lymph node dissection between 2002 and 2006. For each case, both snap-frozen and formalin-fixed paraffin-embedded tumor tissue blocks were available. Breast carcinomas were typed and graded according to the WHO Classification and the Elston and Ellis (13) grading system, respectively.

Quantitative real-time PCR. For quantitative real-time PCR (RT-PCR) analysis, we used frozen tissue blocks that had been stored at −80°C. Breast cancer cases consisted of at least 50% invasive tumor cells. As a control, we included snap-frozen biopsy specimens representing normal premenopausal ($n = 5$) and postmenopausal ($n = 14$) breast tissue obtained from mammoplastic surgery specimens. Total RNA was extracted from eight to ten 20- μ m sections of each frozen tissue block, using the RNeasy kit (Qiagen). One microgram RNA was reverse transcribed and PCR reactions on the resulting cDNA were done in the ABI-prism 7700 sequence detector (Applied Biosystems). PCR primers and fluorogenic probes (FAM or VIC as reporter dyes and TAMRA or BHQ as quencher dyes) for mouse β -actin, mouse *Chk1*, mouse *Claspin*, human *Chk1*, human *Claspin*, human *E2F1*, human *Ki-67*, and human *18S* RNA were purchased from Eurogentec. Sequences of primers and probes are available upon request. Expression of β -actin (for MC3T3-E1 cells) or *18S* RNA (for breast tissue) was used to normalize gene expression.

Immunohistochemistry. All breast cancer samples were examined for the expression of ER (clone SP1, dilution 1/100, Labvision Corporation), PR (clone SP2, dilution 1/500, Labvision Corporation), and HER-2 (clone CB11, dilution 1/40, Novocastra Laboratories). For ER and PR, any nuclear staining of invasive tumor cells was considered as positive. HER-2 immunostaining was scored according to the guidelines for HercepTest (14) and a positive HER-2 status was confirmed by fluorescence *in situ* hybridization (PathVision, Vysis).

Figure 1. Expression of *Chk1* and *Claspin* is growth dependent. **A**, time schedule of cell cycle re-entry after serum addition to serum-starved mouse MC3T3-E1 cells as determined by fluorescence-activated cell sorting analysis. **B**, *Chk1* and *Claspin* transcript levels, normalized to β -actin levels, were measured by quantitative RT-PCR after addition of serum to MC3T3-E1 cells that were synchronized in the G₁ phase of the cell cycle by serum starvation. Points, mean of three independent experiments; bars, SE.

Figure 2. Promoter activity of *Chk1* and *Claspin* is regulated by E2F transcription factors. **A and B, left**, exponentially growing MC3T3-E1 cells were transfected with an E2F expression vector, the corresponding empty control expression vector, or an expression vector for mutant E2F1, with a β -galactosidase expression vector and with a luciferase reporter construct that carried either a mouse *Chk1* (A) or *Claspin* (B) promoter fragment that contained an intact (black columns) or mutated (white columns) E2F-responsive site. **A and B, right**, exponentially growing MCF-7 cells were transfected with an E2F expression vector, the corresponding empty control expression vector, or an expression vector for mutant E2F1, with a β -galactosidase expression vector and with a luciferase reporter construct that carried either a human *Chk1* (A) or *Claspin* (B) promoter fragment that contained the E2F-responsive site. In all transfection experiments, cells were lysed 48 h after transfection. Luciferase activities, normalized for β -galactosidase activities, of the different reporter constructs were standardized against the empty pGL3 vector. Furthermore, induction by the different E2F expression plasmids was expressed relative to the pcDNA3.1. Columns, mean of at least three independent experiments; bars, SE. *, $P < 0.05$, E2F overexpression versus empty vector control pcDNA3.1 (Student's *t* test).

Protein expression of Chk1, E2F1, and Ki67, for which commercial antibodies were available, was determined in a selected series including three grade 1, three grade 2, and nine grade 3 tumors. For E2F1 (Clone KH95, 1/100 diluted, Santa Cruz Biotechnology, Inc.) and Ki-67 (clone Mib-1, 1/100 diluted, DakoCytomation), the percentage of positively stained tumor nuclei was scored as follows: 0%, negative; 1% to 10%, 1+; 11% to 25%, 2+; 26% to 50%, 3+, and >50%, 4+. For Chk1 (clone EP691Y, 1/400 diluted, Epitomics), the intensity of nuclear and/or cytoplasmic staining of invasive tumor cells was scored on a 0 to 4+ scale.

Breast carcinomas with a triple-negative ER-/PR-/HER-2- status were additionally stained for high-molecular-weight cytokeratins CK5.6 (clone D516B4, dilution 1/50, DakoCytomation), CK14 (clone LL002, 1/10 diluted, Novocastra Laboratories), and CK17 (clone E3, 1/20 diluted, DakoCytomation) and for epidermal growth factor receptor (EGFR; PharmDx kit, prediluted, DakoCytomation). For CK5/6, CK14, and CK17, a threshold of $\geq 10\%$ of invasive tumor cells showing cytosolic staining was adopted (15). EGFR immunostaining was scored according to the guidelines for HercepTest (14). Triple-negative tumors positive for at least one high-molecular-weight cytokeratin and/or EGFR were considered to have a basal-like phenotype (16).

Statistics. All statistical analyses were done with the software program Statistica (StatSoft, Inc.). Results from *in vitro* experiments in MC3T3-E1 and MCF-7 cells were expressed as the means and SE of at least three independent experiments. Significance of the overall up-regulation of gene expression after addition of serum to serum-starved cells was assessed by the Fisher's least significant difference multiple comparison test. For the transfection experiments, differences between E2F overexpression and empty vector control (pcDNA3.1) were analyzed by Student's *t* test. Differences in gene expression levels across patient groups, defined by histologic tumor grade; lymph node metastases; ER, PR, or HER-2 status; and tumor size were assessed by the Mann-Whitney *U* (two groups) or Kruskal-Wallis (three or more groups) tests. The Pearson product-moment

correlation coefficient was used to evaluate the associations between *Chk1*, *Claspin*, *E2F1*, and *Ki-67*. Logistic regression was implemented to determine whether *Chk1*, *Claspin*, *E2F1*, or *Ki-67* expression was a significant predictor of histologic tumor grade; lymph node metastasis; or ER, PR, or HER-2 status. Considering grade 3 breast carcinomas separately, we further examined whether *Chk1*, *Claspin*, *E2F1*, or *Ki-67* gene expression levels could predict ER, PR, and/or HER-2 status.

Results

Expression of *Chk1* and *Claspin* is growth-related and regulated by E2F transcription factors. To determine whether the expression of *Chk1* and *Claspin* was growth-regulated, mouse MC3T3-E1 cells were serum starved for 48 h and subsequently stimulated to re-enter the cell cycle by addition of serum (Fig. 1A). As shown in Fig. 1B, mRNA levels of *Chk1* and *Claspin* increased after the addition of serum, suggesting that the expression of these genes was growth regulated. Promoter constructs of mouse *Chk1* and *Claspin*, which carry E2F-responsive sites, were transfected in MC3T3-E1 cells together with E2F expression plasmids to investigate whether exogenous expression of E2F transcription factors could enhance *Chk1* and *Claspin* promoter activities. E2F1, E2F2, and E2F3 were able to transactivate reporter constructs that were driven by the promoter regions of *Chk1* (Fig. 2A, left, black columns; 2.1–2.2-fold induction) or *Claspin* (Fig. 2B, left, black columns; 2.5- to 11.1-fold induction). However, mutant E2F1 was unable to transactivate the mouse *Chk1* or *Claspin* promoter construct. Mutation of the E2F-responsive elements within these promoter constructs also completely abolished their responsiveness to E2F

(Fig. 2A and B, left, white columns). The sequence of the E2F-binding sites was well conserved between mouse and human. Human promoter constructs were transfected in human MCF-7 breast cancer cells together with different E2F family members. E2F1, E2F2, E2F3, and E2F4 were able to transactivate reporter constructs that were driven by the promoter regions of human *Chk1* (Fig. 2A, right; 1.4- to 2.3-fold induction) or *Claspin* (Fig. 2B, right; 1.4- to 4.3-fold induction).

Transcript levels of *Chk1* and *Claspin* are highly correlated with *E2F1* transcript levels in human breast carcinomas. As shown in Fig. 3, expression levels of *Chk1*, *Claspin*, *E2F1*, and *Ki-67* were consistently low in normal postmenopausal breast tissue as opposed to premenopausal tissue. In postmenopausal breast carcinomas, there was a strong and statistically significant correlation between *Chk1* and *E2F1* expression (Pearson product-moment correlation coefficient of 0.66) as well as between *Claspin* and *E2F1* expression (correlation coefficient of 0.74). Transcript levels of *Chk1* and *Claspin* also correlated very well (correlation coefficient of 0.84). Moreover, expression levels of *Chk1*, *Claspin*,

and *E2F1* correlated strongly with the expression level of the proliferation marker *Ki-67* (correlation coefficients of 0.73, 0.78, and, 0.57, respectively).

Evaluation of *Chk1*, *Claspin*, *E2F1*, and *Ki-67* transcript levels in postmenopausal breast carcinomas. Gene expression levels of *Chk1*, *Claspin*, *E2F1*, and *Ki-67* were significantly higher in tumors showing large tumor size (>2 cm), high histologic grade, and lack of ER and/or PR expression (Table 1). No significant expression differences were found between HER-2-positive and HER-2-negative tumors. Neither was there a correlation with the presence of lymph node metastases at diagnosis.

Transcript levels of the four investigated genes were higher in triple-negative ER-/PR-/HER-2- grade 3 breast carcinomas compared with all other grade 3 tumors (Table 2). When corrected for multiple comparisons, significance was only reached for *Chk1* ($P < 0.0001$) and *Ki-67* ($P < 0.01$). In logistic regression analysis, *Chk1* was found to be the most powerful single predictor of the triple-negative status of breast tumors (area under receiver operating characteristic curve of 0.83; $P < 0.01$).

Immunohistochemistry. Protein expression of E2F1, Ki-67, and Chk1 was generally higher in high-grade compared with low-grade carcinomas (Fig. 4). In particular, triple-negative ER-/PR-/HER-2- breast carcinomas tended to show stronger Chk1 expression compared with other grade 3 tumors.

Twelve of 16 (75%) triple-negative tumors showed a basal-like immunophenotype (Supplementary Table). Additional staining for "basal-like markers" in triple-negative breast cancer was not correlated with higher expression levels of *Chk1*, *Claspin*, *E2F1*, or *Ki-67*.

Discussion

In the present study, we investigated the transcriptional regulation of *Chk1* and *Claspin* by the E2F family of transcription factors and examined *Chk1* and *Claspin* transcript levels in a set of 103 primary invasive breast carcinomas. A first indication of the E2F dependency of the *Chk1* and *Claspin* promoter activity was obtained by the observation that *Chk1* and *Claspin* transcript levels were growth-regulated both *in vitro* and *in vivo*. Indeed, *Chk1* and *Claspin* mRNA expression levels were significantly down-regulated in the serum-arrested mouse MC3T3-E1 cells. Likewise, *Chk1* and *Claspin* transcript levels were significantly lower in normal postmenopausal breast tissue in comparison with normal premenopausal breast tissue, which is known to contain a larger proportion of hormone dependent, proliferating cells (16). Transient transfection experiments illustrated that both mouse and human *Chk1* and *Claspin* promoter activities were induced upon cotransfection of activating E2F family members. However, further studies are required to determine the contribution of posttranscriptional stabilization to the transactivation of *Chk1* and *Claspin* by the E2F transcription factors *in vivo*.

In our series of primary invasive breast carcinomas, we found a strong correlation between the expression levels of *Chk1* and *Claspin*. This correlation is in agreement with the finding that Chk1 overexpression increases Claspin levels (17). Furthermore, we found a tight correlation between the expression levels of both *Chk1* and *Claspin* and the proliferation marker *Ki-67*. These findings are consistent with the data of Tort et al. (18), that human lymphomas with high proliferative activity showed higher levels of Chk1 mRNA and protein and confirm recent data of Tsimaratou et al. (19), who suggested the use of Claspin as a proliferation

Figure 3. *Chk1* and *Claspin* are more abundantly expressed in normal premenopausal breast tissue. Transcript levels of *Chk1*, *Claspin*, *E2F1*, and *Ki-67* were determined in normal premenopausal ($n = 5$) and postmenopausal ($n = 14$) breast tissue obtained from mammoplastic surgery specimens. Mann-Whitney U tests were applied to investigate expression differences between premenopausal and postmenopausal breast tissue.

Table 1. Patient and tumor characteristics of 103 cases of primary invasive breast carcinoma analyzed by quantitative RT-PCR

Characteristics	Cases, n (%)	<i>Chk1</i> *		<i>Claspin</i> [†]		<i>E2F1</i> [‡]		<i>Ki-67</i> §	
		Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>
Menopausal status									
Premenopausal	0								
Postmenopausal	103 (100.0)	10.9 ± 1.1		4.6 ± 0.5		40.2 ± 5.3		94.7 ± 9.0	
Tumor grade			<0.0001		<0.0001		<0.0001		<0.0001
Grade 1 (7 ITC and 13 IDC)	20 (19.4)	5.0 ± 1.1		1.3 ± 0.2		11.8 ± 2.1		30.4 ± 4.2	
Grade 2 (11 ILC and 10 IDC)	21 (20.4)	4.2 ± 0.5		1.6 ± 0.2		16.0 ± 2.4		33.0 ± 4.5	
Grade 3 (10 ILC and 52 IDC)	62 (60.2)	15.1 ± 1.6		6.6 ± 0.8		57.6 ± 8.0		138.0 ± 12.3	
Tumor size (cm)			0.0336		0.0006		0.0022		0.0045
≤2	63 (61.2)	8.6 ± 1.6		3.2 ± 0.8		31.6 ± 10.5		71.5 ± 12.3	
>2	40 (38.8)	12.4 ± 1.5		5.5 ± 0.7		45.7 ± 5.6		110.9 ± 12.3	
Lymph node status			0.2548		0.8495		0.5531		0.8125
Negative	42 (40.8)	10.4 ± 1.2		4.5 ± 0.5		37.6 ± 5.5		97.2 ± 12.7	
Positive	61 (59.2)	11.7 ± 2.0		4.7 ± 1.1		43.9 ± 10.4		93.2 ± 12.7	
ER status			<0.0001		<0.0001		<0.0001		<0.0001
Negative	31 (30.1)	18.9 ± 2.2		7.6 ± 1.1		65.4 ± 13.2		162.7 ± 20.3	
Positive	72 (69.9)	7.5 ± 1.0		3.2 ± 0.5		29.3 ± 4.6		66.7 ± 7.5	
PR status			<0.0001		<0.0001		<0.0001		<0.0001
Negative	40 (38.8)	17.0 ± 2.0		6.9 ± 1.0		57.6 ± 10.7		146.5 ± 17.5	
Positive	63 (61.2)	7.1 ± 1.0		3.1 ± 0.7		29.2 ± 5.0		63.3 ± 7.5	
HER-2 status			0.3957		0.0093		0.0352		0.0284
Negative	73 (70.9)	11.5 ± 1.5		4.4 ± 0.7		39.1 ± 7.1		92.2 ± 12.2	
Positive	30 (29.1)	9.5 ± 1.0		4.9 ± 0.5		42.8 ± 5.8		103.8 ± 9.9	

Abbreviations: ITC, invasive tubular carcinoma; IDC, invasive ductal carcinoma—no specific type; ILC, invasive lobular carcinoma.

**Chk1*/18S*1E7.

†*Claspin*/18S*1E7.

‡*E2F1*/18S*1E7.

§*Ki-67*/18S*1E7.

||Unadjusted *P* values according to Mann-Whitney *U* test (two groups) or Kruskal-Wallis test (three groups).

marker in human cancer and normal tissues. Interestingly, we found concurrent up-regulation of all three markers, *Ki-67*, *Chk1*, and *Claspin*, with *E2F1*. Whereas coregulation of *Ki-67* and *E2F1* has been shown before (20, 21), our data strongly support the findings of microarray studies, showing that proliferation-associated

genes are often concurrently up-regulated in breast cancer, which is called a proliferation cluster (22). Many of these proliferation-associated genes are shown to contain binding sites for the E2F transcription factors and *E2F1* itself is a member of this proliferation signature (23).

Table 2. Transcript levels of *Chk1*, *Claspin*, *E2F1*, and *Ki-67* in grade 3 breast tumors

Characteristics	Cases, n (%)	<i>Chk1</i> *		<i>Claspin</i> [†]		<i>E2F1</i> [‡]		<i>Ki-67</i> §	
		Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>	Mean ± SE	<i>P</i>
Tumor type			<0.0001		0.1221		0.2244		0.0020
ER−, PR−, HER-2−negative tumors	16 (26.2)	25.8 ± 3.3		9.1 ± 2.0		74.3 ± 24.0		206.2 ± 33.7	
Other grade 3 tumors	45 (73.8)	11.4 ± 1.7		5.7 ± 0.8		51.9 ± 7.2		114.4 ± 10.1	

**Chk1*/18S*1E7.

†*Claspin*/18S*1E7.

‡*E2F1*/18S*1E7.

§*Ki-67*/18S*1E7.

||Unadjusted *P* values according to Mann-Whitney *U* test (two groups) or Kruskal-Wallis test (three groups).

The expression of *Ki-67*, and also of *Chk1*, *Claspin*, and *E2F1*, was strongly associated with histologic tumor grade and lack of ER and/or PR expression, but not with HER-2 status and lymph node status. Immunohistochemical stainings illustrated that also at the protein level, the highest expression of Chk1, E2F1, and Ki-67 was found in grade 3 tumors. Most of these findings on Ki-67 are in agreement with previous reports (24). Interestingly, we found that the transcript levels of *Ki-67* and especially *Chk1* were significantly higher in grade 3 breast carcinomas showing a triple-negative ER-/PR-/HER-2- status compared with other grade 3 tumors. In parallel, protein levels of Chk1 and Ki-67 tended to be increased in grade 3 triple-negative breast carcinomas. These data fit with the intrinsic breast cancer subtypes revealed by gene expression profiling studies. These have identified at least four distinct breast cancer subgroups that have prognostic as well as predictive implications (25–27): ER-positive or luminal, ER-negative/HER-2-positive, normal breast-like, and basal-like tumors. The latter basal-like tumors show an aggressive clinical behavior. They are characterized by a triple-negative ER-/PR-/HER-2-status, the expression of high-molecular-weight cytokeratins, and overexpression of proliferation-associated genes and/or EGFR (26, 28, 29). Further studies have shown that these tumors encompass as many

as 25% of grade 3 breast carcinomas and that they are associated with a high mitotic index (30, 31). Most of the triple-negative tumors in our study showed a basal-like immunophenotype (12 of 16, 75%) as they expressed one or more high-molecular-weight cytokeratins and/or EGFR, which is in line with the literature (32). However, additional staining for basal-like markers in triple-negative breast cancer was not correlated with higher expression levels of *Chk1*, *Claspin*, *E2F1*, or *Ki-67*.

Whereas ER- and/or PR-positive breast carcinomas can be treated with antiestrogen therapy and HER-2-positive carcinomas with anti-HER-2 monoclonal antibody therapy, no targeted therapeutics are available for triple-negative ER-/PR-/HER-2- tumors, including the basal-like subtype. At present, chemotherapy is the only option for the latter and success rates have been controversial (33, 34). Therefore, novel therapeutic approaches are mandatory for the triple-negative breast cancer patient. In this context, inhibition of EGFR signaling by either anti-EGFR monoclonal antibodies or small-molecule tyrosine kinase inhibitors has been proposed because EGFR overexpression has been reported in a substantial proportion of triple-negative and/or basal-like breast carcinomas (35, 36). In our series of 16 triple-negative tumors, six cases showed EGFR overexpression (37.5%).

Figure 4. Immunohistochemical staining of Chk1, E2F1, and Ki-67 in breast carcinoma. Invasive lobular carcinoma, classic-type (grade 2; A–C) compared with grade 3 invasive ductal carcinoma, triple ER-/PR-/HER-2- (D–F). A, weak cytoplasmic/nuclear Chk1 staining (score 1+) compared with (D) strong, mainly cytoplasmic Chk1 staining (score 4+). B, nuclear E2F1 staining in <10% of invasive tumor cells (score 1+) compared with (E) nuclear E2F1 staining in at least 50% of invasive tumor cells (score 4+). C, nuclear Ki-67 staining in <10% of invasive tumor cells (score 1+) compared with (F) nuclear Ki-67 staining in at least 50% of invasive tumor cells (score 4+). Original magnification, $\times 400$.

Unfortunately, targeted anti-EGFR therapy in breast cancer has been quite disappointing thus far and in contrast to anti-HER-2-targeting, it is as yet unclear which patients are likely to benefit from anti-EGFR therapy (37, 38). In the present study, we found higher expression of the checkpoint proteins Claspin and especially Chk1 in triple-negative breast carcinomas. Interestingly, it has been shown that inhibition of checkpoint signaling enhances the effect of concomitant treatment with DNA-damaging drugs or radiation. Especially, targeting of the kinase Chk1 was suggested to be effective in the treatment of cancer (39). Different Chk1-inhibitors, among which 7-hydroxystaurosporine (UCN-01), the indocarbazole Go6976, isogranulatimide, CEP-3891, and CHIR-124, have been shown both *in vitro* as *in vivo* to increase chemosensitivity and radiosensitivity in tumor cells (ref. 40 and references herein; refs. 41, 42). Triple-negative breast carcinomas frequently harbor alterations in DNA damage response pathways such as p53 and BRCA1, both of which are critically involved in the G₁-S phase checkpoint; as a result, these tumor cells largely depend on the G₂-M checkpoint in case of DNA damage (31, 43, 44). It would therefore be interesting to investigate whether anti-Chk1-containing therapeutic regimens, which sensitize to DNA damage, would be useful in the treatment of triple-negative breast carcinomas. A major challenge in this field would concern specific targeting of tumor cells because Chk1 is essential for the maintenance of genomic integrity also in normal cells. To this end, a new

generation of oncolytic adenoviral vectors, expressing antisense *Chk1* cDNA preferentially in tumor cells, has been used in a preclinical setting and yielded promising results (6, 23). Alternatively, Chen et al. (45) have recently developed two potent and selective Chk1 inhibitors, A-690002 and A-641397, which potentiate the cytotoxicity of DNA-damaging agents in p53-deficient cancer cells but not in p53-proficient normal cells.

In conclusion, expression of the E2F-regulated genes *Chk1* and *Claspin* correlated strongly with several adverse clinicopathologic variables in breast cancer. Moreover, *Chk1* expression was significantly higher in grade 3 breast carcinomas showing a triple-negative ER-/PR-/HER-2- phenotype compared with other grade 3 tumors. Further research is warranted to validate the use of *Chk1* inhibitors in triple-negative breast carcinomas for which treatment strategies are limited at present.

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