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CHFR-Associated Early G_2/M Checkpoint Defects in Breast Cancer Cells

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Cell division is a highly regulated process. Checkpoints can halt cell-cycle progression due to adverse conditions such as misalignment of chromosomes to prevent missegregation. The search for new regulators of the cell cycle revealed the mitotic checkpoint gene *CHFR* (checkpoint with forkhead-associated and ring finger). *CHFR* coordinates an early mitotic phase by delaying chromosome condensation in response to a mitotic stress. Because aneuploidy and chromosome instability are common in malignant breast tumors, we screened 24 breast cancer cell lines for *CHFR* expression and demonstrated that 50% (12 of 24) of breast cancer cell lines had low *CHFR* levels. Expression of *CHFR* was reactivated with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) in two low-*CHFR*–expressing cell lines. Eleven of these 12 (92%) low-*CHFR*–expressing cell lines had an unusually high number of condensed chromosomes and high mitotic indices in response to nocodazole treatment. Transfection of *CHFR* in one of these cancer cell lines lowered the mitotic index after nocodazole treatment. In conclusion, our data suggested that low *CHFR* expression associated with high mitotic indices in response to nocodazole treatment were common in the breast cancer cell lines studied. Additional flow cytometry studies and analysis of a protein that interacts with CHFR in vitro, polo-like kinase 1 (PLK1), suggests that this CHFR-associated early G_2/M checkpoint is complex, involving additional, as yet unidentified, proteins. Further analysis of CHFR in breast cancer cells will be important for understanding the complex mechanisms leading to aneuploidy and chromosomal instability observed in breast cancer.

Key words: CHFR; checkpoint; breast cancer; G_2/M, mitosis

INTRODUCTION

The somatic cell cycle is a highly regulated process with checkpoints that maintain normal progression through each phase and insure that each step takes place without errors. Checkpoints monitor G_1/S and G_2/M transitions where cancer related defects can occur. Mutations that disrupt some checkpoint genes have been linked to genetic instability observed in cancer cells [1]. *CHFR* (checkpoint with forkhead-associated and ring finger) is a recently identified checkpoint gene that functions at the early G_2/M phase [2]. G_2 to M phase progression in the cell cycle is controlled at least in part by the cell division cycle 2 (CDC2)/cyclin B1 complex [3]. Cyclin B1 starts to accumulate and associates with CDC2 to form the mitosis promoting factor (MPF) complex as cells enter prophase [4,5]. The MPF complex is then transported to the nucleus and kept inactive by WEE1 and MYT1 kinases, which phosphorylate CDC2 [6–10]. CDC25C dephosphorylates CDC2 to activate the MPF complex so that the cell can proceed into prometaphase where chromosomes condense, nuclear envelope breaks down, and mitotic spindles form [11–14].

Recent studies demonstrate that CHFR functions as an important checkpoint protein early in the G_2/M transition and its activation delays the cell cycle in prophase, preventing chromosome condensation in response to a mitotic stress by prolonging the inhibitory phosphorylation state (Tyr15) of CDC2 in vitro [2,15]. Initial analysis indicates that the CHFR-associated G_2/M checkpoint is inactivated, as demonstrated by high mitotic indices due to lack of *CHFR* expression or *CHFR* mutations in a neuroblastoma, an osteosarcoma, and two colon cancer cell lines (four of eight different cancer cell lines) [2]. Further studies also found hypermethylation of the *CHFR* promoter in a variety of cancer cell lines, including esophageal, colon, lung, osteosarcoma, central nervous system, leukemic and primary

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Abbreviations: CHFR, checkpoint with forkhead-associated and ring finger; CDC2, cell division cycle 2; MPF, mitosis promoting factor; HPV, human papilloma virus; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CSGE, conformation sensitive gel electrophoresis; RT, reverse transcriptase; 5-aza-dC, 5-aza-2′-deoxycytidine; TSA, trichostatin A; PLK1, polo-like kinase 1.

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tumors of the colon, lung, and esophagus [16–19] suggesting that decreased expression is associated with the observed malignant phenotype.

Currently it is unclear which in vivo events trigger activation of the CHFR-associated G_2/M checkpoint. The fact that nocodazole is a microtubule depolymerizing agent that can activate the checkpoint before the formation of microtubule spindles suggests that early events dependent on microtubules, such as centrosome duplication and centrosome separation, may be monitored by this checkpoint. Defects in centrosome duplication or separation are already known to be associated with genetic instability [20–22].

Because genetic instability and aneuploidy are very common in malignant breast cancers, we hypothesized that defects in the CHFR-associated G_2/M checkpoint could be an important step leading to aneuploidy and genetic instability during breast tumorigenesis. In this study, we examined the CHFR-associated G_2/M checkpoint and expression levels of *CHFR* in breast cancer cell lines to improve our understanding of this early checkpoint and to investigate its potential role in mammary tumorigenesis.

MATERIALS AND METHODS

Cell Lines

SUM149, SUM159, SUM229, SUM52, SUM102, SUM225, SUM1315, SUM185, and human papilloma virus (HPV)-immortalized nontumorigenic mammary cell lines were developed and provided by SP Ethier at the University of Michigan Comprehensive Cancer Center [23–26]. Other cancer cell lines (BT20, CAL51, DU4475, Hs578T, MDA-MB361, MDA-MB435, MCF10A, DLD-1, MCF7, MDA-MB468, MDA-MB157, BT474, T47D, BT549, MDA-MB231, MDA-MB453, and SK-BR-3) were obtained from American Type Culture Collection (Manassas, VA) and grown under recommended conditions.

RNA Isolation and Northern Blot Analysis

RNA isolation and Northern blot analysis were performed as previously described [27]. Polymerase chain reaction (PCR) products of 1.1 kb for *CHFR* (F: CAGGATCAGGAGGATTTGGA, R: AGTCAGGACGGGATGTTACG) and 0.4 kb for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (F: GGGAGCCAAAAGGGTCATCA, R: TTTCTAGACGGCAGGTCAGGT) were used to generate probes by random priming with [α-^{32}P] dCTP [28]. Band intensities were examined by visual inspection and quantified by densitometry (Alpha Innotech IS-1000 Digital Imaging System, Version 2.00).

Conformation Sensitive Gel Electrophoresis (CSGE) Analysis

Primers were designed to amplify *CHFR* coding sequence such that the PCR products were no more than 650 bp and they overlapped with neighboring PCR products (Table 1). Five micrograms of RNA was used to generate cDNA with oligo (dT) primers with the Superscript Preamplification System (GibcoBRL, Rockville, MD). Reverse transcriptase (RT)-PCR products were electrophoresed and visualized on a 3% agarose gel stained with ethidium-bromide. Three to five microliters of breast cancer and HPV11-21 cell line RT-PCR products were mixed to facilitate the heteroduplex formation. The mixtures were denatured at 95°C for 5 min, allowed to reanneal at 68°C for 30 min and analyzed by CSGE as previously described [29]. Bands were visualized after electrophoresis by ethidium-bromide staining. Samples that formed heteroduplexes were electrophoresed on agarose gels, gel purified (Qiagen, Valencia, CA), and sequenced at the University of Michigan Sequencing Core.

Table 1. Primer Pairs Used for the CSGE Analysis of *CHFR**

Sense primer	Antisense primer	Size (bp)	Annealing (T°C)
GGATGTGAATCCCGATGGAG	GTATGCCACGTTGTGTTCCG	386	60/58/56
TGGGGATGTCATCTACTTGG	AGGTCCCCATCTCCTCTCAT	500†	56/54/52
CAGGATCAGGAGGATTTGGA	TCCTGGCATCCATACTTTGC	406	56/54/52
AAGCATACCTCATCCAGCATCC	AGTACAGGTGGCAGAAAGGCTG	486	60/58/56
CAGCAGTCCAGGATTACGTGTG	AGCAGTCAGGACGGGATGTTACGG	501	62/60/58
AGCTTCCGTGAGCTGACCTAT	AAGCTCCACAGAAGAGTCACCC	330	62/60/58
ATACAGAGGCAAGCACGTCAA	GCAGTTTTGGACATTGGAAGGTT	638	62/60/58
CTGGGAAAACCACAGCATTT	CAAGGAGCGACTAACTTGGC	245	58
TCCAAAACTGCTAACCCTCG	GACATGGCAAAGCTAGCACA	323	58
CTTTGCCATGTCATCTGGAA	CAAACGCGGCTCATTTATTA	236	58

*CSGE, conformation sensitive gel electrophoresis; CHFR, checkpoint with forkhead-associated and ring finger.
Some polymerase chain reactions (PCRs) included three cycles with the first annealing temperature, followed by three cycles at the middle temperature and 29 cycles at the lowest annealing temperature or 35 cycles with a single annealing temperature.
†Denotes a PCR product that was initially predicted to be 387 bp according to NM_018223, however, this primer set amplified a 500 bp product which is consistent with AF170724.

Methylation and Histone Modification Analysis

Cells were treated with 10 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St. Louis, MO) for 96 h or with 0.5 μM trichostatin A (TSA) (Sigma) for 24 h. Medium was aspirated off and replaced every 24 h with fresh medium for 5-aza-dC treatment. RNA from treated and untreated cells was collected as described above at the end of incubation and analyzed by Northern blot analysis for *CHFR* expression.

Mitotic Index Analysis

Actively growing breast cancer cell lines at 70–80% confluency were treated with 200 ng/mL nocodazole (Sigma) [1] for 16 h [2]. After 16 h, the media were aspirated off and cells were rinsed and trypsinized according to manufacturer's suggestions prior to incubation with a hypotonic solution of 0.075 M potassium chloride for 20 min. After centrifugation at $800 \times g$ for 5 min, the potassium chloride solution was discarded and cells were fixed in 3:1 methanol and glacial acetic mixture. Cells were dropped onto microscope slides and stained with 0.2 mg/L Giemsa stain (GibcoBRL) for 10 min, after which the slides were rinsed with phosphate buffered saline. Nine hundred cells were counted on average for each cell line. Mitotic indices were calculated based on the percentage of cells that were in any stage of mitosis with visibly condensed chromosomes as analyzed by light microscopy. Mitotic indices less than 50% were considered ''low,'' whereas indices more than 50% were considered ''high.''

Transient CHFR Transfections

A full-length *CHFR* cDNA clone was generously provided by Scholnick and Halazonetis [2]. *CHFR* cDNA was cloned into the HindIII and NotI sites of retroviral pLNCX2 vector (BD Biosciences Clontech, Palo Alto, CA). The packaging cell line PT67 was transfected with 6 μg of DNA and 12 μL of FuGENE6 (Roche, Indianapolis, IN). Hs578T cells were infected with supernatants from PT67 cells transfected with retroviral empty pLNCX2 vector, pLNCX2-*CHFR*, and pLNCX2-*LacZ* constructs. The medium was replaced with fresh medium and nocodazole was added 36 h after infection [2]. The mitotic index was determined as described. RNA was isolated and cDNA was synthesized with RNeasy and Omniscript RT kits (Qiagen) to determine levels of *CHFR* expression. *CHFR* and *GAPDH* were co-amplified in a semiquantitative RT-PCR reaction and *CHFR*/*GAPDH* ratios were calculated following methods previously described [27]. X-gal staining was performed by standard methods as a control for infection efficiency in the pLNCX2-*LacZ* infected Hs578T cells.

Cell Synchronization and Flow Cytometric Analysis of Cell Cycle

Cells were synchronized at G_1/S as described [2]. 1×10^6 control or nocodazole-treated cells were collected and resuspended in 0.1% sodium citrate, 0.1% Triton X, 100 μg/mL Rnase, and 50 μg/mL propidium iodide. Cells were then analyzed for DNA content at the University of Michigan Flow Cytometry Core. Cell-cycle profiles of synchronized cell lines were also confirmed by flow cytometry. G_2-M/G_1 ratios were determined to estimate if cells were in mitosis. Cell lines with a threefold increase of G_2-M/G_1 ratios were considered to be in mitosis after nocodazole treatment.

Western Blot Analysis

Fifty micrograms of whole cell lysates was used from breast cancer cell lines. Western blot analysis was performed as previously described [30]. The following antibodies were used: rabbit antibody specific to the phosphorylated inactive form of CDC2 (phospho-CDC2-Tyr15) was purchased from Cell Signalling (Beverly, MA) and used 1:1000 dilution. Mouse polo-like kinase 1 (PLK1) antibody was received from Zymed Laboratories (San Francisco, CA) and used 1:500 dilution. Mouse actin antibody (used as a loading control) (1:1000), goat antirabbit (1:7500) and rabbit antimouse antibodies (1:5000 for PLK1 and 1:1200 for actin) were purchased from Sigma.

RESULTS

Expression Analysis of *CHFR* in Breast Cancer Cells

Northern blot analysis revealed that 12 of 24 breast cancer cell lines had low or no *CHFR* expression (Figure 1). By RT-PCR, we were able to amplify the *CHFR* coding region even in the cell lines that did not show expression by Northern blot analysis, indicating low level of expression (e.g., DU4475).

Mutation and Methylation Analysis of *CHFR*

To determine if specific mutations inactivate *CHFR* or decrease *CHFR* expression, the coding region of *CHFR* was screened in all 24 breast cancer cell lines by CSGE. We found several sequence variants, but no predicted pathogenic alterations were detected in any of the cell lines (Table 2). Cell lines with low *CHFR* expression were then treated with both the demethylating agent 5-aza-dC and histone deacetylase inhibitor TSA. Northern blot analysis revealed that after 5-aza-dC treatment, *CHFR* expression was reactivated in two cell lines (MDA-MB435 and SUM52) (Figure 2). However, 5-aza-dC and TSA cotreatment did not cause an enhanced response in these cells compared to 5-aza-dC treatment only. In addition, we did not observe any increase of *CHFR* expression in the other cell lines with low *CHFR* levels after TSA treatment (data not shown).

Functional Analysis of the Early G_2/M Checkpoint in Breast Cancer Cells

Mitotic indices were determined in the 24 breast cancer cell lines as the percentage of cells that had

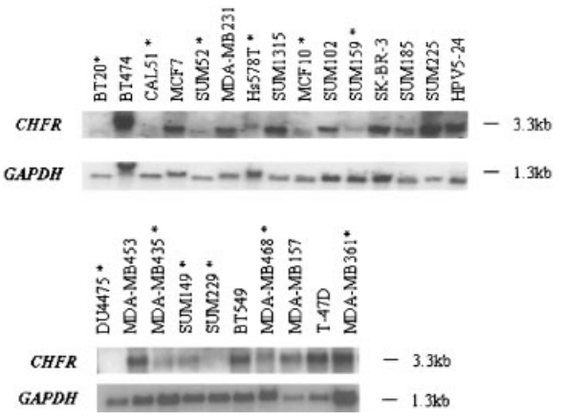

Figure 1. Low *CHFR* (checkpoint with forkhead-associated and ring finger) expression was detected in 50% (12 of 24) of breast cancer cell lines by Northern blot analysis. HPV5-24 was a human papilloma virus (HPV) immortalized mammary cell line and it was used as a positive control for *CHFR* expression but was not included in percentage calculations. Samples with the asterisks had low *CHFR* expression relative to other samples and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression. The mean *CHFR/GAPDH* ratio determined by densitometry was 0.4 for low-*CHFR*–expressing cells, whereas it was two for higher *CHFR*-expressing cells, including HPV5-24. Hybridization of *GAPDH* probe was used to assess equal loading of samples. Ethidium bromide staining of ribosomal RNA on agarose gels was consistent with both running and loading patterns of the samples assessed by *GAPDH* hybridization (data not shown).

condensed chromosomes after nocodazole treatment (Figure 3). Eleven of 12 (92%) cancer cell lines with low *CHFR* expression had high (>50%)-mitotic indices. The MDA-MB468 was the single exception, with low *CHFR* expression and a low mitotic index after nocodazole treatment. To examine whether low levels of *CHFR* expression were associated with high mitotic indices, we transiently transfected three low-*CHFR*–expressing cells with the retroviral empty pLNCX2 vector pLNCX2-*CHFR* and pLNCX2-*LacZ* constructs. Hs578T cells were the only successfully transfected cells. X-gal staining of transient LacZ transfected cells showed approximately 50% transfection efficiency. Although the level of *CHFR* in Hs578T cells as detected by semi-quantitative RT-PCR was not increased to the same endogenous levels in high-*CHFR* cell lines (0.4-fold less), mitotic index in pLNCX2-*CHFR* transfected cells was reduced to 34%, whereas vector-only transfected cells demonstrated 85% mitotic index after nocodazole treatment.

MDA-MB468 had low levels of *CHFR* transcript, but, unlike the other 11 cell lines with low-*CHFR* expression, this cell line demonstrated a low mitotic index after treatment with nocodazole. To delineate further the dynamics of cell-cycle progression through G_2/M in the low mitotic index cells, we analyzed the DNA content in these cells by flow cytometry analysis. The ratios of G_2-M (4 N) to G_1 (2 N) populations were determined to estimate if cells were arrested in mitosis or exited mitosis after nocodazole treatment (Table 3). MCF7, MDA-MB157, BT474, and SUM102 cell lines had more than threefold increase of the G_2-M/G_1 ratios after nocodazole treatment. However, no significant increase was observed in MDA-MB468, T-47D, BT549, or SUM225.

In the case of BT474, the mitotic index was 46%, which was close to our top cut-off value (50%) for categorizing lines as having low mitotic indices. Therefore, this cell line required further molecular characterization. BT474 cells were synchronized at the G_1/S border and nocodazole was introduced 12 h after G_1/S release [2]. The phosphorylation level of CDC2-Tyr 15 did not change in BT474 cells,

Table 2. Sequence Variants Detected by CSGE*

Cell line	Nucleotide change	Codon change	Status
BT20	3175 (A → G)	3′ UTR	Hemi/homozygous†
SUM159	1794 (T → C)	569 (Leu → Leu)	Heterozygous
MDA-MB361	1794 (T → C)	569 (Leu → Leu)	Heterozygous
MDA-MB453	974 (A → C)	295 (Pro → Pro)	Heterozygous
	1794 (T → C)	569 (Leu → Leu)	Heterozygous
MDA-MB468	2714 (G → A)	3′ UTR	Heterozygous
BT474	974 (A → C)	295 (Pro → Pro)	Hemi/homozygous†
	1794 (T → C)	569 (Leu → Leu)	Hemi/homozygous†
SUM225	974 (A → C)	295 (Pro → Pro)	Heterozygous
	1794 (T → C)	569 (Leu → Leu)	Heterozygous
MDA-MB231	1579 (C → T)	497 (Ala → Val)	Heterozygous
SUM185	974 (A → C)	295 (Pro → Pro)	Heterozygous
	1579 (C → T)	497 (Ala → Val)	Heterozygous
	1794 (T → C)	569 (Leu → Leu)	Heterozygous

*UTR, untranslated region.
†The genotypes of these cell lines were not known with respect to the number of alleles present; thus it was not clear if these samples were hemizygous or homozygous for the sequence changes.

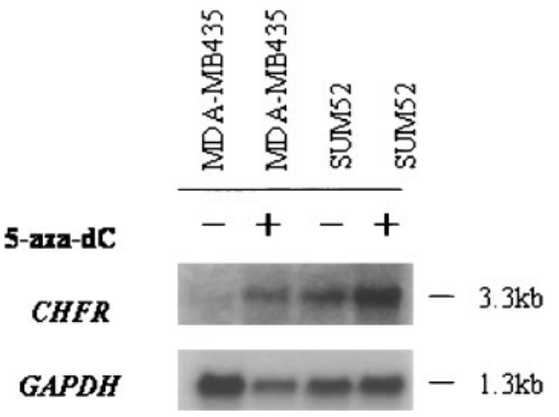

Figure 2. 5-Aza-2′-deoxycytidine (5-aza-dC) treatment reactivated transcription of *CHFR* in two cells lines that had low-CHFR levels. Cells were treated with 10 μM 5-aza-dC for 96 h. RNA was isolated as described and expression of *CHFR* was detected by Northern blot analysis.

whereas in a high mitotic index, low-*CHFR* expressing cell line Hs578T, the level of phosphorylation after nocodazole treatment decreased (Figure 4). Five of 12 (42%) breast cancer cell lines (SUM1315, MDA-MB231, MDA-MB453, SUM185, and SK-BR-3) that had high levels of *CHFR* expression, also demonstrated high mitotic indices in response to nocodazole.

Expression Analysis for the CHFR Interacting Protein PLK1

Because the only known *CHFR* interacting protein is PLK1, we examined PLK1 levels in the same 24 breast cancer cell lines. Variable levels of PLK1 expression were detected in these breast cancer cell lines; however, there were no correlating patterns of PLK1 expression with the response of cells to nocodazole or with *CHFR* levels (Figure 5A). When levels of PLK1 in synchronized cells (Hs578T and BT474) were examined, no changes were detected after nocodazole treatment, regardless of *CHFR* levels in these cells (Figure 5B).

DISCUSSION

Studies report that *CHFR* expression is ubiquitous in normal human tissues and that lack of *CHFR* expression or mutations of *CHFR* is associated with early G_2/M checkpoint defects in various cancers [2]. Here, we report that 50% (12 of 24) of breast cancer cell lines had low levels of *CHFR* expression. No pathogenic sequence alterations were found in any of the 24 breast cancer cells. The sequence variant that caused an amino acid change (Ala497 to Val) in two high-*CHFR*–expressing cells was reported to be a polymorphism [16]. However, we cannot eliminate the possibility of undetected mutations or alterations. The lack of significant sequence alterations in any of the cell lines led us to examine the possibility

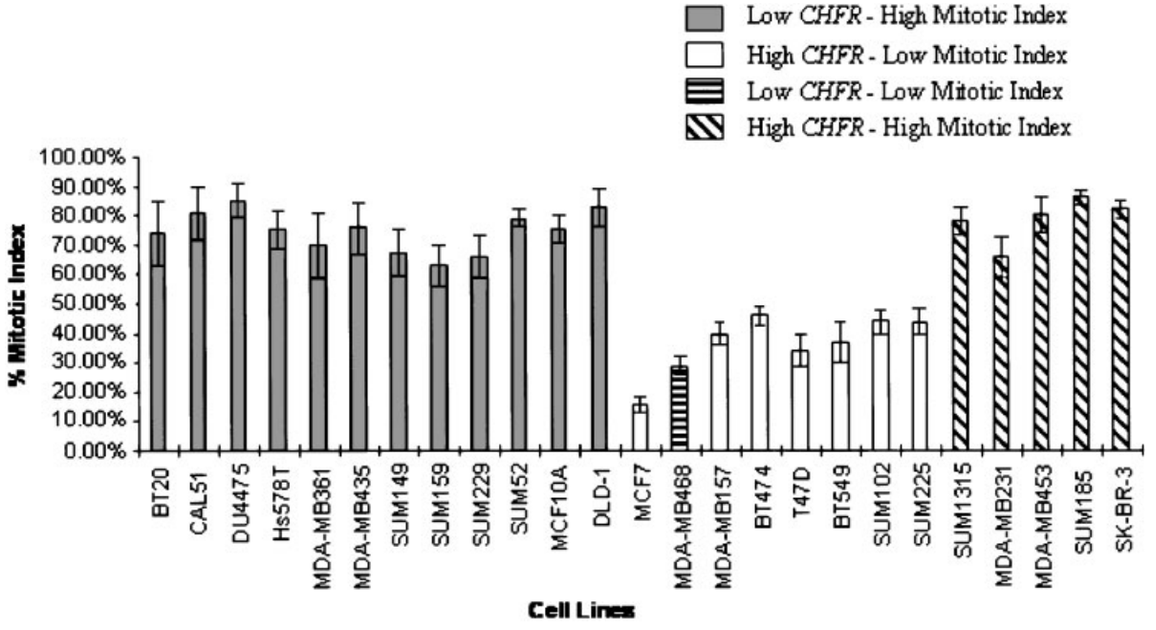

Figure 3. Eleven of 12 (92%) low-*CHFR*–expressing cells had high mitotic indices. Cells were treated with nocodazole and mitotic indices were determined based on the percentage of cells with condensed chromosomes. DLD-1 is a colon cancer cell line that lacks *CHFR* expression, has a high mitotic index in response to nocodazole [2], and was used as a control in this experiment but was not included in percentage calculations. Dark gray bars, cell lines that had low-*CHFR* expression and high (more than 50%)-mitotic indices; white bars, high-*CHFR* expression, low (less than 50%)-mitotic indices; horizontally dashed bar, the only cell line that had low *CHFR* and low (less than 50%) mitotic index; and diagonally dashed bars, high-*CHFR* expression, high (more than 50%)-mitotic indices.

Table 3. G_2-M/G_1 Ratios Before and After Nocodazole Treatment

Cell line (nocodazole)	G_2-M/G_1 ratio −	+	Fold change
MCF7	0.12	0.52	4.3
MDA-MB468	0.89	0.94	1.1
MDA-MB157	0.17	0.71	4.2
BT474	0.29	1.36	4.7
T47D	0.17	0.28	1.6
BT549	0.32	0.37	1.2
SUM102	0.23	0.74	3.2
SUM225	0.11	0.22	2.0

Flow cytometry analysis was used to determine the DNA content of cell lines. Fold change was calculated as the change in G_2-M/G_1 ratios before and after nocodazole treatment.

of hypermethylation or histone modification of the *CHFR* promoter in low-*CHFR*–expressing cell lines because such alterations are reported as common mechanisms of *CHFR* silencing in different cancer types [16–19]. Data showed that *CHFR* was reactivated in two cell lines after treatment with a demethylating agent, suggesting other mechanisms to be more common than epigenetic regulation to account for low *CHFR* expression in breast cancer cells.

Lack of *CHFR* expression or mutations of *CHFR* have been associated with high mitotic indices and abnormal response to mitotic stress in cancer cells [2,19,31]. Consistent with previous data, 11 of 12 (92%) low-*CHFR*–expressing breast cancer cell lines demonstrated high mitotic indices in response to nocodazole treatment. Transient transfection of *CHFR* into Hs578T, a cell line which had low *CHFR* expression and a high mitotic index after nocodazole treatment, lowered the mitotic index after nocodazole treatment, suggesting that the high mitotic index seen in nocodazole treated Hs578T cells was at least in part due to low *CHFR* levels. Among the other low-*CHFR*–expressing cells we tried to transfect, Hs578T was the only successfully transfected cell line. This observation is also consistent with previous studies reporting difficulty of transfecting *CHFR* into mammalian cells and the growth disadvantage presumably resulted from *CHFR* overexpression [15,19].

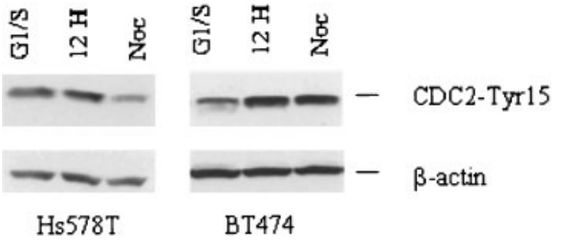

Figure 4. Western blot analysis showed prolonged cell division cycle 2 (CDC2) Tyr15 phosphorylation in early G_2/M checkpoint arrested BT474 cells. Decreased phosphorylation was detected in Hs578T cell line, which had low-*CHFR* and high mitotic index. Hs578T and BT474 cells were synchronized at G_1/S border by thymidine and thymidine/deoxyctidine, aphidicolin block. Lysates were collected at G_1/S border, 12 h after G_1/S release and after mitotic stress was induced. Mitotic stress was induced 12 h after G_1/S block [2].

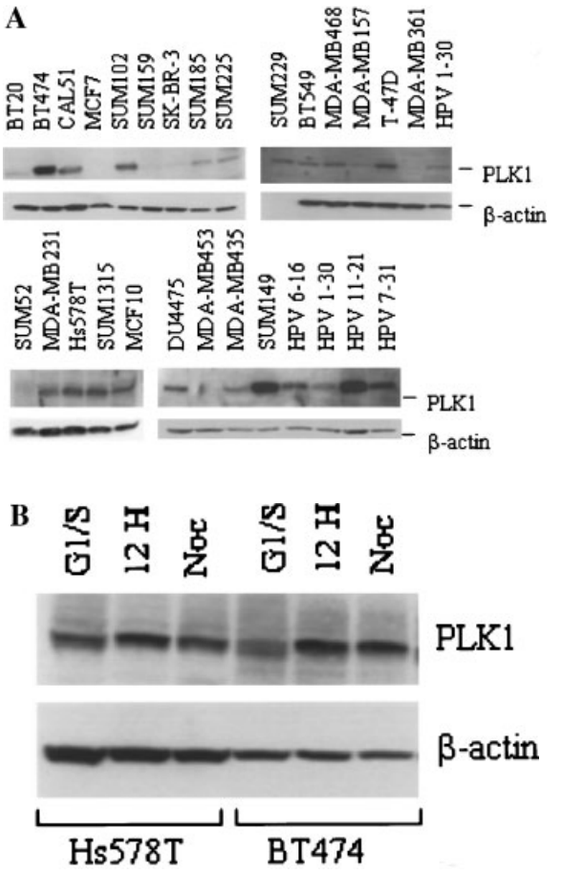

Figure 5. Polo-like kinase 1 (PLK1) levels were independent of *CHFR* levels in breast cancer cells. (A) Western blot analysis showed variable expression of PLK1 in breast cancer cell lines. HPV-immortalized cell lines were used as controls for PLK1 expression. (B) PLK1 levels remained the same in synchronized Hs578T and BT474 cells regardless of *CHFR* levels in these cells after nocodazole treatment.

The only cell line of 12 with low *CHFR* expression that also demonstrated low mitotic index was MDA-MB468. Cell-cycle analysis of low mitotic index cells after nocodazole treatment revealed that four cell lines including MDA-MB468 failed to arrest in mitosis and had exited mitosis possibly due to defects in both the early G_2/M and spindle checkpoints.

To further characterize ''low'' and ''high'' mitotic index cell lines, dephosphorylation of CDC2 as a marker of mitotic progression was investigated in two cell lines from both groups because CHFR delays dephosphorylation of CDC2 in response to a mitotic

stress [15]. Inhibitory Tyr15 phosphorylation of CDC2 was detected in BT474 in support of CHFR-activated early G_2/M checkpoint arrest. The Hs578T cell line, which had low *CHFR* expression and displayed high mitotic index, demonstrated decreased Tyr15 phosphorylation of CDC2, indicating activation of CDC2 and thus mitotic progression.

Interestingly, five of 12 (48%) high-*CHFR* expressing cell lines also had high mitotic indices, suggesting the presence of other proteins involved in this early G_2/M checkpoint or CHFR inactivation due to undetected mutations. The only other protein interacting with CHFR reported to date is PLK1. In *Xenopus* cell-free extracts, CHFR can ubiquitinate PLK1 for degradation through its ubiquitin ligase activity [15,31]. Therefore, we examined the levels of PLK1 in the 24 breast cancer cell lines, but no correlation was found between PLK1 levels and mitotic index or *CHFR*. In addition, no changes were detected in PLK1 levels after nocodazole treatment that would trigger activation of the CHFR-associated early G_2/M checkpoint in synchronized cells. The in vitro ubiquitination of PLK1 by CHFR may actually involve more complex interactions in vivo. As we begin to understand how this checkpoint functions, we will be better able to investigate how the molecular interactions take place in the cell when the checkpoint is activated.

In this study, we report that low *CHFR* expression and high mitotic indices after nocodazole treatment were common in breast cancer cells. The high incidence of low *CHFR* expression associated with an unexpectedly high percentage of condensed chromosomes after nocodazole treatment in breast cancer cell lines may suggest a potential role for CHFR and the early G_2/M checkpoint. CHFR and its role in the early G_2/M checkpoint need to be further explored to reveal the in vivo conditions that activate this pathway, response of the pathway to different drugs, and key proteins that may function in this or related pathways. Analysis of CHFR expression in additional breast cancer cells, including primary breast tumors, with a highly specific CHFR antibody as well as in vivo analysis in animal models, will help elucidate the significance of *CHFR* alterations in human breast cancers. Elucidation of this CHFR checkpoint should help us better understand the genetic instability commonly observed in malignant lesions and may provide important insights for cancer therapy.

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