ORIGINAL ARTICLE

Silencing of syndecan-binding protein enhances the inhibitory effect of tamoxifen and increases cellular sensitivity to estrogen

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ABSTRACT
Objective: Tamoxifen is used as a complementary treatment for estrogen receptor (ER)-positive breast cancer (BCa), but many patients developed resistance. The aim of this study was to examine the role of syndecan-binding protein (SDCBP) silencing in ER-positive BCa cells.

Methods: In MCF-7/T47D cells, the effects of SDCBP silence/overexpression on cell proliferation and estrogenic response were examined. Cell proliferation was examined using the MTT assay and cell cycle regulators were examined by Western blot. Estrogen response was examined from a luciferase activity and evaluation of transcript levels of pS2 and progesterone receptor (PR) upon estrogen administration. Samples of ER-positive BCa were stained with ERα, PR, and SDCBP antibodies, and their expression correlations were analyzed.

Results: We found that SDCBP silencing inhibited the proliferation of ER-positive BCa cells and arrested a greater number of cells in the G1 phase of the cell cycle compared to tamoxifen alone, while SDCBP overexpression limited the anti-cancer effects of tamoxifen. SDCBP silencing and overexpression also enhanced and attenuated the estrogenic response, respectively. Expression of SDCBP was negatively correlated with PR, ERα, and the PR/ERα ratio in ER-positive BCa tissue samples.

Conclusions: SDCBP may be involved in tamoxifen resistance in ER-positive BCa. Tamoxifen treatment combined with SDCBP silencing may provide a novel treatment for endocrine therapy-resistant BCa.

KEYWORDS
Syndecan-binding protein (SDCBP); tamoxifen; breast cancer; endocrine-therapy resistance

Introduction

Breast cancer (BCa) is a heterogeneous disease, and approximately 75% of all BCa cases show overexpression of estrogen receptors (ER) and/or progesterone receptors (PR). The estrogen pathway affects the expression of hundreds of genes involved in proliferation, differentiation, survival, invasion, metastasis, and angiogenesis, all of which are particularly relevant to cancer.

Apart from surgery, endocrine therapy is considered a complementary treatment in most patients and has shown consistent clinical benefits, particularly for ER-positive patients with respect to inducing tumor remission. Among all endocrine therapies, tamoxifen is the most extensively used drug and functions as a selective ER modulator that competitively blocks estrogen binding. However, many breast tumors show either primary resistance to endocrine therapies or develop secondary resistance after initial responsiveness. Approximately 20%–30% of patients who received adjuvant tamoxifen experienced relapse, and most patients with advanced disease who showed an initially positive response to tamoxifen eventually experienced disease progression. The mechanism of this resistance involves cross-talk between ER and alternative signaling pathways involved in cell survival and proliferation, such as those for epidermal growth factor receptor and human epidermal growth factor receptor 2.

Melanoma differentiation-associated gene 9 was discovered through screening of differentially expressed genes upon treatment of melanoma. This protein, also known as syntenin, interacts with syndecan family members and is therefore also known as syndecan-binding protein.
(SDCBP). The syndecan family belongs to a group of cell surface molecules and is involved in cell–cell and cell–matrix adhesion. SDCBP has a total of 298 amino acids and contains two PDZ domains, PDZ-1 (amino acids 110–193) and PDZ-2 (amino acids 194–274). The PDZ domain is found in a family of proteins that controls diverse and central physiologic processes such as migration and lipid binding.

Through cross-talk with protein kinase C alpha via adhesion-mediated activation downstream of the fibronectin signal, SDCBP activates focal adhesion kinase to take part in cellular migration and invasive BCa development. Moreover, activation of integrin β1 and extracellular signal-related kinase 1/2 was shown to be required for syntenin-mediated migration and invasion of BCa cells.

Our previous study showed that SDCBP expression was positively correlated with histologic grade and tumor staging, but negatively correlated with ERα expression. In ER-negative BCa cells, SDCBP silencing increased cell populations in G1 phase of the cell cycle and resulted in up-regulation of p21 and p27 while down-regulating cyclin E, thereby arresting the cell cycle and prohibiting cell proliferation. In the present study, we examined the effects of SDCBP on ER-positive BCa cells. To determine the role of SDCBP expression in ER-positive BCa development and whether SDCBP down-regulation can be used as a targeted treatment, we evaluated the expression profile of SDCBP in ER-positive cases. Using the RNAi technique, we analyzed the mechanisms underlying the involvement of SDCBP in ER-positive BCa development and its correlation with the estrogen-signaling pathway as well as its impact on endocrine therapy.

Material and methods

Sample collection

ER-positive breast tissue samples (n = 99) were obtained from patients who underwent surgical excision at the Department of Breast Cancer Pathology and Research Laboratory at Tianjin Medical University Cancer Institute and Hospital (China) from January to March of 2010. These samples were used in our previous study.

Immunohistochemistry

Staining of ERα, PR, and SDCBP was performed as described in our previous publication. Table S1 lists information regarding the antibodies used.

The expression levels of ERα, PR, and SDCBP were semi-quantified using a modified scoring system, where the intensity score (0 = negative; 1 = low; 2 = medium; 3 = high) was multiplied by the percentage of cells that were stained. This scoring system gives a final score ranging from 0 to 300. In the presence of cytoplasmic staining, SDCBP status was classified according to this modified scoring system: negative (0–50), weak (51–100), moderate (101–200), or strong (201–300). ERα and PR status were categorized in the same manner as SDCBP signals in the presence of nuclear staining. All cases were evaluated by two pathologists independently and any discrepancy was resolved by group discussion. The PR/ERα ratio was calculated as the PR staining score/ERα staining score. The correlation between SDCBP status and pathologic features were analyzed using a non-parametric Spearman correlation test.

Cell culture

The human BCa cell lines MCF-7 and T47D were purchased from American Type Culture Collection (ATCC® HTB-22™ and ATCC® HTB-133 respectively, Manassas, VA, USA). To deplete estrogen, cells were cultured in phenol red-free RPMI 1640 containing 2.5% HyClone Charcoal/Dextran-Treated Fetal Bovine Serum (SH30068.03, Thermo Scientific, Waltham, MA, USA) for 24 h. Next, 17-β estradiol (E2, Sigma-Aldrich, St. Louis, MO, USA) in ethanol was added to the culture medium at a final concentration of 0, 0.1, 1, or 10 nM, and the cells were cultured for another 24 h. Tamoxifen was purchased from Sigma and added to the culture medium at a final concentration of 2 μM.

Real-time quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA extraction was performed as previously reported. Primers for pS2, PR, and SDCBP are listed in Table S2 and β-actin was used as an internal control. The real-time qRT-PCR assay was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). pS2 and PR mRNA transcription levels were normalized against β-actin mRNA expression.

Establishment of SDCBP-silenced MCF-7 cells

The shRNA used to silence SDCBP and negative control shRNA are listed in Table S3 [both were designed by Genepharma Co., Ltd (Shanghai, China)]. The procedures for screening the SDCBP-silenced stable MCF-7 cell line were
performed as previously reported\textsuperscript{23}. Subcultures showing maximal SDCBP silencing were designated as “MCF-7 shRNA”, while control shRNA-transfected subcultures were designated as “MCF-7 NC”.

**SDCBP-overexpression BCa cell line construction**

SDCBP-overexpressing and control cell lines were constructed as described previously\textsuperscript{24}. Corresponding exogenous protein overexpression was evaluated by Western blot after the cells were cultured for 8 and 6 weeks for MCF-7 and T47D cells, respectively, in the appropriate medium containing 0.5 mg/mL of G418 (Sigma-Aldrich).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was performed as previously reported\textsuperscript{23}, except that MCF-7 and T47D cells were seeded at 2,000 and 1,500 cells per well in a 96-well plate, respectively.

**Flow cytometric cell-cycle analysis**

Cell-cycle analysis was performed on a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) as described previously\textsuperscript{25}.

**Western blot assay**

Holoproteins in cell lysates were extracted, quantified, and immunoblotted as previously described\textsuperscript{23}. The information and usage of antibodies are listed in Table S1. Protein expression levels were quantified using intensities normalized to β-actin. The expression levels calculated from three repeated immunoblots of all groups followed a normal distribution and were presented as the mean ± standard deviation. Student’s \(t\)-test was used to examine differences between groups.

**Luciferase assay**

Cells were co-transfected with the ER\(\alpha\) luciferase reporter plasmid PGMER-Lu (Genomeditech Co., Ltd., Shanghai, China) and wide-type Renilla luciferase reporter gene control plasmid pGMR-TK in 24-well plates. Luciferase activities in cell lysates were measured using the Dual-Luciferase Reporter Assay in triplicate and normalized to Renilla luciferase activity. pGM-CMV-Lu-transfected cells were used as positive controls and the average relative luciferase activity of transfected MCF-7 NC/MCF-7 Neo/T47D Neo cells was defined as “1”. Student’s \(t\) test was used to examine the differences between these normally distributed groups.

**Results**

**Silenced/overexpressed SDCBP influences the effects of tamoxifen on BCa proliferation**

As shown in Figure 1A, SDCBP shRNA silenced most target proteins compared to MCF-7 NC in either the presence or absence of tamoxifen. However, SDCBP silencing alone did not affect cellular proliferation in the absence of tamoxifen, but rather enhanced the suppressive effect of tamoxifen (Figure 1B). Although SDCBP silencing did not affect MCF-7 cell-cycle kinetics in the absence of tamoxifen, it consistently contributed to the arrest of more cells in G1 in the presence of tamoxifen (\(P < 0.001\), Figure 1C).

As shown in Figure 1D and 1G, SDCBP was significantly overexpressed in both MCF-7 and T47D cells; SDCBP overexpression accelerated cellular proliferation in both the absence and presence of tamoxifen in both cell lines. Under conditions of SDCBP overexpression, the effect of tamoxifen on cell proliferation was significantly attenuated (Figure 1E and 1H). Accordingly, in both cell lines, SDCBP overexpression reduced cells in G1 phase in both the absence and presence of tamoxifen and weakened the effects of tamoxifen on the cell cycle (Figure 1F and 1I).

**Effects of SDCBP silencing/overexpressing on cell-cycle regulators in MCF-7 cells in the presence of tamoxifen**

In ER-positive MCF-7 cells, tamoxifen treatment alone significantly increased p21 levels but attenuated the levels of phosphorylated Rb and cyclin D1. However, SDCBP silencing alone did not influence levels of p21, p27, cyclin D1, cyclin E, or phosphorylated Rb. In contrast, SDCBP silencing significantly up-regulated the levels of p21 and p27, but down-regulated the levels of phosphorylated Rb and cyclin E beyond that of tamoxifen alone. However, SDCBP silencing failed to further decrease cyclin D1 compared to tamoxifen alone (Figure 2A–2G). SDCBP overexpression alone did not influence p21 levels, but significantly down-regulated p27. Tamoxifen treatment did not recover the levels of p27, but up-regulated the levels of p21 under conditions of SDCBP overexpression (Figure 2H–2K).
Effects of SDCBP silencing/overexpression on estrogen responsiveness in ER-positive BCa cell line

The luciferase assay suggested that SDCBP silencing enhanced the estrogenic response when E2 was administrated at concentrations between 0.1 and 10 nM compared to MCF-7 NC counterparts ($P = 0.017$, $P = 0.020$ and $P = 0.002$, respectively) (Figure 3A). qRT-PCR evaluation showed that SDCBP silencing up-regulated pS2 and PR by 40.0% and

Figure 1  Effect of tamoxifen treatment and SDCBP silencing/overexpressing on proliferation of ER-positive breast cancer cells. (A, D, G) Expression of SDCBP in the absence or presence of 2 μM tamoxifen as shown by Western blot assay. β-actin was used as an internal reference. (B, E, H) Proliferation was examined by the MTT assay. (C, F, I) Cell-cycle progression was determined by flow-cytometric cell-cycle analysis in the absence or presence of 2 μM tamoxifen. Student’s t-test was then used to compare differences ($#P > 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).
**Figure 2** Effect of tamoxifen treatment and SDCBP silencing/overexpression on G1/S cell-cycle regulators. Western blot was conducted to examine the differential expression of p21, p27, phosphorylated Rb (phospho-Rb), cyclin D1, and cyclin E levels in MCF-7 cells with 2 μM tamoxifen treatment and/or SDCBP silencing (A)/overexpression (H). The ratios of Western blot intensities for the examined proteins to β-actin were calculated from triplicate experiments (B-G, I-K); Student’s *t*-test was then used to compare differences (*P < 0.05, **P < 0.01, ***P < 0.001, #P > 0.05).
62.3% at the mRNA level, respectively, compared to those in MCF-7 NC cells incubated with 10 nM E2 ($P = 0.026$ and $P = 0.0011$, respectively) (Figure 3B and 3C). The enhanced effect of SDCBP silencing on pS2 and PR transcription depended upon the presence of estrogen, as transcriptional levels were unaffected in the absence of estrogen ($P = 0.847$ and $P = 0.413$, respectively).

In contrast, the luciferase assay suggested that SDCBP overexpression in MCF-7 or T47D cells attenuated the estrogenic response compared to their MCF-7 and T47D Neo counterparts, respectively (Figure 3D and 3G). qRT-PCR showed that SDCBP overexpression resulted in down-regulation of pS2 and PR in MCF-7 cells (51.6% and 28.1%, respectively) (Figure 3E and 3F) and T47D cells (33.7% and 19.8%, respectively) (Figure 3H and 3I) when incubated with 10 nM E2.

Clinical pathologic characters of ER-positive BCa cases and their correlations with SDCBP expression

Correlations between pathologic characters and SDCBP
expression were examined in ER-positive BCa tissues \((n = 99)\). Among PR-negative tumors, 26.3% (5/19) demonstrated strong SDCBP staining (Table 1), while no tumors staining strongly positive for PR (0/26) showed strong positive staining for SDCBP (Table 1). Negative correlations between SDCBP expression and PR status or the PR/ERα ratio were also established \((R_S = –0.37, P < 0.001; \text{and} \ R_S = –0.24, P = 0.017, \text{respectively})\) (Figure 4 and Table 1). This experiment also showed that SDCBP expression was negatively correlated with ERα \((R_S = –0.29, P = 0.004)\) (Table 1). There were no significant differences among the different levels of SDCBP staining in lymph node involvement and pTNM stage (Table 1). Detailed information for each case is shown in Table S4.

**Discussion**

Tamoxifen is the most commonly used chemotherapeutic agent for patients with ER-positive BCa\(^26\), and tamoxifen resistance poses great challenges to BCa treatment. Some patients have presented with intrinsic resistance regardless of showing high levels of ER, while other patients initially respond to tamoxifen but later develop acquired resistance\(^27\).

Our previous study showed that expression of SDCBP can be attenuated by estrogen\(^23\); in the present study, we found that silencing of SDCBP enhances the inhibitory effect of tamoxifen with regard to cellular proliferation and cell-cycle progression in ER/PR-positive MCF-7 cells. This indicates that SDCBP drives cell proliferation and cell-cycle progression by up-regulating self-expression and activating alternative signaling pathways when estrogen signaling is inhibited. Under conditions of SDCBP overexpression, the function of tamoxifen on cell proliferation was significantly attenuated, suggesting that SDCBP overexpression leads to tamoxifen resistance in ER-positive BCa. Notably, SDCBP silencing alone did not affect cell proliferation or the expression of molecules that control the cell cycle in ER-positive MCF-7 cells; however, SDCBP overexpression

| Pathological features | Cases | Syndecan binding protein (%) | \(r_s\) | \(P^*\) |
|----------------------|-------|-----------------------------|--------|--------|
| Lymph node status    |       | Negative                    | Weak   | Moderate| Strong |
| Negative             | 52    | 15 (28.8)                   | 24 (46.2) | 8 (15.4) | 5 (9.6) | 0.08  | 0.441 |
| Positive             | 47    | 12 (25.5)                   | 18 (38.3) | 14 (29.8) | 3 (6.4) |       |       |
| pTNM stage           |       |                             |        |        |        |       |       |
| I                    | 33    | 10 (30.3)                   | 14 (42.4) | 5 (15.2) | 4 (12.1) | 0.17  | 0.101 |
| II                   | 47    | 14 (29.8)                   | 24 (51.1) | 8 (17.0) | 1 (2.1) |       |       |
| III–IV               | 19    | 3 (15.8)                    | 4 (21.1)  | 9 (47.4) | 3 (15.8) |       |       |
| PR status            |       |                             |        |        |        |       |       |
| Negative             | 19    | 2 (10.5)                    | 5 (26.3)  | 7 (36.8) | 5 (26.3) | –0.37 | <0.001|
| Weak                 | 15    | 4 (26.7)                    | 4 (26.7)  | 5 (33.3) | 2 (13.3) |       |       |
| Moderate             | 39    | 13 (33.3)                   | 17 (43.6) | 8 (20.5) | 1 (2.6) |       |       |
| Strong               | 26    | 8 (30.8)                    | 16 (61.5) | 2 (7.7)  | 0 (0.0) |       |       |
| ERα status           |       |                             |        |        |        |       |       |
| Weak                 | 30    | 7 (23.3)                    | 8 (26.7)  | 8 (26.7) | 7 (23.3) | –0.29 | 0.004 |
| Moderate             | 24    | 5 (20.8)                    | 11 (45.8) | 7 (29.2) | 1 (4.2) |       |       |
| Strong               | 45    | 15 (33.3)                   | 23 (51.1) | 7 (15.6) | 0 (0.0) |       |       |
| PR/ERα ratio\(^3\)  |       |                             |        |        |        |       |       |
| Cases                | 99    | 27                          | 42      | 22      | 8      | –0.24 | 0.017 |
|                     |       | 0.94 (0.72)                 | 0.92 (0.60) | 0.65(1.02) | 0.29 (0.85) |       |       |

\(^*\), \(P\) values were calculated by Spearman’s rank-correlation test \((n = 99)\).

\(^3\), PR/ERα ratio: represented by median (inter-quartile range), i.e. M (QR).
ERα, PR, and SDCBP expression in ER-positive breast cancer tissue. Case 1: the sample was stained with high ERα and SDCBP but low PR (H&E staining, 200 ×, respectively). Case 2: the sample was stained with moderate ERα and low SDCBP but high PR (H&E staining, 200 ×, respectively). Scale bar = 50 μm.

In conclusion, SDCBP promotes cell cycle progression in ER-positive BCa, particularly when the estrogen-signaling pathway is blocked. It also negatively regulates the estrogenic response and may play an important role in developing resistance against endocrine treatment in ER-positive BCa. These results also suggest that SDCBP silencing can be applied as a targeted treatment in ER-positive BCa.
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Conflict of interest statement

No potential conflicts of interest are disclosed.

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**Table S1**  Detailed information for antibodies used in this work.

| Name                          | Type                  | Supplier                                      | Catalog #  | Origin            | Dilution ratio       |
|-------------------------------|-----------------------|-----------------------------------------------|------------|-------------------|----------------------|
| Cyclin D1 Antibody (H-295)   | Rabbit polyclonal     | Santa Cruz Biotechnology, Inc.                | sc-753     | Dallas, TX, U.S.A. | 1:1000               |
| Cyclin E Antibody (M-20)     | Rabbit polyclonal     | Santa Cruz Biotechnology, Inc.                | sc-481     | Dallas, TX, U.S.A. | 1:1000               |
| β-Actin Antibody (C4)        | mouse monoclonal      | Santa Cruz Biotechnology, Inc.                | sc-47778   | Dallas, TX, U.S.A. | 1:1500               |
| p27 Kip1 (D69C12)            | Rabbit monoclonal     | Cell Signaling Technology, Inc.              | #3686      | Danvers, MA, U.S.A | 1:1000               |
| p21 Waf1/Cip1 (12D1)         | Rabbit monoclonal     | Cell Signaling Technology, Inc.              | #2947      | Danvers, MA, U.S.A | 1:1000               |
| ERα antibody (6F11)          | mouse monoclonal      | Thermo Fisher Scientific Inc.                | MA5-13304  | MA, U.S.A         | 1:50                 |
| PR antibody (SP2)            | Rabbit monoclonal     | Thermo Fisher Scientific Inc.                | MA5-14505  | MA, U.S.A         | 1:50                 |
| Syntenin-1 Antibody (N-20)   | goat polyclonal       | Santa Cruz Biotechnology, Inc.                | sc-19379   | Dallas, TX, U.S.A. | 1:500 1:75           |
| Phospho-Rb (S780)            | goat polyclonal       | Santa Cruz Biotechnology, Inc.                | sc-12901   | Dallas, TX, U.S.A. | 1:500               |

* Westernblot**, Immunohistochemistry

**Table S2**  Primers for semi-quantitative and real-time quantitative reverse transcription-PCR.

| Official symbol | Genebank No. | Amplified fragment length (bp) | Annealing temperature (°C) | Primer name | Sequence (from 5’ to 3’) |
|-----------------|--------------|--------------------------------|-----------------------------|-------------|--------------------------|
| pS2             | X00474       | 356                            | 56.2                        | pS2 foward  | CATGGAGAACAAGGTGATCTG    |
|                 |              |                                |                             | pS2 reverse | CAGAAGCGTGTCCTGAGGTGTC   |
| PR              | NM_001278456 | 320                            | 55.6                        | PR foward   | CCATGTGGCGAGATCCCCAGGAGTT|
|                 |              |                                |                             | PR reverse  | TGGAAATCAACACTCAGTGCCGG  |
| β-actin         | NM_001101    | 194                            | 55.9                        | β-actin foward | GTCCAACCTGGGACGACAT |
|                 |              |                                |                             | β-actin reverse | AGCAGAGCCTTGATAGCAAC |

**Table S3**  The target sequence for short-hairpin RNA design of syndecan-binding protein (SDCBP)

| Target site     | Target sequence (from 5’ to 3’) |
|-----------------|----------------------------------|
| Negative control| AATTCTCCGAAAGGTGTCAGCT            |
| 611#            | GGGACCAAGTGACTTCAGATCA            |

#: The numbers represents the position of the 5’ starting site of target sequences in syndecan binding protein mRNA (NM_001007067).
Table S4  Detailed features of 99 consecutive cases of estrogen receptor (ER)-positive breast cancer from January to March of 2010.

| SDCBP status | PR scoring | PR status | ERα scoring | ERα status | PR/ER ratio | Lymph node status | pTNM staging |
|--------------|------------|-----------|-------------|------------|--------------|-------------------|--------------|
| Negative     | 160        | Moderate  | 55          | Weak       | 2.91         | Negative          | I            |
| Negative     | 65         | Weak      | 60          | Weak       | 1.08         | Positive          | II           |
| Negative     | 150        | Moderate  | 70          | Weak       | 2.14         | Negative          | II           |
| Negative     | 30         | Negative  | 80          | Weak       | 0.38         | Negative          | I            |
| Negative     | 80         | Weak      | 85          | Weak       | 0.94         | Positive          | II           |
| Negative     | 20         | Negative  | 90          | Weak       | 0.22         | Positive          | II           |
| Negative     | 120        | Moderate  | 100         | Weak       | 1.2          | Positive          | II           |
| Negative     | 270        | Strong    | 135         | Moderate   | 2            | Positive          | II           |
| Negative     | 180        | Moderate  | 140         | Moderate   | 1.29         | Negative          | I            |
| Negative     | 130        | Moderate  | 150         | Moderate   | 0.87         | Negative          | II           |
| Negative     | 270        | Strong    | 160         | Moderate   | 1.69         | Positive          | III–IV       |
| Negative     | 75         | Weak      | 165         | Moderate   | 0.45         | Negative          | I            |
| Negative     | 270        | Strong    | 210         | Strong     | 1.29         | Negative          | I            |
| Negative     | 270        | Strong    | 210         | Strong     | 1.29         | Negative          | II           |
| Negative     | 180        | Moderate  | 210         | Strong     | 0.86         | Positive          | II           |
| Negative     | 140        | Moderate  | 210         | Strong     | 0.67         | Negative          | I            |
| Negative     | 120        | Moderate  | 210         | Strong     | 0.57         | Negative          | II           |
| Negative     | 120        | Moderate  | 210         | Strong     | 0.57         | Positive          | II           |
| Negative     | 270        | Strong    | 225         | Strong     | 1.2          | Negative          | II           |
| Negative     | 240        | Strong    | 225         | Strong     | 1.07         | Negative          | I            |
| Negative     | 120        | Moderate  | 225         | Strong     | 0.53         | Positive          | III–IV       |
| Negative     | 95         | Weak      | 225         | Strong     | 0.42         | Negative          | I            |
| Negative     | 240        | Strong    | 240         | Strong     | 1            | Negative          | I            |
| Negative     | 190        | Moderate  | 240         | Strong     | 0.79         | Positive          | II           |
| Negative     | 160        | Moderate  | 255         | Strong     | 0.63         | Positive          | II           |
| Negative     | 270        | Strong    | 285         | Strong     | 0.95         | Positive          | III–IV       |
| Negative     | 160        | Moderate  | 285         | Strong     | 0.56         | Negative          | I            |
| Weak         | 165        | Moderate  | 55          | Weak       | 3            | Negative          | II           |
| Weak         | 120        | Moderate  | 55          | Weak       | 2.18         | Positive          | II           |
| Weak         | 140        | Moderate  | 55          | Weak       | 2.55         | Negative          | II           |
| Weak         | 110        | Moderate  | 60          | Weak       | 1.83         | Positive          | II           |
| Weak         | 0          | Negative  | 75          | Weak       | 0            | Negative          | I            |
| Weak         | 90         | Weak      | 80          | Weak       | 1.13         | Positive          | II           |
| Weak         | 25         | Negative  | 80          | Weak       | 0.31         | Positive          | II           |
| Weak         | 55         | Weak      | 90          | Weak       | 0.61         | Positive          | II           |
| Weak         | 70         | Weak      | 110         | Moderate   | 0.64         | Negative          | I            |
| Weak         | 270        | Strong    | 120         | Moderate   | 2.25         | Negative          | II           |
| Weak         | 60         | Weak      | 125         | Moderate   | 0.48         | Negative          | I            |

Continued
| Weak | 255 | Strong | 135 | Moderate | 1.89 | Positive | II |
|------|-----|--------|-----|----------|------|-----------|----|
| Weak | 160 | Moderate | 135 | Moderate | 1.19 | Negative | I  |
| Weak | 210 | Strong | 140 | Moderate | 1.5  | Negative | I  |
| Weak | 180 | Moderate | 150 | Moderate | 1.2  | Negative | II |
| Weak | 50 | Negative | 150 | Moderate | 0.33 | Positive | III–IV |
| Weak | 225 | Strong | 160 | Moderate | 1.41 | Negative | II |
| Weak | 40 | Negative | 165 | Moderate | 0.24 | Negative | II |
| Weak | 120 | Moderate | 180 | Moderate | 0.67 | Negative | I  |
| Weak | 270 | Strong | 210 | Strong | 1.29 | Positive | III–IV |
| Weak | 150 | Moderate | 210 | Strong | 0.71 | Positive | II |
| Weak | 240 | Strong | 210 | Strong | 1.14 | Negative | I  |
| Weak | 0  | Negative | 210 | Strong | 0    | Positive | III–IV |
| Weak | 270 | Strong | 225 | Strong | 1.2  | Positive | II |
| Weak | 255 | Strong | 225 | Strong | 1.13 | Positive | II |
| Weak | 180 | Moderate | 225 | Strong | 0.8  | Negative | I  |
| Weak | 150 | Moderate | 225 | Strong | 0.67 | Positive | II |
| Weak | 255 | Strong | 240 | Strong | 1.06 | Positive | II |
| Weak | 240 | Strong | 240 | Strong | 1    | Positive | II |
| Weak | 180 | Moderate | 240 | Strong | 0.75 | Negative | I  |
| Weak | 180 | Moderate | 240 | Strong | 0.75 | Negative | I  |
| Weak | 140 | Moderate | 240 | Strong | 0.58 | Positive | II |
| Weak | 240 | Strong | 255 | Strong | 0.94 | Negative | II |
| Weak | 140 | Moderate | 255 | Strong | 0.55 | Negative | II |
| Weak | 285 | Strong | 270 | Strong | 1.06 | Negative | I  |
| Weak | 240 | Strong | 270 | Strong | 0.89 | Negative | II |
| Weak | 180 | Moderate | 270 | Strong | 0.67 | Positive | II |
| Weak | 160 | Moderate | 270 | Strong | 0.59 | Positive | III–IV |
| Weak | 285 | Strong | 285 | Strong | 1    | Negative | II |
| Weak | 210 | Strong | 285 | Strong | 0.74 | Negative | I  |
| Weak | 130 | Moderate | 285 | Strong | 0.46 | Negative | I  |
| Weak | 270 | Strong | 285 | Strong | 0.95 | Negative | I  |
| Moderate | 40 | Negative | 60 | Weak | 0.67 | Negative | II |
| Moderate | 40 | Negative | 60 | Weak | 0.67 | Negative | I  |
| Moderate | 0  | Negative | 60 | Weak | 0    | Negative | I  |
| Moderate | 160 | Moderate | 75 | Weak | 2.13 | Positive | II |
| Moderate | 100 | Weak | 75 | Weak | 1.33 | Positive | II |
| Moderate | 20 | Negative | 80 | Weak | 0.25 | Positive | III–IV |
| Moderate | 0  | Negative | 85 | Weak | 0    | Positive | III–IV |

Continued
| Level   | Type   | Value | Comparison | Ratio | Result   | Stage |
|---------|--------|-------|------------|-------|----------|-------|
| Moderate| Moderate| 90    | Weak       | 1.33  | Positive | III–IV |
| Moderate| Moderate| 105   | Moderate   | 1.14  | Positive | III–IV |
| Moderate| Moderate| 120   | Moderate   | 1.25  | Negative | II    |
| Moderate| Strong  | 150   | Moderate   | 1.4   | Positive | III–IV |
| Moderate| Weak    | 150   | Moderate   | 0.6   | Positive | III–IV |
| Moderate| Moderate| 160   | Moderate   | 0.75  | Positive | II    |
| Moderate| Negative| 165   | Moderate   | 0     | Negative | I     |
| Moderate| Strong  | 180   | Moderate   | 1.5   | Positive | II    |
| Moderate| Weak    | 210   | Strong     | 0.43  | Positive | III–IV |
| Moderate| Weak    | 225   | Strong     | 0.36  | Positive | II    |
| Moderate| Negative| 225   | Strong     | 0.18  | Positive | III–IV |
| Moderate| Moderate| 240   | Strong     | 0.63  | Negative | I     |
| Moderate| Weak    | 255   | Strong     | 0.24  | Positive | III–IV |
| Moderate| Moderate| 270   | Strong     | 0.67  | Negative | II    |
| Moderate| Moderate| 285   | Strong     | 0.56  | Negative | I     |
| Strong  | Moderate| 55    | Weak       | 2.18  | Negative | I     |
| Strong  | Negative| 55    | Weak       | 0     | Negative | I     |
| Strong  | Negative| 60    | Weak       | 0.33  | Positive | III–IV |
| Strong  | Weak    | 65    | Weak       | 1     | Negative | I     |
| Strong  | Negative| 75    | Weak       | 0     | Positive | III–IV |
| Strong  | Negative| 80    | Weak       | 0.25  | Negative | I     |
| Strong  | Negative| 95    | Weak       | 0.11  | Negative | II    |
| Strong  | Weak    | 120   | Moderate   | 0.5   | Positive | III–IV |