The synergistic effect of \textit{CDKN2B-AS1} and \textit{SPC25} on triple-negative breast cancer

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**Background:** Accumulating evidence suggests that long non-coding ribonucleic acid (RNA) cyclin-dependent kinase inhibitor 2B antisense RNA 1 (\textit{CDKN2B-AS1}) and messenger RNA (mRNA) spindle component 25 (\textit{SPC25}) contribute to tumorigenesis and progression in various cancers. However, the synergistic effect between \textit{CDKN2B-AS1} and \textit{SPC25} has not yet been fully elucidated in triple-negative breast cancer (TNBC). This study sought to examine the synergistic effect of \textit{CDKN2B-AS1} and \textit{SPC25} and uncover a novel mechanism for the progression of TNBC.

**Methods:** The transcriptome profiles of TNBC in The Cancer Genome Atlas (TCGA) were calculated for differentially expressed genes (DEGs). Gene co-expression networks were constructed via a weighted correlation network analysis. We validated the relationship between \textit{CDKN2B-AS1} and \textit{SPC25} by bioinformatics and \textit{in-vitro} studies (including Cell Counting Kit-8, transwell assays, and quantitative real-time polymerase chain reaction).

**Results:** \textit{CDKN2B-AS1} was found to be carcinogenic and was significantly upregulated and co-expressed with elevated \textit{SPC25} expression levels in the TNBC cells and sequencing profiles. Notably, the \textit{SPC25} mRNA levels were associated with poor clinical outcomes in TNBC patients. Specifically, the knockdown of \textit{CDKN2B-AS1} significantly inhibited TNBC cell proliferation and migration.

**Conclusions:** We identified a novel cancer-promoting regulation axis. The co-expression of \textit{CDKN2B-AS1} and \textit{SPC25} is expected to serve as a powerful candidate biomarker for diagnostic and prognostic purposes in TNBC.

**Keywords:** Triple-negative breast cancer (TNBC); cyclin-dependent kinase inhibitor 2B antisense RNA 1 (\textit{CDKN2B-AS1}); spindle component 25 (\textit{SPC25}); proliferation

Submitted Apr 22, 2022. Accepted for publication Jul 08, 2022.
doi: 10.21037/atm-22-2900

View this article at: https://dx.doi.org/10.21037/atm-22-2900

**Introduction**

In 2020, breast cancer (BC) was one of the most common cancers and the primary cause of cancer-related mortality in women worldwide (1). Despite crucial advances in the accurate diagnosis and early intervention of BC, the survival times of some patients remain short. BC is a heterogeneous disease that manifests in different molecular and clinicopathologic features (2). Triple-negative breast...
cancer (TNBC) is the most aggressive and fatal subtype of BC and accounts for 10–20% of all BC cases (3). Thus, novel therapeutic targets for TNBC desperately need to be identified.

Spindle component 25 (SPC25) is a crucial portion of the NDC80 complex that participates in spindle checkpoint activity and kinetochore-microtubule clustering (4). Recent research has shown that upregulated SPC25 accelerates the proliferation of hepatocellular carcinoma (HCC) and is related to a poor prognosis (5-7). Additionally, previous studies have also reported that SPC25 is an important tumor marker for cell cycle and malignant progression, and stem cell proliferation in prostate cancer (8). Further, SPC25 has been shown to regulate the stemness of lung cancer cells significantly (9).

Long non-coding ribonucleic acids (lncRNAs) belong to a kind of non-coding RNA that modulates gene transcription or protein translation by multiple distinct mechanisms (10). Alterations in IncRNA expression may exhibit anti-oncogenic or oncogenic activities. The IncRNA cyclin-dependent kinase inhibitor 2B antisense RNA 1 (CDKN2B-AS1) is located at chromosome 9p21, and increases the malignancy of renal cancer (11). Additionally, CDKN2B-AS1 has been shown to induce the proliferation and migration of colorectal cancer (12).

In this study, we found that upregulated SPC25 had an oncogenic function and predicted a poor overall survival (OS) time. Notably, CDKN2B-AS1-activated SPC25 enhanced the proliferation of TNBC cells. Consequently, our research findings may enable the exploration of novel targets for TNBC treatment and prognostic evaluation. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amergroups.com/article/view/10.21037/atm-22-2900/rc).

Methods

Retrieving data

We downloaded the transcriptome profiles and survival information of TNBC from The Cancer Genome Atlas Program (https://portal.gdc.cancer.gov/). GSE45827 dataset was obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) database. We further investigated the pan-cancer expression profiles based on the Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) (13). A Clinical Proteomic Tumor Analysis Consortium (CPTAC, https://cptac-data-portal.georgetown.edu/) was conducted to confirm the protein expression analysis. The Human Protein Atlas (https://www.proteinatlas.org/) was used to verify protein expression levels in diverse tissues and the subcellular localization of the SPC25 protein. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Identification of DEGs and the weighted gene co-expression network analysis (WGCNA)

R software DESeq2 (14) and limma (15) packages were used to identify the differentially expressed genes (DEGs) of the transcriptome data. A WGCNA algorithm (16) was conducted to identify the gene modules that were highly correlated with TNBC. Gene significance (GS) and module membership (MM) were applied to quantify the features and configurations of the modules. The protein–protein interaction network was constructed by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) with minimum required interaction score >0.4 and PPI enrichment P value <1×10−16. Hub genes of TNBC were identified and visualized by the Molecular Complex Detection (MCODE) plugins in Cytoscape. LncSEA as a comprehensive IncRNA sets resource and enrichment analysis platform was performed to analyze functions of lncRNAs.

Functional enrichment analysis

A gene set enrichment analysis (GSEA) was conducted to explore the key module genes and detect some crucial biological functions, such as cell cycle and cellular signaling pathway. GSEA software was used for the GSEA with the following parameters: number of permutations =1,000; Normalized Enrichment Score (NES) >2; nominal (NOM) P value <0.05; and false discovery rate <0.05. The STRING (https://cn.string-db.org/) database was used to identify the proteins co-expressed with SPC25. Subsequently, a Gene Ontology (GO) pathway enrichment analysis was conducted using the clusterProfiler (17) packages in R software.

Single-cell analysis and survival analysis

CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/) was applied to reveal the potential function of SPC25 in BC (18). A survival analysis was performed and visualized using the survival and survminer packages in R software.
**Cell culture and transfection**

Normal human breast cell lines (MCF10A) and TNBC cell lines (i.e., BT549, MDA-MB-231, and MDA-MB-468) were obtained from American Type Culture Collection (ATCC). All the cell lines were authenticated by short tandem repeat deoxyribonucleic acid (DNA) profiling and tested for mycoplasma (GENEWIZ Co., Ltd., Suzhou, China) within 6 months. Lipofectamine 2,000 (Invitrogen, USA) was used for transfection. The GeneCreate Biological Engineering Company (Wuhan, China) synthesized small-interfering RNA specifically targeting CDKN2B-AS1 (si-CDKN2B-AS1) and corresponding non-specific siRNA (si-NC). The siRNA sequences used in our research were: 5’-CCACACAUCUCUUGGAGUAAUTT and AUUACUCAAAGGGAUGUGGT-3’.

**RNA extraction and qRT-PCR**

We applied the Trizol reagent (Introvigen, USA) to extract total RNA from the cell lines. Subsequently, the RNAs were reverse transcribed into complementary DNA and quantitative real-time polymerase chain reaction (qRT-PCR) assays were conducted. The $2^{-\Delta \Delta C_t}$ method was used to calculate the expression level of CDKN2B-AS1 and SPC25. The primers were purchased from The GeneCreate Biological Engineering Company (Wuhan, China). The primer sequences of the RNAs are shown in Table S1.

**Cell proliferation**

Cell counting kit-8 (CCK-8) and transwell assays were used to assess cell proliferation viability and migration. First, the MDA-MB-231 cells were transfected with si-CDKN2B-AS1 or si-NC. Second, the CCK-8 solution (Beyotime, China) was supplemented, and the absorbance value at 450 nm was measured at different time points (i.e., 0, 24, 48, and 72 h).

**Cell migration experiments**

The transfected cells (2×10^4/well) were seeded into the upper compartment of a 24-well transwell chamber (Corning Costar, USA). The lower chambers contained 10% fetal bovine serum. After incubation for 48 h, the non-migrated cells were carefully removed from the upper chamber with cotton swabs. We randomly selected the average confluence of the migrated cells from 3 visual fields (captured by a 100× microscope) to count the number of cells.

**Cell apoptosis experiments**

Cells were harvested and washed twice with PBS buffer, 5 μL of Annexin V-FITC and 5 μL of 7-AAD were added to the cells and incubated for 30 min in the dark. Flow cytometer (BD Accuri TM C6) and BD Accuri TM C6 software used to analyze

All of the above experiments were replicated 3 times in laboratory.

**Statistical methods**

The statistical analysis was conducted using R (v.4.0.3) software. The DESeq2 package was used to analyze the DEGs of the transcriptome data. The WGCNA package was used to construct the co-expression networks. The limma package was used to calculate the DEGs of the microarray data. The gene expression correlation analyses were conducted using Pearson’s coefficients. GSEA software and the clusterProfiler package was used for the pathway enrichment analysis. The survival package was used for the survival analysis. The mean ± standard deviation were calculated, and the statistical difference was examined by the Student’s t-test. A P value <0.05 was considered statistically significant.

**Results**

**WGCNA and GSEA**

A total of 12,993 DEGs, including IncRNAs, messenger RNAs (mRNAs), and small-nucleolar RNAs, were identified based on the transcriptome data in TCGA (see Figure 1A), and some representative genes were selected to illustrate this different expression patterns (see Figure 1B). The WGCNA were used to identify the gene modules in the para-cancerous tissues and TNBC. To satisfy the scale-free topology, the soft threshold $\beta$ value was set at 7 (see Figure 1C). In total, 56 gene modules (which were not merged) were constructed by average-linkage hierarchical clustering (see Figure 1D). Additionally, a network heatmap of 600 randomly selected genes revealed a high level of independence across the co-expression clusters (see Figure 1E). The module-trait relationships of different co-expression modules revealed that the brown module showed the highest positive correlation with TNBC (see Figure 1F).
In total, 1,155 hub genes were confirmed to have a MM >0.8 and a GS >0.5 in the brown module (available online: https://cdn.amegroups.cn/static/public/atm-22-2900-1.xlsx and Figure 1G). Additionally, in the brown module, CDKN2B-AS1 and SPC25 were more highly expressed in the TNBC tissues than the corresponding normal tissues (see Figure S1A,S1B). The Spearman correlation analysis also revealed that CDKN2B-AS1 and SPC25 were highly and positively correlated (see Figure 1H). STRING database was utilized to enrich the PPI network of DEGs of TNBC. SPC25 as one of hub genes was identified by the MCODE plugin in Cytoscape (see Figure S2A). CDKN2B-AS1 enrichment analysis on LncSEA indicated to be associated with a variety of tumors, including TNBC (see Figure S2B). According to the GSEA, the mRNAs in the brown module were predominantly enriched in the transcription factor E2F and MYC targets, mitotic spindle, and the G2M checkpoint, which are closely associated with cell-cycle regulation (see Figure 1I-1L).
Expression validation, survival analysis, and protein-protein interactions

The expression levels of CDKN2B-AS1 and SPC25 in more than 30 cancer types from TCGA were extracted from GEPIA database. Notably, CDKN2B-AS1 and SPC25 were upregulated in most cancer types (see Figure 2A,2B). Further, the microarray data of GSE45827, comprising 41 basal samples, 14 cell line samples, 30 human epidermal growth factor receptor 2 (Her2+) samples, 29 Luminal A samples, 30 Luminal B samples, and 11 normal samples, were used to validate the upregulation of mRNA SPC25 between the basal samples and normal samples (see Figure 2C). Additionally, the SPC25 expression confirmed by the Human Protein Atlas was higher in tumors compared to normal tissue (see Figure 2E,2F). The subcellular location of SPC25 is in the cytoplasm and SPC25 co-locates with microtubule proteins, which play an important role in cell division (see Figure 2G).

Based on the OS times of TNBC patients in TCGA, a survival analysis of SPC25 was performed to evaluate the difference between the high and low expression groups. Notably, the OS time was lower in patients who had a higher SPC25 expression (see Figure 2H). The protein-protein interaction network of the SPC25 protein was established via the STRING database. The proteins co-expressed with SPC25 were retrieved, and included SPC24, CDK1, ZWINT, MIS12, TOP2A, NUF2, NDC80, DSN1, BUB1, and CASC5 (see Figure 2I). The GO annotation of the co-expressed genes revealed their relevant biological processes (see Figure 2J), which included chromosome segregation, mitotic cell-cycle checkpoint, and nuclear division.

Functions of co-expressed SPC25 and CDKN2B-AS1

Based on the data of the Braune EB (number cells: 369) in CancerSEA, a single-cell analysis was conducted to examine the functions of SPC25, which showed that SPC25 might participate in the regulation of the cell cycle in BC (see Figure 3A,3B). Additionally, the expression of SPC25 was positively correlated with the above-mentioned biological processes (see Figure 3C-3F). The correlation analysis revealed that SPC25 was highly correlated with the cell cycle-related genes (i.e., CDC25A, CCNE1, CCNA2, CCNB1, MKI67, and CDK1) (see Figure 3G).

In-vitro studies were used to explore the correlation of SPC25 expression with dysregulated IncRNA CDKN2B-AS1. The validation of the differentially expressed CDKN2B-AS1–SPC25 pair was performed by qRT-PCR. We found that CDKN2B-AS1 and SPC25 were more upregulated in the BT549, MDA-MB-231, and MDA-MB-468 cell lines than the MCF10A cell lines (see Figure 3H,3I). MDA-MB-231 was selected for further interfering assays. As expected, the knockdown of CDKN2B-AS1 inhibited SPC25 expression (see Figure 3J). Further, the cell number monitoring assays revealed that the cell proliferation varied (see Figure 3K) and the invasion ability was restrained (see Figure 3L-3N) after the knockdown of CDKN2B-AS1. In addition, flow cytometry cell apoptosis analysis showed that knockdown of CDKN2B-AS1 significantly increased the percentage of apoptotic cells (see Figure 3O).

Discussion

In our study, a WGCNA was conducted to find the co-expression gene modules, and we identified the key gene modules associated with tumorigenesis in the network. The WGCNA package uses bioinformatics approaches to describe clusters of highly correlated genes (19). We discovered that CDKN2B-AS1 and mRNA SPC25 in the brown module were significantly overexpressed in TNBC. We further characterized the synergistic effect of CDKN2B-AS1 and SPC25 and found that they accelerated TNBC. To examine the effects of the upregulation of CDKN2B-AS1 on TNBC proliferation and migration, CCK-8 and transwell assays were conducted, and revealed that the overexpression of SPC25 was positively correlated with the poor OS of TNBC patients. Further, our findings revealed that CDKN2B-AS1 may promote TNBC proliferation by upregulating SPC25 and accelerating the cell cycle.

Recently, studies have revealed that IncRNAs are aberrantly expressed in breast cancer and closely associated with the development and progression of breast cancer (20). IncRNAs can regulate mRNA expression through multiple mechanisms: epigenetic regulation, transcriptional regulation, post-transcriptional regulation, acting as ceRNA and other molecules mechanisms (21). However, the specific mechanism is still unclear and it is a challenge to use IncRNAs as biomarkers for diagnosis or therapeutic targets. We found that CDKN2B-AS1 and mRNA SPC25...
A  

B  

C  

D  

E  

F  

G  

The median expression of CDKN2B-AS1
Figure 2 Validation of SPC25 and functional annotation. (A,B) The mRNA expression of SPC25 and CDKN2B-AS1 is notably increased in most human cancer tissues compared to corresponding normal tissues. Red dots represent SPC25 expression in tumor samples. Green dots represent SPC25 expression in paired normal samples. (C) The upregulation of mRNA SPC25 was validated in GSE45827. (D) The protein expression of SPC25 in the CPTAC database was significantly higher in the TNBC samples than the normal samples. (E,F) SPC25 expression in normal breast tissues and BC tissues by immunohistochemical stain. Magnification, ×100. (G) According to the Human Protein Atlas database, SPC25 was localized in the cytoplasm of multiple human cells, such as U-2 OS and U-251 MG by immunofluorescence staining and photographed by confocal microscopy. Scale bar, 20 μm. (H) High SPC25 expression displayed a significant correlation with poor OS in TNBC patients. (I) SPC25-interaction proteins in TNBC. (J) A GO enrichment analysis was used to identify relevant the biological processes of the co-expressed genes. SPC25, spindle component 25; CDKN2B-AS1, cyclin-dependent kinase inhibitor 2B antisense RNA 1; CPTAC, Clinical Proteomic Tumor Analysis Consortium; OS, overall survival; TNBC, triple-negative breast cancer; BC, breast cancer; U-2 OS, human osteosarcoma cell line; U-251 MG, human glioblastoma cell line; GO, Gene Ontology.

were significantly overexpressed in TNBC. LncRNA CDKN2B-AS1 participates in a variety of regulatory roles, including the cell cycle of tumors (11,22-24). The aberrant expression of CDKN2B-AS1 has been reported in multiple cancers, and it might accelerate proliferation and inhibit the apoptosis of tumor cells (25). Studies have revealed that the mRNA level of CDKN2B-AS1 is increased and exerts carcinogenic effects in various tumors (11,26,27). Research has shown that CDKN2B-AS1 is overexpressed in gliomas and enhances cell proliferation, invasion, and migration, and inhibits the apoptosis of glioma cells (28). CDKN2B-AS1 has also been shown to be significantly overexpressed in renal carcinoma cells, while the downregulation of CDKN2B-AS1 has been shown to suppress the tumor growth involved in
Angiogenesis
Apoptosis
Cell cycle
Differentiation
DNA damage
DNA repair
EMT
Hypoxia
Inflammation
Invasion
Metastasis
Proliferation
Quiescence
Stemness

Skin
Kidney
Breast
Head and neck
Ovary
Bowel

MEL
RCC
BRCA
HNSCC
OV
CRC

Correlation

| Correlation | 1.0 | 0.8 | 0.5 | 0.3 | 0.0 | -0.3 | -0.5 | -0.8 | -1.0 |
|------------|-----|-----|-----|-----|-----|------|------|------|------|

CDK25A
SPC25
CCNE1
CCNA2
CCNB1
MKI67
CDK1

Gene expression

CDK25A relative expression

SPC25 relative expression

CDK2
CDK1
MKI67
CCNB1
CCNA2
CCNE1
SPC25

Group

MCF10A
BT549
MDA-MB-231
MDA-MB-468

NC
si-CDKN2B-AS1

OD450 value

Control
si-CDKN2B-AS1

MDA-MB-231
MDA-MB-468

***

Number of migrated cells

N

Apoptosis, %

MDA-MB-231

si-NC
si-CDKN2B-AS1

si-NC
si-CDKN2B-AS1

MDA-MB-231
proliferation, migration, and invasion (29).

As a part of the Ndc80 complex, the SPC25 homolog plays an important role in the mitosis of cell-cycle processes, including spindle formation and chromosome alignment (30-32). SPC25 and CDKN2B-AS1 expression levels are upregulated in most of the cancers identified by the GEPIA database, and the GEO analysis confirmed that the expression of SPC25 was significantly more elevated in TNBC tissues than normal tissues. SPC25 has been shown to be upregulated and involved in cell proliferation in lung cancer (33). We found that the expression of SPC25 was downregulated by siRNA-mediated CDKN2B-AS1 knockdown. Additionally, the Spearman correlation analysis revealed that the expression levels of SPC25 and CDKN2B-AS1 were highly and positively correlated. The CCK-8 and transwell assays indicated that proliferation and migration were strongly inhibited in the si-CDKN2B-AS1 cell line. We confirmed the synergistic effect of CDKN2B-AS1 and that SPC25 enhances the proliferation and migration capacity of TNBC. Similarly, previous studies have demonstrated that the downregulation of SPC25 significantly reduces the proliferation of prostate cancer (34). Additionally, SPC25 has been shown to interact with checkpoint-related proteins and increase proliferation in lung cancer (33). SPC25 facilitates tumor growth via the tumor protein P53 pathway and may be a novel tumor-promoting factor and a therapeutic target in HCC (7). The downregulation of SPC25 also leads to aberrant spindle organization with misaligned chromosomes (31). Thus, we hypothesize that SPC25 overexpression is involved in genetic alterations in the cell cycle caused by incorrect chromosome segregation, in which motility plays a crucial role, and ultimately leads to tumorigenesis. We also observed that SPC25 was colocalized with microtubule proteins. Previous research has shown that tubulin plays a vital role in cell proliferation (35). Thus, we hypothesize that SPC25 binds to microtubule proteins and then accelerates TNBC tumorigenesis and progression.

Next, the survival analysis confirmed that the SPC25 level was an independent prognostic factor of TNBC. High levels of SPC25 were related to poor OS and recurrence-free survival in lung adenocarcinoma patients (9). Research has indicated that increased SPC25 expression levels are correlated with poor OS in BC patients (36). The high expression of SPC25 has also been shown to be correlated with short survival time and to enhance the proliferation of HCC cells (5). The patient with higher SPC25 expression had higher recurrence rates, which was associated with shorter OS in BC patients (37). Our study was the first to reveal that SPC25 could have potential clinical value in TNBC.

In this study, the GO annotation of the co-expressed genes identified the relevant biological processes, including chromosome segregation, mitotic cell-cycle checkpoint, and nuclear division. We performed a GSEA to evaluate the underlying function of the brown module. WGCNA
and pathway analyses have shown that SPC25 is a potential marker of HCC and is associated with the mitotic cell cycle (38,39). Genes in the brown module were primarily related to cell-cycle regulation, which is a pervasive and complex sequence of events (40). Aberrant cell cycles, which allow cells to divide indefinitely, are hallmarks of cancer. Thus, therapies targeting cell cycles have long been regarded as promising anti-cancer strategies. Genes in the brown module may potentially promote tumor development via the transcription of E2F and MYC to accelerate cell cycles. In the future, the synergistic effect of CDKN2B-AS1 and SPC25 might be important for improving the optimal development and application of cell-cycle targeting drugs in clinical practice.

The single-cell analysis demonstrated that SPC25 may contribute to the progression of BC by accelerating the cell cycle. The downregulation of SPC25 expression is associated with cell-cycle at M-phase arrest (32). The correlation analysis revealed that SPC25 may increase the cell cycle through co-expression with CDC25A, CCNE1, CCNA2, CCNB1, MKI67, and CDK1. Further experiments need be conducted to verify the interaction of SPC25 with these cell cycle-related genes.

In summary, our research shows that the synergistic effect of CDKN2B-AS1 and SPC25 on the occurrence and development of TNBC. CDKN2B-AS1 may play a significant role in the diagnosis, prognosis, and specific therapeutic target in TNBC. Our results provided new insights into the potential biomarkers and mechanisms of TNBC oncogenesis and also suggest a potential application of treatment in the future.

This study had a number of limitations. First, while CDKN2B-AS1 and SPC25 are potential markers, large-scale validations studies urgently need to be conducted to apply these findings in a clinical setting. Second, the WGCNA method has limitations, as its selection criteria and thresholds may affect the ultimate findings.

**Conclusions**

In brief, we used the WGCNA method to identify the synergistic effect of lncRNAs and mRNAs on TNBC progression. We also verified the functional roles and molecular process of CDKN2B-AS1 as a tumor-promoting factor that upregulates SPC25 expression in tumor tissues. Collectively, we identified a number of TNBC co-expression modules and hub genes. Our findings provide a theoretical foundation for future research. The candidate factors may be applied as diagnostic and prognostic biomarkers or clinical treatment targets in the future.

**Acknowledgments**

The authors would like to thank TCGA and the GEO databases and all the contributors to the databases who have provided open-source data for researchers.

**Funding:** None.

**Footnote**

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at [https://atm.amegroups.com/article/view/10.21037/atm-22-2900/rc](https://atm.amegroups.com/article/view/10.21037/atm-22-2900/rc)

*Data Sharing Statement:* Available at [https://atm.amegroups.com/article/view/10.21037/atm-22-2900/dss](https://atm.amegroups.com/article/view/10.21037/atm-22-2900/dss)

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at [https://atm.amegroups.com/article/view/10.21037/atm-22-2900/coif](https://atm.amegroups.com/article/view/10.21037/atm-22-2900/coif)). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Cite this article as: Deng N, Chen K, Fan H, Jin F. The synergistic effect of CDKN2B-AS1 and SPC25 on triple-negative breast cancer. Ann Transl Med 2022;10(14):783. doi: 10.21037/atm-22-2900