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Research paper

## Upregulation of the long non-coding RNA CBR3-AS1 predicts tumor prognosis and contributes to breast cancer progression

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## ABSTRACT

Breast cancer is the most common female malignancy and the major cause of cancer-related death in women. Long non-coding RNAs (lncRNAs), as oncogenic or tumor suppressor factor, involved in the development and progression of various cancers. In this study, we sought to investigate the function of lncRNA CBR3-AS1 in breast cancer. We evaluated the expression pattern of CBR3-AS1 in breast cancer tissues and cell lines, explored the correlation between CBR3-AS1 expression and the survival time of breast cancer patients, and probed the effect of CBR3-AS1 on tumor progression of breast cancer through loss-of-function and gain-of-function strategies. Our results showed that CBR3-AS1 was overexpressed in breast cancer tissues and cell lines and predicted the prognosis of breast cancer patients. And CBR3-AS1 exerted biological function as an oncogenic lncRNA, involved in the regulation of cell proliferation, colony formation, apoptosis and tumor growth in breast cancer. Taken together, CBR3-AS1 was up-regulated in breast cancer and promoted the risk of breast cancer. It may be a novel therapeutic target and potential prognostic marker for breast cancer.

## 1. Introduction

Breast cancer is the most common female malignancy and the major cause of cancer-related death in women (DeSantis et al., 2014). Despite the new advances in cancer diagnosis and treatment, the incidence in developing countries is still increasing (Lancet, 2009). Therefore, understanding the pathogenesis of breast cancer and searching for the novel therapeutic targets for the detection and therapy of breast cancer is urgently required.

The development of breast cancer is a complex multistep process associated with numerous genetic alterations (Lee et al., 2012; Hu et al., 2009). Long non-coding RNAs (lncRNAs), a class of RNA molecules longer than 200 nucleotides in length, possess important functions in a variety of biological processes, such as lymph node invasion and metastasis, cell apoptosis and cell cycle control, cell development and differentiation, transcriptional and translational regulation, and central

metabolism (Ding et al., 2014; Wapinski and Chang, 2011; Clark and Mattick, 2011; Ørom et al., 2010; Ellis et al., 2014). Accumulating evidence confirmed that dysregulation of lncRNAs is often associated with many human diseases, including various types of carcinomas (Cheetham et al., 2013; Li and Chen, 2013; Venkatesh et al., 2015). Many lncRNAs have been shown to exert oncogenic or tumor suppressor properties in common human cancers, e.g. POU3F3, MT1DP, HOTAIR and PRNCR1 (Martens-Uzunova et al., 2014; Tong et al., 2015; Yu et al., 2014). Studies indicated that lncRNA CBR3 antisense RNA 1 (CBR3-AS1, also known as PlncRNA1) was up-regulated in prostate cancer, modulated cell apoptosis and proliferation during the progression of prostate cancer (Cui et al., 2013). In addition, a recent study revealed that up-regulation of CBR3-AS1 promoted esophageal squamous carcinoma cell proliferation and correlated with advanced clinical stage (Wang et al., 2014). However, its role in breast cancer remains poorly understood.

**Abbreviations:** lncRNAs, long non-coding RNAs; ATCC, American Type Culture Collection; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; FACS, fluorescence activated cell sorter; Annexin V<sup>+</sup>/PI<sup>-</sup>, Annexin V positive and PI negative stained; Annexin V<sup>+</sup>/PI<sup>+</sup>, Annexin V and PI positive stained

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In this study, we aim to explore the effect of lncRNA CBR3-AS1 on the cancer carcinogenesis of breast cancer. We evaluated the expression pattern of CBR3-AS1 in breast cancer tissues and cell lines, explored the correlation between CBR3-AS1 expression and prognosis of breast cancer patients, and investigate the function of CBR3-AS1 in tumor progression of breast cancer through loss-of-function and gain-of-function strategies.

## 2. Material and method

### 2.1. Patients, cell lines and cell culture

Seventy breast cancer samples and twenty-nine normal breast tissues were collected from the department of radiotherapy, Changzhou Tumor Hospital, Soochow University. Pathological diagnosis of breast cancer was performed according to World Health Organization criteria (Hanby et al., 2004). Informed written consent was obtained from all patients and donors. The four non-aggressive human breast cancer cell lines MCF-7, MDA-MB-453, T47D, SKBR3 and the normal breast cell line HSS78Bst were purchased from the American Type Culture Collection (ATCC). All cell lines were grown in 5% CO<sub>2</sub> at 37 °C incubator and maintained according to providers' recommendations. This study protocol was approved by the Medical Ethics Committee of Changzhou Tumor Hospital, Soochow University. The clinic pathological features of 70 patients are summarized in Table 1.

### 2.2. Cell transfection

Cell transfection was performed as described previously (Wang et al., 2014). siRNAs targeting CBR3-AS1, negative control siRNA, mimic CBR3-AS1 and mimic negative control were purchased from Life Technologies (Life Technologies, USA). Four breast cell lines (MCF-7, MDA-MB-453, T47D and SKBR3) were grown on six-well plates to confluency and respectively transfected with the vectors mentioned above using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were harvested 48 h post transfection for qRT-PCR to determine the transfection efficiency.

### 2.3. Cell viability and apoptosis assay

Cell viability assay was performed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Cell Proliferation/ Viability Assay kit (Sigma, Germany) according to the guidelines. The absorbance (570 nm) of each well was then measured and recorded. Cell viability was calculated with the following equation:  $(OD_{\text{sample}} / OD_{\text{control}}) \times 100$ .

Apoptosis rate was evaluated using an Annexin-V-Fluos and

Propidium Iodide (PI) Apoptosis Detection Kit (Sigma, Germany) by fluorescence activated cell sorter (FACS) according to the manufacturer's protocol. Annexin V positive and PI negative stained (Annexin V<sup>+</sup>/PI<sup>-</sup>) cells were recorded to be at the early apoptotic stage, whereas Annexin V and PI positive stained (Annexin V<sup>+</sup>/PI<sup>+</sup>) cells were recorded to be at the late apoptotic stage. The experiments are repeated three times.

### 2.4. Colony formation assay

Colony formation assay was based on the previous study (Wang et al., 2014). Briefly, the transfected breast cells were seeded in duplicate at a density of 800 cells/well in 6-well plates and incubated at 37 °C with 5% CO<sub>2</sub> for 14 days. Cells were washed twice with PBS and stained with 0.1% crystal violet. The visible colonies were counted under a stereomicroscope.

### 2.5. Tumor xenograft assay

Xenograft mice experiments were performed as described previously (Wang et al., 2015a). In brief, equal numbers ( $1 \times 10^6$ ) of breast cells expressing either CBR3-AS1 knockdown or over-expression vectors were inoculated subcutaneously into the left back of 6-week-old female BABL/c nude mice (n = 5), respectively. Mice were sacrificed at the end of 21 days. The cancer tissues were harvested and the tumor weights were measured.

### 2.6. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from frozen tissue from tumors and cell lines using TRIzol (Invitrogen, USA) and reversely transcribed into cDNA using SuperScriptIII reverse transcriptase kit (Invitrogen, USA) in accordance to the manufacturer's instructions. The PCR amplification was performed using SYBR Premix Ex Taq™ II kit (Takara, Japan) on an ABI-7500 platform (Applied Biosystems, USA). GAPDH served as an internal standard. Experimental operation and reaction conditions referred to the description previously (Wang et al., 2014). The results are from three independent experiments performed in triplicate.

### 2.7. Statistical analysis

Data were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS software version 16.0. *t*-test was performed to compare the differences between two groups. The Kaplan-Meier method with the log-rank test was used to calculate survival rate. *P* < 0.05 was considered statistically significant.

## 3. Results

### 3.1. CBR3-AS1 is up-regulated in breast cancer tissues and cell lines

As shown in Fig. 1A, CBR3-AS1 was significantly up-regulated in breast cancer tissues compared to the normal tissues (*P* < 0.0001). Consistent with the results in tissues, the expression levels of CBR3-AS1 in breast cancer cell lines (MCF-7, MDA-MB-453, T47D and SKBR3) were increased by 3.03-fold, 3.09-fold, 3.02-fold and 2.99-fold respectively, compared with the normal breast cell line (Fig. 1B, *P* < 0.001), indicating the expression levels of CBR3-AS1 were not associated with the phenotypic differences of the cell lines.

### 3.2. High level of CBR3-AS1 is correlated with poor prognosis of patients with breast cancer

Using the median as cut-off value, the median value (5.87) of CBR3-AS1 expression level was used to categorize patients with breast cancer into a high-level group (n = 37) and a low-level group (n = 33).

**Table 1**

The clinical characteristics of the breast cancer patients.

Variable	Number of patients
Age (years)	
< 50	32
≥ 50	38
Tumor size (cm)	
< 2	41
≥ 2	29
Clinical stages	
I/II	33
III/IV	37
Lymph nodes metastasis	
Negative (N)	43
Positive (P)	27
Histological subtype	
Ductal	62
Lobular	6
Lobular and ductal mixed forms	2

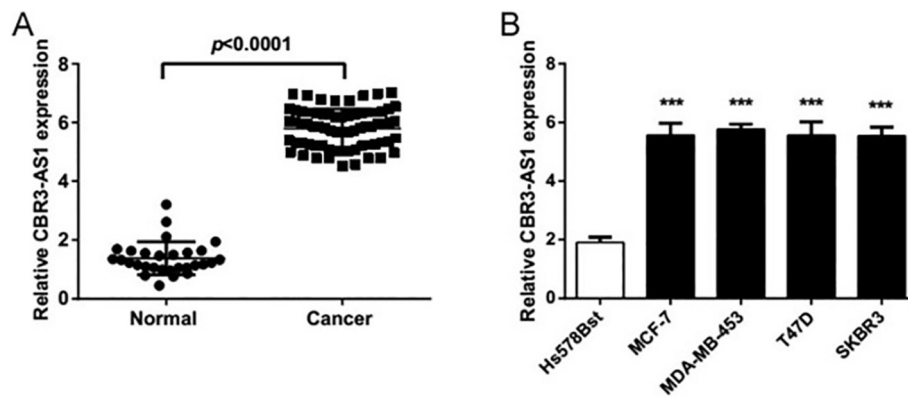


Fig. 1. CBR3-AS1 is up-regulated in breast cancer tissues and cell lines.

(A) The expression of CBR3-AS1 was up-regulated in breast cancer tissues.  $n = 29$  in normal group,  $n = 70$  in cancer group (B) The expression of CBR3-AS1 was up-regulated in breast cancer cell lines. \*\*\* $P < 0.001$ .

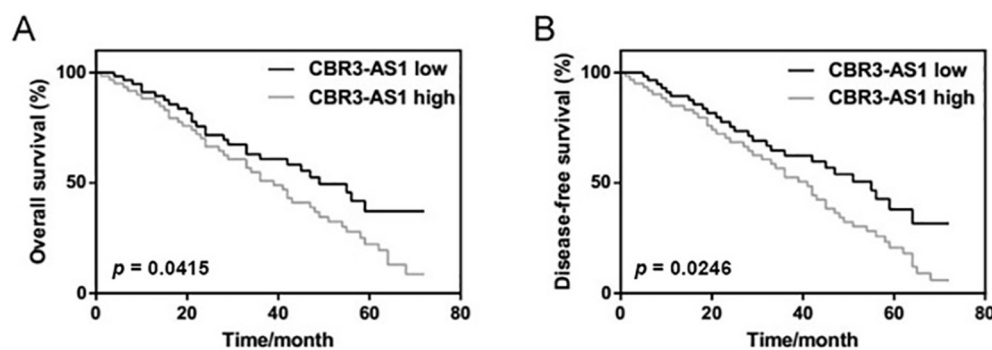


Fig. 2. High level of CBR3-AS1 was correlated with poor prognosis of patients with breast cancer.

(A) High CBR3-AS1 expression predicted poor overall survival. (B) High CBR3-AS1 expression predicted poor disease-free survival. The CBR3-AS1 expression was determined using qRT-PCR. The sample whose CBR3-AS1 expression level was lower than the median value ( $< 5.87$ ) were included in the low group ( $n = 33$ ), while the others were included in the high group ( $n = 37$ ).

Kaplan-Meier survival analysis showed that the patients with high level of CBR3-AS1 were associated with poorer overall survival and disease-free survival than those with low level of CBR3-AS1 (Fig. 2A). These results implied that the expression level of CBR3-AS1 may be associated with the prognosis of breast cancer.

### 3.3. CBR3-AS1 regulates the cell proliferation, colony formation, apoptosis and tumor growth in vitro and in vivo

To further investigate the function of CBR3-AS1 in tumor progression of breast cancer, we knocked-down and overexpressed CBR3-AS1 in the four breast cancer cell lines (MCF-7, MDA-MB-453, T47D and SKBR3), respectively. The expression level of CBR3-AS1 was significantly suppressed when CBR3-AS1 was knocked-down (Fig. 3A,  $P < 0.001$ ); while the expression of CBR3-AS1 was obviously increased in breast cancer cells after treatment with CBR3-AS1 mimic (Fig. 3E,  $P < 0.001$ ).

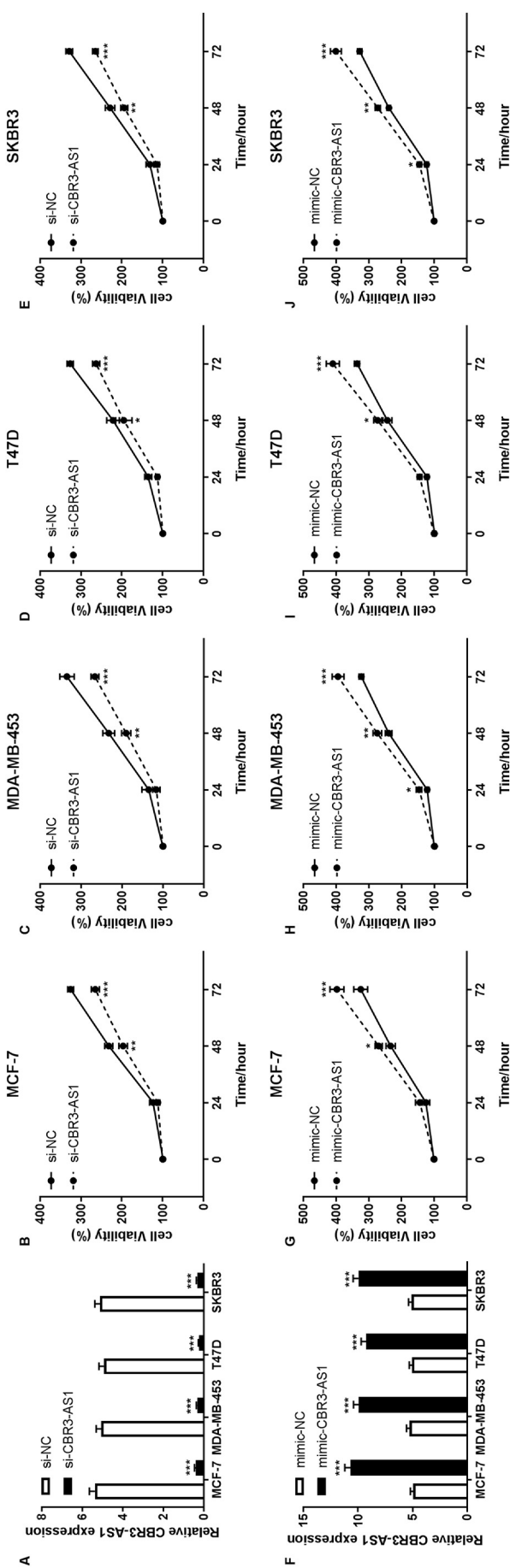
We first examined the effect of CBR3-AS1 on cell proliferation in the four breast cancer cell lines. MTT assay revealed that CBR3-AS1 knockdown significantly inhibited cell viability in breast cancer cells at 48 or 72 h after treatment with si-CBR3-AS1 (Fig. 3B–E). At the same time, ectopic expression of CBR3-AS1 in breast cancer cells obviously accelerated the cell proliferation (Fig. 3G–J). Furthermore, stable knockdown of CBR3-AS1 resulted in decreased colony formation (Fig. 4A,  $P < 0.001$ ), as well as CBR3-AS1 overexpression obviously promoted the colony formation of breast cancer cells (Fig. 4B,  $P < 0.001$ ). In addition, it was also found that CBR3-AS1 was involved in the regulation of cell apoptosis in breast cancer. Stable knockdown of CBR3-AS1 significantly induced cell apoptosis (Fig. 4C,  $P < 0.001$ ), whereas overexpression of CBR3-AS1 possessed an obvious reduction in the proportion of apoptotic cells (Fig. 4D,  $P < 0.001$ ).

In vivo, xenograft assay showed that CBR3-AS1 knockdown

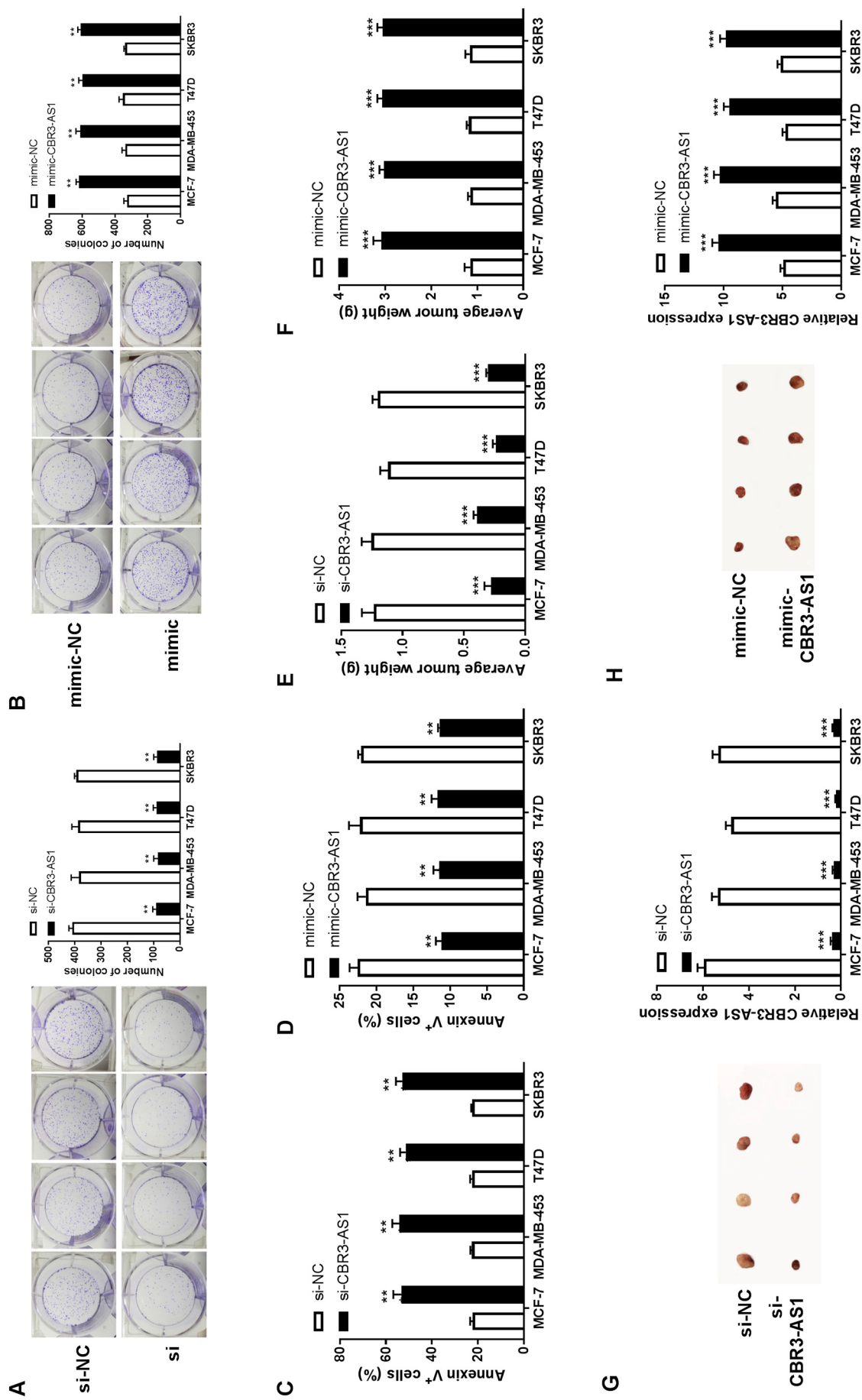
markedly inhibited the tumor growth, while CBR3-AS1 overexpression dramatically promoted the tumor growth (Fig. 4E and F,  $P < 0.001$ ). Taken together, these findings demonstrated that CBR3-AS1 was an oncogene in breast cancer, contributed to the tumor progression of breast cancer.

## 4. Discussion

The sequencing of the human genome revealed that about 98% of transcription products do not code for proteins (Szymanski and Barciszewski, 2002). lncRNAs, a newly discovered class of non-coding genes, have been demonstrated to involve in the regulation of cell development and differentiation, transcription and translation, and metabolism (Clark and Mattick, 2011; Ørom et al., 2010; Ellis et al., 2014). Mounting evidence detected that lncRNAs, as oncogenic or tumor suppressor genes, were correlated with the development and progression of cancers, including gastric cancer, hepatocellular cancer, glioma, lung cancer, prostate cancer, colorectal cancer, and so on (Wang et al., 2015b; Yao et al., 2015; Takahashi et al., 2014; Zhang et al., 2014; Pickard et al., 2013; Rui et al., 2015). Microarray expression profile analysis of lncRNAs in human breast cancer revealed that 790 up-regulated and 637 down-regulated lncRNAs were differently expressed between breast cancer tissues and its paired adjacent tissues (Xu et al., 2015). CBR3-AS1 (also known as PlncRNA-1), a protein coding gene on chromosome 21, locates in the antisense region of carbonyl reductase 3 (CBR3). Previous studies showed that CBR3-AS1 was overexpressed in prostate cancer and esophageal cancer, modulated cell apoptosis and proliferation (Cui et al., 2013; Wang et al., 2014). In this study, we for the first time reported that CBR3-AS1 was significantly up-regulated in breast cancer tissues and cell lines compared to the corresponding normal tissues and cells. And high level of CBR3-AS1 was correlated with poorer overall survival and disease-free survival. These results



**Fig. 3.** CBR3-AS1 regulates cell proliferation in breast cancer. (A) The expression of CBR3-AS1 was inhibited in MCF-7, MDA-MB-453, T47D and SKBR3 cells after treatment with si-CBR3-AS1. \*\*\**p* < 0.001 vs. si-NC. (B) CBR3-AS1 knockdown inhibited cell viability in MCF-7 cells. \*\**p* < 0.01, \*\*\**p* < 0.001 vs. si-NC. (C) CBR3-AS1 knockdown inhibited cell viability in MDA-MB-453 cells. \*\**p* < 0.01, \*\*\**p* < 0.001 vs. si-NC. (D) CBR3-AS1 knockdown inhibited cell viability in T47D cells. \**p* < 0.05, \*\*\**p* < 0.001 vs. si-NC. (E) CBR3-AS1 knockdown inhibited cell viability in SKBR3 cells. \*\**p* < 0.01, \*\*\**p* < 0.001 vs. si-NC. (F) The expression of CBR3-AS1 was increased in MCF-7, MDA-MB-453, T47D and SKBR3 cells after treatment with mimic-CBR3-AS1. \*\*\**p* < 0.001 vs. mimic-NC. (G) CBR3-AS1 overexpression promoted cell viability in MCF-7 cells. \**p* < 0.05, \*\*\**p* < 0.001 vs. mimic-NC. (H) CBR3-AS1 overexpression promoted cell viability in MDA-MB-453 cells. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. mimic-NC. (I) CBR3-AS1 overexpression promoted cell viability in T47D cells. \**p* < 0.05, \*\*\**p* < 0.001 vs. mimic-NC. (J) CBR3-AS1 overexpression promoted cell viability in SKBR3 cells. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. mimic-NC.



**Fig. 4.** CBR3-AS1 regulates colony formation, apoptosis and tumor growth in breast cancer. (A) CBR3-AS1 knockdown inhibited colony formation of MCF-7, MDA-MB-453, T47D and SKBR3 cells. \*\*\*p < 0.001 vs. si-NC. (B) CBR3-AS1 overexpression increased colony formation in MCF-7, MDA-MB-453, T47D and SKBR3 cells. \*\*\*p < 0.001 vs. mimic-NC. (C) CBR3-AS1 knockdown induced cell apoptosis in MCF-7, MDA-MB-453, T47D and SKBR3 cells. \*\*\*p < 0.001 vs. mimic-NC. (D) CBR3-AS1 overexpression promoted tumor growth. \*\*\*p < 0.001 vs. mimic-NC. (E) CBR3-AS1 knockdown suppressed tumor growth. \*\*\*p < 0.001 vs. si-NC. (F) CBR3-AS1 overexpression increased CBR3-AS1 expression level in the xenograft tumor. \*\*\*p < 0.001 vs. si-NC. (G) CBR3-AS1 knockdown reduced CBR3-AS1 expression level in the xenograft tumor. \*\*\*p < 0.001 vs. mimic-NC. (H) CBR3-AS1 overexpression increased CBR3-AS1 expression level in the xenograft tumor. \*\*\*p < 0.001 vs. mimic-NC.



suggested that CBR3-AS1 might be involved in the tumor progression of breast cancer and could serve as a potential prognostic marker.

Our study further demonstrated the function of CBR3-AS1 in tumor progression of breast cancer through loss-of-function and gain-of-function strategies. It was revealed that stable knockdown of CBR3-AS1 significantly inhibited the cell proliferation, colony formation and tumor growth *in vitro* and *in vivo*, and induced the apoptosis of breast cancer cells; whereas opposite results were found when CBR3-AS1 was overexpressed. Our results testified that CBR3-AS1 as an oncogenic lncRNA played a key role in the development and progression of breast cancer. However, the target gene of CBR3-AS1 and the potential mechanism in breast cancer carcinogenesis were still unknown. Studies indicated that lncRNA MALAT1 as the target of miR-1 involved in the regulation of cell proliferation, apoptosis and motility of breast cancer (Liu et al., 2015). In addition, it was found that knockdown of lncRNA XIST suppressed tumor growth in human glioblastoma stem cells by up-regulating miR-152 (Yao et al., 2015). lncRNA-miRNA functional network might play an important role in the development and progression of breast cancer. Yan et al. found that miR-143 regulated ERBB3 to suppress cell proliferation and invasion in breast cancer (Yan et al., 2014). Moreover, StarBase v 2.0 (<http://starbase.sysu.edu.cn/>) has been used to predict the possible binding sites between CBR3-AS1 and miR-143. Further studies investigating the effects of CBR3-AS1-miR-143 pathway on the development of breast cancer will help to clarify the molecular mechanisms of CBR3-AS1 in the breast cancer.

In summary, our study demonstrated that CBR3-AS1 was up-regulated in breast cancer tissues and cell lines and predicted the prognosis of breast cancer patients. In addition, CBR3-AS1 exerted biological function as an oncogenic lncRNA, involved in the regulation of cell proliferation, colony formation, apoptosis and tumor growth in breast cancer. It may be as a novel therapeutic target and potential prognostic marker for breast cancer.

#### Declaration of interest statement

The authors declare that they have no competing interests.

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