Long non-coding RNAs XIST and MALAT1 hijack the PD-L1 regulatory signaling pathway in breast cancer subtypes

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Abstract. Long non-coding RNAs (lncRNAs) have attracted widespread attention as potential biological and pathological regulators. lncRNAs are involved in several biological processes in cancer. Triple negative breast cancer (TNBC) is characterized by strong heterogeneity and aggressiveness. At present, the implication of microRNAs (miRs) and lncRNAs in immunotherapy has been poorly studied. Nevertheless, the blockade of immune checkpoints, particularly that of the programmed cell-death protein-1/programmed cell-death ligand-1 (PD-L1) axis, is considered as a principle approach in breast cancer (BC) therapy. The present study aimed to investigate the interaction between immune-modulatory upstream signaling pathways of the PD-L1 transcript that could enhance personalized targeted therapy. MDA-MB-231 cells were transfected with miR-182-5p mimics followed by RNA extraction and cDNA synthesis using a reverse transcription kit, and the expression levels of the target genes were assessed by reverse transcription-quantitative PCR. Furthermore, the expression levels of target genes were measured in tissues derived from 41 patients with BC, including patients with luminal BC and TNBC, as well as their adjacent lymph nodes. The results revealed that the expression levels of miR-182-5p, PD-L1 and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) were upregulated in MDA-MB-231 cells and BC tissues. However, X-inactive specific transcript (XIST) expression was downregulated in cancer tissues and TNBC cells. Following co-transfection of cells with small interfering RNAs specific for each target gene and miR-182-5p antagomirs, the effect of miR-182-5p was abolished in the presence of lncRNAs. Therefore, the results of the present study indicated that although miR-182-5p exhibited an oncogenic effect, XIST exerted a dominant effect on the regulation of the PD-L1 signaling pathway via the inhibition of the oncogenic function of MALAT1.

Introduction

In the context of tumor biology, the six hallmarks of cancer have been proposed to be associated with progressively growing tumors and to be responsible for the complexity of neoplastic diseases, and these are limitless replicative potential, evading apoptosis, self-sufficiency in growth signals, sustained angiogenesis, insensitivity to anti-growth signals, invasion and metastasis (1). In the last decade, two emerging hallmarks have been added to this list, namely deregulating cellular energetics and evading immune destruction (2). Evasion of immune destruction, resulting in the formation of the tumor microenvironment through a theory known as 'cancer immune-editing', remains a major concern (2). This theory comprises three distinct phases: Elimination, equilibrium and escape (2). Tumor cells induce the immune system, and, in turn, tumor-infiltrating lymphocytes migrate to the tumor site to eradicate the transformed cells (3). However, some of these transformed cells can escape the immune destruction and can progressively grow and give rise to a clinically apparent tumor (3). Therefore, the immune system is considered as a dual weapon; it either suppresses tumor formation or facilitates tumor progression by sculpting the immunogenicity of the tumors (4). This has brought immunotherapy to the forefront of oncology, aiming to inhibit tumor growth and activate antitumor activity. Among the different approaches of immunotherapy, immune checkpoints serve an important role. Notably, programmed cell-death protein-1 (PD-1) is expressed by T-lymphocytic cells (T-cells) during the effector phase to limit its function via binding to its ligand, known as programmed cell-death ligand-1 (PD-L1) on the surface of tumor cells, thus leading to T-cell exhaustion (5). Nivolumab and pembrolizumab are two anti-PD-1 immunotherapies that have been approved for the treatment of melanoma and non-small cell lung cancer (NSCLC), respectively (6,7). Alternative oncogenic signaling pathways promote PD-L1 expression in tumor cells, which is the 'innate immune response'. The induction of PD-L1 expression in response to

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IFN-γ is known as the 'adaptive immune response' (8). Based on these important signaling pathways, the regulation of PD-L1 expression is a broad area of investigation in several types of cancer, including breast cancer (BC).

In 2018, BC was the most common type of cancer among women worldwide and ranked first among Egyptian women (9). BC is associated with a poor prognosis due to the strong heterogeneity of its pathogenesis. Disease complexity has prompted researchers to investigate what is beyond the genetic disruption of the disease. The results of these studies revealed that the epigenetic regulation of the disease pathogenesis and progression also serves an important role in BC. Emerging evidence has suggested that the newly discovered non-coding RNAs (ncRNAs) greatly contribute to carcinogenesis (10). microRNAs (miRNAs/miRs), a subtype of ncRNAs, may lead to gene silencing via binding to the 3' untranslated region (3'UTR) of target mRNAs, either through translational repression or mRNA cleavage (11). Several miRNAs, such as Let-7a and miR-145, have been reported to be tumor suppressors in BC, resulting in decreased cellular proliferation and metastasis (12). Another class of miRNAs that contribute to cancer cell proliferation are oncomiRs, such as miR-10b and miR-21 (13). The present study focused on miR-182-5p, which has been reported to serve as either an oncogene or tumor suppressor in numerous types of cancer. Previous studies have demonstrated that the inhibition of miR-182-5p attenuates cell proliferation and invasion in BC (14), hepatocellular carcinoma (HCC) (15) and oral squamous cell carcinoma (16). Furthermore, miR-182-5p suppresses renal cell carcinoma cell proliferation by regulating the AKT/FOXO3a signaling pathway (17).

Another important group of ncRNAs are long non-coding RNAs (lncRNAs). lncRNAs serve a pivotal role in gene silencing and disease progression (18). The lncRNA X-inactive specific transcript (XIST) is exclusively expressed from the X-inactivation center of the inactive X chromosome and is essential for the initiation and spread of X chromosome inactivation (19). A previous review article reported that XIST exerts contradictory functions in different types of cancer (20). For example, in invasive pituitary adenoma, XIST acts as an oncogene (21). In addition, XIST could promote brain metastasis following its silencing in BC (22). Our previous study demonstrated that XIST combined with PD-L1 expression could serve as a potential biomarker in patients with BC (23). Furthermore, a recent study has supported the role of PD-L1 as a useful biomarker for immunotherapy (24). Another study revealed that PD-L1 expression is positively associated with that of lncRNA T cell leukemia/lymphoma 6 (TCL6) (25). In addition, it has been reported that lncRNA TCL6 is associated with a poor prognosis in patients with BC and increased immune cell infiltration (25). Additionally, lncRNA GATA binding protein 3 antisense RNA 1 induces the deubiquitination of PD-L1, thus resulting in PD-L1 stabilization and enhanced triple-negative breast cancer (TNBC) progression (26). TSIX transcript, XIST antisense RNA (TSIX) is considered to orchestrate the initiation of X chromosome inactivation, thus determining which X chromosome remains active by blocking the expression of the antisense XIST RNA (27). Another lncRNA, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), was originally identified as a prognostic marker for metastatic lung cancer (28); however, it is also associated with several other human tumors, such as HCC (29) and glioblastoma (30). Therefore, the current study aimed to identify potential ncRNAs regulating PD-L1 expression in TNBC cell lines.

Materials and methods

Egyptian patients. The present study included 41 patients with BC (4.88% males and 95.12% females) who underwent tumor resection surgery between September 2016 and April 2018 at the following hospitals: Demerdash, Cleopatra, Queens and Nozha Hospitals (Cairo, Egypt). BC tissues biopsies as well as their adjacent non-cancerous tissues together with their metastatic lymph nodes (LNs) were removed. Tissues were subdivided into luminal BC (n=30; 73.1%), TNBC (n=7; 17.07%) and HER-2-positive (n=4; 9.75%) subtype. The age of patients ranged between 28 and 70 years with a mean of 49 years. Pathological examination was performed to assess tumor grade and stage (The Eighth Edition of the American Joint Committee Cancer Staging Manual) using the TNM staging system (31). Immunohistochemistry was performed to analyze receptors (estrogen receptor, progesterone receptor and HER2) and Ki67. Tumor molecular subtyping was performed for all tumor tissues by a pathologist during the surgical resection, it was not performed at our laboratory. This was performed at Elia Laboratory (Cleopatra Hospital, Heliopolis, Cairo, Egypt). Furthermore, CEA and CA15-3 were analyzed before surgery. All human biopsies were obtained with written informed consent. Patients were subjected to clinical assessment as shown in Table I. The Ethical Committee of the German University in Cairo and Ain Shams University (Cairo, Egypt) approved the present study. The inclusion criteria were: All molecular subtypes of BC, all ages and all types of treatment. The exclusion criteria were: Male sex.

Cell culture. MDA-MB-231 cells (Vacsera) were cultured and maintained in DMEM (Lonza Group, Ltd.) supplemented with 4.5 g/l glucose + L-Glutamine, 10% FBS (Lonza Group, Ltd.) and 1% penicillin/streptomycin (Lonza Group, Ltd.) at 37°C in a 5% CO_2 atmosphere.

Bioinformatics. To detect the potential miRNAs targeting the 3'UTR of PD-L1 mRNA, the TargetScan (release number, 7.2; http://www.targetscan.org/vert_72/) bioinformatics target prediction algorithm was used. Based on binding scores and number of hits, miRNAs with good scores were selected. PD-L1 upstream targets were predicted. RNA22 software version 2.0 (http://cm.jefferson.edu/rna22/Interactive/) competing endogenous RNA (ceRNA; version 2.0; https://web.archive.org/web/20130922123437/http://starbase.sysu.edu.cn/mrnaCeRNA.php) and TargetScan prediction software were used to analyze the potential binding of miR-182-5p to lncRNAs XIST, MALAT1 and PD-L1 (position 1193-1199 in the UTR). Furthermore, lnCedb (Gencode 19 version; http://gyanxet-beta.com/lncedb/index.php) and Diana tools software (version 7.0; http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software) were used to predict the potential binding of lncRNAs XIST and MALAT1 to PD-L1.

Table I. Characteristics of patients with breast cancer.

No.	Size, cm	Type	Grade	Stage	Axillary lymph node	Treatment	Duration of cancer since diagnosis	Molecular subtype	Ki67, %
1	4.0	IDC	3	4	Positive	N/A	2 months	TNBC	35
2	2.0	IDC	3	1	Positive	N/A	2 months	Luminal B, HER2$^-$	40
3	2.4	IDC	2	2	Positive	N/A	6 months	Luminal B, HER2$^+$	40
4	4.0	IDC	3	2	Positive	N/A	6 months	Luminal B, HER2$^-$	15
5	2.5	IDC	2	3	Negative	Neoadjuvant chemotherapy (6 cycles)	8 months	Luminal B, HER2$^+$	25
6	2.5	IDC	2	2	Positive	N/A	2 months	Luminal B, HER2$^-$	23
7	1.4	IDC	2	1	Negative	N/A	2 months	Luminal A	12
8	2.1	IDC	2	2	Negative	N/A	4 months	Luminal B, HER2$^-$	23
9	1.4	IDC	2	1	Negative	N/A	3 months	Luminal B, HER2$^-$	30
10	3.5	IDC	2	2	Negative	N/A	1 year	Luminal A	17
11	5.0	IDC	3	3	Negative	N/A	6 months	HER2$^+$	50
12	0.3	IDC	2	2	Negative	Chemotherapy and radiotherapy	1 month	TNBC	30
13	2.0	IDC	2	2	Negative	N/A	1 month	TNBC	35
14	2.7	IDC	2	2	Positive	N/A	2 weeks	Luminal B, HER2$^+$	30
15	4.2	IDC	2	2	Negative	N/A	6 months	HER2$^+$	40
16	2.0	IDC	2	2	Negative	N/A	2 months	Luminal B, HER2$^+$	14
17	2.0	IDC	2	2	Negative	Chemotherapy and radiotherapy	1 month	Luminal A	18
18	4.0	IDC	2	2	Positive	N/A	8 months	Luminal B	24
19	2.5	IDC	2	2	Negative	N/A	3 months	Luminal B, HER2$^-$	50
20	3.0	IDC	2	2	Positive	N/A	4 months	TNBC	85
21	0.3	IDC	2	1	Positive	N/A	1 month	HER2$^+$	30
22	3.0	IDC	2	2	Positive	N/A	4 months	Luminal A	20
23	4.0	ILC	2	2	Positive	N/A	6 months	Luminal A	18
24	1.5	IDC	1	1	Negative	N/A	2 years	Luminal A	14
25	6.0x3.0	IDC	2	2	Positive	N/A	6 months	Luminal A	5
26	2.0x2.5	IDC	3	3	Positive	N/A	10 months	TNBC	18
27	2.5	IDC	2	2	Negative	N/A	2 years	Luminal A	10
28	2.5x2.3	IDC	2	2	Positive	N/A	7 months	Luminal B	35
29	2.5x2.0	IDC	2	3	Positive	N/A	8 months	Luminal B	22
30	4.0	IDC	3	4	Negative	N/A	6 months	Luminal B	60
31	1.0x1.0	IDC	3	N/A	Positive	N/A	6 months	Luminal B, HER2$^-$	35
32	9.0	IDC	2	N/A	Negative	N/A	1 year	Luminal B	50
33	4.2	IDC	2	2	Positive	N/A	1 month	Luminal B	30
34	1.6	ILC	1	2	Negative	N/A	2 months	Luminal B	22
35	2.5x2.0	IDC	2	2	Positive	N/A	6 months	HER2$^+$	35
36	1.5x1.0	IDC	2	1	Positive	N/A	4 months	Luminal A	8
37	3.5x2.5	IDC	2	2	Positive	N/A	3 months	TNBC	30
38	1.8	IDC	2	1	Negative	N/A	1 month	Luminal A	7
39	2.0x1.5	IDC	2	2	Positive	N/A	4 months	TNBC	30
40	2.5x2.0	IDC	2	2	Positive	N/A	2 months	Luminal A	18
41	4.0x3.0	IDC	2	4	Positive	N/A	6 months	Luminal A	15

IDC, invasive ductal carcinoma; TNBC, triple-negative breast cancer.

Transfection of MDA-MB-231 cells using miRNA and small interfering RNA (siRNA/si) oligonucleotides. MDA-MB-231

cells were transfected with 1 nmol mimics (GeneGlobe, cat. no. 219600) and inhibitors (antagomiRs) of 1 nmol

miR-182-5p (GeneGlobe Id-MIN0000259; 5'-UUUGGCAAU GGUAGAACUCACACU-3'; cat. no. 219300; Qiagen GmbH) at 25°C for 1 h to examine the effect of miR-182-5p on PD-L1, MALAT1, XIST and TSIX transcript expression. In addition, a parallel experiment was carried out for transfection efficiency analysis. This was followed by a series of transfection experiments using 5 nmol siRNAs (predesigned siRNA; Qiagen GmbH) for each lncRNA, MALAT1 (NR_002819), XIST (NR_001564) and TSIX (NR_003255). Co-transfection experiments were performed to examine the combined effect of the upstream manipulators (miR-182-5p and lncRNAs XIST and MALAT1) of PD-L1 on its expression levels. All transfection experiments were carried out in quadruplicate using HiPerfect Transfection Reagent (Qiagen GmbH) according to the manufacturer's protocol. A group of scrambled (non-specific) siRNAs (cat. no. 1022076; Qiagen GmbH) and scrambled miRNA mimics and antagomirs: Mixtures of mimics of miR-15a-5p (cat. no. 219600) and miR-122 (cat. no. 219600) for scrambled miRs and mixtures of anti-miR-15a-5p and anti-miR-122 for scrambled anti-miRs (hsa-miR-15a-5p; MIMAT0000068; 5'-UAGCAGCACAUAAUGGUUUGUG-3'; cat. no. 219300; and hsa-miR-122-5p; MIMAT0000421; 5'-UGGAGUGUG ACAAUGGUGUUUG-3'; cat. no. 219300; Qiagen GmbH) were used as negative controls in gene knockdown and miRNA gain/loss of function experiments, respectively. Cells that were only exposed to transfection reagent were designated as mock cells, cells transfected with miR-182-5p were referred to as miR-182-5p cells and cells transfected with miR-182-5p inhibitor were referred to as anti-miR-182-5p cells. Cells transfected with siRNAs of MALAT1, XIST and TSIX were referred to as siMALAT1, siXIST and siTSIX, respectively. The cells were transfected and incubated under normal culture conditions (37°C with 5% CO_2) for 48 h.

mRNA and miRNA extraction from breast biopsies and MDA-MB-231 cells (TNBC cell lines). Breast samples (healthy, cancerous and adjacent LN tissues) were collected during surgery and were immediately snap-frozen (-196°C) in liquid nitrogen. The specimens were manually pulverized in liquid nitrogen. Subsequently, ~100 mg tissue powder was used for large and small RNA extraction using Biozol reagent (BioFlux) according to the manufacturer's protocol. MDA-MB-231 cells were harvested 48 h after transfection according to the HiPerfect Transfection Reagent protocol. RNA was isolated using Biozol reagent, followed by cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 37°C for 135 min. Subsequently, RNA was quantified using reverse transcription-quantitative PCR (RT-qPCR). Experiments were performed in quadruplicate.

miRNA and mRNA quantification. The extracted miRNAs were reverse transcribed into single stranded cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Bio Basic, Inc.). mRNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Bio Basic, Inc.) according to the manufacturer's protocol at 37°C for 75 min. Relative expression levels of miR-182-5p and RNU6B (housekeeping gene) were measured using specific primers for hsa-miR-182-5p and RNU6B. Their assay IDs were 002334

and 001093, respectively, as well as MALAT1, XIST, PD-L1, TSIX and β-2 microglobulin (as a housekeeping gene for normalization) were quantified using TaqMan Real-Time Q-PCR (Applied Biosystems; Thermo Fisher Scientific, Inc.). The ABI Assay IDs for MALAT1, XIST, PD-L1, TSIX and B2M were Hs00273907_m1, Hs01079824_m1, Hs01125301_m1, Hs03299334_ml and Hs00187842_m1, respectively. A StepOne™ System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. The $2^{-\Delta\Delta Cq}$ method was used for quantification (32). The thermocycling conditions were as follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, 4°C for infinity consisting of 40 cycles of denaturation, annealing and extension, respectively.

Statistical analysis. All data are presented as the mean relative quantitation ± SEM and repeated in quadruplicates. The statistical method used for multiple groups was one-way ANOVA and multiple comparisons were analyzed by Tukey's multiple comparison test (when the mean of each column was compared with every other column) and Dunnett's multiple comparison test (when the mean of each column was compared with the mean of the control column). Analysis was performed using GraphPad Prism 7.02 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Screening of PD-L1 in breast tissues. Statistically significant upregulation of PD-L1 transcript expression was observed in all BC subtype tissues (P=0.0217) compared with healthy tissues, whereas this change was not significant in the LNs compared with adjacent normal tissues (P=0.0360; Fig. 1A). However, when patients were categorized into luminal molecular subtypes and TNBC, a marked difference in PD-L1 expression was observed. Patients with TNBC exhibited significant upregulation of PD-L1 expression (P=0.0037) compared with patients with luminal subtypes (Fig. 1B).

Selection of potential upstream regulators of PD-L1 mRNA. *In silico* predictions were performed using all aforementioned software. According to bioinformatics analysis, miR-182-5p was predicted to target PD-L1, MALAT1 and XIST. Additionally, MALAT1 and XIST were identified to target PD-L1 mRNA.

Screening of miR-182-5p, MALAT1 and XIST expression in BC tissues. miR-182-5p expression was identified to be upregulated in tumor tissues and LNs (P=0.0061 and P=0.0014, respectively) compared with in healthy tissues (ANOVA P=0.0013; Fig. 2A). A marked increase in MALAT1 mRNA expression was observed in patients with all subtypes of BC compared with normal adjacent tissue controls (P=0.00001), whereas there was no significant difference observed for the expression in LNs (ANOVA P<0.0001; Fig. 2B). In our previous study, XIST expression was decreased in tissues of patients with BC and adjacent LN samples from these patients, and markedly downregulated in TNBC (23).

Transfection efficiency for gene knockdown and miRNA ectopic expression. In order to assure successful transfection
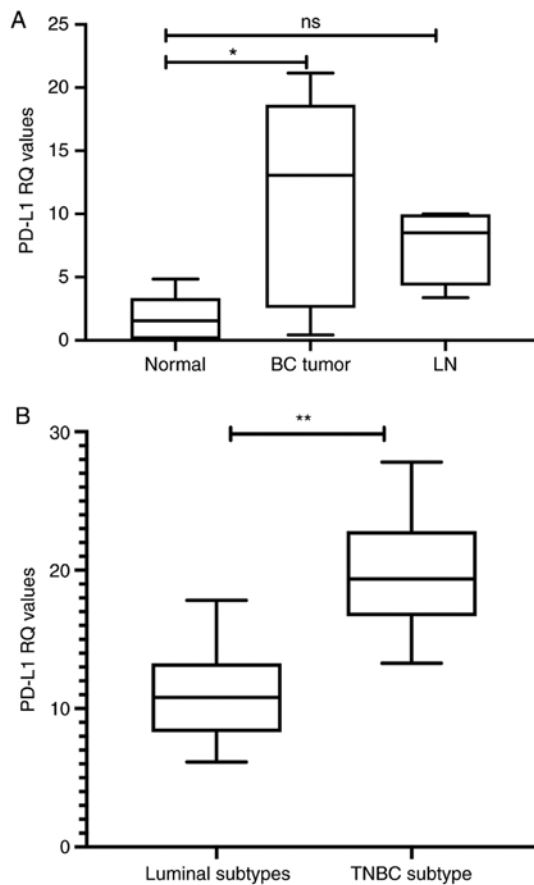
Figure 1. PD-L1 expression in breast tissues. (A) PD-L1 expression was revealed to be significantly upregulated in tumor tissues compared with adjacent normal tissues and non-significant in LNs. Analysis was performed using one-way ANOVA for multiple groups with Dunnett's multiple comparison test. (B) Following further separation of TNBC from other luminal subtypes, PD-L1 expression was identified to be significantly higher in TNBC samples compared with luminal subtypes. CD274 is the cluster of differentiation for PD-L1. Analysis was performed using GraphPad Prism 7.02 software. The expression levels were compared using an unpaired Student's t-test. *P<0.05, **P<0.01. Experiments were performed in quadruplicate. BC, breast cancer; LN, lymph node; ns, not significant; PD-L1, programmed cell-death ligand-1; RQ, relative quantitation; TNBC, triple-negative breast cancer.
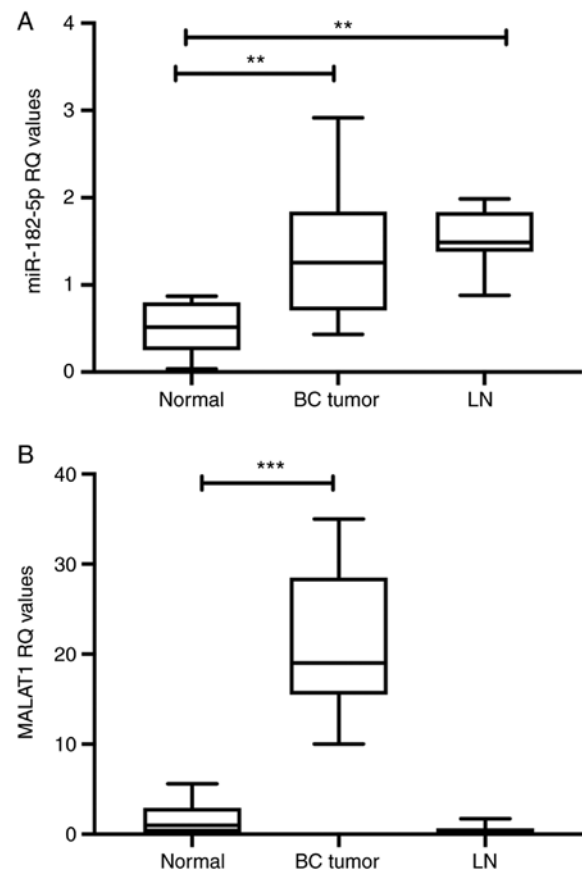

Figure 2. miR-182-5p, MALAT1 expression in BC tissues. (A) miR-182-5p was analyzed using RT-qPCR and normalized to RNU6B as an endogenous control. BC samples, as well as LN samples, exhibited upregulation of miR-182-5p expression. Analysis was performed using one-way ANOVA for multiple groups and Tukey's multiple comparison test. (B) lncRNA MALAT1 expression was analyzed using RT-qPCR and normalized to B2M as an endogenous control. MALAT1 expression was upregulated in BC tissues compared with healthy tissues, whereas no significant difference in expression was observed in LNs. Analysis was performed using one-way ANOVA for multiple groups and Dunnett's multiple comparison test. Experiments were performed in quadruplicate. **P<0.01, ***P<0.001. BC, breast cancer; LN, lymph node; lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, microRNA; RQ, relative quantitation; RT-qPCR, reverse transcription-quantitative PCR; XIST, X-inactive specific transcript.

of siRNAs, transfection efficiency was first assessed at 48 h after transfection using RT-qPCR. The mRNA expression levels of MALAT1 (Fig. S1A), XIST (Fig. S1B) and Tsix (Fig. S1C) were markedly decreased in cells transfected with their siRNAs compared with their respective mock cells (P=0.0021, P=0.0056 and P=0.0051, respectively). Additionally, the expression levels of miR-182-5p were assessed in MDA-MB-231 cells. miR-182-5p expression was markedly increased in miR-182-transfected cells compared with mock cells (P=0.0044; Fig. S1D).

Effect of ectopic miR-182-5p expression on its downstream targets in MDA-MB-231 cells. Ectopic miR-182-5p expression in MDA-MB-231 cells was assessed. MDA-MB-231 cells transfected with miR-182-5p mimics exhibited significant upregulation of PD-L1 expression, as well as MALAT1 transript expression (P=0.0062 and P=0.0007, respectively), compared with mock untransfected cells (ANOVA P=0.0065 and 0.0004, respectively; Fig. 3A and B). Inhibitors of miR-182-5p significantly decreased PD-L1 and MALAT1

transcript expression (P=0.034 and P=0.0053, respectively) compared with miR-182-transfected cells (Fig. 3A and B). However, transfection of mimics of miR-182-5p was associated with a significant decrease in XIST expression (P=0.0026) compared with that in the scrambled miRs group of cells, while miR-182-5p antagomirs increased XIST expression compared with that in cells transfected with mimics (P=0.0434; ANOVA P=0.0001; Fig. 3C). Since Tsix is the anti-sense of XIST, the expression levels of lncRNA Tsix were examined. In MDA-MB-231 cells, overexpression of miR-182-5p increased Tsix expression compared with that in mock cells (P=0.0004; ANOVA P<0.0001; Fig. 3D).

Effect of lncRNAs MALAT1 and XIST on PD-L1 expression in MDA-MB-231 cells. At 48 h after transfection with specific siRNAs against MALAT1 and XIST, PD-L1 expression was analyzed and normalized to that of B2M. PD-L1 mRNA expression was decreased significantly in MALAT1-silenced MDA-MB-231
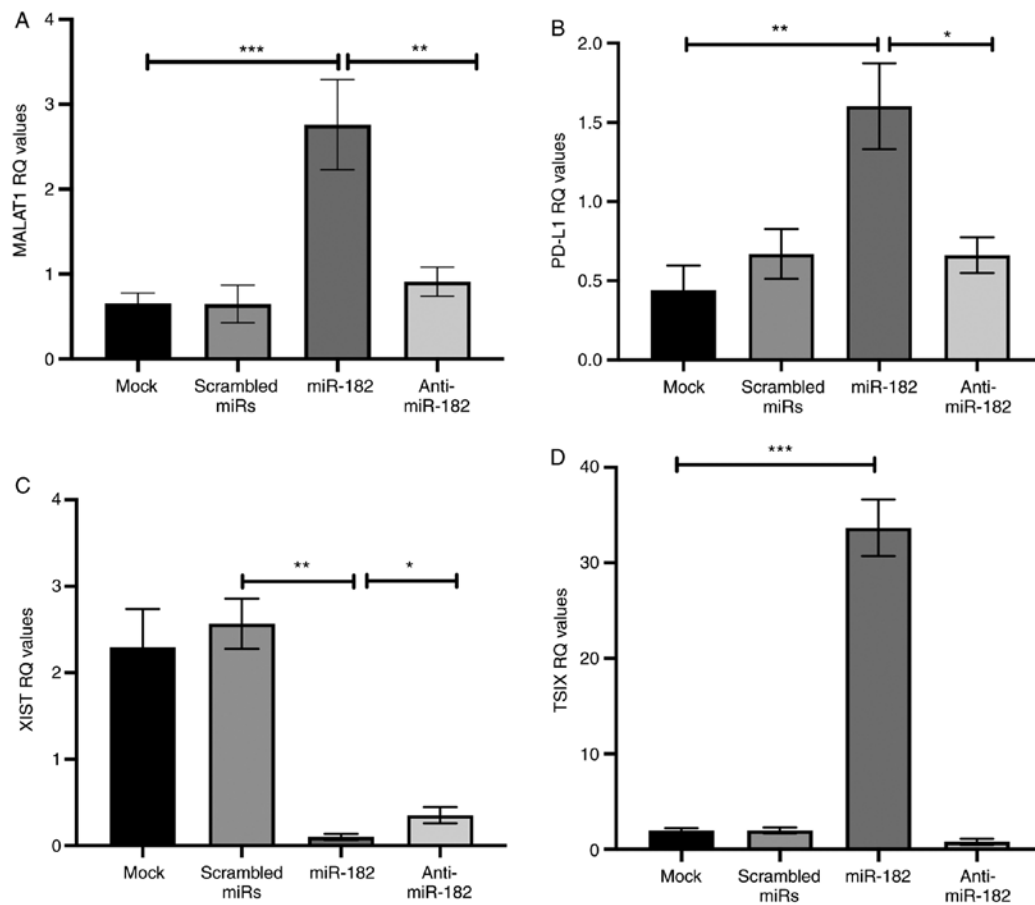
Figure 3. Effect of ectopic miR-182-5p expression on its downstream targets in MDA-MB-231 cells. (A) miR-182-5p mimic transfection of MDA-MB-231 cells resulted in significant upregulation of MALAT1 mRNA expression compared with that in mock cells, whereas anti-miR-182-5p transfection decreased MALAT1 expression compared with mimic-transfected cell lines. (B) PD-L1 mRNA expression was assessed using reverse transcription-quantitative PCR and normalized to B2M as an endogenous control. miR-182-5p mimic transfection of MDA-MB-231 cells resulted in significant upregulation of PD-L1 mRNA expression compared with that in mock cells, whereas anti-miR-182-5p transfection decreased PD-L1 expression compared with that in miR-182-transfected cells. (C) XIST mRNA expression was decreased significantly in miR-182-transfected cells compared with scrambled miRs. Furthermore, anti-miR-182-5p transfection resulted in an increase in XIST expression compared with that in mimic-transfected cells. (D) miR-182-5p mimic transfection resulted in significant upregulation of TSIX expression compared with that in mock cells. Analysis was performed using one-way ANOVA for multiple groups and Tukey's multiple comparison test. Experiments were performed in quadruplicate. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, microRNA; PD-L1, programmed cell-death ligand-1; RQ, relative quantitation; XIST, X-inactive specific transcript; Tsix, TSIX transcript, XIST antisense RNA.

cells (P=0.0007) compared with mock cells (ANOVA P=0.0004; Fig. 4A). In contrast to overexpression of PD-L1, in XIST-silenced MDA-MB-231 cells, PD-L1 mRNA expression was increased significantly in BC cells (P=0.0178) compared with untransfected mock cells (ANOVA P=0.0071; Fig. 4B).

Combined effect of the ncRNAs on PD-L1 expression in MDA-MB-231 cells. In three groups of MDA-MB-231 cells, co-transfection of miR-182-5p mimics was performed once with siXIST and PD-L1 mRNA expression was significantly increased compared with that of mock cells (P=0.00004). In order to induce XIST expression in BC cells, another group of cells was transfected with siRNAs of Tsix, a negative regulator of XIST, combined with mimics of miR-182-5p. miR-182-siTsix co-transfection resulted in a significant decrease in PD-L1 mRNA expression in BC cells, compared with that in mock cells (P=0.0357). Additionally, the third group of cells was co-transfected with miR-182-5p mimics combined with siMALAT1, and the expression levels of PD-L1 were significantly decreased compared with those in mock cells (P=0.0331; ANOVA P=0.0003; Fig. 5).

Effect of combined knockdown of lncRNAs on PD-L1 expression in MDA-MB-231 cells. PD-L1 expression was analyzed at 48 h after transfection of MDA-MB-231 cells. Following transfection with MALAT1 siRNAs combined with XIST siRNAs, a marked decrease in PD-L1 expression was observed compared with that of mock cells (P=0.006). Furthermore, following silencing of MALAT1 combined with Tsix knockdown, significant downregulation of PD-L1 mRNA expression was observed compared with mock cells (P=0.0002; ANOVA P=0.0004; Fig. 6).

Effect of miR-182-5p mimic transfection combined with lncRNAs on MALAT1 expression in MDA-MB-231 cells. MALAT1 expression was evaluated at 48 h after transfection of MDA-MB-231 cells with miR-182-5p mimics combined with XIST siRNAs. Based on the results of RT-qPCR, transfection with mimics of miR-182-5p and XIST siRNAs resulted in a significant increase in MALAT1 expression compared with that of mock cells (P=0.0004). Additionally, combined knockdown of Tsix and ectopic miR-182-5p expression was associated with significant upregulation of MALAT1
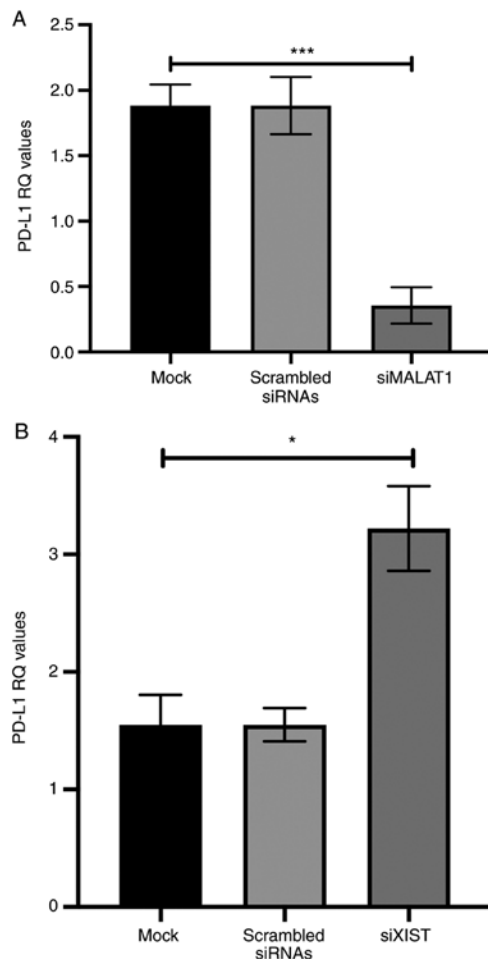
Figure 4. Effects of lncRNAs MALAT1 and XIST on PD-L1 expression in MDA-MB-231 cells. (A) MALAT1 knockdown decreased PD-L1 expression compared with that in mock cells. (B) XIST knockdown increased PD-L1 mRNA expression significantly compared with that in mock cells. Analysis was performed using one-way ANOVA for multiple groups and Dunnett's multiple comparison test. Experiments were performed in quadruplicate. *P<0.05, ***P<0.001. lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; PD-L1, programmed cell-death ligand-1; RQ, relative quantitation; si, small interfering RNA; XIST, X-inactive specific transcript.

expression compared with that of mock cells (P<0.0001; ANOVA P<0.0001; Fig. 7).

Discussion

In the last decade, significant progress and previous advances in cancer immunology have provided novel therapeutic approaches for the treatment of cancer (33). The clinical response observed in patients treated with antibodies blocking the immune checkpoints, namely the expression of cytotoxic T-lymphocyte-associated protein 4 and the PD-1/PD-L1 signaling pathway, led to their approval by the Food and Drug Administration for the treatment of melanoma in 2011 and 2014, respectively (34). The antibody against PD-1, nivolumab, was approved in 2015 for squamous lung cancer treatment (6). In addition, it has been reported that antibodies targeting PD-1 or PD-L1 are effective and safe in treating several types of tumors, including bladder cancer, Hodgkin's lymphoma and renal cell carcinoma (35). The effects of immune checkpoint inhibitors in
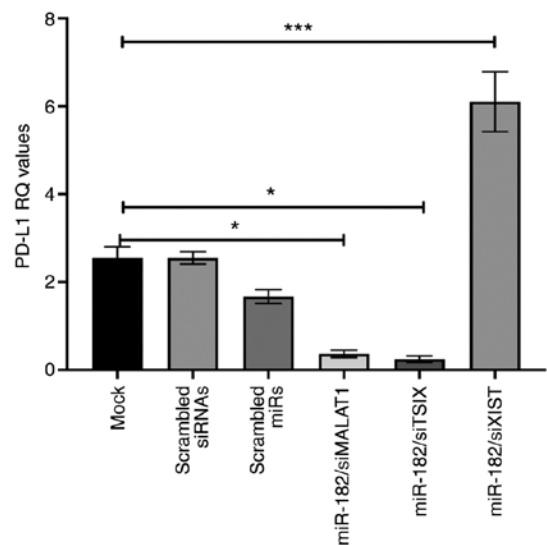

Figure 5. Combined effect of the two upstream regulators of the PD-L1 promoter (miR-182-5p and lncRNAs; XIST and MALAT1) in MDA-MB-231 cells. Following transfection of miR-182-5p mimics and silencing of XIST, PD-L1 expression was increased compared with that of mock cells. However, following transfection with miR-182-5p mimic and knockdown of Tsix, PD-L1 expression was identified to be significantly downregulated, and PD-L1 expression was revealed to be downregulated following transfection with miR-182-5p mimic and silencing of MALAT1. Analysis was performed using one-way ANOVA for multiple groups and Dunnett's multiple comparison test. Experiments were performed in quadruplicate. *P<0.05, ***P<0.001. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, microRNA; PD-L1, programmed cell-death ligand-1; RQ, relative quantitation; si, small interfering RNA; XIST, X-inactive specific transcript; Tsix, TSIX transcript, XIST antisense RNA.
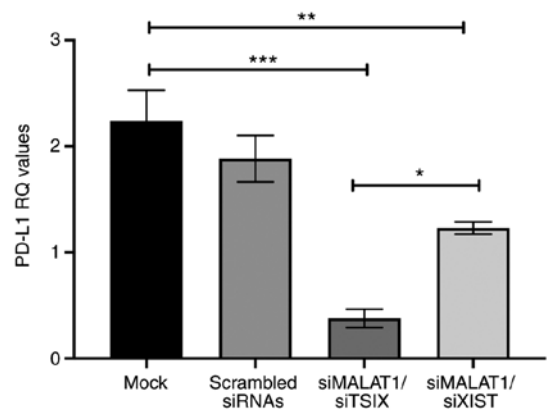

Figure 6. Effect of combined knockdown of lncRNAs on PD-L1 expression in MDA-MB-231 cells. The change in PD-L1 expression as a response to co-transfection of the cells using siRNAs of MALAT1 and XIST was assessed using TaqMan reverse transcription-quantitative PCR and normalized to B2M. The results revealed a marked decrease in PD-L1 expression compared with that in mock cells, in addition to a significant decrease in PD-L1 expression following silencing of the oncogenic MALAT1 and silencing of Tsix, the antisense of XIST. Analysis was performed using one-way ANOVA for multiple groups and Tukey's multiple comparison test. Experiments were performed in quadruplicate. *P<0.05, **P<0.01, ***P<0.001. lncRNA, long on-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; PD-L1, programmed cell-death ligand-1; RQ, relative quantitation; si, small interfering RNA; XIST, X-inactive specific transcript; Tsix, TSIX transcript, XIST antisense RNA.

different types of cancer have promoted the targeting of these signaling pathways in other tumors, such as BC (36). The association between PD-L1 and the prognosis of
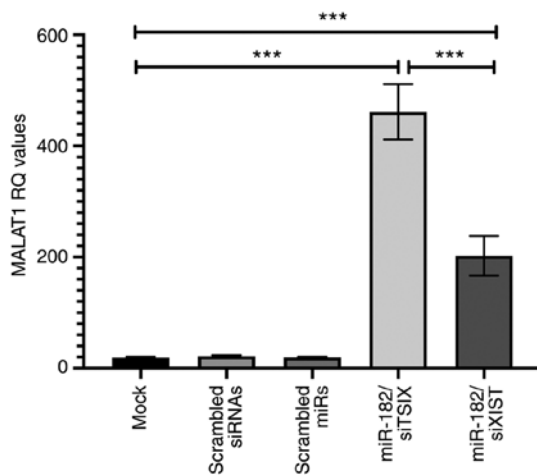
Figure 7. Effect of miR-182-5p mimic transfection combined with lncRNAs on MALAT1 regulation in MDA-MB-231 cells. Using reverse transcription-quantitative PCR, the relative expression levels of MALAT1 were analyzed and normalized to B2M. A significant increase in MALAT1 expression was observed in breast cancer cells when XIST siRNAs were co-transfected with miR-182-5p mimics compared with mock cells. Notably, MALAT1 mRNA expression was increased when breast cancer cells were co-transfected with siTsix combined with miR-182-5p mimics compared with that of mock cell. Analysis was performed using one-way ANOVA for multiple groups and Tukey's multiple comparison test. Experiments were performed in quadruplicate ***P<0.001. lncRNA, long on-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; miR, microRNA; RQ, relative quantitation; si, small interfering RNA; XIST, X-inactive specific transcript; Tsix, TSIX transcript, XIST antisense RNA.

patients with different types of cancer has been a research topic of considerable interest. However, the prognostic value of PD-L1 in patients with BC remains controversial. Therefore, the current study aimed to investigate the differential expression of lncRNAs in tissues derived from patients with BC and the MDA-MB-231 cell line. In addition, the miRNA-mediated regulation of PD-L1, XIST and MALAT1 expression remains poorly investigated, particularly in BC. Therefore, the present study also aimed to reveal novel miRNA/lncRNA interactions in BC and their immune-modulatory effects on PD-L1 expression, in order to provide novel possible immunotherapeutic targets for the treatment of different subtypes of BC, particularly TNBC. The results demonstrated that PD-L1 expression was upregulated in BC tissues, with higher expression levels observed in TNBC compared with the luminal subtypes. A previous study has demonstrated that PD-L1 expression is associated with LN metastasis and TNBC, suggesting that PD-L1 could serve as a promising biomarker for monitoring prognosis in patients with BC and selecting the appropriate immunotherapy (37). Consistent with a previous study that demonstrated that PD-L1 expression is upregulated in the MDA-MB-468 cell line (38), the PD-L1 expression levels were increased in MDA-MB-231 cells in the present study. Early clinical trials have revealed that treatment with PD-1/PD-L1 inhibitors is efficient against BC tumors, and particularly against TNBC (39). Furthermore, a previous study has demonstrated that patients with metastatic TNBC present a positive clinical response to the blockade of PD-1 or PD-L1 with specific antibodies, such as pembrolizumab or atezulizumab (40). This finding was consistent with the

results of the present study. Notably, PD-L1 expression is associated with several clinicopathological parameters, including tumor size, grade and invasion (41). PD-L1 expression is associated with higher tumor grade (42), shortened survival rate and poor therapeutic outcome (43,44).

Since the present study demonstrated that PD-L1 expression was upregulated in TNBC, bioinformatics tools were subsequently applied to identify the upstream modulators of its expression. It has been reported that ncRNAs are involved in BC and the regulation of gene expression. The majority of studies have focused on determining the functions of miRNAs and lncRNAs, and only a few have investigated how their expression is transcriptionally regulated. Furthermore, numerous studies have reported the targeting effect of miRNAs on lncRNAs in different types of cancer. For example, a study demonstrated that miR-130a could directly target FOS-like antigen 1, thus inhibiting cancer cell migration and invasion in TNBC (45). In addition, lncRNAs can compete with miRNAs for the same target-gene and they serve as precursors for miRNAs. Emerging evidence has suggested that miRNAs are critical key players in cancer immunotherapy as they act as crucial regulators of immune responses under physiological and pathological conditions (46). It has been demonstrated that miRNAs are involved in cell transformation and multiplication by acting as oncomiRs or tumor suppressors in various types of cancer (47). Several miRNAs have been identified as regulators of PD-L1 expression. Tumor suppressors, such as miR-15a and miR-16, are predicted to target PD-L1, thus resulting in downregulation of PD-L1 expression in malignant pleural mesothelioma (48). In the present study, miR-182-5p was selected based on the results of the bioinformatics analysis, and it was demonstrated that miR-182-5p exerted strong binding affinity with PD-L1, MALAT1 and XIST mRNAs. The results revealed that miR-182-5p expression was upregulated in TNBC, as well as in luminal subtypes. This finding was consistent with previous studies, showing that miR-182-5p expression is increased in TNBC (49,50). Additionally, another study revealed that treatment with miR-182-5p inhibitors attenuates cell apoptosis and proliferation via regulation of CRISPR associated protein 9 expression in MCF-7 human BC cells (51). Additionally, miR-182-5p has been identified to be upregulated in TNBC and luminal A breast tumors (52). Furthermore, miR-182-5p was highly expressed in a panel of human BC samples, highlighting its role as a potential oncomiR in BC that could positively regulate metastasis and promote cell colonization (53).

lncRNA MALAT1 serves an important oncogenic role in different types of cancer. The present study demonstrated that MALAT1 mRNA expression was significantly elevated in BC tissues. These findings were in agreement with previous studies showing that lncRNA MALAT1 could promote cell proliferation and invasion in TNBC (54) and lung cancer (55). Additionally, miR-182-5p could regulate MALAT1 expression in MDA-MB-231 cells. A previous study revealed that MALAT1 overexpression is associated with poor prognosis in patients with colorectal carcinoma (53). In addition, the expression levels of MALAT1 are positively associated with the LN status, tumor stage and histological grade in BC (54).

MALAT1 downregulation suppresses the progression of osteosarcoma (56). Furthermore, miR-129-5p could upregulate MALAT1, resulting in cell proliferation and the progression of colon cancer (57). Previous studies have demonstrated that MALAT1 serves a protumorigenic role in pancreatic cancer (58), NSCLC (59) and ovarian cancer (60). Furthermore, accumulating evidence has suggested that MALAT1 contributes to the initiation and progression of bladder cancer via regulation of the expression levels of miRNAs (61). A previous study suggested that MALAT1 could serve as a therapeutic target or a novel diagnostic biomarker for BC (62). Consistent with the findings of the present study, another study demonstrated that MALAT1 knockdown inhibits BC cell proliferation, migration and invasion, and induces apoptosis (63). The present results revealed that silencing of MALAT1 decreased PD-L1 expression. Furthermore, it has been demonstrated that lysine demethylase 5B expression could promote BC aggressiveness via MALAT1 overexpression and downregulation of miR-448 (64). In TNBC, MALAT1 expression is upregulated, and patients with increased levels of MALAT1 exhibit poor overall survival (65). It has been reported that lncRNA XIST serves an important role as a tumor suppressor or oncogene in several types of cancer, such as prostate cancer where it acts as a tumor suppressor (66). In the present study, XIST expression was downregulated in MDA-MB-231 cells following miR-182-5p overexpression. Similarly, a previous study revealed that XIST expression is downregulated in BC (67). By contrast, another study demonstrated that the expression levels of XIST were increased in BRCA1-positive BC, suggesting that XIST expression could be used as a marker to discriminate between BRCA1-positive and -negative breast tumors (68). Additionally, XIST upregulation promotes osteosarcoma (69), HCC (70) and bladder cancer (71) cell proliferation, while it acts as an oncogene in NSCLC via regulation of the miR-37a/la-related protein 1 downstream signaling pathway (72).

Since PD-L1 and MALAT1 act as immune-modulatory targets in the scope of the downstream signaling pathway, their expression pattern was investigated following manipulation of miR-182-5p expression. Ectopic miR-182-5p expression was assessed in MDA-MB-231 cells using RT-qPCR and resulted in the upregulation of PD-L1 and TSIX expression. In addition, elevated mRNA expression levels of MALAT1 were observed in MDA-MB-231 cells transfected with miR-182-5p mimics compared with mock cells. In contrast to renal cancer, where miR-182-5p mimics decrease MALAT1 expression, resulting in inhibition of cancer cell proliferation (73), the treatment with miR-182-5p inhibitor reversed this effect. Transfection with miR-182-5p mimics markedly downregulated XIST expression. Bioinformatics analysis predicted that PD-L1 could strongly bind with MALAT1 and XIST. Therefore, it was hypothesized that the regulation of PD-L1 expression could be mediated by lncRNAs, as upstream regulators, rather than miR-182-5p. Therefore, the effect of each lncRNA on PD-L1 expression was investigated. The expression levels of PD-L1 were determined in cells transfected with siMALAT1 or siXIST. PD-L1 expression was upregulated following transfection with siXIST; however, it was downregulated following MALAT1 knockdown. This finding was consistent with the results of another study, demonstrating that the expression

levels of MALAT1 are positively associated with PD-L1 expression in NSCLC (59). Additionally, following treatment of B-cell lymphoma human cell lines with short hairpin RNA MALAT1, PD-L1 levels are decreased, resulting in inhibition of tumor cell proliferation (74).

To investigate the regulatory association between miR-182-5p and PD-L1, and to explore the combined effect of the two upstream factors on the regulation of PD-L1 expression, the expression levels of PD-L1 were assessed in cells co-transfected with different modulators. PD-L1 expression was downregulated in cells co-transfected with siMALAT1 and miR-182-5p mimics. Furthermore, silencing of Tsix (a negative regulator of XIST) and miR-182-5p overexpression in MDA-MB-231 cells decreased PD-L1 expression. However, the mRNA expression levels of PD-L1 were increased in MDA-MB-231 cells following co-transfection with siXIST and miR-182-5p mimics. Additionally, it was hypothesized that the effect of miR-182-5p on PD-L1 expression was abolished in the presence of lncRNAs. Therefore, the combined effect of lncRNA expression on that of PD-L1 was further investigated. MDA-MB-231 cells were co-transfected with siMALAT1 and siXIST or siMALAT1 combined with siTsix to upregulate XIST expression. For both co-transfection conditions, PD-L1 mRNA expression levels were evaluated. The results revealed that following the silencing of both MALAT1 and Tsix, PD-L1 expression was downregulated compared with that of control cells. In addition, the expression levels of PD-L1 were decreased in MDA-MB-231 cells transfected with siXIST and siMALAT1. These opposing forces on the regulation of PD-L1 expression indicated that XIST could augment the inhibitory effect of MALAT1 knockdown on PD-L1 expression. This hypothesis prompted an investigation into the effect of the main lncRNAs, MALAT1 and XIST, on PD-L1 expression. Therefore, the expression levels of MALAT1 were determined in cells co-transfected with miR-182-5p mimics and siXIST. The results revealed that MALAT1 expression was upregulated in the aforementioned cells. Additionally, MALAT1 expression was upregulated in cells co-transfected with miR-182-5p mimics and siTsix. Notably, PD-L1 expression was downregulated in these cells, suggesting that XIST could be the dominant endogenous competitor in the regulation of PD-L1 expression. Nevertheless, the lack of experiments in additional TNBC cell lines should be considered to be a potential limitation of the present study.

In conclusion, the present study introduced a novel immune-modulatory miRNA-lncRNA interaction network in BC, namely the MALAT1/XIST/miR-182-5p/PD-L1 axis. A previous study (75) has demonstrated that miR-182 acts as an oncomiR, since its expression increases cell migration and proliferation *in vitro*. *In vivo* assays in mice have demonstrated that the expression of miR-182 significantly increases tumor volume and enhances instant metastasis in the lungs (75). The results of the present study suggested that the upregulation of miR-182-5p could act as an oncomiR in BC tissues and MDA-MB-231 cells, and highlighted its molecular effects on pivotal immunomodulatory signaling pathways by promoting the upregulation of oncogenic lncRNAs PD-L1 and MALAT1 in the MDA-MB-231 BC cell line. In addition, miR-182-5p

downregulated the expression of the tumor suppressor gene XIST in the same cells. These findings supported the key role of the ceRNA network, MALAT1/XIST, in regulating PD-L1 expression in BC, and suggested their potential role as immunotherapeutic targets. Overall, both molecules could be utilized as promising biomarkers in clinical diagnosis and prognosis of aggressive BC tumors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AS performed the practical work, data analysis, writing and revision of the manuscript. RAT was the sample provider. RAT was also involved in the acquisition of data, editing and revising the manuscript, and contributed to the conception and design of the study, and to obtaining materials and analysis tools, as well as assessing the authenticity of all the raw data. HMET was the principle investigator of the project who supervised the work. HMET made substantial contributions to conception and design, in addition to analysis and interpretation of data, was involved in drafting the manuscript, assessing the authenticity of all the raw data and revising it critically for important intellectual content and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent for participation in the study or use of their tissue was obtained from all participants. The Ethical Committee of the German University in Cairo and Ain Shams University (Cairo, Egypt) approved the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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