Article

Discovery of Two Targets of Dexmedetomidine in Breast Cancer

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Article

Dexmedetomidine Impacts IDO1 and CHRM3 in Breast Cancer

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Abstract: Background: As the most invasive breast cancer (BrCa), triple-negative BrCa (TNBC) has the worst survival. The use of dexmedetomidine potentially affected BrCa surgery and dexmedetomidine was reported to have direct effects on TNBC cells. The objective of this study is to explore the mechanisms underlying the effect of dexmedetomidine on TNBC. **Methods:** Dexmedetomidine targets were predicted using The Cancer Genome Atlas data SwissTargetPrediction. Cell lines MDA-MB-231, MCF7, and MCF10A were used to validate the targets in TNBC with both clinical samples and cell lines. Cancer cell lines and normal breast cell lines were grouped in cancer and normal groups respectively. Both groups were exposed to dexmedetomidine treatment. Cell Counting Kit-8 was used to determine the effect of dexmedetomidine on cells with target silencing. The binding model of the candidate targets was docked and critical amino acids were mutated to validate the binding model. **Results:** Dexmedetomidine selectively inhibits cancer cells. Catalytic subunit of the DNA-dependent protein kinase (PRKDC), indoleamine 2,3-dioxygenase 1 (IDO1), opioid receptor kappa 1 (OPRK1), glutaminyl-peptide cyclotransferase (QPCT), macrophage migration inhibitory factor (MIF), potassium voltage-gated channel, subfamily H (Eag-related), member 2 (KCNH2), cholinergic receptor, muscarinic 3 (CHRM3), and potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (KCNN4) were identified as dexmedetomidine targets in TNBC. The expression levels of PRKDC, IDO1, MIF, KCNH2, CHRM3, and KCNN4 were found to be upregulated in TNBC tissues compared to non-TNBC tissues($p<0.05$). Silencing of these genes was found to reduce the sensitivity of TNBC cells to dexmedetomidine($p<0.05$). This effect was counteracted when the silenced genes were overexpressed, resulting in an increase in the sensitivity of cells to dexmedetomidine ($p<0.05$). Furthermore, a direct interaction between dexmedetomidine and IDO1 and CHRM3 was observed, which regulated the sensitivity of cells to dexmedetomidine($p<0.05$). **Conclusion:** IDO1 and CHRM3 are direct targets of dexmedetomidine in TNBC.

Keywords: triple-negative breast cancer; dexmedetomidine; IDO1; CHRM3

Introduction

According to the American Cancer Society statistics, the most prevalent type of cancer in females is breast cancer (BrCa), and it is the second leading cause of cancer-related death among women [1]. There were 290,560 new BrCa cases which resulted in 43,780 deaths in the US last year[1]. BrCa is a cancer type that is very heterogeneous. Based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), BrCa was classified into three major subtypes: hormone receptor-positive BrCa, HER2-enriched BrCa, and triple-negative BrCa (TNBC)[2].

Among these BrCa types, TNBC is the most invasive BrCa that is ER^-, PR^-, and $HER2^-$ [2]. TNBC is a heterogeneous and aggressive form of BrCa that lacks expression of ER, PR, and HER2 and is associated with poor prognosis and limited therapeutic options. The molecular mechanisms underlying TNBC pathogenesis and progression are not fully understood[3–6]. A number of studies have suggested that genetic and epigenetic alterations, alterations in signal transduction pathways, and alterations in the tumour microenvironment may play a role in TNBC pathogenesis[3,7–10]. Studies have also suggested that tumour-associated macrophages, neutrophils, and other immune cells may contribute to TNBC progression and metastasis. A number of studies have investigated the genetic alterations associated with TNBC pathogenesis and progression. Studies have identified mutations in a number of genes that are associated with TNBC[2]. In addition, studies have identified

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epigenetic alterations, including DNA methylation, and microRNA expression, that may play a role in TNBC pathogenesis and progression[11–13]. As of yet, TNBC is the most challenging type of BrCa to treat. However, researchers are steadily progressing toward a more effective treatment [14]. Overall, only 77% of female patients with TNBC can survive five years after diagnosis[15]. One of the major treatments of TNBC is the resection of the tumour. But studies have suggested that the use of perioperative anaesthetics might exert potential effects on surgical outcomes and recurrence [16,17]. Further, apart from the systematic effects on the immune system and the nervous system, anaesthetics might have direct impacts on cancer cells[18–21]. For instance, lidocaine was found to inhibit cancer cell viability[22]. This study focused on dexmedetomidine, an anaesthetic that has not been reported to have a direct effect on BrCa cells.

Dexmedetomidine is an α2-adrenoceptor agonist with sedative, anxiolytic, sympatholytic, and analgesic-sparing effects, and minimal depression of respiratory function[23]. Dexmedetomidine is widely accepted to be potent and highly selective for its target in the nervous system, which exerts hypnotic action by activating central synaptic α2-receptors in the locus coeruleus, thereby inducing unconsciousness in patients [24]. In hepatically, dexmedetomidine is primarily glucuronidated and hydroxylated into inactive metabolites [25]. Due to its properties, dexmedetomidine has become a commonly used anaesthetic in the perioperative period in major surgeries, including BrCa surgeries. Studies reported dexmedetomidine decreased the serum levels of cytokine thereby suppressing inflammatory [26,27]. However, so far, the mechanisms underlying the direct effect of dexmedetomidine on TNBC cell remains largely unknown.

The identification of pharmacological targets [28] and disease therapeutic targets [29] is one of the best strategies for cancer studies. Recently, The Cancer Genome Atlas (TCGA) is an initiative funded by the National Cancer Institute that seeks to catalog and discover cancer-causing genetic alterations in order to create a comprehensive "atlas" of cancer genomic profiles, has been widely used to study potential targets and biomarkers for human cancers [30–42]. TCGA data-based included TNBC and normal breast expression data that allows the identification of potential cancer-specific drug targets that were overexpressed in TNBC. Computational docking, however, provides a method for predicting small molecule targets' most likely macromolecular targets [43–45]. In this study, we took advantage of both large-scale mRNA sequence data of TNBC and molecular dynamics docking to identify direct targets of dexmedetomidine in TNBC. The candidate targets were subsequently screened and validated by experimental evidence. Our study was conducive to a better understanding of dexmedetomidine in TNBC treatments. We think that the direct effects of dexmedetomidine on cancer cells would be easier than the complex indirect effects, and enable a further understanding of dexmedetomidine in cancer treatments.

Materials and Methods

1. Drug

Dexmedetomidine hydrochloride was provided by Gibco (Waltham, MA, USA), which was dissolved in ddH_2O at a concentration of 1 mM in stock and then further diluted to final concentrations in the cell culture medium when used.

2. Cell lines

MDA-MB-231, MCF7, and MCF10A were provided by The American Type Culture Collection (Waltham, MA, USA). Cell lines were authenticated by analyzing the cell line through short tandem repeat (STR) profiling. STR profiling is a method of analyzing the DNA of a cell line to ensure its identity. Using this method, the entire genome of a cell line can be compared to a reference DNA profile to verify its identity. MDA-MB-231 and MCF7 are BrCa cell lines and MCF10A is normal breast cell lines. All cell lines were cultured in a DMEM (D5796, Gibco, MA, USA) containing 10% Fetal Bovine Serum (FBS) at 37 °C and 5% CO_2 condition. Cell lines were tested for mycoplasma contamination using the TransDetect PCR Mycoplasma Detection Kit (FM311-01, TransGen Biotech, Beijing, China).

3. Cell viability assay

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To evaluate the effect of the gene on cell viability, the cell viability was measured using the CCK8 assay. Cells were collected and seeded in 96-well plates at 10^3 cells/well. The next day, dexmedetomidine (at testing concentrations) was added and cells were cultured for 48 h. Then Cell Counting Kit-8 (CCK8) solution (K1018, APExBIO, China) was added to each well. After 60 min incubation, the absorbance at 450 nm was measured using a microplate reader (DTX 800, Beckman Coulter, MA, USA). The OD blank was calibrated using the following formula:

cell viability = (OD value of experimental group - OD value of blank group) / OD value of blank group × 100%

The data is then normalized with the control group with no treatment. We defined the fold-change normalized absorbance at 450 nm as "cell viability". The normalization method has been used in a previous paper[22].

4. **Identification and analysis of dexmedetomidine targets**

To identify the potential role of the gene, differential expression genes (DEG) analyses were used to identify DEG between TNBC and normal breast tissues from TCGA datasets (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). DEG was identified by the unpaired Student's t-test, within the DESeq2 (3.8) package [46]. Genes with a P-value <0.05 and an absolute fold change (FC) larger than 1.3 were considered to be statistically significant. All the DEG was presented in a volcano plot. To identify the target of the drug, the drug targets of dexmedetomidine were predicted using the SwissTargetPrediction(http://www.swisstargetprediction.ch/) [47,48]. To identify the common targets, the intersection analysis was conducted using the EVenn (http://www.ehbio.com/test/venn/) [49]. The protein-protein interaction (PPI) network analysis was conducted using GeneMANIA(https://genemania.org/) [50]. Gene annotations of dexmedetomidine candidate targets in TNBC were from the GeneCard(https://www.genecards.org/) [51].

5. **QPCR (quantitative polymerase chain reaction)**

TNBC tissue samples were collected from patients with surgery previously by the Department following related regulations and laws with ethical approval. The total RNA of tissue or cell samples was isolated with QIAzol (79306, Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with a reverse transcription kit (A3500, Promega, Madison, WI, USA). Reverse transcription is a process in which an enzyme, reverse transcriptase, is used to create a complementary DNA (cDNA) strand from an RNA template. The protocol typically involves the following steps: 1. Prepare the reaction mix: The reaction mix should contain reverse transcriptase, buffer, $MgCl_2$, dNTPs, and the RNA template. 2. Incubate the reaction mix at the optimal temperature for reverse transcriptase (usually around 37°C). 3. Add the reverse transcriptase to the reaction mix and incubate for the recommended time (usually around 30 min). 4. Add the appropriate enzyme inhibitor (such as RNase inhibitor) to the reaction mix and incubate for the recommended time (usually around 10 min). 5. Heat inactivate the reverse transcriptase, usually by incubating at 75°C for 10 min. 6. Perform a final centrifugation step to separate the cDNA from the reaction mix. 7. Collect the cDNA and store it at -20°C until ready to use. RT-qPCR was performed with the LightCycler TM instrument (LightCycler-® 480 Instrument II, Roche Applied Sciences, Indianapolis, IN, USA) according to the manufacturer's protocol and the primer property. The mRNA level of each target gene was normalized by internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Catalytic subunit of the DNA-dependent protein kinase (PRKDC), indoleamine 2,3-dioxygenase 1 (IDO1), opioid receptor kappa 1 (OPRK1), glutaminyl-peptide acyltransferase (QPCT), macrophage migration inhibitory factor (MIF), potassium voltage-gated channel, subfamily H (Eag-related), member 2 (KCNH2), cholinergic receptor, muscarinic 3 (CHRM3), and potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 (KCNN4) were identified as dexmedetomidine targets in TNBC, hence their expression were determined. The relative expression levels were calculated using the $2^{(-\triangle\triangle CT)}$ method. The primer was designed using the Eurofins Genomic primer design tool. Primer sequences were as follows:

PRKDC:

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F: 5'- CAGAAGGCTGCCACAGGAA -3'
R: 5'- GCTGATCGTCTGAAGACCTC -3'

IDO1:
F: 5'- TGCCTTCAGCTCCGTCACTT -3'
R: 5'- GATCCCAGGTGAGGGGACAA -3'

MIF:
F: 5'- CACATGCTGCCTGGCATTCT -3'
R: 5'- GTCACTGTCTGCTGCTGCCA -3'

KCNH2:
F: 5'- AGTGGCATCCATTGCTTCTC -3'
R: 5'- AGTGCTGCCCAATGTCTGTC -3'

CHRM3:
F: 5'- CAGCTAAAACCGGTGCTCCA -3'
R: 5'- GCGTCTTGCATTGCTCACCA -3'

KCNN:
F: 5'- CTCGGTACTTGGCCTTCATG -3'
R: 5'- GCTTGGTGGTGGTCTTGCAT -3'

GAPDH
F: 5'- TGAAGGTCGGAGTCAACGGATTTGGT -3'
R: 5'- TGTAGACCATGTAGTTGAGGTCAATG -3'

6. **Western blotting**

TNBC tissue samples were collected from patients with surgery previously by the Department following related regulations and laws with ethical approval. Tissue and cell samples were lysed in RIPA buffer. Protein samples were quantified by using a Bio-Rad DC protein assay kit II (5000112, Bio-Rad, Hercules, CA), separated by electrophoresis on 8–15% SDS-PAGE gel (NW0012C, Invitrogen, NY, USA), and electrotransferred onto a Hybond enhanced chemiluminescence (ECL) transfer membrane (Cytiva RPN2235, Amersham Pharmacia, Piscataway, NJ). After blocking with 3–5% non-fat skim milk, the membrane was probed with primary against Anti-PRKDC antibody (1:1000; abx011674, abbexa, MA, USA), Anti-IDO1 antibody (1:1000; HPA023149, Sigma-Aldrich, CA, USA), Anti-OPRK1 antibody (1:1000; STJ115970, St John's Laboratory Ltd, London, UK), Anti-QPCT antibody (1:1000; HPA008406, Sigma-Aldrich, CA, USA), Anti-MIF antibody (1:3000; orb11051, Biorbyt Ltd, Cambridge, UK), Anti-KCNH2 antibody (1:5000; PA5-33867 Thermo Fisher Scientific Inc., MA, USA), Anti-CHRM3 antibody (1:1000; PA1-86940, Thermo Fisher Scientific Inc., MA, USA), and Anti-KCNN4 antibody (1:1000; NBP2-33694, Novus Biologicals, CO, USA) or GAPDH (1:10,000; #HC301-02; TransGen Biotech) overnight at 4 °C, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies. Goat Anti-Mouse IgG, Peroxidase-Conjugated (1:1000; BL001A; Biosharp, Hefei, China) and Goat Anti-Rabbit IgG, Peroxidase-Conjugated (1:30,000; BL003A; Biosharp, Hefei, China) were used as secondary antibodies. Protein expression was measured by using the ECL system. The western blot image was analyzed using the Volume Box Tools method with GelDoc Go Imaging System, and the protein expression was normalized with the expression level of GAPDH.

7. **Immunostaining**

To validate the protein expression in the tissue samples, immunostaining was used to observe the expression of proteins in TNBC tissues and normal breast tissues (adjacent tissue paired with the TNBC tissues). The breast cancer samples and para-cancer normal breast tissue samples were

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collected from 33 TNBC patients with surgical treatment or biopsy from the Second Hospital University of South China. The supplementary materials provided the details of the patients involved in the study. The samples were preserved by fixing, embedding them in paraffin wax, and keeping them at 4°C. All donors were aged 18 or over and had provided written consent for the use of their samples. The project had received approval from the Ethics Committee at The Second Hospital University of South China. All the samples in paraffin were sliced into slides with paraffin sections which were deparaffinized and dehydrated. Deparaffinization and dehydration of paraffin sections typically involve the use of a sequence of solvents and alcohols of increasing strength. The solvents used in this process usually include xylene and a graded series of ethanol solutions ranging from 50-100%. Incubated 0.3% hydrogen peroxide for 15 min, then slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave, incubated 10% normal rabbit serum (Gibco, MA, USA) for 30 min, and finally, incubated slides with antibodies overnight at 4 °C. The tissues were stained with primary antibodies at 1:100 overnight at 4 °C followed by the incubation of secondary antibodies at 1:500 overnight at 4 °C. DAB (brown) was used to stain the proteins.

8. **Antibodies**

Anti-PRKDC antibody (abx011674, abbexa, MA, USA), Anti-IDO1 antibody (HPA023149, Sigma-Aldrich, CA, USA), Anti-OPRK1 antibody (STJ115970, St John's Laboratory Ltd, London, UK), Anti-QPCT antibody (HPA008406, Sigma-Aldrich, CA, USA), Anti-MIF antibody (orb11051, Biorbyt Ltd, Cambridge, UK), Anti-KCNH2 antibody (PA5-33867 Thermo Fisher Scientific Inc., MA, USA), Anti-CHRM3 antibody (PA1-86940, Thermo Fisher Scientific Inc., MA, USA), and Anti-KCNN4 antibody (NBP2-33694, Novus Biologicals, CO, USA).

9. **Plasmids and transfection of cells**

To overexpress or knock down gene expression, all the plasmids were designed and constructed by GenScript (Nanjing, China). The plasmids were customized and modified pCMV (item number 44154214). The silencing of endogenous proteins was achieved by transfecting plasmids expressing short hairpin RNA (shRNA- TGGAGATGTCCGTAAGGTCTTGCCAAGAAATATTGCTGTTC). The compensation of genes was achieved by transfecting plasmids expressing the exogenous mRNA. The overexpression of genes was conducted as the compensation experiment. All the plasmids were transfected into MDA-MB-231, MCF7, and MCF10A cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48h before experiments. Lipofectamine 3000 is a transfection reagent used to introduce DNA or RNA into eukaryotic cells. It is used in a two-step process. First, mix the DNA or RNA with Lipofectamine 3000 reagent in a serum-free medium. Then, combine the mixture with the cells in a culture dish. Incubate for the recommended amount of time. Finally, change the medium and incubate for the desired amount of time before harvesting the cells or analyzing them. Wild-type genes (such as wild-type IDO1), mean the gene sequence was not changed.

10. **Protein-ligand docking**

To predict the binding of the protein and drug, the protein-ligand docking was used to dock the binding of dexmedetomidine candidate targets and dexmedetomidine in TNBC. The protein structures were downloaded from the Alpha-Fold [52]. The protein used was IDO1. The AutoDock Vina (1.1.2) [53,54] was used to dock the protein-ligand binding. Protein-ligand interactions were analyzed using the PLIP [55].

11. **Statistical analysis**

The results were expressed as means ± standard deviation from at least three independent experiments. Statistical analyses of comparisons between groups were conducted by One-way ANOVA or Student's t-test using SigmaPlot version 12 (Systat Software Inc., San Jose, CA, USA). Turkey's post hoc comparisons were conducted. P-value < 0.05 was considered statistically significant.

Results

1. **Dexmedetomidine selectively inhibits TNBC cells**

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To test the hypothesis that dexmedetomidine inhibited TNBC specifically, we determined and compared the IC_{50} of TNBC cell lines MDA-MB-231 and MCF7, and normal breast cell lines MCF10A. IC_{50} is the half-maximal inhibitory concentration. It is a measure of the amount of a drug or other substance that is needed to inhibit a biological or chemical process by half. It is commonly used to measure the effectiveness of a drug in inhibiting the growth of cancer cells. As dexmedetomidine hydrochloride (the salt form of dexmedetomidine) is freely soluble in water (Fig.1A), we exposed cells to dexmedetomidine-containing mediums for 48 h. Results showed that TNBC cell lines MDA-MB-231 and MCF7 had an IC_{50} of 0.56 and 3.52 μM respectively, while normal breast cell lines MCF10A had an IC_{50} of 60 μM, more than 10 times higher than that of TNBC cell lines (Fig.1 B). Although this was evidence from only cell line models, the hypothesis that dexmedetomidine inhibited TNBC specifically was supported. MDA-MB-231 had a lower IC_{50} compared to MCF7, thus it was used for the subsequent study.

2. PRKDC, IDO1, OPRK1, QPCT, MIF, KCNH2, CHRM3, and KCNN4 were identified as dexmedetomidine targets in TNBC

In order to identify dexmedetomidine candidate targets in TNBC, we conducted a DEG analysis to obtain up-regulated genes in TNBC by comparing TNBC tissues and normal breast tissues from TCGA datasets. Results identified 1092 up-regulated genes in TNBC, which were potential TNBC treatment targets (Fig.1C yellow). On the other hand, dexmedetomidine's most probable macromolecular targets are estimated using SwissTargetPrediction. The results of the prediction revealed 100 potential macromolecular targets for dexmedetomidine, with the most common being Family A G Protein-Coupled Receptors (36%), Kinase (13%), Protease (10%), Cytochrome P450 (9%) and so on (Fig.1D). After that, we conducted an intersection analysis of 1084 up-regulated genes in TNBC and 100 probable macromolecular targets of dexmedetomidine. The intersection analysis further narrowed the targets to 8 candidate genes, including PRKDC, IDO1, OPRK1, QPCT, MIF, KCNH2, CHRM3, and KCNN4. In addition, we conducted a PPI network analysis of these 8 candidate genes and constructed a PPI network (Fig.1E).

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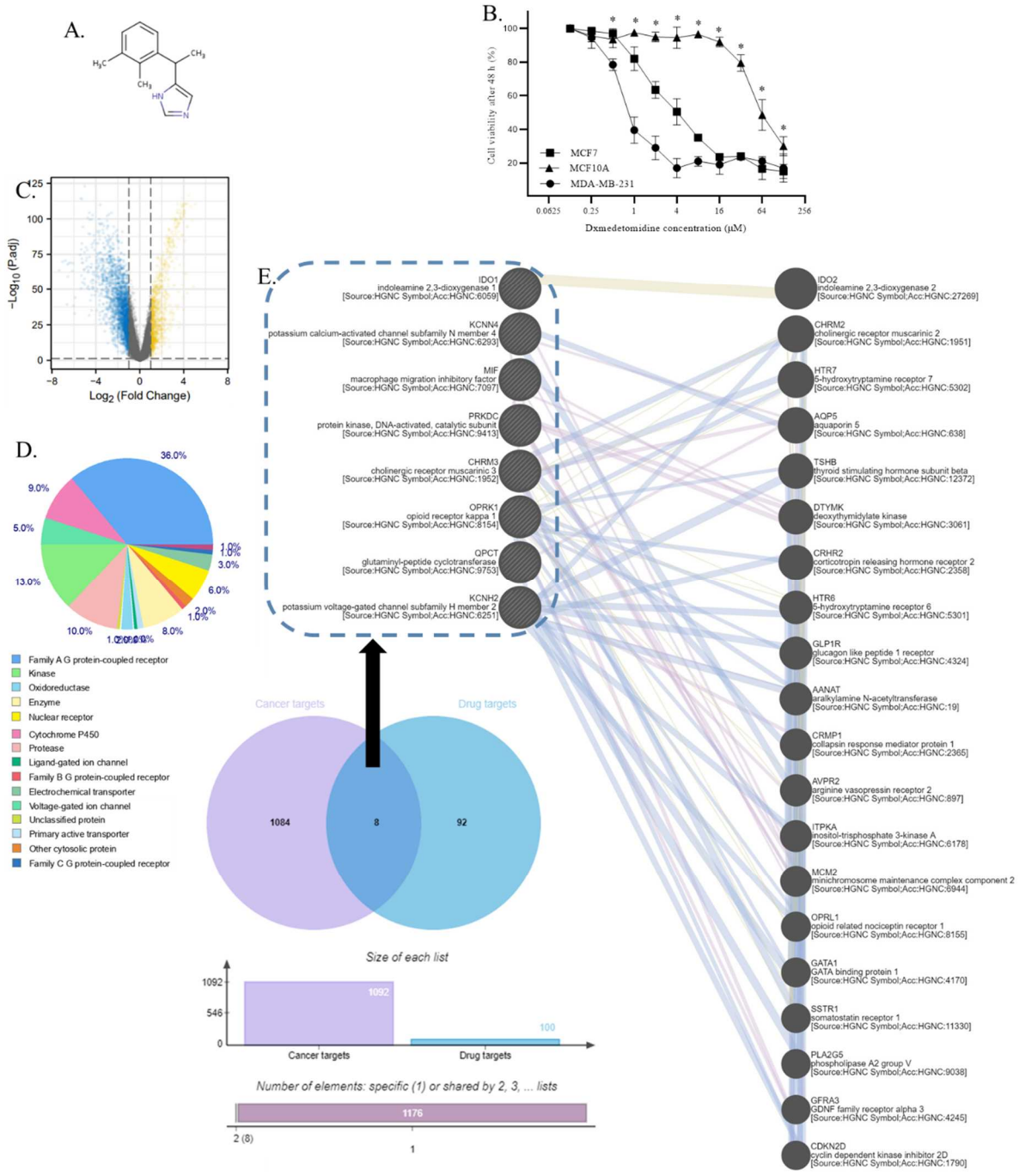

Figure 1. Identification of dexmedetomidine candidate targets in TNBC. **A.** The chemical structure of dexmedetomidine. **B.** The effect of dexmedetomidine on the viability of TNBC cancer cell lines. * Represent the difference between cancer groups and MCF-10A group P<0.05. **C.** DEG of TNBC. Yellow points represent up-regulated genes of fold change over 2, blue points represent down-regulated genes of fold change over 2. **D.** Drug targets of dexmedetomidine. **E.** Intersection analysis of DEG of TNBC and drug targets of dexmedetomidine with the PPI network.

3. PRKDC, IDO1, MIF, KCNH2, CHRM3, and KCNN4 were overexpressed in TNBC tissues

To validate the overexpression of dexmedetomidine candidate targets in TNBC, we conducted qPCR and western blotting experiments to determine the expression of these candidate targets identified in the previous study in normal breast tissues and TNBC tissue. Results showed that TNBC expressed higher levels of all 8 targets tested compared to normal breast tissues ($p<0.05$) at both mRNA and protein levels (Fig.2A-C). To observe the protein expression of these targets, we also

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conducted immunostaining of these proteins. Not all the 8 targets were significantly overexpressed in TNBC compared to normal breast tissue. Results showed these candidate targets were significantly overexpressed in TNBC compared to normal breast tissues except for OPRK1 and QPCT (Fig.2D).

4. **PRKDC, IDO1, MIF, KCNH2, CHRM3, and KCNN4 were overexpressed in TNBC cells**

To further validate the overexpression of these targets and determine the property of the TNBC cell models for the subsequent study, we analyzed the expression of these targets in MCF10A and MDA-MB-231 cells using qPCR and western blotting experiments. Results showed that PRKDC, IDO1, MIF, KCNH2, CHRM3, and KCNN4 were expressed significantly higher in MDA-MB-231 compared to MCF10A at both mRNA and protein levels, while OPRK1 and QPCT showed no differences($p>0.05$) (Fig.2E-G). The protein in the tissues was stained brown; the darker the color, the greater the expression of the protein in the tissues. These results were consistent with the results of tissue samples. In addition, as the IC_{50} of dexmedetomidine in MCF10A and MDA-MB-231 were remarkably different, the differences in these target genes might contribute to the difference in the viability inhibition.

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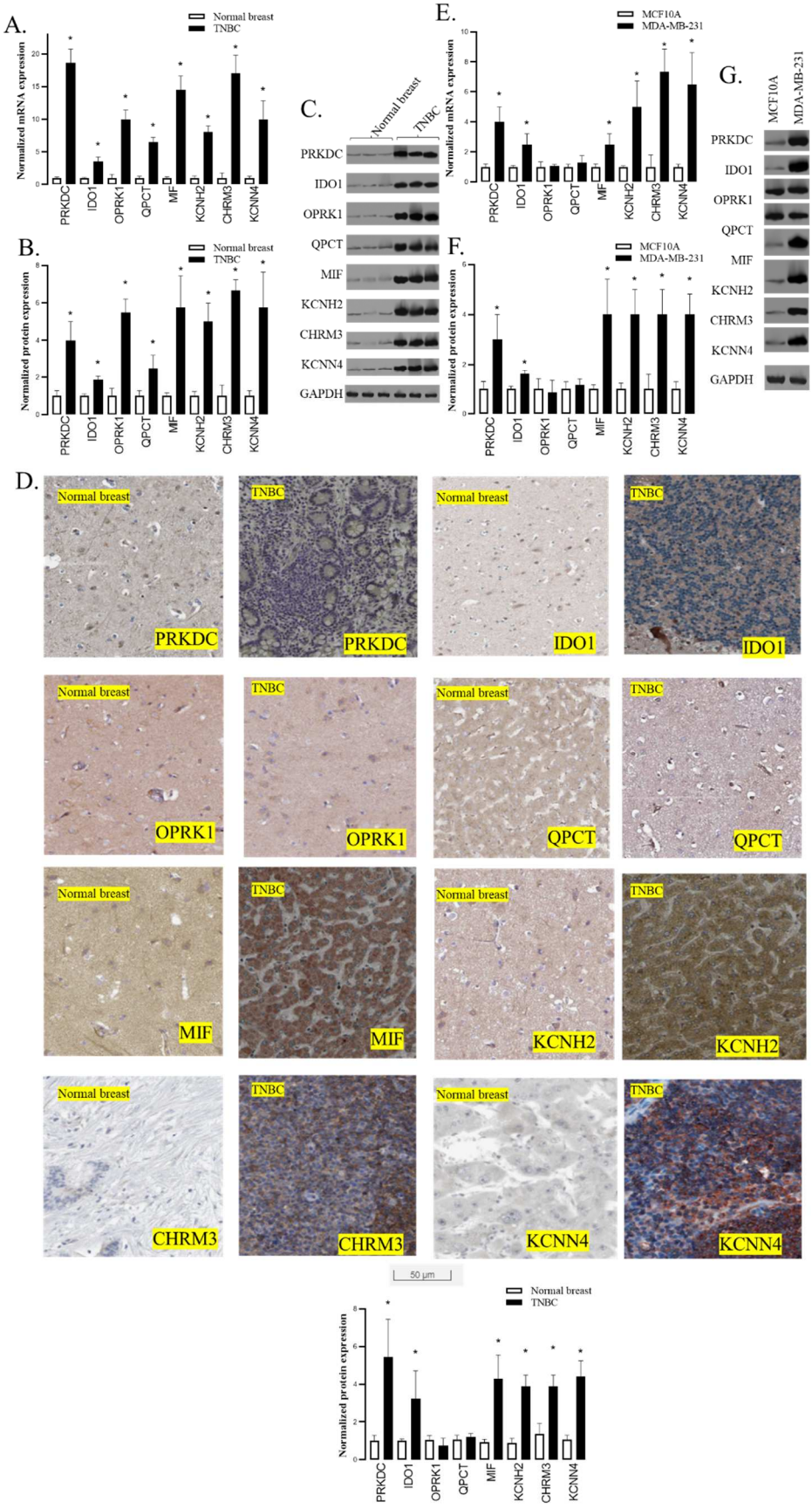

Figure 2. The validation of the overexpression of dexmedetomidine candidate targets in TNBC. **A.** The mRNA expression of candidate targets in TNBC and normal breast tissues. **B.** The protein expression of candidate targets in TNBC and normal breast tissues. **C.** Representative images of

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candidate targets in TNBC and normal breast tissues in western blotting. **D.** Representative images of candidate targets in TNBC and normal breast tissues in immunostaining. The brown color of the picture represents the expression of the protein. Scale bar and Quantifications of images of candidate targets in TNBC and normal breast tissues in immunostaining are shown below. **E.** The mRNA expression of candidate targets in TNBC and normal breast cells. **F.** The protein expression of candidate targets in TNBC and normal breast cells. **G.** Representative images of candidate targets in TNBC and normal breast cells in western blotting. (*p<0.05 compared to normal breast or MCF10A) (n=33).

5. Gene annotation from the GeneCard of PRKDC, IDO1, MIF, KCNH2, CHRM3, and KCNN4

To understand the potential effect of these 6 candidate genes, we downloaded gene annotation from the GeneCard (Table 1). We believe that all these six target proteins might have potential roles in TNBC cell viability and dexmedetomidine sensitivity that have not been studied, hence, we conducted further studies for these six targets.

Table 1. Gene annotation of candidate targets from the GeneCard.

Gene symbol	Entrez gene summary
PRKDC	It functions with the Ku70/Ku80 heterodimer protein in DNA double-strand break repair and recombination. The protein encoded is a member of the PI3/PI4-kinase family. [provided by RefSeq, Jul 2010]
IDO1	It is a heme enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine. This enzyme acts on multiple tryptophan substrates including D-tryptophan, L-tryptophan, 5-hydroxy-tryptophan, tryptamine, and serotonin. This enzyme is thought to play a role in a variety of pathophysiological processes such as antimicrobial and antitumor defense, neuropathology, immunoregulation, and antioxidant activity. Through its expression in dendritic cells, monocytes, and macrophages this enzyme modulates T-cell behavior by its peri-cellular catabolization of the essential amino acid tryptophan. [provided by RefSeq, Feb 2011]
MIF	This gene encodes a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation. It plays a role in the regulation of macrophage function in host defense through the suppression of anti-inflammatory effects of glucocorticoids. This lymphokine and the JAB1 protein form a complex in the cytosol near the peripheral plasma membrane, which may indicate an additional role in integrin signaling pathways. [provided by RefSeq, Jul 2008]
KCNH2	This gene encodes a voltage-activated potassium channel belonging to the eag family. It shares sequence similarity with the Drosophila ether-a-go-go (eag) gene. Mutations in this gene can cause long QT syndrome type 2 (LQT2). Transcript variants encoding distinct isoforms have been identified. [provided by RefSeq, Jul 2008]
CHRM3	The muscarinic cholinergic receptors belong to a larger family of G protein-coupled receptors. The functional diversity of these receptors is defined by the binding of acetylcholine and includes cellular responses such as adenylate cyclase inhibition, phosphoinositide degeneration, and potassium channel mediation. Muscarinic receptors influence many effects of acetylcholine in the central and peripheral nervous systems. The muscarinic cholinergic receptor 3 controls smooth muscle contraction and its stimulation causes secretion of glandular tissue. Alternative promoter use and alternative splicing results in multiple transcript variants that have different tissue specificities. [provided by RefSeq, Dec 2016]
KCNN4	The protein encoded by this gene is part of a potentially heterotetrameric voltage-independent potassium channel that is activated by intracellular calcium. Activation is followed by membrane hyperpolarization, which promotes calcium influx. The encoded

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protein may be part of the predominant calcium-activated potassium channel in T-lymphocytes. This gene is similar to other KCNN family potassium channel genes, but it differs enough to possibly be considered as part of a new subfamily. [provided by RefSeq, Jul 2008]

6. **Silencing PRKDC, IDO1, and CHRM3 decreased the sensitivity of TNBC cells to dexmedetomidine**

We designed silencing and compensation experiments to test their effects on dexmedetomidine sensitivity using MDA-MB-231. In the silencing groups, we transfect shRNA plasmids to silence the endogenous expression of the target proteins. We validated the overexpression of proteins by western blotting assay. Results showed that all 6 proteins were successfully silenced in MDA-MB-231 cells (Fig.3A). The CCK-8 assay revealed that the silencing of KCNH2 and KCNH4 had almost no effect on the IC_{50}, while the IC_{50} was significantly increased in the PRKDC, IDO1, MIF, and CHRM3 silencing groups. The IC50 was slightly increased after MIF silencing, which was 3.88 μM. The IC_{50} of PRKDC, IDO1, and CHRM3 silencing groups were 21.76, 38.32, and 15.55 μM respectively (Fig.3B). These results indicate that PRKDC, IDO1, and CHRM3 might mediate the inhibition of dexmedetomidine in MDA-MB-231. Therefore, we further explore the effect of PRKDC, IDO1, and CHRM3 in dexmedetomidine sensitivity in the subsequent study.

7. **Compensation of IDO1 and CHRM3 recovered the sensitivity of TNBC cells to dexmedetomidine**

In the compensation groups, we co-transfected the shRNA plasmids and the expression plasmid to express the exogenous expression of the target proteins while silencing the endogenous expression of the proteins. To avoid the silencing of the exogenous mRNAs, the codons of the shRNA targeting sequences in the exogenous expression plasmid were replaced by the synonymous codons encoding the same amino acids. We validated the silencing and the compensation of the proteins using western blotting, then tested the IC_{50} of dexmedetomidine in MDA-MB-231. Results showed that the compensation of PRKDC failed to recover the sensitivity of MDA-MB-231 to dexmedetomidine($p>0.05$) (Fig.3C). These results indicated that The silencing of PRKDC might trigger unreversible mechanisms that complexed the PRKDC regulation of dexmedetomidine sensitivity. Thus, we excluded PRKDC from the subsequent study. However, the compensation of IDO1 and the compensation of CHRM3 significantly reduced the IC_{50} of MDA-MB-231 to dexmedetomidine. The compensation of IDO1 almost completely recovered the sensitivity of MDA-MB-231 to dexmedetomidine($p>0.05$) (Fig.3D) while the compensation of CHRM3 reduced the IC_{50} to 2.98 μM (Fig.3E).

8. **Overexpression of IDO1 and CHRM3 increased the sensitivity of breast cells to dexmedetomidine**

To further validate the critical roles of IDO1 and CHRM3 in the sensitivity of breast cells to dexmedetomidine, we overexpressed IDO1 and CHRM3 in MCF10A normal breast cells that expressed low levels of IDO1 and CHRM3 and had relatively low sensitivity to dexmedetomidine. Western blotting showed that the expression of IDO1 and CHRM3 was overexpressed in MCF10A by about 6 and 18 times respectively. The overexpression of IDO1 and CHRM3 remarkably decreased the IC_{50} of MCF10A to dexmedetomidine to 5.61 and 4.22 μM respectively (Fig.3F). These results further supported that IDO1 and CHRM3 were critical in dexmedetomidine sensitivity.

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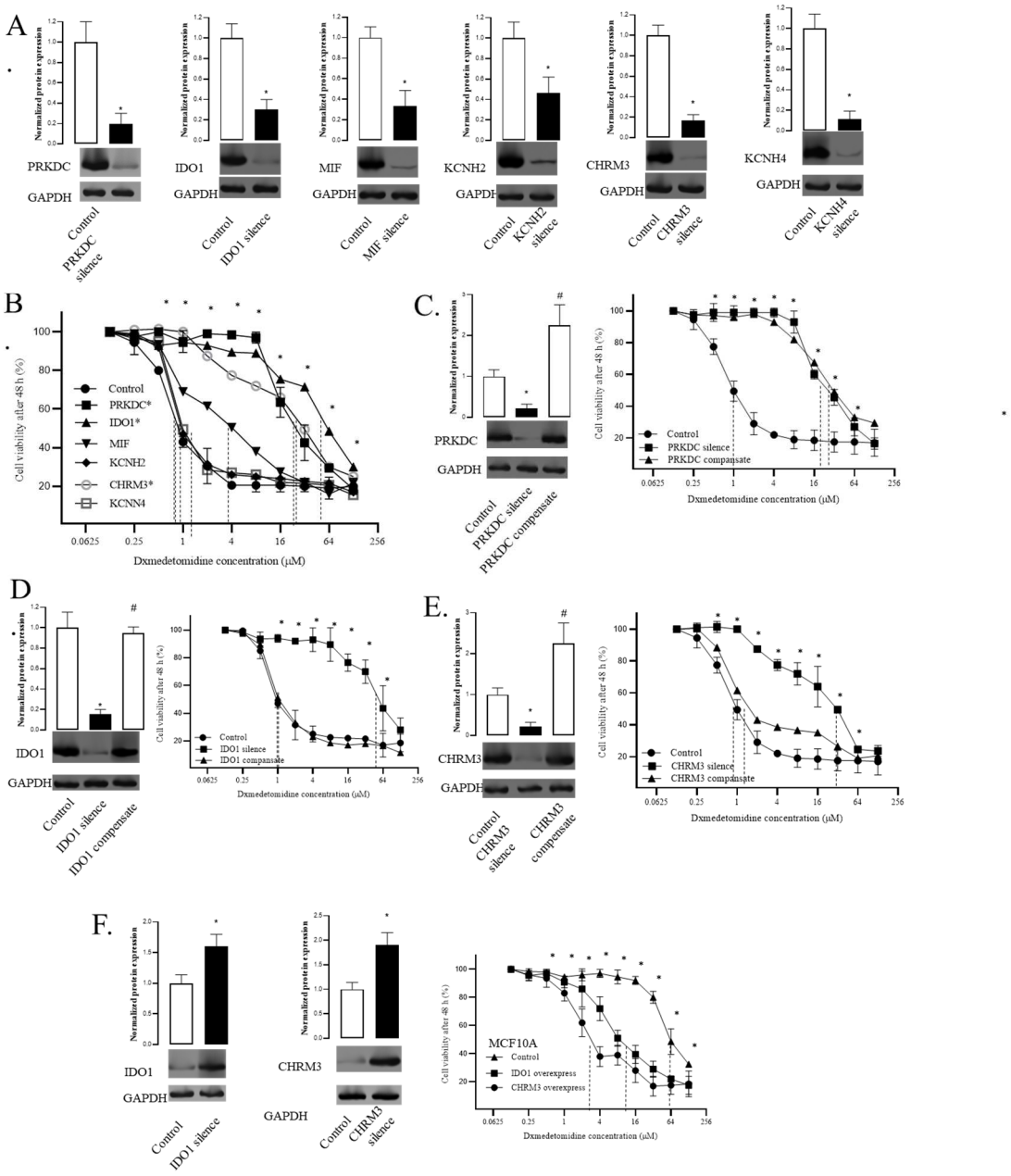

Figure 3. The effect of candidate targets on the dexmedetomidine sensitivity of TNBC cells. **A.** The protein expression of candidate targets in MDA-MB-231 after candidate targets silencing with representative images in western blotting. **B.** The effect of dexmedetomidine on the viability of MDA-MB-231 with candidate targets silencing. **C.** Left panel: The protein expression of candidate targets in MDA-MB-231 after PRKDC silencing with compensation. Right panel: The effect of dexmedetomidine on the viability of MDA-MB-231 with PRKDC silencing with compensation. The same is true for IDO1D and CHRM3 in **D.** and **E.** respectively. **F.** Left panel: The protein expression of IDO1D and CHRM3 in MCF10A after IDO1 and CHRM3 overexpression respectively. Right panel: The effect of dexmedetomidine on the viability of MCF10A after IDO1 and CHRM3 overexpression respectively. * Represent the significant difference between the treatment group and the control group $P<0.05$. The curves in C, D, E, and F show the viability of cells under drug exposure at different concentrations. Data are presented as the mean ± SD of three independent experiments (n=3).

9. Direct interaction of dexmedetomidine to IDO1 and CHRM3 regulated the sensitivity of cells to dexmedetomidine

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Since we have demonstrated that IDO1 and CHRM3 proteins were critical for the sensitivity of TNBC cells to dexmedetomidine, in this study, we proposed that dexmedetomidine directly interacted with IDO1 and CHRM3. To validate this hypothesis, we conducted computational protein-ligand interaction docking using the AutoDock Vina. The docking predicted the potential binding site of protein-ligand where we replaced the interacting amino acids to further validate the interaction.

The dexmedetomidine-IDO1 interaction model showed that there were ten hydrophobic interactions between dexmedetomidine and IDO1 involving eight amino acids (Fig.4A-B). These eight amino acids were replaced with serine, which we called mutated IDO1 (Table 2). Serine has side chains that are polar but not charged, thus it does not form hydrophobic interaction with dexmedetomidine. Then we conducted compensation experiments and tested the IC_{50} as in previous experiments. Results showed that MDA-MB-231 cells compensated with mutated IDO1 failed to recover the IC_{50} as MDA-MB-231 cells compensated with wild-type (WT) IDO1 (Fig.4C). These results validated the model and indicated that the direct interaction of dexmedetomidine and IDO1 contributes to the sensitivity of MDA-MB-231 cells to dexmedetomidine. To further validate this conclusion, we also overexpressed mutated IDO1 in MCF10A cells. Results showed that the mutated IDO1 expression did not affect the IC_{50} as the WT IDO1 (Fig.4D).

For the other target, CHRM3, the dexmedetomidine-CHRM3 interaction model showed that there were two hydrophobic interactions and one hydrogen bond between dexmedetomidine and CHRM3 involving three amino acids (Fig.4E-F). The two amino acids with hydrophobic interactions were replaced with serine and the asparagine that formed a hydrogen bond was replaced by serine and alanine (Table 3). Alanine has no hydrogen donor or acceptor atoms in its side chains and thus does not form hydrogen bonds. Then we conducted compensation experiments and tested the IC_{50} as in previous experiments. Results showed that MDA-MB-231 cells compensated with mutated CHRM3 did not recover the IC_{50} as MDA-MB-231 cells compensated with WT CHRM3 (Fig.4G). These results validated the dexmedetomidine-CHRM3 interaction model and indicated that the direct interaction of dexmedetomidine and CHRM3 contributes to the sensitivity of MDA-MB-231 cells to dexmedetomidine. To further validate this conclusion, we also overexpressed mutated CHRM3 in MCF10A cells. Results showed that the mutated CHRM3 expression did not affect the IC_{50} as the WT IDO1(p0.05) (Fig.4H).

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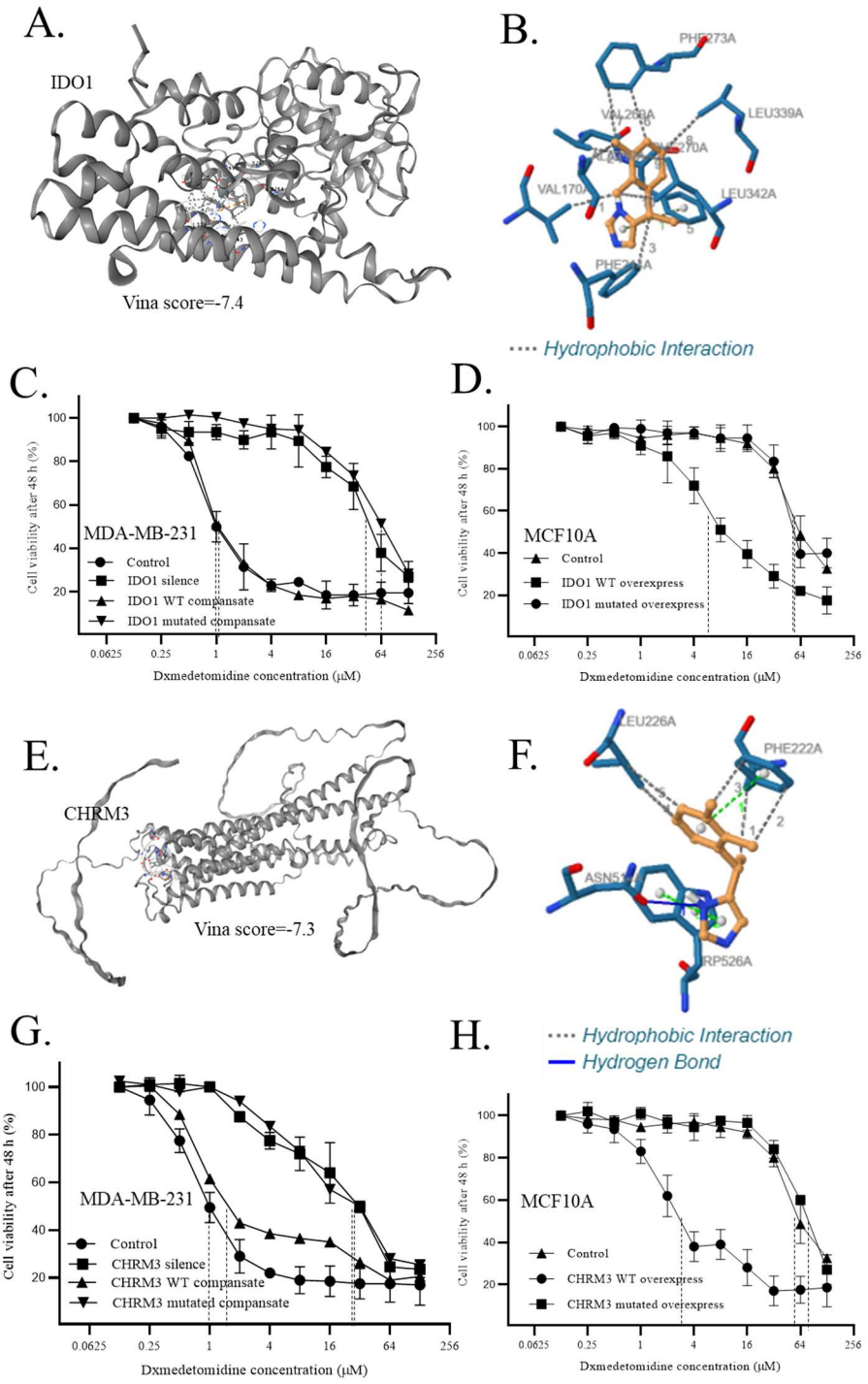

Figure 4. Direct interactions of dexmedetomidine and candidate targets. **A.** The binding model of dexmedetomidine and IDO1. **B.** The interactions of dexmedetomidine and IDO1. **C.** The effect of dexmedetomidine-IDO1 interactions elimination on the viability of MDA-MB-231. **D.** The effect of dexmedetomidine-IDO1 interactions elimination on the viability of MCF10A. **E.** The binding model of dexmedetomidine and CHRM3. **F.** The interactions of dexmedetomidine and CHRM3. **G.** The effect of dexmedetomidine-CHRM3 interactions elimination on the viability of MDA-MB-231. **H.** The effect of dexmedetomidine-CHRM3 interactions elimination on the viability of MCF10A. Data are presented as the mean ± SD of three independent experiments (n=3). P<0.05 represents a significant difference between the mutated group and the control group. *One-way ANOVA p<0.05.

Table 2. Dexmedetomidine-IDO1 interactions.

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Interactions	Residue	Amino acid	Distance	Amino acid changes to
Hydrophobic Interactions1	170A	VAL	2.92	SER
Hydrophobic Interactions2	210A	ALA	3.69	SER
Hydrophobic Interactions3	214A	PHE	3.76	SER
Hydrophobic Interactions4	269A	VAL	3.41	SER
Hydrophobic Interactions5	270A	PHE	3.79	SER
Hydrophobic Interactions6	273A	PHE	3.55	SER
Hydrophobic Interactions7	273A	PHE	3.95	SER
Hydrophobic Interactions8	339A	LEU	3.61	SER
Hydrophobic Interactions9	342A	LEU	3.31	SER
Hydrophobic Interactions10	342A	LEU	3.43	SER

Table 3. Dexmedetomidine-CHRM3 interactions.

Interactions	Residue	Amino acid	Distance	Amino acid changes to
Hydrophobic Interactions1	222A	PHE	3.56	SER
Hydrophobic Interactions2	222A	PHE	3.76	SER
Hydrophobic Interactions3	222A	PHE	3.67	SER
Hydrophobic Interactions4	226A	LEU	3.58	SER
Hydrophobic Interactions5	226A	LEU	3.92	SER
Hydrogen Bonds	514A	ASN	2.44	ALA

Discussions

This study's major finding is the effect of dexmedetomidine on TNBC cells. When we compared the sensitivity between the TNBC cells and normal breast cells, a striking finding was that the MDA-MB-231 and MCF7 were more sensitive to dexmedetomidine compared to MCF10A. Dexmedetomidine is an α2-adrenoceptor agonist and studies showed that α2-adrenoreceptors are present in the human mammary gland [56]. Data suggested that human breast cell lines exhibit

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functional α2-adrenoceptors and it is associated with cell proliferation[57]. For BrCa, α2-adrenoceptors were found to promote tumour growth [57]. However, these results did not explain the distinguish sensitivity between the TNBC cells and normal breast cells in this study, because both TNBC and normal breast cells expressed functional alpha2-adrenoceptors which were targeted by dexmedetomidine. Thus, we believed that dexmedetomidine might have molecular targets besides alpha2-adrenoceptors that were differently expressed in TNBC and normal breasts.

Compared with previous studies in the field, medical drugs and bioinformatics have been studied for the mechnisms and therapy of many human diseases[16,31,38,40,42,58–66], especially for drugs that might have multiple effects[67,68]. The identification of targets of drugs is one of the challenges in pharmacological studies[69]. We used the SwissTargetPrediction [70] to predict the potential molecular targets of dexmedetomidine. These results provide a list of potential targets that can directly interact with dexmedetomidine, which was also a base of the subsequent detailing docking calculations. On the other hand, Big data analysis and high throughput analysis have been very helpful for human disease studies[42,61,63]. TCGA data help us to find the overexpressed genes in TNBC and the overexpression of the targets we were interested in was validated in both clinical tissue samples and TNBC cell lines. We eventually narrowed it down to just two targets: IDO1 and CHRM3. The strength of this study is that, for the first time, IDO1 and CHRM3 are identified to be targets for dexmedetomidine, providing promising results that can be conducive to the future development of clinical TNBC drugs and instruct the use of dexmedetomidine for clinical cancer treatment. However, one of the limitations of this study is that it is not certain if these two targets only work in TNBC or if they are common targets across cancer types.

IDO1 is a rate-limiting metabolic enzyme that converts the essential amino acid tryptophan (Trp) into downstream catabolites known as kynurenines[71]. It is highly expressed in multiple types of human cancer [72] and has been associated with mediating potently immunosuppressive effects in cancer [73], but its immune regulation has been reported to depend on other immune checkpoints [74,75]. In this study, a novel finding is that IDO1 regulated the sensitivity of TNBC cells to dexmedetomidine in vitro even without the presence of immune cells. These effects might be mediated by the IDO1/TDO2-KYN-AhR pathway[76]. The activation of the kynurenine pathway was reported to associate with a worse prognosis of multiple tumour types, such as colon cancer[77]. TDO2-KYN-AhR pathway regulated the tryptophan metabolism which affected multiple critical pathways in cancers, such as the phosphoinositide-3 kinase (PI3K) pathway, Wnt/β-catenin pathway [78], extracellular signal-regulated kinase (ERK) pathway, cyclooxygenase 2 (COX-2) pathway, P53, cyclin-dependent kinase (CDK) pathway, collagen type XII α1 chain (COL12A1) pathway, and bridging integrator 1 (BIN-1) pathway [79]. The extracellular signal-regulated kinase (ERK) pathway plays a critical role in human cells[80], while the inhibition of the kynurenine pathway was also reported to induce cell death in colon cancer [81]. A study also found that the kynurenine pathway and COL12A1/integrin β1 reciprocally promoted each other and their interaction eventually boosted extracellular signal-regulated kinase to facilitate the growth [82]. In addition, the kynurenine pathway is closely associated with neuroinflammation [83] which might involve ion channels on cells that have been found to play a role in cancer cells [84–86]. Hence, these mechanisms might account for the alteration in the viability inhibition sensitivity of TNBC cells to dexmedetomidine we observed in this study.

In this study, dexmedetomidine was identified as a target of the protein CHRM3, which has been relatively less studied. A study suggests that in prostate cancer, autocrine activation of CHRM3 may be involved in facilitating cancer cell proliferation and castration resistance through the phosphorylation of Akt, which is mediated by CaM/CaMKK signalling [87]. In addition, CHRM3 has been reported as a prognostic biomarker for the prognosis of patients with endometrial carcinoma [88]. However, so far, the association of CHRM3 and TNBC cells has not been studied. In this study, we found that CHRM3 was overexpressed in TNBC and its binding to dexmedetomidine affects the sensitivity of TNBC cells to dexmedetomidine. Further studies are required to explore the potential mechanism underlying these effects.

Conclusion

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This study's major finding is that this study investigated the effect of dexmedetomidine on TNBC cells. Our results provide a list of potential targets that can directly interact with dexmedetomidine, which was also a base of the subsequent detailing docking calculations. We eventually narrowed it down to just two targets: IDO1 and CHRM3. We also proved that IDO1 and CHRM3 are direct targets of dexmedetomidine in TNBC.

Authors' contributions: Jiangzhong Liang designed the research and edited the paper; Jingxia Liang and Jing Li carried out the research and edited the paper; Lianzhong Liu analyzed the data and edited the paper; Jing Li, Lianzhong Liu, and Jiangzhong Liang wrote, reviewed, and edited the manuscript.

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Availability of data and materials: All data, models, and codes generated or used during the study are included in the submitted article or are accessible from the corresponding author upon request. The expression data of TNBC and normal breast tissue samples were obtained from the TCGA datasets (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga).

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Competing interests: The authors declare that there are no competing interests.

Ethics approval and consent to participate: All procedures were carried out in accordance with the relevant guidelines and regulations of Helsinki. Experimental protocols were approved by the ethics committee Review Board of The Second Hospital University of South China. Informed consent was obtained from all participants and/or their legal guardians. The ethical approval number is 2022022121A.

Consent for publication: Not applicable.

Abbreviations

BrCa (breast cancer)
TNBC (Triple-negative breast cancer)
DEG (Differential expression gene)
DMEM (Dulbecco's Modified Eagle Medium)
CCK8 (Cell Counting Kit-8)
TCGA (The Cancer Genome Atlas)
FBS (Fetal Bovine Serum)
ECL (Enhanced Chemiluminescence)
GAPDH (Glyceraldehyde 3-phosphate Dehydrogenase)
PRKDC (protein kinase, DNA-activated, catalytic polypeptide)
IDO1 (indoleamine 2,3-dioxygenase 1)
OPRK1 (opioid receptor kappa 1)
QPCT (glutaminyl-peptide cyclotransferase)
MIF (macrophage migration inhibitory factor)
KCNH2 (potassium voltage-gated channel, subfamily H (Eag-related), member 2)
CHRM3 (cholinergic receptor, muscarinic 3)
KCNN4 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4)

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