Monoclonal Antibody Against Sortilin Induces Apoptosis in Human Breast Cancer Cells

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Abstract

Sortilin has an important role to play in various malignances and can be used as a promising target to eradicate cancer cells. The expression of sortilin in 4T1 and MDA-MB231 was evaluated by flow cytometry and immunocytochemistry. Apoptosis test was also performed. Based on cell surface flow cytometry results 2D8-E3 mAb could recognize sortilin molecules in 45.9% and 90.3% of 4T1 and MDA-MB231 cells respectively. The immunocytochemistry staining results verified sortilin surface expression. Apoptosis assay indicated that 2D8-E3 mAb could induce apoptosis in 4T1 and MDA-MB231 cell lines. Our study revealed the important role of surface sortilin in breast carcinoma cell survival and its possible usage as a therapeutic agent in cancer targeted therapies.

Introduction

Breast cancer is among the most common cancers especially in women worldwide [1]. In the Middle East, breast cancer is the most common malignancies among women. Similarly, in our country, Iran, the incidence of breast cancer is estimated at 22.4 (Summary of Report on Cancer Incidence in Iran, 2000) per 100,000 women. Although chemotherapy combined with surgical excision is the mainstay of treatment for patients with breast cancer [2]. The frequently encountered resistance to chemotherapeutic drugs is a crucial point of breast cancer recurrence and metastasis that subsequently leads to high mortality rates [3].

Currently monoclonal antibodies (mAbs) are broadly utilized for clinical diagnosis and treatment of cancers. Albeit, antibody-based therapeutic strategy in solid tumors is well established [4-6], the same is yet to be fully developed in the context of breast cancer.

The expression of proteins related to the nervous system in cancer is an attractive feature of several carcinomas that may be due to the common developmental basis of neurons and epithelial cells, both of them are from the neuroepithelial layer of the embryo [7-9]. Sortilin, a neuronal type-1 membrane protein encoded by the SORT1 gene on chromosome 1 [10]. This type I membrane glycoprotein is a part of mammalian Vacuolar Protein Sorting 10 protein (VPS10P) family of receptors that highly expressed in both central and peripheral nervous systems [11]. The crystal structure of sortilin reveals that the sortilin ectodomain in complex with neurotensin shapes a ten-bladed beta-propeller structure with an inner tunnel that comprises multiple ligand binding sites [12], and largely more involved in protein transfer through a complex pattern between the, endosome, lysosome, Golgi apparatus and plasma membrane, leading to its contributions in multiple, qualitatively different biological processes including lipid and glucose metabolism besides neuraldevelopment, survival and cell death [13-17].

Besides its role as regulator of neuronal viability, sortilin may also attributed to cancer pathogenesis. Involvement of the sortilin 1 receptor and its ligand, NT3 in prostate, colon, and pancreas tumors cell growth has been elucidated before [18].

Indeed, remarkable increase of sortilin has been reported in human cancerous epithelial cells as compared to normal epithelial tissues [19].

It appears that sortilin participates in the progression of HT29 colon cancer cells by regulation of brain-derived growth factor, via interacting with its tyrosine kinase receptor TrkB [20]. in the A549 lung cancer cell line, sortilin induces the release and transfer of exosomes [21] and contributes to tumor cell adhesion and invasion in breast cancer cells [19]. Furthermore, sortilin promote prostate cancer cell growth through progranulin activity regulation [22]. Additionally, sortilin has been identified as a co-receptor for pro-nerve growth factor (proNGF), and acts in a complex with the neurotrophin receptor p75NTR to induce melanoma cancer cell invasion [23].

In spite of its strong relationship with cancer progression, sortilin has been shown to express in few cancer cell lines. In the investigation presented here, we examined the expression of sortilin, using Anti-sortilin monoclonal antibody (Padza Co., Iran) targeting extracellular domain in triple-negative MDA-MB231 and 4T1 breast cancer cells and its inhibitory effects on tumor cell growth in-vitro.

Materials And Methods

Cell line and culture conditions

The Brest carcinoma cell lines, 4T1 and MDA-MB231(National Cell Bank of Iran) were cultured in their optimal conditions in RPMI-1640 (Gibco, Paisley, Scotland), comprising 10% FBS (Gibco, Paisley, Scotland), 100 *units/ml* penicillin (ICN Biomedicals, Ohio) and 100 *µg/ml* streptomycin (Sigma, St. Louis, MO) at 37 *°C* in a humidified incubator with 5% CO2 atmosphere.

Detection of Sortilin cell surface expression by Flow cytometry

Cell lines were cultured in tissue culture flask. After 24 h when cells reached 70-80% confluency, were detached by 0.25% trypsin-EDTA (Gibco) and washed three times with cold Phosphate Buffered Saline (PBS), transferred to flow cytometry tubes and blocked with 5% sheep serum for 30 min at 4 °C. Then cells were incubated with the 10 µg/ml anti-sortilin monoclonal antibody clone 2D8-E3 for 1h at 4 °C (Padza Co). After 1 hour the cells were washed with PBS containing 0.05% BSA and 0.1% NaN_3, and the bound antibodies were detected by incubating the cells with a fluorescein isothiocyanate (FITC)–labeled sheep anti–mouse IgG (Padza Co.) (1:50) at 4 °C for 30min in a dark place. The results were examined by a PAS III flow cytometer (Partec, GmbH, Germany) and analyzed with FlowJo software (v.10). The calculation of average FITC intensities carried out by multiplication of the Percentage of Positive cells (POP) to Mean Fluorescence Intensity (MFI) (POP×MFI)

Immunocytochemistry (ICC)

After 24 h incubation at 37 °C in a humidified incubator, cells were transferred to slides using Cytospin 4 Cytocentrifuge (Thermo Scientific, USA) (400 rpm, 5 min) at a density of 1×105 cells and fixated in

neutral buffered formalin (NBF) for 10 min. The slides were washed three times (3×5 min) with Phosphate-buffered saline (PBS) (pH=7.4). The slides were blocked with 10% sheep serum containing 3% bovine serum albumin (BSA) and 0.01% PBS-Tween 20 for 30 min at Room Temperature (RT). Slides were stained using Primary antibody sortilin (Padza Co.) at 10 µg/ml in 1% BSA+ 0.01% PBS-Tween 20 and the cells were incubated for 1 h and 30 min in a humid chamber at room temperature. After washing for three times, the secondary sheep anti-mouse FITC-labeled antibody was added and incubated for 45 min at room temperature. After further washing for three times, the cell nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Calbiochem, USA) at 1 µg/ml concentration for 10 min. Staining cells without primary antibody utilized as negative. Finally, after washing with PBS, the slides were mounted in 50% PBS-glycerol. Images were captured using a fluorescent microscope (Olympus, Tokyo, Japan).

Study of apoptosis

Apoptosis in 4T1, MDA-MB231 and control cells treated with sortilin Ab was measured at three different time points (24, 48 and 72 *hr* after treatment).

Cells were scraped and washed in PBS (2x). After centrifuge, pelleted cells were incubated with 1× binding buffer containing FITC-annexin V (Padza Co., Iran) and stored in dark for 1 hr. Then the cells were washed twice and propidium iodide was added. Finally, apoptotic cells were examined using a BD FACSLyric™ Flow Cytometer (BD Life Sciences, San Jose, CA, USA) and analyzed using the FlowJo software (version 10).

Results

Cell surface expression by flow cytometry and ICC

The expression of sortilin in 4T1, MDA-MB231 and Human Fetal Foreskin Fibroblast (HFFF) (as negative control) was investigated by flow cytometry. The 2D8-E3 mAb recognized sortilin molecules in 79.2% and 90.3% of 4T1 and MDA-MB231 cells while the expression of sortilin in HFFF was 2.1% as shown in **Fig 1**. The average of FITC intensity (MFI×POP) values were 799.92 in 4T1, 517.42 in MDA-MB231 and 5.52 in HFFF (**Fig1**, Table 1). For further confirmation, cell surface staining of 4T1 and MDA-MB231 cell lines were performed. It demonstrated that they are expressing sortilin on their surfaces. The sortilin Ab indicated binding to cancerous cells at a concentration of 10 µg/mL (**Fig 2**)

Apoptosis induction by sortilin Ab

Next, the effect of sortilin Ab on survival of 4T1 and MDA-MB231 cells was measured by FACS analysis of annexin V staining 24, 48, and 72 *hr* post-induction. In each experiment, the percentage of apoptotic cells was examined as the sum of percentages of cells in early (*i.e.*, annexin V positive PI-negative) and late (*i.e.*, annexin V-positive, PI-positive) stages of apoptosis and was subtracted from negative control cells. The results showed that incubation with anti-sortilin antibody induced 8.45% early and 4.66% late

apoptosis of 4T1 cells 24 *hr* after induction. This amount of apoptosis was markedly higher in 48 *hr*, 73.3% early and 6.43% late apoptosis. 25.6% and 23.3% of cells dropped to early and late apoptosis, respectively in 72 *hr* (**Fig 3A**).

The results of 24 *hr* of incubation demonstrated that 2D8-E3 mAb could induce apoptosis in MDA-MB231 cell line with 12.3 and 8.11% of the cells for early and late apoptosis, respectively. The values for 48 *hr* of incubation were 20.3 and 39.7% for early and late apoptosis. 72 *hr* results were 5.06% and 6.1% for early and late apoptosis respectively in this cell line (**Fig 3B**), meanwhile it is obvious that the level of apoptosis increased over time after antibody induction in both cell lines. As compared to control cells, the 2D8-E3-treated cells showed 10.31% (24 *hr*) and 28.92% (48 *hr*) and 8.95(72 *hr*) apoptosis (**Fig 3C**). Meanwhile The isotype control mAb don't induce significant apoptosis in all examined cells.

Discussion

Breast cancer accounts for 23% of all cancers diagnosed worldwide (2). Conventional cancer therapies are the gold standard treatment for cancers. Scientists have been looking for novel strategies with boost efficacies and more durable response in cancer patients. However, immunotherapy opened a new era in cancer treatment, it is still struggling with limited therapeutic effectiveness and life-threatening adverse reactions [24]. Hence there is an urgent need for finding new targets for improving immunotherapeutic results.

This study is the first to report sortilin over expression in 4T1(murine breast cancer cell line) and MDA-MB231(human breast cancer cell line), by means of flow cytometry. Our results indicate an ectopic expression of sortilin on the surface of breast cancer cells in comparison with HFFF as normal control cells and subsequent ICC analysis confirmed this finding. In terms of gene expression, sortilin mRNA abundance has been reported not only in breast but also prostate, colon, and pancreatic cancer cell lines [25]. Human renal carcinoma 786-O and ACHN cells over express sortilin in mRNA and protein levels and present in TrkB/p75NTR complex in RCC cells but its function is not associated with cell apoptosis induction or survival [26]. On the other hand, sortilin in complex with two receptors containing a tyrosine kinase domain, TrkB and EGFR might have an important role in angiogenesis and cell survival by induction of this pathway in human umbilical vein endothelial cells (HUVECs) exposed to sortilin-containing exosomes, as it blocks when exploiting sortilin-depleted exosomes. Wilson, Cornelia M., et al. proved that the angiogenesis process is impaired in A549 cells, a lung cancer cell line, in case of sortilin downregulation in exosomes [21]. Also, sortilin in complex with TrkA, another tyrosine kinase receptor for NGF, mediates breast cancer aggressiveness and associated with tumor cell adhesion and invasion. The impact of sortilin in lymph node invasion of breast tumor cells stems from the fact that sortilin 1 as a type I receptor is an essential part of a receptor complex for pro-NGF induced neural cell death and this former was produced and secreted by malignant cells [16]. In another study the cleavage of luminal part of sortilin by matrix metalloproteinases produced soluble sortilin (sSortilin) which activates the Focal Adhesion Kinase pathway and regulates the downstream pathways involving either cell migration or

metastasis in HT29 colorectal cancer cell line [27]. Furthermore sortilin enhanced invasive capacity of glioblastoma through GSK-3β/β-catenin/twist pathway [28].

Considering the fact sortilin can serve as an essential regulator of cell growth, according to above findings, there is no doubt that sortilin affects the incidence and growth of different type of tumor cells, so it could be intriguing target for cancer eradication. Recently scientists exploited theratechnologies peptides conjugated with anticancer drugs to eradicate tumor cells. In a study carried out by B. Annabi and et.al, a novel curcumin-peptide conjugate called TH1901 inhibits cancer cell proliferation in sortilin-positive cancers including ovary (ES2), breast (MD-MB231, HCC-1569 and HCC-1954), skin (SK-MEL-28 and A-375) and colorectal (HT-29) [29]. Another two peptide conjugates to docetaxel (TH1902) and doxorubicin (TH1904) specifically designed to target Sortilin receptor (SORT1). The former showed anti-proliferative and anti-migratory activities in MDA-MB-231 cells and lead to tumor regression in murine MDA-MB-231 xenograft tumor model [30]. Also, TH1904 inhibits the formation of 3D-tubular structures in an *in vitro* model of ovarian and triple negative breast cancer cells. This VM 3D-tubular structures are strongly associated with ES-2 ovarian cancer and MDA-MB-231 TNBC cells progression and invasion, hence interfere with their formation by targeting SORT-1 has a potent therapeutic values in cancer treatment [31]. Moreover, sortilin receptor targeted by siRNAs abolish the stem cell propagation, both in vitro and in vivo breast cancer models, decrease either the aggressive or metastatic value of tumor cells [32].

Despite of the fact that the majority of investigators mainly focus on intracellular domain of molecule, perhaps because to localization of the great part of sortilin in cytoplasmic region [33], and develop monoclonal antibodies or inhibitory siRNAs against intracellular domain of this molecule, or amino acids peptides which provides specific binding to surface sortilin molecule, Rabbani and colleges generates a mAb targeting surface expressed sortilin. Consequently sortilin antibody could bind to extracellular epitope of sortilin protein in number of ovarian carcinoma cell lines [34]. Also, In another study again carried out by Rabbani and colleges this mAb can detects the surface sortilin in malignant B cells unlike previous studies, none of them report the surface expression of sortilin in normal or malignant B cells [35]. This difference findings originates from unique design of 2D8-E3 mAb that targets the very N-terminal part of sortilin [34].

Furthermore, this report was the first that demonstrates invasive breast cell growth in vitro was blocked by the 2D8-E3 mAb, which detects the cell surface expressed sortilin. In a former study on CLL cells using the same antibody, similar results were found in case of apoptosis induction [36]. However the significant role of sortilin in apoptosis induction has been shown through $p75^{NTR}$-sortilin complex that bound to pro-BDNF in high affinity manner in CRC cell lines [20], the exact mechanism of 2D8 mAb action is not clear; however, it was proved that sortilin in complex with Neurotensin Receptor 1 (NT-R1) induces growth signaling pathways through neurotensin internalization [25]. Hence one possible explanation of 2D8 mAb direct cell destruction would be its preventive effect on heterodimerization of sortilin with NTR1. Undoubtedly further studies should be done in order to prove this hypothesis. In addition, beyond of this

killing mechanism of action, mAbs have the capacity to directly eradicate cancer cells or boost the chemo- or radio-therapeutics on cancer cells by modulating anti-apoptotic pathways [37].

Recently the increased level of sortilin has been shown in ovarian cancer, its silencing through siRNA led to inhibition of proliferation (40.1%) in Caov-4 cells [38]. Similarly, sortilin silencing in CAL-62 thyroid cancer cell line hampered the activation of extracellular signal-regulated kinases (ERK) which subsequently resulted in cell survival inhibition and decrease in migration of malignant cells [39].

Analysis of apoptosis assays indicates that in spite of the fact that sortilin shows higher expression in MDA-MB231 cells when compared to 4T1 cells (90.3 vs. 79.2), the rate of apoptosis is significantly lower in human breast cancer cell line. This finding highlights the importance of arbitrary value in apoptosis induction. Arbitrary value represents the number of receptors on the tumor cells [40]and calculated by multiplication of MFI by the percentage. as shown in table 1, the arbitrary value for 4T1 cells is higher hence the treatment of two breast cell lines with equal amount of 2D8-E3 antibody resulted in stronger apoptosis in 4T1 cancer cells. For both cell lines the viability of the cells was reduced to 19.2% for 4T1 cells and 23.7% for MDA-MB231 cells in 48 *hr* after induction. To the best of our knowledge this study was the first that reports the functional significance of surface sortilin in breast cancer and can considered as stepping stone for further studies for implication of this mAb in cancer targeted therapeutics.

Overally, in this study we investigate whether we could see the increased expression of surface sortilin in breast cancer cell line. Roselli, Séverine, et al [19] study gives empirical support to the increased level of sortilin in breast cancer cell line as well as malignant tissues as compared to normal tissues. We report here that a surface protein, sortilin, is overexpressed in 4T1 and MDA-MB231 breast cancer cell lines and mAb against sortilin inhibits breast cancer cell growth in vitro, induces apoptosis in these cells, Although the inhibition of cell growth is often important in vitro, the effector functions of mAbs can just as important in vivo. This highlights the significance of further investigation on the utility of this antibody in animal experiments, that will provide more evidence that sortilin is a promising agent for the treatment of tumor.

Conclusion

This study indicates that sortilin expressed on the surface of breast cancer cells at high level and have a crucial role in these cells' survival; hence it can be served as a promising target in anti-cancer therapeutic agents' field. It is apparent that further studies including other carcinoma cell lines and in vivo experiments is required to apply anti-Sortilin mAb as a solo therapeutic target not only in breast cancer but also in the other malignancies as well.

Future Perspective

The antibody therapy field represents a promising era in the field of cancer treatment. Based on impressive results in vitro and in vivo, it is not to far from expectations that these novel therapies can

substitute the classic methods in near future, where the cancerous patients may treat with either more effective and less painful approaches.

Declarations

Conflicts of interest/Competing interests :

The authors declare that they have no conflict of interests.

Financial disclosure:

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Consent to participate

N/A

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References

1. Mcguire A, Brown JA, Malone C, Mclaughlin R, Kerin MJ. Effects of age on the detection and management of breast cancer. *Cancers* 7(2), 908-929 (2015).
2. Bouchard H, Viskov C, Garcia-Echeverria C. Antibody–drug conjugates—a new wave of cancer drugs. *Bioorganic Med. Chem. Lett.* 24(23), 5357-5363 (2014).

3. *****Li Z, Qian J, Li J, Zhu C. Knockdown of lncRNA-HOTAIR downregulates the drug-resistance of breast cancer cells to doxorubicin via the PI3K/AKT/mTOR signaling pathway*. Exp Ther Med* 18(1), 435-442 (2019). The siginificant challenges that hampers the effectiveness of claasic cancer tretment methods.
4. Chen R, Zhang D, Mao Y *et al*. A human Fab-based immunoconjugate specific for the LMP1 extracellular domain inhibits nasopharyngeal carcinoma growth in vitro and in vivo. *Mol. Cancer Ther.* 11(3), 594-603 (2012).
5. Jubb AM, Miller KD, Rugo HS *et al*. Impact of exploratory biomarkers on the treatment effect of bevacizumab in metastatic breast cancer*. Clin. Cancer Res.* 17(2), 372-381 (2011).
6. Yardley DA, Hart L, Bosserman L *et al*. Phase II study evaluating lapatinib in combination with nab-paclitaxel in HER2-overexpressing metastatic breast cancer patients who have received no more than one prior chemotherapeutic regimen*. Breast Cancer Res Treat* 137(2), 457-464 (2013).
7. Li ZJ, Cho CH. Neurotransmitters, more than meets the eye—neurotransmitters and their perspectives in cancer development and therapy*. Eur J Pharmacol.* 667(1-3), 17-22 (2011).
8. Mancino M, Ametller E, Gascón P, Almendro V. The neuronal influence on tumor progression*. Biochim Biophys Acta Rev Cancer BBA-REV CANCER* 1816(2), 105-118 (2011).
9. Mehlen P, Delloye-Bourgeois C, Chédotal A. Novel roles for Slits and netrins: axon guidance cues as anticancer targets? *Nat. Rev. Cancer* 11(3), 188-197 (2011).
10. Petersen CM, Nielsen MS, Nykjær A *et al*. Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography*. J. Biol. Chem. J BIOL CHEM* 272(6), 3599-3605 (1997).
11. Willnow TE, Petersen CM, Nykjaer A. VPS10P-domain receptors—regulators of neuronal viability and function*. Nat. Rev. Neurosci* 9(12), 899-909 (2008).
12. Quistgaard EM, Madsen P, Grøftehauge MK, Nissen P, Petersen CM, Thirup SS. Ligands bind to Sortilin in the tunnel of a ten-bladed β-propeller domain*. Nat. Struct. Biol.* 16(1), 96-98 (2009).
13. Huang G, Buckler-Pena D, Nauta T *et al*. Insulin responsiveness of glucose transporter 4 in 3T3-L1 cells depends on the presence of sortilin*. Mol Biol Cell* 24(19), 3115-3122 (2013).
14. Kjolby M, Nielsen MS, Petersen CM. Sortilin, encoded by the cardiovascular risk gene SORT1, and its suggested functions in cardiovascular disease*. Curr Atheroscler Rep* 17(4), 18 (2015).
15. Nielsen MS, Madsen P, Christensen EI *et al*. The sortilin cytoplasmic tail conveys Golgi–endosome transport and binds the VHS domain of the GGA2 sorting protein*. EMBO J* 20(9), 2180-2190 (2001).
16. Nykjaer A, Lee R, Teng KK *et al*. Sortilin is essential for proNGF-induced neuronal cell death*. Nature* 427(6977), 843-848 (2004).
17. Patel KM, Strong A, Tohyama J *et al*. Macrophage sortilin promotes LDL uptake, foam cell formation, and atherosclerosis*. Circ. Res.* 116(5), 789-796 (2015).
18. *****Mazella J, Vincent J-P. Functional roles of the NTS2 and NTS3 receptors*. Peptides* 27(10), 2469-2475 (2006). The role of sortilin in differnet cancers.

19. Roselli S, Pundavela J, Demont Y *et al*. Sortilin is associated with breast cancer aggressiveness and contributes to tumor cell adhesion and invasion. *Oncotarget* 6(12), 10473 (2015).
20. Akil H, Perraud A, Mélin C, Jauberteau M-O, Mathonnet M. Fine-tuning roles of endogenous brain-derived neurotrophic factor, TrkB and sortilin in colorectal cancer cell survival. *PloS one* 6(9), e25097 (2011).
21. Wilson CM, Naves T, Vincent F *et al*. Sortilin mediates the release and transfer of exosomes in concert with two tyrosine kinase receptors. *J. Cell Sci.* 127(18), 3983-3997 (2014).
22. Tanimoto R, Morcavallo A, Terracciano M *et al*. Sortilin regulates progranulin action in castration-resistant prostate cancer cells. *Endocrinology* 156(1), 58-70 (2015).
23. Truzzi F, Marconi A, Lotti R *et al*. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. *J. Invest. Dermatol. J INVEST DERMATOL* 128(8), 2031-2040 (2008).
24. Tan S, Li D, Zhu X. Cancer immunotherapy: Pros, cons and beyond. *Biomed. Pharmacother.* 124 109821 (2020).
25. Dal Farra C, Sarret P, Navarro V, Botto JM, Mazella J, Vincent JP. Involvement of the neurotensin receptor subtype NTR3 in the growth effect of neurotensin on cancer cell lines. *Int J Cancer* 92(4), 503-509 (2001).
26. Miguel A, Berger J, Sánchez-Prieto R *et al*. p75 neurotrophin receptor and pro-BDNF promote cell survival and migration in clear cell renal cell carcinoma. *Oncotarget* 7(23), 34480 (2016).
27. Béraud-Dufour S, Devader C, Massa F, Roulot M, Coppola T, Mazella J. Focal adhesion kinase-dependent role of the soluble form of neurotensin receptor-3/sortilin in colorectal cancer cell dissociation. ***Int***. *J. Mol. Sci.* 17(11), 1860 (2016).
28. Yang W, Wu P-F, Ma J-X *et al*. Sortilin promotes glioblastoma invasion and mesenchymal transition through GSK-3β/β-catenin/twist pathway. *Cell Death Dis* 10(3), 1-15 (2019).
29. *****Annabi B, Charfi C, Demeule M *et al*. TH1901, a novel curcumin-peptide conjugate for the treatment of Sortilin-positive (SORT1+) cancer. (2020). A novel peptide conjugate which targets surface sortilin.
30. ******Marsolais C, Charfi C, Demeule M *et al*. A novel Sortilin-targeted docetaxel peptide conjugate (TH1902), for the treatment of Sortilin-positive (SORT1+) triple-negative breast cancer. (2020). The utilization a peptide conjugate that tagets surface sortilin and had antiproliferative impact on MDA-MB231 cells.
31. *****Currie J-C, Demeule M, Larocque A *et al*. Sortilin receptor-mediated novel cancer therapy: A targeted approach to inhibit vasculogenic mimicry in ovarian and breast cancers. (2020). A novel peptide conjugate which inhibits 3D-tubular structures via targeting surface sortilin.
32. Berger K, Rhost S, Hughes E *et al*. Abstract P2-06-11: Sortilin targeted therapy in breast cancer with elevated progranulin expression. (2019).
33. Ni X, Canuel M, Morales CR. The sorting and trafficking of lysosomal proteins. *Histol. Histopathol.* (2006).

34. Ghaemimanesh F, Bayat AA, Babaei S *et al*. Production and characterization of a novel monoclonal antibody against human sortilin*. Monoclon. Antibodies Immunodiagn. Immunother.*34(6), 390-395 (2015).
35. Fauchais A-L, Lalloué F, Lise M-C *et al*. Role of endogenous brain-derived neurotrophic factor and sortilin in B cell survival*. J. Immunol.* 181(5), 3027-3038 (2008).
36. **Farahi L, Ghaemimanesh F, Milani S, Razavi SM, Akhondi MM, Rabbani H. Sortilin as a novel diagnostic and therapeutic biomarker in chronic lymphocytic leukemia*. Avicenna J Med Biotechnol* 11(4), 270 (2019). The usage of sortilin mAb in identification of mslignsnt B-cells.
37. Ludwig DL, Pereira DS, Zhu Z, Hicklin DJ, Bohlen P. Monoclonal antibody therapeutics and apoptosis*. Oncogene* 22(56), 9097-9106 (2003).
38. Ghaemimanesh F, Ahmadian G, Talebi S *et al*. The effect of sortilin silencing on ovarian carcinoma cells*. Avicenna J Med Biotechnol* 6(3), 169 (2014).
39. Faulkner S, Jobling P, Rowe CW *et al*. Neurotrophin receptors TrkA, p75NTR, and sortilin are increased and targetable in thyroid cancer*. Am. J. Pathol.* 188(1), 229-241 (2018).
40. Tsai Y-C, Tsai T-H, Chang C-P, Chen S-F, Lee Y-M, Shyue S-K. Linear correlation between average fluorescence intensity of green fluorescent protein and the multiplicity of infection of recombinant adenovirus*. J. Biomed. Sci.* 22(1), 1-9 (2015).

Tables

Table 1. Flow cytometry on breast cancer and normal cell lines

Cell line	Antibody	MFI**	POP***	MFI*POP
4T1				
	Anti-sortilin mAb*	10.1	79.2	799.92
	Isotype control	4.25	4.42	18.75
MDA-MB231				
	Anti-sortilin mAb	5.73	90.3	517.42
	Isotype control	4.54	18.9	85.5
HFFF				
	Anti-sortilin mAb	2.63	2.1	5.52
	Isotype control	3.19	2.28	7.27

* Monoclonal antibody. ** Mean fluorescence intensity. *** Percentage of positive cells.

Figures

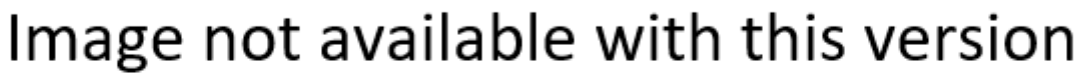

Figure 1

Reactivity of anti-sortilin monoclonal antibody clone 2D8-E3 to breast cancer and normal cell lines using flow cytometry. Left panel: A) 2D8-E3 could react with sortilin in 79.2% of 4T1 and 90.3% of MDA-MB231 cells, compared to HFFF cell (2.1%) as a normal sample. The values for isotype controls in all three cell lines have also illustrated. Middle panel: B) The same results illustrated as bars for better visualization. Right panel: C) The average FITC intensities were calculated through multiplying the mean fluorescence intensity by percentage of positivity (MFI×POP).

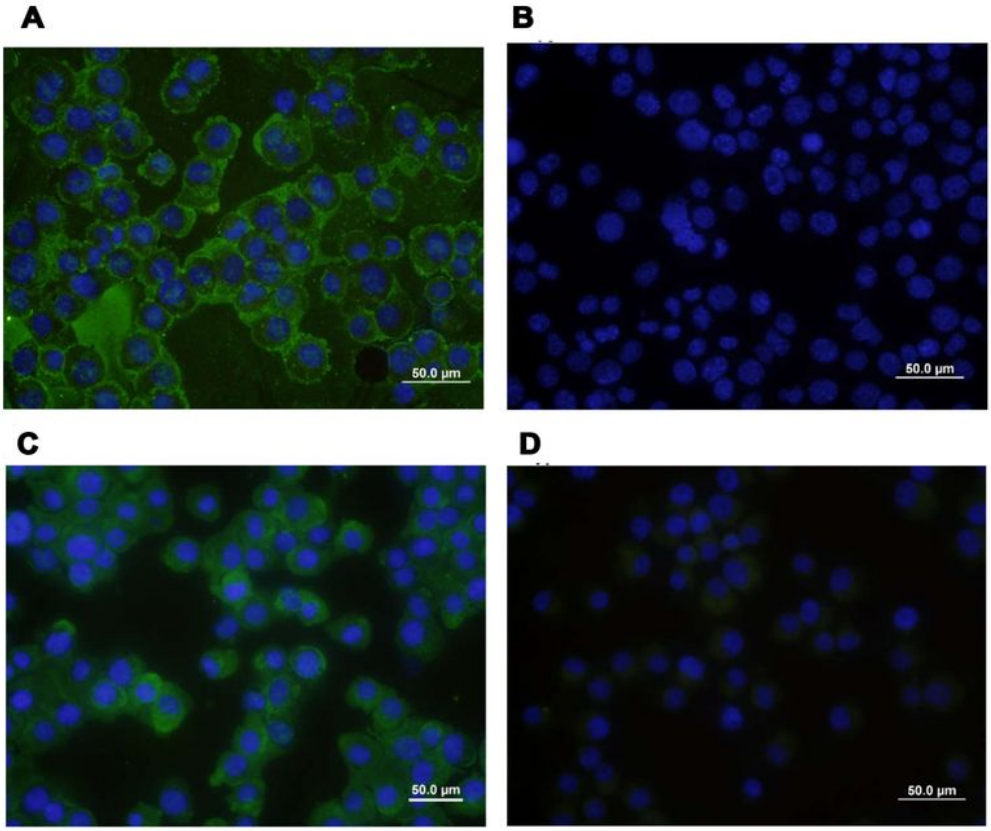

Figure 2

Immunocytochemistry (ICC) assay on breast carcinoma cell lines. Mouse monoclonal anti-sortilin antibody 2D8-E3 was used as a primary antibody and FITC-conjugated sheep anti-mouse antibody as secondary antibody (Green). DAPI was used for counterstaining the nucleus (Blue). A (4T1 cells), C (MDA-MB231 cells), mouse IgG isotype controls (B and D).

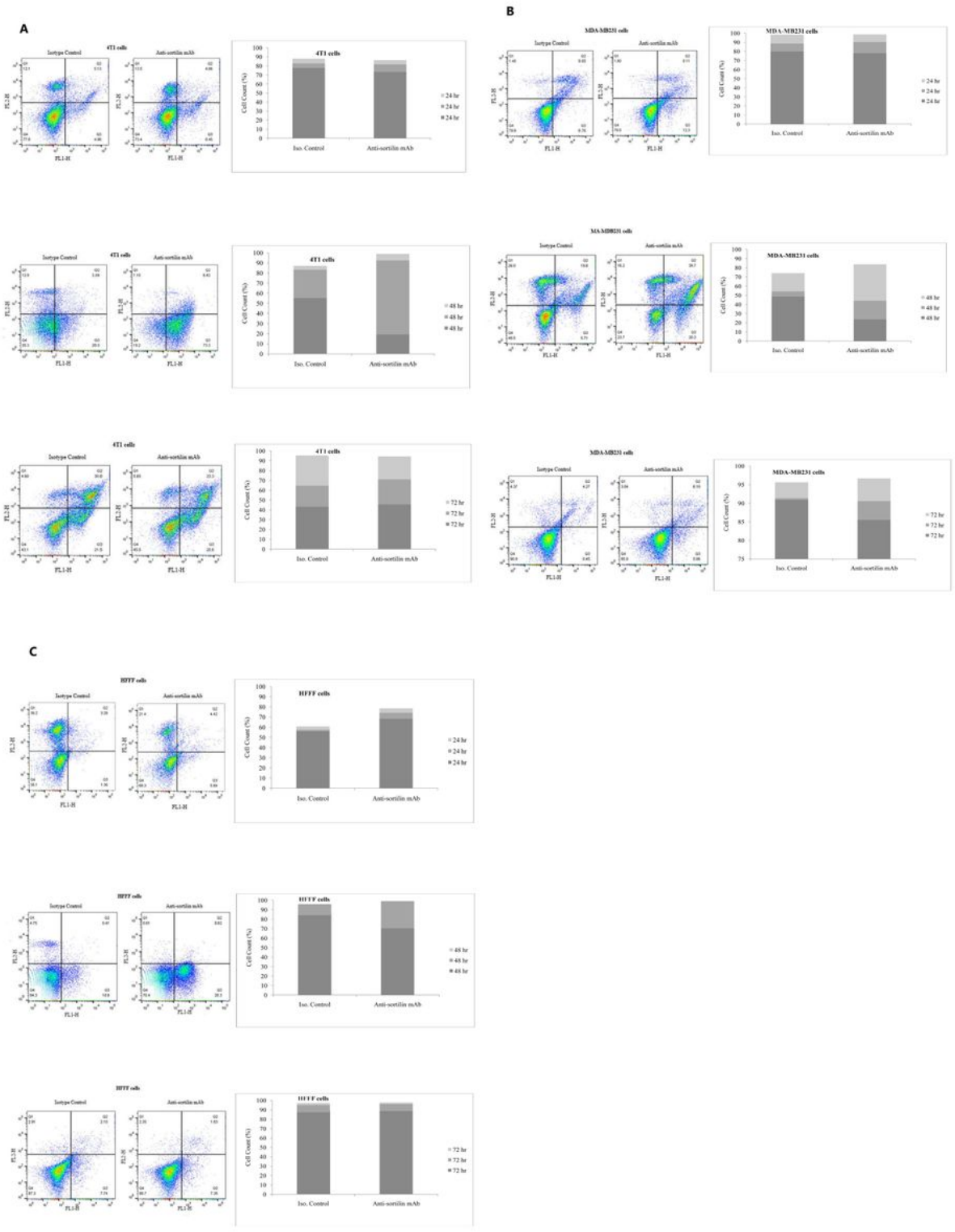

Figure 3

A flow cytometric apoptosis assay was performed by anti-sortilin monoclonal antibody (2D8-E3) on breast cancer cell lines for 24, 48 and 72 hr. A) and B) The antibody could induce apoptosis (Early and late apoptosis) in 4T1 and MDA-MB231 cells after 48 hr. For better visualization of results a bar graph was drawn. The percentage of viable cells were 73.4 (24 hr), 19.2 (48 hr) and 45.5 (72 hr) for 4T1 cells

and 78 (24 hr), 23.7 (48 hr) and 85.5 (72 hr) for MDA-MB231. The viability of cells remains almost unchanged in both lines after 24, 48 and 72 hr of induction in HFFF cells as a normal cell line (C).