Identification of new breast cancer-related biomarkers via integrated bioinformatics analysis and experimental validation

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1 Introduction

1.1 Triple negative breast cancer (TNBC)

Breast cancer (BC) is the most diagnosed cancer and is considered the fifth leading cause of cancer-related deaths worldwide. It is estimated that 2.3 million cases and 685,000 deaths occurred in 2020 [32]. The projections for future trends are even more alarming, with estimates suggesting that BC cases could reach 4.4 million by 2070 [30]. The main risk factors associated with BC include older age, obesity, exposure to tobacco, physical inactivity, high-fat dietary, early age menarche, late age at first full-term pregnancy, shorter breastfeeding periods, use of hormonal menopausal therapy or oral contraceptives, high breast density and BC family history [4].

BC is a highly heterogenous neoplasm with four subtypes classified based on the immunohistochemical expression of hormone receptors: estrogen receptor positive (ER+), progesterone receptor positive (PR+), human epidermal growth factor receptor positive (HER2+), and triple negative [28]. Triple negative breast cancer (TNBC) was first referred in the mid-2000s to identify the subset of BCs lacking ER, PR, and HER2 [10, 3]. TNBC accounts for approximately 20 % of all BCs and it is most common among women under 40 years old of age [18]. It is characterized by its aggressive nature and early relapse. In addition, TBNC is poorly differentiated, highly proliferative, and a heterogenous neoplasm [24].

To date, chemotherapy remains the standard of care for both early and advanced TBNC, usually involved combination regimens of taxanes, anthracyclines, cyclophosphamide, cisplatin and fluorouracil [26, 34]. Despite the high clinical response rates to neoadjuvant chemotherapy, TNBC patients show an elevated risk of recurrence and visceral metastasis, leading to a significantly higher mortality than any other BC subtypes [6]. The poor outcomes regarding TNBC treatments, demand the research for new therapy strategies, more specific and targeted to improve the current bad prognosis, and ultimately enhance patients' survival chance.

1.2 Aptamers as an emerging strategy in tumor-targeted therapies

The development of high-performance recognition tools for cancer is urgently needed to achieve early diagnosis and precise therapy [35]. In line with this, over the past decades, a varied array of molecular recognition tools has been broadly explored, namely antibodies, peptides, and nucleic acids [36, 29, 15]. An emerging strategy in tumor-targeted therapies is based on the use of specific ligands, such as aptamers, which are capable to bind a variety of targets including proteins, small molecules, viruses, bacteria and live cells with high affinity, specificity and selectivity [40, 31, 41].

The term "aptamer" was first reported by Ellington et al. [8], in 1990. Nucleic acid aptamers are single-stranded DNA/RNA oligonucleotides that bind to target cargos by folding into specific secondary/tertiary conformations [39]. Aptamers are derived from random oligonucleotide libraries through an in vitro iterative method so-called Systematic Evolution of Ligands by EXponential Enrichment (SELEX) [33, 8]. Importantly, the development of cell-SELEX technology has allowed the use of intact living cells as targets to select cell type-specific aptamers [40, 27]. A remarkable feature of cell-SELEX is that it allows the identification of specific aptamers for cells of interest without prior knowledge of the target molecules on cell surface or the available targetable biomarkers [25]. The aptamers developed by cell-SELEX have been widely used in cancer research, including for in vivo tumor imaging [37], as vehicles for the targeted delivery of miRNA/siRNA or anticarcinogens [42], and as anti-cancer agents [43].

1.3 Aptamers in TBNC therapies

Aptamers targeting BC cells have been successfully used in research for the development of novel therapeutic strategies, including for TNBC [2, 16, 17, 9]. For example, a study performed by Wan et al. [34] demonstrated that the synthetic aptamer PDGC21T, previously identified and used to recognize poorly differentiated gastric cancer tissue, was capable of binding to the surface of TNBC cells with high affinity. Camorani et al. [5] published another remarkable study in which they identified six aptamers capable of distinguishing TBNC from non-TNBC cells and non-malignant cells. In another work, Luo et al. [19] selected the aptamer 5TR1, which binds to the tumor biomarker MUC1 in MDA-MB-231 cells and conjugated it to doxorubicin to enhance specificity and mitigate known side effects such as cardiotoxicity . Similarly, He et al. [11] developed an aptamer-drug-conjugate with high specificity and cytotoxicity against the MDA-MB-231 cell line, demonstrating in vivo anti-TNBC efficacy with negligible side effects on healthy organs.

Among these promising advances in aptamer research for TNBC therapy, the focus of this project builds upon a previous study initiated by Ferreira et al. [9]. In that study, the authors identified novel aptamers targeting TNBC cells, identified through cell-SELEX with Apt2 outperforming the other identified candidates. Apt2 demonstrated high binding affinity to the surface of MDA-MB-231

cells, a highly metastatic TNBC cell line, highlighting its potential for targeted therapy and diagnostic applications in TNBC [9]. However, fully realize this potential, additional key studies are required. As cell-SELEX was employed, the specific target protein recognized by this aptamer remains unknown. This is precisely where the current project is positioned – leveraging computational approaches to identify and characterize Apt2's interaction with potential protein targets on TNBC cells, thus advancing its development toward clinical applications.

2 Objectives

This work focuses on studying how the experimentally developed aptamer, Apt2, binds to TNBC cells. Specifically, this project aims to analyze potential targets identified in the lab and determine the most suitable candidate for binding to Apt2.

To achieve this, computational methodologies - including structure prediction AI-models, docking, and molecular dynamics – will be leveraged to complement and validate the experimental findings. The approach included AI-based structure prediction methods, protein-aptamer docking, and molecular dynamic simulations combined with free binding energy calculations using the molecular mechanics–generalized born surface area (MM-GBSA) methodology. These analyses aim to identify potential biomarkers targeted by Apt2.

The specific aims for this project are:

- Determine 2D and 3D models of Apt2 using bioinformatics tools and molecular modeling techniques to ensure its stability and functionality. Models will be generated both with and without the primer zones used in its experimental generation;
- Study the 3D structure of target proteins expressed on the surface of TNBC cells to estimate their binding sites. Additionally, some modeling work will be performed such as homology search to better understand the structural features of these target proteins;
- Characterize the molecular interactions between Apt2 and target proteins by employing molecular docking simulations to predict the binding affinities of Apt2-protein complexes.

3 Methodology

To accomplish the proposed specific aims, this work will be divided into the following tasks:

3.1 Generation of Apt2 aptamer secondary structure

The first step in generating the three-dimensional structure for the aptamer involves predicting its secondary structure, following a well-established methodology previously described [14]. Initially, the DNA sequence of the aptamer will be input into the Mfold web server, which is currently part of the UnaFold web server [44]. The secondary structure will be predicted using free energy minimization algorithms. The sequence will be treated as linear, with the folding temperature set at 37 ° C. The default ionic conditions will include 187 mM Na^+ and 0.5 mM Mg^{2+}, at pH 7.4, with adjustments made for oligomers [9]. Only foldings within 5 % of the minimum free energy will be calculated, with a maximum of 50 foldings computed. No constraints will be applied regarding the maximum distance between paired bases. The resulting structure with the lowest free energy will be utilized in subsequent steps. Additionally, the secondary structure will be extracted in dot-bracket notation (Vienna format) for further analysis in task 2.

3.2 Generation of three-dimensional structures for Apt2

The resulting secondary structure will be used as input for the 3dRNA/DNA tool to predict aptamer's three-dimensional structure, as described by [38]. This tool uses a template-based or a distance-geometry method to model three-dimensional conformations from the secondary structure. The model outputs ten structures, each scored through an internal scoring. The top three best scoring structures will be considered and downloaded in PDB format for the further analysis in subsequent steps.

3.3 Aptamer refinement by molecular dynamics

To prepare Apt2 for molecular docking simulations, its conformational flexibility will be evaluated using the LEAP module of the AMBER20 software package [7]. Aptamer flexibility will be studied under the effect of an explicit solvent by molecular dynamics simulations. In this case, the aptamer will be solvated using a TIP3P rectangular box of water molecules [22], ensuring that each atom is at least 12 Angstrom from the edge. To achieve physiological concentration of NaCl within the simulation, the SPLIT method will be used [20], allowing precise calculation of the total number of Na+ and Cl- ions. The system will be described using the BSC1 force field, developed for atomistic DNA simulations [13].

After solvation, a sequence of minimization steps of the system will be undertaken to remove atomic clashing during molecular dynamics: (1) water molecules; (2) hydrogen atoms; (3) side chains of the DNA structure; and (4) the complete

system. Following a step of system equilibration, the final stage will be the production run using an NPT ensemble. To constrain all covalent bonds involving hydrogen atoms, the SHAKE algorithm will be employed [23].

3.4 Target proteins data integration

A previous proteomic study identified six potential target proteins of interest to which Apt2 binds: P09493, P13984, Q14141, Q9BTC0, Q9NSK0, and Q8N8S7 (primary protein accession IDs). Before generating the three-dimensional structure of these targets in the next task, preliminary modeling work must be conducted. This step aims to perform a data retrieval process from databases such as UniProt, NCBI Protein, and PDB, integrating information on important features and key aspects that may influence the modeling process itself.

3.5 Three-dimensional protein structure prediction

To predict the three-dimensional structure for each protein, AlphaFold3, a state-of-the-art deep learning-based method for protein structure prediction developed by DeepMind [1], will be used. The amino acid sequences of the selected proteins will serve as input for the AlphaFold prediction server. By leveraging AlphaFold's advanced neural network architecture and extensive training data, highly accurate 3D structural models of the proteins will be generated.

3.6 Prediction of Apt2 binding site by docking

To identify representative aptamer structures from the molecular dynamics' simulations suitable for molecular docking with the target proteins, cluster analysis will be conducted. The K-means clustering method will be used, and clusters will be generated based on the root mean square deviation (RMSD) of all non-hydrogen atoms. The representative structure of each cluster will be selected for further analysis. Next, the complexes between proteins and Apt2 will be predicted using the HADDOCK 2.4 web server [12]. Ambiguous interaction restraints (AIRs) will be randomly determined from residues. The ranking of the different complexes for each protein will be determined based on the HADDOCK score (HS), which is calculated as a linear weighted sum of energetic terms and buried surface area:

$$HS = 1.0Evdw + 0.2Eelec + 1.0Edesol + 0.1Eair \quad (1)$$

Each term in Equation 1 represents van der Waals (Evdw), Coulomb electrostatics (Eelec), desolvation (Edesol), and restraints energy (Eair), respectively.

3.7 Molecular dynamics simulations to evaluate the docked Apt2-protein complexes

The most likely Apt2-protein complexes, as suggested by docking studies, will be evaluated by molecular dynamics following a similar protocol to that described in task 3. All parameters will remain consistent, except for the use of the ff14SB force field - specifically designed for protein simulations [21]- in addition to the force field applied to the aptamer. Free energy calculations with the MM-GBSA method will then be employed to estimate the binding free energy of the aptamer to each target and characterize the most likely binding regions of the protein target and aptamer.

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