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Recombinant Expression and Purification of Adenocarcinoma GPR161 Receptor

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Abstract. Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer and nowadays very few therapeutic options are available for its treatment. Interestingly, G-protein coupled receptor 161 (GPR161), is expressed in TNBC cells, and can activate the mammalian target of the rapamycin complex 1 signaling pathway. GPR161 and Ras GTPase-activating-like protein, a protein involved in intracellular signaling, proliferation, and cellular adhesion, have been shown to genetically interact in human breast cancer cells. Targeting of GPR161 by monoclonal antibodies may therefore be a strategy to develop diagnostics and therapeutics for TNBC. Thus, to obtain such monoclonal antibodies, we synthesized the GPR161 gene de novo, cloned it into the pET32 expression plasmid and used the recombinant plasmid to transform the competent BL21 (DE3) strain of Escherichia coli. The recombinant GPR161 gene was designed to contain an N-terminal thioredoxin tag, a thrombin site, the GPR161 sequence, and a C-terminal hexa-histidine tag to facilitate purification by metal-affinity chromatography. Following purification of the recombinant GPR161 (rGPR161) protein using a HisTrap column, we characterized the protein by western blotting and mass spectrometry. The rGPR161 protein had a molecular mass of ~49 kDa and its identity as rGPR161 was confirmed by mass spectrometry data using the SwissProt database and the Mascot program. Future studies will involve the development of monoclonal antibodies using rGPR161 as the immunogen.
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1. Introduction

Triple-negative breast cancer (TNBC) accounts for approximately 15% of breast cancer diagnoses in women and currently lacks an effective targeted therapy. TNBC cells are characterized by a lack of estrogen (ER), progesterone (PR), and (human epidermal growth factor 2) HER2 receptor, and are associated with a poor outcome. The lack of ER, PR, and HER2 receptors on TNBC tumor cells restricts the use of hormone-based drugs for treatment. However, in breast cancer, there may be different types of cancer that meet the definition of TNBC, for example, basal-like breast cancers. This type of breast cancer is characterized by the absence or low levels of expression of the ER and low levels of expression of HER2. Both types of cancers (TNBC and basal-like breast cancers) are characterized by the presence of a mutation in the BRCA1 gene and a high histologic grade (Foulkes *et al.*, 2010; Lachapelle and Foulkes, 2011; Rakha and Reis-Filho, 2009).

Breast cancer is generally highly heterogeneous and the different types are identified by their histopathological features, genetic alterations, and gene-expression profiles (Sotiriou and Pusztail, 2009). Kreike *et al.*, 2007 characterized TNBC at the gene expression and histopathological levels. Based on their gene expression profiling study, they found that TNBC is itself heterogeneous and can be subdivided into at least five distinct subgroups. Because of the high levels of genetic heterogeneity, TNBC lacks both effective targeted therapies and diagnostics.

An important goal in studying triple-negative tumors is to identify novel therapeutic and diagnostic targets within this group of tumors. This is especially important as these tumors do not respond to ER- and HER2-targeted therapies since 73% of TNBC tumors are EGFR-negative. Haffty *et al.,* 2006 used simple commonly available markers for ER, PR, and HER2/neu and found that patients with triple-negative breast cancers have a relatively poor prognosis. Since they lack the receptors for ER, PR, and HER2/neu, patients with triple-negative tumors are not candidates for adjuvant hormonal therapy or trastuzumab.

G-protein coupled receptors (GPCRs) mediate numerous physiological processes and represent the targets for a vast array of therapeutics for different diseases. GPCRs transduce extracellular signals from a variety of ligands through the activation of heterotrimeric G-proteins and downstream second messengers. Through this mechanism, rapid signaling events occur via the generation of short-lived second messengers. GPCRs can also act as regulators of oncogenesis. One such mechanism involves the transactivation of other signaling molecules, including the epidermal growth factor receptor (EGFR). Because they can be activated by a wide variety of ligands, GPCRs play critical roles in the pathogenesis of hormone-responsive tumors. For example, somatic mutations found in the thyrotropin receptor result in the activation of adenylyl cyclase, leading to hyperactivation of thyroid adenomas. Similarly, activating mutations have been found in the luteinizing hormone receptor in Leydig-cell testicular tumors. Several recent studies have also demonstrated the role of GPCRs in controlling cancer cell invasion and metastasis (Feigin, 2013).

Using large-scale genomic analysis, Feigin *et al*., 2014 discovered a poorly characterized receptor referred to as GPR161 that was up-regulated in TNBC. The GPR161 receptor was characterized as a prognostic biomarker for TNBC, and was found to regulate the proliferation and migration of breast cancer cells. The authors also demonstrated the role for GPR161 in the pathogenesis of TNBC by providing evidence that GPR161 promotes proliferation by activating mTORC1. The GPR161 receptor also regulates migration and invasion by disrupting the localization of E-cadherin (E-cad). The authors also demonstrated that GPR161 induces proliferation and migration in an IQGAP1-dependent manner. On the basis of these data, GPR161 appears to promote cancer cell proliferation and migration and is a promising drug target for the treatment of TNBC.

2. Materials and methods

Bacterial strain, plasmids, and antibodies

E. coli DH5α, BL21(DE3) (Novagen, Madison, WI, USA), and plasmids pET28 and pET32 (Novagen) were used in this study. All *E. coli* strains were cultured in lysogeny broth (LB) medium. An anti His-tag mouse monoclonal antibody (Sigma-Aldrich, Taufkirchen, Germany) and a peroxidase conjugated secondary antibody (Sigma-Aldrich, Taufkirchen, Germany) were used for western blotting.

Gene synthesis

The amino acid sequence of the GPR161 receptor isoform 1 (Homo sapiens) was extracted from PubMed with the NCBI sequence reference NP_001254538.1. Part of the sequence of the 7tm_GPCR (seven-transmembrane G protein-coupled receptor) encompassing amino acids 64-344 was selected for further study. The gene sequence was codon-optimized for expression in *E. coli* using soft Vector NTI 11.5 and the GPR161 receptor cDNA was synthesized by Macrogen Inc., Korea. The synthetic GPR161 cDNA was cloned into pET28 and pET32 plasmids using the NcoI and XhoI restriction sites so that the final pET32 plasmid construct contained an N-terminal thioredoxin tag, a thrombin site, the GPR161 receptor, and six C-terminal His-Tags. The final pET28 based construct contained the same sequences except for the N-terminal thioredoxin tag. The predicted molecular weights of the His-tagged recombinant GPR161 in pET32 and pET28 were ~49 kDa and ~36 kDa, respectively.

Transformation of *E. coli* and expression of rGPR161

Competent BL21 (DE3) *E. coli* were transformed with the pET28 and pET32 plasmid vectors, with or without the gene insert, by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA) under the following conditions: 100 ng of plasmid per 50 µL of cell suspension, at 2.5 kV, 25 µF, and 200 Ω. Electroporation duration was 5.2 ms. The transformed cells were incubated in 950 µL of superoptimal broth (SOC) at 37°C for 1 h with rotary shaking at 200 rpm. Following this, 50 µL of cells were seeded onto LB agar containing ampicillin as the selection antibiotic and grown at 37°C for 16 h. Single colonies of transformants were cultured in 2 × YT broth containing ampicillin. In the middle of the logarithmic growth phase of the bacterial mass (OD_{600} = 0.6), 0.1 mmol/L of the inducer, isopropyl-β-D-1-galactopyranoside (IPTG), was added and the culture incubated for 6 h at 26°C. Cells were then pelleted by centrifugation at 6,000 × *g* for 7 min at 4°C. For sequencing, the GPR161 gene product was transformed into the DH5α *E. coli* strain. *E. coli* colonies were grown on solid agar medium and analyzed by PCR using Taq polymerase and M13 primers. Four positive clones were used for DNA purification and sequencing using the BigDye Terminator reagent kit (Thermo Fisher Scientific, Austin, TX, USA)

Cell lysis and chromatographic purification

For purification of the recombinant receptor we used a protocol based on that reported by Attrill *et al*., 2009. Cells were lysed in an ice-cold buffer (20 mmol/L NaCl, 20 mmol/L HEPES, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5% CHAPS, pH 7.5) using a UP200S ultrasonic disintegrator set at 24 kHz in a pulsating mode (10 pulses, 10 s per pulse). Cell debris was pelleted at 60,000 × *g* for 1 h. The detergent solubilized fraction

was filtered through a 0.2 µm syringe filter (Millipore). Recombinant protein was purified using metal-chelate chromatography on Ni^{2+} ions using a 1-mL HisTrapTM HP column (GE Healthcare, Piscataway, NJ, USA).

The protein solution was loaded onto the Ni^{2+}-NTA column (2 mL bed volume) equilibrated with the same buffer. The column was washed with 10 bed volumes of the equilibration buffer (20 mmol/L Tris-HCl, pH 8.0, containing 500 mmol/L NaCl, 50 mmol/L imidazole, 0.5% CHAPS, and 0.1 PMSF). The GPR161 protein was then eluted with a 20–500 mmol/L linear imidazole gradient. The protein concentrations in the cell lysate and purified fractions were determined using the Bradford assay with bovine serum albumin as the standard (Bradford, 1976).

Western blotting

Fractions containing the GPR161 receptor were separated by electrophoresis on 11% polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to the Laemmli method using a Bio-Rad electrophoresis apparatus (Bio-Rad) (Laemmli, 1970). Proteins were electrophoretically transferred onto nitrocellulose membranes using an immunoblotting device (Bio-Rad) according to a previously published method (Towbin *et al.*, 1979).

For immunochemical detection, the nitrocellulose membranes were first incubated in a blocking solution (phosphate buffered saline (PBS), pH 7.4, containing 1% BSA) overnight at 4°C and then washed three times in PBS, pH 7.4, containing 0.05% Tween-20 (PBST). Membranes were incubated for 1.5 h at 37°C in a blocking buffer containing 1:2000 dilution of a mouse monoclonal antibody against the His-tag. Subsequently, the membranes were rewashed as above and incubated in a blocking buffer with peroxidase-conjugated secondary antibody at 1:10000 dilution for 1 h at 37°C. The substrate solution was prepared immediately before use as follows: 0.01 g of 4-chloro-naphthol (Sigma, St. Louis, MO, USA) dissolved in 2 mL of methanol, and mixed with 18 mL of buffer 1; finally, 0.01 mL of 3% (v/v) hydrogen peroxide was added. This substrate solution was applied to the blots for visualization of immunoreactive protein bands. The blot was placed in the substrate solution and incubated for 15 min at room temperature until the stained bands appeared.

NanoLC and tandem mass spectrometry (nanoLC-MS/MS)

Workbench surfaces were routinely cleaned of dust using a clean, damp paper towel to avoid sample contamination with keratin. To ensure sample purity, we also used keratin-free Eppendorf tubes and barrier tips during sample preparation. Purified GPR161 samples were fractionated by 11% SDS-PAGE and the gels were stained with Coomassie blue. The protein bands were precisely excised and transferred to keratin-free Eppendorf tubes. The excised bands were then divided into small gel fragments of 1 × 1 mm dimension. To destain the gel pieces of Coomassie blue, 100 µL of 100 mM ammonium bicarbonate in acetonitrile (1:1) was added and the gel fragments were incubated at 37°C for 30–40 min. After removing the supernatant, 5 mM DTT was added to each tube and incubated at 60°C for 10 min. The DDT solution was then removed and 100 µL of 100 mM iodoacetamide was then added and incubated at 37°C for 15 min to alkylate the reduced cysteine residues in the proteins. Excess reagent was then removed, and the gel pieces were washed twice in 100 µL of 50 mM ammonium bicarbonate. To remove any residual iodoacetamide, the gel pieces were subjected to two cycles of dehydration in 200 µL of 100% acetonitrile and rehydrated in 50 mM ammonium bicarbonate in water. The gel pieces were then dehydrated in 100% acetonitrile for 3–5 min to reduce their size, after which the acetonitrile was removed and the tubes dried for 5 min. Finally, 2 µL of 100 ng/µL trypsin and 50 µL of 50 mM ammonium bicarbonate were added, and the tubes were incubated overnight at 37°C to allow trypsin digestion to occur. On the following day, the supernatant containing the peptide mixtures resulting from trypsin digestion were transferred to clean Eppendorf tubes. For the second extraction of the digested peptides, the remaining gel pieces were washed in 50 µL of 50 mM ammonium bicarbonate, incubated for 15–20 min, and the supernatant transferred into the same tube containing the peptide mixture from the previous extraction. The contents of the tubes were dried using a vacuum concentrator at 45°C for 30–60 min. After complete removal of water, the resultant residue (containing the tryptic peptides) was dissolved in 10 µL of 0.1% trifluoroacetic acid, and the soluble peptide mixture was desalted using a Zip-tip kit (Millipore Ziptips Micro-C18, 0.2 µL, Sigma).

The resulting mixture of tryptic peptides was separated using high-performance liquid chromatography (HPLC) and analyzed by in-line tandem mass spectrometry. For LC-MS/MS, an Acclaim™ PepMap™ 100 C18 pre-column (5 mm × 300 cm; 5 µm particle size; Thermo Fisher Scientific) was used with a Dionex HPLC pump (Ultimate 3000 RSLCnano System, Thermo Fisher Scientific). The peptide mixture was separated on an Acclaim™ Pep-Map™ RSLC column (15 cm × 75 µm, 2 µm particle size; Thermo Fisher Scientific) using a 75-min multistage acetonitrile gradient (buffer A, 0.1% formic acid; buffer B, 90% acetonitrile/10% H_2O in 0.1% formic acid) at a flow rate of 0.3 µL/min. The gradient program for buffer B was: 0 min—2%, 10 min—2%, 58 min—50%, 59 min—99%, 69 min—

99%, 70 min—2.0%, 75 min—2.0%. The unmodified CaptiveSpray ion source (Capillary 1300 V, dry gas 3.0 L/min, dry temperature 150 °C) was used to interface the chromatography system with the Impact II (Bruker). Subjecting the mixture of digested peptides to chromatography ensured the removal of low-molecular-weight impurities. The tandem MS/MS conditions were as follows: two of the most intense precursor ions to obtain sample data were selected for subsequent fragmentation with a full-time cycle of 3 s. The mass range was from 150 to 2,200 m/z under the positive ion mode.

The Mascot software was used to search the SwissProt 2016_10 database (552,884 sequences; 197,760,918 residues). Search parameters included variable modifications, including cysteine carbamidomethylation and methionine oxidation, a fragment ion mass tolerance of 0.6 Da, and a mass tolerance of the parent ion of 1.20 Da.

3. Results

Design and construction of the expression vector

Comparison of the amino acid sequence of the GPR161 receptor isoform 1 with all GPR161 sequences present in the NCBI database revealed that the GPR161 isoform 1 receptor is homologous to other members of this family of receptors (Fig.1).

Figure 1. Homology of the GPR161 receptor isoform 1 with other members of the GPR161 family

The codon-optimized nucleotide sequence of the selected region (length 897 bp including the restriction sites) (Fig. 2) was inserted into the pET28 and pET32 plasmids. Then, DH5α bacteria transformed with the vector containing the correct insert were identified and further confirmed by DNA sequencing to contain the insert encoding the fragment of recombinant transmembrane receptor GPR161 (rTMGPR161).

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      BamHI EcoRI  NcoI
      ~~~~~~~~~~~~~~~~~~
  1   GGATCCGAAT TCCCATGGAG GGCAATCTGG TGATTGTGGT GACCCTGTAT AAAAAAAGCT ATCTGCTGAC CCTGAGCAAT AAATTTGTGT TTAGCCTGAC
      CCTAGGCTTA AGGGTACCTC CCGTTAGACC ACTAACACCA CTGGGACATA TTTTTTTCGA TAGACGACTG GGACTCGTTA TTTAAACACA AATCGGACTG
 101  CCTGAGCAAT TTTCTGCTGA GCGTGCTGGT GCTGCCGTTT GTGGTGACCA GCAGCATTCG TCGTGAATGG ATTTTTGGCG TGGTGTGGTG CAATTTTAGC
      GGACTCGTTA AAAGACGACT CGCACGACCA CGACGGCAAA CACCACTGGT CGTCGTAAGC AGCACTTACC TAAAAACCGC ACCACACCAC GTTAAAATCG
 201  GCGCTGCTGT ATCTGCTGAT TAGCAGCGCG AGCATGCTGA CCCTGGGCGT GATTGCGATT GATCGTTATT ATGCGGTGCT GTATCCGATG GTGTATCCGA
      CGCGACGACA TAGACGACTA ATCGTCGCGC TCGTACGACT GGGACCCGCA CTAACGCTAA CTAGCAATAA TACGCCACGA CATAGGCTAC CACATAGGCT
 301  TGAAAATTAC CGGCAATCGT GCGGTGATGG CGCTGGTGTA TATTTGGCTG CATAGCCTGA TTGGCTGCCT GCCGCCGCTG TTTGGCTGGA GCAGCGTGGA
      ACTTTTAATG GCCGTTAGCA CGCCACTACC GCGACCACAT ATAAACCGAC GTATCGGACT AACCGACGGA CGGCGGCGAC AAACCGACCT CGTCGCACCT
 401  ATTTGATGAA TTTAAATGGA TGTGCGTGGC GGCGTGGCAT CGTGAACCGG GCTATACCGC GTTTTGGCAG ATTTGGTGCG CGCTGTTTCC GTTTCTGGTG
      TAAACTACTT AAATTTACCT ACACGCACCG CCGCACCGTA GCACTTGGCC CGATATGGCG CAAAACCGTC TAAACCACGC GCGACAAAGG CAAAGACCAC
 501  ATGCTGGTGT GCTATGGCTT TATTTTTCGT GTGGCGCGTG TGAAAGCGCG TAAAGTGCAT TGCGGCACCG TGGTGATTGT GGAAGAAGAT GCGCAGCGTA
      TACGACCACA CGATACCGAA ATAAAAAGCA CACCGCGCAC ACTTTCGCGC ATTTCACGTA ACGCCGTGGC ACCACTAACA CCTTCTTCTA CGCGTCGCAT
 601  CCGGCCGTAA AAATAGCAGC ACCAGCACCA GCAGCAGCGG CAGCCGTCGT AATGCGTTTC AGGGCGTGGT GTATAGCGCG AATCAGTGCA AAGCGCTGAT
      GGCCGGCATT TTTATCGTCG TGGTCGTGGT CGTCGTCGCC GTCGGCAGCA TTACGCAAAG TCCCGCACCA CATATCGCGC TTAGTCACGT TTCGCGACTA
 701  TACCATTCTG GTGGTGCTGG GCGCGTTTAT GGTGACCTGG GGCCCGTATA TGGTGGTGAT TGCGAGCGAA GCGCTGTGGG GCAAAAGCAG CGTGAGCCCG
      ATGGTAAGAC CACCACGACC CGCGCAAATA CCACTGGACC CCGGGCATAT ACCACCACTA ACGCTCGCTT CGCGACACCC CGTTTTCGTC GCACTCGGGC
                                                                                          XhoI HindIII
                                                                                          ~~~~~~~~~~~~
 801  AGCCTGGAAA CCTGGGCGAC CTGGCTGAGC TTTGCGAGCG CGGTGTGCCA TCCGCTGATT TATCATCATC ATCATCATCA TTAACTCGAG AAGCTTT
      TCGGACCTTT GGACCCGCTG GACCGACTCG AAACGCTCGC GCCACACGGT AGGCGACTAA ATAGTAGTAG TAGTAGTAGT AATTGAGCTC TTCGAAA
```

Figure 2. Nucleotide sequence of the codon optimized GPR161 fragment encoding amino acids 64-344 (rTMGPR161)

Transformation with the gene construct and establishment of an E. coli strain producing rTMGPR161

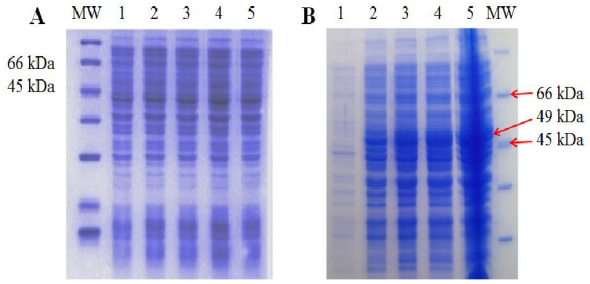

Figure 3. SDS-PAGE electrophoresis of total proteins expressed in the strains BL21/pET28/rTMGPR161 (A) and BL21/pET32/rTMGPR161 (B). Lane 1 — *E. coli* culture without IPTG; Lane 2 — *E. coli* culture 2 h after IPTG addition; Lane 3 — *E. coli* culture 4 h after IPTG addition; Lane 4 — *E. coli* culture after 6 h incubation with IPTG; Lane 5 — *E. coli* culture after 24 h incubation with IPTG; MW — molecular-weight markers

The expression vectors pET28/rTMGPR161 and pET32/rTMGPR161 were transformed into *E. coli* BL21. The strains obtained were then analyzed for the expression of the recombinant GPR161 protein. To detect protein expression, *E. coli* strains were cultured in LB medium supplemented with 0.2 mM IPTG at 26°C. After the addition of IPTG, samples of the *E. coli* culture were taken at different time points (at 2, 4, 6, and 24 hours), sonicated, lysed, and subjected to SDS-PAGE followed by Coomassie Blue staining. There was no recombinant protein expression in BL21 *E. coli* transformed with pET28/rTMGPR161 suggesting that this recombinant GPR161 protein was toxic to *E. coli* (Fig. 3(A)). In contrast, there was expression of rTMGPR161 in BL21 *E. coli* transformed with pET32/rTMGPR161 (Fig. 3(B)). The rTMGPR161 protein was expressed 2 h after IPTG addition and had an approximate molecular mass of ~49 kDa.

Isolation and purification of rTMGPR161

A representative SDS-PAGE analysis of soluble and insoluble fractions from BL21/pET32/rTMGPR161 is shown in Fig. 4. Following cell lysis and centrifugation to remove insoluble debris, the rTMGPR161 fusion protein remained present in the soluble fraction (Fig. 4(B)) of the cell lysate, while it was not detectable in the pellet (Fig. 4(A)).

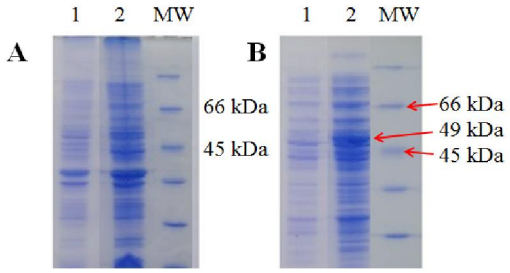

Figure 4. SDS-PAGE electrophoresis of pellet (A) and supernatant (B) from lysates of *E. coli* BL21/pET32/rTMGPR161. Lane1—*E. coli* culture without IPTG; Lane 2— *E. coli* culture after 24 h incubation with IPTG; MW — molecular-weight markers

To optimize the isolation and purification of rTMGPR161, the BL21/pET32/rTMGPR161 *E. coli* were cultured in 2 × YT medium containing ampicillin and various IPTG concentrations (0.05 mM, 0.1 mM, 0.2 mM, and 0.4 mM), at different time points (at 2, 4, 6, and 24 hours) and at a temperature of 26°C. We found that the conditions for the optimal expression of rTMGPR161 were media containing 0.2 mM IPTG and 24 h incubation (Table 1).

Table 1. Optimization of the expression of rTMGPR161

Strain	Media	IPTG (mM)	OD_{600}	Protein (μg/L)
E.coli BL21	2 × YT	0.05	3.1	30
E.coli BL21	2 × YT	0.1	2.9	125
E.coli BL21	2 × YT	0.2	2.1	250
E.coli BL21	2 × YT	0.4	1.8	60

After purification of the rTMGPR161 protein by Ni^{2+}–Sepharose chromatography and elution using a buffer containing 200 mM imidazole, we used SDS-PAGE and Coomassie Blue staining to visualize the purified rTMGPR161 protein. The results of the purification are shown in Fig. 5.

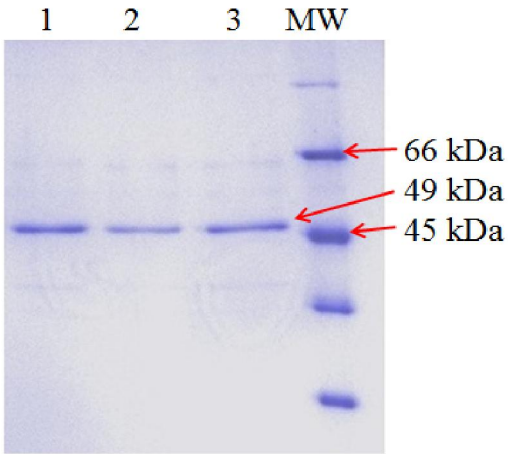

Figure 5. SDS-PAGE analysis of purified rTMGPR161. Lanes 1–3 – purified fractions containing rTMGPR161; MW – molecular-weight markers

As can be seen in Figure 5, chromatography yielded fractions containing purified rTMGPR161 with an expected molecular weight of about 49 kDa.

To further confirm expression of the correct protein, we used an anti-His-tag monoclonal antibody in western blotting of the rTMGPR161 samples (Fig. 6(A)) separated by SDS-PAGE electrophoresis (Fig. 6(B)). This western blotting analysis confirmed the presence of the hexa-histidine tag on a protein with a molecular mass of ~49 kDa, which corresponds to the predicted molecular mass of rTMGPR161.

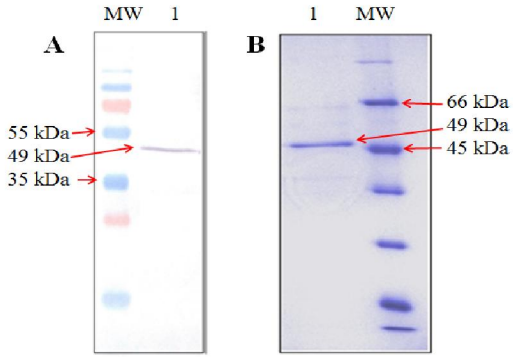

Figure 6. Western blot (A) and SDS-PAGE electrophoresis (B) of purified proteins expressed and extracted from the BL21/pET32/rTMGPR161 *E. coli*. Lane 1 – purified rTMGPR161; MW – molecular-weight markers

LC-MS/MS analysis of rTMGPR161

LC–MS/MS was used to confirm the identity of rTMGPR161. The MS/MS spectra of peaks corresponding to the fragmented ions of peptides derived from trypsin-digested rTMGPR161 were identified following SDS-PAGE, trypsin digestion, and chromatographic separation. Trypsin-digested peptides are characterized by the presence of lysine or arginine residues at the C-terminus of the peptide. The MS/MS spectra were converted to mgf files using the DataAnalysis program. These files were submitted to the Mascot search engine, which compares the experimental data with theoretical mass spectra using available sequence databases of amino acids, such as NCBI or SwissProt.

The score (86.8) corresponded to only one protein, namely GPR161. Representative MS/MS spectra of EGNLVIVVTLYKKSYLLTLSNKF peptide of rGPR161 and their fragmentation ions are shown in Fig. 7.

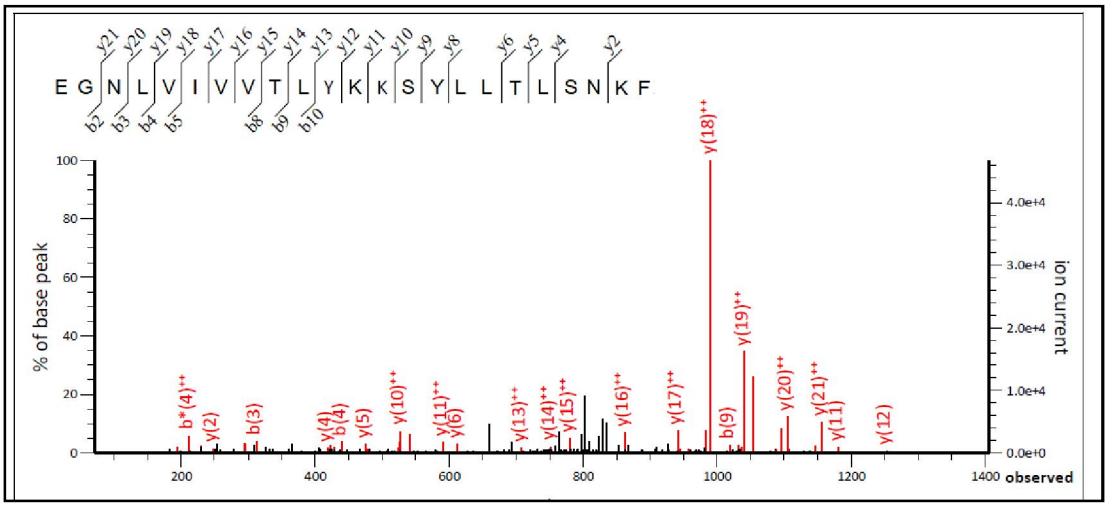

Figure 7. MS/MS spectra of fragmented peptides derived from trypsin-digested rTMGPR161

4. Discussion

The GPR161 receptor is a prognostic biomarker of triple negative breast cancer and an important regulator of the proliferation and migration of breast cancer cells. The GPR161 receptor decreases IQGAP1 serine phosphorylation and activates the mTOR/p70S6K signal pathway. Genetic analyses of the GPR161 receptor in triple negative breast cancer cells have identified two mutations. The first mutation (R91G) is found within the first extracellular loop and may therefore play a role in ligand binding. The second mutation, S251G, is found within the third intracellular loop, a region known to be phosphorylated in response to the activation of many GPCRs and the site of interaction of β-arrestins (Feigin *et al.*, 2014). Genetic analyses of other cancer types have uncovered the recurrent amplification of GPR161 genes in bladder urothelial carcinoma, lung adenocarcinoma, and melanoma. It is therefore likely that developing methods to target GPR161 may have diagnostic and therapeutic applications beyond breast cancer.

Electron microscopy research of this family of receptors has shown that these receptors consist of seven transmembrane α-helices. Furthermore, each transmembrane helix has several characteristic residues and a common 3D structure that is conserved throughout all GPCRs (Lomize *et al.*, 1999). The transmembrane protein structure contains seven stretches of 20–30 hydrophobic amino acids which form the membrane-spanning α-helices. The protein has an extracellular N-terminus and a cytoplasmic C-terminus. The N-termini of GPCRs vary greatly in length ranging from seven amino acids for the adenosine receptor to over 300 amino acids residues for the glycoprotein hormone receptors. The C-terminus has one phosphorylation site, which can influence signal transduction across the membrane. Furthermore, the C-terminal cysteine may be palmitoylated, thereby forming an additional cytoplasmic loop, which may influence receptor mobility or G-protein coupling (Probst *et al.*, 1992).

The ligand binding sites of GPCRs have been partly delineated through both biochemical and molecular biological approaches. Helical amino acids have been classified as (A) amino acids that are in contact with membrane lipids and (B) amino acids that are not in contact with membrane lipids (Baldwin, 1993). Amino acids residues that are conserved within

GPCRs contribute to the specificity of ligand binding. Example, two conserved serines in TM 5 of the β2-adrenergic receptor (Ser204 and Ser207) have been implicated in forming hydrogen bonds with the meta- and para-hydroxyl groups of adrenergic agonists. In many hormone GPCRs, the N-terminus is glycosylated and is rich in cysteine residues that may form disulfide bridges and help maintain the three-dimensional structure of the protein. However, in the absence of the extracellular N-terminus, ligands can still bind to the seven transmembrane components of the receptor, albeit with a lower affinity (Ji I.H. and Ji T.H., 1991). This suggests that GPCRs receptors may contain both a high-affinity extracellular binding site and a low-affinity site within the transmembrane domains. It is possible that the high-affinity extracellular binding site serves to capture the ligand and present it to the intramembranous binding pocket for signal transduction.

On the basis of this background, in the present study, we prepared the recombinant GPR161 receptor aiming to obtain monoclonal antibodies which can be used for developing diagnostics and therapeutics for TNBC. We generated a GPR161 fragment encompassing amino acids 64-344 (length 897 bp including restriction sites) and cloned it into two different expression vectors and transformed them into *E. coli* strain BL21 (DE3). We assessed and confirmed the expression of rTMGPR161 using one of the vectors, pET32/rTMGPR161, and optimized the conditions for the isolation and purification of rTMGPR161.

We solubilized rTMGPR161 in a solubilization buffer and purified it by metal-affinity chromatography on a Ni^{2+}-Sepharose column. As a result, a high initial concentration of the protein, which was effectively refolded while simultaneously being separated from high-molecular-weight protein aggregates, was obtained. Purification was achieved by spatially separating the rTMGPR161 molecules from each other through the pores of the column and a gradual increase in the concentration of the imidazole. The LC-MS-MS analysis showed that rTMGPR161 was a fusion protein and contained thioredoxin and the TMGPR161 protein fragment.

The most widely used strategy to express GPCRs in *E. coli* is to use a fusion protein containing the receptor and a bacterial protein. The first example of this was the β2-adrenergic receptor, which had the first 279 residues of β-galactosidase, a cytosolic protein naturally expressed in *E. coli*, fused to its N-terminus (Marullo *et al.*, 1988). Fusion of the receptor to membrane proteins found in *E. coli* such as MBP or LamB increases the expression levels of the receptor by 10-fold. Nevertheless, identical expression levels of the receptor can be obtained by using a strong IPTG-inducible promoter (gene 10 of the T7 bacteriophage) (Breyer *et al.*, 1990; Nahmias *et al.*, 1991). This difference clearly suggests that expression levels are receptor dependent and that for each GPCR, a variety of genetic constructs must be systematically employed because the results depend on the exact primary sequence of the construct (Chapot *et al.*, 1990).

In summary, we successfully expressed a recombinant form of GPR161, which we believe will be useful for the generation of either diagnostic or therapeutic monoclonal antibodies and can also be used as a protein therapeutic.

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