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 very few therapeutic options are currently 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.

Keywords: Breast cancer, GPR161 receptor, monoclonal antibodies, recombinant protein, tumor diagnostics

INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for approximately 15% cancer of the breast diagnosis in women and does not currently have effective targeted therapy. TNBC cells don't express the estrogen (ER), progesterone (PR) and human epidermal growth factor 2 receptor (HER2), and are associated with a poor prognosis. 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 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 histological grade (Foulkes *et al.*, 2010; Lachapelle and Foulkes, 2011; Rakha and Reis-Filho, 2009).

Breast cancer is generally highly

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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 epidermal growth factor receptor (EGFR)-negative. Haffty *et al.* (2006) used simple commonly available markers for ER, PR, and HER2/neu and found that patients with TNBC have a relatively poor prognosis. Since they lack ER, PR, and HER2/neu receptors, patients with triple-negative breast cancer are not candidates for adjuvant hormonal therapy or trastuzumab.

G protein-coupled receptors (GPCRs) mediate different physiological processes and introduce the targets for a broad array of drugs 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 formation of short-lived secondary signals. GPCRs can also act as regulators of oncogenesis. Transactivation of signaling molecules, including EGFR, occurs by this mechanism. Because they can be activated by a wide variety of ligands, GPCRs play important roles in the pathogenesis of hormone-responsive tumors. For example, somatic mutations found in the thyrotropin receptor result in the activation of adenyl cyclase, leading to hyperactivation of thyroid adenomas. Thus, initiating mutations have been found in the luteinizing hormone receptor in Leydig-cell testicular tumors. Some new studies have also demonstrated the role of GPCRs in regulating 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 importance of 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.

MATERIALS AND METHODS

Bacterial strain, plasmids, and antibodies

E. coli DH5 α and 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) 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 GPCR) encompassing amino acids 64-344 was selected for further study. The gene sequence was codon-optimized for expression in *E. coli* using Vector NTI 11.5 software, 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 μ L of cell suspension, at 2.5 kV, 25 μ F, and 200 Ω . Electroporation duration was 5.2 ms. The transformed cells were incubated in 950 μ L of superoptimal broth at 37°C for 1 h with rotary shaking at 200 rpm. Following this, 50 μ L 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 $\times$ 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 $6,000 \times g$ for 7 min at 4°C. For sequencing, the *GPR161* gene product was transformed into the DH5 α *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 $60,000 \times g$ for 1 h. The detergent-solubilized fraction was filtered through a 0.2- μ m syringe filter (Millipore, Burlington, MA, USA). Recombinant protein was purified using metal-chelate chromatography on Ni^{2+} ions using a 1-mL HisTrap™ HP column (GE Healthcare, Piscataway, NJ, USA).

The protein solution was placed 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% bovine serum albumin) 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 a 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) was 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.

Nano liquid chromatography 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- polyacrylamide gel electrophoresis (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 dithiothreitol (DTT) was added to each tube and incubated at 60°C for 10 min. The DTT solution was then removed and 100 µL of 100 mM iodoacetamide was 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 ZiptipsMicro-C18, 0.2 µL, Sigma).

The resulting mixture of tryptic peptides was separated using high-performance liquid chromatography (HPLC) and analyzed by in-line MS/MS. 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 RSLC nano 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% H₂O in 0.1% formic acid) at a flow rate of 0.3 µL/min. The gradient program for buffer B was as follows: 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, Billerica, MA, USA). 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.

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 (Figure 1). The codon-optimized nucleotide sequence of the selected region (length 897 bp including the restriction sites) (Figure 2) was inserted into the pET28 and pET32plasmids. Then, DH5 α 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).

	1	80
GPR161 isoform 1 Homo sp.	(1)	MSARGVQHALPTPRRGALTMSLNSLSLCKKELSNLTTEEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
GPR161 isoform 2 Homo sp.	(1)	MSLNSLSLCKKELSNLTTEEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
GPR161 isoform 3 Homo sp.	(1)	---MKVVQHALPTPRRGALTMSLNSLSLCKKELSNLTTEEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
GPR161 isoform 4 Homo sp.	(1)	-----
GPR161 isoform 5 Homo sp.	(1)	-----
GPR161 isoform 6 Homo sp.	(1)	-----
GPR161 Cricetulus griseus	(1)	MSLNSLSLPHYKELSNLTATEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
Gpr161 Mus musculus	(1)	---MDPVQHALLTASRGALTMSLNSLSLCKKELSNLTTEEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
Consensus	(1)	MSLNSLSLCKKELSNLTTEEKGEGGVITQFIATIIITIEVCLGNLIVIVTLYKKSYLLT
	81	160
GPR161 isoform 1 Homo sp.	(81)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
GPR161 isoform 2 Homo sp.	(61)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
GPR161 isoform 3 Homo sp.	(78)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
GPR161 isoform 4 Homo sp.	(1)	-----
GPR161 isoform 5 Homo sp.	(1)	-----
GPR161 isoform 6 Homo sp.	(1)	-----
GPR161 Cricetulus griseus	(60)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
Gpr161 Mus musculus	(77)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
Consensus	(81)	LSNKFVFSLTLSNFFLSVLVLPFVVVTSIRREWIFGVVWCNFSALLYLLISSASMLTLGVIAIDRYAVILYPMVPMKIT
	240	320
GPR161 isoform 1 Homo sp.	(161)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 isoform 2 Homo sp.	(141)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 isoform 3 Homo sp.	(158)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 isoform 4 Homo sp.	(9)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 isoform 5 Homo sp.	(63)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 isoform 6 Homo sp.	(27)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
GPR161 Cricetulus griseus	(140)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
Gpr161 Mus musculus	(157)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
Consensus	(161)	GNRAVMALVYIWLHSLIGCLPFLFGWSSVEFDEFKNCVAAHREPGYTAFWQINCALFFFLMLVVCYGFIFRVARVKAR
	241	320
GPR161 isoform 1 Homo sp.	(241)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 isoform 2 Homo sp.	(221)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 isoform 3 Homo sp.	(238)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 isoform 4 Homo sp.	(89)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 isoform 5 Homo sp.	(143)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 isoform 6 Homo sp.	(107)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
GPR161 Cricetulus griseus	(220)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
Gpr161 Mus musculus	(237)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
Consensus	(241)	KVHCGTVVVEEDAQRGRKNSSTSTSSGSRNNAFGQVYYSANQCKALITILVVLGAFMVTWGPYIMVVIASEALWGFSS
	321	400
GPR161 isoform 1 Homo sp.	(321)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 isoform 2 Homo sp.	(301)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 isoform 3 Homo sp.	(318)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 isoform 4 Homo sp.	(169)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 isoform 5 Homo sp.	(223)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 isoform 6 Homo sp.	(187)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
GPR161 Cricetulus griseus	(300)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
Gpr161 Mus musculus	(317)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
Consensus	(321)	VSPLEETWATWLSFASVCHPLIYGLWNKTVRKELLMGCFGDRYYREFVQRTSRLFSISNRTDLGLSPHLTALMAG
	401	480
GPR161 isoform 1 Homo sp.	(401)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 isoform 2 Homo sp.	(381)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 isoform 3 Homo sp.	(398)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 isoform 4 Homo sp.	(249)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 isoform 5 Homo sp.	(303)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 isoform 6 Homo sp.	(267)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
GPR161 Cricetulus griseus	(380)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
Gpr161 Mus musculus	(397)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
Consensus	(401)	GQPLGHSSTGDTGFSCQDSGTIMLLEDYTSDDNPPSHCTCPFKRRSSVTFEDEVEQKEAAKNSILHVKAHVHKSLLD
	481	549
GPR161 isoform 1 Homo sp.	(481)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 isoform 2 Homo sp.	(461)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 isoform 3 Homo sp.	(478)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 isoform 4 Homo sp.	(329)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 isoform 5 Homo sp.	(383)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 isoform 6 Homo sp.	(347)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
GPR161 Cricetulus griseus	(460)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
Gpr161 Mus musculus	(477)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR
Consensus	(481)	SYAASLAKAIEAEAKINLFGEEALPGVLVARTVFGGPGGRRGSRITLVSRQLQSQIEEGDWLAEEQR

Figure 1. Homology of the GPR161 receptor isoform 1 with other members of the GPR161 family. Yellow color - the complete match of the nucleotide sequence of all receptor isoforms; blue and green colors - variations of nucleotide sequences.

A	BamHI EcoRI NcoI										
	1	GGATCCGGAAT	TCCCATGGAG	GGCAATCTGG	TGATTGTGGT	GACCCCTGTAT	AAAAAAGCT	ATCTGCTGAC	CCTGAGCAAT	AAATTTGTGT	TTAGCCTGAC
		CCTAGGCTTA <td>AGGGTACCTC</td> <td>CCGTTAGAAC</td> <td>ACTAACACCA</td> <td>CTGGGACATA</td> <td>TTTTTTTCGA</td> <td>TAGACGACTG</td> <td>GGACTCGTTA</td> <td>TTTAACACCA</td> <td>AATCGGACTG</td>	AGGGTACCTC	CCGTTAGAAC	ACTAACACCA	CTGGGACATA	TTTTTTTCGA	TAGACGACTG	GGACTCGTTA	TTTAACACCA	AATCGGACTG
101		CTGAGCAAT	TTTCTGCTGA	GGCTGCTGGT	GCTGCCGTTT	TGGTGAACCA	CAGCATTCCG	TCGTGAATGG	ATTTTGGGCG	TGGTGTGGTG	CAATTTTAGC
		GGACTCGTTA	AAAGACGACT	CGCAGACGCA	CGACGGCAAA	CACCATCTGG	CGTCGTGAAC	AGCATTACAC	TAAAAACCCG	ACGACACGAT	GTAAAACTGA
201		GGCTGCTGT	ATCTGCTGAT	TACGAGCGCG	AGCATGCTCG	CTCTGGGCGT	GATTGCGATT	GATCGTTATT	GTGCGGTGCT	GTATCCGATG	GGTGATCCGA
		CGCGACGAC	TACAGCAGTA	ATCGTCGGCG	TGCTACGACT	GGGACCCGCA	CTAACGCTAA	CTAGCAATAA	TACGGCACGA	CATAGGCTAC	CACATAGGCT
301		TGAAAATGAC	CGGCAATCGT	GGCGTGGTGG	TGTTTGGTGA	TATTTGGGTC	CATAGCTGTA	TGGGCTGCC	CGCGCGCTCG	TTTGGCTGGA	GCAGCGTGA
		ATTTTAAATG	GGCGTTAGCA	CGCCATCACT	GGGACACCAT	ATAAACCCAG	GTCATCGGCT	AACCGCACGA	CGCGCGCGAC	AAACCGGACT	CGTCGCACT
401		CTTTTAAATG	GGCGTTAGCA	TGTGGCTGGC	GGGACGACAT	CGTGAACCGC	GCTATACCGC	GTTTTGGGAC	ATTTTGGGCG	CGCTGTTTTC	CTTTCGGTGT
		TAAACTACTT	TTAATTTACT	ACACGACGCT	CGGACCGCTG	CGCATCTGCC	GATATGGGCG	CAAAAACCCG	TAAACACCGC	CGGACAAAGG	CAAGAGGACT
501		CTCTGGTGT	GCTATGGCTT	TATTTTCGCT	TGGGCGCGTG	GCAAAGCGCG	TAAAGTGATG	TGGGCGACCG	TGGTGAATGT	GGAAGAAGAT	GGCGAGCGTA
		TACGACGACA	CGATAGCGAA	ATAAAAAAGC	CGACCGCGAC	ACTTGTGGCG	ATTTTCAAGTA	ACGCGGCTGG	ACCATCAACA	CTTTCTCTCA	CGCGTCGAT
601		CGCGCGGTAA	AAATAGACAG	ACGACGACCA	CGACGACGCG	CAGCGGCTGT	AATGGGTTTC	AGGGCGGCTG	GTATAGCGCG	AATCAGTGCA	AAGCGCTGAT
		CGCGCGGACT	TTATTCGCTG	TGGTGTGGTG	CGTCGTCGCC	CTGGGACAGA	TGCGCAAAAG	TCGCGCACCA	CATATCGCGC	TTAGTCAGCT	TTTGGGCTAT
701		TACCATCTCG	GTGGTGGCTG	GGCGGTTTGT	GGTACGTTAT	GGGCGGCTGG	TTGGTGGTAT	TGGGCGGATG	TGGGCGGCTG	AAAGAACGAC	CTGGAGCGCG
		ATGGTAAGAC	CACACGACCC	CGCGCAATA	CACTGGGACC	CGGGGCATAT	ACCACCACTA	ACGCTCGCTT	CGCGACACCC	CGTTTTCGCT	CGACTCGGG
										XhoI HindIII	
										801	
		AGCCTGGAAA	CTGGGCGCAG	CTGGCTGAGC	TTTGCAGGCG	CGGTGTGCCA	TCGCGTGATT	TATCATCATC	ATCATCATCA	TAAACTCGAG	AAGCTTTT
		TCGGACCTTT	GGACCCGCTG	GACCGACTCG	AAACGCTCGC	GCACACCGGT	AGGCGACTAA	ATAGTAGTAG	TAGTAGTAGT	AATTGAGCTC	TTTGAAA
B	BamHI EcoRI NcoI										
	1	ATGAGCGATA	AAATTAATCA	CCTGACTGAC	GACAGTTTTG	ACACGGATGT	ACTCAAGGCG	GACGGGCGCA	TGCTGTGCGA	TTTCTGGGCA	GAGTGGTGCG
		TACTGCTAT	TTTAATAAGT	GGACTGACTG	CTGTCAAAC	TGTGCTACA	TSAGTTTCGC	CTGCCCCGCT	AGGACGAGCT	AAAGACCGGT	CTACCAACGC
										Clal	
101		TGCGGTGCAA	AATGATCGCC	CGGATCTGGG	ATGAAATGCG	TGACGAATAT	CAGGCGCAAC	TGACGTTTGC	AAACTGTGAC	ATGATCAAAA	ACCTGGGAC
		CAGGCACTGT	TTACTAGCGG	GGCTAAGACC	TACTTTAGCG	ACTGCTTATA	GTCCGTTTGC	ACTGCGAAGC	TTTTGACTTG	TAGTAGTATT	TGGAGCGGTG
										ApalI	
										201	
		TGCGGCGAAA	TATGGCATGT	GTGGTATCCC	GACTCTGCTG	CTGTTCAAAA	ACGGTGAAGT	CGCGGCAACC	AAAGTGGGTTG	GACTGTCTAA	AGTTCAGTTG
		ACGCGGCTTT	ATACGCTAGG	CAGACGACTT	CTGAGCGAGC	CATGAGTTTGT	TGCCACTTCA	CGCGGTGCGG	CGATCAATCA	CATGATCATC	TCAGTCAAC
301		AAAGAGTTCC	TGCGACGTAA	CTCGCCCGGT	CTGTGTTCTG	GCATATATGA	CCATCATAGT	CATCATCTTT	GTGTCTGCTG	CGACGCGGCT	CTGGTATGTA
		TTTCTCAAGG	AGCTGGGATG	AGACGACGCA	AGACGATACG	CGGTATAGCT	GATAGTAGTA	GATAGTAGAA	GACGACCAAA	CGGTCGCCCA	AGACCATACT
										NcoI	
										401	
		AAAGAAACGC	TGCTGTCTAA	TTGCAAGCGC	AGACATATGC	CAGCCGACGA	CTGGGTGCTG	ACGAGCGAGT	CAGGCGCATG	GAGGCGAATC	TGGTGAATGT
		TTCTTTGGCG	AGCAGCAATT	TGAGGCTGGG	TGCTGTAACT	TGGGCTGCTA	GACCATATGC	TGCTGTGCTG	TTCTGCTGAT	CTCCGCTAGC	ACCACTAATG
										501	
		GTGTAGCCTG	TATAAAAAAA	GCTATCTGCT	GACCTGAGC	ATAAAATTTG	TGTTTAGGCT	GACCTGAGC	AAATTTCTGC		

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

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 (2, 4, 6,

and 24 h), 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 (Figure 3(A)). In contrast, there was expression of rTMGPR161 in BL21 *E. coli* transformed with pET32/rTMGPR161 (Figure 3(B)). The rTMGPR161 protein was expressed 2 h after IPTG addition and had an approximate molecular mass of ~49 kDa.

Figure 3. SDS-PAGE 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.

Isolation and purification of rTMGPR161

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

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 (2, 4, 6, and 24 h) 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 (Figure 5).

After purification of the rTMGPR161 protein by Ni²⁺-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. As can be seen in Figure 6(A), chromatography yielded fraction containing purified rTMGPR161 with an expected molecular weight of about 49 kDa. To confirm expression of the correct protein, we used an anti-His-tag monoclonal antibody in Western blotting of the separated by SDS-PAGE rTMGPR161 samples (Figure 6(B)). This Western blotting analysis confirmed the presence of the hexahistidine tag on a protein with a molecular mass of ~49 kDa, which corresponds to the predicted molecular mass of rTMGPR161.

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 the EGNLVIVVTLYKKSYLLT LSNKF peptide of rGPR161 and its fragmentation ions are shown in Figure 7.

Figure 4. SDS-PAGE of supernatant (A) from lysates of *E. coli* BL21/pET32/rTMGPR161 and pellet (B). Lane 1 - *E. coli* culture without IPTG; Lane 2 - *E. coli* culture after 24 h incubation with IPTG; MW - molecular weight markers.

Figure 5. Graph of rTMGPR161 concentration versus IPTG concentration.

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

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

DISCUSSION

The GPR161 receptor is a prognostic biomarker of TNBC 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 signaling pathway. Genetic analyses of the GPR161 receptor in TNBC cells have identified two mutations. The first mutation (R91G) is located within the first extracellular loop and may, therefore, play a role in ligand coupling. The second mutation, S251G, is found

within the third intracellular loop, a region which is phosphorylated in response to the activation of many GPCRs and is the site of interaction of β -arrestins (Feigin *et al.*, 2014). Genetic analyses of other cancer types have uncovered their current amplification of the *GPR161* gene 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 determined that these receptors include seven transmembrane α -helices.

Moreover, each transmembrane helix has several specific residues and a common three-dimensional structure that is conserved throughout all GPCRs (Lomize *et al.*, 1999). The transmembrane protein structure contains seven stretches of 20–30 hydrophobic amino acids that form the membrane-spanning α -helices. The protein has an extracellular N-terminus and a cytoplasmic C-terminus. The N-termini of GPCRs varies greatly in length, ranging from seven amino acids for the adenosine receptor to over 300 amino acid 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, through building an additional cytoplasmic loop, which may affect receptor mobility or G-protein coupling (Probst *et al.*, 1992).

The ligand-binding sites of GPCRs have been partly described through both biochemical and molecular biological methods. Helical amino acids have been classified as (A) amino acids that are in association with membrane lipids and (B) amino acids that are not in association with membrane lipids (Baldwin, 1993). Amino acid residues that are conserved within GPCRs contribute to the specificity of ligand binding. For example, two conserved serines in transmembrane helix 5 of the β 2-adrenergic receptor (Ser204 and Ser207) have been involved in building 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 connect to the seven-transmembrane components of the receptor, albeit with a more inferior affinity (Ji and Ji, 1991). This suggests that GPCRs may include both a site extracellular binding with high affinity and a site in the within transmembrane domains of low affinity. It is possible that the site of the extracellular binding with high affinity created 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 that 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), 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. Despite the many benefits of recombinant protein production in *E. coli*, this expression system has problems such as low expression levels or even lack of proteins expression (Soleimani *et al.*, 2016). The lack of expression of rTMGPR161 in *E. coli* when using the pET28 vector is likely due to the formation of inclusion bodies. At the same time, the pET32 series is designed for cloning and high-level expression of peptide sequences fused with the thioredoxin protein. Thioredoxin significantly increases the solubility of proteins, while maintaining their biological activity (LaVallie *et al.*, 1993).

We solubilized rTMGPR161 in a solubilization buffer and purified it by metal-affinity chromatography on a Ni^{2+} -Sephrose 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 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 β 2-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 regularly used because the results depend on the correct original sequence of the construct (Chapot *et al.*, 1990).

CONCLUSION

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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