Experimental Cell Research 356 (2017) 40–47

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Regulation of AGR2 expression *via* 3'UTR shortening

Eva Matoulkova, Lucia Sommerova, Michal Pastorek, Borivoj Vojtesek, Roman Hrstka*

Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czechia

ARTICLE INFO

Keywords:
Anterior gradient 2
3' untranslated region
3'UTR shortening
mTOR

ABSTRACT

One recently discussed general mechanism affecting gene expression is 3'-untranslated region (3'UTR) length. Events such as shortening, translocation or loss of 3'UTRs are observed within oncogenes and are proposed to associate with increased expression. Thus, increased efforts are being made to understand constitutive and differential transcript 3'end formation. Investigation of *AGR2* mRNA revealed a direct impact of its 3'UTR length on *AGR2* expression. *In silico* analyses identified several regulatory sequences within the distal part of *AGR2* mRNA that may regulate 3'UTR length and associated protein levels. Short 3'UTRs were observed in a panel of AGR2-positive cancer cell lines and in human breast cancer specimens, in which more extensive 3'UTR shortening correlated with increased AGR2 protein levels. AGR2 is an important member of PI3K/AKT signalling pathway, which along with the proposed involvement of mTOR in the regulation of alternative polyadenylation, prompted us to study the role of mTOR in relation to *AGR2* mRNA 3'UTR shortening. A direct impact of mTOR signalling on *AGR2* 3'UTR shortening associated with increased protein synthesis was found, which led to the identification of a novel molecular mechanism involved in upregulation of AGR2 levels in mTOR-activated cells *via* modulating the 3'UTR length of *AGR2* mRNA.

1. Introduction

Recently, molecular research on the regulation of gene expression has become more focused on non-coding sequences such as UTRs (untranslated regions) and introns. The impact of these sequences and their length has been recognized as one of the major regulatory mechanisms of protein expression [1]. 5'UTRs are studied predominately in relation to their impact on the initiation of translation due to localization of 5'cap or internal ribosome entry sites (IRES), usually situated upstream to open reading frames (uORFs). The 3'UTR is considered to be the main stabilization part of a mRNA, that influences translation efficiency through transcript cleavage, alternative polyadenylation and the presence of binding sites for numerous regulatory proteins and non-coding RNAs including microRNAs (miRNAs) [2]. The most well-known specific inhibitory sequences within 3'UTRs were discovered approximately two decades ago and have been characterized as specific miRNA binding sites [3]. Other regulatory options are cis elements or specific secondary structures affecting speed of translation and hence the rate of protein synthesis. Alternative polyadenylation sites and/or cleavage sites within the 3'UTR have been described as expression-enhancing elements leading to shortening of 3'UTR and loss of inhibitory elements [4,5]. Alternatively, specific chromosomal translocations have been characterized as a genetic mechanism responsible for translocation of specific 3'UTR regions, causing significant changes in the expression of the affected genes [5,6].

The role of 3'UTR shortening is often discussed in relation to tumor development, more precisely with over-expression of many oncogenes *e.g. HMGA2* [4], *CCND1* [7] and *CCND2* [5], in which upregulated expression may lead to increased cell proliferation and survival resulting in cancer development and progression. The most likely explanations for aberrant activation linked with 3'UTR shortening would be the loss of regulatory sequences such as miRNA binding sites, or loss of potential secondary structures affecting rate of translation [8].

Increased *AGR2* expression was detected in various tumors including breast, ovarian, prostate, pancreatic, lung and liver [9]. *AGR2* over-expression stimulates cell adhesion [10], cell proliferation and invasion [11], cell survival and metastatic potential followed by aggressive tumorigenic progress [12–15], which is also supported by reports showing significant association of AGR2 over-expression with poor patient outcomes [16–19].

AGR2 has also been characterized as a gene generating alternative isoforms having various 5'UTR, coding and 3'UTR sequences. Interestingly, different levels of distinct splice variants of *AGR2* in

Abbreviations: 3'UTR, 3' untranslated region; AGR2, Anterior Gradient 2; BRCA1, Breast cancer 1; HPRT1, Hypoxanthine phosphoribosyltransferase 1; mTOR, Mechanistic target of rapamycin; TORC1, protein complex containing at least TOR (target of rapamycin) and Raptor (regulatory-associated protein of TOR)

* Corresponding author.
E-mail address: hrstka@mou.cz (R. Hrstka).

http://dx.doi.org/10.1016/j.yexcr.2017.04.011
Received 22 December 2016; Received in revised form 28 March 2017; Accepted 8 April 2017
Available online 11 April 2017
0014-4827/

urine exosomes were confirmed as sensitive markers for prostate cancer [20]. To date, seven different *AGR2* splice variants have been reported in Ensembl [21]; four transcripts could be potentially translated into a protein, but only two have been confirmed experimentally. These two translated splice variants are denoted as *AGR2-001* consisting of 175 amino acids and *AGR-005* consisting of 188 amino acids. The most well-known protein coding transcript, *AGR2-001* (hereinafter referred to as *AGR2*), consists of eight exons with a length of 1778 bases. *AGR2-001* is more highly expressed compared to the other *AGR2* transcripts and possesses the longest 3'UTR of 1137 bases. However, highly expressed genes generally use proximal poly(A) sites and have shorter 3'UTRs than genes with low expression levels [22]. Since AGR2 is highly expressed in some cancers, regulatory mechanism(s) influencing *AGR2* mRNA 3'UTR length to modulate its expression may be present.

Indeed, our study analysing *AGR2* mRNA revealed different 3'UTR sequence lengths in a panel of tumor cell lines. A direct link between 3'UTR length and AGR2 protein determined by immunohistochemical staining was confirmed in a cohort of breast cancer specimens. The effect of 3'UTR shortening in relation to AGR2 protein level was further confirmed functionally by transient transfection of plasmids carrying the AGR2 coding sequence with different 3'UTR lengths, as well as by a luminescence reporter system measuring activity of shorter *AGR2* 3'UTRs. We also demonstrate a role for mTOR signalling in regulating *AGR2* 3'UTR length and AGR2 protein levels.

2. Materials and methods

2.1. Cloning

AGR2-001 cDNA including 5'UTR and 3'UTR sequences was obtained by reverse transcription of RNA isolated from MCF-7 cells using primers specific for the beginning of the 5'UTR and the end of the 3'UTR of *AGR2-001*. cDNAs of *AGR2-001* with specifically shortened 3'UTRs were obtained using a specific primer for the beginning of the *AGR2* coding sequence and primers that bind to the 3'UTR at positions 283, 350, 474 and 819 bp after the stop codon. Constructs with respective UTR(s) and shortened variants were cloned into pcDNA3 expression vector using *Bam*HI and *Xho*I restriction sites according to the manufacturer's instructions (New England Biolabs). Restriction enzymes, T4 DNA polymerase and calf-intestinal alkaline phosphatase were obtained from New England Biolabs.

2.2. Cell cultures, transfections and treatments

All AGR2 positive human cancer cell lines (A549, MCF-7, T-47D, SK-BR-3, PANC-1, ZR 75.1, BxPC3, BT474, MDA-MB-468) and AGR2 negative cell lines (H1299, ARN8, HCT 116, HEK-293FT) were cultured in high glucose Dulbecco´s Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 300 μg/ml L-glutamine at 37 °C and humidified 5% CO_2 atmosphere.

Cells were seeded at 50% confluence one day prior to transfection in D-MEM and were transfected using PEI (Polyethylenimine). Amount of DNA was calculated for the same copy number. To determine mRNA stability, cells were exposed to 5 ng/ml of Actinomycin D (Tocris Bioscience) for 2, 4, 6 and 8 h after 24 h post transfection. Torin1 (Tocris Bioscience) was applied at 50 nM and 100 nM for 24 h. Control cells were treated with the corresponding amount of DMSO.

For adhesion assay 20,000 cells were seeded per well on 96-well plates. Cells were left to adhere for 1 h at 37 °C and then adherent cells were fixed and stained with crystal violet staining solution.

Three-dimensional multicellular spheroid cultures were created using a hanging drop method. Cells were grown to approximately 80% confluence on standard tissue-culture plates. The cells were subsequently trypsinized, resuspended, either in complete medium enriched with extracellular AGR2 (eAGR2) or without eAGR2 and counted. The cell suspension was diluted to a concentration 100,000 cells/ml, and 20 μl of the cell suspension were pipetted onto the underside of a sterile 5 cm tissue-culture plate lid. After loading the droplets, the lid was placed onto a tissue culture plate containing 4 ml of sterile PBS and incubated for 24 h to facilitate cellular aggregation and spheroid formation and recorded using Nikon microscope.

2.3. Immunochemical analyses and pulse immunoprecipitation (IP)

Cells for Western blot analysis were harvested 24 h post-transfection into lysis buffer (50 mM TrisHCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3CO_4, 1% Nonidet P40) containing protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using Bradford assay (Bio-Rad), 10 μg of total protein was separated on 10% polyacrylamide gels. Membranes were incubated overnight at 4 °C with anti-AGR2 K31 (rabbit polyclonal, [23]) and anti-β-actin as a loading control (A-2066, Sigma-Aldrich), washed and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Dako). Proteins of interest were detected using ECL (0,5 M EDTA pH 8.0, 90 mM coumaric acid, 1 M luminol, 200 mM Tris-HCl pH 9.4, Na-perborate×4H_2O, 50 mM Na-acetate pH 5.0).

Pulse IP: Cells were pulse-labelled with 100 μCi/60 mm dish of EXPRE35S Protein Labelling Mix (PerkinElmer) for 30 min. Pulse-labelled cells were washed twice with PBS. Lysis of the cells and concentration determination was conducted as well as for Western blot analysis. After pre-clearing using protein G Sepharose (GE Healthcare Bio-Sciences), lysates were incubated overnight with anti-AGR2 antibody (1:1000, Abnova) at 4 °C. The beads were then added to the immune complexes and precipitated for 40 min at 4 °C with gentle rotation and washed five times with lysis buffer. Immunoprecipitates were eluted with Laemmli sample buffer containing 50 mM DTT for 10 min at 70 °C. The proteins were analyzed by immunoblotting and detected as mentioned above.

2.4. Functional evaluation of AGR2 3'UTR

Differentially shortened *AGR2* 3'UTRs were cloned into the 3'UTR reporter vector pLightSwitch_3'UTR (Switchgear Genomics). 24 h after PEI co-transfection of 3'UTR constructs bearing particular AGR2 3'UTR sequences (*Renilla* luciferase) and pGL4.50 vector (*Firefly* luciferase; used for data normalization), cells were harvested using 0.125% trypsin into PBS, washed 3 times with PBS, then resuspended and frozen at −80 °C in 10 mM Tris-HCl pH 8.0. After thawing, supernatants were divided into two sets of triplicates and the activity of *Firefly* luciferase was measured using 0.2 mM luciferin (Applichem) as substrate and of *Renilla* luciferase using 0.25 mM coelenterazine h (NanoLight Technology).

2.5. Real-Time PCR

Total cellular RNA was extracted by TRI-Reagent (MRC). cDNA synthesis was carried out from 0.5 μg RNA using random hexamers and SuperScript VILO Master Mix (Invitrogen). Triplicate samples were subjected to quantitative PCR analysis using SYBR Green (Roche) for *AGR2*, *ACTB* (*β-actin*) and *GAPDH* (*glyceraldehyde 3-phosphate dehydrogenase*) both serving as endogenous control. In parallel plasmid carrying coding sequence followed by whole 3'UTR was used as an internal control of similar PCR efficiency for all *AGR2* specific primers. The primers for amplification of specific *AGR2* sequences were as follows: *AGR2* coding sequence/beginning of 3'UTR (at position from −29 to +110 bases from stop codon, CDS/3'UTR): Forward 5'-GCTCTCAAGTTGCTGAAGACTG-3', Reverse 5'-AACCTAATCAGTGTGTTCACTATGC-3'. Proximal part of 3'UTR (at position from +272 to +414 bases from stop codon; prox-3'UTR): Forward 5'-GTCTTTCACAGTGGTTCGTTTACC-3', Reverse 5'-TAGTGAGAACCTGTGGTGTGG-3'. Middle part of 3'UTR (at position