The biological role of the long non-coding RNA Link-A in ovarian carcinoma

Running title: Link-A in ovarian carcinoma

Article type: Experimental study

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Abstract

Aim: To analyze the biological role of the long non-coding RNA Link-A.

Materials and methods: An 850bp segment from the second exon of Link-A was removed using the CRISPR/Cas9 system in OVCA 433 ovarian serous carcinoma cells. Spheroid formation, migration, invasion, proliferation, matrix metalloproteinase (MMP) activity and expression of cell signaling proteins were assessed *in vitro*.

Results: OVCA 433 cells with Link-A deletion were more invasive ($p=0.0008$), but had reduced migration and MMP-9 secretion compared to controls ($p=0.003$ and $p=0.005$, respectively). Link-A deletion did not affect proliferation, but induced ERK1/2 phosphorylation of (10-fold; $p=0.005$). Link-A KO additionally reduced spheroid formation.

Conclusion: Link-A inactivation via CRISPR/Cas9 affects invasion, migration, MMP secretion, cell signaling and spheroid formation in ovarian serous carcinoma cells. Added to our previous data from analysis of clinical specimens, the sum effect of this molecule is likely as a tumor suppressor.

Keywords: Link-A; long non-coding RNA; CRISPR/Cas9; high-grade serous carcinoma; tumorigenesis

Introduction

Ovarian cancer, consisting predominantly of ovarian carcinoma (OC), is the most aggressive gynecologic cancer. Ovarian cancer was predicted to be diagnosed in 22,530 women and lead to 13,980 fatalities in the U.S. in 2019 [1]. Globally, the disease was diagnosed in 295,414 women and led to 184,799 deaths in 2018, making OC the 8th most common and 8th most lethal cancer in women [2]. Late diagnosis and chemotherapy resistance are two major factors contributing to the fact that prognosis remains dismal for the majority of patients [3].

Recent years have brought growing interest in non-coding RNA (ncRNA). Long non-coding RNAs (lncRNAs) are a family of ncRNAs longer than 200 nucleotides that are involved in many different cellular processes and pathways. In cancer, lncRNAs have been found to be involved in posttranscriptional modifications and decoy of mRNA, proliferation and immortality, maintenance of genomic stability, invasion and metastasis, and drug resistance [4]. Link-A (also known as LOC339535, LINC01139 and NR_015407) is a ~1.5kb long intergenic ncRNA located on chromosome 1 at the 238,480,372-238,486,05 site on the antisense strand. It consists of two exons, of which the second contains functional sites consisting of a phosphatidylcholine (PC) binding site, breast tumor kinase (BRK) binding sites #1 and #2, a PIP3-binding loop and a leucine-rich repeat kinase 2 (LRRK2) binding site. This lncRNA was first described in triple-negative breast cancer, where it was shown to phosphorylate and stabilize HIF1 α , and thereby promote tumorigenesis [5]. In a subsequent study by the same group, Link-A was shown to hyperactivate AKT and mediate resistance to AKT inhibitors [6]. The ability of Link-A to activate HIF1 α was subsequently demonstrated also in ovarian cancer (OC) [7]. In OC, Link-A was further found to activate the TGF- β pathway and to promote migration and invasion [8].

High-grade serous carcinoma (HGSC) is the most common histotype of ovarian carcinoma, and is responsible for the majority of deaths from this disease. In a recent study of clinical HGSC specimens, Link-A was overexpressed in solid metastases compared to HGSC effusions, the

ovarian tumors and exosomes derived from HGSC effusions. However, higher Link-A levels in post-chemotherapy HGSC effusions were significantly related to longer progression-free and overall survival, the latter finding retained as independent prognosticator in multivariate Cox analysis [9]. Given this apparent discrepancy, we wished to study the biological role of this molecule in OC. In the present study, we used CRISPR/Cas9 technology to knock out (KO) Link-A and study the effect of this change *in vitro* in OC cells.

Methods and Materials

Cell culture

OC cell lines included in this study consisted of OVCAR-3 (HGSC), OVCAR-8 (HGSC), OVCA 433 (serous adenocarcinoma), OC-238 (serous cystadenocarcinoma), OVCA 429 (cystadenocarcinoma), DOV13 (adenocarcinoma) and ES-2 (clear cell carcinoma). All cell lines were cultured in 37°C, 5% CO₂ in the following media:

OVCAR-3, OC-238, ES-2: Dulbecco's Modified Eagle's medium – high glucose (DMEM; Sigma-Aldrich, St. Louis, MO) 10% fetal calf serum (FCS; Sigma-Aldrich).

OVCA 433, OVCA 429, DOV13: MEM-EAGLA Earle's Salts Base (MEM; Biological Industries, Kibbutz Beit-Haemek, Israel) 10% FCS.

OVCAR-8: RPMI Medium 1640 (GIBCO, Thermo Fisher Scientific, Waltham, MA) 5% FCS.

All media were supplemented with 1% MEM-Eagle Non-essential amino acids solution, 1% L-Glutamine Solution, 1% MEM Vitamins Solution, 1% Sodium Pyruvate Solution, 1% Penicillin-Streptomycin-Amphotericin B Solution (Biological Industries).

CRISPR/Cas9 KO

Guides: Link-A active sites are found on the second exon, and we therefore decided to delete it.

To achieve this goal, we designed two guides that cut out ~870 bases of the second exon. The guides were designed with the help of MIT (<https://zlab.bio/guide-design-resources>) and UCSC (<http://genome.ucsc.edu/index.html>) web sites. Each guide was cloned into a PX330 plasmid (pX330-U6-Chimeric_BB-CBh-hSpCas9; a kind gift from Dr. Yosef Buganim's lab, The Hebrew University of Jerusalem).

Transfection: PX330 plasmid transfection into OVCA 433 cells was made using Avalanche-Everyday (EZ Biosystems, College Park, MD). 1.25µg of each guide plasmid and 0.5µg of puromycin-resistant plasmid (Fuw-original-puro-2A-EGFP was a kind gift from Dr. Yosef Buganim's lab) were co-transfected for 24 hours, followed by puromycin selection for another 48 hours. The transfected cells were then seeded as single cells in a 96-well plate.

Spheroid formation

Control and KO OVCA 433 cells (n=400,000) were seeded on a 6-well plate dish and agitated for 24 hours and photographed. The medium was collected for RNA isolation.

Scratch assay / migration assay

OVCA 433 cells (n=100-200,000) were seeded on a 12-well plate dish and cultured to confluence. A scratch was made with a 1000µl pipette tip and images were taken at 0, 6 and 24 hours. Results were analyzed using the ImageJ program (NIH, Bethesda, MD).

Cell proliferation assay

OVCA 433 cells (n=100,000) were seeded on a 12-well plate. At 0 and 24 hours, the cells were treated with 2mg/ml of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Calbiochem, San Diego, CA) for 20 minutes at 37°C, in a 5% CO₂ incubator. Cells were subsequently lysed using 150µl Dimethyl Sulfoxide (DMSO; Sigma-Aldrich) and absorption at 570nm was measured by a Cytation 3 instrument (BioTek Instruments, Inc., Winooski, VT).

Matrigel invasion assay and motility assay

Nuclepore polycarbonate filters (13mm, 8µm pore size, polyvinylpyrrolidone [PVP]-free; Whatman International Ltd, Maidstone, UK) were coated with basement membrane components extract (Matrigel, 25µg per filter) for the chemo-invasion assay or with 5µg collagen IV for the motility assay, and placed in Boyden chambers. OVCA 433 cells (n=200,000) were resuspended in serum-free medium and placed in the upper compartment of the Boyden chambers. Chemoattractant, fibroblast-conditioned medium (obtained from NIH-3T3 cells) was used. After 5 or 24 hours of incubation at 37°C in a 5% CO₂ incubator, the filters' lower surface was stained in DiffQuik (Medion Diagnostics International Inc., Miami, FL) and 5 random fields were counted.

Matrix metalloproteinase (MMP) activity assay (zymography)

The assay was performed on medium collected from Boyden chambers at the end of the Matrigel invasion experiment. Medium from OVCA 433 control and KO cells was loaded onto 10% sodium-dodecyl-sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) gels with 1mg/ml gelatin (Sigma-Aldrich), as previously described [10].

RNA extraction and cDNA generation

RNA was extracted from OVCA 433 OC cells using the Bio-Tri reagent (Bio-Lab ltd, Jerusalem, Israel) according to the manufacturer's protocol. RNA concentration was measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

Prior to cDNA formation, 1µg of extracted RNA was subjected to DNA degradation by PerfeCTa DNase I (Quanta Biosciences, Gaithersburg, MD). cDNA was created by qScript cDNA synthesis kit (Quanta Biosciences).

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and RT-PCR

qRT-PCR was performed using the Fast SYBRTM Green Master Mix (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA) with specific primers in CFX Connect Real-Time system (Bio-Rad Laboratories, Hercules, CA). RT-PCR was carried out using Hy-Taq Ready Mix (2X) (Hylabs, Rehovot, Israel) with specific primers in PCR TOUCH T960 (Hangzhou Jingle Scientific Instrument Co., Ltd, Hangzhou, China). PCR products were loaded on 1.5% Agarose gel with 100bp DNA ladder (GeneDireX Inc., Taoyuan, Taiwan). Gene expression was normalized to RPLP0.

Primer sequences were as follows:

LINK-A: Forward: AACCAGTCACCCAACCAGAG

Reverse: CACAGGCCAGATGGAGTTTT

RPLP0: Forward: CCAACTACTTCCTTAAGATCATCCAACTA

Reverse: ACATGCGGATCTGCTGCA

Western blot

Total protein isolation was performed using RIPA (1% NP-40, 20mM Tris-HCl (pH 7.5), 137mM NaCl, 0.5mM EDTA, 10% glycerol, 1% protease inhibitor cocktail (Millipore, Burlington, MA) and 1mM sodium Orthovanadate and 0.1% sodium dodecyl sulfate (SDS). 25µg of protein was loaded onto 10% SDS-PAGE gel. The proteins were then transferred to Immobilon PVDF membrane (Millipore). Subsequently, the membrane was blocked by 5% Difco skim milk (BD Biosciences, San Jose, CA) for 1 hour, and then incubated for 16 hours at 4°C with one of the following rabbit monoclonal antibodies (all from Cell Signaling Biotechnology, Danvers, MA):

GAPDH antibody, clone 14C10, cat# 2118

p44/42 MAPK (ERK1/2), clone 137F5, cat# 4695

p-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), clone 197G2, cat# 4377

Results

Link-A expression in OC cell lines

Link-A expression was analyzed in 7 OC cell lines - OVCAR-3, OVCAR-8, OC-238, OVCA 429, OVCA 433, DOV13 and ES2, in 2 forms of culture- 2D monolayer and 3D spheroids. Link-A was expressed in the OVCA 433, DOV13 and ES2 cell lines. In OVCA 433 cells, expression was low in 2D monolayer and high in spheroids ($p < 0.0001$; **Figure 1**).

Link-A CRISPR/Cas9 KO

We performed KO of the second exon of Link-A using CRISPR/Ca9 system in OVCA 433 cells. **Figure 2** shows the change in Link-A secondary structure following the KO.

Link-A KO affects invasion and migration

The invasiveness of KO cells was strongly upregulated following the loss of Link-A to 452% of control level ($p = 0.0008$; **Figure 3-A**). In contrast, in wound healing assay, KO cells closed the gap less efficiently than control cells, with closure at 54% vs. 70%, respectively, after 24 hours ($p = 0.003$; **Figure 3-B**).

Link-A KO decreases MMP secretion

We examined MMP secretion in two states - native cells cultured in serum-free medium for 24 hours and cells in the invasion assay, studied at 5 hours. No MMPs were found in medium from the native cells, whereas the medium from the invasion assay, obtained from both the OVCA 433 control and KO cells, had MMP2 and MMP9 activity. Significant difference was found only for MMP9, which was decreased to 70% in the KO cells ($p = 0.005$; **Figure 3-C**).

Link-A does not affect cell proliferation

To find whether Link-A is involved in cell proliferation we performed a MTT assay for 24 hours. No significant difference was found between the numbers of control and KO cells ($p > 0.05$; **Figure 3-D**).

Suppression of Link-A leads to a change in spheroids structure and size

As Link-A was highly expressed in spheroids, we assessed the KO effect on their formation.

KO cell spheroids were much smaller and less spherical compared to those formed by OVCA 433 control cells (**Figure 4**).

Link-A suppresses ERK1/2 phosphorylation

In KO cells, ERK 1/2 expression levels were not significantly altered, but the phosphorylation was elevated about 2.5-fold ($p=0.0015$; **Figure 5**).

Discussion

Research focusing on lncRNAs is a relatively new field in molecular biology, but an increasing number of studies has in recent years documented the important role of these molecules in the evolution and progression of cancer. In OC, lncRNAs have been shown to be involved in invasion, proliferation, cell cycle regulation, apoptosis, and drug resistance [11-13]. Moreover, association between lncRNA expression levels and patient survival has been reported by us and others [9,14].

In the current study, we focused on the lncRNA Link-A. Link-A has, in addition to its above-discussed role in breast carcinoma and OC, been recently reported to have a biological and clinical role in other cancers. Link-A promotes migration, invasion and proliferation in pancreatic adenocarcinoma, apparently through upregulation of Rho-associated protein kinase 1 (ROCK1) [15]. It was similarly reported to promote migration and invasion in non-small cell lung carcinoma (NSCLC). In this tumor, Link-A plasma levels were higher in patients with metastatic disease compared to those with non-metastatic NSCLC or controls [16].

Similar results have been reported in non-epithelial cancers. Link-A plasma levels were higher in patients with glioma and mantle cell lymphoma compared to controls, and this molecule prevented apoptosis through upregulation of survivin *in vitro* [17,18]. Promotion of migration and invasion through increased HIF1 α levels *in vitro*, combined with higher plasma levels compared to controls, were also seen in osteosarcoma [19].

Using the CRISPR/Cas9 system, we deleted the majority of the second exon and thereby eliminated Link-A functional sites and disrupted its secondary structure. We found that Link-A KO suppressed migration and MMP secretion, though it increased invasion, with no effect on proliferation. Importantly, we observed that OVCA 433 KO cells did not form spheroids. Seen together with the high levels of Link-A initially found in spheroids, we hypothesize that this lncRNA is critical for their formation. Additionally, Link-A KO increased ERK1/2 phosphorylation. A previous study showed that Link-A can activate AKT [3], but in our KO cells, we found no significant change in AKT levels or phosphorylation (data not shown).

In summary, the present study shows that Link-A is involved in critical cellular processes in ovarian serous carcinoma. While Link-A KO has both tumor-promoting and tumor-suppressing effect in an experimental model of OC, combination of the present findings with our earlier analysis of clinical specimens may favor a tumor-suppressor role in this cancer. This does not concur with the above-discussed studies of different malignant tumors or with the recent report of Hu and co-workers, in which Link-A was reported to negatively regulate cancer cell antigen presentation and intrinsic tumor suppression [20]. This discrepancy may suggest that Link-A has different roles in different cancers. In clinical OC, it may promote invasion and metastasis in early-stage disease, but mediate undefined tumor-suppressing activities later in the clinical course, when tumorcells are metastatic. Analysis of the role of this molecule in early-stage disease, including in the primary fallopian tube lesion in HGSC, may shed light on this issue.

Declarations

Funding: None declared.

Conflict of interest: We have no conflict of interest

Authors' contributions

NFL: Performed the experiments and wrote the manuscript.

BD: Participated in designing the study and co-wrote the manuscript.

RR: Designed the study, supervised the experiments and critically read the manuscript.

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Figure legends

Figure 1: Link-A expression in the OVCA 433 cell line cultured as monolayer and spheroids (* $p < 0.0001$).

Figure 2: The secondary structure of Link-A before (A) and after (B) the deletion in exon 2, predicted using the "RNAfold web server" website (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). The phosphatidylcholine (PC) binding site is in nucleotides 241-300, the breast tumor kinase (BRK) binding site #1 at nucleotides 471-550, BRK binding site #2 at nucleotides 771-850, PIP3 binding loop at nucleotides 1081-1140, and leucine-rich repeat kinase 2 (LRRK2) binding site at nucleotides 1251-1330.

Figure 3: (A) Matrigel invasion assay showing the percentage of OVCA 433 control and KO cells that invaded through matrigel (* $p = 0.0008$).

(B) Wound healing assay. Left: Images at 0 and 24 hours of OVCA 433 KO cells and control cells; Right: Graph showing the % of the gap left after 24h for control (30%) and KO (46%) cells (* $p = 0.003$).

(C) MMP levels in KO cells compared to controls, with reduction to 76% and 70% for MMP2 and MMP9, respectively (* $p = 0.05$).

(D) MTT assay. A graph showing the O.D of OVCA 433 control cells and KO at 0 hours and after 24 hours ($p > 0.05$).

Figure 4: (A-B) spheroids formed by OVCA 433 control cells; (C-D) spheroids formed by KO cells.

Figure 5: Protein levels (after normalization to GAPDH) of p-ERK in OVCA 433 control and KO cells (* $p=0.0015$).

Figure 1

Figure 2

Figure 3-A

Figure 3-B

Figure 3-C

Figure 3-D

Figure 4

Figure 5