Cancer Letters 354 (2014) 455–466

Contents lists available at ScienceDirect

Cancer Letters

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

miR-21-3p is a positive regulator of L1CAM in several human carcinomas

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ARTICLE INFO

Article history:
Received 1 July 2014
Received in revised form 13 August 2014
Accepted 14 August 2014

Keywords:
miR-21-3
Kidney carcinoma
Endometrial carcinoma
Ovarian carcinoma

ABSTRACT

Expression of L1 cell adhesion molecule (L1CAM) occurs frequently in human cancers and is associated with poor prognosis in cancers such as ovarian, endometrial, breast, renal cell carcinoma and pancreatic ductal adenocarcinoma. L1CAM promotes cell motility, invasion, chemoresistance and metastasis formation. Elucidating genetic processes involved in the expression of L1CAM in cancers is of considerable importance. Transcription factors such as SLUG, β-catenin/TCF-LEF, PAX8 and VHL have been implicated in the re-activation of L1CAM in various types of cancers. There is increasing evidence that micro-RNAs can also have strong effects on gene expression. Here we have identified miR-21-3p as a positive regulator of L1CAM expression. Over-expression of miR-21-3p (miR-21*) but not the complementary sequence miR-21-5p (miR-21) could strongly augment L1CAM expression in renal, endometrial and ovarian carcinoma derived cell lines by an unknown mechanism involving transcriptional activation of the L1CAM gene. In patient cohorts from renal, endometrial and ovarian cancers we observed a strong positive correlation of L1CAM and miR-21-3p expressions. Although L1CAM alone was a reliable marker for overall and disease free survival, the combination of L1CAM and miR-21-3p expressions strongly enhanced the predictive power. Our findings shed new light on the complex regulation of L1CAM in cancers and advocate the use of L1CAM/miR-21-3p for diagnostic application.

Introduction

L1CAM is a 200–220 kDa cell surface molecule playing an important role both for the development of the nervous system and for the malignancy of human tumors [1,2]. L1CAM can interact with itself (homophilic) or with heterophilic ligands such as integrins, CD24, neurocan, neuropilin-1 and other members of the neural cell adhesion family [3,4]. L1CAM is over-expressed in many human carcinomas such as pancreatic, colorectal, ovarian, endometrial, gastric and renal cancers and its expression is associated with poor patient prognosis [5–11]. Furthermore, high levels of L1CAM were found associated with increased grade of malignancy [5,12], epithelial–mesenchymal transition (EMT) [13], worse response to chemotherapy [7,14,15] and activation of the NFkB signaling pathway [16,17]. Thus, due to its ability to trigger cell motility, invasion and metastasis formation L1CAM is considered as a driver of tumor progression [18].

An important question is by which genetic mechanisms L1CAM is up-regulated in tumors. Earlier work has implicated that in non-neuronal tissues the *L1CAM* gene is suppressed by repressor element 1 silencing transcription factor (REST) and a neural restrictive silencer element (NRSE) within the second intron of the L1CAM gene prevented expression [19]. Interestingly, L1CAM expression has been linked to a loss of REST in non-small lung cancer [20]. Several other TFs such as SLUG in pancreatic and endometrial cancers [12,21,22], β-catenin/TCF-LEF in colorectal cancer [23] or PAX8 and VHL in RCC [24] were implicated to regulate L1CAM expression in cancer. Interestingly, recent reports have shown that the methylation status of the L1CAM promoter is critical for L1CAM expression [25–27]. Thus, published data suggest that up-regulation of L1CAM at the transcriptional level can be achieved by various mechanisms.

Abbreviations: DSF, disease free survival; EC, endometrial carcinoma; EMT, epithelial to mesenchymal transition; IHC, immunohistochemistry; L1CAM, L1 cell adhesion molecule; OS, overall survival; RCC, renal cell carcinoma; TF, transcription factor; VHL, von Hippel–Lindau.

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http://dx.doi.org/10.1016/j.canlet.2014.08.020
0304-3835/

Micro-RNAs (miRNAs) are 20–24 nucleotide non-coding RNAs that are powerful regulators of gene expression. These small RNAs can be isolated from blood, serum, and plasma, or from cells and tissues. Dys-regulation of miRNAs in cancer cells are known to be important in different steps of the tumorigenesis, from initiation and development to progression toward EMT induction and the acquisition of a metastatic phenotype [28–30]. We have recently shown that miR-34a can bind to specific binding sites in the 3′UTR of L1CAM mRNA and thereby target it to degradation [31]. These results provided proof of principle that L1CAM expression can be regulated by miRs.

In the present report we searched for miRs that can augment the expression of L1CAM in cancer. We identified miR-21-3p as a strong positive regulator in RCC cell lines. This microRNA is derived from MIR-21 and was previously named miR-21* [32]. We found a strong correlation of L1CAM and miR-21-3p in clinical samples of RRC tumor tissues. Importantly, a similar correlation was observed in two independent cohorts of endometrial and ovarian carcinomas. Our results identify essential new players in the complex network that regulates L1CAM expression and suggest that combined determination of L1CAM and miR-21-3p could significantly improve prognosis.

Methods

Cell lines

The RCC lines Foehn, A498, 786-0 [24], HK2 [33], the EC cell lines EFE124, MFE 296, EN1 [27,31] and the ovarian carcinoma cell line OVMz [34,35] were maintained in DMEM medium (PAA Laboratories, Pasching, Austria). The A498 neo and VHL were described earlier [24,36]. The medium was supplemented with 10% fetal calf serum as described before. For primary fibroblasts and HK2 cells the medium was also supplemented with non-essential amino acids and glutamine.

Chemicals and antibodies

Antibodies to the ectodomain of L1CAM (monoclonal antibody (mAb) L1-11A, a subclone of UJ127.11) were described before [34,37]. The antibodies for detection in Western blot against β-actin were from Santa Cruz Biotechnology (Heidelberg, Germany). Suramin and actinomycin D were obtained from Sigma-Aldrich (St. Louis, USA).

Treatment of cells and biochemical analysis

Cells were lysed for 15 min at 4 °C in NuPage LDS sample buffer (LifeTech, Carlsbad, CA) and sonified. To prevent proteinase activities a cOmplete™ mini tablet (Roche) was added to 10 mL buffer. After centrifugation at 10000× *g* for 10 min at 4 °C, supernatant was collected and protein concentrations were determined (Pierce, BCA protein assay, Thermo Scientific, Waltham, USA). 30 µg of protein per lane was separated by 10% SDS-polyacrylamide gel electrophoresis. The SDS gel electrophoresis and Western blotting were performed as described before [34].

FACS analysis

The cells were stained with a directly conjugated mAb against L1CAM. Stained cells were analyzed with a FACS Canto II, using FlowJo software (Becton-Dickinson, Heidelberg, Germany) [34,37].

siRNA, mimic and miRNA-inhibitor transfection

24 h before the treatment 0.5×10^5 cells were seeded in a 6-well plate. The transfection was performed with oligofectamine (QIAGEN) following manufacturer protocol. The final oligonucleotide concentration was 20 nM. After the transfection the cells were incubated for 72 h under standard conditions and were then analyzed. The transfected mimic RNA, mimic control, miRNA inhibitor and inhibitor control were obtained from Thermo Scientific. For siRNA transfection we used the FlexiTube siRNA from QIAGEN that contained a pool of four specific siRNAs that target one gene.

Plasmid transfection

Transient transfection of plasmids was done using the reagent FuGene (Roche, Switzerland). 24 h before the treatment 1.0×10^5 cells were seed per 12-well plate. For luciferase assay cells were transfected with the corresponding renilla and luciferase constructs. Cells were harvested after 48 h of transfection.

Migration assay using xCELLigence system RTCA

For the migration assay the cells were transfected as described above. Cells were cultured in serum free medium for 24 h. After starving, 8×10^4 cells in 100 µl were seeded in the upper chamber of CIM-plates 16 containing serum free medium. In the lower chamber, the medium with serum was used as a chemoattractant. The xCELLigence RTCA (real-time cell analyzer) instrument measures migration in a label-free real-time setting. Electrical impedance (cell index, CI) increases when cells migrate through the 8 µm membrane pores and adhere to the electronic sensors (gold electrodes) on the bottom of the membrane. The continuous migration of the cells over 25 h was documented and analyzed in a time-resolved manner with the xCELLigence RTCA DP instrument and the RTCA Software 1.2 (Roche Diagnostics, Mannheim, Germany) was used to measure the CI values. Depicted are the values for every 2.5 h.

cDNA synthesis and real-time PCR

RNA from cultured cells was isolated using the RNA Easy Kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). Equal amounts of total cellular RNA (1 µg) were reverse-transcribed with random primer by the use of M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). Transcribed cDNAs were used for polymerase chain reaction (PCR) with specific primers for L1CAM (5′-GACTACGAGATCCACTTGTTTAAGGA-3′ and 5′-CTCACAAAGCCGATGAACCA-3′) and β-actin (5′-GGACTTCGA GCAAGAGATGG-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′). The PCR reactions were performed with the SYBRgreen Master Mix from Applied Biosystems in an ABI 7300 analyzer. Specific primers for

Fig. 1. miR-21-3p positively regulates L1CAM in renal cancer cell lines. **(A)** Relative qPCR of L1CAM to β-Actin expression in A498, Foehn and 786-0 cells that were transfected for 72 hours with mimics of miR-182, miR-124, miR-34a, miR-21-3p, miR-21-5p or control RNA (ctrl-RNA). **(B)** Relative qPCR of L1CAM to β-actin expression in A498, Foehn and 786-0 cells. **(C)** Relative qPCR of miR-21-3p to U48 expression in A498, Foehn and 786-0 cells. Western blot **(D)** and FACS **(E)** analyses of L1CAM expression in A498, Foehn and 786-0 cells that were transfected for 72 hours with miR-21-3p or control RNA (ctrl-RNA) (n = 3). Relative qPCR of L1CAM to β-actin expression in Foehn **(F)** and 786-0 **(G)** cells that were transfected for 24, 48 and 72 hours with miR-21-3p or control RNA (ctrl-RNA). **(H)** Western blot analysis for L1CAM and β-actin of Foehn and 786-0 cells that were transfected for 24, 48 and 72 hours with miR-21-3p or control RNA (ctrl-RNA). **(I)** Western blot analysis for L1CAM and β-actin in primary fibroblasts and HK2 cells that were transfected for 72 hours with miR-21-3p, control RNA (ctrl-RNA) or mock.