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Clinical implications of aldo-keto reductase family 1 member C3 and its relationship with lipocalin 2 in cancer of the uterine cervix

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HIGHLIGHTS

- Silencing of AKR1C3 gene increased LCN2 expression and decreased migratory and invasive abilities and changed cytoskeleton in cervical cancer cells.
- AKR1C3 over-expression inhibited LCN2 promoter activity and increased migration, then MMP-2 mRNA expression and activity increased in silenced LCN2 cells.
- Positive AKR1C3 and negative LCN2 predicted higher recurrence and poorer survival in patients with cervical cancer.

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ABSTRACT

Objective. Over-expression of the aldo-keto reductase family 1 member C3 (AKR1C3) has been demonstrated in many human cancers. Lipocalin 2 (LCN2) is reported to inhibit cervical cancer metastasis but little is known regarding its relationship with AKR1C3 in the development and progression of uterine cervical cancer. This study aimed to investigate the involvement of AKR1C3 and its relationship with LCN2 in cervical cancer.

Methods. The roles of AKR1C3 and LCN2 were investigated using the lentivirus shRNA system in SiHa and Caski cervical cancer cells. LCN2 and matrix metalloproteinase-2 (MMP-2) promoters were constructed to demonstrate transcriptional regulation by shAKR1C3 and shLCN2, respectively. The influences of metastatic phenotypes were analyzed by wound healing, Boyden chamber, and immunofluorescence assays. The activity of MMP-2 was determined by zymography assay. The impacts of AKR1C3 and LCN2 on patient prognosis were evaluated using tissue microarrays by Cox regression and Kaplan–Meier models.

Results. Silencing of the AKR1C3 gene increased the expression of LCN2 and decreased the migratory and invasive abilities and changed the cytoskeleton of cervical cancer cells. When AKR1C3 was over-expressed, it decreased LCN2 promoter activity and LCN2 expression and increased cell migration. The mRNA level and enzyme activity of MMP-2 increased in silenced LCN2 cells. Positive AKR1C3 and negative LCN2 were correlated with higher recurrence and poorer survival of cervical cancer patients.

Conclusions. Silencing of AKR1C3 increases LCN2 expression and inhibits metastasis in cervical cancer. Both AKR1C3 and LCN2 serve as molecular targets for cancer therapy to improve the clinical outcome of cervical cancer patients.

Introduction

The aldo-keto reductase family 1 member C3 (AKR1C3), also known as type 5 17β-hydroxysteroid dehydrogenase, belongs to the aldo-keto reductase enzymes of ~37 kDa [1]. AKR1C3 catalyzes the NAD(P)H-dependent reduction of carbonyl groups on a wide variety of substrates and has diverse physiologic roles. In humans, 13 isoforms of aldo-keto

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reductases have been found, including four isotypes (AKR1C1–AKR1C4) that reduce ketosteroids to hydroxysteroids [2]. In several recent studies, AKR1C has been shown to modify steroid hormones and prostaglandins, and has been implicated in the development of human cancer [3–5]. Its over-expression has also been demonstrated in many human cancers, such as breast and prostate cancers [6]. Furthermore, truncated human papillomavirus (HPV16) E6 transactivates AKR1C3 expression in cervical cancer cells [7]. AKR1C3 exhibits cisplatin resistance in colon cancer and radio-resistance in lung cancer [8,9].

LCN2, also called neutrophil gelatinase associated lipocalin (NGAL), is a 25 kDa secretory glycoprotein originally identified in human neutrophil granules [10]. LCN2 belongs to a large family of lipocalins, concerned with iron trafficking, induction of apoptosis, inflammation, cancer and cell proliferation and differentiation [11]. A previous investigation has demonstrated that increased LCN2 expression reduces the migratory and invasive potentials of cervical cancer cells [12]. Moreover, cervical cancer patients with high LCN2 levels have significantly less deep stromal invasion of uterine cervix and significantly better overall survival.

Previous studies indicate that AKR1C3 exerts anti-differentiation action, relying on its ability to perform an 11β-ketoreduction of prostaglandin D_2 (PGD_2) to 11β-PGF2α and prevent the ultimate generation of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), which inhibits NF-κB signaling and activates the peroxisome proliferator-activated receptor γ (PPAR-γ) [13]. The low expression of dihydrodiol dehydrogenase (DDH, AKR1C) in patients with squamous cell esophageal carcinoma correlates with significantly lower incidence of tumor recurrence and better survival compared to those with DDH over-expression [14].

Considering the roles of AKR1C3 and LCN2 in the metastatic phenotypes of cervical cancer and in patient prognosis, it is necessary to delineate their correlations and implications in cervical cancer. With the hypothesis that AKR1C3 and LCN2 interact inversely and are concerned with the metastatic potentials of cervical cancer cells, this study aimed to determine if AKR1C3 influenced LCN2 and was associated with cell motility, migration, invasiveness, and cytoskeleton. Molecular interrogation of these biomarkers points to the need for therapeutic intervention and further definition of the clinical implications of AKR1C3 and LCN2 in cervical cancer.

Materials and methods

Cell culture

SiHa and Caski cancer cell lines of the uterine cervix and human embryonic kidney cell line 293T were obtained from the American Type Tissue Culture Collection (ATCC; Rockville, MD, USA). The SiHa and 293T cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) supplemented by 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY). The Caski cell line was grown in Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco, Grand Island, NY) medium with 10% heat-inactivated FBS.

Lentivirus production and transduction

The 293T cells were transfected with 5 μg short hairpin RNA (shRNA) plasmid, 4 μg pCMVDR8.91, and 0.4 μg pMD.G by jetPEI DNA transfection reagent following the manufacturer's protocol (PolyPlus-transfection, 101-10; Strasbourg, France). Pre-designed shRNA and target sequences were purchased from the National RNAi Core Facility as follows: shAKR1C3 #564 (TRCN0000026564): 5′-CCGGAGTAAATTGC TAGATTT-3′; shAKR1C3 #350 (TRCN0000278350): 5′-CCGGAGTAAA TTGCTAGATTT-3′; shLCN2 #289 (TRCN0000060289): CCAGCATGCTAT GGTGTTCTT; shLCN2 #290 (TRCN0000060290): GTACTTCAAGATCACC CTCTA; shLuc (TRCN0000072246): CAAATCACAGAATCGTCGTAT; and shGFP (TRCN0000072178): CAACAGCCACAACGTCTATAT.

Plasmids and cell transfection

To establish the 3× flag-AKR1C3 over-expressing cervical cancer cell lines, nos. 6 and 9 AKR1C3 genes containing the full CDS (from +70 to +1041) were amplified using PCR and the following primer sets: 5′-primer, AKR1C3 (+70) 5′-GGATCCATGGATTCCAAACACCAGTG-3′ and 3′-primer, AKR1C3 (+1041) 5′-CTCGAGTTAATATTCATCTGAATATGG-3′.

Isolation of RNA, reverse transcription-PCR and quantitative real time PCR

Total cellular RNA was extracted from cells using RareRNA (Genepure Technology, Taiwan) according to the manufacturer's protocol. For PCR amplification, the primer sets were as follows: for AKR1C3, 5′-GTGCTCTGGGATCTCAACGAG-3′ (forward) and 5′-ACCTGCACGTTC TGTCTGATGC-3′ (reverse); for LCN2, 5′-GAGTTACCCTGGATTAACGA-3′ (forward) and 5′-CTCCTTTAGT TCCGAAGTCA-3′ (reverse); for β-actin, 5′-CAGGGAGTGATGGTGGGCA-3′ (forward) and 5′-CAAACATCATCTGG TCATCTTCTC-3′ (reverse).

Real time PCR was performed using the ABI PRISM 7000 real time PCR system with gene-specific primers and the Smart Quant Green Master Mix (Protech, Taipei, Taiwan).

Western blot analysis

An equivalent amount of total protein was processed by 12% SDS-PAGE and electroblotted onto Hybond ECL PVDF membranes, and incubated with primary antibodies against human AKR1C3 (ABGENT, San Diego, California, NP_003730), LCN2 (R&D Systems, Abingdon, UK, AF1757), and β-actin (Sigma, St. Louis, MO, A5441).

Wound healing assay

Seventy microliters of SiHa or Caski cells (3×10^5 cells/mL) was seeded and cultured onto 24-well plate with culture insert (ibidi GmbH, Munich, Germany). After 16 h incubation, the culture inserts were removed and appeared as linear "wound" in the cell monolayer of each well. The cells were photographed under a light microscope at the indicated times.

Cell migration and invasiveness assay

Cell migration and invasiveness assays were performed using modified Boyden chambers with 25 × 80 mm porous (12 μm size) polycarbonate membrane or 10× Matrigel precoated polycarbonate membrane (for cell invasiveness) using a 48 well chamber from Neuro Probe, Inc. The cells, which passed through the membrane, were stained in 20% Giemsa stain (Merck, Darmstadt, Germany). The cells on per well were in full photograph in microscopic fields at a magnification of ×40 and then were counted for the total migrated or invasion cells in triplicate.

Immunofluorescence

The shAKR1C3#350, #564, and shLuc of SiHa cells were seeded onto 24-well plates (5×10^4cells/well) with coverslips. After 24 h, the cells were fixed in a 3.7% paraformaldehyde–PBS solution, permeabilized with a solution of 0.1% Triton X-100 in PBS, and stained with Texas Red-X phalloidin (2 units/mL). Alexa Fluor 488 DNase I conjugate (9 μg/mL) was used to localize the filamentous actin (F-actin). The coverslips were mounted on a microscope slide with Prolong Gold anti-fade reagent with DAPI (Invitrogen, Paisley, Scotland).

Reporter assay

The 2×10^5 cells/well were seeded onto a 24-well plate and then transfected with 0.8 μg reporter plasmids (pGL3-basic, LCN2