

World Journal of *Clinical Oncology*

World J Clin Oncol 2019 June 24; 10(6): 222-246



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RESPONSIBLE EDITORS FOR THIS ISSUE

Responsible Electronic Editor: Yan-Xia Xing

Proofing Production Department Director: Yun-Xiaoqian Wu

NAME OF JOURNAL

World Journal of Clinical Oncology

ISSN

ISSN 2218-4333 (online)

LAUNCH DATE

November 10, 2010

FREQUENCY

Monthly

EDITORS-IN-CHIEF

Hiten RH Patel

EDITORIAL BOARD MEMBERS

<https://www.wjgnet.com/2218-4333/editorialboard.htm>

EDITORIAL OFFICE

Jin-Lei Wang, Director

PUBLICATION DATE

June 24, 2019

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<https://www.wjgnet.com/bpg/gerinfo/242>

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ONLINE SUBMISSION

<https://www.f6publishing.com>

Basic Study

Leptin-induced Notch and IL-1 signaling crosstalk in endometrial adenocarcinoma is associated with invasiveness and chemoresistance

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Supported by the National Cancer Institute at the National Institutes of Health, No. S21 MD000101, No. 5G12 MD0076021, No. G12 RR026250-03, No. NIH RR030341C06, No. RR18386 to Morehouse School of Medicine; the National Institute of General Medical Sciences, National Institutes of Health, No. 5T32HL103104-04 to DDB.

Conflict-of-interest statement: The

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Abstract**BACKGROUND**

Obesity is a recognized risk factor for endometrial cancer (EmCa) and other cancer types. Leptin levels are significantly increased in obese individuals. Leptin-induced signaling crosstalk [Notch, Interleukin-1 (IL-1) and leptin outcome, NILCO] has been associated with breast cancer progression. This complex signaling crosstalk affects cancer cell proliferation, migration, invasion, angiogenesis, apoptosis and chemoresistance. NILCO expression was previously detected in human EmCa. However, it is unknown whether leptin regulates NILCO and alters EmCa's response to chemotherapeutics. It is hypothesized that leptin induces NILCO and increases aggressiveness and chemoresistance in EmCa cells.

AIM

To determine whether leptin induces NILCO molecules in EmCa affecting cell proliferation, aggressiveness and chemoresistance.

METHODS

Leptin's effects on the expression of NILCO molecules [mRNAs and proteins for Notch receptors (Notch1-4), ligands (JAG1 and DLL4) and downstream effectors (survivin, Hey2), and leptin (OB-R) and IL-1 (IL-1R tI) receptors] was examined in EmCa cells (type I: Ishikawa, and HEC-1A, and type II: An3Ca and KLE) using Real-time PCR and Western blot analysis, respectively. In addition, the effects of leptin on cell cycle, proliferation and cell invasion were determined using cytometric analysis (Cellometer Vision CBA system), MTT cell proliferation and

authors declare to have no conflict of interest.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

Received: September 7, 2018

Peer-review started: September 7, 2018

First decision: October 26, 2018

Revised: March 14, 2019

Accepted: March 27, 2019

Article in press: March 28, 2019

Published online: June 24, 2019

P-Reviewer: Piché A, Luo WR, Bonanno E

S-Editor: Dou Y

L-Editor: A

E-Editor: Xing YX



Matrigel-based invasion assays, respectively. Inhibitors of leptin (nanoparticle-bound leptin peptide receptor antagonist-2, IONP-LPrA2), IL-1 (anti-IL-1R t1 antibody) and Notch (siRNA interference RNA) were used to investigate NILCO's effects on cell proliferation and invasion. Leptin's effects on Paclitaxel cytotoxicity in EmCa cells was determined by the CCK8 and Cellometer-based Annexin V assays.

RESULTS

For the first time it was shown that leptin is an inducer of Notch in EmCa. Experimental data suggest that leptin induced the expression of NILCO molecules, promoted proliferation and S- phase progression, and reduced Paclitaxel cytotoxicity on EmCa cells. Leptin's effects were higher in type II EmCa cells. The progression of this more aggressive form of the disease is associated with obesity. Remarkably, the use of the leptin signaling antagonist, IONP-LPrA2, re-sensitized EmCa cells to Paclitaxel.

CONCLUSION

Present data suggest the notion that leptin-induced NILCO could be a link between obesity and EmCa progression and chemoresistance. Most aggressive type II EmCa cells were higher sensitive to leptin, which appears to increase proliferation, cell cycle progression, aggressiveness, and chemoresistance to Paclitaxel. Therefore, leptin and NILCO could be novel therapeutic targets for type II EmCa, which does not have targeted therapy. Overall, IONP-LPrA2 has a potential as a novel adjuvant drug to enhance the effectiveness of type II EmCa chemotherapy.

Key words: Endometrial cancer; Leptin; Notch; Interleukin-1; Notch, IL-1 and Leptin crosstalk outcome; Chemoresistance

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Core tip: It's the first time we report that leptin induces Notch expression in endometrial cancer (EmCa) cells. Moreover, leptin induction of Notch, Interleukin-1 (IL-1) and leptin outcome (NILCO) molecules was higher in type II EmCa cells that showed increased aggressiveness and resistance to Paclitaxel's cytotoxic effects. Remarkably, the use of a leptin inhibitor, IONP-LPrA2, re-sensitizes EmCa cells to Paclitaxel that suggests targeting NILCO could be a new strategy to increase chemotherapeutic efficiency in type II EmCa that lacks targeted therapies.

Citation: Daley-Brown D, Harbuzariu A, Kurian AA, Oprea-Ilie G, Gonzalez-Perez RR. Leptin-induced Notch and IL-1 signaling crosstalk in endometrial adenocarcinoma is associated with invasiveness and chemoresistance. *World J Clin Oncol* 2019; 10(6): 222-233
URL: <https://www.wjnet.com/2218-4333/full/v10/i6/222.htm>
DOI: <https://dx.doi.org/10.5306/wjco.v10.i6.222>

INTRODUCTION

Endometrial cancer (EmCa) accounts for 7% of new cancer cases among women in the United States. In 2017, approximately 61400 EmCa cases have been reported with about 11000 women that succumbed to the disease^[1]. Obesity is a major risk factor for EmCa^[2,3]. Indeed, the incidence of EmCa is increasing in developing countries where obesity is on the rise^[4]. Obese individuals exhibit elevated serum levels of the adipokine leptin. Leptin's primary role is to signal as a long term satiety factor that helps to maintain the food intake and energy balance^[5]. However, leptin signaling controlling appetite is impaired in obese individuals, which suggests a kind of leptin-resistance status^[6]. Leptin is mainly secreted by adipocytes but also by the gastric mucosa, heart, placenta, and skeletal muscle. In addition, cancer cells secrete leptin that induces tumor proliferation, inflammation and angiogenesis in various cancer types^[6,7].

Previous studies have demonstrated that leptin up-regulates various signaling pathways involved in cancer progression^[8]. Studies have shown that leptin up-

regulates Interleukin-1 (IL-1) signaling in breast and EmCa cells, and Notch in breast and pancreatic cancer cells^[8-12]. In particular, leptin-induced Notch and IL-1 pathways are associated with breast cancer cell proliferation, migration, invasion as well as chemoresistance^[7,10]. Notch signaling is an evolutionarily conserved pathway that affects cell differentiation, proliferation and apoptosis across various cell types at different stages of development^[5]. Consequently, mutations of Notch genes can lead to many diseases within organs and tissues^[13]. Likewise, IL-1 is known to be up-regulated in many tumor types, where promotes tumor angiogenesis, growth and metastasis^[14]. A novel signaling crosstalk between leptin, Notch and IL-1 (NILCO) was associated with breast cancer progression^[10].

Chemotherapy is a standard therapy in multiple cancers. While chemotherapy is often capable of inducing apoptosis in cancer cells that eventually leads to a reduction in the tumor size, cancer recurrence is still a major issue. Recurrence rates often result in death because of treatment failure^[15].

NILCO expression was previously found in human EmCa^[16], but whether leptin can regulate its expression and whether is involved in EmCa proliferation, aggressiveness and chemoresistance remain to be determined. We hypothesize that NILCO is essential in the regulation of leptin-mediated induction of proliferation, migration/invasion and chemoresistance in EmCa cells, and that leptin's effects will be more evident in the more aggressive and poorly differentiated EmCa cells. In the present study, we found that leptin mediated the expression of Notch and IL-1 signaling components, which was higher in type II EmCa cells. In addition, NILCO impacted on the proliferation and invasiveness of type I and II EmCa cells. Moreover, leptin impaired paclitaxel cytotoxic effects on EmCa cells.

MATERIALS AND METHODS

EmCa cell lines and reagents

EmCa cell lines HEC-1A, KLE and An3Ca, and 1% penicillin-streptomycin solution were purchased from American Type Culture Medium (ATCC). EmCa Ishikawa cell line, monoclonal antibodies anti-Notch1 and GAPDH, dimethyl sulfoxide (DMSO) and other common reagents were obtained from Sigma (St. Louis, MO). Polyclonal Notch4, JAG1, IL-1R t1, and OB-R antibodies were obtained from Santa Cruz Biotechnology. Polyclonal anti-Notch2, Notch3 and DLL4 antibodies were purchased from Abcam. Monoclonal survivin antibody was obtained from Cell signaling, and anti-Hey2 antibody was purchased from Millipore. Vybrant 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation kit was from LifeTechnologies (Grand Island, NY). Cell Counting Kit- 8 (CCK-8) was from Dojindo Molecular Technologies Inc. (Japan). Leptin was purchased from R and D Systems (Minneapolis, MN). The leptin peptide receptor antagonist 2 (LPrA2) was synthesized and purified as previously described^[17]. LPrA2 was bound to iron oxide nanoparticles (IONP; Ocean Nanotech, San Diego, CA) as described elsewhere^[18,19]. Specific small interfering RNA (siRNA) for Notch1, Notch3 and Notch4 were obtained from (Qiagen). Annexin V/ Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI) for cell cycle analysis were obtained from Nexcelom Bioscience Boston, MA. Paclitaxel (PTX) was obtained from SelleckChem Houston, TX.

Cell cultures

Type I (Ishikawa, and HEC-1A) and type II EmCa cells (An3Ca and KLE) were cultured in Dulbecco's Modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS from Gemini Bioproducts) and 1% penicillin-streptomycin (ATCC) until they were 80% confluent. Then, cells were serum-deprived by culturing in basal medium for 24 h, and treated for additional 24 h in basal medium containing increasing leptin doses (0, 0.6, 1.2, and 6.25 nM, equivalent to 0, 10, 20 and 100 ng/mL, which characterized leptin levels in overweight, obese and morbid obese patients, respectively) and inhibitors of leptin (IONP-LPrA2), Notch (siRNA Notch1, Notch3 and Notch4) and IL-1 signaling (antibody anti-IL1 R t1) or with the chemotherapeutic paclitaxel.

Western blotting

Expression levels of Notch receptors, ligands and targets were determined by Western blotting (WB) analysis in EmCa cells cultured under different treatments. Cellular protein content after treatments was obtained using RIPA lysis buffer. Protein concentrations of cell lysates were determined using the Bradford Assay. Membrane blocking was performed using 5% non-fat milk in TBST for 30 min at room temperature. A 1:200 dilution of primary antibodies was used overnight at 4 °C and

incubated in properly diluted secondary antibody. Positive controls from Santa Cruz Biotech for each primary antibody were used for more accurate identification of specific antigens. WB results were normalized using GAPDH as loading control. Detection of antigen bands was displayed by WB chemiluminescent substrate (Thermo Fisher). The NIH Image program (Image J) was used for quantitative analysis of antigen bands. Representative data were derived from biological triplicates (mean \pm standard error; SE).

Real-time PCR

RNA was extracted and purified from An3Ca and Ishikawa cell cultures to synthesize cDNA to determine the expression of NILCO components *via* qPCR as previously described^[8]. The following primers (Invitrogen, Carlsbad, CA) were used: Notch1 forward: 5'-cactgtgggcggtcc-3' and reverse: 5'-gttgattggttcggcaccat-3'; Notch2 forward: 5'-aatccctgactccagaacg-3' and reverse: 5'-tggtagaccaagtctgtgatg-3'; Notch3 forward: 5'-tgaccgtactggcgagact-3' and reverse: ccgcttgctgcatcag-3'; Notch4 forward: 5'-tagggctcccgactctc-3' and reverse: 5'-ggcaggtgccccatt-3'; JAG1 forward: 5'-gactcatcagccgtgtctca-3' and reverse: 5'-tggggaacactcacactcaa-3'; DLL4 forward: tgctgctggtggcattt-3' and reverse: 5'-cttgtaggtgctgctgtt-3'; survivin forward: 5'-gcccagtggttcttctgctt-3' and reverse: 5'-cctccaaagtgtggtatt-3'; Hey2 forward: 5'-aaaaagctgaaatattgcaaat-3' and reverse: 5'-gtaccgcgaacttctgtt-3'. GAPDH forward: 5'-agggtgcttttaactctggt-3' and reverse: 5'-cccacttgatttggaggga-3'. qPCR conditions and relative expression values (R) were calculated as described previously^[12]. Representative data were derived from triplicates (mean \pm SE).

MTT cell proliferation assay

HEC-1A, Ishikawa, An3Ca, and KLE cells were seeded in 96-well plates (5×10^3 cells per well). Cells were serum-starved for 24 h and incubated for additional 24 h in medium containing several leptin concentrations. Then, 10 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/mL) were added to each well. After 4 h of incubation at 37 °C, cells were lysed by addition of 50 μ L DMSO per well. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices, CA)^[9].

Cell cycle assay

Ishikawa and An3Ca cells were cultured in 6 well plates and starved in serum-free medium as described above. After starvation, cells were treated with leptin (1.2 nM) and IONP-LPrA2 (0.0036 pM) for 24 h. The cells were trypsinized, washed with 1 \times PBS, and resuspended in cold 100% ethanol. Next, EmCa cells were fixed with 100% ethanol, washed and incubated with 50 μ L PI staining solution for 40 min at 37°C. The cells were centrifuged to remove the PI and resuspended in PBS. Then, cell cycle progression was analyzed using a Cellometer Vision CBA system (Nexcelom Biosciences, Lawrence, MA)^[8,9].

Blockade of IL-1 and leptin signaling

Ishikawa and An3Ca cells were serum-starved as described above and incubated with basal medium containing 1.2 nM leptin, 0.1 mg/mL rabbit IL-1R tI antibody and 0.0036 pM IONP-LPrA2 for 24 h. Notch expression levels were analyzed by WB as previously described.

Small interfering RNA

Specific small interfering RNAs (siRNA, Qiagen) were used to suppress the gene expression of Notch1, Notch3, and Notch4 in Ishikawa and An3Ca cells. Cells were seeded at a density of 1×10^5 /mL in 12 well plates and cultured in DMEM medium containing 10% FBS and 1% penicillin and streptomycin solution until they were 60% confluent. Conditioned media were removed, and cells were cultured for 6 h in serum-free basal medium containing Notch1, Notch3, or Notch4 oligonucleotides (10 nM) and controls. siRNAs were composed of four specific siRNA targets of 19-25 nucleotide length and negative controls (SiControl) included scramble siRNAs (Qiagen). After siRNA transfection, cells were cultured at 37°C for an additional 24 h in DMEM with 10% FBS and antibiotics. Cell lysates were obtained and analyzed for WB using specific antibodies to determine Notch1, Notch3, and Notch4 expression.

Cell invasion assay

Ishikawa and An3Ca cells (8×10^4) were transfected with siRNA for Notch1, Notch3 and Notch4 as described above. Cells were suspended in DMEM-serum free medium (basal medium) and added to the upper chamber of an insert coated with matrigel (6.4-mm diameter, 8-mm pore size; BD Biosciences). The inserts were placed in 24-well plates containing basal medium with or without leptin (1.2 nM). Invasion assays

were carried out for 24 h. Then, the cells in the upper side of the insert were whipped out with a cotton swab and cells in the lower side of the insert were fixed with 3.7% formaldehyde and stained with hematoxylin. Six randomly selected fields ($\times 10$ objective) were photographed, and the migrated cells were counted^[10].

PTX cytotoxicity

To assess whether leptin is a survival factor for EmCa treated with chemotherapeutics, Ishikawa and An3Ca cell lines were treated with Paclitaxel (PTX).

CCK8 and annexin IV assays

First, to determine the dose-cytotoxic effects of PTX in EmCa cells, Ishikawa and An3Ca cells were cultured in 96 well plates and starved as described and with treated different concentrations of PTX (50-1000 nM) in medium containing leptin (1.2 nM) and IONP-LPrA2 (0.0036 pM) for 3 d. Then, cells were incubated with 10 μ L per well of the reagent from Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc.) for 4 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, CA) to determine cell viability.

Additionally, apoptotic and viable cells were determined using the Annexin V FITC/PI Assay (Nexcelom) after treatment. Ishikawa and An3Ca cells were cultured in 6 well plates until approximately 80% confluency. Cells were starved as described above. Then, cells were treated with PTX as described above for 3 d. After that, cells were treated with Annexin V-FITC and propidium iodide (PI) staining solution. The number of apoptotic, necrotic and live cells were measured using Cellometer Vision CBA System (Nexcelom Biosciences, Lawrence, MA).

Statistical analysis

ANOVA and student *t*-test were used for data analysis. Representative results were performed in triplicate. Values for $P < 0.05$ were considered statistically significant based on the F-values and Tukey's multiple comparisons between group means as determined using SigmaPlot (Systat Software, Inc.). Mean + SE are indicated in the graphical analysis, based on replicates of densitometry analysis of WB, the percentage of cell cycle sub-phases, or percentage of proliferating cells as indicated in the figures.

RESULTS

Leptin induces Notch protein and mRNA expression in EmCa cells

Overall, leptin increased protein levels of Notch receptors, ligands and downstream targets in a dose dependent manner in EmCa cell lines (Figure 1). Remarkably, at least a two-fold increase in levels of Notch receptors, ligands and targets were seen after leptin treatment of poorly differentiated cell lines [An3Ca (Figure 1C) and KLE (Figure 1D)]. Notch1, Notch3, and Notch4 receptors, Notch ligands (JAG1 and DLL4) and Notch targeted molecules (survivin and Hey2) were also significantly increased but to lesser extent by leptin in more differentiated type I EmCa cells: Ishikawa (Figure 1A) and HEC-1A cells (Figure 1B). Similarly, leptin induced 1.2-2.4 and 2.0-5.5 fold mRNA expression of Notch receptors, ligands and targets in type I (Figure 2A) and type II EmCa cells (Figure 2B), respectively.

Leptin up-regulates IL-1R tI and OB-R expression in EmCa cells

In basal culture conditions, type II EmCa cell lines (Figure 3C and 3D) expressed significantly higher IL-1 (IL-1R tI) and leptin (OB-R) receptors than type I EmCa cells (Figure 3A and 3B). Leptin increased more the expression of OB-R and IL-1R tI in type II EmCa cells (2.7-3.7 fold) compared to type I EmCa cells (1.6-2.1 fold) (Figure 3).

Leptin induces cell cycle progression and proliferation of EmCa cells

Leptin significantly increased S-phase progression (Figure 4A) and proliferation (Figure 4B) of EmCa cells. However, leptin-induced S-phase progression was higher in type II EmCa cells (2.0-fold increase) compared to type I EmCa cells (1.5-fold increase) (Figure 4A). Moreover, type I EmCa cells were less responsive to leptin-induced proliferation (2.0-2.7 fold) compared to type II EmCa cells (3.0-3.5 fold) (Figure 4B).

Inhibition of IL-1R tI decreases leptin-induced Notch expression in EmCa cells

Next, we assessed whether a functional crosstalk between leptin and IL-1 signaling could be involved in the regulation of Notch expression in EmCa cells. The blockade of IL-1 signaling by an anti-IL-1R tI antibody significantly reduced leptin-induced up-regulation of Notch receptors and ligands in type I (Figure 5A) and type II EmCa cells (Figure 5B). To further assess the specificity of leptin's effects in EmCa, we

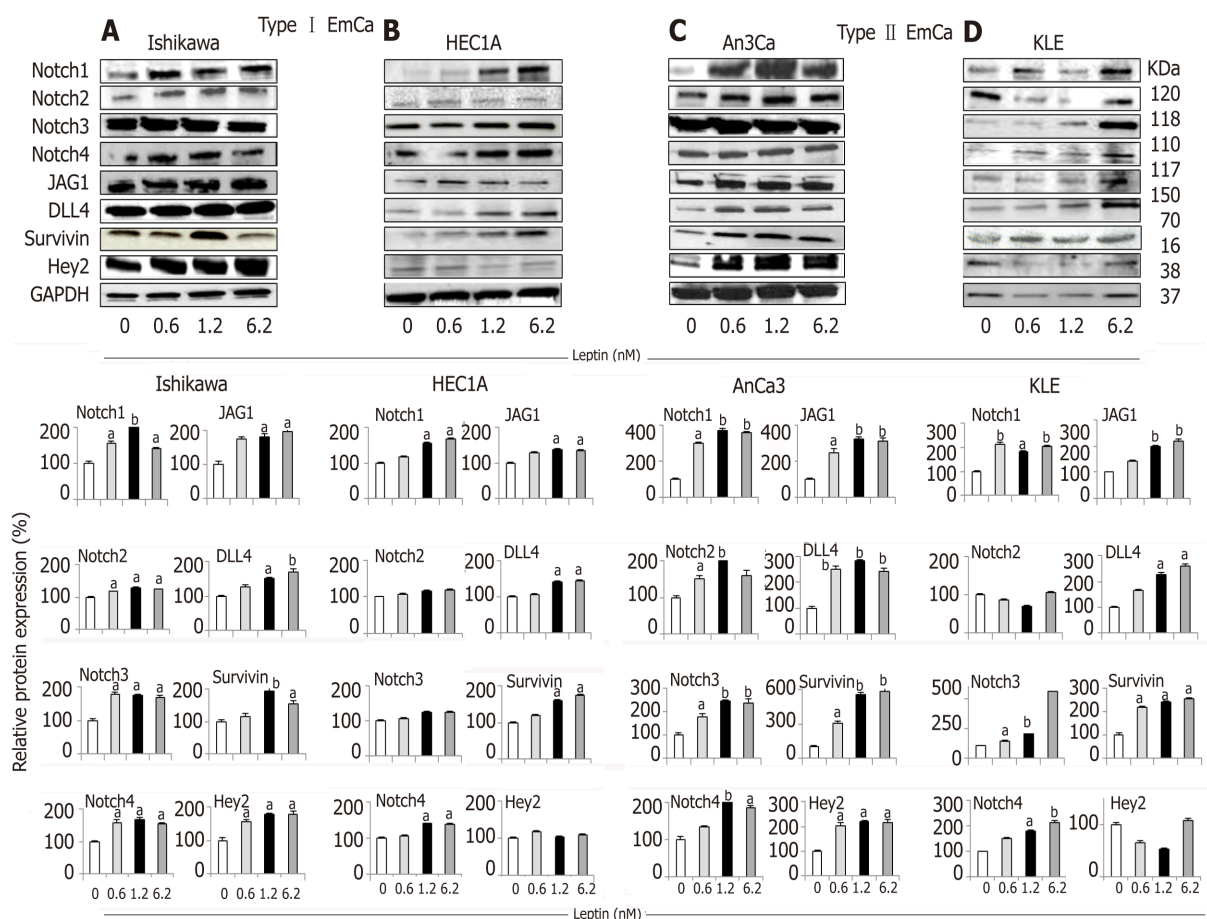


Figure 1 Leptin-induced Notch protein expression in endometrial cancer cells. A-D: Representative results from Western Blot (WB) analysis of leptin dose-response effects on levels of Notch proteins in A and B [type I endometrial cancer (EmCa) cells] and B and C (type II EmCa cells). Leptin increases Notch protein levels (receptors: Notch1, Notch2, Notch3 and Notch4; ligands: JAG1 and DLL4 and targets: Survivin, Hey2) in type I EmCa (A, Ishikawa and B, HEC1A) and type II EmCa cells (C, An3Ca and D, KLE); Cells were cultured for 24h. Quantitative WB data (relative protein expression %) were calculated from densitometric analysis of bands with the NIH image program. The values were normalized to GAPDH as protein loading control. Data (mean \pm SE) are representative results derived from a minimum of three independent experiments. ^a $P < 0.05$ and ^b $P < 0.01$ vs basal (no leptin).

determined whether the inhibition of leptin signaling via IONP-LPrA2 could affect Notch expression. IONP-LPrA2 abrogated leptin-induced effects on Notch expression in EmCa cells (Figure 5A and 5B). These results indicate that a functional crosstalk between leptin, IL-1 and Notch (NILCO) occurs in EmCa.

Notch signaling is involved in leptin-induced EmCa cancer cell invasion

We previously showed that the addition of DAPT (an inhibitor of gamma-secretase, the rate-limiting enzyme of Notch activation) reduced leptin-induced EmCa migration^[16]. Similarly, leptin-induced migration of EmCa was abrogated by using anti IL-1R tI antibodies in EmCa cells^[16]. To additionally examine the role of NILCO in EmCa, we determined whether leptin-induced cell migration is affected by the specific inhibition of Notch signaling via SiRNA. It was further accessed that leptin significantly induced invasion of EmCa cells that was more evident in the more aggressive An3Ca cells (type II EmCa). Notably, leptin-induced cell invasion was significant reduced by Notch siRNA knockdown (data not shown). However, no significant differences were found by silencing specific Notch receptors.

Leptin reduces PTX cytotoxicity of EmCa cells

PTX significantly reduced the viability of Ishikawa (PTX EC₅₀ approximately 392 nM; Figure 6A) and An3Ca cells (PTX EC₅₀ approximately 356 nM; Figure 6B). Remarkably, the addition of leptin significantly increased the number of live cells treated with PTX [type I EmCa (PTX-leptin EC₅₀ approximately 458 nM; Figure 6A) and type II EmCa (PTX-leptin EC₅₀ approximately 445 nM; Figure 6B)]. Interestingly, IONP-LPrA2 re-sensitized EmCa cells to the cytotoxic effects of PTX. Moreover, the addition of IONP-LPrA2 could allow for using a reduced quantity of PTX to achieve EC₅₀ dose in both cell lines [type I EmCa (PTX-leptin-IONP-LPrA2 EC₅₀

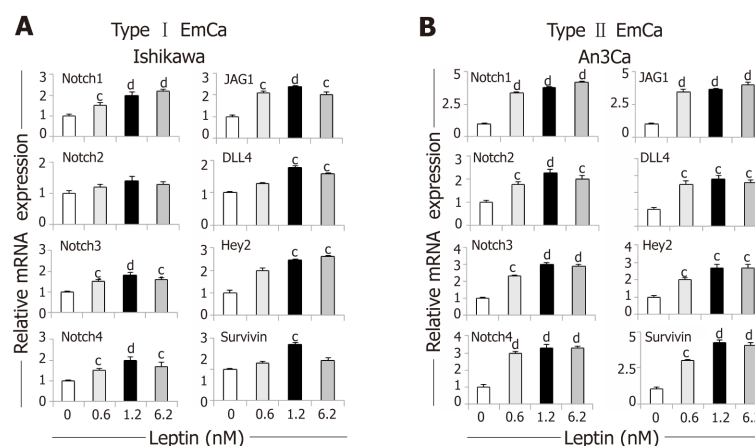


Figure 2 Dose-response effects of leptin on the levels of Notch mRNA in endometrial cancer cells. A, B: Notch mRNA levels (receptors: Notch1, Notch2, Notch3 and Notch4; ligands: JAG1 and DLL4 and targets: Survivin, Hey2) induced by leptin (0, 0.6, 1.2 and 6.2nM) in type I endometrial cancer (EmCa) (A, Ishikawa) and type II EmCa cells (B, An3Ca). Cells were cultured for 24h. Notch mRNA levels were quantified by real-time RT-PCR. Cells were cultured for 24h. RNA expression was calculated by normalizing values to GAPDH mRNA. Data (mean \pm SE) representative results derived from a minimum of 3 independent experiments. ^c $P < 0.05$ and ^d $P < 0.01$ vs basal (no leptin).

approximately 167 nM; **Figure 6A**) and type II EmCa (PTX-leptin-IONP-LPrA2 EC50 approximately 240nM; **Figure 6B**)]. These results suggest a potential for the use of IONP-LPrA2 as a chemotherapeutic adjuvant for EmCa.

DISCUSSION

We earlier reported that the expression of NILCO molecules in EmCa tissues was associated with obesity. Furthermore, type II EmCa tissues expressed higher NILCO molecules, which suggests the signaling crosstalk is linked to the progression of the more aggressive EmCa phenotype^[6]. However, whether leptin signaling could induce Notch expression and oncogenic changes in EmCa cells is poorly understood^[20-22]. Here we found that leptin is an inducer of Notch and a survival factor for EmCa cells. Moreover, NILCO was linked to proliferation, invasion and drug resistance (PTX), especially in type II EmCa.

Although, the categorical classification of EmCa in two major types (type I and II) it is still a point of discussion, endometrioid adenocarcinoma is known as type I EmCa, which is a more differentiated cancer that resembles normal endometrial morphology. Approximately 85% of all EmCa are type I and have better prognosis. Type I EmCa shows higher incidence and dependence of hormonal stimuli. In contrast, type II EmCa shows more aggressive phenotype, poor prognosis and is associated with high recurrences. Type II EmCa comprises less differentiated tumors showing lower incidence, but their growth and progression are independent of sex hormone stimuli. Additionally, there are other less abundant EmCa types that include the mixed mesenchymal Mullerian malignant tumor (MMMT) or carcinosarcoma, mucinous, clear cell, squamous cell, mixed and undifferentiated^[2].

Obesity is a risk factor for EmCa, albeit through not very well-defined mechanisms^[2,3]. High adiposity is accompanied with deregulated levels of many molecules (*i.e.*, estrogen, leptin, leptin induced-molecules, Notch, cytokines and growth factors) that affect EmCa progression. Basal-like breast cancer and type II EmCa present similar genomic features and lack targeted therapies^[2,14].

We have previously shown that the crosstalk between leptin, Notch and IL-1 can induce important oncogenic actions in breast cancer. Moreover, a functional leptin-Notch axis was also reported essential for pancreatic cancer progression and growth^[5,8,9]. Aberrant Notch activation has been reported in many solid tumors. However, Notch signaling pathway can exert divergent impact on cancer tissues. Indeed, it is known that Notch signaling may be oncogenic or suppressive in different tumors^[5]. Accumulating evidence suggest a role for Notch signaling in EmCa^[2,15]. However, the role of Notch in EmCa is still poorly understood. We hypothesize that NILCO could be a link between obesity and EmCa progression.

Here, we present data supporting the notion that leptin can induce the expression

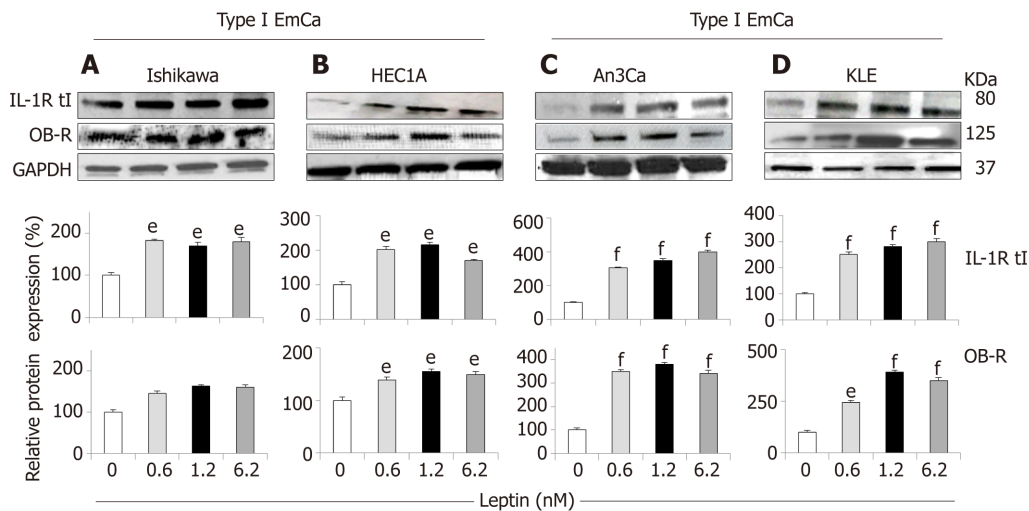


Figure 3 Leptin-induced IL-1R tI and OB-R protein expression in endometrial cancer cells. A-D: Representative results from Western Blot (WB) analysis of leptin dose-response effects on levels of IL-1R tI and OB-R proteins in A and B [type I endometrial cancer (EmCa) cells] and B and C (type II EmCa cells). Leptin increases IL-1R tI and OB-R protein levels in type I EmCa (A, Ishikawa and B, HEC1A) and type II EmCa cells (C, An3Ca and D, KLE). Cells were cultured for 24h with leptin (0, 0.6, 1.2 and 6.2nM). Quantitative WB data (relative protein expression %) were calculated from densitometric analysis of bands with the NIH image program. The values were normalized to GAPDH as protein loading control. Data (mean \pm SE) are representative of the results derived from a minimum of three independent experiments. ^e $P < 0.05$ and ^f $P < 0.01$ vs basal (no leptin).

of NILCO molecules in EmCa affecting cell proliferation, aggressiveness and chemoresistance. Present data suggest that leptin is an inducer of Notch [Notch receptors (Notch1-4), ligands (JAG1 and DLL4) and downstream effectors (survivin, Hey2)] and leptin (OB-R) and IL-1 (IL-1R tI) receptors in EmCa cells. Leptin showed higher impact on the poorly differentiated and more aggressive cell lines, An3Ca and KLE, which resemble type II EmCa. Remarkably, leptin treatment significantly increased mRNA expression of NILCO molecules in type II EmCa cells. Additionally, leptin also increased the expression of Notch1, Notch3, and Notch4 receptors in the more differentiated EmCa, HEC-1A and Ishikawa cells that resemble type I EmCa.

It was earlier reported that OB-R expression correlated with estrogen (ER) and progesterone receptor expression in EmCa^[23] that suggests a potential crosstalk between leptin and estrogen signaling could occur in type I EmCa, which is more dependent on hormonal cues. We earlier reported that type II EmCa expresses higher levels of OB-R^[16]. Present data show that OB-R and ER are co-expressed in EmCa. Additionally, we have previously found that leptin upregulates the IL-1 system in EmCa cells^[11]. Here we extended these observations by showing that leptin greater increased OB-R and IL-1R tI expression in type II EmCa cells. Consequently, these cells were more responsive to leptin stimulus than type I EmCa cells in term of cell cycle progression, proliferation and invasion. Interestingly, as it was previously reported in breast^[11], pancreatic^[8,9], and some EmCa cells^[16], leptin's effects on cell invasion were dependent of Notch signaling. Therefore, NILCO could be related to type II EmCa progression^[5].

Surgery is the main treatment for EmCa. Among the more significant poor prognostic factors identified for EmCa treatment failure are initial stage II to IV, type 2 histology, positive cytology, and recurrence at multiple sites^[24]. Adjuvant chemotherapy using single agent or combination is advised for metastatic, recurrent, or high-risk EmCa. PTX and combination therapies with platinum drugs or anthracyclines are commonly prescribed for EmCa aggressive disease^[25]. Type II EmCa are independent of ER and PR signaling, very aggressive, have poor prognosis, and no targeted therapies^[2]. Therefore, type II EmCa are mainly treated with chemotherapeutics, which eventually reduce their effectiveness due to the development of drug resistance. We hypothesize that leptin could be an important survival factor for EmCa treated with chemotherapeutics. Indeed, leptin's actions significantly reduced PTX cytotoxicity on EmCa cells. Moreover, inhibition of leptin signaling via IONP-LPrA2 re-sensitized EmCa to PTX's effects and allowed its dose reduction while achieving similar cytotoxicity on EmCa. These results encourage the notion for a potential use of leptin signaling inhibitors (*i.e.*, IONP-LPrA2) as chemotherapeutic adjuvants for EmCa.

In conclusions, we report here for the first time that leptin is an inducer of Notch in EmCa. Leptin and Notch are part of a complex crosstalk (NILCO) that involves IL-1

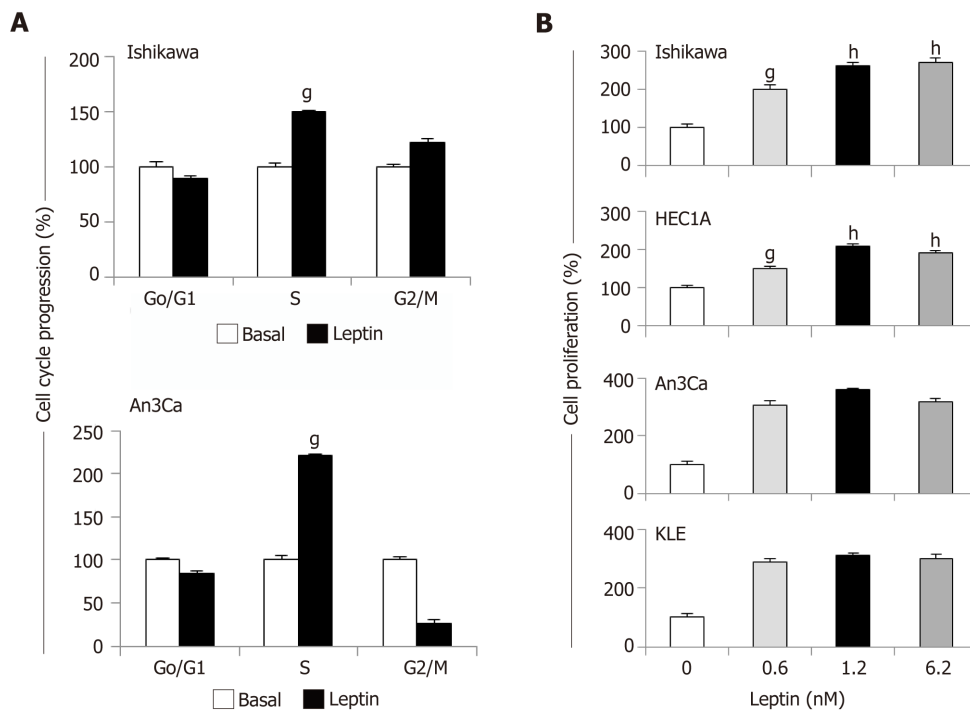


Figure 4 Leptin induces cell cycle progression and proliferation of endometrial cancer cells. A: Leptin induces S-phase progression of endometrial cancer (EmCa) cells. Cells were cultured with leptin (0 and 1.2nM) for 24 h. Cell cycle progression was determined by image cytometry using a Cellometer Vision CBA system (Nexcelom Biosciences, Lawrence, MA); B: Results of MTT assay from EmCa cells (type I: Ishikawa and HEC1A, and type II: An3Ca and KLE) cultured with leptin (0, 0.6, 1.2 and 6.2 nM) for 24 h. Absorbance was determined at 540 nm, and data was evaluated with Spectramax software. Data (mean \pm SE) are representative of the results derived from a minimum of three independent experiments. ^g $P < 0.05$ and ^h $P < 0.01$ vs basal (no leptin).

signaling. Obesity (characterized by high levels of leptin) is a modifiable risk factor for EmCa that is linked to poor treatment outcome^[2]. Thus, present data suggest that leptin through the induction of NILCO can increase EmCa aggressiveness and proliferation, and reduce PTX efficacy in type II EmCa cells, which showed higher responsiveness to leptin by overexpressing OB-R and Notch. Remarkably, the inhibition of leptin signaling re-sensitized EmCa cells to PTX. Since a strong association between central obesity and EmCa^[3] has been found, present observations support the hypothesis that strategies leading to the inhibition of leptin signaling and NILCO in EmCa could be of paramount importance to design new adjuvant treatments to boost chemotherapy outcomes, and reduce chemotherapeutic dosage, especially in obese EmCa patients.

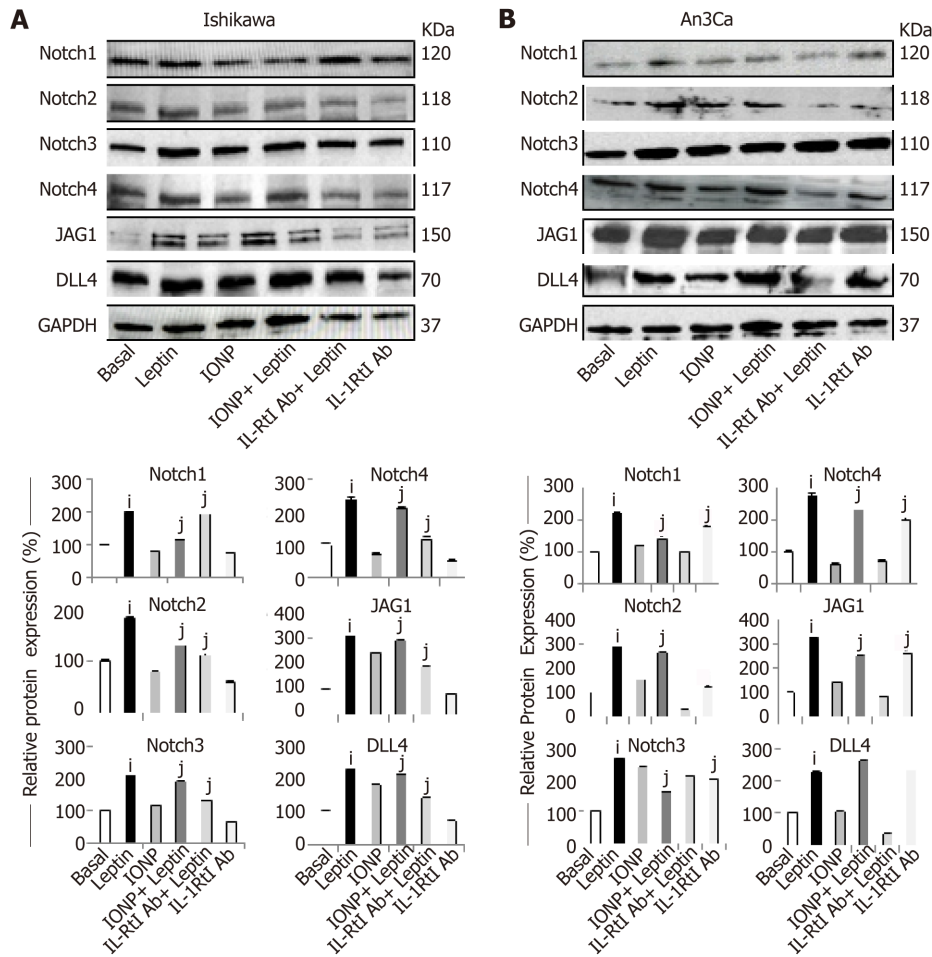


Figure 5 Inhibition of IL-1 R tI abrogates leptin induction of Notch in endometrial cancer cells. A, B: Representative results from Western Blot (WB) analysis of the effects of IL-1R tI antibodies on leptin-induced levels of Notch proteins (receptors: Notch1, Notch2, Notch3 and Notch4, and ligands: JAG1 and DLL4) in Ishikawa [A, type I endometrial cancer (EmCa)] and An3Ca cells (B, type II EmCa). Cells were cultured for 24h with leptin (0 and 1.2nM), leptin signaling inhibitor IONP-LPrA2 (IONP), and IL-1R tI antibodies (IL-1R tI Ab). Quantitative WB data (relative protein expression %) were calculated from densitometric analysis of bands with the NIH image program. The values were normalized to GAPDH as protein loading control. Data (mean \pm SE) are representative of the results derived from a minimum of three independent experiments. ⁱ $P < 0.05$ and ^j $P < 0.01$ vs basal (no leptin).

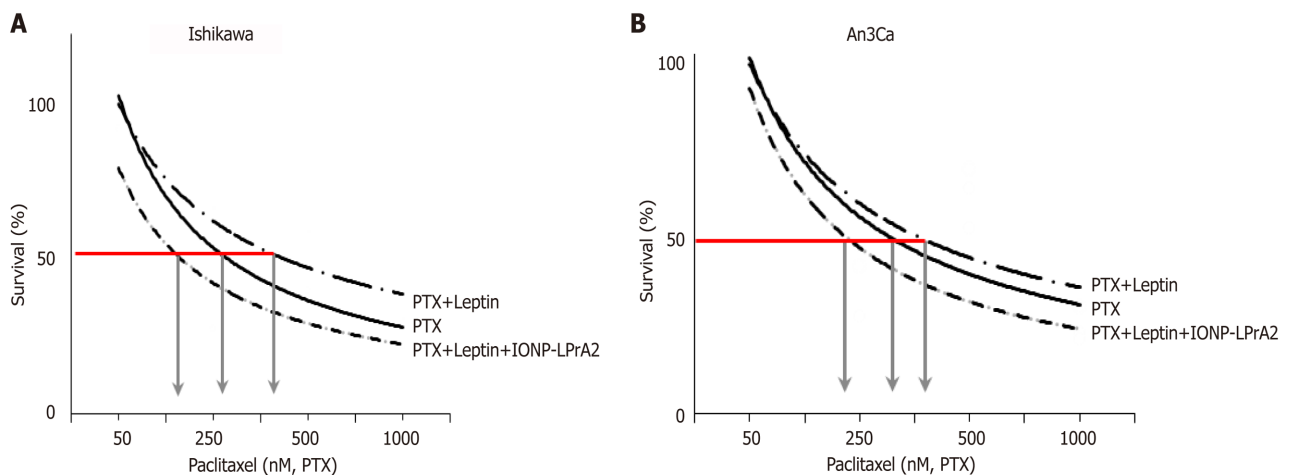


Figure 6 Leptin increases survival of endometrial cancer cells treated with paclitaxel. A, B: Dose-response effects of paclitaxel (PTX) on survival of Ishikawa (A) and An3Ca cells (B) cultured with leptin (0 and 1.2nM) and leptin signaling inhibitor (IONP-LPrA2; 0.0036pM) for 3 d. The relative number of live cells (Survival% compared to basal) was calculated using Annexin V/FITC/PI assay by image cytometry using a Cellometer Vision CBA system (Nexcelom Biosciences, Lawrence, MA) and CCK8 assay (Cell counting kit-8 (Dojindo Molecular Technologies, Inc., Japan). The red line indicates the PTX EC50 calculated for each cell line. Arrows indicate PTX concentrations required to reduce 50% cell viability. Note that EC50 for PTX increases when leptin was added but was reduced when IONP-LPrA2 was added. Data (mean \pm SE) are representative of the results derived from a minimum of three independent experiments.

ARTICLE HIGHLIGHTS

Research background

The expression of Notch, Interleukin-1 (IL-1) and leptin outcome (NILCO) molecules (mRNAs and proteins) was previously detected in breast cancer and endometrial cancer (EmCa) from African-American and Chinese patients. Although, obesity status of Chinese patients was unknown, it looked like that NILCO was higher expressed in obese patients. However, NILCO molecules were expressed higher in type II EmCa, regardless of ethnic background or obesity status of patients. Leptin levels are high in obese patients that may suggest this adipokine is involved in the progression of the more aggressive EmCa phenotype (type II) and chemoresistance.

Research motivation

EmCa is the most frequent gynecological malignancy of the female reproductive tract and is the fourth most commonly diagnosed new cancer among women in the United States. Hormone nonresponsive breast cancer and type II EmCa have no targeted therapies, are mainly treated with chemotherapeutics and eventually develop drug resistance. Because leptin is a known inducer of NILCO in breast cancer and has been related to chemoresistance, it was hypothesized that comparable leptin's actions could occur in EmCa. The validation of this hypothesis may suggest that NILCO plays essential roles in tumor progression and chemoresistance, and thus, may represent a new EmCa target, particularly for type II EmCa.

Research objectives

To investigate whether leptin mediates the expression of NILCO signaling components, and impairs paclitaxel cytotoxic effects, and whether leptin's proliferative, invasion and chemoresistant actions are more prominent in type II EmCa cells.

Research methods

Two representative type I and type II (more aggressive and estrogen independent) EmCa cell lines were investigated for the potential leptin regulation of NILCO mRNA and proteins [Notch receptors, ligands and downstream effectors, and leptin (OB-R) and IL-1 (IL-1R tI) receptors] *via* Real-time PCR and Western blot analysis. Leptin's proliferative and invasion effects were assessed by cytometric analysis (Cellometer Vision CBA system), and MTT and Matrigel-based invasion assays. NILCO inhibitors included nanoparticle-bound leptin peptide receptor antagonist-2 (IONP-LPrA2), anti-IL-1R tI antibody and Notch siRNA. The CCK8 assay was used to investigate leptin-mediated Paclitaxel drug resistance. Additionally, apoptotic and viable Paclitaxel-treated EmCa cells were determined by the Annexin V FITC/PI Assay (Nexcelom).

Research results

Leptin increased at least two-fold mRNA and protein levels of Notch receptors, ligands and downstream targets, and almost four-fold protein levels of OB-R and IL-1R tI in a dose dependent manner, mainly in type II EmCa cells. Leptin stimulated higher the progression of cell cycle, and the proliferation and invasion of type II EmCa cells, which were Notch-signaling dependent. The inhibition of IL-1R tI impaired the effects of leptin on Notch. Abrogation of Notch signaling *via* siRNA negatively affected leptin-induced EmCa invasiveness. Additionally, leptin acted as a survival factor for EmCa cells by significantly reducing the cytotoxic effects on Paclitaxel, which was more prominent in type II EmCa cells. The inhibition of OB-R *via* IONP-LPrA2 allowed the resensitization of EmCa cells to Paclitaxel. Thus, IONP-LPrA2 has a potential as a novel neo-adjuvant that may allow reducing Paclitaxel dosage and its undesirable side effects.

Research conclusions

For the first time, it was found that leptin is an inducer of Notch and targets in EmCa. Leptin-induced NILCO could be specifically related to the progression, invasiveness and drug resistance of type II EmCa. Moreover, obesity could increase the progression of EmCa and the development of chemoresistance *via* leptin signaling and NILCO, which may be greater for type II EmCa. Leptin-induces NILCO, which could be a common signaling crosstalk that stimulates the progression and chemoresistance of several obesity-related cancers. Present data suggest that NILCO plays essential roles in tumor progression and chemoresistance, and thus, may represent a new EmCa target, particularly for type II EmCa.

Research perspectives

In vitro data suggest that EmCa requires leptin signaling and NILCO for proliferation and invasion, and to increase drug resistance and survival. Future research should investigate the role of NILCO in EmCa progression and chemoresistance using animal models. Spontaneous, syngeneic, xenograft and PDX EmCa-mouse models should be used to validate the hypothesis tested in the present paper.

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Metastatic potential and prognostic significance of SOX2: A meta-analysis

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Author contributions: Javaeed A performed the literature search, study design and conception and data extraction, and provided final approval of the manuscript; Ghauri SK performed the data extraction, analysis and interpretation of the data, and drafting of the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Manuscript source: Unsolicited manuscript

Received: January 21, 2019

Revised: March 31, 2019

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Abstract

BACKGROUND

SOX2 is a regulator of pluripotent cellular transcription, yet it has been recently integrated in cancer biology. The present study provides an analytic insight into the correlation of SOX2 overexpression with cancer metastasis and patient survival.

AIM

To investigate the association of SOX2 overexpression with metastasis and its implication in the prognosis of cancer patients.

METHODS

A meta-analysis was conducted including studies that compared the association of low or high SOX2 expression with lymph node metastasis (LNM) and/or distant metastasis (DM). The following data were additionally extracted: survival, including the overall survival (OS) and disease-free survival (DFS), and prevalence of high and low SOX2 expression. Odds ratios (commonly known as ORs) and their respective 95% confidence intervals (CIs) were used to investigate the association between SOX2 expression and LNM and DM, while hazard ratios (commonly known as HRs) and 95% CIs were applied to evaluate the prognostic markers.

RESULTS

In a total of 2643 patients (60.88% males), the pooled prevalence of SOX2 overexpression was 46.22% (95%CI: 39.07%-53.38%) in different types of cancer. SOX2 overexpression significantly correlated with DM (OR = 1.79, 95%CI: 1.20-3.25, $P < 0.008$) compared to low SOX2 expression. In subgroups analyses, a high SOX2 expression was associated with LNM in cancers of the lung, breast, and colon and associated with DM in hepatic, head and neck, and colon cancers. SOX2 overexpression was also associated with a shorter OS (HR = 1.65, 95%CI:

Accepted: April 8, 2019
Published online: June 24, 2019

P-Reviewer: Kim HS, Huo Q,
 Morelli F, Zhou J
S-Editor: Dou Y
L-Editor: A
E-Editor: Xing YX



1.34-2.04, $P < 0.001$) and DFS (HR = 1.54, 95% CI: 1.14-2.08, $P = 0.005$).

CONCLUSION

A remarkable role of SOX2 overexpression was observed in cancer biology and metastasis. However, many questions in the regulatory pathways need to be addressed to reveal as many functional aspects as possible to tailor new targeted therapeutic strategies.

Key words: Tumor progression; SOX2; Cancer; Cancer stem cell markers; Lymphatic metastasis

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Core tip: SOX2 overexpression is associated with both lymph node and distant metastasis predominantly in cancers of the colon and head and neck. Targeting the biological pathways of SOX2 seems to provide a promising rationale for developing relevant therapeutic interventions.

Citation: Javaeed A, Ghauri SK. Metastatic potential and prognostic significance of SOX2: A meta-analysis. *World J Clin Oncol* 2019; 10(6): 234-246

URL: <https://www.wjgnet.com/2218-4333/full/v10/i6/234.htm>

DOI: <https://dx.doi.org/10.5306/wjco.v10.i6.234>

INTRODUCTION

The mechanisms by which the metastatic cascade is instigated and developed have merited the attention of the researchers worldwide. Tumor metastasis involves a sequential process, comprising cell invasion through the basement membrane and extracellular matrix followed by intravasation to access the vascular or lymphatic circulation, extravasation out of the circulatory system, and eventually distant localization and proliferation^[1]. Failure to manage distant dissemination of cancer cells causes a significant mortality burden, with approximately 1500 cancer spread-related deaths reported daily^[2]. A plethora of studies has been conducted on animal models to outline the metastatic phenotype. These studies usually employ intravenous injections for metastatic initiation. However, such an approach lacks the appropriate characterization of the origin of metastasis and, thus, multiple triggering factors are either still hypothesized or their contribution in metastasis is not fully delineated.

Of these factors, the transcription factor sex determining region Y-box 2 (SOX2) has been recently integrated in cancer biology^[3]. SOX2 is one of the SOX proteins that has $\geq 50\%$ similarity in the amino acid structure to a specific domain (HMG domain) of the sex-determining region located on the Y chromosome (*Sry*)^[4]. The encoding gene was initially discovered in 1994 in human, being located on chromosome 3q26.3-q27^[5]. Heterozygous mutations of the *SOX2* gene result in the development of anophthalmia syndrome, which includes an aberrant development of endodermal and ectodermal tissues^[6]. SOX2 is an important regulator of pluripotent cellular transcription and is a crucial factor implicated in the development and maintenance of undifferentiated embryonic stem cells. Besides, it contributes to somatic cell reprogramming back towards pluripotent features and this process was shown to be co-induced by ectopic expression of *cMyc*, *Klf4*, and *Oct4*^[3].

Intriguingly, this discovery highlighted the role of SOX2 expression in different types of cancers. *SOX2* gene amplification or increased expression has been demonstrated in malignancy, yet its involvement in the most important aspects of patient survival and metastasis remains relatively unclear. Herein, we provide analytic insight into the association between metastasis and SOX2 expression in cancer patients, along with its relation to the prognostic profile.

MATERIALS AND METHODS

A meta-analysis was conducted on studies that investigated the relationship between

development of a metastatic phenotype as well as the prognostic significance of such expression. The general outline of the study was based on the guidelines in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (commonly known as PRISMA)^[7].

Eligibility criteria

The included studies investigated at least the association of SOX2 expression, reported as “low” or “high” expression levels, and either lymph node metastasis (LNM) or distant metastasis (DM). When appropriate, patients’ prognosis should have been evaluated using survival analysis, where the overall survival (OS) and/or disease-free survival (DFS) were reported. Only peer-reviewed studies conducted on male or female human patients were considered. The cancer patients should have been diagnosed using pathological examinations. Studies were excluded if they provided insufficient data to be extracted or had employed animal subjects solely. Furthermore, non-English articles, case reports, reviews and cell experiments were omitted.

Information sources

The following scientific databases were used for the search process: MEDLINE, Embase, Cochrane Library, and Google Scholar. Additionally, the bibliographies of the screened articles were thoroughly searched for eligible studies.

Search strategy

Two authors performed the search process using the following key words: (“transcription factor sex determining region Y-box 2” OR “SOX2”) AND (“cancer” OR “metastasis”) AND (“prognosis” OR “survival” OR “hazard”). The publication dates of the included studies were limited to those articles published between 2010 and 2018, to attain the most recent evidence. Screening of the titles and abstracts was performed and the relevant articles were obtained for subsequent assessment of the full-texts. Any disagreement was resolved by discussion.

Data collection

A specifically-designed form was used in Microsoft Excel spreadsheet software (version 2016) to extract data. The extracted data for each study were categorized into four main categories: (1) Study and patients’ characteristics, including the name of the first author, publication date, methods of detection of SOX2 expression, method of survival analysis, reported outcomes, number of samples, type of primary cancer, sex of patients, frequency of SOX2 expression (low or high), and clinical stage (I and II or III and IV); (2) Primary outcomes, including the frequency of reported samples with low or high SOX2 expression in the regional lymph nodes (*i.e.* LNM) or distant organs (*i.e.* DM); (3) Clinicopathological parameters, including SOX2 expression levels according to patients’ age, sex, clinical stage, and tumor size and differentiation; (4) Prognostic data, including direct extraction of the hazard ratios (HRs) and their respective 95% confidence intervals (CIs) of the OS and/or DFS. When both univariate and multivariate analyses were used for survival analysis, multivariate tests were preferentially used.

Quality assessment

A specialized scale for quality assessment was used [*i.e.*, Newcastle-Ottawa scale (commonly known as NOS)]^[8], which employs a scoring system comprising nine items and yielding a total score of 0 to 9 for low-quality to high-quality articles, respectively. The assignment of such scores is based on the selection, comparability, and outcomes of the groups under investigation. A score of ≥ 6 indicated a high-quality study.

Statistical analysis

The following formula was used for calculation of the prevalence rate of high SOX2 expression: (number of samples with high SOX2 expression/total number of samples) $\times 100$; the standard error and 95% CIs were calculated as described previously^[9]. The RevMan 5.3 software was used for statistical analysis (Review Manager, the Cochrane Collaboration, Oxford, United Kingdom). For dichotomous data (*e.g.*, LNM, DM and clinicopathological data), odds ratios (ORs) and their respective 95% CIs were applied. Regarding prognostic markers (OS and DFS), HRs and their respective 95% CIs were integrated in the meta-analysis. Statistical heterogeneity was assessed using the I^2 statistical test, which was interpreted as a significant heterogeneity at $I^2 > 50\%$. In such instance, a random effects model was applied. On the other hand, a fixed effects model was used when the heterogeneity was insignificant. Subgroup analyses were performed according to sample size, the type of primary cancer, and quality score.

RESULTS

Results of the search process

Figure 1 shows the outcomes of the employed search process. The initial search yielded a total of 1120 records, from which 45 records were considered duplicates, while an additional 5 records were identified from the bibliographies of the resultant records. Therefore, 1080 records were screened for eligibility through their titles and abstracts. Subsequently, the full-text versions of 23 articles were examined for inclusion. Ultimately, 20 studies were included in the meta-analysis.

Characteristics of the included studies

Table 1 demonstrates the main characteristics of the included studies. A total of 2643 patients (60.88% males) were investigated for eight types of cancer, including cancers of the breast^[10,11], cervix^[12], colon^[13], esophagus^[14-16], head and neck^[17-23], liver^[24], lung^[25], and stomach^[26-29]. The sample sizes ranged between 20 and 307 patients. Excluding three studies in which the clinical stage was not reported^[10,13,24], 1265 patients (54.98%) were at stage III or IV. Immunohistochemical staining was utilized in all studies to detect SOX2 expression. With respect to outcomes, only two studies did not report LNM^[12,17], while DM was reported in 11 studies^[12-14,17,19,20,22-24,26,27], OS in 11 studies^[12,15-23,26], and DFS in 3 studies^[17-19]. Two studies were performed in European countries^[13,19], one in Africa^[10], and the remaining studies in Asia. All of the included studies scored a NOS equal to or greater than 6, indicating the inclusion of high-quality studies.

Prevalence of high SOX2 expression and its relationship to the clinicopathological parameters

The pooled prevalence of high SOX2 expression in different types of cancer was 46.22% (95%CI: 39.07%-53.38%) with a significant heterogeneity among the included studies ($I^2 = 94\%$). Subgroup analysis was performed to detect the sources of heterogeneity, yet it remained significant for most of the subgroups. As for the clinicopathological parameters, SOX2 expression was not significantly correlated to patient's age, sex and clinical stage as well as the size and differentiation of tumors (**Table 2**).

Relationship between SOX2 expression and metastasis

Although applying a random effects model yielded no remarkable association between high SOX2 expression and LNM (**Figure 2A**), the relationship was significant in the subgroup analyses for specific types of tumors, including cancers of the colon (OR = 4.15, 95%CI: 1.43-12.09, $P = 0.009$), breast (OR = 2.78, 95%CI: 1.50-5.50, $P = 0.002$, $I^2 = 0\%$), and lung (OR = 1.74, 95%CI: 1.02-2.89, $P = 0.04$) (**Table 3**).

Interestingly, SOX2 was highly expressed in distant metastatic tumors (OR = 1.79, 95%CI: 1.20-3.25, $P < 0.008$; **Figure 2B**) despite the existence of a significant heterogeneity between studies ($I^2 = 57\%$). Such relationship was also consistent for hepatic (OR = 4.80, 95%CI: 1.56-14.76, $P = 0.006$), colon (OR = 3.04, 95%CI: 1.15-8.04, $P = 0.03$), and head and neck tumors (OR = 2.46, 95%CI: 1.63-3.72, $P < 0.001$) (**Table 3**).

Relationship between SOX2 expression and patient prognosis

As compared to low SOX2 expression, high SOX2 expression was significantly associated with shorter OS and the studies showed no heterogeneity (HR = 1.65, 95%CI: 1.34-2.04, $P < 0.001$, $I^2 = 44\%$; **Figure 3A**). Similarly, the included studies showed a significantly shorter DFS when SOX2 was highly expressed without heterogeneity (HR = 1.54, 95%CI: 1.14-2.08, $P = 0.005$, $I^2 = 15\%$; **Figure 3B**).

DISCUSSION

Cancer biology involves a number of hallmarks that enable cellular division and spread, including continued proliferative signaling, initiation and maintenance of metastasis, and evasion of cell death^[30]. SOX2 has exhibited various roles in these hallmarks *via* promoting cellular proliferation through the induction of cyclin D3 transcription and enabling S-phase entry^[31] as well as evading apoptotic signals by precluding *ORAI1* expression and subsequently reducing store-operated Ca^{2+} entry^[32]. The present meta-analysis showed that SOX2 contributed to the development of a metastatic phenotype to distant organs and its high expression was associated with shorter OS and DFS in patients with cancer.

SOX2 is one of the embryonic cell fate determinants which have been linked to increasing tumor aggressiveness, metastasis, and poor prognosis. In addition to SOX2,

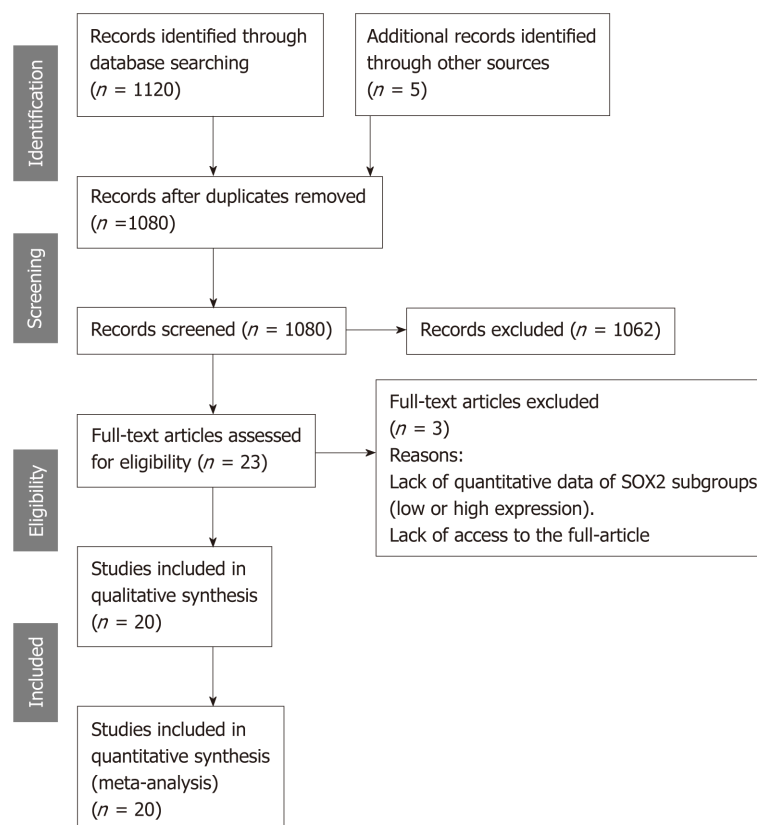


Figure 1 Flow diagram showing the search process used in the present study.

these factors, namely the Yamanaka factors^[3], include *Oct4*, *Klf4*, and *Myc*, among others. SOX2 determines distinct cell fate decisions during embryonic development *via* antagonization of CDX2, NKX2-1, MITF, and other tissue-specific factors^[33]. In cancer, evidence from experimental studies has revealed different mechanisms of SOX2-mediated metastasis. Girouard *et al*^[34] showed that the transduction of G361 cells, which normally express low SOX2 levels, to enhance SOX2 overexpression led to a 3.8-fold increase in invasiveness ($P = 0.0004$) and, on the other hand, SOX2 knockdown in melanoma cells resulted in a significant reduction of invasiveness and diminished matrix metalloproteinase (MMP)-3 expression by 87.8%. Furthermore, invasion and migration of cancer cells in colorectal cancer (CRC), malignant glioma, and laryngeal squamous cell carcinoma were attributed to MMP-2-mediated effects and this was associated with SOX2 overexpression^[35-37]. Therefore, in the current study, it was not surprising that SOX2 overexpression was associated with LNM and DM in colon cancer and DM in head and neck cancer. MMP-2 is a type of IV collagenase that has been implicated previously in carcinogenesis and metastasis^[38]. In general, MMPs comprise a family of zinc-dependent endopeptidases that degrade extracellular matrix proteins and they are involved in metastasis *via* several aspects, including tumor invasion, angiogenesis, and establishing metastatic foci^[39].

In hepatocellular carcinoma, SOX2 induced cell invasion and hence was associated with DM. SOX2 expression was low in non-carcinogenic liver cells, moderate in non-invasive cells, and highest in the invasive cells^[24]. Such an effect is mediated by enhancing cancer stemness properties through activation of the epithelial-to-mesenchymal transition (EMT) process, which is characterized by remarkable activation of distinct transcriptional factors, including Snail, Twist, and Slug. The SOX2-Slug axis has been consistently reported not only in hepatocellular carcinoma but also in esophageal squamous cell carcinoma and muscle-invasive bladder cancer^[24,40,41]. SOX2-mediated Slug expression was suppressed by inhibition of STAT3/HIF-1 α signaling using siRNA, indicating a role of such pathway in the co-expression of SOX2 and Slug^[40]. Nonetheless, the exact regulatory mechanisms between EMT and SOX2 and other stemness-related transcriptional factors, considering their contribution to cancer metastasis, have yet to be clearly revealed. Through its common activating effect on EMT, SOX2 exerts its metastatic potential in CRC *via* a different mechanism. Activation of the WNT pathway, which is thought to

Table 1 Characteristics of the included studies

Study	YOP	Patients, M/F/T	SOX2 expression, high/low	Clinical stage, I+II/ III +IV	Cancer type	Outcomes	Survival analysis	NOS
Abd El-Maqsoud <i>et al</i> ^[10]	2014	0/126/126	42/84	NA	IBC	LNM	NA	7
Chen <i>et al</i> ^[26]	2016	59/18/77	28/49	26/51	GC	DM, LNM, OS	Mu	8
Chen <i>et al</i> ^[27]	2016	233/74/307	96/211	95/212	GC	DM, LNM	NA	8
Chuang <i>et al</i> ^[14]	2015	72/3/75	40/35	21/27	ESCC	DM, LNM	NA	6
Dai <i>et al</i> ^[17]	2014	75/56/131	82/49	77/54	ACC	DM, OS, DFS	Mu	7
Ge <i>et al</i> ^[18]	2010	84/1/85	67/18	21/64	HSCC	LNM, OS, DFS	Mu	7
González-Márquez <i>et al</i> ^[19]	2014	203/17/220	74/146	24/196	SCC	DM, LNM, OS, DFS	Mu	6
Liu <i>et al</i> ^[23]	2018	35/26/61	39/22	46/15	TSCC	DM, LNM, OS	Mu	7
Liu <i>et al</i> ^[11]	2018	0/237/237	122/115	167/70	BC	LNM	NA	7
Luo <i>et al</i> ^[20]	2013	92/30/122	68/54	33/89	NPC	DM, LNM, OS	Mu	6
Neumann <i>et al</i> ^[13]	2011	58/56/114	24/90	NA	CC	DM, LNM	NA	7
Saigusa <i>et al</i> ^[15]	2011	18/2/20	8/12	10/10	ESCC	LNM, OS	U	7
Shen <i>et al</i> ^[12]	2014	0/132/132	83/49	70/62	CSCC	DM, OS	Mu	8
Sun <i>et al</i> ^[24]	2013	65/10/75	46/29	NA	HCC	DM, LNM	NA	6
Tang <i>et al</i> ^[21]	2013	152/9/161	88/73	65/96	LSCC	LNM, OS	Mu	8
Wang <i>et al</i> ^[16]	2018	87/30/117	54/63	48/69	ESCC	LNM, OS	Mu	7
Wang <i>et al</i> ^[28]	2015	132/71/203	48/155	65/138	GC	LNM	NA	6
Yang <i>et al</i> ^[25]	2013	140/82/222	124/98	173/49	SCLC	LNM	NA	7
Yoshihama <i>et al</i> ^[22]	2016	69/39/108	63/45	64/44	OSCC	DM, LNM, OS	Mu	7
Zhang <i>et al</i> ^[29]	2010	35/15/50	16/34	31/19	GC	LNM	NA	6

ACC: Adenoid cystic carcinoma of the salivary gland; CC: Colon cancer; CSCC: Cervical squamous cell carcinoma; DFS: Disease-free survival; DM: Distant metastasis; ESCC: Esophageal squamous cell carcinoma; F: Female; GC: Gastric cancer; HCC: Hepatocellular carcinoma; HSCC: Hypopharyngeal squamous cell carcinoma; IBC: Invasive breast cancer; LNM: Lymph node metastasis; LSCC: Laryngeal squamous cell carcinoma; M: Male; Mu: Multivariate; NOS: Newcastle-Ottawa scale; NPC: Nasopharyngeal carcinoma; OS: Overall survival; OSCC: Oral squamous cell carcinoma; SCLC: Small cell lung cancer; T: Total; TSCC: Tongue squamous cell carcinoma; U: Univariate; YOP: Year of publication.

be critical for CRC tumorigenesis, was demonstrated in CRC and cisplatin-resistant lung adenocarcinoma cells^[35,42].

In contrast to our results, in ovarian cancer cells, SOX2 overexpression increased phosphorylation of Src and FAK proteins^[43], both of which are well-established as prometastatic proteins through their abilities to mediate EMT^[44]. Additionally, SOX2 functionality was linked to the Hedgehog signaling pathway in controlling different hallmarks of prostate cancer, such as reduction of apoptotic death, increasing cellular proliferation, and augmenting the metastatic ability^[45]. This was also confirmed by the effects of SOX2 silencing, where it lowered the migration capabilities of different prostate cancer cell lines by 11%-31%^[45]. Similarly, SOX2 gene knockout or silencing led to loss of tumorigenic properties and reduced cellular proliferation in lung cancer^[46], glioblastoma^[47], and pancreatic cancer^[31]. Paradoxically, SOX2 overexpression was found in gastric cancer and could contribute to cellular invasion^[48], while it was found to be significantly downregulated in the same type of cancer in other studies^[28,49,50].

In the present meta-analysis, SOX2 correlated with poor prognosis in different types of cancers. Other investigations have also indicated higher rates of recurrence and that its expression was associated with the parameters of worse outcome in primary cancers of the head and neck^[51]. Furthermore, SOX2 was associated with advanced tumor stages in head and neck adenoid cystic carcinoma^[52]. However, we failed to prove such a relationship, possibly because the included studies had to report at least one parameter of metastasis (LNM and/or DM) in addition to the prognostic data. Thus, several prognosis-relevant studies might have escaped inclusion.

Table 2 Pooled effect of the relationship between SOX2 expression (high vs low) and the clinicopathological parameters of cancer patients in the included studies

Variable	Studies	Model (I^2 %)	OR (95%CI)	P-value
Age	17	F (0)	1.07 (0.90-1.28)	0.43
Sex	16	F (0)	1.13 (0.90-1.41)	0.29
Clinical stage	18	R (79)	1.51 (0.97-2.37)	0.07
Tumor size	9	F (27)	1.06 (0.84-1.33)	0.64
Tumor differentiation	14	R (54)	1.19 (0.80-1.75)	0.39

CI: Confidence interval; F: Fixed effects model; OR: Odds ratio; R: Random effects model.

Given that SOX2 expression was detected not only in the nuclei of cancer cells but also in the their cytosols in hepatic cancer, pancreatic cancer and CRC^[24,32,35], it is plausible that the intranuclear existence might explain its transcriptional role; yet, its functional role in the cytosol remains only fairly understood. In light of the confirmed transcriptional roles, functional-depletion of SOX2, *via* knockdown approaches using siRNA, is possible experimentally; although, its clinical application is challenging. Alternatively, researchers should strive to identify the possible regulatory mechanisms of SOX2 to tailor distinct targeted therapies accordingly.

In conclusion, SOX2 has exhibited a significant metastatic potential in various types of cancer, including colorectal, head and neck, liver and breast. It can also be regarded as a poor prognostic indicator, and recurrence may be expected in patients with SOX2 overexpression. Different transcriptional regulation mechanisms of SOX2 are involved, yet promotion of the EMT was apparently of paramount importance. Implementing targeted therapies aimed to counteract high SOX2 expression might be beneficial to treat this deadly disease.

Table 3 Subgroup analysis of the association between SOX2 expression (high vs low) and LMN and DM

Variable	LMN				DM			
	Studies	Model (I^2)	OR (95%CI)	P-value	Studies	Model (I^2)	OR (95%CI)	P-value
Sample size								
< 150	13	R (83)	1.85 (0.96-3.56)	0.06	12	F (47)	2.16 (1.61-2.91)	< 0.0001
≥ 150	5	R (85)	0.93 (0.43-2.01)	0.11	1	N/A	0.24 (0.05-1.15)	0.007
Cancer type								
Head and neck	6	R (88)	1.89 (0.72-4.96)	0.19	7	F (39)	2.46 (1.63-3.72)	< 0.0001
Esophagus	3	R (79)	0.98 (0.24-4.04)	0.98	1	N/A	0.56 (0.09-3.57)	0.54
Cervix	-	-	-	-	1	N/A	3.12 (0.99-9.82)	0.05
Stomach	4	R (85)	0.68 (0.21-2.24)	0.53	2	R (56)	0.56 (0.16-1.96)	0.36
Breast	2	F (0)	2.87 (1.50-5.50)	0.002	-	-	-	-
Liver	1	N/A	1.68 (0.39-7.32)	0.49	1	N/A	4.80 (1.56-14.76)	0.006
Lung	1	N/A	1.74 (1.02-2.89)	0.04	-	-	-	-
Colon	1	N/A	4.15 (1.43-12.09)	0.009	1	N/A	3.04 (1.15-8.04)	0.03
Quality score								
≥ 7	12	R (88)	1.64 (0.82-3.27)	0.16	7	R (69)	2.23 (1.04-4.77)	0.04
< 7	6	R (79)	1.38 (0.60-3.18)	0.45	6	F (35)	1.71 (0.89-3.27)	0.11

DM: Distant metastasis; F: Fixed effects model; LNM: Lymph node metastasis; OR: Odds ratio; R: Random effects model.

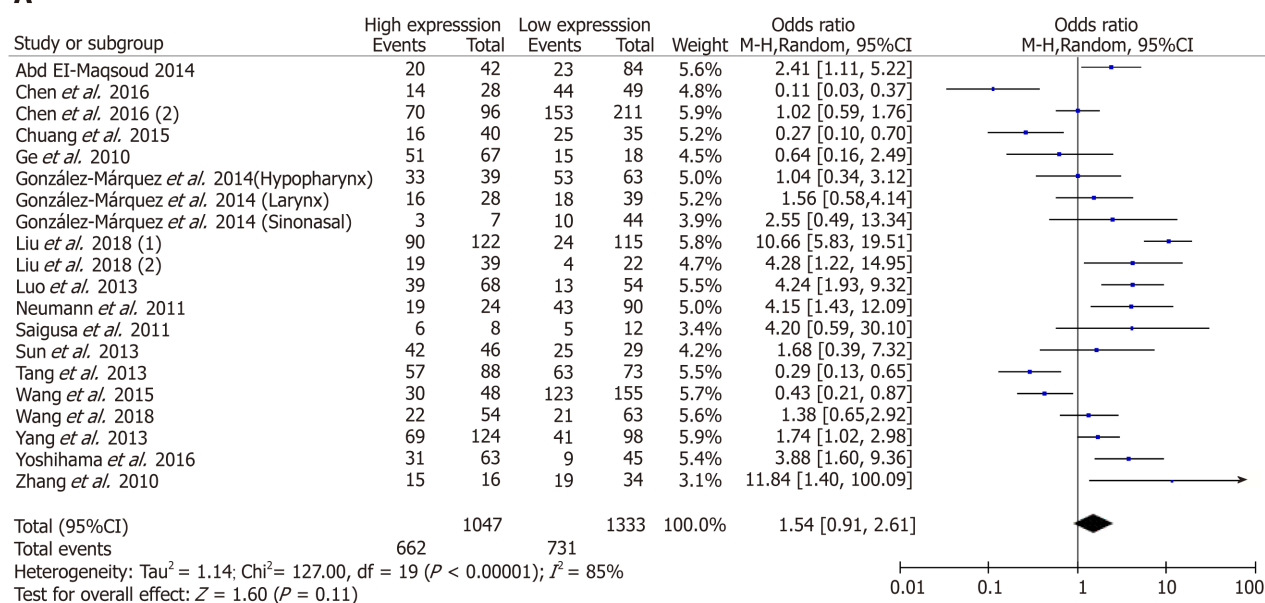
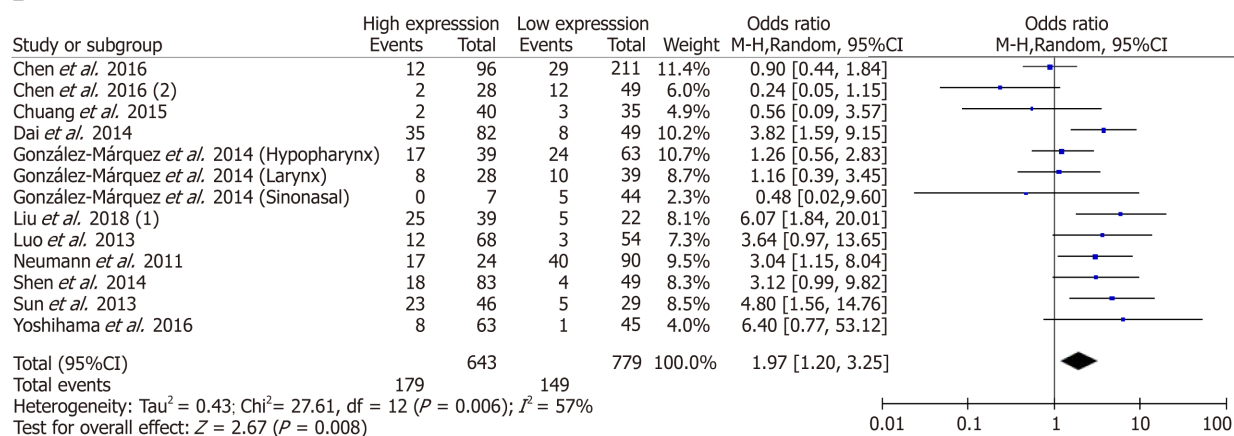
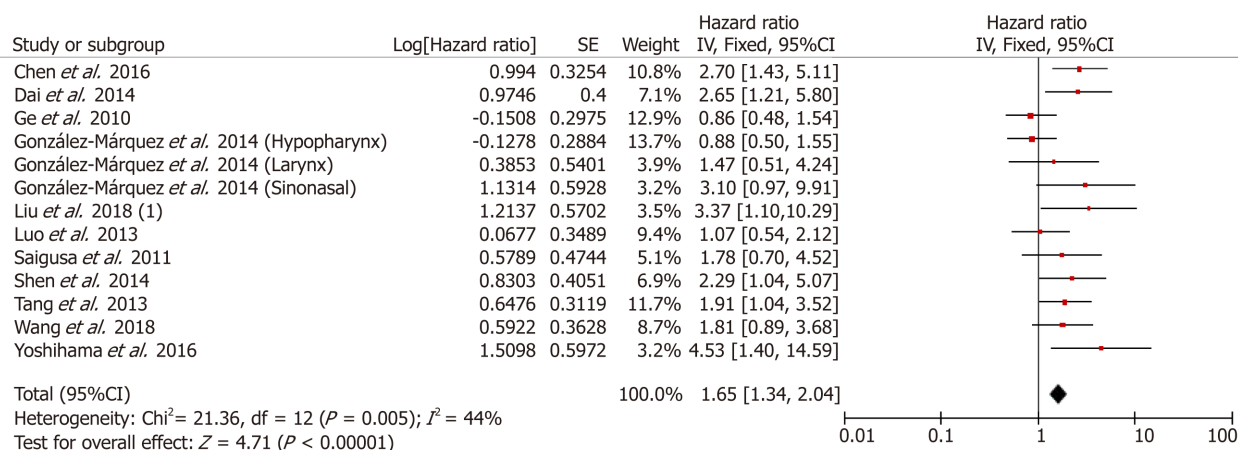
A**B**

Figure 2 Forest plot of the association between SOX2 expression and lymph node metastasis and distant metastasis. A: Lymph node metastasis; B: Distant metastasis.

A



B

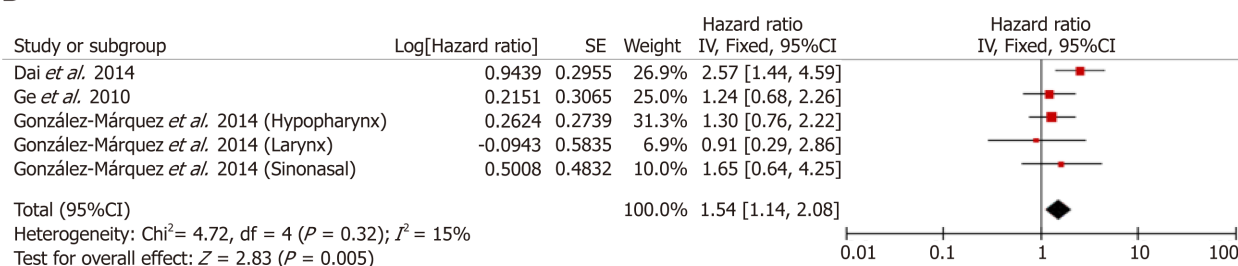


Figure 3 Forest plot of the association between high SOX2 expression and lymph node metastasis and distant metastasis. A: Lymph node metastasis; B: Distant metastasis.

ARTICLE HIGHLIGHTS

Research background

SOX2 is a significant regulator of pluripotent cellular transcription and it helps in the reprogramming of somatic cells to the pluripotent properties. The involvement of SOX2 in cancer biology has been recently demonstrated.

Research motivation

Metastasis comprises the most important aspect of cancer-related mortality. Linking SOX2 expression to metastasis and subsequently to patient's survival may open novel horizons for the implementation of future therapeutic strategies that target SOX2 biological pathways.

Research objectives

To investigate the association between SOX2 overexpression and the development of a metastatic phenotype as well as the survival patterns of patients with increased SOX2 expression.

Research methods

A meta-analysis was conducted, including studies that recruited patients with different types of cancer and reporting SOX2 expression as either "low" or "high", and evaluating patient survival using the relevant analytical methods [overall survival (OS) and/or disease-free survival (DFS)]. A comprehensive search of articles published between 2010 and 2018 was performed in distinct scientific databases.

Research results

A total of 20 studies involving 2643 patients (60.88% males) were included. SOX2 overexpression was significantly associated with distant metastasis (odds ratio = 1.79, 95%CI: 1.20-3.25, $P < 0.008$), while it was associated with lymph node metastasis only in subgroup analyses of cancers of the colon, breast, and lung. Both OS and DFS were shorter in patients expressing high SOX2, as compared to those with low SOX2 expression (hazard ratio = 1.65, 95%CI: 1.34-2.04, $P < 0.001$ and hazard ratio = 1.54, 95%CI: 1.14-2.08, $P = 0.005$, respectively).

Research conclusions

The present study adds a comprehensive insight into the significant role of SOX2 in distant metastasis of different types of cancers and its correlation to poor prognosis rather than the

outcomes obtained by individual studies. In line with the increased research interest in SOX2, we showed that it can be used as a prognostic marker in cancer patients, while, on the other hand, new therapeutic strategies are urgently needed to target the biological pathways implicated in SOX2 overexpression for more effective cancer treatment.

Research perspectives

Targeting SOX2 expression in cancer regimens is warranted. Future research studies should focus on developing novel drugs as well as the identification of the cut-off values of poor prognosis.

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