OLR1 Promotes Pancreatic Cancer Metastasis via Increased c-Myc Expression and Transcription of HMGA2

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ABSTRACT

Pancreatic cancer is one of the most lethal human malignancies, partly because of its propensity for metastasis. However, the mechanisms of metastasis in pancreatic cancer remain unclear. Oxidized low-density lipoprotein receptor 1 (OLR1), a lectin-like scavenger receptor that recognizes several ligands, such as oxidized low-density lipoprotein, was previously reported in cardiovascular and metabolic diseases. The role and mechanism of OLR1 in pancreatic cancer is unclear. In this study, we found that OLR1 expression was significantly higher in pancreatic cancer tissues than that in adjacent normal tissues and closely associated with reduced overall survival. OLR1 promoted proliferation and metastasis of pancreatic cancer cells in vitro and in vivo. Mechanically, OLR1 increased HMGA2 transcription by upregulating c-Myc expression to promote the metastasis of pancreatic cancer cells. In addition, patients with pancreatic cancer with high expression of OLR1–c-Myc–HMGA2 axis showed worse prognosis compared with patients with low expression of OLR1–c-Myc–HMGA2 axis.

Implications: Our findings suggested that the OLR1–c-Myc–HMGA2 axis promotes metastasis of pancreatic cancer cells and may serve as potential therapeutic targets and prognosis markers for patients with pancreatic cancer.

Introduction

Pancreatic cancer is the fourth and sixth leading cause of cancer-related death in the United States and China, respectively, with an overall survival (OS) rate of less than 9% in both countries (1, 2). Furthermore, the mortality rate of pancreatic cancer is increasing, and pancreatic cancer is projected to be the second most common cause of cancer-related death by 2030 in the United States (3). Because of the atypical clinical symptoms of pancreatic cancer, the majority of patients already show metastases at diagnosis and thus are unable to be treated by radical operation (4). Despite treatment with surgical resection, most patients with pancreatic cancer eventually develop metastases because of recurrence or chemoresistance. The current understanding of the underlying mechanism of metastasis in pancreatic cancer remains limited. Therefore, clarifying the metastasis mechanisms is important for developing new treatment strategies for pancreatic cancer and prolonging patient survival.

Oxidized low-density lipoprotein receptor 1 (OLR1) is a lectin-like scavenger receptor that recognizes several ligands, such as oxidized low-density lipoprotein, polyanionic chemicals, and anionic phospholipids (5). OLR1 is mainly distributed in vascular endothelial cells and tissues with abundant blood vessels such as renal, pulmonary, and neuronal tissues. Previous studies on OLR1 mainly focused on its role in cardiovascular and metabolic diseases, such as atherosclerosis, hypertension, diabetes, and hyperlipemia (6–8). Upregulation of OLR1 in cardiovascular and metabolic diseases increased DNA damage caused by intracellular reactive oxygen species, promoted the release of inflammatory cytokines, and enhanced hypoxia and angiogenesis (5, 9). Notably, these processes are also closely related to cancer development (10). Indeed, upregulation of OLR1 was recently found in multiple cancers and shown to be involved in tumorigenesis, cancer development, and metastasis (11–14). For example, activation of the TNFα/NF-κB pathway upregulates OLR1 to promote the migration of breast cancer cells (11, 15). In return, upregulated OLR1 also activates NF-κB signaling to induce cellular transformation in breast cancer and trigger epithelial–mesenchymal transition (EMT) to induce metastasis in prostate cancer (13, 16). OLR1 also facilitates metastasis of gastric cancer through driving PI3K/Akt/GSK3β activation (17). Recently, our group found that OLR1 was upregulated by lncRNA GSTM3TV2 through competitively sponging let-7 to promote gemcitabine resistance in pancreatic cancer (18). Together these studies suggest that OLR1 may be a link between metabolic diseases and cancer. However, the role of OLR1 in pancreatic cancer has not been completely revealed.

The proto-oncogene c-Myc regulates DNA synthesis, cellular proliferation, differentiation, survival, and immortalization through its function as a transcription factor that binds canonical DNA-binding motifs (E box 5′-CAGGTG-3′) in the promoters of target genes to regulate gene expression. c-Myc plays an important role in the initiation and development of cancer by its placement at the crossroads of many cancer-related signaling pathways (19, 20). c-Myc promotes cell metastasis in many cancers and is regulated by many factors (19). However, the relationship between OLR1 and c-Myc is unclear.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Mol Cancer Res 2020;18:685–97
doi: 10.1158/1541-7786.MCR-19-0718
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Published OnlineFirst February 4, 2020; DOI: 10.1158/1541-7786.MCR-19-0718
High mobility group AT-hook 2 (HMGA2), a member of high mobility group A, plays a critical role in growth during embryonic development but is expressed at low levels in adult tissues (21). However, HMGA2 has been reported to be upregulated in many cancers (21–23). HMGA2 mainly functions as a transcription regulator by modifying chromosomal architecture or recruiting other transcription-associated proteins to regulate the differentiation and proliferation of cells (24). HMGA2 was recently shown to promote metastasis in cancer by regulating EMT and enhancing TGFβ signaling (21–23, 25).

In this study, we investigated the role and potential function of OLR1 in pancreatic cancer. We found that OLR1 expression was highly expressed in pancreatic cancer tissues and that OLR1 promoted metastasis of pancreatic cancer cells through increasing transcription of HMGA2 through c-Myc. Our results identified a novel OLR1-c-Myc-HMGA2 axis and show its potential as a combined biomarker of pancreatic cancer patient prognosis.

Materials and Methods

Cell lines and clinical specimens

The human pancreatic cancer cell lines Bxpc-3 and Mia PaCa-2 were purchased from the ATCC and cultured in DMEM or RPMI1640 with 10% FBS (HyClone) in a 5% CO2 cell culture incubator at 37°C. All cell lines were authenticated using high-resolution small tandem repeats (STR) profiling at Department of General Surgery Laboratory. The mycoplasma of cells was examined by MycoSensor PCR Assay Kit (Catalog No. C00315; Beyotime). Cells were grown for 15 passages and then replated with fresh stocks. The 99 clinical pancreatic cancer specimens were obtained from patients who received surgery in the Peking Union Medical College Hospital from January 2004 to November 2008; the specimens were used for the construction of a TMA. This research was approved by the Ethics Committee of Peking Union Medical College Hospital and written informed consent was obtained from each patient.

IHC analysis

Antibodies against OLR1, HMGA2 (Proteintech Group), and c-Myc (Cell Signaling Technology) were used to perform IHC analysis in the TMA as described previously (26). Two experienced pathologists independently assessed the results. Staining intensities were graded as 0 (negative), 1 (low), 2 (medium), or 3 (high), whereas the staining extent was scored from 0% to 100%. The final IHC staining score was calculated as intensity score × percentage score × 100.

TCGA, GEO, and ENCODE datasets

Data from the TCGA, the GEO database (accession numbers: GSE28735 and GSE71729) and ENCODE were used (27).

Constructs and cell transfection

siRNA targeting OLR1 and a scramble control siRNA were purchased from Guangzhou RiboBio Co., Ltd. The full-length OLR1 cDNA was subcloned into the pDNA 3.1 empty vector to generate the pDNA3.1-OLR1 expression construct. Transient transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The OLR1 overexpression construct and OLR1 shRNA were packaged into lentivirus, and cells were infected to establish cells that stably express OLR1 or shRNA targeting OLR1. Cells were then selected by puromycin (1 μg/mL) for 7 days. The siRNA sequences are listed in Supplementary Materials and Methods.

Western blot analysis

Western blot analysis was performed as described previously (28). Briefly, protein lysates (20 μg) were separated using SDS-PAGE, and primary and secondary antibodies were applied to detect target proteins. An enhanced chemiluminescence assay was used to visualize protein bands. Primary antibodies targeting OLR1 and HMGA2 were purchased from Proteintech Group and c-Myc and β-actin antibodies were obtained from Cell Signaling Technology.

qRT-PCR

TRizol reagent (Invitrogen) was used for the extraction of total RNA and cDNA synthesis was performed using the Takara First Strand cDNA Synthesis Kit following the manufacturer's instructions. qRT-PCR assays were performed independently at least three times. The PCR primers are listed in the Supplementary Materials and Methods.

RNA-sequencing and data analysis

A RNasy MINI KIT (Qiagen) was used for the extraction of total RNA. The directional (stranded) libraries were generated using an Illumina platform for the paired-end sequencing of Bxpc-3 cells. Cuffdiff was used to obtain the sequence count data [fragments per kilobase of transcript per million mapped reads (FPKM) values] to conduct differential expression analysis. Shanghai Biotechnology Corporation performed the library construction and sequencing.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP Assay Kit (Millipore, 17-295) according to the manufacturer's instructions. Briefly, c-Myc protein was immunoprecipitated from whole cell lysates by A/G magnetic beads conjugated to anti-c-Myc antibody (Cell Signaling Technology). Quantitative ChIP was performed using an ABI StepOne Plus with SYBR Green dye. Primers spanning every region comprising c-Myc binding elements 1 or 2 were designed and used for ChIP-qPCR. The primer sequences are listed in the Supplementary Materials and Methods.

Dual luciferase reporter assay

A Dual Luciferase Reporter Assay System (Promega) was performed as described previously (22). Briefly, cells (1 × 10⁴ cells/well) were plated in a 96-well plate. After 24 hours, the cells were cotransfected with 0.2 μg expression vector plasmid or 0.02 nmol siRNA, 0.2 μg promoter reporter plasmid, and 0.002 μg SV40 plasmid using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. After 48 hours, cells were lysed using lysis buffer and the lysates were centrifuged at maximum speed for 1 min in an Eppendorf microcentrifuge. The Dual Luciferase Reporter Assay System was used to detect the relative luciferase activity of the samples, and the transfection efficiencies were normalized according to Renilla activity.

Proliferation assays

Cell Counting Kit-8 (CCK-8) reagent (Dojindo) was used to perform growth assays following the manufacturer's protocol. Cells were seeded into 96-well plates (1,000 cells/well). At 0, 24, 48, 72, and 96 hours later, CCK-8 (10 μL/well) was added and cells were cultured for an additional 2.5 hours at 37°C. The optical density (OD) was measured as the relative cell number at a wavelength of 450 nm (OD450) with a microplate reader.
**OLR1-c-Myc-HMGA2 Promotes Metastasis in Pancreatic Cancer**

**Cell migration and invasion assays**

Cell migration and Matrigel cell invasion assays were conducted as described previously (26). In brief, polycarbonate membranes with 8-μm pores were coated with 0.5% FBS. After 24 hours, the membranes were rehydrated, and 4.0 × 10^4 pancreatic cancer cells in DMEM without FBS were placed into the upper chamber of a Transwell unit, and DMEM with 10% FBS was applied as a chemoattractant in the lower chamber. After 24 hours, the migrated or invaded cells were fixed by 4% paraformaldehyde and stained with hematoxylin and eosin. The membranes were dried, and the numbers of migrated/invaded cells were counted in 10 low-power fields (magnification, ×100). The mean values were determined in triplicate assays. All experiments were performed at least twice independently.

**Wound-healing assay**

Cells were seeded into a 12-well plate and then incubated overnight in complete medium. A scratch wound was created in the cell monolayer with a sterile 200-μL pipette tip, and the detached cells were removed. The cells were then incubated in 1% FBS medium, and images were captured at 6, 12, 18, and 24 hours later. The relative distance of cell migration from the wound at 0 hour was determined as the migration distance, as published previously (26).

**Xenograft tumor mouse models**

All animal experiments were performed according to the institutional ethical guidelines of Peking Union Medical College (Beijing, China). For in vivo imaging, human pancreatic cancer cells were infected by lentivirus carrying a firefly luciferase cassette as well as the puromycin resistance gene. Cells expressing firefly luciferase (1 × 10^6) were inoculated in situ or intravenously or intrasplenically into nude mice and monitored by an IVIS imaging system, as published previously (26, 29).

**Statistical analysis**

Each experiment was repeated at least three times. Data were measured by Student t test (two-tailed) and analyzed using one-way ANOVA. All statistical tests were performed using the Statistical Program for Social Sciences (SPSS16.0 for Windows) and were two-sided. P value of <0.05 was regarded as statistically significant. GraphPad Prism 6 software (GraphPad) was used to create all graphs.

**Ethics approval and consent to participate**

All experiments were approved by the institutional ethical committee of Peking Union Medical College Hospital (Beijing, China).

**Results**

**OLR1 expression is upregulated in pancreatic cancer specimens**

We first detected the expression of OLR1 in a tissue microarray (TMA) containing 99 pancreatic cancer tissues and 62 normal pancreatic tissues using IHC. OLR1 was mainly expressed in the cell cytoplasm and cell membrane (Fig. 1A) consistent with the cellular immunofluorescence result (Supplementary Fig. S1A). The expression of OLR1 was significantly higher in the cancer tissues than in adjacent normal pancreatic tissues according to the IHC scores (P < 0.001; Fig. 1B). The paired analysis of OLR1 IHC scores in 62 of the 99 cases with both cancer and adjacent tissues showed the same result (P < 0.001; Fig. 1C). We also investigated the expression of OLR1 in pancreatic cancer samples from public databases (GSE28735 and GSE71729) and found that the level of OLR1 mRNA was upregulated in pancreatic cancer tissues compared with adjacent normal tissues (Fig. 1D and E).

We next evaluated the correlation between the level of OLR1 and clinicopathologic features. There was no significant correlation of OLR1 expression with gender, age, tumor location, or T stage in patients with pancreatic cancer. However, high expression of OLR1 was correlated with poor differentiation (P < 0.05), increased lymph node metastasis (P < 0.001), and advanced TNM staging (P < 0.001; Table 1), indicating that OLR1 level is related to metastasis.

Kaplan–Meier analysis revealed that patients with pancreatic cancer with high OLR1 expression had a significantly worse median OS than those with low OLR1 expression (10 months vs. 35 months, respectively; P = 0.0095; Fig. 1F). Analysis of patients with pancreatic cancer in the Cancer Genome Atlas (TCGA) and GSE28735 also revealed that high OLR1 mRNA expression correlated with poor prognosis of patients with pancreatic cancer (P < 0.05; Fig. 1G and H). Univariate and multivariate analyses showed that OLR1 level was negatively correlated with OS in both analyses, indicating that high OLR1 expression is an independent prognostic risk factor for patients with pancreatic cancer (Table 2).

**OLR1 promotes proliferation, migration, and invasion of pancreatic cancer cells**

To determine the effect of OLR1 overexpression on cell proliferation, CCK-8 assay was performed as described in Materials and Methods by measuring the OD at a wavelength of 450 nm as the relative cell number after adding CCK-8 agent into cells and culturing for an additional 2.5 hours at 37°C. The growth rates of Bxpc-3 and Mia PaCa-2 pancreatic cancer cells transfected with OLR1 overexpression plasmids were significantly faster compared with control cells (P < 0.0001; Fig. 2A) and the growth was inhibited when OLR1 was knocked down (P < 0.0001; Fig. 2B). Transwell migration and invasion assays showed that OLR1 upregulation promoted migration and invasion of Bxpc-3 and Mia PaCa-2 cells and OLR1 knockdown in Bxpc-3 and Mia PaCa-2 cells inhibited cell migration and invasion (P < 0.0001; Fig. 2C). Wound-healing assays also revealed that OLR1 overexpression increased but OLR1 knockdown decreased the migratory activity of both Bxpc-3 and Mia PaCa-2 cells compared with control cells (Fig. 2D and E). The effect of OLR1 knockdown and upregulation on OLR1 protein levels was verified by Western blot analysis (Fig. 2F).

Together these findings demonstrated that OLR1 promotes the proliferation, migration, and invasion of pancreatic cancer cells in vitro.

**OLR1 promotes the growth and invasion of pancreatic cancer cells in subcutaneous pancreatic cancer mouse models**

To explore the effect of OLR1 inhibition of pancreatic cancer proliferation and invasion in vivo, we used a lentivirus system expressing shRNA to inhibit OLR1 expression (Lenti-shOLR1) or control lentivirus (Lenti-shCon) in Bxpc-3 cells. Lenti-shOLR1 and Lenti-shCon-infected Bxpc-3 cells were subcutaneously injected into the posterior flanks of nude mice. The growth of xenograft tumors in mice was monitored, and the mice were sacrificed 5 weeks after transplantation. Compared with control mice, the Lenti-shOLR1 group showed significantly suppressed tumor growth (P < 0.05; Fig. 3A). Tumor weights were also lower in the OLR1 silenced group than in the control group (P < 0.0001; Fig. 3B). H&E staining showed that OLR1 down-regulation decreased the invasion of pancreatic cancer cells to adjacent

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muscle tissues (Supplementary Fig. S1B). We performed similar experiments using a lentivirus system overexpressing OLR1 (Lenti-OLR1). The tumor growth and tumor weights in the OLR1 upregulated mouse group were significantly higher than those in the control group (Fig. 3C and D). The invasion of pancreatic cancer cells to adjacent muscle tissues was also more apparent when OLR1 was increased (Supplementary Fig. S1B). OLR1 overexpression and knockdown was verified by IHC staining and Western blot analysis (Supplementary Figs. S1B–S1D).

Together, these data demonstrated that OLR1 promotes the proliferation and invasion of Bxpc-3 pancreatic cancer cells in subcutaneous xenograft mouse models.

OLR1 inhibition suppresses the metastasis of pancreatic cancer cells in orthotopic, intrasplenic, and intravenous pancreatic cancer models

To investigate whether OLR1 inhibition suppresses pancreatic cancer metastasis, we injected Bxpc-3 cells infected with Lenti-shOLR1 or Lenti-shCon orthotopically into the pancreas of SCID mice and monitored the growth of transplanted tumors by bioluminescence imaging. After 5 weeks, the group with OLR1 knockdown showed suppressed xenograft tumor growth compared with controls (Fig. 3E).

In mice injected with Lenti-shOLR1-expressing Bxpc-3 cells, the numbers of metastases in the liver, kidney, lung, spleen, abdominal, and gut decreased dramatically compared with control mice (Fig. 3F).
and G). All the metastases were validated by H&E staining (Fig. 3H) and the downregulation of OLR1 expression was verified by IHC staining (Supplementary Fig. S1E).

To confirm the above results, we further injected Bxpc-3 cells infected with Lenti-shOLR1 or Lenti-shCon into the spleen of mice. The bioluminescence imaging and dissection results revealed fewer and smaller metastases in liver when OLR1 was knocked down compared with controls (Fig. 3I and J). And the metastases were validated by H&E staining (Fig. 3K and L). Similarly, the bioluminescence imaging and dissection results of the intravenous mice also showed fewer and smaller metastases in lung when OLR1 was knocked down compared with controls (Supplementary Fig. S2A–S2D).

Together, these results indicate that inhibition of OLR1 leads to the decrease of metastatic capacity in pancreatic cancer cells.

OLR1 promotes the metastasis of pancreatic cancer cells by regulating c-Myc and HMGA2

To identify the transcriptome signature that may be involved in OLR1-mediated pancreatic cancer metastasis, we performed whole transcriptome RNA-sequencing (RNA-seq) analysis of Bxpc-3 cells with OLR1 overexpression or inhibition. In pancreatic cancer cells with OLR1 overexpression, there were 181 upregulated and 203 downregulated genes compared with control cells (by ≥2.0-fold change and \( P < 0.05 \); Fig. 4A; Supplementary Table S1). In cells with downregulation of OLR1, 179 genes were upregulated and 166 genes were downregulated compared with controls (Fig. 4A; Supplementary Table S1). To narrow down the genes specifically related to OLR1 expression, 26 genes positively correlated with OLR1 and 39 genes negatively correlated with OLR1 in both Lenti-OLR1 and

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Note: \( P < 0.05 \) indicates the statistical significance of differences. Abbreviations: HR, hazard ratio; CI, confidence interval.
Figure 2.
OLR1 promotes proliferation, migration, and invasion of pancreatic cancer cells. A, Comparison of the growth of Bxpc-3 (top) and Mia PaCa-2 cells (bottom) transfected with pcDNA3.1 control or pcDNA3.1-OLR1 plasmids using CCK8 assay. B, Comparison of the growth of Bxpa-3 (top) and Mia PaCa-2 (bottom) cells transfected with siRNA control (siNC) or siOLR1 for 48 hours using CCK8 assay. C, In vitro transwell migration and invasion assays were performed in Bxpc-3 (top) and Mia PaCa-2 cells (bottom) with OLR1 plasmid overexpression or silencing by siRNA (siOLR1) for 24 hours. Representative images are shown. Magnification, ×100. Cell numbers of migrated and invasive cells are shown in the right panels. D, Wound-healing assays were performed in Bxpa-3 (left) and Mia PaCa-2 cells (right) with OLR1 overexpression. Wounds were photographed, and the wound closure percentage from a representative experiment (n = 3) was measured using AxioVision software. Magnification, ×100. E, Wound-healing assays were performed in Bxpa-3 (left) and Mia PaCa-2 (right) cells with OLR1 silencing. Wounds were photographed, and the percentage of wound closure (n = 3) was measured using AxioVision software. Magnification, ×100. F, Western blot analysis of OLR1 in cells with OLR1 knockdown and upregulation (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
OLR1 promotes the growth of pancreatic cancer cells in pancreatic cancer mouse models. A, Photographs of dissected tumors from nude mice injected with Lenti-shCon- and Lenti-shOLR1-expressing Bxpc-3 cells (left); the tumor volumes are shown in the right panel. B, Tumor weights were calculated at the end of the experiment (right, n = 7). C, Photographs of dissected tumors from nude mice injected with Lenti-OLR1 and Lenti-Con-expressing Bxpc-3 cells (left); the tumor volumes are shown in the middle panel (n = 7). D, Tumor weights were calculated at the end of the experiment (right, n = 7). E, Knockdown of OLR1 decreased the tumor growth in orthotopic xenograft models established using Bxpc-3 cells. Representative IVIS images of nude mice are shown (n = 5). F, Dissected primary pancreas tissues, livers, lungs, and kidneys. G, Numbers of metastatic nodes and invasive organs are shown. H, Metastatic and invasive organs were examined by H&E staining. Magnification, ×50; scale bars, 200 μm. The images in the top right or bottom right panels represent magnified views of the boxed regions. Magnification, ×200; scale bars, 100 μm. I and J, Inhibition of OLR1 decreased the metastasis of Bxpc-3 cells as observed in intrasplenic pancreatic cancer xenograft models. Representative IVIS images of nude mice are shown (n = 5). K, Representative dissected liver images in Lenti-shOLR1 and Lenti-shCon groups. L, Representative H&E images of the liver are shown in Lenti-shOLR1 and Lenti-shCon groups; black arrows show the metastases in livers. Magnification, ×40; scale bars, 500 μm. The images in the right panels represent magnified views of the boxed regions. Magnification, ×100; scale bars, 200 μm (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
OLR1 increases the metastasis of pancreatic cancer cells by regulating c-Myc and HMGA2. 

A. Analysis of gene expression profiles identified 26 genes positively correlated with OLR1 levels and 39 genes negatively correlated with OLR1 levels in Bxpc-3 cells.

B. Cluster heatmap of mRNA expression profiles in Bxpc-3 cells with OLR1 overexpression or knockdown.

C. RT-qPCR of c-Myc (C) and HMGA2 mRNA (D) in Bxpc-3 cells. GAPDH mRNA served as a loading control.

E. Western blot analysis of c-Myc and HMGA2 in Bxpc-3 cells.

F and G. c-Myc (F) and HMGA2 (G) expression positively correlated with OLR1 expression in the TCGA database of patients with pancreatic cancer (n = 178).

H–K. Rescue assays were performed in vitro transwell and invasion migration experiments in Bxpc-3 cells. Representative images are shown. Magnification, ×100. Cell numbers of migrated and invasive cells are shown in the right panels (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
c-Myc promotes the transcription of HMGA2

Our results showed that OLR1 enhances c-Myc and HMGA2 expression at both mRNA and protein levels, c-Myc is a classical transcription factor and oncogene (32). Therefore, we speculated that OLR1 may promote the transcription of HMGA2 through c-Myc. To verify our hypothesis, we examined the expression of HMGA2 in Bxpc-3 and Mia PaCa-2 cells with c-Myc silencing or overexpression and found that c-Myc increased the expression of HMGA2 at both mRNA and protein levels (Fig. 5A and B; Supplementary Figs. 5A and 5B). TCGA data also showed a positive correlation between c-Myc and HMGA2 expression (r = 0.24; P < 0.001; Fig. 5C). To determine whether c-Myc directly binds to the promoter of HMGA2, we first analyzed the human HMGA2 promoter region (−2,000 bp to +1 bp) and identified two potential c-Myc binding sites containing the E-box 5'–CACGTG–3' sequence (Fig. 5D). ENCODE (Encyclopedia of DNA Elements) c-Myc ChiP-seq data showed that c-Myc was enriched at HMGA2 promoter regions in MCF10A human breast cells, HepG2 hepatocellular carcinoma cells and Gm12878 lymphocyte cells (Fig. 5E; ref. 27). To verify whether c-Myc binds directly to these potential binding sites in the HMGA2 promoter, we performed a ChIP-qPCR assay in 293T, Bxpc-3 and Mia PaCa-2 cells. In chromatin fractions pulled down by anti-c-Myc antibody, the c-Myc binding elements 1 and 2 in the HMGA2 promoter were significantly increased compared with samples immunoprecipitated with control antibody (Fig. 5F and G).

To further determine whether c-Myc activates the promoter of HMGA2, we constructed three luciferase reporter vectors driven by the HMGA2 wild-type promoter (WT) or HMGA2 mutant promoters (Mut1 and Mut2; Fig. 5D). Dual luciferase assays showed that the activities of HMGA2 WT and Mut2 promoters significantly increased when c-Myc was overexpressed (Fig. 5H). However, activity of the HMGA2 Mut1 promoter showed no significant change with coexpression of c-Myc. In cells cotransfected with HMGA2 WT and Mut2 reporters and tic-Myc, the luciferase activities were significantly inhibited, whereas the luciferase activity of HMGA2 Mut1 showed no changes with coexpression of tic-Myc (Supplementary Fig. 5C).

Together, these results show that c-Myc promotes the transcription of HMGA2 by directly binding to binding element 1 in the promoter of HMGA2.

OLR1 upregulates HMGA2 through c-Myc to promote pancreatic cancer metastasis

We next examined whether OLR1 increased transcription of HMGA2 through c-Myc is involved in the effects of OLR1 in promoting pancreatic cancer metastasis. We first examined whether c-Myc promotes invasion and metastasis of pancreatic cancer through increasing HMGA2. We transfected siHMGA2 in c-Myc-overexpressing Bxpc-3 and Mia Paca-2 cells and found that the increased migration and invasion activities from c-Myc overexpression were significantly inhibited in the absence of HMGA2 (Fig. 6A; Supplementary Fig. 5A). HMGA2 overexpression also increased migration and invasion activities of Bxpc-3 and Mia Paca-2 cells with c-Myc knockdown (Fig. 6B; Supplementary Fig. 5B). These results suggested that HMGA2 is required for the effect of c-Myc in promoting metastasis of pancreatic cancer cells. Previous studies indicated that EMT is closely related to metastasis, and therefore we explored whether HMGA2 regulates EMT to promote metastasis of pancreatic cancer cells. The results demonstrated that HMGA2 enhanced the expression of β-catenin and snail and inhibited E-cadherin level in Bxpc-3 and Mia PaCa-2 cells (Fig. 6C), indicating that HMGA2 may promote metastasis by inducing EMT of pancreatic cancer cells.

We next examined whether c-OLR1 regulation of HMGA2 occurred through c-Myc. The rescue result showed that HMGA2 induction by OLR1 overexpression was compromised by tic-Myc in Bxpc-3 and Mia PaCa-2 cells (Fig. 6D; Supplementary Fig. 5C).

Together these data indicated that OLR1 increases the invasion and metastasis of pancreatic cancer cells by promoting the transcription of HMGA2 through c-Myc activity.

High OLR1, c-Myc, and HMGA2 expression is associated with poor prognosis of patients with pancreatic cancer

We next examined the clinical relationship of OLR1, c-Myc, and HMGA2 levels by analyzing the expression of c-Myc and HMGA2 in TMAs containing the same patient tissues as the TMA used for OLR1 analysis. The expressions of c-Myc and HMGA2 were significantly higher in the OLR1 highly expressed group, and HMGA2 was significantly increased in the c-Myc highly expressed group, indicating that they are positively correlated with each other (Fig. 6E–H). IHC staining of subcutaneous and orthotopic xenograft tumors also revealed that the expressions of c-Myc and HMGA2 were positively correlated with the expression of OLR1 (Supplementary Figs. 6A and 6B). Kaplan–Meier survival analysis demonstrated that high expression of c-Myc and HMGA2 were each negatively correlated with the prognosis of patients with pancreatic cancer in our cohort and TCGA (Supplementary Fig. S7A–S7D). When OLR1, c-Myc, and HMGA2 were combined, Kaplan–Meier survival analysis showed the prognosis of patients with high expression of all three proteins was worse than that of patients with two highly expressed proteins, and the prognosis of patients with high expression of two of these proteins was worse compared with patients with high expression of only one or none of these proteins (Fig. 6I; Supplementary Figs. S7E–S7G). Analysis of TCGA data also revealed a similar result (Fig. 6J; Supplementary Figs. S7H–S7I). These data revealed that the OLR1–c-Myc–HMGA2 axis may serve as prognostic markers for patients with pancreatic cancer.
c-Myc promotes the transcription of HMGA2. A and B, The expression of HMGA2 regulated by c-Myc was validated in Bxpc-3 cells by RT-qPCR (A) and Western blot analysis (B). C, HMGA2 expression positively correlated with c-Myc expression in the TCGA database of patients with pancreatic cancer ($r = 0.24; P < 0.001$). D, Schematic of the HMGA2 gene promoter. Two potential c-Myc binding elements and their respective locations (top) and the design of mutants for each binding element are shown (middle and bottom). E, Evaluation of ENCODE c-Myc ChIP-sequencing data. Peaks represent c-Myc enrichment at the HMGA2 promoter in immortalized MCF10A mammary epithelial cells, HepG2 hepatocellular carcinoma, and Gm12878 lymphocyte cells. F and G, ChIP with antibodies against c-Myc or isotype control IgG and qRT-PCR for c-Myc binding element 1 (F) and 2 (G) was performed in 293T, Bxpc-3, and Mia PaCa-2 cells. H, Dual luciferase assay was performed in 293T, Bxpc-3, and Mia PaCa-2 cells with c-Myc overexpression and control cells transfected with pGL3 empty vector, pGL3-HMGA2 WT, pGL3-HMGA2-Mut1, or pGL3-HMGA2-Mut2. The results are shown as relative luciferase activity to pGL3 empty vector in corresponding cells after normalization with Renilla luciferase activity ($*, P < 0.05; **, P < 0.01; ***, P < 0.001$).
Figure 6.
OLR1 upregulates HMGA2 through c-Myc to promote pancreatic cancer cell metastasis, and combined OLR1, HMGA2, and c-Myc are associated with poor prognosis of patients with pancreatic cancer. A and B, Rescue assays were performed with in vitro transwell and invasion experiments in Bxpc-3 cells. Representative images are shown. Magnification, ×100; numbers of migrated and invasive cells are shown in the right panels. C, E-cadherin, Snail, and β-catenin level were assessed by Western blot analysis in Bxpc-3 and Mia PaCa-2 cells with HMGA2 overexpression or knockdown. D, c-Myc and HMGA2 protein levels were assessed in Bxpc-3 cells by rescue assay using Western blot analysis. E, Representative images of IHC staining of OLR1, c-Myc, and HMGA2 in the same pancreatic cancer tissue. All proteins are expressed at low levels in the two cases on the left and overexpressed in the two cases on the right. Magnification, ×50; scale bars, 200 μm. F–H, IHC score analysis results revealed that OLR1, c-Myc, and HMGA2 positively correlated with each other. I and J, Kaplan-Meier prognosis analysis of patients with pancreatic cancer with combined OLR1, c-Myc, and HMGA2 in TMA (H) and TCGA database (I). Group 1, one protein (OLR1, c-Myc, or HMGA2) is highly expressed; Group 2, two proteins (OLR1, c-Myc, and/or HMGA2) are highly expressed; Group 3, all three proteins (OLR1, c-Myc, and HMGA2) are highly expressed (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Discussion

In this study, we explored the expression and role of OLR1 in pancreatic cancer. We first examined the expression of OLR1 in pancreatic cancer tissues and found that OLR1 expression was significantly higher in pancreatic cancer than in adjacent normal tissues. Clinicopathologic analysis showed that increased OLR1 expression was closely associated with poor differentation, increased lymph node metastasis, and TNM staging, as well as reduced OS. Inhibition of OLR1 expression decreased the proliferation and metastasis of pancreatic cancer cells both in vitro and in vivo. Mechanistically, OLR1 upregulated the expression of c-Myc to increase the transcription of HMGA2 gene, promoting the metastasis of pancreatic cancer. Finally, we revealed that the expression of OLR1, c-Myc, and HMGA2 were positively correlated with each other in pancreatic cancer tissues and negatively correlated with the prognosis of patients with pancreatic cancer. Altogether, these data demonstrated that OLR1 increases the proliferation and metastasis of pancreatic cancer cells by promoting the transcription of HMGA2 through c-Myc.

OLR1 has been closely related with cardiovascular and metabolic diseases. However, OLR1 was also recently reported to play an important role in cancer development, and the overexpression of OLR1 has been reported in multiple cancers, including pancreatic cancer. For example, Jie and colleagues reported that OLR1 is a poor prognostic indicator for pancreatic cancer and induced EMT, which is consistent with our result. However, the authors did not explore the function and mechanism of OLR1 in pancreatic cancer metastasis (14). To elucidate the mechanism of OLR1 in the metastasis of pancreatic cancer cells, we performed whole transcriptome RNA-seq analysis of Bxpc-3 cells with OLR1 stable overexpression or inhibition and found that c-Myc and HMGA2 were positively correlated with the level of OLR1. We further established that OLR1 promotes the metastasis of pancreatic cancer through c-Myc and HMGA2.

Previous reports showed that c-Myc and HMGA2 promote cancer metastasis as oncogenic factors (22, 30), but the relationship between c-Myc and HMGA2 is rarely reported. Because c-Myc is a transcription factor, we speculated that OLR1 increased HMGA2 expression through c-Myc to promote metastasis of pancreatic cancer. We first demonstrated that the mRNA and protein levels of HMGA2 were regulated by c-Myc. Dual luciferase assay and ChIP assay confirmed that c-Myc promoted the transcription of HMGA2 by directly binding to its promoter through one of two potential binding sites. We also showed that HMGA2 was required for c-Myc-promoted invasion and metastasis of pancreatic cancer cells and that HMGA2 promoted EMT in pancreatic cancer cells. Together these results revealed that OLR1 increases invasion and metastasis of pancreatic cancer cells by promoting the transcription of HMGA2 through c-Myc.

The clinical significance of OLR1, c-Myc, and HMGA2 was evaluated in pancreatic cancer tissues and TCGA database and the results showed that OLR1, c-Myc, and HMGA2 were positively correlated with each other and negatively correlated with the prognosis of pancreatic cancer patients. In addition, patients with pancreatic cancer with high expression of all proteins showed worse prognosis compared with patients with high expression of two or fewer proteins. These data suggest that OLR1, c-Myc, and HMGA2 might serve as potential prognosis markers for patients with pancreatic cancer.

This study has several limitations. For example, the mechanism by which OLR1 promotes the expression of c-Myc is still unclear. Previous studies reported that OLR1 as a metabolism- and inflammation-related factor activates the NF-κB and PI3K/Akt pathways (11, 17), which is related to the regulation of c-Myc (20), indicating OLR1 may regulate c-Myc through these pathways. For example, previous studies identified OLR1 as one of the most upregulated lipid metabolism genes during cellular transformation, and inhibition of OLR1 reduced the activation of NF-κB through inhibition of IκBα phosphorylation in cancer cells (16). Furthermore, another study showed that overexpression of OLR1 induced the activation of NF-κB (9). However, the transcription of c-Myc is directly regulated by NF-κB, the activation of which could promote the transcription of c-Myc (20, 33). OLR1 was reported to drive PI3K/Akt/GSK3β activation (17), which could enhance the expression of c-Myc (20). In addition, OLR1 may regulate the expression of HMGA2 and c-Myc through other mechanisms, such as miRNAs and IncRNAs (34). For instance, HMGA2, c-Myc, and OLR1 are targets of let-7 (18, 35), and OLR1 may exert its inhibitory effect on c-Myc and HMGA2 by competitive binding with let-7, thus promoting the expression of c-Myc and HMGA2. However, further studies are required to explore this possibility.

Although some lipid-lowering agents were reported to inhibit the level of OLR1, as a potential link between metabolic-cardiovascular diseases and cancer, no effective targeted drug for OLR1 is available (36). The development of specific inhibitors targeting OLR1 will be useful to study the feasibility of OLR1 as a therapeutic target for pancreatic cancer in the future.

In conclusion, we identified the OLR1–c-Myc–HMGA2 axis as a pathway that promotes metastasis of pancreatic cancer and that may serve as potential therapeutic targets of metastasis and prognosis markers for patients with pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: G. Yang, T. Zhang, Y. Zhao
Development of methodology: G. Xiong, M. Feng, H. Wang, G. Yang, T. Zhang, Y. Zhao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Yang, J. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Yang, F. Zhao, J. Qiu, Z. Cao
Writing, review, and/or revision of the manuscript: G. Yang, H. Wang, Y. Zhao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Xiong, M. Feng, F. Zhao, Y. Liu, L. You, L. Zheng
Study supervision: Y. Zhao

Acknowledgments

T. Zhang was supported by grants from the National Natural Science Foundation of China (Nos. 81772269 and 81972258) and CAMS Innovation Fund for Medical Sciences (CIFMS, No. 2016-I2M-1-001). Y. Zhao was supported by grants from the National Natural Science Foundation of China (No. 81973576) and the Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (Nos. 2018PT32014 and 2018PT32002). G. Yang was supported by grants from CAMS Innovation Fund for Medical Students (Nos. 2017–1002–1–16), PUMC Youth Fund and the Fundamental Research Funds for the Central Universities (No. 2017320027). We thank Dr. Y. Zhao for assistance with the ChIP assay.

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Received July 13, 2019; revised November 23, 2019; accepted January 31, 2020, published first February 4, 2020.


OLR1 Promotes Pancreatic Cancer Metastasis via Increased c-Myc Expression and Transcription of HMGA2

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