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Estrogen receptor alpha regulates the Wnt/β-catenin signaling pathway in colon cancer by targeting the NOD-like receptors

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ABSTRACT

It has been reported that estrogen receptors (ERs) participate in carcinogenesis by directly regulating NOD-like receptors (NLRs). However, the expression profiles of ERs and NLRs in tumor and the ER-NLR regulated signaling pathway are not clear. In this study, we summarized gene expression profiles of ERs and NLRs across normal and tumor tissue by comprehensive data mining. Then we explored the ER-NLR regulated signaling pathway by RNA sequencing (RNA-seq). The results showed that the NLRs and ERs were differentially expressed in different neoplasm tissues. Such expression discrepancies might influence inflammatory regulation and tumorigenesis. Importantly, we identified that ER-NLR regulate Wnt/β-catenin pathway in colon cancer. Taking colon adenocarcinoma (COAD) as example, we found that Wnt2b/LRP8/Dvl1/Axin2/GSK3a/APC/β-catenin genes were differentially expressed in $ER^{-/-}$ mouse colon tissue and colon cancer cells. The selective ERα antagonist could significantly decrease Wnt2b/LRP8/Dvl1 expression, increase destruction complex (Axin2/GSK3a/APC) expression, and promote degradation of β-catenin in colon carcinoma cell by inhibited NLRP3 expression. In short, the research demonstrates that NLRs are potential biomarkers for cancer, and ERs can regulate the Wnt/β-catenin signaling pathway in cancer by targeting the NLRs. Our results provide a possible signaling pathway in which ER-NLR is correlated with Wnt/β-catenin.

1. Introduction

NOD-like receptors (NLRs) are a newly discovered family of pattern recognition receptors (PRRs) which play a key role in immune regulation of innate immunity and adaptive immunity [1]. NLRs contain 3 domains, including N-terminal signal transduction domain for protein-protein interaction, central NACHT domain for oligomerization, and C-terminal ligand recognition domain with leucine-rich repeats. The leucine-rich repeats domain also acts as a repressor of NLR signaling by masking the N-terminal domain in the absence of ligand stimulation [2]. As reported, mammalian NLRs can be classified into four types based on their N-terminal domain: CIITA, NAIP, NLRCs (including NOD1, NOD2, NLRC3–5, NLRX1), and NLRPs (including NLRP1-NLRP14) [3]. Due to the active role of NLRs in regulating pro-inflammatory signals and recruiting the adaptive arm of the immune system, NLRs have been reported to influence disease outcomes and tumorigenesis. For instance, NLRP3 is involved in lymphangiogenesis and metastasis [4]. CIITA plays an important role in lymphoid cancers [5]. NLRC5 can regulate cell proliferation, migration, and invasion by targeting the Wnt/β-catenin signaling pathway in hepatocellular carcinoma [6]. However, expression profiles of NLRs across normal and tumor tissue have not been researched.

Nuclear receptors (NRs), acting as transcription factors, can bind to DNA and influence the adjacent genes expression. It has been reported that NOD proteins, such as NOD1, NOD2, and NOD4, are positively related to NRs [7]. Therefore, it is obvious that the regulation of NRs plays a key role in NLR-related neoplasms. ERs (including ERα and ERβ) are members of the NRs. It has been reported that estrogen can attenuate local inflammasome activation during spinal cord injury and suppress hepatocellular carcinoma cells through upregulation of the NLRP3 inflammasome-mediated with ERβ [8,9]. In our previous study, we have demonstrated that ERs participate in carcinogenesis by directly regulating NLRs in colon cancer cells [10]. However, the downstream regulatory pathway of ER-NLR in colon cancer remained unknown. Considering the important role of ERs and NLRs in neoplasm, further research on this signaling pathway may benefit neoplasm prevention

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and therapy. In this research, based on data mining, we found that NLRs and ERs were differentially expressed in neoplasm tissues. Taking COAD as an example, the mRNA of both NLRP3 and ERα were significantly increased in tumor tissues relative to the matched normal tissues. Furthermore, a possible signaling pathway in which ER-NLR was related with Wnt/β-catenin was provided.

2. Materials and methods

2.1. Expression profiles of NLRs in different neoplasm tissues

The expression profiles of NLRs in different neoplasm tissues were acquired based on the expression values of each gene in RNA-SEQv2 of the Cancer Genome Atlas (TCGA) Data Portal (https://tcga-data.nci.nih.gov/tcga/) according to a previous method [11]. The NLRP3 and ERα expression profiles in colon adenocarcinoma tissues from Gene Expression across Normal and Tumor tissue database (http://medical-genome.kribb.re.kr/GENT/search/search.php), The Human Protein Atlas database (https://www.proteinatlas.org/) and Oncomine™ database (https://www.oncomine.org/resource/main.html).

2.2. Animals and colonic epithelial cell culture

Animals were used in accordance with the guidelines for care and use of experimental animals issued by the Nanjing Agricultural University (Permission Number: SYXK (Su) 2017-0007). Male wild-type mice and ERα-deficient mice of 6–8 weeks old back-crossed on a C57BL/6 background were used in the experiment. Mice were fed a normal diet and allowed to drink tap water ad libitum.

Human colon cancer cell line (HCT116) and human normal colon cells (NCM-460) were purchased from GuanDao Biological Engineer Corporation (Shanghai, China). ER antagonist (AZD9496, MCE) treated cells were cultured in DMEM with supplemented with 10% normal fetal bovine serum (Gibco, US). ER agonists (PPT, Abcam) treated cells were cultured in DMEM supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, UT).

2.3. RNA-seq and data analysis

Total RNA was extracted from colon tissue by Trizol reagent (Invitrogen) separately. The integrity of the purified RNA was analyzed by the Agilent 2200 Electrophoresis Bioanaly zer System (Agilent Technologies, USA). The RNA with RIN > 6.0 is right for mRNA seq. Enrichment of poly(A)-RNA preparation for RNA-seq was performed using NEBNext Poly(A) mRNA Magnetic Isolation Module(NEB) kit. The cDNA libraries were constructed for each pooled RNA sample using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina according to the manufacturer' s instructions. The tagged cDNA libraries were pooled in equal ratio and used for 150 bp paired-end sequencing in a single lane of the Illumina HiSeqXTen. Pathway analysis was used to find out the significant pathway of the differential genes according to the KEGG database. We turn to Fisher's exact test to select the significant pathway, and the threshold of significance was defined by *P*-value.

2.4. Lentiviral production and infection

Control shRNA lentivirus and NLRP3 shRNA lentivirus were used for lentiviral production and infection. The sequences were listed in Supplemental Table 2. Control and sh-NLRP3 cell lines were selected with puromycin (4 μg/mL) for 2 weeks and maintained in medium containing 1 μg/mL puromycin. The level of NLRP3 expression was analyzed by quantitative western blot.

2.5. RNA extraction and PCR analysis

RNA was extracted from the cells using Trizol reagent and reverse transcribed into cDNA. Then, 1 μL of the cDNA sample was extracted in nonuplicate. Subsequently, PCRMix (10 μL), primers (2 μL) of each Wnt2b, LRP8, Dvl1, Axin-2, APC, GSK3b, β-catenin, and GAPDH primers (see Supplemental Table 3) and ddH_2O (7 μL) were added. To semi-quantitate the PCR results, the Image J software was used for intensity analysis of amplified bands. The band intensity was normalized with the corresponding GAPDH.

2.6. Western blotting (WB)

Lysis buffer (200 μL) was added into cells and the system was centrifuged with 12,000 r/min for 5 min. Then the supernatants were dissolved in Laemmli buffer and separated using SDS-PAGE. Separated proteins were transferred to a 0.45 mm PVDF membrane and blotted with primary antibodies against NLRP3, ERα, Wnt2b, β-catenin, and anti-β-actin (omnimabs, US). Immunoglobulin G (IgG) anti-rabbit-HRP was then applied, and an Amersham Imager 600 (GE Healthcare) was used to visualize chemo-luminescence.

2.7. Statistical analysis

All data were processed using GraphPad Prism 6. Data were analyzed by a two-tailed Student's test and two-way ANOVA test. *P* value < 0.05 was considered significant. The plot of sensitivity versus 1-Specifity was used to generate receiver operating characteristic curves with the respective areas under the curves. The optimal cut-off value was represented highest Youden's index (sensitivity + specificity − 1).

3. Results

3.1. NLRs and ERs are differentially expressed in neoplasm tissues

To determine whether NLRs are differentially expressed in neoplasm tissues, we explored the expression of 22 NLR genes in 33 neoplasm tissues. The four most significant changed neoplasm tissues of different NLRs were shown in Fig. 1. The threshold of significant changes in mRNA levels was set at > 2 or < 1/2 fold difference compared with control. As shown in the figure, NLRPs were expressed in many neoplasm tissues. For example, NLRP2 was expressed more highly in cervical squamous cell carcinoma, endocervical adenocarcinoma, and cholangiocarcinoma cancer tissues than in normal tissues. NLRP3 was expressed more highly in normal tissues than in lung squamous cell carcinoma tissues. NLRP4 had higher expression in endometrial and hepatocellular carcinoma tissues. NLRP8–10 and NLRP13–14 expression were lower than those of other NLRPs. However, NLRP11 and NLRP12 had high expression in both cancer and normal tissues. In addition, CIITA also had higher expression in cervical squamous cell carcinoma and endocervical adenocarcinoma and glioblastoma multiforme, which was similar to NOD2, the NLRC member. NAIP was found frequently higher in colorectal adenocarcinoma than in normal tissues. The NLRC family NLRs, NOD1, NLRC3, and NLRC5, were highly expressed in both cancer tissue and normal tissue, while NLRC4 had higher expression in clear cell renal cell carcinoma tissues. Receiver operating characteristic curve and the area under the curve (AUC) was further used to evaluate the diagnostic ability of NLRs in different neoplasm tissue (Supplemental Fig. 1). The AUC, cut-off points, sensitivity, specificity, Youden's index, and positive and negative likelihood ratios, were exhibited in Supplemental Table 1. As demonstrated in the table, the AUC ranged from 0.70–0.96 (95%CI: 0.603–0.993) with high sensitivity and specificity. These data showed that certain types of NLRs were differentially expressed in tumor tissues. The expression profiles may provide valuable insight into potential role of NLRs in neoplasms.

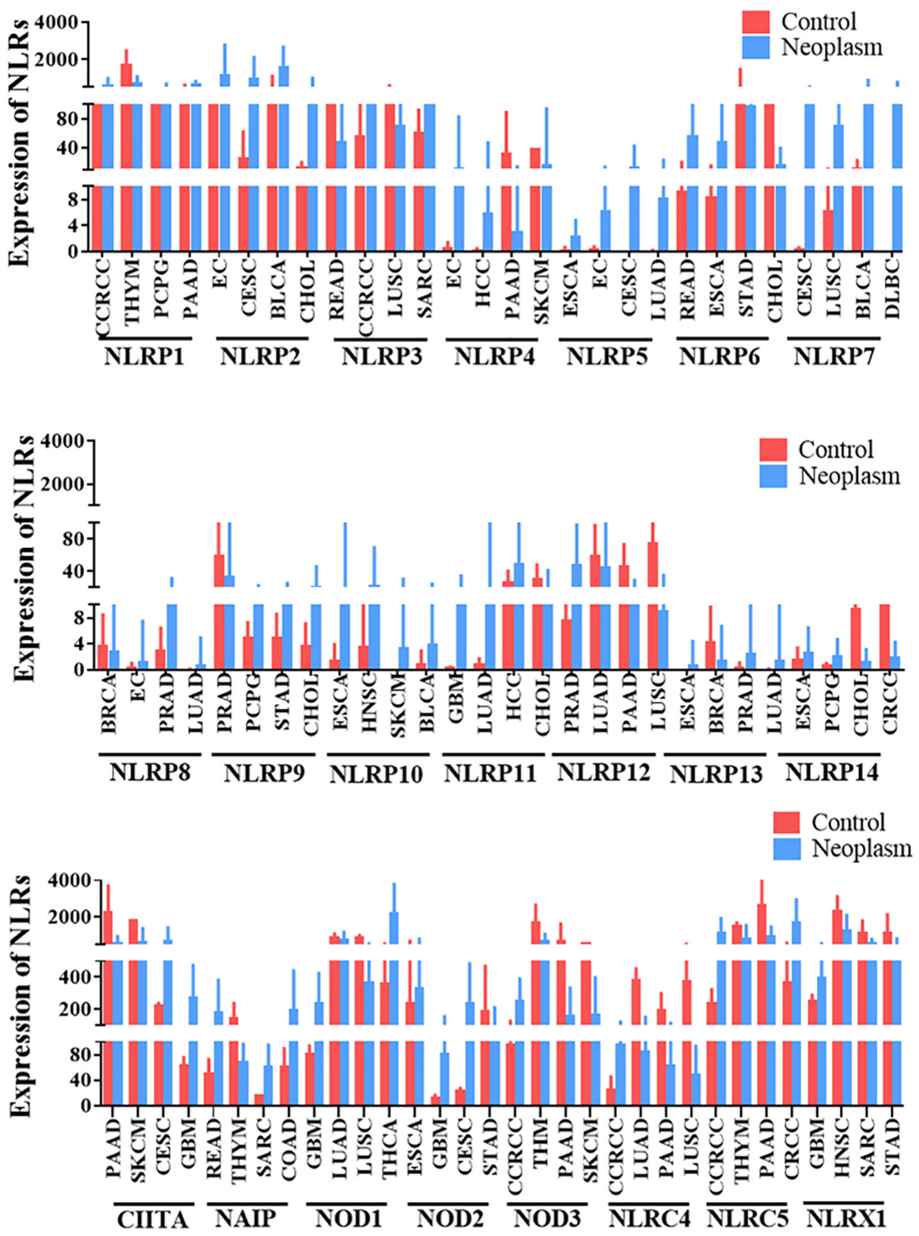

Fig. 1. NLR expression in different neoplasm tissues. The Cancer Genome Atlas (TCGA) database was used to analyze the expression profiles of NLRs. According to the barcodes of TCGA samples, the samples were classified into neoplasm or normal tissue. The mRNA expressions of NLRs in normalized results were provided by RNA-SEQv2 data (normalized count: upper quartile normalized RSEM count estimates). ($0 < n \leq 200$, Data represent mean values ± SEM.) The threshold of significant changes in mRNA levels was set at > 2 or < 1/2 fold difference compared with control. The full names of different neoplasm types are listed in electronic supplementary material 1.

Detailed NLR expression data in neoplasm tissue is provided in electronic supplementary material 1. We have shown that ERs, as a kind of nuclear receptor, could directly regulate NLRs, which has been of considerable biological interest in neoplasms [10,12]. In this study, we also explored the expression pattern of ER genes in 33 neoplasm tissues and found that ERs were differentially expressed in tumor tissues (Supplemental Fig. 2). Therefore the interaction between ER and NLR in neoplasm tissues may exist. Thus, the anti-inflammatory and anti-neoplasm functions of the ER in various diseases might be explained, at least in part, by regulation of NLRs [13–15].

3.2. *Expressions of NLRP3 and ERα in COAD patients*

We have demonstrated NLRP3 was differentially expressed in normal and colon cancer cells, while selective ERα antagonist could significantly decrease pro-inflammatory cytokines expression, suppress colon cancer cells proliferation and promote apoptosis by inhibited NLRP3 expression and inflammasome activity [10]. In this study, we investigated the NLRP3 and ERα expression profiles in colon adenocarcinoma tissues using COAD as an example (Source: http://medical-genome.kribb.re.kr/GENT/search/search.php; https://www.proteinatlas.org/ and https://www.oncomine.org/resource/main.html). As expected, the mRNA of both NLRP3 and ERα were significantly increased ($P < .01$) in tumor tissues relative to matched normal tissues (Fig. 2A and B, Supplemental Fig. 3). Moreover, high expression of NLRP3 and ERα in tumors were confirmed at the protein level by immunochemistry results (Supplemental Fig. 3). To further investigate the clinical significance of NLRP3 and ERα in COAD patients, we performed a survival analysis of COAD patients. We found that the overall survival rate of patients with low NLRP3 expression was higher than that of high expression of NLRP3 (66% versus 61%, $P = 0.14$). The less significance could be ascribed to the small size of the database. Similarly, the patients in the ERα high expression group had a significantly poorer survival rate compared to those with low ERα expression (57% versus 66%, $P = 0.0087$) (Fig. 2C and D, Supplemental Fig. 3). These results suggest that ERα might function as a potential prognostic marker in COAD patients.

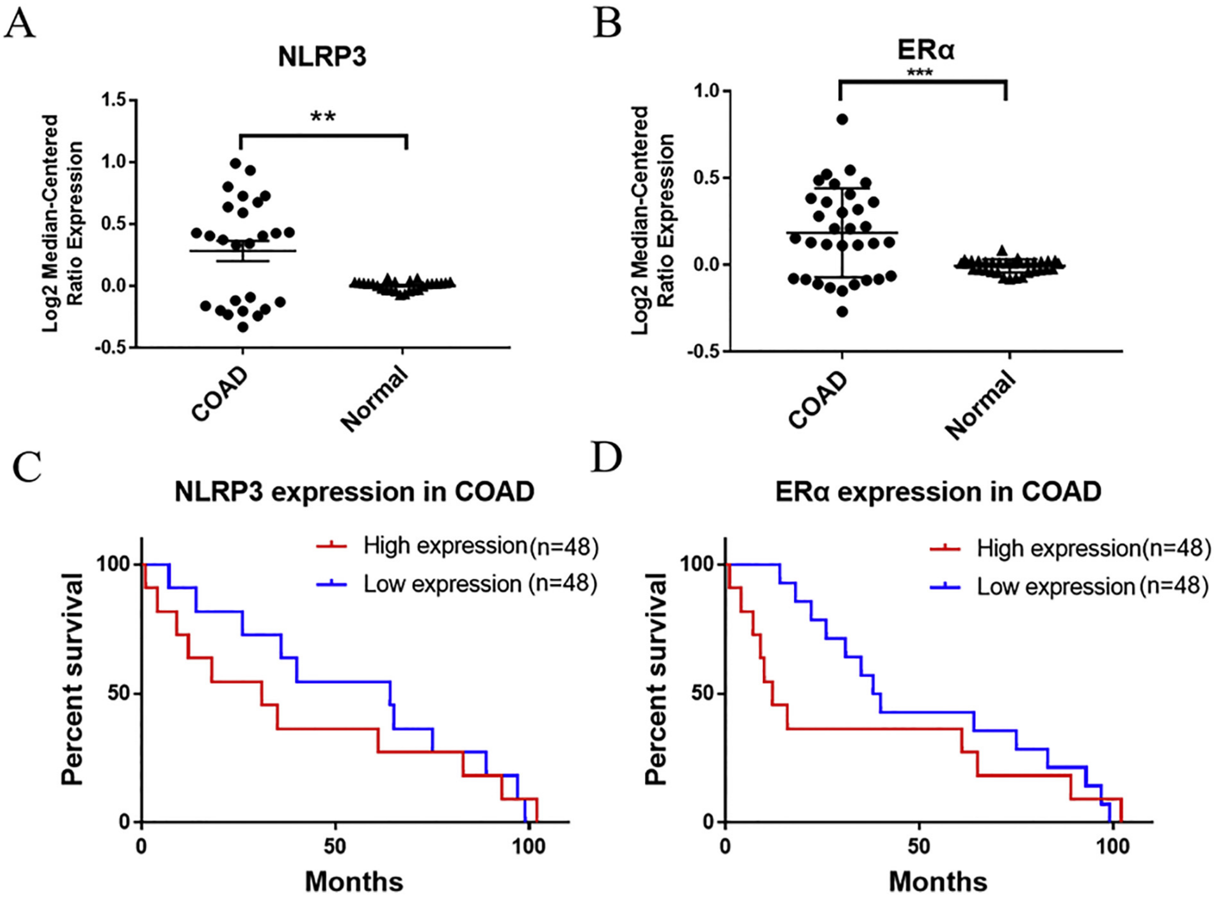

Fig. 2. Expression of NLRP3 and ERα and their clinical significance in COAD patients. Analysis of NLRP3 (A) and ERα (B) expression in TCGA cohort of patients with colon adenocarcinoma (COAD). The overall survival rate of COAD patients with different NLRP3 (C) and ERα (D) expression. Source: Oncomine (www.oncomine.org/resource/main. HTML). **, $p < 0.01$. ***, $p < 0.001$.

3.3. ER regulates the Wnt/β-catenin signaling pathway in colon

To confirm the signaling pathway of ER in the colon, we did genome-wide expression analysis, which is not influenced by deductive assumptions and can provide unbiased information about aberrant gene expression in different samples. We employed RNA-seq to evaluate whole transcriptomic changes in colon tissue of ER$^{-/-}$. Pathway enrichment analysis based on the KEGG pathway database was carried out in this study. Significantly enriched pathways (top 20) are listed in Fig. 3A, in which, DNA replication, Wnt signaling pathway, and pathways in cancer are included. This is consistent with our previous research that ERα antagonist could induce G2/M arrest and inhibit cell proliferation in colon cancer cells [10]. Due to Wnts are growth stimulatory factors, which can impact the cell cycle at various points, therefore we speculated that ERα might regulate the Wnt/β-catenin signaling pathway in cancer. Additionally, the down-regulated gene related pathway includes NOD-like receptors signaling (Fig. 3B). This result also confirmed our recent report that ERs can directly regulate NLRs gene transcription [10].We then analyzed the mRNA levels of Wnt/β- catenin signaling pathway and NLRP3 genes. Of note, Wnt2b, LRP12, LRP8, Dvl1, Axin-2, GSK3b, APC, β-catenin genes were differentially expressed in ER$^{-/-}$ mouse colon tissue and the NLRP3 expression was also significantly decreased (Fig. 3C and D).

3.4. ERα regulates the Wnt/β-catenin signaling pathway by targeting the NLRP3 in colon cancer

We then detected the Wnt/β-catenin pathway genes expression in colon cells (NCM460 and HCT116). The results showed the mRNA expressions of Wnt2b, LRP8, Dvl1, and β-catenin were significantly increased in HCT116. Meanwhile, Axin-2, GSK3b and APC expressions were similar in both cell lines (Fig. 4A, Supplemental Fig. 5A). To confirm the role of NLRP3 in the Wnt/β-catenin pathway, we knock-down the NLRP3 in NCM-460 cells. We found Wnt2b, LRP8, Dvl1, GSK3b, APC, and β-catenin expression all significantly decreased except Axin-2, which suggested that NLRP3 was involved in regulation of Wnt/β-catenin pathway (Fig. 4B, Supplemental Fig. 5B). Additionally, we found ERα agonist and antagonist had different effects on the Wnt/β-catenin pathway in HCT116 cells. As shown in Fig. 4C and Supplemental Fig. 5C, Wnt2b, LRP8, Dvl1, and β-catenin expression could be enhanced by ERα agonist but decreased by ERα antagonist. However, the chemicals couldn't influence the GSK3b, APC, and Axin-2 expression (Fig. 4C, Supplemental Fig. 5C). Accordingly, the influence of ERα agonist and antagonist on the Wnt/β-catenin pathway would be counteracted by the knock-down of NLRP3 in HCT116 cells (Fig. 4D, Supplemental Fig. 5D). Finally, we detected the Wnt/β-catenin protein expressions under different conditions. The changed protein expressions were similar to mRNA expression (Fig. 4E and F). All these results suggested that ERα can regulate NLRP3 and then activate the Wnt/β-catenin signaling pathway in colon cancer.

4. Discussion

Within the past 10 years, NLR-mediated inflammation has been linked to the tumor-related behavior, such as tumor initiation, progression, metastasis, and survival, even alterations in the anti-tumor adaptive immune response [16]. In this study, we found different types of NLRs were differentially expressed in different tumor tissues. For

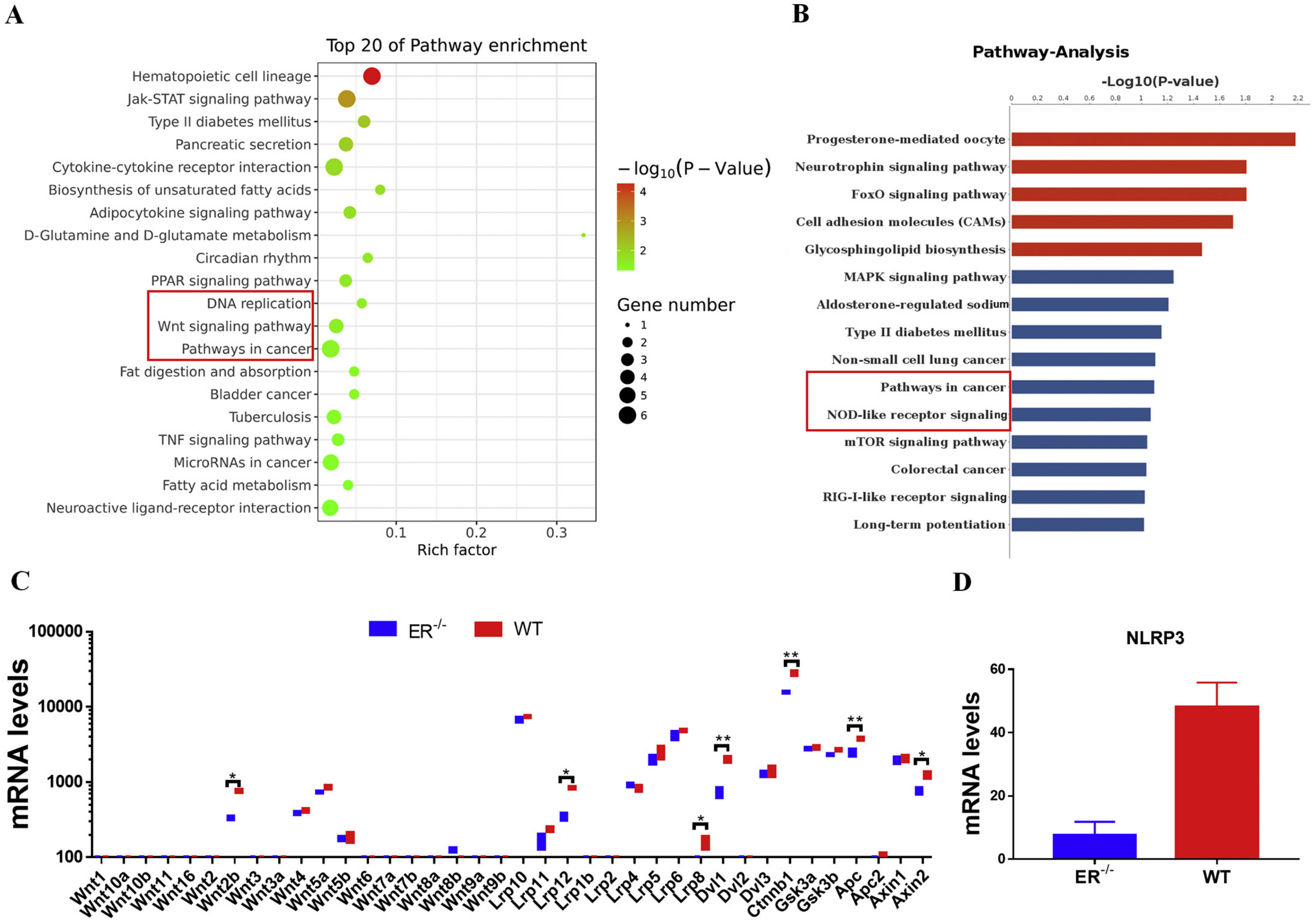

Fig. 3. RNA-Seq profiling of ER $^{-/-}$ mice. Six colon tissues from ER $^{-/-}$ and Wild-type (WT) mice were used for RNA-seq. (A) KEGG enrichment analysis showing the list of the top 20 pathways. The enrichment map is colored by the gradient level of the -log10(*P* value). (B) The downregulated pathways. The red represents significantly enriched pathways. (C) mRNA levels of Wnt/β-catenin complex genes. (D) mRNA levels of NLRP3 gene. Data are shown as mean ± SEM of 3 samples per group. Significance was calculated in relation to the WT group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (unpaired two-tailed Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

example, NLRP2–3, NLRP5, NLRP7–10, and NOD2 were highly expressed in cancer tissue, and NLRP14 and NLRC4 behaved in the opposite way; while NLRP1, NOD1, NLRC3, NLRC5, and NLRPX1 were highly expressed in both normal and neoplasm tissue. Due to the discrepancies, it is uncertain how dysregulation of these innate immune sensors induces inflammation and leads to carcinogenic transformation of cells [1]. However, our study provides some helpful hints for neoplasm prevention or therapy. For example, currently, the research of NLRC4 is mainly focused on anti-infection against Salmonella, Shigella, Pseudomonas and Legionella, or promotion of host intestinal immunity [17,18]. In our research, NLRC4 and NLRC5 were highly expressed in clear cell renal cell carcinoma tissue. Some studies have reported that genovariation of NLRC5 could be observed in all renal cell carcinoma (RCC) cell lines, and high expression of NLRC5 is associated with better RCC-free survival and overall survival [19]. Thus, similar to NLRC5, NLRC4 might be used as a prognostic marker to predict patient outcomes in RCC. We also predicted regulatory networks (interacting protein, lncRNA and miRNA) of NLRs, and the results are submitted as supplementary materials (Supplemental Fig. 4 and electronic supplementary material 2). Considering the important role of NLRs, this information could facilitate the investigation of cancers.

To ascertain how ERs and NLRs work in cancer, we investigated ERα and NLRP3 expression profile, using COAD as an example. The results showed both ERα and NLRP3 were overexpressed in colon cancer tissue. Interestingly, the high expression of ERα is significantly correlated with the survival of COAD patients. All these data suggest that ERα and NLRP3 play a critical role in colon tumorigenesis and progression. So far, compared to the ERβ, the exact function of ERα and its biological effects on COAD is unclear [20–22]. It has been proven that hypomethylation of the ERα gene was found in most human colon cancers [23]. Furthermore, the ERα/ERβ ratio has been positively correlated with the colon carcinomas proliferation and decreased apoptosis [24]. Our study obtained a similar result, which provided the new and important information for exploring the role of ER in colon cancer. To confirm the regulatory mechanism of ER in the colon, we did RNA-seq analysis using ER $^{-/-}$ colon tissue and found that the Wnt signaling pathway might be regulated by ER. As we know, the core Wnt/β-catenin pathway components including Wnt, Fzd/LRP, Dvl, Axin/APC/GSK3/CK1, β-catenin and TCF [25], which control the life cycle of Wnt signaling molecules, their interactions with key receptors, and the actions of target cells [26]. Since Wnt signals are crucial for the activity of cells, it is not surprising that the Wnt pathway is involved in carcinomas [27–30]. Our results indicated ERα and NLRP3 had effects on the Wnt pathway. Briefly, they could increase Wnt2b, LRP8, Dvl1 expression, which could be abolished by NLRP3 knockdown. Besides, ERα and NLRP3 have a subtle influence on APC/Axin-2/GSK3b expression, however, they might inhibit the action of destruction complex to keep stabilization of β-catenin. These results were consistent with

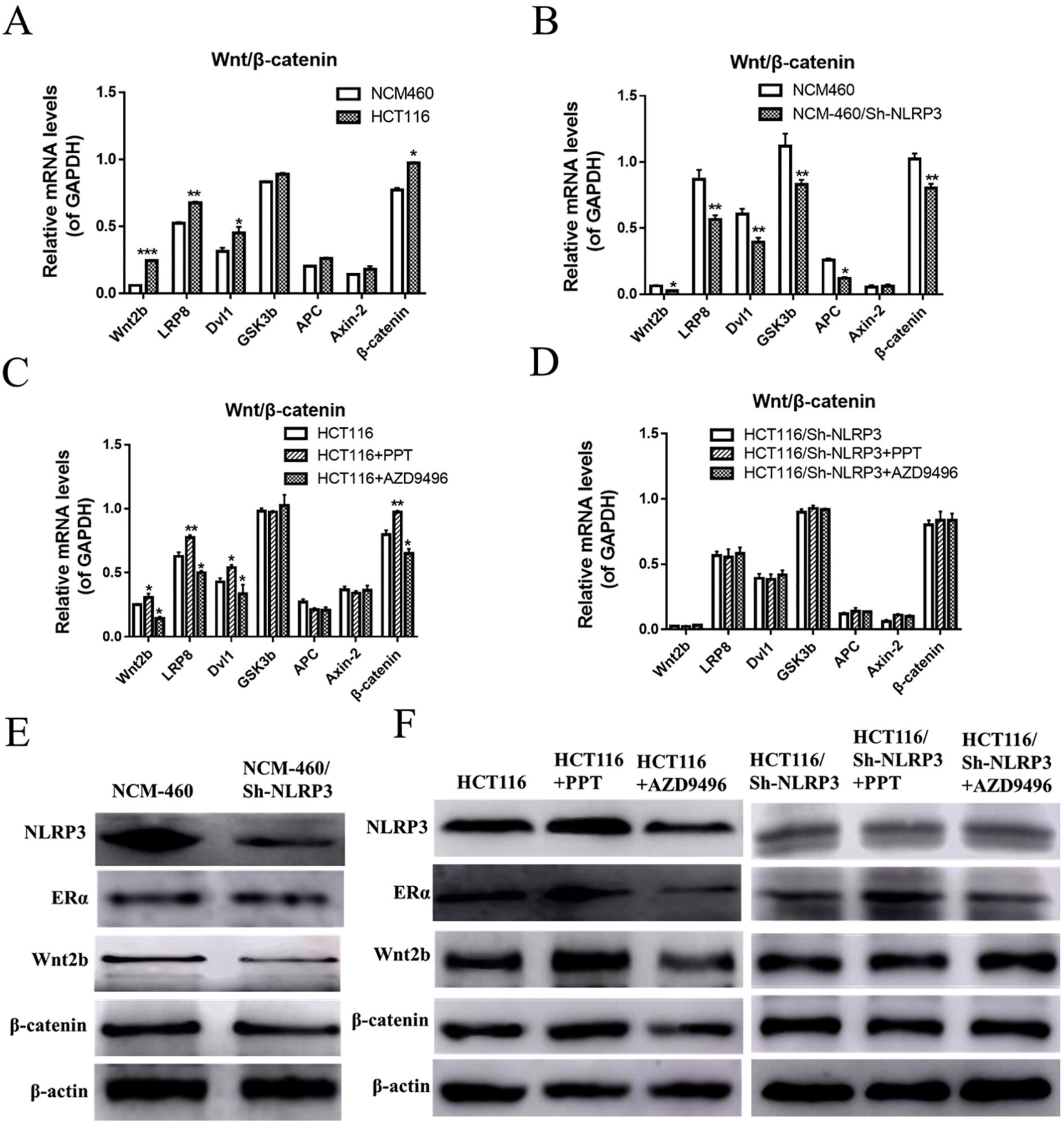

Fig. 4. Expression of NLRP3, ER, and Wnt/β-catenin genes in colon cells. (A) mRNA expressions of Wnt/β-catenin pathway genes in normal colon cells (NCM-460) and colon cancer cells (HCT116). (B) mRNA expressions of Wnt/β-catenin pathway genes in normal colon cells (NCM-460) and in NLRP3 knockdown NCM-460 cells (NCM-460/Sh-NLRP3). (C) mRNA expressions of Wnt/β-catenin pathway genes with ERα agonist/antagonist induction in HCT116 cells. (D) mRNA expressions of Wnt/β-catenin pathway genes with ERα agonist/antagonist induction in NLRP3 knockdown HCT116 cells (HCT116/Sh-NLRP3). (*E*-F) Western blot results of NLRP3, ER, Wnt2b, and β-catenin different cells. Images are from one experiment representative of three independent experiments. Data are shown as mean ± SEM of per group and are from one experiment representative of three independent experiments. Significance was calculated in relation to the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired two-tailed Student's t-test).

another report that ER helps to maintain the intestinal stem cell microenvironment by modulating cytokine and Wnt availability [31]. Furthermore, this result further explained our previous study that ERα antagonist could inhibit HCT116 cell proliferation [10]. In conclusion, our study suggested that distinct NLRs show varied roles in tumorigenesis in different tissues, which might serve as valuable biomarkers. Most important, a possible regulatory mechanism of Wnt/β-catenin in cancer may be identified in this study.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.05.009.

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