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ORIGINAL RESEARCH

Fusobacterium nucleatum prevents apoptosis in colorectal cancer cells via the ANO1 pathway

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Objective: Chemotherapy failure derived from drug resistance is the most important reason causing the recurrence in colorectal cancer patients. Therefore, it is necessary to shed light on the mechanism of chemotherapy resistance in colorectal cancer patients.
Methods: We looked into the contribution of *Fusobacterium nucleatum* and ANO1 to chemoresistance in the human colorectal carcinoma cell lines. We silence and overexpress *ANO1* in HCT116 and HT29 cells with lentivirus and siRNA knockdown technique in the absence or presence of *F. nucleatum*, oxaliplatin or 5-fluorouracil (5-FU). ANO1, p-pg, cleaved PARP, cleaved caspase-3, and EGFR expression was measured by Western blot. Cell apoptosis was measured by flow cytometry.
Results: We found that *F. nucleatum* promoted ANO1 expression on colon cancer cells. Moreover, ANO1 prevent colon cancer apoptosis from oxaliplatin and 5-FU. Additionally, knockdown *ANO1* expression could block *F. nucleatum* protective effects and increase the apoptosis effects induced by oxaliplatin and 5-FU. Therefore, *F. nucleatum* might be biologically involved in the development of colon cancer chemoresistance via ANO1 pathway.
Conclusions: Taken together, our findings provide a valuable insight into clinical management and therapy, which may ameliorate colorectal cancer patient outcomes.
Keywords: colorectal cancer, *F. nucleatum*, chemoresistance, 5-fluorouracil, oxaliplatin, ANO1

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death and the third most common cancer in the world.[1,2] Chemotherapy drugs can generally shrink tumor size, reduce tumor growth, and inhibit tumor metastasis in advanced CRC patients. For example, 5-fluorouracil (5-FU) and oxaliplatin are commonly used active cytotoxic drugs for CRC patients. 5-FU inhibits the enzyme activity of thymidylate synthase during DNA replication.[3] Oxaliplatin inhibits tumor cell growth and causes cell G2 phase arrest by covalently binding DNA and forming platinum-DNA adducts.[4] These chemotherapeutic agents were widely combined to treat CRCs[1] and are initially effective in most cases. However, patients finally suffer tumor recurrence due to drug resistance, and more than 90% advanced CRC patients die in 5 years.[5] Unfortunately, novel immune checkpoint therapy is generally useless for colon cancer patients.[6] Therefore, it is necessary to shed light on the mechanism of chemotherapy resistance in CRC patients.

CRC chemoresistance was caused by the complex interplay between the environment and gene regulation. The micropopulation influences tumor-related signaling

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pathways and intestinal inflammation, which is associated with CRC initiation and progression.[7–10] Recent studies have demonstrated that the gut micropopulation may regulate local immune responses and successively impact immunotherapy[11,12] and chemotherapy.[13,14] *Fusobacterium nucleatum* (*F. nucleatum*) is important micropopulation in CRC patients. The amounts of *F. nucleatum* are gradually augmented from normal tissues to adenoma tissues and to adenocarcinoma tissues in colorectal carcinogenesis.[15,16] Furthermore, the abundance of *F. nucleatum* in CRC tissues is linked to the lower survival rate.[17] Especially, compared to those with nonrecurrence post-chemotherapy, CRC patients with recurrence post-chemotherapy contained more *F. nucleatum*. Another report shows that *F. nucleatum* can modulate several cellular signal pathways and activate the autophagy pathway which may play a key role in mediating CRC chemoresistance to small drug chemotherapeutics.[18]

Anoctamin-1 (ANO1) is one of the human chloride channel proteins and is encoded by the *ANO1* gene located on 11q13,[19] which is frequently amplified in different types of human carcinomas.[20,21] The expression of ANO1 is usually upregulated in several cancers including breast cancer,[22] prostate cancer,[23] gastrointestinal stromal tumor,[21,24] colorectal cancer,[25,26] esophageal squamous cell carcinoma[27] and so on. It also plays an important role in the development of distant metastasis and poor prognosis of cancer patients.[23,28,29] Recently, ANO1 has been reported to activate the mitogen-activated protein kinase (MAPK) signaling pathway, which promoted tumorigenesis and invasion.[24] Furthermore, ANO1 has been reported to induce the activation of EGFR and calmodulin-dependent protein kinase II and subsequently activate serine-threonine protein kinase (AKT) and MAPK signaling in breast cancer, which contribute to cancer progression.[22] Researches from two different groups have demonstrated that high ANO1 expression was a significant prognostic factor for overall survival of patients with CRC and inhibition of ANO1 expression suppresses growth and invasion in human CRC cells.[25,26] However, a detailed analysis of the role of ANO1 in CRC and its contribution to chemoresistance is missing. In this study, we demonstrated that *F. nucleatum* could upregulate ANO1 expression in CRC cells and prevent apoptosis induced by chemotherapy drugs. Through constructing *ANO1* overexpression and silencing cell lines, we found that ANO1 plays an important role in the process of chemotherapy drugs which induces CRC cell apoptosis. Our data indicate that *F. nucleatum* could prevent CRC apoptosis from chemotherapy drugs via the ANO1 pathway.

Materials and methods

Chemicals

5-FU and oxaliplatin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA, F6627, O9512). TRIzol™ was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Cell and cell culture

The human colorectal carcinoma cell lines HCT116 and HT29 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in a culture medium consisting of RPMI1640 (Thermo Fisher Scientific) supplemented with 10% FBS (lot No. 40K2368, Sigma-Aldrich or lot No. 1248850, Thermo Fisher Scientific), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific) in an atmosphere of 95% air and 5% CO_2 at 37°C.

Recombinant lentiviron packaging

The cDNA (CGAAGAAGATGTACCACAT (837–855 nt, NM_018043.5) targeting siRNA site of *ANO1* gene, the pervasive disturbing sequence designed as negative-control shRNA and the gene coding ANO1 (NM_018043.5) were cloned into lentiviral nuclear vectors which produced pLKO.1-shANO1, pLKO.1-siNC and pLVX-Puro-ANO1 by JRDUN Biotechnology Co., Ltd (Shanghai, China), respectively. 293 T cells (ATCC) were transfected with the pLKO.1-shANO1, pLKO.1-siNC or pLVX-Puro-ANO1 mixing the lentiviral packaging plasmids (psPAX2 and pMD2G) using lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After 6 hours, the culture medium was changed to complete medium. The supernatant was concentrated and collected after culturing for 48 hours. The virus titer of the 293 T cells was determined using the dilution gradient method. The transfected cells were stored in a –80°C refrigerator for later use.

Examination of cell transfection

Three packaged recombinant lentivirus were separately diluted into three groups with culture medium with 5 µg/mL polybrene and were transfected into HCT116 and HT29 cells, respectively. The culture medium was changed to complete medium at 12 hours after transfection. Cells were cultured for 72 hours, following which the expression level of GFP in the cells was observed using an inverted fluorescence microscope. The transfection rate was also analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The lentivirus-infected cells with the GFP^+ were

sorted with flow cytometry. Knockdown and overexpression of *ANO1* gene in both cell lines were assessed by Western blot and RT-PCR.

Quantitative RT-PCR

Total RNA was extracted from the GFP+ lentivirus-infected cells using TRIzol reagent (Thermo Fisher Scientific) and quantified using a Merinton SMA1000 instrument (Merinton; Ann Arbor, MI, USA). The first strand of cDNA was synthesized from 2.5 μg of total RNA using the iScript cDNA Synthesis kit (Bio-RadLaboratories Inc., Hercules, California, USA) according to the manufacturer's instructions. The qRT-PCR was performed using the Quantifast SYBR Green PCR kit (Qiagen NV, Venlo, the Netherlands) in an ABI 7500 system. The primers used for PCR of *ANO1* were as follows: forward: 5′-AACGGGACCATGCACGGCTT-3′, reverse: 5′-TGTTGTGGTGGTTGCACGGC-3′. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The reaction cycles for all genes were initiated as follows: 95°C 30 seconds, 95°C 5 seconds, 64°C 34 seconds, 95°C 15 seconds, 60°C 60 seconds. Forty amplification cycles were necessary to achieve exponential amplification. All experiments were repeated three times. Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.[30]

Western blot

Cells were cultured in a 6-well plate at a density of 2×10^5 cells/well and incubated with oxaliplatin (10 μM) and 5-FU (2 μM) for 24 hours. Following treatments, cells were collected. Total protein was extracted from cells lysed by RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) and separated by 10% SDS-PAGE before being transferred onto PVDF membranes (Amersham). The PVDF membranes were blocked with 5% BSA in Tris-buffered saline with TWEEN 20 (TBST) at room temperature (RT) for 2 hours and probed using the primary antibodies against ANO1, p-pg, cleaved PARP, cleaved caspase-3, EGFR and GAPDH at 4°C for 1 hour (1:2,000; all from Cell Signaling Technology, Beverly, MA, USA). The detection was performed using a secondary antibody (Sigma-Aldrich Co.) at room temperature for 1 hour. After washing, the bound antibody was detected with immobilization western chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA). Then, chemiluminescent emission was captured on Kodak XAR film, and images were analyzed by ImageJ software. The relative expression of interest protein was represented as the grayscale ratio of the protein to GAPDH. All experiments were repeated four times.

Flow cytometry analysis

Cell apoptosis was detected by two-color immunofluorescence staining in the flow cytometric analysis. Cells (2×10^5 cells/well) were plated into 6-well plate and then were treated with oxaliplatin (10 μM) and 5-FU (2 μM) for 12 hours. The wells added by PBS were used as negative control. Adherent cells were trypsinized without EDTA before two washing steps with PBS. Cells were then incubated with 5 μL of fluorescein isothiocyanate, annexin V (BD Pharmingen, San Jose, CA, USA) and propidium iodide (BD Pharmingen) in 400 μL of 1× binding buffer for 15 minutes at RT. Data were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) equipped with CellQuest Software (BD Biosciences).

Statistical analyses

All the results are expressed as the mean ± SD. Data were analyzed by single factor analysis of variance and the *t*-test using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Silencing and overexpression of *ANO1* with shRNA lentivirus

To explore the function of ANO1 in colon cancer cells, HT29 and HCT116 cell lines were separately transfected by lentivirus to silence and overexpress *ANO1*. Briefly, both the stable silencing of *ANO1* and overexpression *ANO1* cell lines, which are named siANO1 and ANO1, respectively. Corresponding negative control groups constructed with empty vector lentivirus were named Vector and siNC. The silencing and overexpression efficacy at mRNA and protein levels in colon cancer cell lines were determined by real-time PCR and Western blot, respectively. The results of Western blot showed that the expression levels of ANO1 in the overexpression groups were 1.8–2.5-fold higher than control groups, and the silencing groups ANO1 reduced to 13–28% of that of the control groups (Figure 1A, B). And real-time PCR revealed that the mRNA levels of overexpressed groups were approximately 15.91±1.377 in HT29 and 16.53±0.5425 in HCT116, the silencing groups were approximately 0.1116±0.00761 in HT29 and 0.08067±0.01176 in HCT116 (Figure 1C). The difference in *ANO1* mRNA levels between the assay groups and control groups were statistically

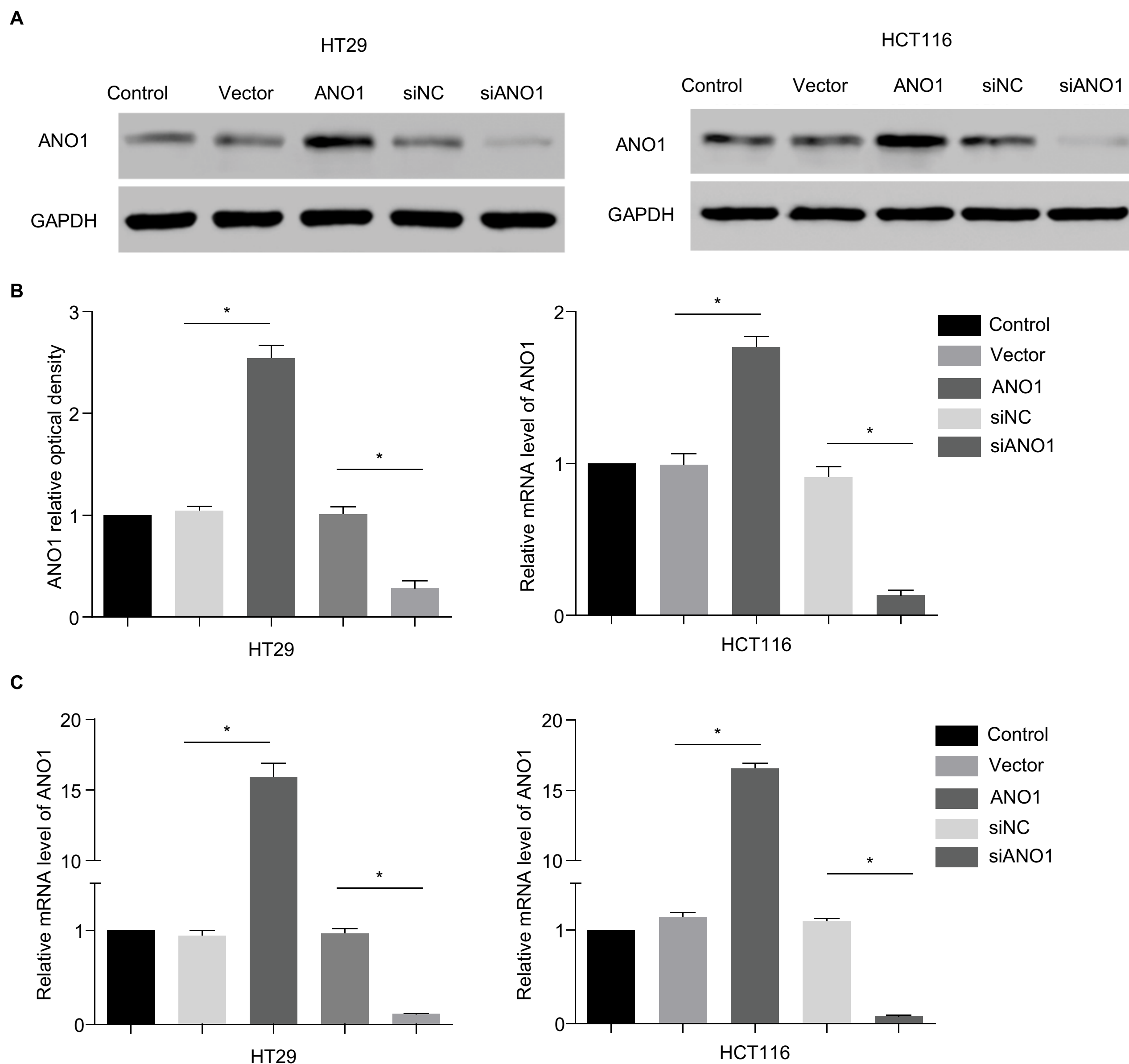

Figure 1 *ANO1* was silenced and overexpressed with shRNA lentivirus.
Notes: (A) Representative Western blots analysis of ANO1 protein expression in HT29 and HCT116 cell lines. **(B)** Graph illustrates the optical density of ANO1 protein bands expressed relative to GAPDH control. (**C)** Real-time PCR analysis of *ANO1* mRNA levels in HCT116 and HT29 cell lines. Data are expressed as mean ± SD (N=3). *P<0.01.

significant. The results of both Western blot and real-time PCR demonstrated that lentiviral vectors were effective for ANO1 expression.

F. nucleatum promotes ANO1 expression on colon cancer cells

RNA-sequencing data confirmed that *ANO1* was expressed in colon cancer cell line HT29, which was in line with most reports while *F. nucleatum* promoted the *ANO1* mRNA levels (Figure 2A, data from GSE90944). We hypothesized that *F. nucleatum* was biologically involved in the development of colon cancer via ANO1 pathway. To test this hypothesis, we co-cultivated colon cancer cell lines HT29 and HCT116 with *F. nucleatum*, performed real-time PCR analysis and Western blots analysis, and compared the ANO1 expression profiles between the colon cancer cell lines cocultured with or without *F. nucleatum*. The rate of *ANO1* mRNA expression increased significantly in co-culture groups, and the difference was statistically significant in both cell lines (Figure 2B). Further, protein expression of ANO1 was detected in HT29

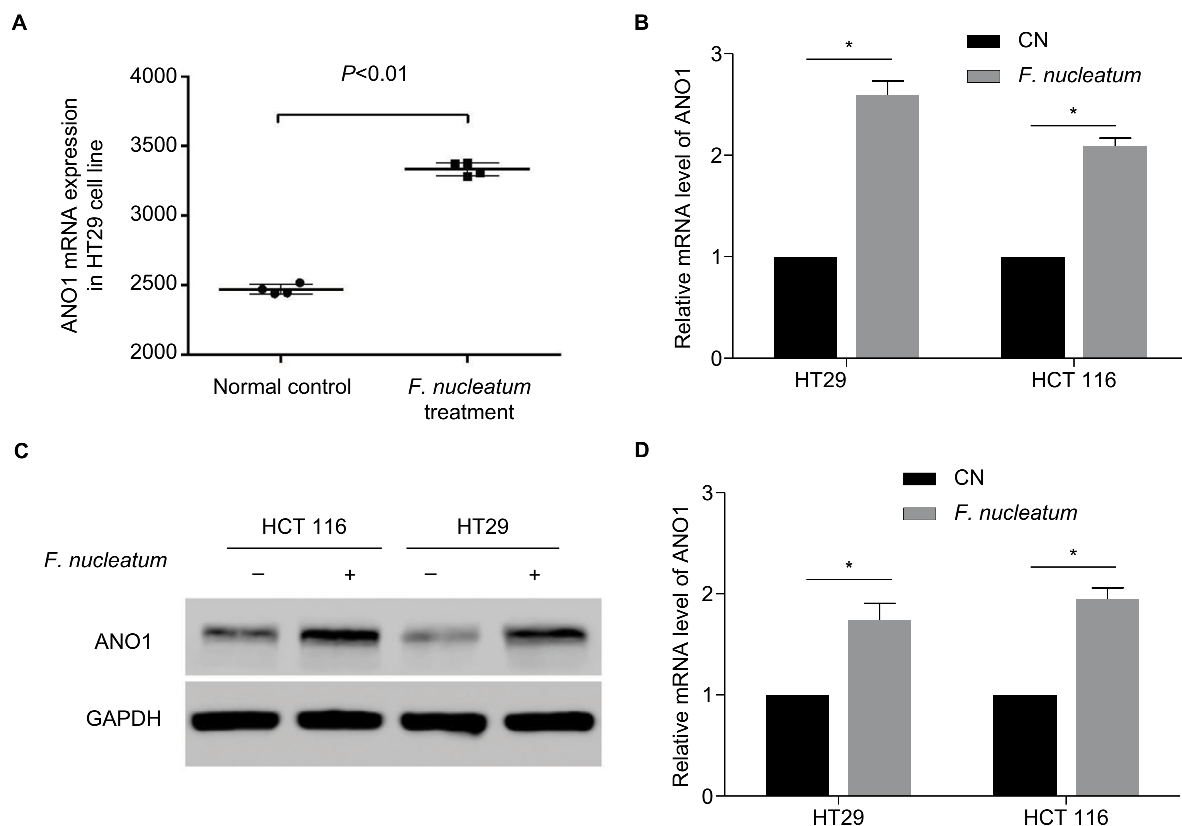

Figure 2 *F. nucleatum* promoted ANO1 expression on colon cancer cells.

Notes: (A) Real-time PCR analysis of *ANO1* mRNA levels in HT29 cell lines with *F. nucleatum* co-culture. (**B)** Real-time PCR analysis of *ANO1* mRNA levels in HCT116 and HT29 cell lines with or without *F. nucleatum* co-culture. (**C)** Representative Western blots comparing the expressions of ANO1 protein in HCT116 and HT29 cell lines with or without *F. nucleatum* co-culture. (**D)** Graph illustrates the optical density of ANO1 protein bands expressed relative to GAPDH control. Data are expressed as mean ± SD (N=3). *$P<0.01$.

Abbreviations: *F. nucleatum, Fusobacterium nucleatum;* CN, control,

and HCT116 cell lines. The rate of ANO1 protein expression was higher in *F. nucleatum* cocultured cells (Figure 2C, D). Given the fact of ANO1 upregulation in several cancers, and overexpression correlated with the development of distant and poor prognosis of cancer patients, our data suggest that *F. nucleatum* may cause ANO1 pathway activation and potentially support cancer chemoresistance.

ANO1 prevents colon cancer apoptosis from chemotherapy drugs

We next hypothesized that *F. nucleatum* was biologically involved in the development of colon cancer chemoresistance via ANO1 pathway. As expected, oxaliplatin and 5-FU induced HT29 and HCT116 cell apoptosis (Figure 3A, B). Oxaliplatin induced 47.8% ± 0.8% and 51.6% ± 0.6% apoptosis in HT29 and HCT116, while 5-FU induced 43.7% ± 1% and 42.6% ± 0.9% apoptosis. Co-culture with *F. nucleatum* reduced HT29 and HCT116 apoptosis induced by these chemotherapeutic agents. Oxaliplatin induced 27.9% ± 0.2% and 33.1% ± 0.6% apoptosis in HT29 and HCT116 cocultured with *F. nucleatum*, while 5-FU induced 26.9% ± 0.4% and 21.3% ± 1.5% apoptosis. The apoptosis ratio between CRC cell lines cocultured with or without *F. nucleatum* was statistically significant. These data indicate that *F. nucleatum* induces CRC resistance to oxaliplatin and 5-FU.

To address whether *F. nucleatum* prevents CRC apoptosis from chemotherapeutic agents via the ANO1 pathway, we treated *ANO1* silencing and overexpression cell lines with chemotherapy drugs. Comparing control groups with *F. nucleatum* cocultured empty vectors group, *F. nucleatum* co-culture could reduce the apoptosis ratio. *ANO1* silencing could block *F. nucleatum* protective effects and increase apoptosis ratio. *ANO1* overexpression could further reduce the apoptosis ratio and have the lowest apoptosis ratio (Figure 4A, B). Moreover, Western blot data showed that oxaliplatin and 5-FU induced the cleavage of caspase-3 and PARP and downregulation of P-pg and EGFR in HT29 and HCT116 cells (Figure 5A, B). *F. nucleatum* cocultured

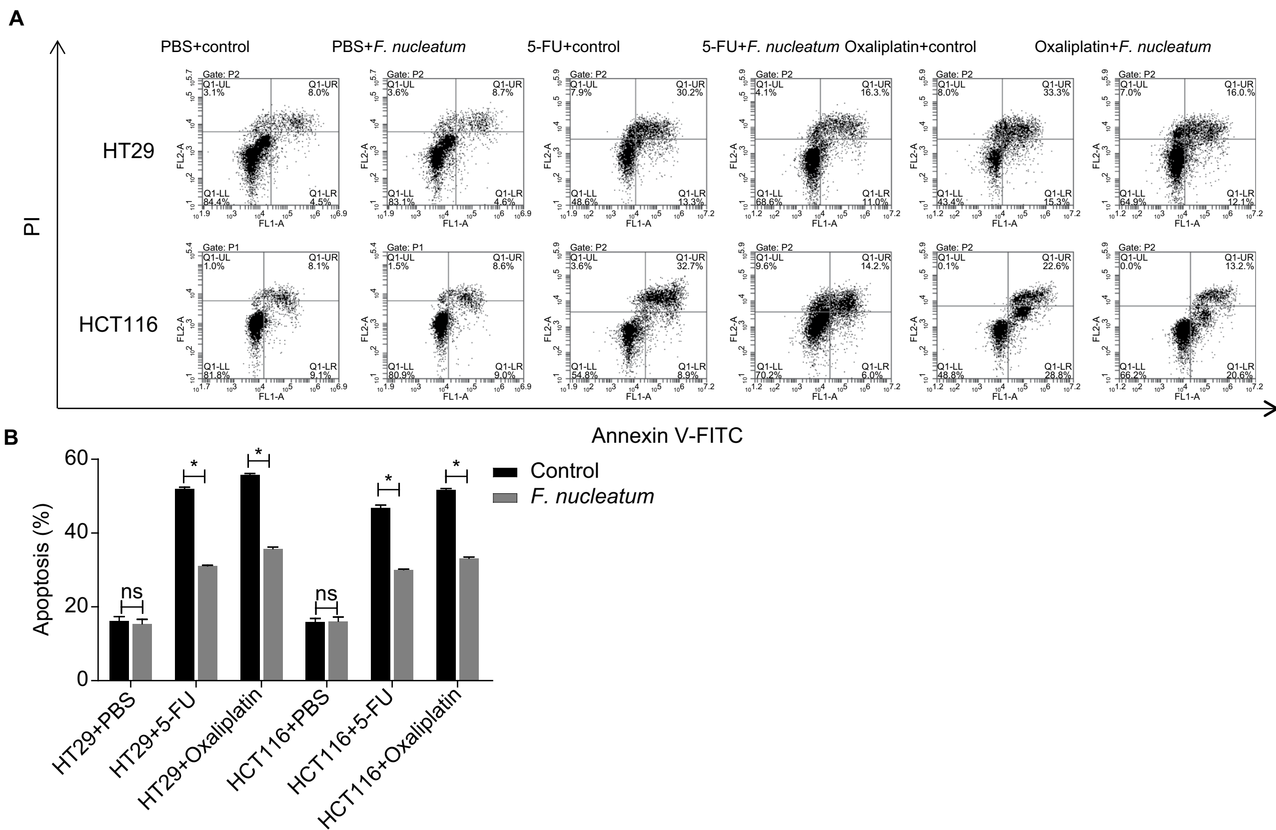

Figure 3 *F. nucleatum* reduced HT29 and HCT116 apoptosis induced by oxaliplatin and 5-FU.
Notes: (A) Representative flow cytometric images of cell apoptosis. **(B)** The statistical analysis for (**A**). Data are expressed as mean ± SD (N=3). **P*<0.01.
Abbreviations: PI, propidium iodide; 5-FU, 5-fluorouracil; *F. nucleatum, Fusobacterium nucleatum;* ns, not significant.

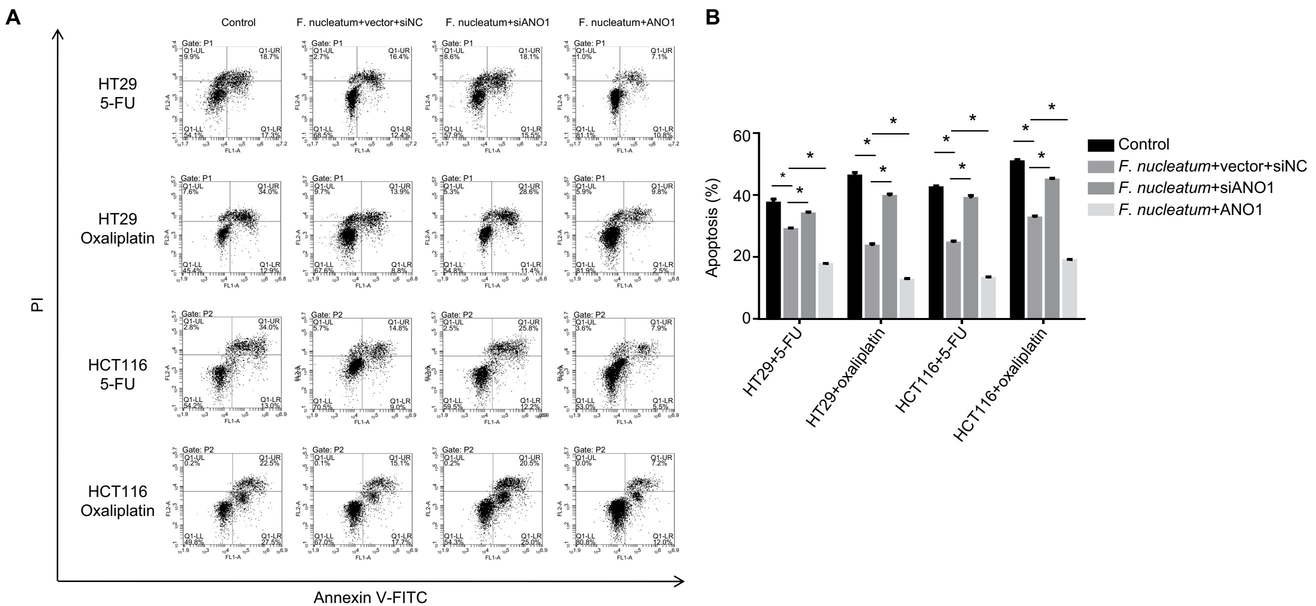

Figure 4 Silencing and overexpression of *ANO1* changed the effects of *F. nucleatum* on HT29 and HCT116 apoptosis induced by oxaliplatin and 5-FU.
Notes: (A) Representative flow cytometric images of cell apoptosis. **(B)** The statistical analysis for (**A**). Data are expressed as mean ± SD (N=3). **P*<0.01.
Abbreviations: 5-FU, 5-fluorouracil; *F. nucleatum, Fusobacterium nucleatum.*

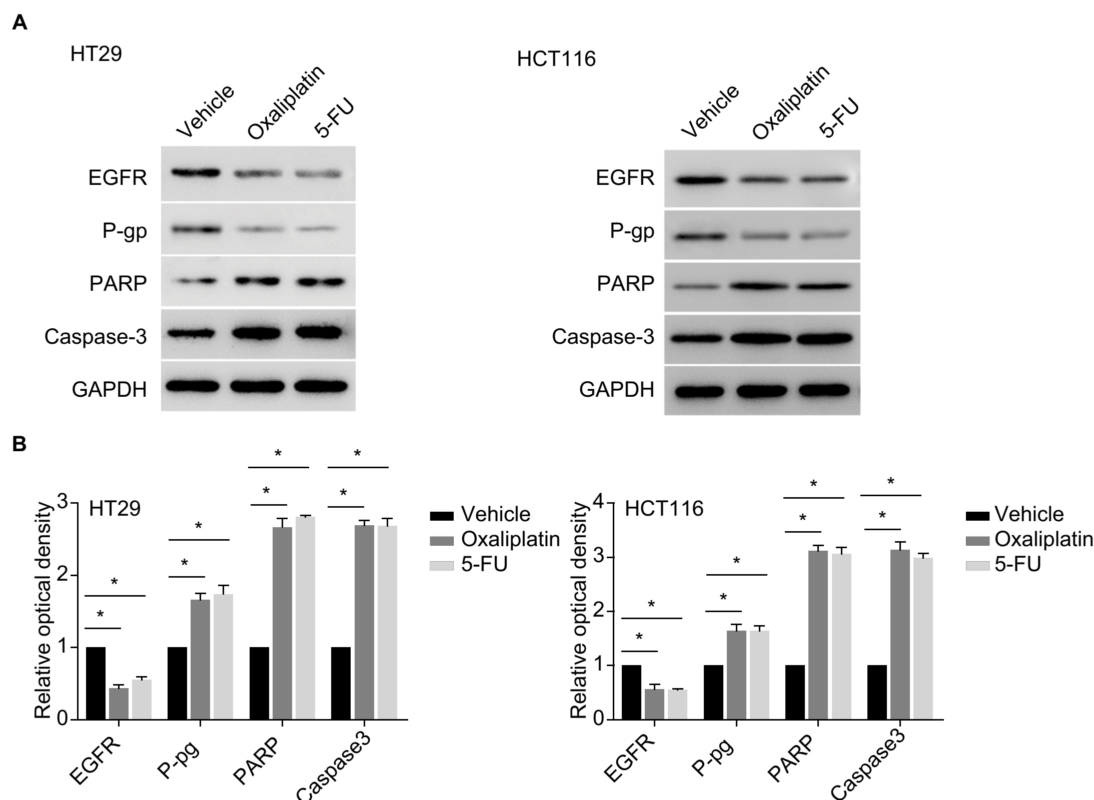

Figure 5 Oxaliplatin and 5-FU changed the expression levels of P-pg, EGFR, cleaved-Caspase 3, cleaved-PARP in HT29 and HCT116.
Notes: (A) Representative Western blot images. **(B)** The statistical analysis for (**A**). Data are expressed as mean ± SD (n=3). *P<0.01.

ANO1 silencing groups treated with chemotherapy drugs expressed more cleaved caspase-3 and PAPR, while ANO1 overexpression groups expressed less cleaved caspase-3 and PAPR, compared with empty vector group (Figure 6A, B). Meanwhile, *F. nucleatum* co-culture could induce P-pg and EGFR upregulation. *F. nucleatum* cocultured *ANO1* silencing groups treated with chemotherapy drugs expressed less P-pg and EGFR than *ANO1* empty vectors groups. *F. nucleatum* cocultured *ANO1* overexpression groups treated with chemotherapy drugs expressed more P-pg and EGFR than *ANO1* empty vector groups. These data suggested the apoptosis effects induced by oxaliplatin and 5-FU could be prevented by ANO1 and *F. nucleatum* induced ANO1 expression and *ANO1* overexpression can further decrease the apoptosis effects. Knockdown *ANO1* expression could block *F. nucleatum* protective effects and increase chemotherapeutic drug-induced apoptosis.

Discussion

CRC is one of the most prevalent carcinomas throughout the world.[1,2] CRC patients were usually treated with capecitabine and 5-FU combined with platinum-based chemotherapy[4] and initially effective. Unfortunately, drug resistance usually exists and patients will finally die due lack of efficient therapy.[2,31] Although novel immune checkpoint therapy can generally cure many other cancer patients,[6] it does little to cure patients with CRC and conventional chemotherapy is the first choice of treatment for the latter. Therefore, it is necessary to clarify the mechanisms of chemoresistance in CRC to optimize current treatment strategies. Through a combination of lentivirus and siRNA knockdown technique plus co-culture, we have demonstrated that *F. nucleatum* was biologically involved in the development of colon cancer chemoresistance via ANO1 pathway.

Cancer epigenetic and modification in CRC chemotherapeutic response have been widely reported.[2,32,33] Calcium-activated chloride channel ANO1, also known as transmembrane member 16A (TMEM16A), has been suggested to drive 11q13 amplification by providing growth or metastatic advantage to tumors. ANO1 is rarely expressed in corresponding normal tissues while it is amplified and highly expressed in a large number of cancers. Moreover, transcriptomic and metagenomic examination have showed that *F. nucleatum* is related to CRC development[15,16] and facilitates colorectal

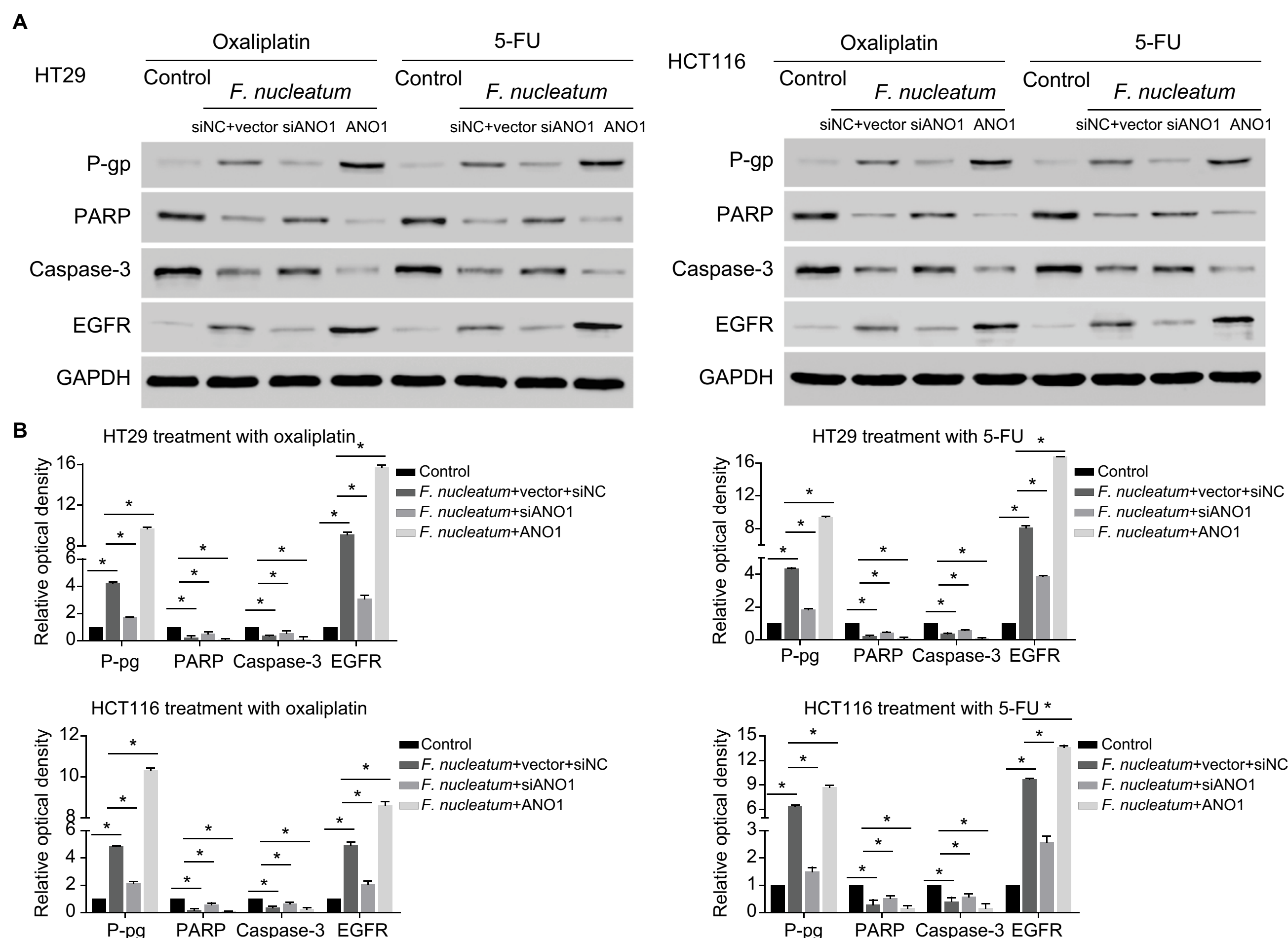

Figure 6 *F. nucleatum* and ANO1 changed the effects of oxaliplatin and 5-FU on expression levels of P-pg, EGFR, cleaved caspase-3 and cleaved-PARP.
Notes: (A) Representative Western blot images. **(B)** The statistical analysis for (**A**). Data are expressed as mean ± SD (N=3). **P*<0.01.

carcinogenesis by attaching to the host epithelial E-cadherin via the fusobacterial adhesin FadA[34] and fusobacterial lectin Fap2.[35] In addition, the amount of *F. nucleatum* is increased in CRC patients with recurrence postchemotherapy.[18] However, it is unknown whether the increasing amount of *F. nucleatum* and ANO1 are related. In this study, we demonstrated, for the first time, *F. nucleatum* promotes ANO1 expression on colon cancer cells via co-culture colon cancer cell lines HT29 and HCT116 with *F. nucleatum*.

Apoptosis is a highly regulated cellular process critical for cell growth and tissue development.[36] Loss of apoptosis can result in tumor initiation, growth, and progression.[37] 5-FU and oxaliplatin can induce tumor cell apoptosis by disrupting and blocking DNA replication.[3,4] In agreement, our results showed that oxaliplatin and 5-FU induced HT29 and HCT116 cell apoptosis. Nevertheless, co-culture with *F. nucleatum* can reduce this effect. Consistently, *ANO1* silencing could block *F. nucleatum* protective effects and increase apoptosis ratio. These facts further demonstrated that *F. nucleatum* induced ANO1 expression. Several different groups in the world have reported that genetic or pharmacological inhibition of ANO1 can induce apoptosis in different types of cancer cells like gastrointestinal stromal tumor cells,[38] human procarcinoma cell[27] and so on.[39] In line with these findings, we demonstrated that the apoptosis effects induced by oxaliplatin and 5-FU could be prevented by ANO1, *F. nucleatum* induced ANO1 expression and ANO1 overexpression. Thus, *F. nucleatum* prevents apoptosis in CRC by the chemicals via the ANO1 pathway. That is to say that *F. nucleatum* was biologically involved in the development of colon cancer chemoresistance via ANO1 pathway based on the facts that *ANO1* is a target of miR-132 that has a crucial role in CRC progression[26] and *F. nucleatum* can inhibit apoptosis via a selective loss of miR-18a* and miR-4802.[18] We guess that the putative mechanism by which *F. nucleatum* prevents apoptosis in CRC by the chemicals via the ANO1 pathway might involve modulation of the amounts of

miRNA. *ANO1* overexpression induced cell proliferation and inhibition of *ANO1* induces apoptosis in prostate carcinoma cells by TNF-α signaling pathway.[27] The mechanism is perhaps modulated by cell-specific factors or by the abundance of other anoctamins. Therefore, further investigations are necessary to elucidate the detailed mechanism.

Our work may be helpful for the clinical management of CRC patients besides its biological importance. As *F. nucleatum* was biologically involved in the development of colon cancer chemoresistance via ANO1 pathway, the conventional chemotherapeutic methods including capecitabine plus oxaliplatin are not appropriate for CRC patients with a high amount of *F. nucleatum* and anti-*F. nucleatum* treatment should add to the standard therapy strategies. Further, it also is important to detect *F. nucleatum* and its associated pathway and to manage patients differentially with different levels of *F. nucleatum*.

Conclusion

We revealed that *F. nucleatum* could prevent colon cancer apoptosis from chemotherapy drugs via the ANO1 pathway. Our data suggest that further study is essentially important to reveal the relationship between *F. nucleatum*, ANO1 and chemoresistance of CRC as well as the potential of *F. nucleatum* and ANO1 as a therapeutic target of CRC patients.

Disclosure

The authors report no conflicts of interest in this work.

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