Progesterone suppresses the progression of colonic carcinoma by increasing the activity of the GADD45α/JNK/c-Jun signalling pathway

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Abstract. Colorectal cancer (CRC) is the third most diagnosed cancer worldwide. Progesterone is associated with a decreased risk of CRC and leads to a favourable prognosis. However, the specific mechanism by which progesterone suppresses malignant progression remains to be elucidated. In the present study, the level of progesterone was first analysed in 77 patients with CRC, and immunohistochemistry was performed to detect the expression of progesterone receptor (PGR) in the paired specimens. The correlations between progesterone, PGR and CRC prognosis were assessed. A Cell Counting Kit-8 assay was then used to detect proliferation of the CRC cells. Flow cytometry was performed to estimate apoptosis and to evaluate the cycle of the CRC cells. A xenograft tumour model was established in nude mice to assess the role of progesterone in tumour growth. Finally, a PCR microarray was used to screen differentially expressed genes to further interpret the mechanism by which progesterone inhibits the malignant progression of CRC. It was found that low expression of progesterone and PGR were significantly associated with poor prognosis of CRC. In addition, progesterone suppressed CRC cell proliferation by arresting the cell cycle and inducing apoptosis *in vitro*. Moreover, the inhibitory role of progesterone in tumour growth was verified *in vivo*. Further investigation showed that the level of growth arrest and DNA damage-inducible protein α (GADD45α) was up-regulated by progesterone, and this was followed by the activation of the JNK pathway. Progesterone increased the activity of the JNK pathway via GADD45α to inhibit proliferation by arresting the cell cycle and inducing apoptosis, thereby suppressing the malignant progression of CRC. Therefore, it can be concluded that progesterone and PGR might act as inhibiting factors for poor prognosis of CRC.

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer worldwide, with 935,000 annual deaths in 2020 and an incidence estimated to increase to 2.5 million new cases by 2035 (1,2). CRC has incidence and mortality rates of 9.8 and 9.2%, respectively, globally with ~25% fewer cases in females compared with in males in 2020 (1). This difference suggests that sex hormones might have an influence on the development of CRC, and this role is most likely protective in females (3). Thus, an improved understanding of the effect of sex hormones on CRC progression is urgently needed to facilitate the exploration of new therapeutic targets and the development of effective treatment strategies for CRC.

Progesterone, an important natural sex hormone, is involved in the menstrual cycle, pregnancy and embryogenesis of humans (4). Its cellular effects are mediated by binding to the progesterone receptor (PGR) and regulating hormone response target genes in several cancer types, such as liver (5), ovarian (6), gastric (7) and breast (8) cancer. Furthermore, progesterone regulates various cancer cell phenotypes, including proliferation, apoptosis, angiogenesis and autophagy (9). Studies have suggested that progesterone promotes apoptosis and an inhibitory effect is observed on cell proliferation in endometrial cancer (10,11). Progesterone-induced apoptosis occurs by

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Abbreviations: BCL-2, B cell lymphoma-2; CCK-8, Cell Counting Kit-8; CRC, colorectal cancer; FACS, fluorescence-activated cell sorting; IHC, immunohistochemistry; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; PBS, phosphate buffer saline; PGR, progesterone receptor; RT-qPCR, reverse transcription-quantitative PCR; PRE, progesterone response element; TNM, Tumor-Node-Metastasis

Key words: progesterone, colorectal carcinoma, proliferation, c-Jun N-terminal kinases, GADD45α

arresting the progression from the G_1 phase to S phase (12). Thus, in clinical settings, progesterone has been used in patients who exhibit well-differentiated endometrial cancer, or patients with recurrence (13). Although there is accumulating data suggesting that progesterone and other hormone replacement therapy contributes to the decreased progression of CRC (11,14,15), the individual effect of progesterone on CRC progression or cell lines has been minimally studied. Progesterone-related gene variants increase the risk of developing CRC in women, predicting a therapeutic target (11). During the treatment of CRC with folic acid, PGR activation is required for its anti-proliferative effect, which results from cell cycle arrest (16). Although progesterone has been preliminarily confirmed to have an inhibiting effect on CRC cell proliferation and tumor progression, respectively, the specific mechanism by which progesterone inhibits CRC progression remains to be elucidated (17).

Through triggering a variety of molecular responses leading to cell proliferation in CRC, the mitogen activated protein kinase (MAPK) pathway has recently been proposed to be involved in apoptosis and cell cycle regulation by various compounds (18). As a member of MAPK family of proteins, the c-Jun N-terminal kinase (JNK) has a specific role in mediating apoptosis in several types of cancer cell (19). G_2/M phase arrest and apoptosis are induced via ROS-mediated p38 and JNK signalling pathways in human colon cancer cells (20). Human CRC cells are inhibited by JNK1 pathway-induced apoptosis (21). The levels of phosphorylated ERK and JNK reportedly increase when treated with a combination of 17β-estradiol and progesterone (22). The up-regulation of progesterone-induced decidual protein is also accompanied by the activation of the ERK pathway, leading to senescence in colon cancer cells (23). However, whether anticancer activity against CRC is induced by progesterone through the JNK pathway has not been determined.

The present study aimed to reveal the potential mechanism by which progesterone inhibits CRC progression. First, the expression of progesterone and PGR was evaluated. Second, the role of progesterone in several cancer-related processes *in vitro* and *in vivo* were investigated. Finally, the specific mechanism by which progesterone inhibited CRC progression was analysed.

Materials and methods

Samples from patients with CRC. In total, 77 pre-existing blood and paraffin-embedded tissue samples were used from patients with CRC who underwent colectomy at The General Surgery Center, the General Hospital of Western Theater Command (Chengdu, China) between January 2015 and January 2020. All CRC tissue samples had paired adjacent non-cancerous tissues. Patient follow-up time ranged from 0.54 to 60 months, with a median follow-up time of 25.24 months. The clinical features of patients with CRC were collected, including age, gender, tumor size, tumor number, differentiation, vascular invasion, and tumor node metastasis (TNM) (24). Written informed consent was obtained from all patients during the admission. The study was performed in accordance with clinical study protocols and the principles of the Declaration of Helsinki (modified 2018) and was approved by The Research Care and Ethics Committee of the General Hospital of Western Theater Command (approval no. SPPHCT2015-0117) (25).

Cell culture. LoVo, SW620, HT29, HCT116 and SW480 CRC cell lines were purchased from The Cell Bank of Shanghai Institute of Cell Biology. The cell lines were tested and were free from mycoplasma contamination, and had also passed STR identification without errors. The cell lines were maintained in complete Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% foetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37˚C in 5% CO_2. Trypsin-EDTA (0.05%) (Thermo Fisher Scientific, Inc.) was used for cell passages.

Flow cytometry. Flow cytometry was performed to observe cell cycle distribution and apoptosis of LoVo and SW620 cells (26). Cells were treated with 250 μM progesterone for 48 h before the start of the experiment to ensure cell synchronisation. The cells were collected through trypsinization and fixed in 90% 4˚C ethanol for 30 min. The fixed cells were washed in phosphate-buffered saline (PBS) and stained with 50 μg/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. A cell cycle detection kit (cat. no. KGA511; Nanjing KeyGen Biotech Co., Ltd.) was used to detect changes in cell cycle progression in each group. Fluorescence-activated cell sorting (FACS) was performed using a FACSCalibur flow cytometer. The proportion of apoptotic cells in each group was measured using an Annexin V-kFluor647/PI double-stained Apoptosis Detection kit (cat. no. KGAV113; Nanjing KeyGen Biotech Co., Ltd.). The cells were detached using 0.25% trypsin without EDTA, washed twice in cold PBS, and resuspended in 100 μl binding buffer. The cells were then incubated with 5 μl Annexin V-kFluor647 and 5 μl PI for 10 min at room temperature in the dark and detected with a FACSCalibur flow cytometer. The data of cell cycle analysis and apoptosis was analysed using CELLQuest (version no. 0.9.13α; BD Biosciences).

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay (cat. no. BS350B, Anhui Biosharp Co., Ltd.) was performed to monitor cell proliferation. Cells were plated at a density of 5,000 cells/well in 96-well plates. After 24 h in culture, the cells were treated with increasing concentrations of progesterone (0, 125, 250 and 500 μM) for 72 h. The control group was treated with PBS. Samples were collected at 24, 48 and 72 h. After adding 10 μl of CCK-8 solution per well and incubating for 1 h, absorbance was measured at 450 nm using an enzyme calibrator (BioTek Instruments, Inc.). Experiments were performed in triplicate.

Small interfering (si)RNA transfection. siRNA targeting GADD45α (5'-GGAGGAAGUGCUCAGCAAA-dTdT-3') and scrambled siRNA (5'-UUCUCCGAACGUGUCACG U-3') were synthesised by GenScript Co., Ltd.. The cells were transfected with siGADD45α or scrambled siRNA using Entranster™-R4000 (Engreen Biosystem Co., Ltd.) according to the manufacturer's instructions. Cells were confluent to be 80% for transfection. One μl Entranster™-R4000 Reagent was diluted in 25 μl Opti-MEM™ medium (cat. no. 11058021, Gibco Co., Ltd.) and mixed well. The siRNA (0.6 μg or 50 pmol) was diluted in 25 μl Opti-MEM™ medium, then

diluted siRNA was added to tube of diluted Entranster™-R4000 Reagent (1:1 ratio) and incubated for 10 min at room temperature. Cells were incubated subsequently for 2 days at 37˚C. Then, the transfected cells were used for subsequent experimentation.

Histology and immunohistochemistry (IHC) analysis. Tissue samples from patients with CRC were cut into 4-μm thick sections, deparaffinized with xylene and rehydrated through graded ethanol (cat. no. 0012036210; Fuyu Chemistry Co., Ltd.) washes (from 100, 100, 95, 85 and 75%). Then the sections were immersed in sodium citrate antigen retrieval solution (cat. no. C1032; Solarbio) at 100˚C for 5 min, and then were cooled to room temperature. Tumour tissues from mice were directly fixed in OCT Compound (cat. no. 4583; Sakura Finetek, Inc.) at room temperature for 4 h, frozen at -80˚C for 2 h and sectioned into 4-μm thick sections using a freezing microtome (Leica Microsystems GmbH). Tissues were blocked with 5% BSA (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 20 min. To detect the expression of PGR and Ki67 in CRC tissue, the sections were incubated with an anti-PGR antibody (1:500; cat. no. ab32085; Abcam) or an anti-Ki67 antibody (1:500; cat. no. ab21700; Abcam) overnight at 4˚C. Slides were rinsed in PBS and the immunoreactive signals were visualised using a DAKO EnVision Detection system (Fuzhou MXB® Biotechnology Development Co., Ltd.). IHC staining scores demonstrated variable expression levels of PGR in tissue samples from patients with CRC and were used to define high and low expression of PGR in CRC tissues. Scores (1-3 and 4) were used to represent at least 10, 30, 50 and 70% of malignant cells exhibiting positive PGR staining, respectively. A score of 0 was used to define <10% of cells showing positive PCR staining. A high expression level was defined as a staining score of ≥3 with at least 50% of the malignant cells exhibiting positive PGR staining, while a low expression level was defined as a staining score ≤2 with <50% of the malignant cells showing nuclear staining. Four views were randomly collected from each sample and images were captured using a light microscope (n=3). PGR expression data extracted from The Cancer Genome Atlas (TCGA) database (UALCAN, http://ualcan.path.uab.edu/) was analysed to display its relationship with normal tissue, primary tumours and the stage of cancer.

Experimental animals and xenograft model. The study was approved by The Animal Research Ethics Committee of the General Hospital of Western Theater Command (Chengdu, China) and complied with the Guidelines for Animal Experiments on Laboratory Animals (27). In total, 12 nude mice, aged 7 weeks old and weighing 18-22 g were purchased from Chengdu Dashuo Biotechnology Co., Ltd. (China; license no. SCXK 2008-24). All mice were maintained on a 12/12-h light dark cycle with free access to standard laboratory feed and water. In order to reduce the suffering in nude mice, xenograft model was established under sodium pentobarbital anaesthesia (50 mg/kg) by intraperitoneal injection. SW620 cells ($1x10^5$) were implanted into the dorsa subcutaneous tissue of mice to study tumour growth. Tumour volume was monitored by measuring the diameter each week (volume=length x width^2/2) for 7 weeks after which the mice were sacrificed. Lack of responsiveness to manual stimulation and an inability to eat or drink in mice, a tumor burden >10% body weight, and ulcerated, nectrotic or infected tumors were considered as humane endpoints. Inhalation of CO_2 (20% of the displacement volume per min) was used for euthanasia, and death was confirmed by parameters including no movement, no breathing or dilated pupils. Tumour weights were measured at the end of the 7-week observation. Harvested tumours were fixed in 4% paraformaldehyde for 24 h at room temperature, and stored in 75% ethanol at 4˚C. After this, the tissues were embedded in paraffin and subsequently sliced into 4-μm thick sections for further analysis.

Enzyme-linked immunosorbent assay (ELISA). The concentration of progesterone was measured using an ELISA kit (cat. no. JL45435; Jianglai Biology; http://www.jonln.com/191804/). All serum samples from patients with CRC were placed at room temperature (22-25˚C) for 2 h, and then centrifuged at 1,000 x g for 20 min at 4˚C, and the supernatant was collected and stored at -80˚C. ELISA was performed according to the manufacturer's instructions and each sample was evaluated in triplicate. The conjugate reagent was then incubated for 2 h at 4˚C, followed by incubation with the substrate solution for 30 min at room temperature in the dark. The reaction was terminated using a stop solution, and the optical density was measured at a wavelength of 450 nm using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Inc.). The best standard curve was constructed. Progesterone levels were determined based on the standard curve.

Western blot analysis. Total protein was extracted from tumour samples collected from patients with CRC, mice or cells. The total protein was extracted with the protein extraction kit (cat. no. KGP250; Nanjing KeyGen Biotech Co., Ltd.), and the protein content was detected using the BCA method. The protein (10 μg per lane) was separated using 10% SDS-PAGE gels. The samples were separated and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature and then incubated overnight at 4˚C with one of the following primary antibodies: Mouse monoclonal anti-PGR (cat. no. ab32085; Abcam); rabbit monoclonal anti-B cell lymphoma-2 (BCL-2; cat. no. ab182858 Abcam), rabbit monoclonal anti-BCL2-Associated X (BAX; cat. no. ab182733; Abcam), rabbit monoclonal anti-cleaved caspase-3 (cat. no. ab2302; Abcam), rabbit polyclonal anti-GADD45α (cat. no. ab180768; Abcam), mouse monoclonal anti-c Jun N terminal kinase 1/2 (JNK1/JNK2 cat. no. AHO1362; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit polyclonal anti-phosphorylated-JNK1/JNK2 (cat. no. 44682G; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit monoclonal anti-c-Jun(cat. no. ab280089; Abcam) and rabbit monoclonal anti-Ki67 (cat. no. ab15580; Abcam). All antibodies were diluted by TBST (cat. no. T1086; Solarbio) to 1:1,000. After washing, the membranes were incubated for 2 h with goat anti-mouse IgG antibody (cat. no. A9917; Sigma-Aldrich; Merck KGaA) and goat anti-rabbit IgG antibody (cat. no. SAB3700870; Sigma-Aldrich; Merck KGaA). Protein intensity was determined using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) and measured using Image Lab™ software (version 2.0.1; cat. no. 1709690; Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR analysis. The SW620 cell line was treated with progesterone (250 μM) for 48 h. Total RNA was extracted using TRIzol® reagent (Invitrogen;

Table I. Primers used for reverse transcription-quantitative PCR.

Primer name	Sequence, 5'-3'	Product length, bp
Cyclin A1		82
Forward	GAGGTCCCGATGCTTGTCAG	
Reverse	GTTAGCAGCCCTAGCACTGTC	
Cyclin B1		111
Forward	AATAAGGCGAAGATCAACATGGC	
Reverse	TTTGTTACCAATGTCCCCAAGAG	
Cyclin D1		135
Forward	GCTGCGAAGTGGAAACCATC	
Reverse	CCTCCTTCTGCACACATTTGAA	
Cyclin E1		80
Forward	AAGGAGCGGGACACCATGA	
Reverse	ACGGTCACGTTTGCCTTCC	
C-Myc		175
Forward	ATGGCCCATTACAAAGCCG	
Reverse	TTTCTGGAGTAGCAGCTCCTAA	
c-Jun		78
Forward	TCCAAGTGCCGAAAAAGGAAG	
Reverse	CGAGTTCTGAGCTTTCAAGGT	
JNK1		179
Forward	GGGTATGCCCAAGAGGACAGA	
Reverse	GTGTTGGAAAAGTGCGCTGG	
JNK2		207
Forward	GAAACTAAGCCGTCCTTTTCAGA	
Reverse	TCCAGCTCCATGTGAATAACCT	
GADD45α		68
Forward	GGATGCCCTGGAGGAAGTG	
Reverse	CTTCGTACACCCCGACAGTGA	
P15		97
Forward	GGGACTAGTGGAGAAGGTGC	
Reverse	CATCATCATGACCTGGATCGC	
BAX		155
Forward	CCCGAGAGGTCTTTTTCCGAG	
Reverse	CCAGCCCATGATGGTTCTGAT	
BCL2L1		136
Forward	CATGCTGGGAGCGTCACAT	
Reverse	CTCCACTGAACTCGTACAAACTT	
BCL2		147
Forward	GCTACCGTCGTGACTTCGC	
Reverse	CCCCACCGAACTCAAAGAAGG	
Ki67		104
Forward	ATCATTGACCGCTCCTTTAGGT	
Reverse	GCTCGCCTTGATGGTTCCT	
GAPDH		197
Forward	GGAGCGAGATCCCTCCAAAAT	
Reverse	GGCTGTTGTCATACTTCTCATGG	

JNK, c-Jun N-terminal kinase.

Thermo Fisher Scientific, Inc.), and reverse-transcribed using the PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Bio, Inc.) according to the manufacturer's instructions. The sequences of the primers used are shown in Table I. The optimal primer concentrations were determined based on the optimisation protocols provided in the Applied Biosystems SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) manual. Each PCR amplification and detection was carried out using a CFX96 Real Time PCR Detection system (Bio-Rad Laboratories, Inc.). The cycling conditions used were as follows: 95˚C For 130 sec, 60˚C for 30 sec, 72˚C for 30 sec and 72˚C for 40 cycles. GAPDH was used as an internal control to normalise the mRNA expression of each gene. All reactions were performed in triplicate. The results were calculated as previously described using the $2^{-\Delta\Delta Cq}$ method (28).

PCR microarray. PCR microarray was performed to screen 80 genes involved in the regulation of progesterone-inhibited CRC progression by Wcgene Biotechnology. SW620 cells were cultured in a 10-cm^2 dish and incubated overnight. After intervention with progesterone (250 μM) for 48 h, the cells were washed and RNA was extracted and purified using TRIzol. Then, 1 μg RNA was used to synthesise cDNA using a reverse transcription kit (Thermo Fisher Scientific, Inc.), and stored at -20˚C. First strand cDNA was added to the PCR microarray precoated with specific primers. PCR array was performed under the following conditions: 95˚C For 10 min, then 40 cycles at 95˚C for 15 sec and finally at 60˚C for 1 min. GAPDH was used for homogenisation. PCR microarray analysis was performed using the GeneAmp7300 RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The results were calculated as previously described and the level of change (fold-change >1 or <-1) was identified as the different significance (28).

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 (IBM, Corp.). All data are expressed as mean ± standard deviation (unless otherwise shown). The correlations and associations between clinical characteristics in patients with CRC were analysed using Pearson's correlation, Spearman's correlation, unpaired Student's t-test or χ^2 test. Survival data were used to draw Kaplan-Meier curves, and differences between the groups were analysed using the log-rank test. Student's t-tests were used to determine the significance of differences between two groups, and one-way ANOVA and Tukey's post hoc test were used to determine differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Progesterone and PGR levels were negatively correlated with CRC prognosis. To examine the correlation between progesterone levels and CRC prognosis, the concentration of progesterone was measured in 77 patients (median, 1.23; interquartile range, 0.737-1.715 nmol/l) (Fig. 1A). Progesterone plays an essential role in physiological and pathological processes through activation of the PGR (5). Thus, IHC staining was performed in colorectal tumour tissues (Fig. 1B), and the scores demonstrated the variable expression level of PGR and were used to define high and low PGR levels (Fig. 1C).

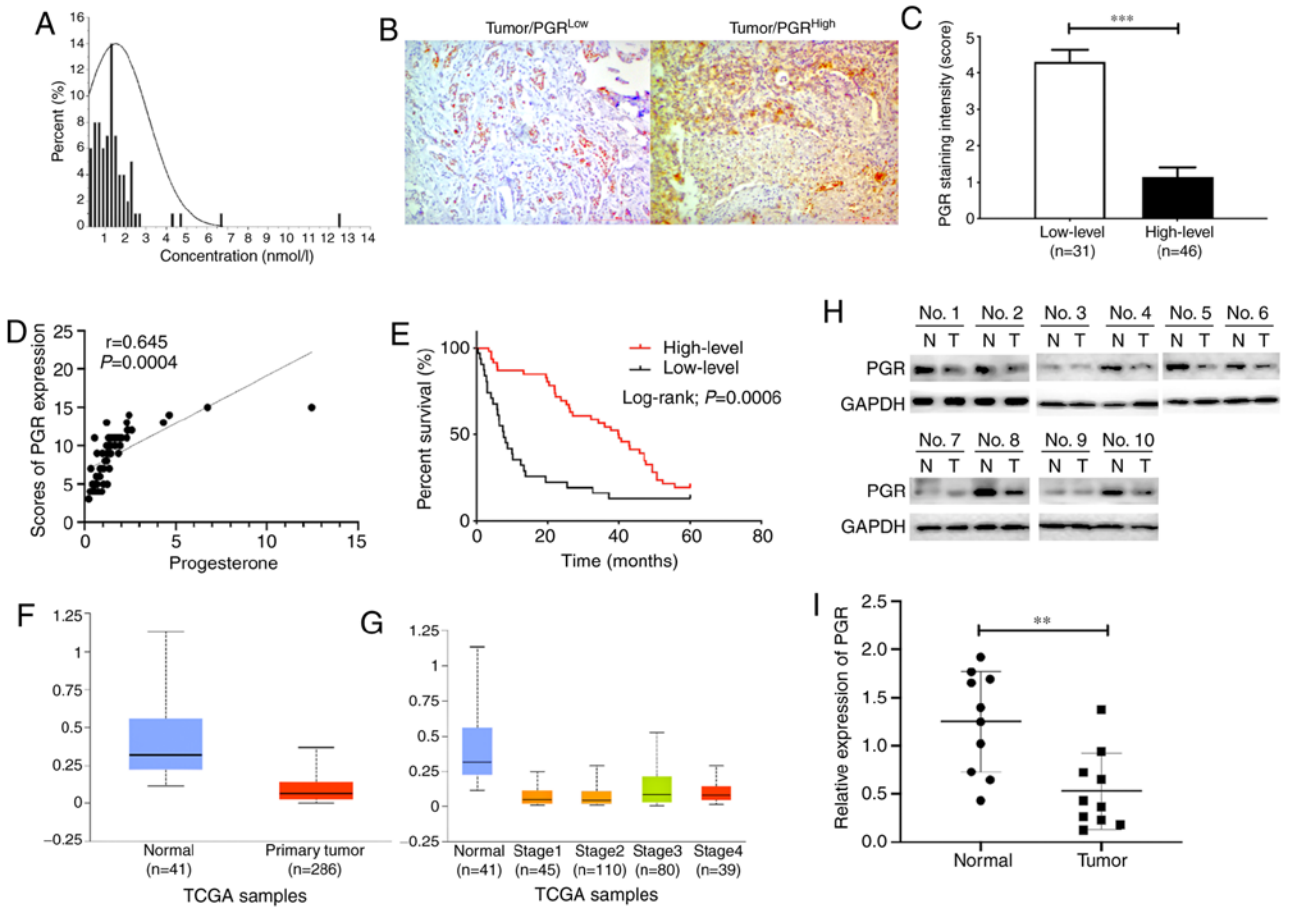

Figure 1. Expression of progesterone and PGR is negatively correlated with prognosis of CRC. (A) Distribution of progesterone levels in 77 patients with colorectal cancer. (B) A representative image showing the expression level of PGR in colorectal cancer tissues. (C) Immunohistochemical staining scores were used to define low and high expression levels of PGR in colorectal cancer tissues. (D) Progesterone was positively correlated with PGR in colorectal cancer tissues. (E) Kaplan-Meier survival analysis of patients with CRC. (F and G) Expression of PGR based on tumour and normal tissue, and individual cancer stages from TCGA database. (H) Levels of PGR in 10 randomly selected paired colorectal cancer tissues were determined via western blot. (I) Semi-quantitative analysis of the western blot results. **P<0.01 and ***P<0.001 vs. low-level or normal groups. CRC, colorectal cancer; PGR, progesterone receptor; TCGA, The Cancer Genome Atlas; P4, progesterone.

Table II. Correlation analysis of progesterone expression and clinical characteristics of 77 patients with colorectal cancer.

Variable	r or t-value	P-value
Age	-0.158	0.169
Sex, male/female	0.682[a]	0.499
Tumor size	-0.170	0.139
Number of tumors	-0.243	0.033[b]
Differentiation, high/moderate/low	-0.254	0.026[b]
Vascular invasion, +/-	0.313[a]	0.755
TNM, I/II/III/IV	-0.198	0.084
Survival time	0.383	0.001[b]

[a]t-value, [b]P<0.05, TNM, tumor node metastasis.

Furthermore, correlation between the expression of progesterone and its receptor was analysed. The results indicated that progesterone level was positively associated with the IHC staining scores of PGR in patient samples (r=0.645, P=0.0004; Fig. 1D). Correlations between progesterone and clinical characteristics were also analysed (Table II). Statistical analysis showed significant correlations in the number of tumours (r=-0.243, P=0.033), tumour differentiation (r=-0.254, P=0.026) and survival time (r=0.383, P=0.001). When association between PGR and clinical characteristics of CRC patients were analysed, it was found that high levels of PGR expression were associated with tumour size (P=0.001), differentiation (P=0.011), vascular invasion (P=0.005) and tumour stage (P=0.001) (Table III). In addition, the results indicated that patients with higher expression levels of PGR had longer short-term survival times (P=0.0006; Fig. 1E). The 1-year, 3-year and 5-year survival rates were 86.96, 78.26, and 19.57%, respectively, in the high expression group, and 35.48, 16.13 and 12.90%, respectively, in the low expression group. The results from UALCAN showed that PGR expression was higher in normal tissues compared with that in primary tumours, regardless of tumour stage (Fig. 1F and G). Further analyses revealed that PGR protein levels were lower in 10 randomly selected paired specimens, which were analysed via western blotting (P<0.01; Fig. 1H and I). In summary, these data confirmed that low progesterone levels in serum and low PGR expression in CRC tissue led to poor prognosis in patients with CRC.

Table III. Relationship between progesterone receptor and clinical characteristics of 77 patients with colorectal cancer.

Variable	Value, n	Expression of the PGR		P-value
		Low level, n=31	High level, n=46	
Age, years				
<60	28	14	14	0.188
≥60	49	17	32	
Sex				
Male	43	13	30	0.044[a]
Female	34	18	16	
Tumor size, cm				
<5	38	8	30	0.001[b]
≥5	39	23	16	
Number of tumors				0.279
Single	34	16	18	
Multiple	43	15	28	
Differentiation				0.011[a]
High	29	13	16	
Moderate	26	14	12	
Low	22	4	18	
Vascular invasion				0.005[b]
No	40	10	30	
Yes	37	21	16	
TNM stage				0.001[b]
I/II	42	10	32	
III/IV	35	21	14	

[a]P<0.05, [b]P<0.01. PGR, progesterone receptor; TNM, tumor node metastasis.

Progesterone inhibited CRC cell proliferation by arresting the cell cycle and inducing apoptosis. The study sought to further identify the biological function of progesterone in CRC cell proliferation by studying the association between progesterone and CRC prognosis. First, PGR expression was analysed in various cell lines. LoVo cells exhibited relatively higher PGR expression levels, while expression in SW620 cells was lower (Fig. 2A). When treated with gradually increasing concentrations of progesterone, PGR expression was enhanced in LoVo and SW620 cell lines (Fig. 2A). When treated with different concentrations of progesterone (125, 250 and 500 μM), proliferation at 72 h was significantly inhibited in both LoVo and SW620 cells (both P<0.001; Fig. 2B and C, respectively), and proliferation at 48 h was significantly inhibited in SW620 cells compared with the control (P<0.01; Fig. 2C). Cell cycle analyses were performed using a flow cytometer following propidium iodide staining (Fig. 2D), and the cell phase distribution at 48 h was analysed (Fig. 2E). Increasing doses of progesterone significantly increased the percentage of LoVo cells in the G_2/M phase (Fig. 2F) and SW620 CELLS (Fig. 2I). To verify cell cycle arrest, cell cycle-related cytokines, including CCNA1, CCNB1, CCND1, CCNE1 and c-myc, were detected at the transcriptional level. The results showed that CCNA1 and CCNB1 were significantly up-regulated in progesterone-treated LoVo (P<0.01; Fig. 2J) and SW620 (P<0.01; Fig. 2K) cells.

Furthermore, flow cytometry was used to analyse the apoptotic process in LoVo and SW620 cell lines 48 h after progesterone intervention (Fig. 3A and B). A significant increase in the apoptosis rate was observed in LoVo cells, with 2-, 6- and 10-fold higher apoptosis rates in the 125, 250 and 500 μM progesterone groups, respectively, compared with the control group (P<0.001; Fig. 3C). Significant increases in apoptosis rates were also observed in SW620 cells, with 3-, 5- and 10-fold higher rates in the 125, 250 and 500 μM progesterone groups, respectively, compared with the control group (P<0.01; Fig. 3D). To verify this variation in apoptosis, the apoptosis inhibitory protein BCL-2, apoptosis-promoting protein BAX and cleaved caspase-3 levels were measured. The results showed down-regulated BCL-2 and up-regulated cleaved caspase-3 in LoVo cells, and down-regulated BCL-2 and BAX and up-regulated cleaved caspase-3 in SW620 cells (Fig. 3E and F). The aforementioned results indicated that progesterone arrested the cell cycle mainly in the G_2/M phase and promoted apoptosis, inducing the inhibition of CRC cell proliferation.

Progesterone inhibits tumour growth in vivo. A xenograft tumour model was established to determine whether progesterone affected tumour growth *in vivo* (Fig. 4A). The volume of implanted tumours reduced significantly following 40 days

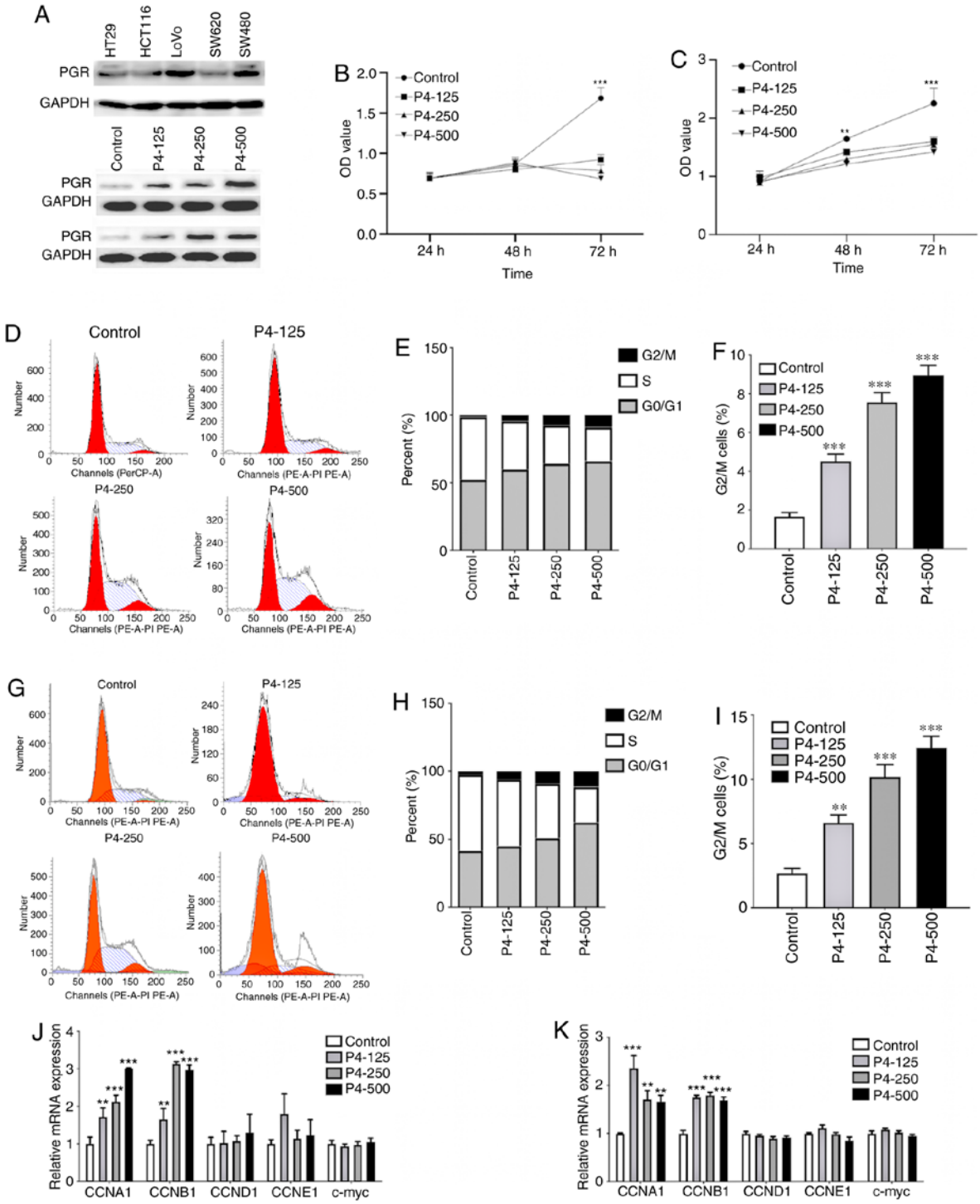

Figure 2. Progesterone inhibits the proliferation of CRC cell lines and arrests the cell cycle. (A) Expression of progesterone receptor in different CRC cell lines (HT29, HCT116, LoVo, SW620 and SW480) and expression of PGR in LoVo (line 1) and SW620 (line 2) cells treated with different concentrations of progesterone (125, 250 and 500 μM) were determined using western blot. Proliferation of (B) LoVo and (C) SW620 cells was inhibited when these cells were treated with varying concentrations of progesterone. Effects of progesterone on (D and E) cell cycle distribution and (F) arrest at the G_2/M phase following treatment with increased concentrations of progesterone in LoVo cell, as well as in (G-I) SW620 cells. Cytokines related to cell cycle in (J) LoVo and (K) SW620 cells were analysed using reverse transcription-quantitative PCR. **P<0.01 and ***P<0.001 vs. control. CRC, colorectal cancer; P4, progesterone.

of progesterone intervention (P<0.01; Fig. 4B). Progesterone treatment also resulted in tumours with lower weights (P<0.05; Fig. 4C) and light staining of Ki-67 in tumours (P<0.05; Fig. 4D and E). It was also observed that the number of TUNEL-positive cells in the tumour tissue was prominently enhanced in the progesterone-treated mice compared with control mice (P<0.05; Fig. 4F and G). Collectively, these data showed that progesterone was essential for inhibiting CRC growth.

Progesterone up-regulates the JNK pathway via GADD45α to inhibit CRC progression. The JNK signalling pathway plays an essential role in regulating cell proliferation, migration and invasion. It is associated with reduced cell proliferation,

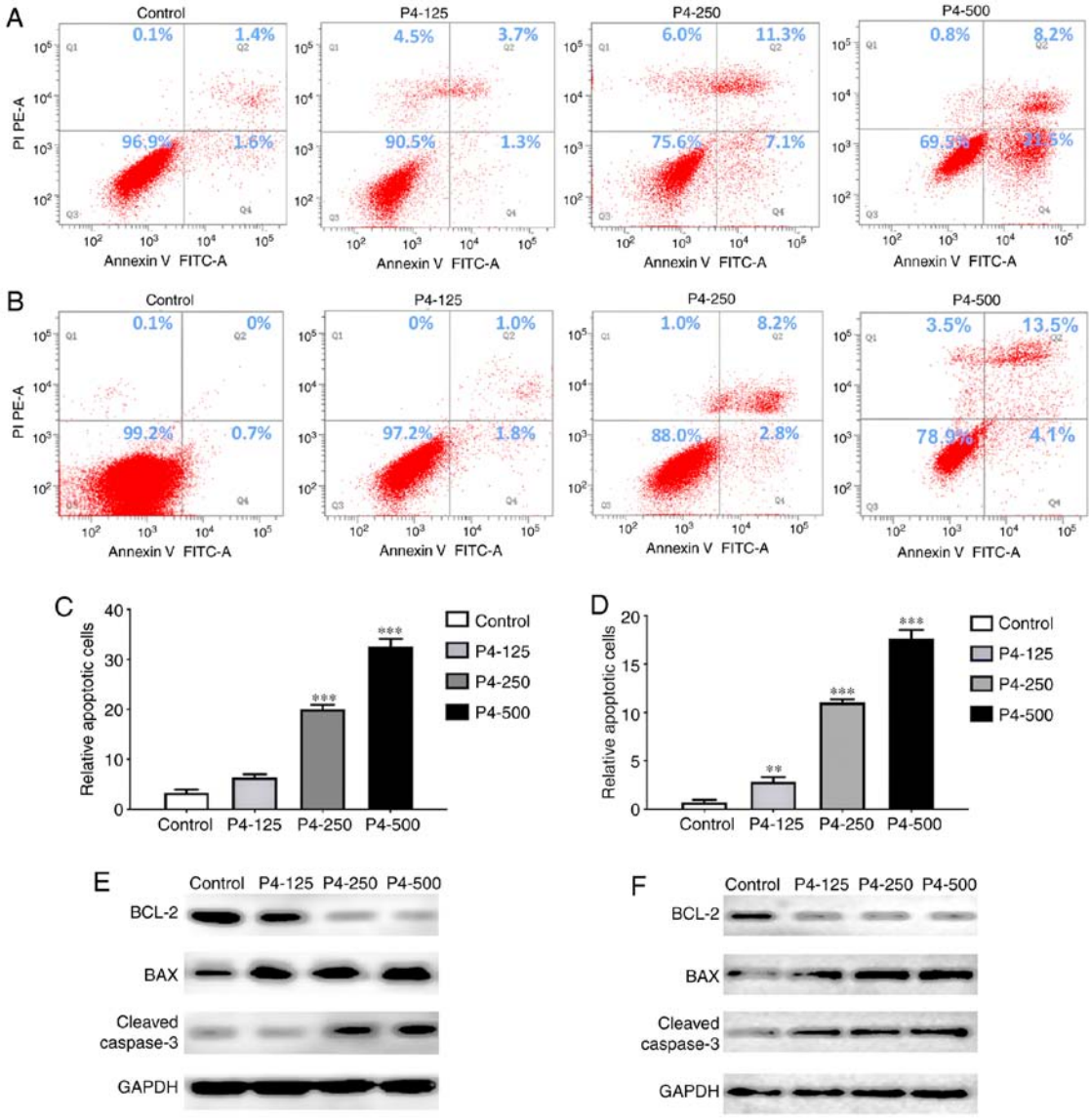

Figure 3. Progesterone induces the apoptosis of colorectal cancer cell lines. Apoptosis rates in (A) LoVo and (B) SW620 cells were analysed using flow cytometry. Treatment with increasing concentrations of progesterone significantly promoted the process of apoptosis in (C) LoVo and (D) SW620 cell lines. BCL-2, BAX and cleaved caspase-3 in (E) LoVo and (F) SW620 cell lines were analysed using western blotting. **P<0.01 and ***P<0.001 vs. control. P4, progesterone; PI, propidium iodide; BCL-2, B cell lymphoma-2.

arrested cell cycle and increased apoptosis following overactivation with progesterone (16). A PCR microarray was performed to investigate progesterone-induced changes in the expression of genes related to cell proliferation. As shown in Fig. 5A, a noticeable number of genes were altered by progesterone intervention (250 μM). In total, 34 genes whose levels changed the most (fold-change >1 or <-1) are depicted in Fig. 5B. In SW620 cells, changes in the expression of P15, c-Jun, GADD45A, CCL18, neutrophil cytosol factor 1 and matrix metalloproteinase 3 were most significant. Progesterone considerably increased the expression of P15 (5.0-fold change), c-Jun (4.0-fold change) and GADD45A (3.9-fold change). RT-qPCR was performed to verify the differences in the genes. The gene with the most considerable change was GADD45α, with a 2.5-fold increase in expression. Increased expression of c-Jun (2.3-fold change) and JNK1 (2.2-fold change), and decreased expression of BCL2L1 (2.0-fold change), were also noted (Fig. 5C). The similar results were also observed in LoVo cells (Fig. 5D). Furthermore, the transcription levels of GADD45α, JNK1, JNK2, c-Jun, BCL-2 and Ki67 were detected with the intervention of progesterone (250 μM) in SW620 (Fig. 5E) and LoVo (Fig. 5G) cells. GADD45α, JNK1 and c-Jun were significantly up-regulated, while BCL-2 and Ki67 were significantly down-regulated. To verify that GADD45α was the main factor involved in the regulation of the JNK pathway, siGADD45α and progesterone were simultaneously used to detect the significant changes. Inhibition of GADD45α strongly suppressed GADD45A and c-Jun and promoted BCL-2 and Ki67 compared with progesterone only treatment. The aforementioned cytokines, which showed significant changes, were analysed at the protein level in SW620 (Fig. 5F) and LoVo (Fig. 5H) cells. GADD45α, phosphorylation of JNK and c-Jun were enhanced with progesterone intervention but decreased when siGADD45α was added. In contrast to this, decreased levels of BCL-2 and Ki67 were observed and were increased with the addition of siGADD45α. Collectively,

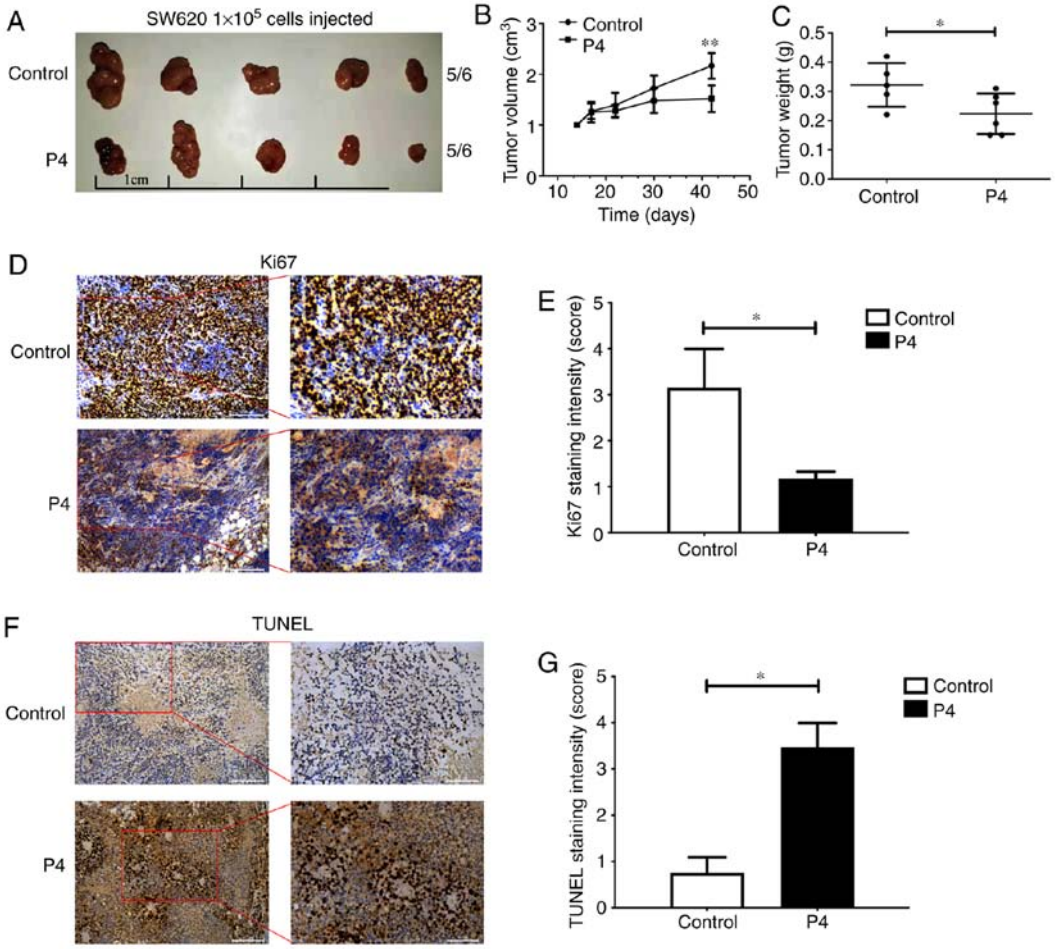

Figure 4. Progesterone facilitates tumour growth *in vivo*. (A) Tumour images. (B) Tumour volume and (C) weight were measured in each group. (D) Representative histopathology of xenograft tumours for Ki67 staining. (E) Quantified Ki67 staining. (F) TUNEL assay and (G) quantified results. *P<0.05 and **P<0.01 vs. control. P4, progesterone.

these data suggested that progesterone inhibited CRC cell proliferation by arresting the cell cycle and inducing apoptosis through the activated GADD45α/JNK/c-Jun pathway.

Discussion

Although some evidence indicates that progesterone is associated with decreased incidence of CRC (3), other reports have correlated progesterone with later cancer stage and increased mortality from CRC (15). Data explaining this contradiction is currently lacking. This may be because the effects of synthetic progestins are different from those of natural progesterone (29). Progesterone has been shown to participate in various cancer-associated processes in different cancer types. Progesterone activates the angiogenesis pathway involved in vascular endothelial growth factor stimulation to promote breast cancer progression (30), and interfers with the progression of ovarian cancer by down=-regulating the phosphorylation of Src and focal adhesion kinase (31). However, information on its cellular effects on CRC cells is limited (32). The present study reported that low levels of progesterone in patients with CRC were consistent with the PGR level in CRC tissues and correlated with poor prognosis of CRC. Treatment with an increased concentration of progesterone resulted in the inhibition of CRC cell proliferation *in vitro* and *in vivo*. Furthermore, progesterone up-regulated GADD45α and c-Jun and promoted the phosphorylation of JNK. These results suggested that progesterone inhibited the proliferation of CRC cells to reduce the malignant progression of CRC by up-regulating the JNK pathway via GADD45α.

Progesterone is dysregulated in several types of cancer and is associated with cancer progression (29). Studies in humans and monkeys have suggested that oral micronized progesterone has a more favourable effect on risk biomarkers for postmenopausal breast cancer (33-35). Progesterone prevents high-grade serous ovarian cancer by inducing necroptosis, and inhibits migration and invasion, revealing its mechanism of action (36). Progesterone also reduces the invasive potential of endometrial cancer cells (37). However, the specific mechanism by which progesterone inhibits the malignant progression of CRC is unclear. In line with previous reports, the current results confirmed that low progesterone expression was associated with poor prognosis of CRC (11,38). Moreover, the relationship with PGR was revealed, and the growth inhibitory effects in CRC were analysed. PGR has been identified in normal and malignant tissues and is a highly structured transcription factor that regulates diverse physiological processes (39,40). However, the reported relationship between PGR expression and cancer progression is still contradictory. In ovarian cancer, higher levels of PGR predict favourable survival (41).

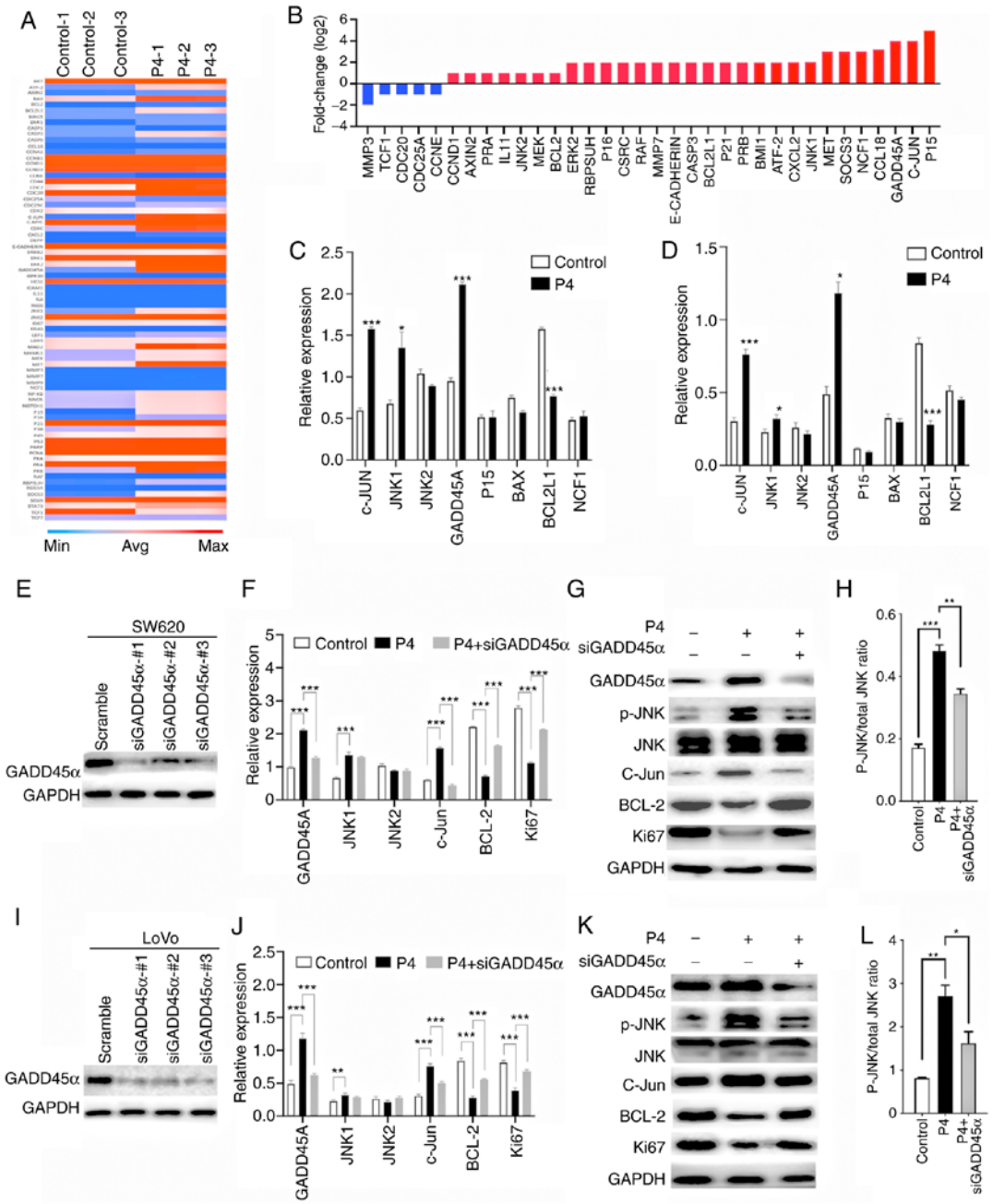

Figure 5. Progesterone up-regulates the JNK pathway via GADD45α activation to inhibit progression of colonic carcinoma. (A) Heat map depicting progesterone-induced changes in the expression profile of genes that were assessed on the PCR microarray. Blue and red represent low and high gene expression levels in pinnae, respectively. (B) Selection of genes that were altered the most in SW620 cells. Genes are presented alphabetically. RT-PCR was used to investigate the effect of progesterone on proliferation-related genes such as c-Jun, JNK1, JNK2, GADD45α, P15, BAX, BCL2L1 and NCF1 in (C) SW620 and (D) LoVo cells Efficiency of knockdown GADD45α in (E) SW620 and (I) LoVo cells, respectively. Expression of GADD45α, JNK1, JNK2, c-Jun, BCL-2, and Ki67 in (F) SW620 and (J) LoVo cells was analysed using RT-PCR, and GADD45α, phosphorylation of JNK, JNK, c-Jun, BCL-2 and Ki67 in (G) SW620 and (K) LoVo cells were analysed using western blot. Relative expression of phosphorylation of JNK to total protein of JNK in (H) SW620 and (L) LoVo cells. *P<0.05, **P<0.01 and ***P<0.001 vs. control. GADD45α, growth arrest and DNA damage-inducible protein α; JNK, c-Jun N-terminal kinase; BCL-2, B cell lymphoma-2; NCF1, neutrophil cytosol factor 1.

However, PRG expression is decreases by 30-40% in prostate cancer-associated stroma compared with benign stroma (42). Higher tumour grades are associated with increased PGR expression in astrocytomas (43). This variation may be due to differences in the way specimens were prepared, the method or antibodies used, the location of the lesions or the level of staining required to consider a sample positive (44). In CRC tumours, low PGR levels are inversely associated with extensive primary tumours, poor prognosis and high recurrence rate of CRC (38,39,45). In addition, mifepristone, the PGR agonist, protects against CRC by modulating the effects of oestrogen on carcinogenesis (46). In contrast, PGR is detected in normal and cancer tissues, but without any significant relationship with clinicopathological tumour characteristics and patient outcome (47,48). Recently, lower PGR expression levels are associated with more extensive CRC primary tumours and poorer prognosis (39). As the range of actions of PGR have limited its clinical use thus far, the present results revealed

a positive correlation with progesterone level and a negative correlation with clinical characteristics. PGR expression in specimens (normal and tumour tissue) was also confirmed, which was consistent with results from TCGA database.

A number of investigations have shown that progesterone signals negatively regulates cell cycle progression by suppressing S and G_2/M phases and downregulating cell cycle phase-specific cyclins/CDKs (49). Progesterone also inhibits CRC by regulating proliferation and apoptosis (16,50). In parallel with a previous study, progesterone also inhibited CRC cell proliferation in this study (17). Cell growth factors were also analysed. CCNA1, CCNB1 and cleaved caspase-3 were up-regulated, while BCL-2 and BAX were down-regulated. These data indicated that progesterone inhibited CRC proliferation by arresting the G_2/M phase and inducing apoptosis. In contrast, previous studies indicated that progesterone does not inhibit the proliferation of CRC cell lines (51,52). These contradictory results may be due to the use of different types of cell lines, different concentrations of progesterone or different PGR expression patterns.

Although the role of progesterone in the progression of CRC has been evaluated, the specific mechanism by which progesterone regulates CRC remains to be explored. Progesterone has the ability to bind to nuclear or membrane receptors to regulate cancer development via the classical and non-classical pathways (34). The classical signalling induced by progesterone leads to the decomposition of heat shock protein, PGR dimerization, and progesterone response element (PRE), and initiates the transcription of downstream effector targets cyclin D1 and P4 mediator receptor activator of nuclear factor κB ligand. In non-classical pathway, PRE binds to proto-oncogene tyrosine-protein Src, mitogen activated protein kinase and protein kinase B, and then activates downstream effector targets wingless-type MMTV integration site family member 1, cyclin D1, epidermal growth factor receptor and transcription of p21. Progesterone exerts a tumour suppressive effect by inducing apoptosis via activation of the MAPK pathway (10). The apoptosis of CRC cells is due to the up-regulation of GADD45α and activation of MAPKs (JNK, p38 and ERK) (53). In addition, GADD45α was also reported to act as a regulator of DNA damage and S-phase arrest in hepatoma cells (54). The present additional investigation using a PCR microarray revealed that GADD45α JNK, and c-Jun expressions were up-regulated following progesterone intervention. Since the mechanism of JNK-induced apoptosis and GADD45α-mediated cell cycle have been widely verified (21,55,56), it was hypothesised that intervention with progesterone regulated the link between cell cycle arrest and GADD45α and apoptosis in response to the activation of JNK/c-Jun pathway. Some studies have shown that GADD45α activates the JNK pathway (56,57), whereas others have suggested that GADD45α is downstream of JNK/p38 pathways, and is associated with cell viability, DNA damage and the cell cycle (58,59). In addition, an *in vitro* experiments have indicated the existence of a feedback loop between GADD45α and the JNK pathway (53). The current study observed enhanced expression of GADD45α, phosphorylation of JNK and c-Jun and inhibition of BCL-2 in progesterone-treated SW620 cells. The down-regulated phosphorylation of JNK and c-Jun and up-regulated expression of BCL-2 was verified when these cells were transfected with GADD45α siRNA. These results implied that the JNK pathway was activated by progesterone-induced GADD45α to inhibit the malignant progression of CRC.

Overall, the present investigations demonstrated that progesterone and PGR acted as inhibiting factors for poor prognosis of CRC. Progesterone intervention in CRC cells suppressed proliferation by arresting the cell cycle and inducing apoptosis. Moreover, progesterone-induced inhibition of CRC progression was regulated by GADD45α/JNK/c-Jun signalling. These findings suggested that progesterone plays an efficient role in CRC inhibition, which might be used in the treatment of CRC patients with progesterone deficiency. However, several missing links remain to be filled in future studies, including the molecular mechanism responsible for progesterone-mediated upregulation of GADD45α.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The datasets generated and/or analyzed during the current study are available in the UALCAN repository (http://ualcan.path.uab.edu/).

Authors' contributions

YLZ, LFZ and YHH designed the study. XDW, XG and PTZ contributed to the acquisition, analysis and interpretation of data from patients. YLZ, XDW, XG, WL, TTW, PTZ and SQH performed the experiments. LFZ was in charge of statistical analysis. YLZ, XDW and XG drafted the manuscript. SQH, TTW and PTZ took charge of critical revision of the manuscript and language polishing. YHH and LFZ provided administrative and technical supervision. XDW and PTZ confirm the authenticity of all raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was performed in accordance with clinical study protocols and the principles of the Declaration of Helsinki (modified 2000) and was approved by The Research Care and Ethics Committee of the General Hospital of Western Theater Command (approval no. SPPHCT2015-0117). Patients or their families gave signed informed consent to participate in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin: Feb 4, 2021 (Epub ahead of print).
2. Dekker E, Tanis P, Vleugels JLA, Kasi PM and Wallace MB: Colorectal cancer. Lancet 394: 1467-1480, 2019.
3. Folkerd EJ and Dowsett M: Influence of sex hormones on cancer progression. J Clin Oncol 28: 4038-4044, 2010.
4. Fonseca EB, Celik E, Parra M, Singh M and Nicolaides KH; Fetal Medicine Foundation Second Trimester Screening Group: Progesterone and the risk of preterm birth among women with a short cervix. N Engl J Med 357: 462-469, 2007.
5. Tsai HW, Ho CL, Cheng SW, Lin YJ, Chen CC, Cheng PN, Yen CJ, Chang TT, Chiang PM, Chan SH, *et al*: Progesterone receptor membrane component 1 as a potential prognostic biomarker for hepatocellular carcinoma. World J Gastroenterol 24: 1152-1166, 2018.
6. Syed V, Mukherjee K, Lyons-Weiler J, Lau KM, Mashima T, Tsuruo T and Ho SM: Identification of ATF-3, caveolin-1, DLC-1, and NM23-H2 as putative antitumorigenic, progesterone-regulated genes for ovarian cancer cells by gene profiling. Oncogene 24: 1774-1787, 2005.
7. Kim HW, Kim JH, Lim BJ, Kim H, Kim H, Park JJ, Youn YH, Park H, Noh SH, Kim JW and Choi SH: Sex disparity in gastric cancer: Female sex is a poor prognostic factor for advanced gastric cancer. Ann Surg Oncol 23: 4344-4351, 2016.
8. Aktas B, Kasimir-Bauer S, Müller V, Janni W, Fehm T, Wallwiener D, Pantel K and Tewes M; DETECT Study Group: Comparison of the HER2, estrogen and progesterone receptor expression profile of primary tumor, metastases and circulating tumor cells in metastatic breast cancer patients. BMC Cancer 16: 522, 2016.
9. Núñez C, Capelo JL, Igrejas G, Alfonso A, Botana LM and Lodeiro C: An overview of the effective combination therapies for the treatment of breast cancer. Biomaterials 97: 34-50, 2016.
10. Kong X, Li M, Shao K, Yang Y, Wang Q and Cai M: Progesterone induces cell apoptosis via the CACNA2D3/Ca2+/p38 MAPK pathway in endometrial cancer. Oncol Rep 43: 121-132, 2020.
11. Lin JH, Manson JE, Kraft P, Cochrane BB, Gunter MJ, Chlebowski RT and Zhang SM: Estrogen and progesterone-related gene variants and colorectal cancer risk in women. BMC Med Genet 12: 78, 2011.
12. Liu Y, Tian LB, Yang HY and Zhang HP: Effects of estradiol and progesterone on the growth of HeLa cervical cancer cells. Eur Rev Med Pharmacol Sci 21: 3959-3965, 2017.
13. Park JY, Kim DY, Kim TJ, Kim JW, Kim JH, Kim YM, Kim YT, Bae DS and Nam JH: Hormonal therapy for women with stage IA endometrial cancer of all grades. Obstet Gynecol 122: 7-14, 2013.
14. Simon MS, Chlebowski RT, Wactawskiwende J, Johnson KC, Muskovitz A, Kato I, Young A, Hubbell FA and Prentice RL: Estrogen plus progestin and colorectal cancer incidence and mortality. J Clin Oncol 30: 3983-3990, 2012.
15. Schürmann R, Cronin M and Meyer JU: Estrogen plus progestin and colorectal cancer in postmenopausal women. N Engl J Med 350: 2417-2419, 2004.
16. Kuo CT and Lee WS: Progesterone receptor activation is required for folic acid-induced anti-proliferation in colorectal cancer cell lines. Cancer Lett 378: 104-110, 2016.
17. Motylewska E and Mełeń-Mucha G: Estrone and progesterone inhibit the growth of murine MC38 colon cancer line. J Steroid Biochem Mol Biol 113: 75-79, 2009.
18. Mauri G, Bonazzina E, Amatu A, Tosi F, Bencardino K, Gori V, Massihnia D, Cipani T, Spina F, Ghezzi S, *et al*: The evolutionary landscape of treatment for BRAF (V600E) mutant metastatic colorectal cancer. Cancers (Basel) 13: 137, 2021.
19. Sui X, Kong N, Ye L, Han W, Zhou J, Zhang Q, He C and Pan H: p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. Cancer Lett 344: 174-179, 2014.
20. Zhu X, Jiang X, Duan C, Li A, Sun Y, Qi Q, Liu Y, Li S and Zhao Z: S-Allylmercaptocysteine induces G2/M phase arrest and apoptosis via ROS-mediated p38 and JNK signaling pathway in human colon cancer cells in vitro and in vivo. RSC Adv 7: 49151-49158, 2017.
21. Chen K, Chu BZ, Liu F, Li B, Gao CM, Li LL, Sun QS, Shen ZF and Jiang YY: New benzimidazole acridine derivative induces human colon cancer cell apoptosis in vitro via the ROS-JNK signaling pathway. Acta Pharmacol Sin 36: 1074-1084, 2015.
22. Zhou WJ, Hou XX, Wang XQ and Li DJ: Fibroblast growth factor 7 regulates proliferation and decidualization of human endometrial stromal cells via ERK and JNK pathway in an autocrine manner. Reprod Sci 24: 1607-1619, 2017.
23. Wang Z, Ma L, Su M, Zhou Y, Mao K, Li C, Peng G, Zhou C, Shen B and Dou J: Baicalin induces cellular senescence in human colon cancer cells via upregulation of DEPP and the activation of Ras/Raf/MEK/ERK signaling. Cell Death Dis 9: 217, 2018.
24. Jin M and Frankel W: Lymph node metastasis in colorectal cancer. Surg Oncol Clin N Am 27: 401-412, 2018.
25. World Medical Association Inc.: Declaration of Helsinki. Ethical principles for medical research involving human subjects. J Indian Med Assoc 107: 403-405, 2009.
26. Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto M, Goldberg M, Goralczyk EM, *et al*: MIFlowCyt: The minimum information about a flow cytometry experiment. Cytometry A 73A: 926-930, 2010.
27. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, *et al*: The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. J Cereb Blood Flow Metab 40: 1769-1777, 2020.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using Real-Time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
29. Lieberman A and Curtis L: In defense of progesterone: A review of the literature. Altern Ther Health Med 23: 24-32, 2017.
30. Zhou L, Zhou W, Zhang H, Hu Y, Yu L, Zhang Y, Zhang Y, Wang S, Wang P and Xia W: Progesterone suppresses triple-negative breast cancer growth and metastasis to the brain via membrane progesterone receptor α. Int J Mol Med 40: 755-761, 2017.
31. Lima MA, Silva SV, Jaeger RG and Freitas VM: Progesterone decreases ovarian cancer cells migration and invasion. Steroids 161: 108680, 2020.
32. Tanaka Y, Kato K, Mibu R, Uchida S, Asanoma K, Hashimoto K, Nozaki M and Wake N: Medroxyprogesterone acetate inhibits proliferation of colon cancer cell lines by modulating cell cycle-related protein expression. Menopause 15: 442-453, 2008.
33. Bluming AZ: Progesterone and breast cancer pathogenesis. J Mol Endocrinol 66: C1-C2, 2021.
34. Pedroza DA, Subramani R and Lakshmanaswamy R: Classical and non-classical progesterone signaling in breast cancers. Cancers (Basel) 12: 2440, 2020.
35. Wood CE, Register TC, Lees CJ, Chen H, Kimrey S and Cline JM: Effects of estradiol with micronized progesterone or medroxyprogesterone acetate on risk markers for breast cancer in postmenopausal monkeys. Breast Cancer Res Treat 101: 125-134, 2007.
36. Wu NY, Huang HS, Chao TH, Chou HM, Fang C, Qin CZ, Lin CY, Chu TY and Zhou HH: Progesterone prevents high-grade serous ovarian cancer by inducing necroptosis of p53-defective fallopian tube epithelial cells. Cell Rep 18: 2557-2565, 2017.
37. Waheed S, Dorjbal B, Hamilton CA, Maxwell GL, Rodriguez GC and Syed V: Progesterone and calcitriol reduce invasive potential of endometrial cancer cells by targeting ARF6, NEDD9 and MT1-MMP. Oncotarget 8: 113583-113597, 2017.
38. Liu D: Gene signatures of estrogen and progesterone receptor pathways predict the prognosis of colorectal cancer. FEBS J 283: 3115-3133, 2016.
39. Abd ElLateef AAE, Mohamed AES, Elhakeem AA and Ahmed SF: Estrogen and progesterone expression in colorectal carcinoma: A clinicopathological study. Asian Pac J Cancer Prev 21: 1155-1162, 2020.
40. Kim JJ, Kurita T and Bulun SE: Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. Endocr Rev 34: 130-162, 2013.
41. Zhao D, Zhang F, Zhang W, He J, Zhao Y and Sun J: Prognostic role of hormone receptors in ovarian cancer: A systematic review and meta-analysis. Int J Gynecol Cancer 23: 25-33, 2013.
42. Yu Y, Yang O, Fazli L, Rennie PS, Gleave ME and Dong X: Progesterone receptor expression during prostate cancer progression suggests a role of this receptor in stromal cell differentiation. Prostate 75: 1043-1050, 2015.

43. Tavares CB, Gomes-Braga FD, Costa-Silva DR, Escórcio-Dourado CS, Borges US, Conde-Junior AM, Barros-Oliveira Mda C, Sousa EB, Barros Lda R, Martins LM, *et al*: Expression of estrogen and progesterone receptors in astrocytomas: A literature review. Clinics (Sao Paulo) 71: 481-486, 2016.
44. Jiang H, Teng R, Wang Q, Zhang X, Wang H, Wang Z, Cao J and Teng L: Transcriptional analysis of estrogen receptor alpha variant mRNAs in colorectal cancers and their matched normal colorectal tissues. J Steroid Biochem Mol Biol 112: 20-24, 2008.
45. Qasim BJ, Ali HH and Hussein AG: Immunohistochemical expression of estrogen and progesterone receptors in human colorectal adenoma and carcinoma using specified automated cellular image analysis system: A clinicopathological study. Oman Med J 26: 307-314, 2011.
46. Koo JH and Leong RW: Sex differences in epidemiological, clinical and pathological characteristics of colorectal cancer. J Gastroenterol Hepatol 25: 33-42, 2010.
47. Berta L, Fronticelli Baldelli C, Fazzari A, Radice E, Bargoni A, Frairia R and Gaetini A: Sex steroid receptors, secondary bile acids and colorectal cancer. A possible mechanism of interaction. Panminerva Med 45: 261-266, 2003.
48. Ye SB, Cheng YK, Zhang L, Wang XP, Wang L and Lan P: Prognostic value of estrogen receptor-α and progesterone receptor in curatively resected colorectal cancer: A retrospective analysis with independent validations. BMC Cancer 19: 933, 2019.
49. Yazdani S, Kasajima A, Onodera Y, McNamara KM, Ise K, Nakamura Y, Tachibana T, Motoi F, Unno M and Sasano H: Progesterone arrested cell cycle progression through progesterone receptor isoform A in pancreatic neuroendocrine neoplasm. J Steroid Biochem Mol Biol 178: 243-253, 2018.
50. Sasso CV, Santiano FE, Campo Verde Arboccó F, Zyla LE, Semino SN, Guerrero-Gimenez ME, Pistone Creydt V, López Fontana CM and Carón RW: Estradiol and progesterone regulate proliferation and apoptosis in colon cancer. Endocr Connect 8: 217-229, 2019.
51. Nakayama Y, Sakamoto H, Satoh K and Yamamoto T: Tamoxifen and gonadal steroids inhibit colon cancer growth in association with inhibition of thymidylate synthase, survivin and telomerase expression through estrogen receptor beta mediated system. Cancer Lett 161: 63-71, 2000.
52. Lointier P, Wildrick DM and Boman BM: The effects of steroid hormones on a human colon cancer cell line in vitro. Anticancer Res 12: 1327-1330, 1992.
53. Su MQ, Zhou YR, Rao X, Yang H, Zhuang XH, Ke XJ, Peng GY, Zhou CL, Shen BY and Dou J: Baicalein induces the apoptosis of HCT116 human colon cancer cells via the upregulation of DEPP/Gadd45a and activation of MAPKs. Int J Oncol 53: 750-760, 2018.
54. Li D, Dai C, Yang X, Li B, Xiao X and Tang S: GADD45a regulates olaquindox-induced DNA damage and S-phase arrest in human hepatoma G2 cells via JNK/p38 pathways. Molecules 22: 124, 2017.
55. Song L, Li J, Zhang D, Liu ZG, Ye J, Zhan Q, Shen HM, Whiteman M and Huang C: IKKbeta programs to turn on the GADD45alpha-MKK4-JNK apoptotic cascade specifically via p50 NF-kappaB in arsenite response. J Cell Biol 175: 607-617, 2006.
56. Ueda T, Kohama Y, Kuge A, Kido E and Sakurai H: GADD45 family proteins suppress JNK signaling by targeting MKK7. Arch Biochem Biophys 635: 1-7, 2017.
57. Camilleri-Robles C, Serras F and Corominas M: Role of D-GADD45 in JNK-dependent apoptosis and regeneration in drosophila. Genes 10: 378, 2019.
58. Choi HJ, Kang KS, Fukui M and Zhu BT: Critical role of the JNK-p53-GADD45α apoptotic cascade in mediating oxidative cytotoxicity in hippocampal neurons. Br J Pharmacol 162: 175-192, 2011.
59. Yin F, Bruemmer D, Blaschke F, Hsueh WA, Law RE and Herle AJ: Signaling pathways involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells. Oncogene 23: 4614-4623, 2004.