GANT61 exerts anticancer cell and anticancer stem cell capacity in colorectal cancer by blocking the Wnt/β-catenin and Notch signalling pathways

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Received June 28, 2020; Accepted May 13, 2021

DOI: 10.3892/or.2022.8397

Abstract. The present study aimed to assess the anticancer cell and anticancer stem cell (CSC) effects of GANT61, and its regulatory influence on the Wnt/β-catenin and Notch signalling pathways in colorectal cancer (CRC). HT-29 and HCT-116 cells were treated with 0, 2.5, 5, 10, 20 or 40 μM GANT61, after which relative cell viability and the expression of Gli1, β-catenin and Notch1, as well as the percentage of $CD133^+$ cells, were detected. Subsequently, HT-29/HCT-116 cells and CSCs were treated with 20 μM GANT61, 10 mM of the Wnt/β-catenin pathway agonist HLY78, and 30 mM of the Notch pathway agonist JAG1 (alone or in combination), which was followed by the assessment of cell viability and apoptosis. In both cell lines, GANT61 reduced relative cell viability in a time- and dose-dependent manner, inhibited Gli1, β-catenin and Notch1 expression, and decreased the percentage of $CD133^+$ cells in a dose-dependent manner. Furthermore, HLY78 and JAG1 were both found to improve the relative viability, while downregulating the apoptosis of untreated and GANT61-treated HT-29 and HCT-116 cells. Moreover, Wnt/β-catenin and Notch signalling pathway activity were upregulated in CSCs isolated from HT-29 and HCT-116 cells, compared with the associated control groups. GANT61 also reduced the viability of HT-29 and HCT-116 cells and increased apoptosis, whereas HLY78 and JAG1 treatment resulted in the opposite effect. Moreover, both HLY78 and JAG1 attenuated the effects of GANT61 on cellular viability and apoptosis. In conclusion, GANT61 was found to effectively eliminate cancer cells and CSCs by blocking the Wnt/β-catenin and Notch signalling pathways in CRC.

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Abbreviations: CRC, colorectal cancer; CSCs, cancer stem cells; Hh, Hedgehog; Gli1, GLI family zinc finger 1; $CD133^+$, CD133 positive; VEGF, vascular endothelial growth factor; MEK, methyl ethyl ketone; SOCS3, suppressor of cytokine signalling 3

Key words: CRC, GANT61, CSCs, Wnt/β-catenin pathway, Notch pathway

Introduction

Over the past several decades, colorectal cancer (CRC) has become one of the most common solid tumour types, and is predicted to become more prevalent in the future, with a projected global incidence of 2,500,000 in 2035 (1,2). Furthermore, CRC has become increasingly common among individuals <50 years of age, which is partially due to unhealthy dietary habits, a sedentary lifestyle and obesity (3). Therefore, many patients are in the advanced stages of disease before receiving treatment. In a clinical setting, the management of patients with CRC primarily includes extensive surgery, ablation, radiotherapy, chemotherapy, targeted therapy and immunotherapy (4,5). However, despite the development of novel drugs and precision medicine, the overall patient prognosis has not improved considerably, as patient survival is largely dependent on the disease stage at first diagnosis. Additionally, though rectal bleeding is a common clinical feature, the majority of CRC cases are asymptomatic, which further complicates early detection. Moreover, cancer stem cells (CSCs) also play a critical role in promoting CRC progression. CSCs are a class of highly undifferentiated cancer cells that share features with normal stem cells, are pluripotent and have the ability to self-renew (6). To date, CSCs have been reported to be involved in multiple pathogenic processes in CRC, and the most predominant findings include the correlation of CSCs with tumour growth, metastasis and chemoresistance (7-10). Therefore, the discovery of novel and efficient therapies is critical to enhancing or facilitating the treatment response, and ultimately prolonging the survival of patients with CRC.

The Hedgehog (Hh) signalling pathway is responsible for promoting the progression of various malignancies, such as lung cancer and clear renal cell carcinoma (11-13). Notably, the Hh pathway is also critically involved in the regulation of CRC pathogenesis, via, for example, regulating drug resistance to fluorouracil and irinotecan, cancer cell metastasis and stemness. Studies have also illustrated that this pathway is a potential biomarker for the management of patients with CRC (14-16). GLI family zinc finger 1 (Gli1; also known as glioma-associated oncogene-1) is an important transcription factor in Hh signalling whose levels represent Hh signalling

activity (17,18). As a selective inhibitor of Gli1, GANT61 inhibits the transactivation modulated by Gli1, and experiments have identified GANT61-D as its bioactive form (19,20). GANT61 has increased in popularity as a potential targeting agent for the treatment of carcinomas. For instance, it has been revealed that GANT61 promotes the sensitivity of prostate cancer cells to ionizing radiation *in vitro* and *in vivo* (21). In addition, GANT61 also exerts anticancer and anti-CSC effects in triple-negative breast cancer (22). Hence, based on the fact that GANT61 is a promising potential therapeutic target for carcinoma, and the selective inhibitor of Gli (a transcription factor in the Hh signalling pathway that is closely involved in CRC pathology), we hypothesised that GANT61 may effectively diminish both CRC cells and CRC-CSCs.

Therefore, the aim of the present study was to elucidate the cytotoxic effects of GANT61 on cancer cells and CSCs, and its regulatory role on the Wnt/β-catenin and Notch signalling pathways in CRC.

Materials and methods

Cell culture. The HT-29 and HCT-116 human CRC cell lines (both ATCC) were cultured in McCoy's 5A medium supplemented with 10% foetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37˚C in a humidified incubator containing 95% air and 5% CO_2. Both cell lines were authenticated by STR profiling.

Treatment of CRC cells with GANT61. GANT61 was dissolved in dimethyl sulfoxide (DMSO) (both MilliporeSigma) to prepare stock solutions of 2.5, 5, 10, 20 and 40 mM. HT-29 and HCT-116 cells were cultured with different concentrations of GANT61 for 24, 48 and 72 h, and cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). At 48 h, the expression levels of Gli1, β-catenin and Notch pathway proteins were evaluated by western blotting, and the percentage of $CD133^+$ cells was determined by flow cytometry.

Co-treatment of CRC cells with GANT61, HLY78 and JAG1. The Wnt/β-catenin pathway agonist HLY78 (MilliporeSigma) was dissolved in DMSO to produce a 10-mM solution. The Notch pathway agonist JAG1 peptide (23-25) (amino acid sequence, CDDYYYGFGCNKFCRPR; Sino Biological, Inc.) was dissolved in phosphate-buffered saline (PBS) to a concentration of 30 mM. The cells were treated with HLY78 and JAG1 at concentrations of 10 (26,27) and 30 μM (25,28,29), respectively. For co-treatment, 20 μM GANT61 was also added. Then, 48 h after incubation, cell viability was determined by CCK-8 assay, and the apoptotic rate was assessed by annexin V/propidium iodide (AV/PI) staining. Finally, the expression levels of β-catenin, transcription factor 7 (TCF7), Notch1 and Hes1 were detected by western blotting at 48 h.

CSC culture. HT-29 and HCT-116 cells were cultured in DMEM/F12 medium supplemented with 2% B27 and 20 ng/ml bFGF (all Gibco; Thermo Fisher Scientific, Inc.), as well as 20 ng/ml EGF and 4 ug/ml heparin (MilliporeSigma), for 10 days. The spheres were then harvested, trypsinized and cultured as CSCs (30,31). Next, the expression of CD133 was assessed by immunofluorescence (IF), while the expression levels of β-catenin, TCF7, Notch1 and Hes1 were detected by western blotting.

Co-treatment of CRC-CSCs with GANT61, HLY78 and JAG1. After isolation, the CSCs were treated with GANT61, HLY78 and JAG1, and subsequent detection was performed in the same manner as that described in the section on 'co-treatment of CRC cells with GANT61, HLY78 and JAG1'.

CCK-8 assay. In brief, cells were incubated in 100 μl DMEM mixed with 10 μl CCK-8 reagent for 2 h at 37˚C. The optical density value was recorded using a microplate reader (BioTek Instruments, Inc.).

Flow cytometry. Cells were harvested and washed with PBS, prior to incubation with a rabbit CD133 monoclonal antibody (1:50 dilution) (1:50; cat. no. ab216323; Abcam) at room temperature for 30 min. After further washing with PBS, the cells were incubated with Alexa Fluor® 488-conjugated anti-rabbit IgG (H+L) (1:500; cat. no. #4412; Cell Signaling Technology, Inc.,) at room temperature for 30 min in the dark. Finally, a FACSCalibur 2 flow cytometer (BD Biosciences) and FlowJo 7.6 software (FlowJo LLC) were used to assess the percentage of $CD133^+$ cells.

Western blotting. After harvesting, total protein was extracted from the cells using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Inc.), after which the protein was quantified using a BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.). Then, gel electrophoresis was performed using 4-12% NuPAGE Bis-Tris Gels (20 μg total protein/lane) and the proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA (MilliporeSigma) at 37˚C for 1.5 h), and then incubated with primary antibodies (4˚C, overnight) followed by secondary antibodies (37˚C, 1.5 h); the bands were detected using enhanced chemiluminescence (ECL Plus western blotting Substrate, Pierce; Thermo Fisher Scientific, Inc.) in the dark. The antibodies used for the western blot analysis are listed in Table I.

Apoptosis analysis. An Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific, Inc.) was used for co-annexin V/propidium iodide (AV/PI) detection, per the manufacturer's instructions. Briefly, the cells were collected and incubated with AV and PI for 15 min at room temperature. Next, the apoptotic rate was evaluated by flow cytometry and analysed with FlowJo software, as aforementioned.

IF. Briefly, the cells were fixed in 4% paraformaldehyde (at room temperature for 10 min) and permeabilized with 0.5% Triton X-100 (both MilliporeSigma) (room temperature for 3 min). After blocking nonspecific protein binding with 5% BSA (MilliporeSigma; room temperature for 30 min), the cells were incubated with a rabbit mAb against CD133 (1:200 dilution) for 2 h at room temperature, followed by incubation with Alexa Fluor® 488-conjugated anti-rabbit IgG (H+L) (1:500 dilution) at room temperature for 1.5 h in the

Table I. Antibodies.

Antibody	Cat. no.	Dilution
Primary		
Gli rabbit mAb	3538	1:1,000
β-catenin rabbit mAb	9582	1:1,000
TCF7 rabbit mAb	2206	1:1,000
Notch1 rabbit mAb	3608	1:1,000
Hes1 rabbit mAb	11988	1:1,000
GAPDH rabbit mAb	5174	1:1,000
Secondary		
Goat Anti-Rabbit IgG-HRP	14708	1:3,000

All antibodies were from Cell Signaling Technology, Inc. TCF7, transcription factor 7; mAb, monoclonal antibody.

dark. Images were then obtained using an inverted microscope (Olympus, Japan).

Statistical analysis. All data are expressed as the mean ± standard deviation. GraphPad Prism Software version 7.0 (GraphPad Software Inc.,) was used for data analysis and graph plotting. Comparisons between the control and other treatment groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. Multiple comparisons among groups were determined by one-way ANOVA followed by Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Impact of GANT61 on viability, Gli1, β-catenin, Notch1 and CD133⁺ proportion in CRC cells. GANT61 decreased the relative viability of both HT-29 and HCT-116 cells in a time- and dose-dependent manner ($P<0.05$; Fig. 1A and B). Furthermore, GANT61 decreased the protein expression levels of Gli1, β-catenin and Notch1 in a dose-dependent manner in HT-29 (Fig. 1C) and HCT-116 (Fig. 1D) cells. In addition, in HT-29 cells, GANT61 reduced the proportion of $CD133^+$ cells in a dose-dependent manner ($P<0.05$; Figs. 1E and S1A). Similarly, in HCT-116 cells, GANT61 also decreased the percentage of $CD133^+$ cells in a dose-dependent manner ($P<0.05$; Figs. 1F and S1B).

GANT61 exhibits killing effects on CRC cells by regulating the Wnt/β-catenin pathway. Furthermore, GANT61 decreased, while HLY78 increased, the protein expression levels of β-catenin and TCF7 in HT-29 and HCT-116 cells; HLY78 also reversed the decrease in β-catenin and TCF7 protein expression in GANT61-treated HT-29 and HCT-116 cells ($P<0.05$) (Fig. 2A-D). Also, GANT61 decreased ($P<0.01$), while HLY78 increased ($P<0.01$), the relative viability of HT-29 cells, and HLY78 also retained the viability of GANT61-treated HT-29 cells ($P<0.05$) (Fig. 2E). In addition, GANT61 promoted ($P<0.001$), but HLY78 inhibited ($P<0.05$) the apoptosis of HT-29 cells, which was prevented by HLY78 in GANT61-treated HT-29 cells ($P<0.001$) (Fig. 2G and I). Furthermore, GANT61 downregulated ($P<0.001$), while HLY78 upregulated ($P<0.05$) the relative cell viability of HCT-116 cells, and HLY78 also retained the viability GANT61-treated HCT-116 cells ($P<0.01$) (Fig. 2F). Furthermore, GANT61 ($P<0.001$) increased HCT-116 cell apoptosis in, which was decreased by HLY78 ($P<0.05$); subsequently, HLY78 also reduced apoptosis in GANT61-treated HCT-116 cells ($P<0.001$) (Fig. 2H and J). These data suggested that HLY78 curtailed the effects of GANT61 on CRC cell viability and apoptosis.

GANT61 exhibits killing effects on CRC cells by regulating the Notch pathway. GANT61 decreased, while JAG1 increased Notch1 and Hes1 protein expression in HT-29 and HCT-116 cells, and JAG1 also retained the expression of these proteins in GANT61-treated HT-29 and HCT-116 cells (Fig. 3A-D). In regard to relative cell viability and apoptosis, GANT61 decreased ($P<0.001$), while JAG1 increased ($P<0.05$) the relative viability of HT-29 cells; in addition, JAG1 retarded the reduction in viability of GANT61-treated HT-29 cells ($P<0.05$) (Fig. 3E). Furthermore GANT61 increased ($P<0.001$), while JAG1 had no significant impact on apoptosis in HT-29 cells. However, JAG1 did reduce apoptosis in GANT61-treated HT-29 cells ($P<0.001$) (Fig. 3G and I). In addition, GANT61 downregulated ($P<0.001$), but JAG1 upregulated ($P<0.05$) the relative viability of HCT-116 cells, and JAG1 revived the viability of GANT61-treated HCT-116 cells ($P<0.01$) (Fig. 3F); additionally, GANT61 increased apoptosis in HCT-116 cells ($P<0.001$), while JAG1 decreased apoptotic rate ($P<0.05$). As with HT-29 cells, JAG1 also reduced apoptosis in HCT-116 cells following treatment with GANT61 ($P<0.001$) (Fig. 3H and J). These data implied that JAG1 reduced the effects of GANT61 on CRC cell viability and apoptosis.

β-catenin, TCF7, Notch1 and Hes1 expression in CRC-CSCs. Considering that GANT61 was found to regulate stemness markers, as well as the Wnt/β-catenin and Notch1 signalling pathways, activation of the Wnt/β-catenin and Notch signalling pathways was evaluated in CRC-CSCs. The results showed that CD133 was abundantly expressed in CSCs derived from both HT-29 and HCT-116 cells, compared with the control cells (Fig. 4A and C), suggesting that CSC generation was successful. Moreover, the protein expression levels of β-catenin, TCF7, Notch1 and Hes1 were upregulated in CRC-CSCs compared with the control cells (Fig. 4B, D, E and F), suggesting that the Wnt/β-catenin and Notch1 signalling pathways were activated in CRC-CSCs.

GANT61 exhibits killing effects on CRC-CSCs by modulating the Wnt/β-catenin pathway. GANT61 was found to decrease the protein expression levels of β-catenin and TCF7 in HT-29 and HCT-116 CSCs, which were increased by HLY78; HLY78 also increased the expression of β-catenin and TCF7 in HT-29 and HCT-116 CSCs following GANT61 treatment (Fig. 5A and B). Furthermore, the relative viability of HT-29 CSCs was decreased by GANT61, but increased by HLY78 (both $P<0.05$), and HLY78 had no significant impact on the viability of GANT61-treated HT-29 CSCs (Fig. 5C). In addition, GANT61 elevated ($P<0.001$), while HLY78 decreased ($P<0.05$) the number of apoptotic HT-29 CSCs; HLY78 also decreased apoptosis in GANT61-treated HT-29 CSCs ($P<0.05$)

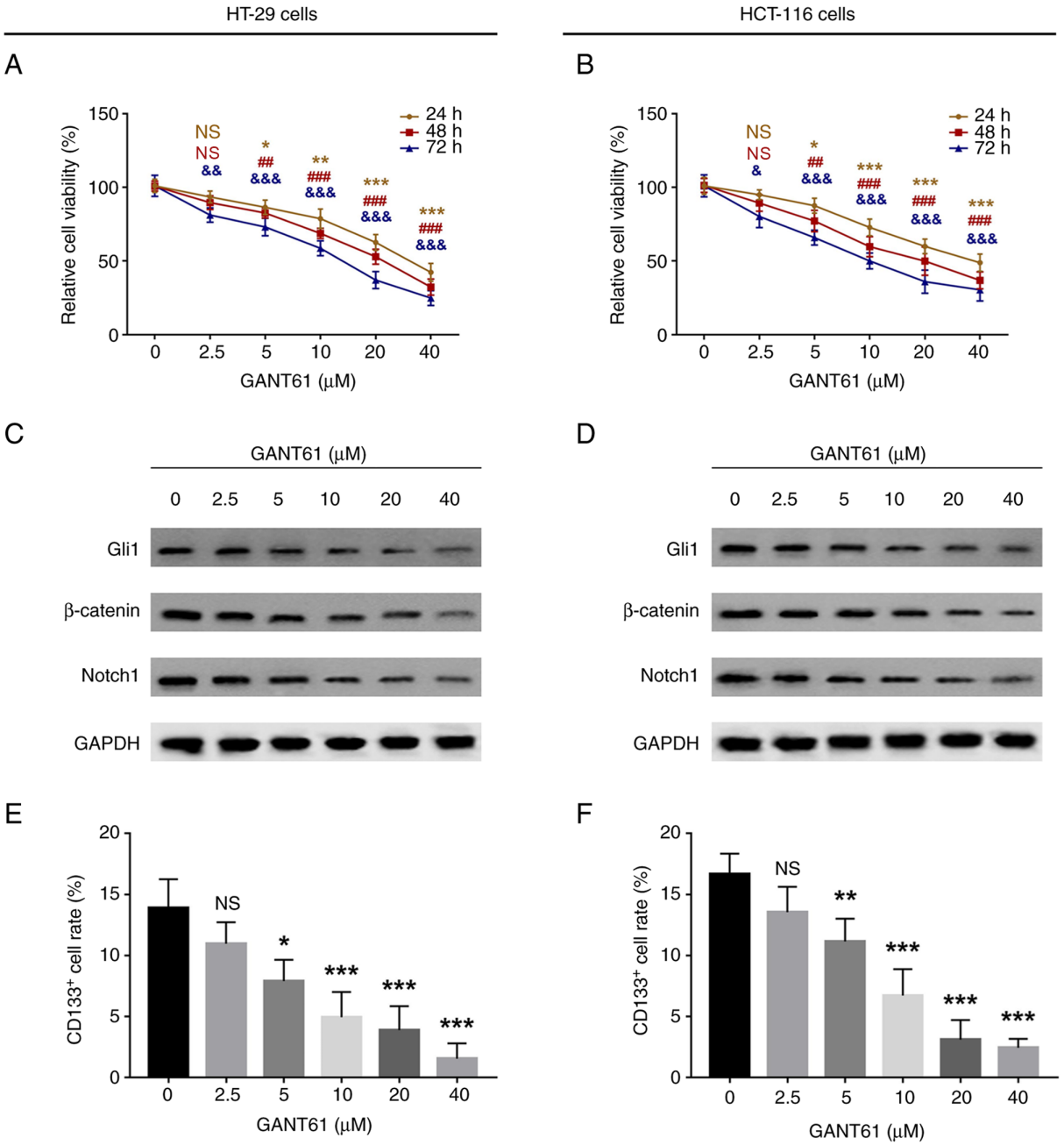

Figure 1. Regulatory role of GANT61 on relative viability, β-catenin and Notch1 expression, and stemness in colorectal cancer cells. Relative viability of (A) HT-29 and (B) HCT-116 cells; Gli1, β-catenin and Notch1 protein expression by (C) HT-29 and (D) HCT-116 cells; and $CD133^+$ rate among (E) HT-29 and (F) HCT-116 cells following treatment with GANT61. *P<0.05, **P<0.01 and ***P<0.001 vs. untreated group at 24 h. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. untreated group at 48 h. $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. untreated group at 72 h. Gli1, glioma-associated oncogene-1; ns, not significant.

(Fig. 5E and G). In HCT-116 CSCs, GANT61 decreased (P<0.01) while HLY78 increased (P<0.05) relatively viability; HLY78 also increased the viability of GANT61-treated HCT-116 CSCs (P<0.05) (Fig. 5D). Additionally, GANT61 upregulated (P<0.001), while HLY78 downregulated (P<0.05) apoptosis in HCT-116 CSCs, and HLY78 also downregulated apoptosis in GANT61-treated HCT-116 CSCs (P<0.01) (Fig. 5F and H). These data suggested that the cytotoxic effects of GANT61 in CRC-CSCs were exerted via the regulation of the Wnt/β-catenin pathway.

Killing effects of GANT61 on CRC-CSCs via modulation of the Notch pathway. GANT61 reduced, while JAG1 elevated Notch1 and Hes1 protein expression levels in HT-29 and HCT-116 CSCs, and JAG1 also elevated the levels of these proteins in GANT61-treated HT-29 and HCT-116 CSCs (Fig. 6A and B). Furthermore, GANT61 decreased relative cell viability (P<0.01), while JAG1 had no significant effect on HT-29 CSCs; however, JAG1 did elevate the relative viability of GANT61-treated HT-29 CSCs (P<0.05) (Fig. 6C). Moreover, GANT61 elevated apoptosis in HT-29 CSCs (P<0.001), while JAG1 decreased this (P<0.05), as well as reducing apoptosis in GANT61-treated HT-29 CSCs (P<0.05) (Fig. 6E and G). In addition, GANT61 downregulated (P<0.001), while JAG1 did not significantly impact relative HCT-116 CSC or GANT61-treated HCT-116 CSC viability (Fig. 6D). Moreover,

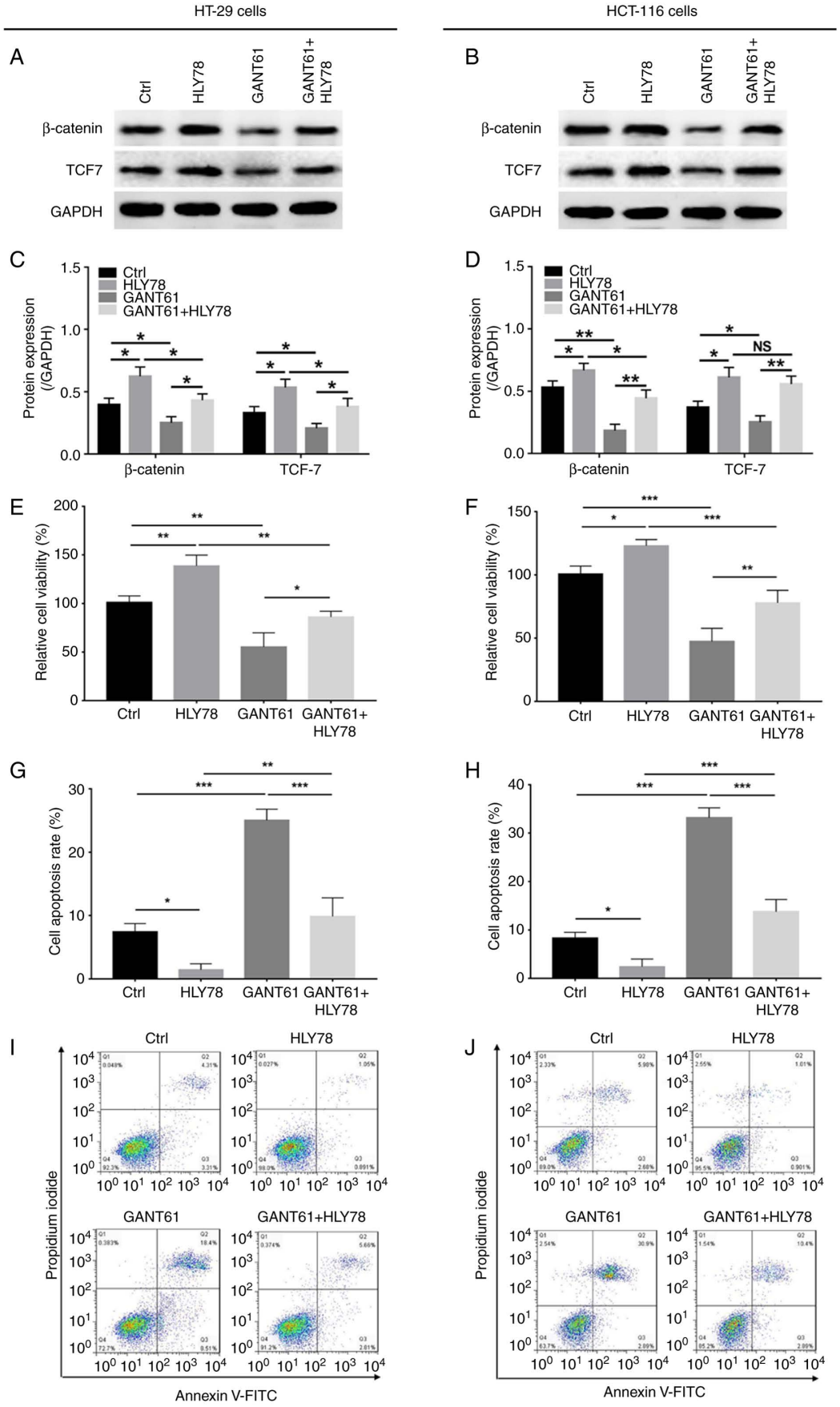

Figure 2. HLY78 enhances viability and reduces apoptosis in GANT61-treated colorectal cancer cells. (A-D) Protein expression levels of β-catenin and TCF7. Representative western blot images for (A) HT-29 and (B) HCT-116 cells and quantified results for (C) HT-29 and (D) HCT-116 cells treated with GANT61 and/or HLY78. Relative cell viability in HT-29 cells (E) and HCT-116 cells (F) among groups. (G-J) Apoptosis of cells in the different groups. (G) Apoptosis rate of (G) HT-29 and (H) HCT-116 cells and representative flow cytometry dot plots for (I) HT-29 and (J) HCT-116 cells. *P<0.05, **P<0.01 and ***P<0.001. NS, not significant; TCF7, transcription factor 7; Ctrl, control.

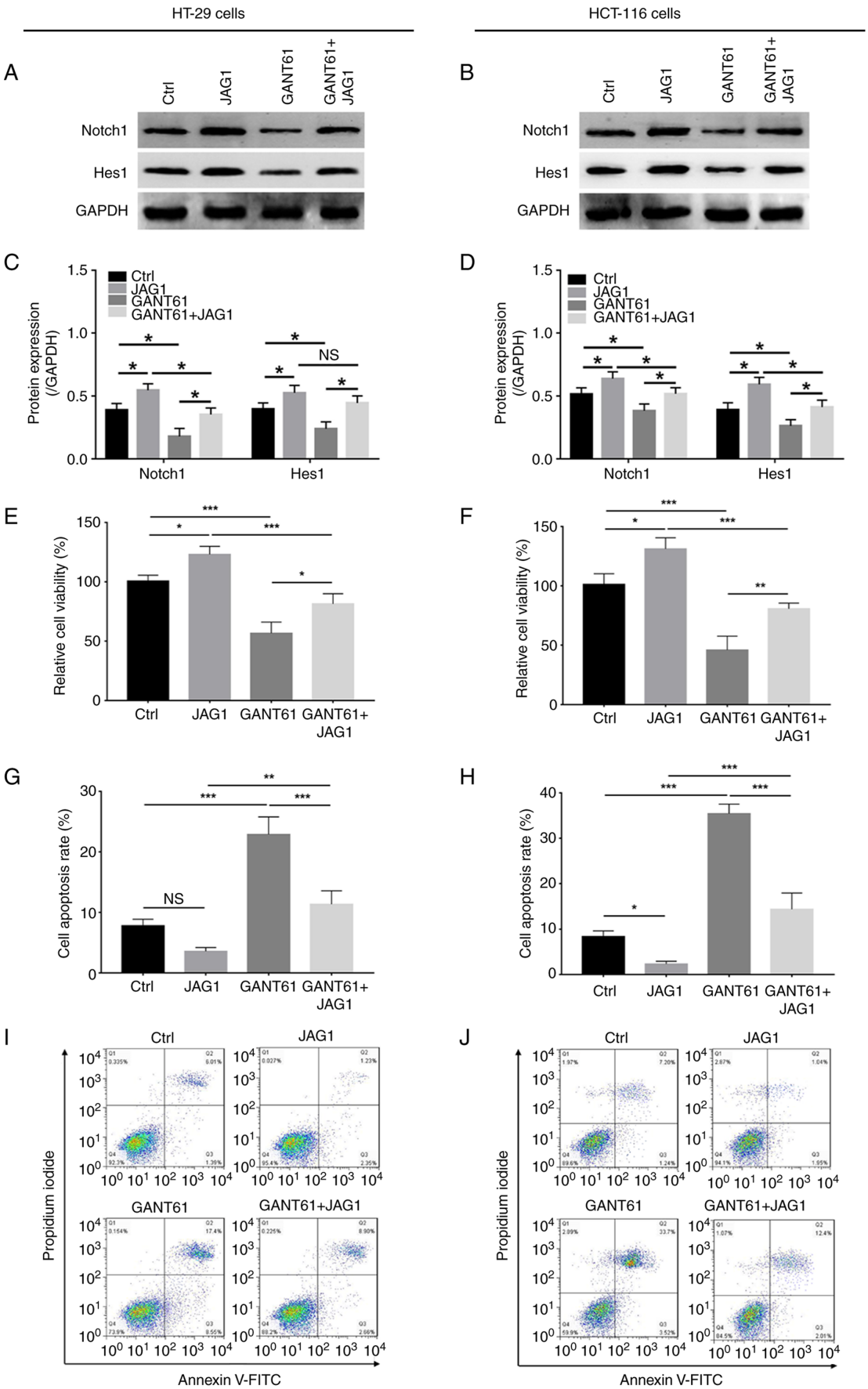

Figure 3. JAG1 promotes viability and reduces apoptosis in GANT61-treated colorectal cancer cells. (A-D) Protein expression levels of Notch1 and Has1. Representative western blot images for (A) HT-29 and (B) HCT-116 cells and quantified results for (C) HT-29 and (D) HCT-116 cells treated with GANT61 and/or JAG1. Relative viability of (E) HT-29 and (F) HCT-116 cells in the different groups. (G-J) Apoptosis of cells in the different groups. Apoptosis rate of (G) HT-29 and (H) HCT-116 cells and representative flow cytometry dot plots for (I) HT-29 and (J) HCT-116 cells. *P<0.05, **P<0.01 and ***P<0.001. Ctrl, control; NS, not significant.

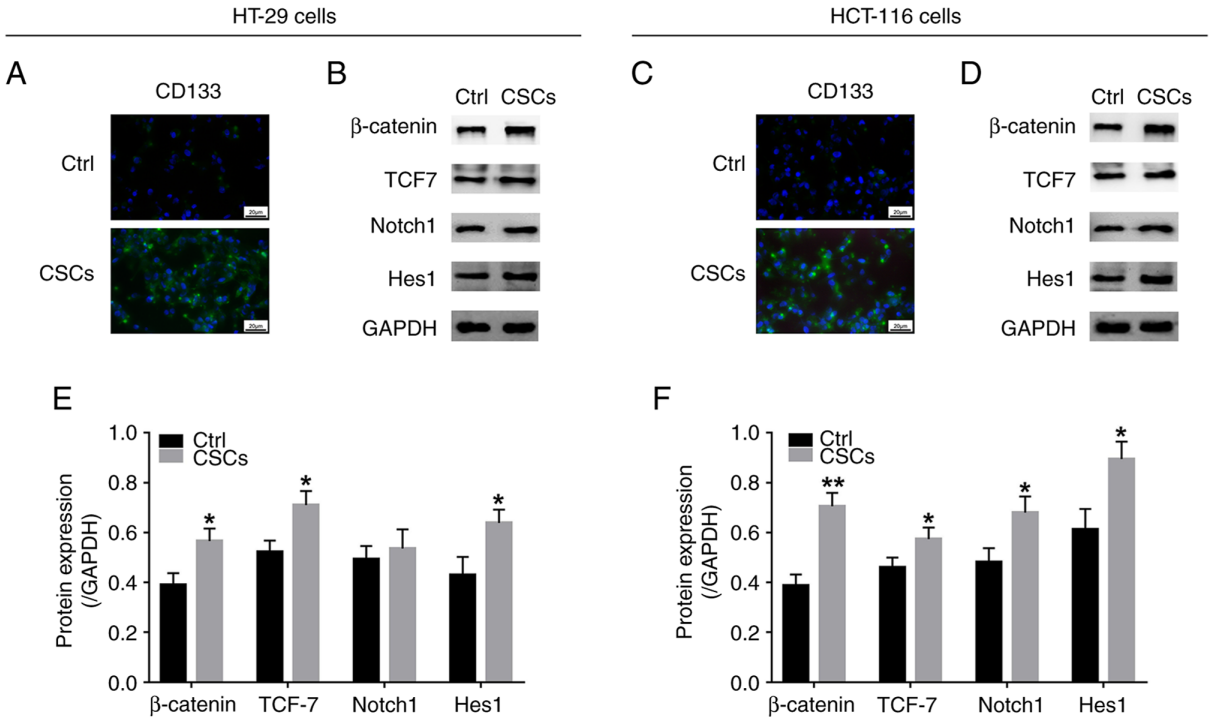

Figure 4. Wnt/β-catenin and Notch signalling pathways in colorectal cancer CSCs. (A) CD133$^+$ cells in Ctrl cells and HT-29 cell-derived CSCs and (B) western blot to assess the protein expression levels of β-catenin, TCF7, Notch1 and Hes1. (C) CD133$^+$ cells and (D) protein expression levels of β-catenin, TCF7, Notch1 and Hes1 in Ctrl cells and HCT-116 cell-derived CSCs of the line. Quantified expression levels of β-catenin, TCF7, Notch1 and Hes1 in in Ctrl cells and derived CSCs of (E) HT-29 and (F) HCT-116 cells. *P<0.05, **P<0.01. CSC, cancer stem cell; TCF7, transcription factor 7; Ctrl, control.

GANT61 upregulated the levels of apoptosis (P<0.001), while JAG1 did not significantly affect the apoptotic rate of HCT-116 CSCs. However, JAG1 did reduce apoptosis in GANT61-treated HCT-116 CSCs (P<0.05) (Fig. 6F and H). The results indicated that JAG1 eliminated the effects of GANT61 on CSC viability and apoptosis.

Discussion

Although CRC patient prognosis has improved, the poor survival rate of patients with metastatic disease necessitates the discovery of novel drugs and additional therapies, among which biological (such as anti-VEGF agents) and targeted therapies are the most promising. In a clinical setting, several novel drugs, such as anti-VEGF agents and MEK inhibitors, have demonstrated favourable therapeutic effects; however, these are not sufficient to fulfil the need for more efficacious therapies (32,33). Previous studies have clarified that the Hh signalling pathway promotes progression and mediates chemoresistance in CRC (14,34,35). However, a limited number of studies have been published on the role of the Hh pathway and its inhibitor GANT61 in CRC. Therefore, the present study was performed to evaluate the effects of GANT61 on CRC cells and CRC-CSCs, which revealed the following: i) GANT61 treatment decreased CRC cell viability and the expression of Gli1, β-catenin and Notch1, as well as the proportion of CD133$^+$ CRC cells, in a dose-dependent manner; and (ii) GANT61 exhibited good cytotoxic activity against CRC cells and CRC-CSCs by regulating the Wnt/β-catenin and Notch signalling pathways.

Studies performed on multiple carcinomas have suggested GANT61 as a potentially promising antitumour drug, since the increasingly important role of the Hh signalling pathway has been established in cancer. For instance, a study performed in nude mice involving the implantation of HeLa cells revealed that GANT61 represses the growth and apoptosis of allograft tumours, and that this compound is also tolerated in mice, as shown by white blood cell count, haemoglobin level, platelet count, and the levels of alanine aminotransferase, aspartate aminotransferase and creatine (36). Moreover, in a Ewing's sarcoma family-derived tumour cell line (SK-N-LO), GANT61 treatment induced cellular and morphological changes (such as cell shrinkage, chromatin condensation and nuclear fragmentation) in a dose-dependent manner; GANT61 also increased the percentage of apoptotic cells (37). Another study illustrated that GANT61 decreased cell viability, but enhanced apoptosis in three T-cell lymphoma cell lines (Jurkat, Karpass299 and Myla3676 cells) by reducing the levels of STAT3 phosphorylation and suppressor of cytokine signalling 3 (SOCS3) (38). These studies indicate the cytotoxic effect of GANT61 in multiple cancers. As for its role in enhancing drug sensitivity, it has been reported that GANT61 increases the response of prostate cancer cells to ionizing radiation both *in vitro* and *in vivo* (21). Another study revealed that the combination of phospholipase Cε-knockdown and GANT61 administration in castration-resistant prostate cancer cells enhanced their sensitivity to enzalutamide via inhibition of the androgen receptor pathway (39). Collectively, these findings indicate that in combination with other antitumour drugs, GANT61 can sensitize carcinomas to various treatment types.

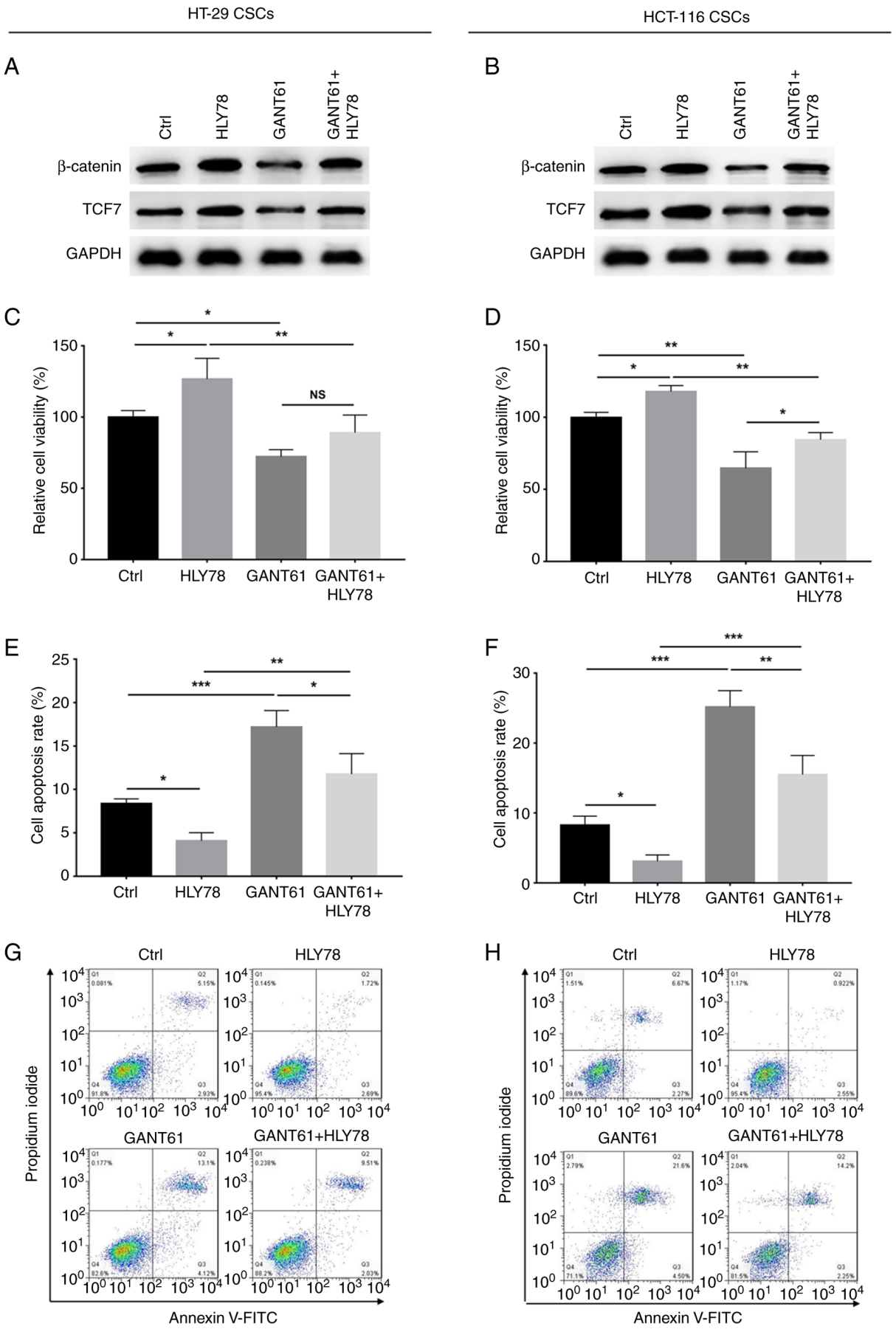

Figure 5. HLY78 enhances viability, while reducing apoptosis in GANT61-treated colorectal cancer CSCs. β-catenin and TCF7 protein expression levels in (A) HT-29 and (B) HCT-116 cells; relative viability of (C) HT-29 and (D) HCT-116 cells in the Ctrl, HLY78, GANT61 and GANT61+HLY78 groups. (E-H) Apoptosis of cells in the different groups. Apoptosis rate of (E) HT-29 and (F) HCT-116 cells and representative flow cytometry dot plots for (G) HT-29 and (H) HCT-116 cells. *P<0.05, **P<0.01 and ***P<0.001. CSC, cancer stem cells; TCF7, transcription factor 7; Ctrl, control; NS, not significant.

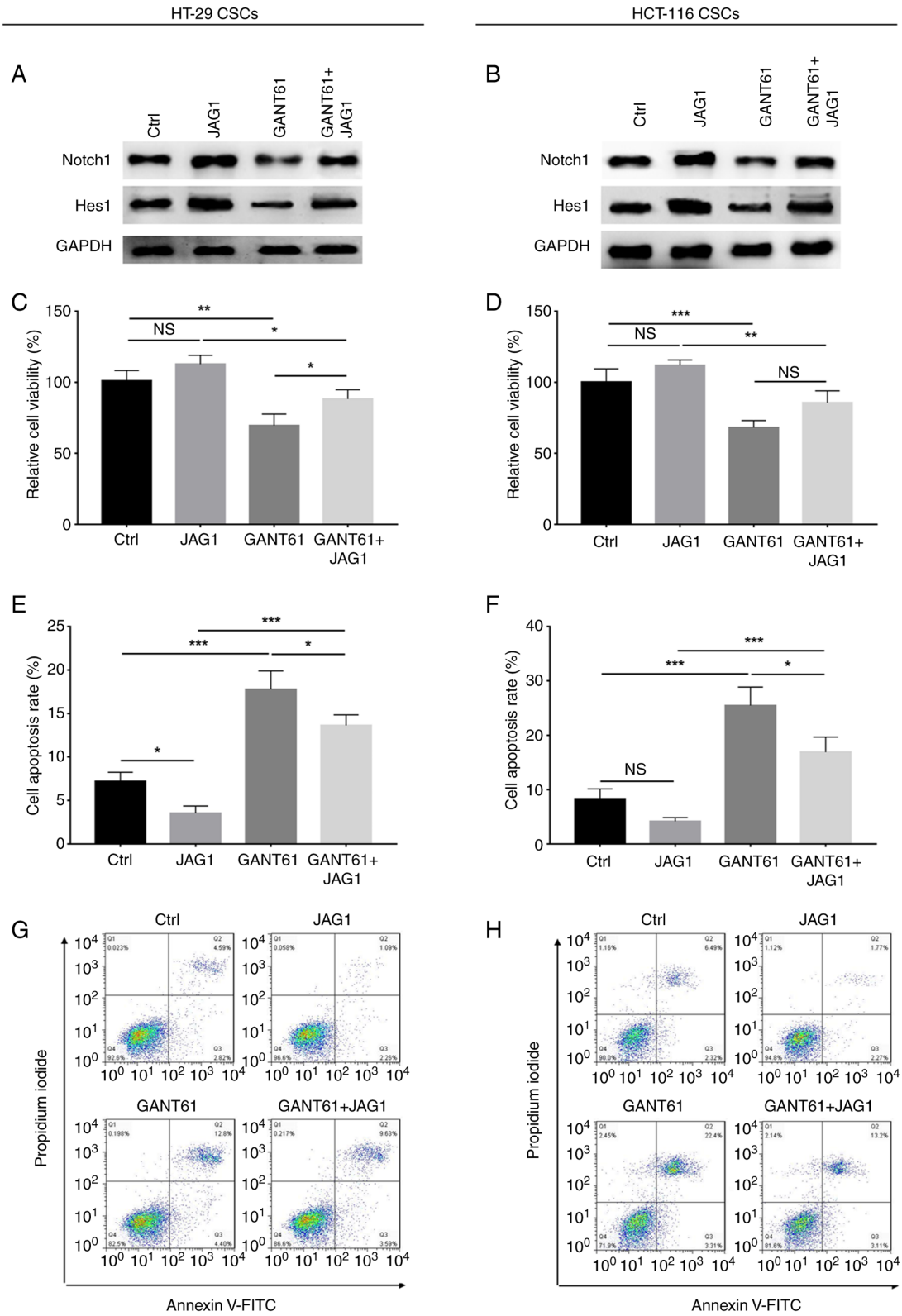

Figure 6. JAG1 promotes viability while supressing apoptosis in GANT61-treated colorectal cancer CSCs. Protein expression levels of Notch1 and Hes1 in (A) HT-29 and (B) HCT-116 cells; relative viability of (C) HT-29 and (D) HCT-116 cells in the Ctrl, JAG1, GANT61 GANT61+JAG1 groups. (E-H) Apoptosis of cells in the different groups. Apoptosis rate of (E) HT-29 and (F) HCT-116 cells and representative flow cytometry dot plots for (G) HT-29 and (H) HCT-116 cells. *P<0.05, **P<0.01 and ***P<0.001. CSC, cancer stem cell; Ctrl, control; NS, not significant.

Compared with the present study, these previous studies were primarily conducted in cancers other than CRC; the present study revealed that GANT61 reduced CRC cell viability, β-catenin and Notch1 protein expression levels, and the percentage of $CD133^{+}$ CRC cells, in a dose-dependent manner. Some possible explanations for these results are as follows: i) GANT61 potentially inhibited the malignant behaviour of CRC cells, including decreasing viability and

stemness, and enhancing apoptosis by targeting Gli1, which subsequently reduced the Hh pathway activity involved in the promotion of CRC pathology (14,34,35); ii) GANT61 may also have regulated the functions and stemness of CRC cells by mediating p-STAT3, SOCS3 or other related factors, as reported in previous studies (21,22,37-42); and iii) in later experiments, it was shown that GANT61 decreased the relative viability of CRC cells, while enhancing apoptosis by regulating the Wnt/β-catenin and Notch signalling pathways, which could provide another explanation of the current results.

Several pathways have been implicated in the regulation of CRC pathogenesis, including those assessed in the current study, the Wnt/β-catenin and Notch signalling pathways. With regards to Wnt/β-catenin, a study reported that *Fusobacterium nucleatum* promotes the progression of CRC by enhancing annexin A1, which is a Wnt/β-catenin modulator (43). It has also been reported that MEK inhibitors, including those of MEK1 and 2, activate Wnt signalling and increase the plasticity of CSCs (44). Furthermore, the epithelial Notch signalling pathway was found to be associated with a poor-prognosis subtype and metastasis in CRC, due to its ability to rewire the tumour microenvironment *in vitro* (including regulating multiple processes, such as neutrophil recruitment) (45). Given the aforementioned findings, and those of the present study suggesting that GANT61 regulates Wnt/β-catenin and Notch signalling pathways in CRC cells, rescue experiments when then performed, which indicated that GANT61 induced cytotoxicity in CRC cells by downregulating the Wnt/β-catenin and Notch signalling pathways. These results suggest a potential therapeutic mechanism of GANT61 in the treatment of CRC.

As for the modulatory role of GANT61 in CSCs, a study revealed that GANT61 and GDC-0449 (a SMO receptor inhibitor of the Hh pathway) elevate apoptosis in prostate CSCs by repressing the GLI family of transcription factors in a direct or indirect manner; moreover, GANT61 induced apoptosis in prostate CSCs more effectively than GCD-0449 (42). Additionally, in CRC organoid culture, GANT61 treatment suppressed the expression of stem cell markers, including c-Myc, CD44 and Nanog, possibly by inhibiting the expression of its transcription factor Gli1 (14). Another study revealed that combining GANT61 administration with mTOR suppression markedly reduces the proportion of CSCs among pancreatic cancer cells (46). Collectively, these findings indicate that GANT61 inhibits stemness and diminishes CSC numbers in multiple carcinomas.

Studies have also revealed that the Notch pathways are involved in the regulation of stemness in CRC and CRC-CSCs. For instance, β-catenin interacts with Tribbles homolog 3 and TCF4 to increase CSC-related gene expression in intestinal cells (47). In addition, another study reported that inhibition of the Notch signalling pathway with a γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) resulted in a killing effect on CRC-CSCs (48). In consideration of the cytotoxic effect of GANT61 on CSCs found in other cancers, and the impact of Wnt/β-catenin and Notch signalling pathways on CRC-CSCs discovered in other studies and in the present study (where GANT61 presented with anticancer ability by blocking Wnt/β-catenin and Notch signalling pathways in CRC), we hypothesized that GANT61 may also effectively destroy CRC-CSCs via the Wnt/β-catenin and Notch signalling pathways. Therefore, further rescue experiments were performed, and the results showed that GANT61 demonstrated good cytotoxicity in CRC-CSCs by blocking the Wnt/β-catenin and Notch signalling pathways. Possible explanations for these findings include the following: GANT61 might inhibit its target Gli1 (as reported in studies conducted in other cancers), which subsequently blocks the Wnt/β-catenin and Notch signalling pathways, and then represses stemness in CRC cells, reduces viability, but also enhances apoptosis of CRC-CSCs (21,22,37-42). To further clarify these conclusions, *in vivo* experiments may be required to validate the present study findings; however, due to insufficient financial support and limited lab conditions, these *in vivo* experiments were not performed.

In conclusion, the Hh signalling pathway/Gli1 inhibitor, GANT61, effectively eliminates cancer cells and CSCs by blocking the Wnt/β-catenin and Notch signalling pathways in CRC. These findings suggest that GANT61 may serve as a potential treatment option for patients with CRC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HT conceived and designed the experiments, analysed the data and revised the manuscript. YS and LL collected and analysed the data. WZ and XL performed data analysis and provided interpretation. QL provided technical support and analysed and interpreted the results. BL critically revised the article and interpreted the data. YS and HT confirm the authenticity of all the raw data. All authors approved the final version of the article.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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