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Abteilung für Allgemein-, Viszeral-, Gefäß- und Transplantationschirurgie, Universitätsmedizin

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Supervisor: PD. Dr. Michael Linnebacher

Immunoglobulin G mediates chemoresistance to oxaliplatin in colon cancer cells by inhibiting the ERK signal transduction pathway

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Submitted by Yuru Shang

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Reviewers

Reviewer#1

PD. Dr. rer. nat. Dietmar Zechner, Universitätsmedizin Rostock, Rudolf-Zenker-Institut für Experimentelle Chirurgie

Reviewer#2

PD. Dr. med. Armin Wiegering, Universitätsklinikum Würzburg, Klinik für Allgemein-, Viszeral-, Transplantations-, Gefäß- und Kinder-chirurgie

Reviewer#3

PD Dr. rer. nat. Michael Linnebacher, Universitätsmedizin Rostock, Abteilung für Allgemein-, Viszeral-, Gefäß- und Transplantationschirurgie

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List of abbreviations

CEA	Carcinoembryonic antigen
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementing group 1
ERKs	Extracellular-signal-regulated kinases
FBS	Filtrated bovine serum
5-FU	Fluorouracil
LV	leucovorin
IgG	Immunoglobulin G
IRI	Irinotecan
MAPK	Mitogen-activated protein kinase
OXA	Oxaliplatin
PBS	Phosphate buffered solution
PI	Propidium iodide
RNA	Ribonucleic acid
SD	Standard derivation
SDS	Sodium dodecyl sulfate

Abstract

Background

Intravenous human immunoglobulin G (IgG) has been a standard therapy for some inflammatory and autoimmune diseases. Recent evidence demonstrates that intravenous IgG can impair cancer cells. However, some evidence prove that cancer cells can produce and secret IgG, and the tumor cell-derived IgG promotes tumor initiation. This suggests that there might be an unrecognized effect of IgG on cancer cells. In addition, there is no study investigating whether or not IgG application benefits cancer patients receiving chemotherapeutics. Thus, the aim of this study was to investigate the effect of therapeutically used IgG on colon cancer in the presence of oxaliplatin, a tranditional therapy of colon cancer.

Methods

A series of low-passage patient-derived colon cancer cell lines and patient-derived xenograft carrying mice were treated with IgG (PRIVIGEN®) and oxaliplatin. The cell viability and tumor volume were evaluated, respectively. In order to evaluate cell death, cells were stained by Annexin V and propidium iodide and flow cytometry cell sorting analyses were performed. In addition, to evaluate tumor cell-derived IgG, colon cancer cells were stained by goat anti-human IgG antibody, the levels of cell-derived IgG were determined by flow cytometry, and the activity of ERK-signaling was evaluated by western blot.

Results

The present study demonstrated that human normal IgG (PRIVIGEN®) failed to significantly influence tumor cell viability and tumor growth; however, it significantly inhibited the cytotoxic activity of oxaliplatin *in vitro* but fail to significantly impair the anti-tumor effect of oxaliplatin *in vivo.* In addition, this study proved that colon cancer cells produced IgG and the levels of tumor

cell-derived IgG were positively correlated with oxaliplatin resistance. When evaluating the ERK activity, this study proved that oxaliplatin induced the activation of ERK; however, PRIVIGEN® IgG inhibits ERK activity. Interestingly, inhibition of ERK signaling by PD98059 and U0126, two traditional inhibitors of the ERK pathway, also reduced oxaliplatin-induced cell death.

Conclusions

The present study demonstrates that IgG impairs the anti-cancerous effect of oxaliplatin and this might dependent on the interaction between IgG and ERK: IgG inhibits ERK activity. Thus, the present study implies that application of therapeutical IgG to treating patients, which are receiving oxaliplatin, might have harmful side effects.

1. Introduction

1.1 Colon cancer

1.1.1 Epidemiology

Following lung cancer (11.6% of the total cases), female breast cancer (11.6%), and prostate cancer (7.1%), colorectal cancer (6.1%) is the fourth common cancer in the world [1]. Interestingly, it has been demonstrated that the incidence of colorectal cancer varies widely worldwide (Fig. 1). For example, Arnold et al. reported that in 2012, the estimated incidence of colorectal cancer in males varied from 1.5 per 100,000 in some African countries, such as Gambia and Mozambique, to over 40 per 100,000 in several countries in Europe and Asia, such as Slovakia (61.6 per 100,000), Hungary (58.9 per 100,000) and the Republic of Korea (58.7 per 100,000) [2]. In addition, the incidence and mortality of colorectal cancer is rising rapidly in many low-income and middle-income countries; however, it is stabilizing or decreasing in highly developed countries [2]. This might be due to the changes in lifestyle and dietary patterns in these areas [3, 4].

According to the UK national statistics, two-third of colorectal cancers are colon cancer [5, 6]. In contrast to the decreasing incidence of rectal cancer, the incidence of colon cancer increased during the last decades in a number of western countries, such as USA, Canada, Australia, New Zeeland, and European countries [5]. In addition, until 2035, the mortality of colon cancer in USA is predicted to increase from 1.8 per 100,000 to 2.3 per 100,000 and in UK, the mortality will increase from 4.2 per 100,000 to 4.9 per 100,000 [7].

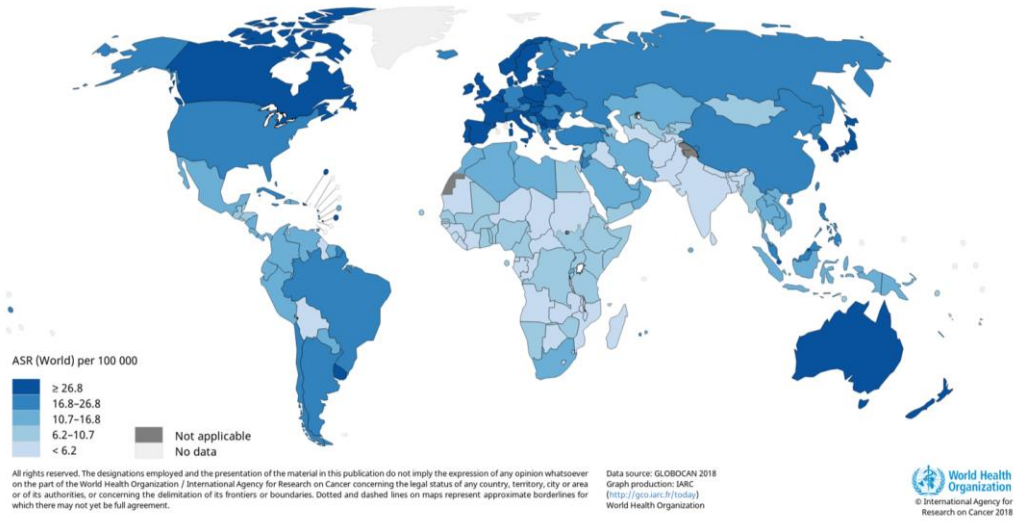

Fig. 1. Estimated age-standardized incidence rates of colon cancer in 2018, both sexes and all ages (https://gco.iarc.fr/). The incidence of colon cancer in Canada, Australia, and several countries in Europe is higher than that in African countries.

1.1.2 Mechanisms of colon cancer development

Several studies prove that some life style factors, such as smoking [8], dietary patterns [9], and lack of physical activity [10], are risk factors of colon cancer. For example, Cheng et al. investigated the relationship between cigarette smoking and the incidence of colon cancer; they observed that cigarette smoking was associated with an increased risk of colon cancer, and current smokers had a significantly higher risk of proximal colon cancer than distal colon cancer [8]. In addition, Kyu et al. systematic reviewed 19 studies, including 53,929,648 patients, and they observed that the risk of colon cancer among individuals in the low active, moderately active, and highly active categories was decreased by 10%, 17%, and 21%, respectively, when they were compared to insufficiently active individuals [10]. Unfortunately, the mechanisms, how these life style factors contribute to the development of colon cancer, is still unclear and genetic alteration might be involved [11, 12]. For example, Limsui et al demonstrated that smoking was

positively correlated with the mutation of *BRAF,* a master regulator of the initiation of colon cancer [13].

A large body of evidence proves that colon cancer is not a single disease but rather a collection of several distinct diseases with diverse molecular background and clinicopathological manifestations [14]. According to this theory, two pathways are invovled in the development of colon cancer: the adenoma-carcinoma pathway and the serrated neoplasia pathway [15].

Based on the concept of traditional adenoma-carcinoma pathway, colon cancer cells begin as a noncancerous growth and develop into a tubular adenomatous polyp. Subsequently, the tubular adenomatous polyp can evolve into an early adenoma, an advanced adenoma, and finally develops into colon cancer [16]. Usually, in this pathway, the colon cancer develops slowly. In addition, the adenoma-carcinoma pathway is characterized by chromosomal instability [17]. This is supported by the fact that the mutation of tumor suppressor genes, such as *p53* [18], and tumor-promoting genes, such as *KRAS* and *BRAF* [19], have been observed in the majority of early neoplastic lesions [20], and several studies have demonstrated that this is a master molecular and pathophysiological event in the initiation and formation of colon cancer [18-21].

Compared to the adenoma-carcinoma pathway, the serrated neoplasia pathway is a novel concept [22, 23]. According to this concept, colon cancer evolves from a subset of polyps, termed sessile serrated polyps, which account for about 5%-10% of all polyps [24]. Distinct from the adenoma-carcinoma pathway, tumors from the serrated neoplasia pathway are characterized by microsatellite and epigenetic instability [24]. Microsatellite instability is caused by the inability of DNA mismatch repair and it manifests as increase or decrease of the length of microsatellite repeats [25]. In addition, epigenetic instability of colon cancer is characterized by aberrant DNA methylation [15].

1.1.3 Diagnosis and chemotherapy

Colonoscopy and tissue biopsy is still the “gold standard” method for diagnosis of colon cancer, and the sensitivity of this method is about 94.7% (95% confidence interval, 90% and 97%) [26]. In addition, colonoscopy in combination with barium enema, computed tomography colonography and the level of carcinoembryonic antigen (CEA) might improve the accuracy of colonoscopy [26].

CEA is derived from endoderm, and it is the most commonly used tumor marker in the diagnosis of colon cancer [27, 28]. It has been reported that overexpression of CEA is occurring in more than 90% of colorectal cancer patients [29]. However, CEA also expresses in other tumors, such as gastric cancer [30, 31], lung cancer [32], and ovarian cancer [30, 33]. This limits the diagnostic accuracy of CEA.

Since early colon cancer usually has no symptoms, it is important to perform screening to detect the tumor. Usually, the individual risk statuses decide when and how start to screen [34]. For example, for people at an average risk for colon cancer, the screening should be offered at age 50 years by one of the following options: Fecal occult blood testing, sigmoidoscopy, fecal occult blood testing in combination with flexible sigmoidoscopy, colonoscopy or double-contrast barium enema [34]. In addition, people with a family history of colorectal cancer or hereditary nonpolyposis have a high risk of developing colon cancer [35]. These people should be suggested to be screened by colonoscopy at 10 years younger than the earliest diagnosis in their family [34]. In order to determine appropriate strategies for distinct patients, the tumor (T), node (N), metastasis (M) classification (Table 1) has been developed by Union for International Cancer Control [36, 37]. For patients without distant metastasis, such as Stage 0 and Stage I, surgical resection is the main or first-line treatment. However, for patients with metastasis, such as Stage

III and Stage IV, surgery is unlikely to cure these cancers and chemotherapy is the main treatment.

Table 1. UICC staging system of colon carcinoma (AJCC 8th edition)

UICC	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1-T2	N0	M0
Stage II	T3 or T4a or T4b	N0	M0
Stage IIIA	T1-T2 T1	N1/N1c N2a	M0 M0
Stage IIIB	T3-T4a T2-T3 T1-T2	N1/N1c N2a N2b	M0 M0 M0
Stage IIIC	T4a T3-T4a T4b	N2a N2b N1-N2	M0 M0 M0
Stage IV	any T	any N	M1a or M1b or M1c

Tis: carcinoma in situ, intramucosal carcinoma; T1: tumor grows through the muscularis mucosa but not invades through the muscularis propria; T2: tumor invades muscularis propria; T3: tumor grows through the muscularis propria and invades the pericolorectal tissues; T4a: tumor grows through the visceral peritoneum; T4b: tumor directly invades or adheres to other adjacent organs or structures; N0: no regional lymph node metastasis; N1: 1-3 regional lymph node metastases; N1a: 1 regional lymph node metastasis; N1b: 2-3 regional lymph node metastases; N1c: no regional lymph node metastasis but there are tumor deposits in the subserosa, mesentery or nonperitonealized pericolic or perirectal/mesorectal tissues; N2: 4 or more regional lymph nodes metastases; N2a: 4-6 regional lymph node metastases; N2b: 7 or more regional lymph node metastases; M1: no other site or organ metastases; M1a:metastasis in one organ or site without peritoneal metastasis; M1b: metastases in two or more sites

or organs without peritoneal metastasis; M1c: metastasis to the peritoneal surface alone or with other site or organ metastases [38].

1.2 Oxaliplatin

1.2.1 Platinum compounds

Platinum compounds and DNA can form platinum-DNA adducts and inhibit DNA synthesis and repair. In 1970s, it has been proven that cisplatin, the first-generation of platinum compound, could impair cancer cells [39]. Subsequently, around 3,000 platinum derivatives were synthesized [39]. Unfortunately, only 30 compounds were tested in clinical trials and most of them failed to impair tumors in patients [39]. Today, only four compounds are approved and used clinically (Fig. 2): Cisplatin, the first generation compound; carboplatin and nedaplatin, the second-generation compounds; oxaliplatin, the third-generation platinum compound. In addition, some platinum complexes, such as triplatin tetranitrate, phenanthriplatin, picoplatin, and satraplatin are still under clinical investigation [39].

1.2.2 Mechanisms of the cytotoxic activity of oxaliplatin

Like other platinum derivatives, the cytotoxic effect of oxaliplatin (transl-L-1,2-diaminocyclohexane, $C_8H_{14}N_2O_4Pt$) is dependent on the platinum-DNA adduct (Fig. 2). However, compared to cisplatin, the amine groups are replaced by diaminocyclohexane (Fig. 2). This leads to a more stable binding between platinum and DNA [40]. Indeed, several preclinical studies proved that, compared to cisplatin, oxaliplatin is more effective in inhibiting DNA synthesis [41]. In addition, previous studies demonstrated that diaminocyclohexane enhances the cytotoxicity of oxaliplatin, and reduces cross-resistance between oxaliplatin and other platinum derivatives *in vitro*. Notably, the oxaliplatin-induced DNA damage can lead to the expression of excision repair cross complementation group 1 (ERCC1), which triggers cancer cell resistance to oxaliplatin [42, 43].

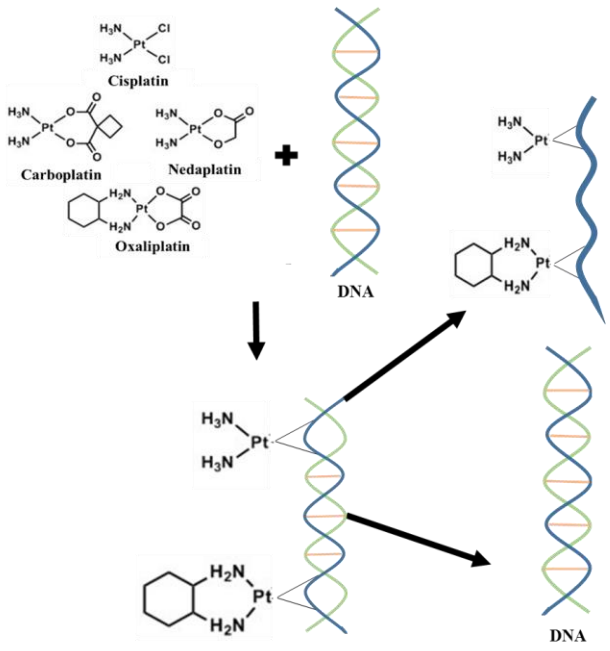

Fig. 2. Platinum leads to DNA damage. Platinum compounds, such as cisplatin, carboplatin, nedaplatin and oxaliplatin, can bind to strands of DNA and form a platinum-DNA adducts. This can impair the replication of DNA.

Several studies proved that DNA damage is not the sole mechanism of oxaliplatin cytotoxicity [44, 45]. For example, Santoro et al. reported that oxaliplatin could activate p38 mitogen-activated protein kinase (p38); a master protein of the mitogen-activated protein kinases (MAPKs) pathway and inhibition of p38 activity significantly impairs the toxic effects of oxaliplatin. Interesting, the same research group observed that oxaliplatin can induce the activity of extracellular signal-regulated kinases 1 and 2 (ERK), another master protein of MAPKs pathway. Surprisingly, inhibition of ERK significantly enhances the cytotoxicity of oxaliplatin. This suggests that MAPKs pathway has ambivalent functions in regulating oxaliplatin cytotoxicity.

1.3 Fluorouracil

Fluorouracil (5-FU), an analogue of uracil, was synthesized by Duschinsky et al. in 1957 [46]. Various mechanisms, which contribute to the cytotoxic effect of 5-FU, have been reported [47]. For example, 5-fluoro-deoxyuridine-monophosphate (5-FdUMP), a metabolite of 5-FU, can form a complex with thymidylate synthase and inhibit the synthesis of DNA [48]. 5-fluoro-deoxyuridinetriphosphate (5-FdUTP), another metabolite of 5-FU, incorporates into DNA and impairs DNA stability [49]. In addition, 5-FU can also be transformed into 5-fluorouridine-triphosphate (5-FUTP), which incorporates into RNA instead of uridine triphosphate (UTP) and inhibits the synthesis of RNA [50].

Although numerous preclinical studies proved that 5-FU is a promising chemotherapy for several types of cancers, such as colon cancer [51], breast cancer [52]and pancreatic cancer [53], there are some serious adverse events of high dose 5-FU, such as central nervous system neutropenia

and febrile neutropenia [54, 55]. Thus, to reduce the dose, 5-FU is clinically given in combination with other chemotherapeutics, such as irinotecan and oxaliplatin [56].

1.4 Irinotecan

Irinotecan and topotecan are camptothecin analogues, and irinotecan can be hepatically metabolized and converted to SN-38 which is 1,000 times more active than irinotecan itself [57]. Subsequently, SN-38 inhibits the activity of topoisomerase I and leads to breaks in both DNA strands and subsequently causes cell death. It has been reported that irinotecan can impair several cancers, such as colon cancer, lung cancer, and gastric cancer [58-60]. In addition, irinotecan in combination with leucovorin and fluorouracil (FOLFIRI) has been clinically used to treat colon cancer [61].

1.5 Treatment of cancer patients with immunoglobulin G

Immunoglobulin G (IgG) is produced and released by B-lymphocytes and it is a master regulator of humoral immunity [62]. IgG is composed of four peptide chains, two light chains and two heavy chains. The heavy chains link to each other by disulfide bonds and each of them binds to a light chain by another disulfide bond (Fig. 3) [63]. In addition, the heavy chains and the light chains can be divided into variable (V) region and constant (C) region. The variable domains of the heavy (VH) and light (VL) chains make up the variable regions of IgG and together form the paratope. This allows for IgG to specifically bind to antigenic epitopes.

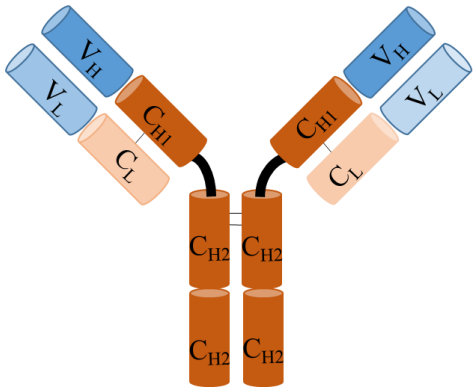

Fig. 3. Structure of IgG. IgG is composed of four peptide chains, two light (L) chains and two heavy (H) chains. Each chain can be divided into variable (V) region and constant (C) region. The light chain (C_L) binds to heavy chain (C_{H1}) by a disulfide bond and the two heavy chains (C_{H2}) bind to each other via two disulfide bonds.

It is a well-accepted concept that intravenous human normal IgG is helpful to treat inflammatory diseases [64, 65]. In addition, cytotoxic chemotherapy impairs the immune system and leads to severe immune deficiency in some cancer patients [66]. Thus, in order to improve the immunity of cancer patients receiving chemotherapeutics, intravenous human normal IgG might be a promising strategy. Interestingly, some preclinical studies proved that IgG can inhibit cell proliferation and induce cell death [67, 68]. Moreover, Shoenfeld and Fishman evaluated the anti-cancerous effect of IgG by several experimental animal models, such as inoculation of melanoma or sarcoma cells to naive mice, inoculation of melanoma cells to the footpad, and inoculation of human tumor cells to severe combined immunodeficient mice. They observed that IgG significantly decreased metastatic lung foci and prolonged survival [69]. However, there is no study which evaluated if and how IgG regulates the cytotoxic effect of chemotherapy.

1.6 The aim of this dissertation

The purpose of this study was to evaluate if intravenous human normal IgG regulates the viability of colon cancer cells. In addition, this study investigated the effects of IgG in combination with 5-FU, irinotecan or oxaliplatin; three clinically used chemotherapeutic agents of colon cancer.

2. Material and Methods

2.1 Patient-derived colon carcinoma cell lines

The fresh colon tumor tissues were obtained from colon cancer patients who underwent surgical resection and signed the written informed consent. This was approved by the Ethics Committee of the University of Rostock (reference number II HV 43/2004) in accordance with the declaration of Helsinki.

The details of the processes have been described in detail in a previous study [70]. 3 × 3 × 3mm tumor tissue(s) was implanted into one or both sides of the flank(s) of immunodeficient mice, such as NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ and NMRI-Foxn1^{nu} mice. The surveillance of the tumor growth was started 15 days after the implantation. Mice were euthanized when the volumes of tumors reached 500-1,000 mm^3, and the tumor tissues were stored at -80°C. In order to develop colon carcinoma cell lines, the fresh human colon tumor tissues or the patient-derived colon carcinoma tissues were dissected into pieces and passed through a cell strainer [71]. Primary cell lines, which directly established from human fresh tumor tissues, are indicated with the prefix HROC (Hansestadt Rostock colorectal cancer) and the ID number of the patient, such as HROC370. Cell lines, which were derived from patient derived xenograft (PDX), are denoted

with additional indices. For example, Met indicates metastatic tumor, T indicating the times of transfer in an immunodeficient mouse, and M indicates the ID number of mice, such as HROC278Met T2 M2.

HROC18, HROC46 T0 M1 (HROC46), HROC50 T1 M5 (HROC50), HROC87 T0 M2 (HROC87), HROC131 T0 M3 (HROC131), HROC147Met (HROC147), HROC277 T0 M1 (HROC277), HROC277Met2, HROC278Met T2 M2 (HROC278), HROC285 T0 M2 (HROC285), HROC370, HROC374 were cultured in DMEM/F12 (1:1) (PAN-Biotech, Aidenbach, Germany, code P04-41500) and supplemented with 10% fetal bovine serum (FBS, PAN-Biotech, code P40-47500) at 37 °C in a humidified atmosphere of 5% CO_2.

In order to determinate appropriate amounts of cells for each experiment, the colon cancer cells were washed by phosphate buffer saline (PBS, PAN-Biotech, code P04-53500). Subsequently these cells were trypsinized by 1mL 0.025% trypsin/EDTA (trypsin, PAN-Biotech, code P10-023100) at 37 °C for 5-10 minutes, and the activity of trypsin was blocked by 3-4mL medium. The appropriate amount of cells were determined with the help of a Neubauer chamber (Thermo Fisher Scientific,Waltham, USA). In addition, the materials used for cell culture were summarized in Table 2.

Table 2. Material for cell culture

Devices		
Product	**Company**	**Headquarters**
Centrifuge 5415 D	Eppendorf	Germany
Cryo Freezing Container: Mr. Frosty™	Thermo Scientific	USA
Pasteur IPS Type A Miroplate Incubator	Bio-Rad	Germany
Incubator	Memmert GmbH+Co.KG	Germany
Laminar Flow MSC Advantage	Thermo Scientific	USA
Light microscope: Olympus CKX 41	Olympus Deutschland GmbH	Germany
Multistepper	Eppendorf	Germany
Neubauer - Counting chamber	Marienfeld	Germany
Pipetboy	Integra Bioscience	Germany
Pipettes	Eppendorf and Gilson	Germany
Regents		
Product	**Company**	**Headquarters**
Dimethyl sulfoxide	AppliChem	Germany
Isopropanol	Walter CMP	Germany
L-glutamine	Pan-Biotech	Germany
Trypan blue (0.02 %)	Sigma-Aldrich	Germany

2.2 Evaluation of the expression of IgG in colon cancer cells

In order to evaluate the accumulation of tumor-derived IgG in colon cancer cells, 4.0×10^5 HROC277, HROC285, HROC370, or HROC374 cells were transferred into a tube and centrifuged at 300× g for 8 minutes. Subsequently, the cells were fixed with 400µL 2% formafix (Grim MED. Logistik GmbH, Torgelow Germany code1117264) for 15 minutes and washed by 400µL 1× PBS (Table 3). Then these cells were incubated with 400µL 1× buffer P (Table 3) for 10 minutes and centrifuged at 300× g for 8 minutes, followed by incubated with 200µL 1× buffer

P, 5µL goat anti-human IgG-heavy and light chain antibody FITC conjugated (Bethyl Laboratories, Montgomery, USA, code A80-119F) and 4µL mouse anti-human CD19 PE-conjugated (ImmunoTools, Friesoythe, Germany, code 21270194) for 30 minutes. The fluorescent intensity of cells was measured by a BD FACSCalibur (Becton Dickinson, New Jersey, USA) and the data were analyzed by the software, BD CellQuest™ Pro (Becton Dickinson).

Table 3. Required components of phosphate buffer saline (10×) and buffer P (100×)

phosphate buffer saline (10×)		
Component	**Company**	**Branch establishment**
80g Sodium chloride	Carl Roth GmbH	Karlsruhe, Germany
2g Potassium chloride	MERCK	Darmstadt, Germany
18.05g Di-sodium hydrogen phospate dihydrate	MERCK	Darmstadt, Germany
1L Aqua dest	-	-
buffer P (100×)		
Component	**Company**	**Branch establishment**
0.5mL FBS	Pan-Biotech	Aidenbach, Germany
5mL 1% saponin	Carl Roth GmbH	Karlsruhe, Germany
5mL 0.1M HEPES	AppliChem	Darmstadt, Germany
39.5mL 1× PBS	-	-

2.3 Evaluation of cell viability

In order to evaluate if human normal IgG can regulate cell viability, 1.2×10^4 HROC18, HROC46, HROC50, HROC87, HROC131, HROC147, HROC277, HROC277Met2, HROC278, HROC285, HROC370, HROC374 cells per well were seeded in a 96 well plate. In addition, to investigate if the human normal IgG influences the cytotoxic effect of oxaliplatin, 1.2×10^4 HROC277,

HROC285, HROC370, and HROC374 cells per well were seeded in a 96 well microplate. After 24 hours, these cells were treated by Sham, PRIVIGEN® IgG, oxaliplatin, or the combination therapy, PRIVIGEN® IgG plus oxaliplatin, with the indicated concentrations given at each figure. After 5 days, these cells were washed by PBS and then stained by 0.2% crystal violet solution (Table 4) for 10 minutes, and the images were obtained with the help of a Plate scanner (Immunospot DC Suite Cellular Technology Ltd., Bonn, Germany). Subsequently, they were washed by PBS and incubated with 1% sodium dodecyl sulfate (SDS, Thermo Fisher Scientific,Waltham, USA, code 15525017) for 10 minutes and the optical density was measured at 570nm with the help of a Tecan Infinite 200 Microplate Reader (Tecan, Männedorf, Switzerland) and I-control 1.9 (Tecan Austria GmbH, Grödig, Austria).

Table 4. Required components of 0.2% crystal violet solution

Component	Company	Branch establishment
200mg Crystal violet	AppliChem	Darmstadt, Germany
2mL Ethanol absolute	Walter CMP	Kiel, Germany
100mL Aqua dest.	-	-

2.4 Evaluation of cell death

In order to evaluate cell death, 2.0 $\times 10^5$ HROC277, HROC285, HROC370, and HROC374 cells per well were plated in a 6 well plate and grown for 24 hours. Subsequently, these cells were treated by Sham, 5mg/mL PRIVIGEN® IgG, 6.25µM oxliaplatin, and the combinational therapy, PRIVIGEN® IgG plus oxaliplatin. After 48 hours, the supernatant was transferred to a FACS-tube and the cells were washed by 200µL PBS. After transferring the PBS into the tube, the

HROC277, HROC285, HROC370, and HROC374 cells were trypsinized by 200µL 0.025% trypsin at 37 °C. After 5-10 minutes, the cells were transferred into the FACS-tube and centrifuged at 300× g for 8 minutes. Then, the cells were washed with 500µL 1× binding buffer (Table 5) and centrifuged again. Re-suspension of cells was done with 70µL binding buffer, and staining with 5µL Annexin V-FITC (ImmunoTools, code 31490013) for 15 minutes at room temperature in the dark. Counter-staining of cells was done with 50µL propidium iodide (PI, PanReac AppliChem, Darmstadt, Germany, code A2261) and measuring intensity of Annexin V-FITC at an excitation and emission wavelength of 488nm and 519nm, respectively, with the help of the BD FACSCalibur (Becton Dickinson). The intensity of PI was measured at an excitation and emission wavelength of 488nm/615nm, respectively. The percentage of dead cells, 100% × (Annexin V^+ PI^- + Annexin V^+PI^+ + Annexin V^-PI^+) cells/total cells, was determined with the help of the BD FACSCalibur and BD CellQuest™ Pro. In order to evaluate if oxaliplatin-induced cell death was dependent on the activity of ERK, 2.0 $\times 10^5$ HROC277 and HROC285 cells were seeded in a 6 well plate. After 24 hours, these cells were treated by 50µM PD98059 (Cell Signaling, Massachusetts, USA, code 9900) or 10µM U0126 (Cell Signaling, code 9903S) for 1 hour. Subsequently, these cells were washed by PBS and treated by 6.25µM oxaliplatin for 48 hours, and the percentages of dead cells were determined as above-mentioned.

Table 5. Required components of binding buffer (10×)

Component	Company	Branch establishment
0.1M HEPES	AppliChem	Darmstadt, Germany
1.4M Sodium chloride	Carl Roth GmbH	Karlsruhe, Germany
25mM Calcium chloride dihydrate	MERCK	Darmstadt, Germany

2.5 Evaluation of cell cycle

In order to evaluate if IgG regulates cell cycle, 1.0×10^5 HROC18, HROC46, HROC50 or HROC87, HROC131, HROC147, HROC277, HROC277Met2, HROC278, HROC285, HROC370, HROC374 cells per well were seeded in a 24 well plate and grown for 24 hours. These cells were treated by Sham or 5mg/mL IgG for 48 hours. Similar to the process of measuring cell death, the supernatant and cells were transferred to a FACS-tube and were centrifuged. Subsequently, these cells were resuspended in 2mL 70% ethanol for 12 hours, and washed by 500µL 1× PBS twice. Then these cells were incubated by 400µL 0.1 % Tween 20 and 5µL 1mg/mL RNase (Sigma-Aldrich, St. Louis, Missouri, USA, code 10109134001) at room temperature. The nuclei were stained by 50µL 0.1mg/ml PI and the intensity of PI was measured at an excitation and emission wavelength of 488nm/615nm with the help of the BD FACSCalibur and the data were analyzed by ModFit LT v3.3 (Becton Dickinson).

2.6 Quantitative real-time PCR

5.0×10^5 HROC285 cells per well grown in a 6 well plate for 24 hours were treated by Sham, 5 mg/mL PRIVIGEN® IgG, 6.25µM oxaliplatin, and PRIVIGEN® IgG plus oxaliplatin for 1 hour and 24 hours. Subsequently, these cells were trypsinized and washed by 1× PBS. The isolation of RNA was performed following the manufacturer instructions of Universal RNA Purification Kit (EURx, Gdansk, Polska, code E3598). In short, cell pellets were re-suspended by 400µL buffer RL and 4µL β-mercaptoethanol (Carl Roth, Karlsruhe, Germany, code 4227.1), and were centrifuged for 3 minutes. Subsequently, the cell lysates were transferred into a fresh tube and mixed with 350µL 70% ethanol and were centrifuged for 3 minutes. Then the cell lysates were washed by 400µL Wash-RB1 buffer, 50µL DNR1, 1U DNase I buffer (Roche, Mannheim,

Germany, code 03539121103), 400µL Wash-RB1 buffer, and 40µL RNase free water (Carl Roth, code 154012146) as indicated in Fig. 4. The quality and quantity of RNA were evaluated by NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA) and RNAs were stored at -80 °C.

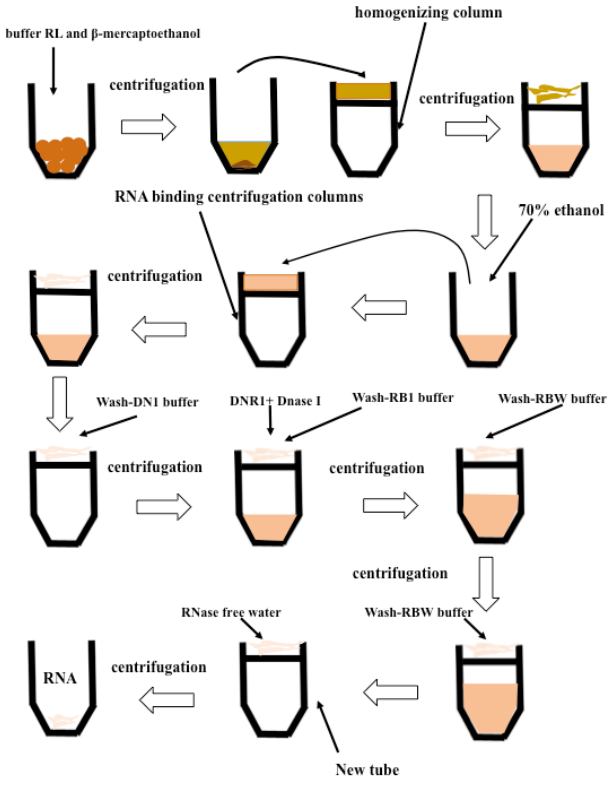

Fig. 4. The process of RNA isolation. The isolation of RNA was performed by the manufacturer instructions of Universal RNA Purification Kit. The cell pellets were thoroughly mixed with 400µL buffer RL with 4µL β-mercaptoethanol and centrifuged. The overlay was carefully transferred to the homogenizing column and centrifuged. The flow was added with 350µL 70% ethanol and mixed thoroughly by pipetting. Then transferred to RNA binding centrifugation columns and centrifuged. The flow was discarded and the column was returned to the collecting vessel then 400µL Wash-DN1 buffer was added before centrifugation. The flow was discarded and the column was returned to the collecting vessel. The column was incubated with 50µL buffer DNR1+1U DNase I for 10min then filled with 400µL buffer Wash-RB1 and centrifuged. Two more wash steps were carried out with 650µL Wash-RBW buffer and 350µL Wash-RBW buffer. The column was placed on a new collecting vessel and 40µL RNase free water were added to the middle of the column membrane and centrifuged. The column was discarded. The purified RNA in the collecting vessel was further analyzed.

In order to synthesize complementary DNA (cDNA), 1µg RNA was diluted by RNase free water, as indicated in Table 6, and incubated with primers at 70 °C for 10 min with the help of a Thermal Cycler (Bio-Rad Laboratories, Munich, Germany). The master mix for one reaction was prepared as following: 4µL 5× RT buffer complete (Bioron, Ludwigshafen, Germany code: 105100), 1µL 10 mM dNTP Mix (Bioron, code: U1511) and 1µL Reverase™ transcriptase (Bioron, code 105100). The synthesis reaction of cDNA was performed in the presence of 14µL diluted RNA and 6µL master mix at 45 °C for 120 minutes and 70 °C for 10 minutes. Finally, the cDNA was diluted by adding 60µL of RNase free water.

Table 6. Dilution of RNA

Component	**Volume (µL)**
mRNA (1µg)	X
Oligo(dT)15 Primer and random primer (Metabion, Planegg, Germany)	1.0
RNase free water	14-X-1
Total volume	**14**

In order to perform PCR, the master mix (Table 7) plus 2µL cDNA or 2µL nuclease free water (negative control) was transferred into a 96 hard shall PCR-plate. Subsequently, the PCR reaction was run with the help of Bio-Rad Real-Time PCR System with the following conditions: 95 °C and 10 minutes for hold stage; 95 °C for 15 seconds and60°C for 30 seconds, 40 cycles for the PCR stage. Relative quantification analysis was performed by the comparative ΔCT method

(ΔΔCT Method) with following formulas: (1) ΔCT = CT $_{\text{target gene}}$ - CT $_{\beta\text{-actin}}$; (2) ΔΔCT = ΔCT $_{\text{treatment}}$ - ΔCT $_{\text{control}}$; (3) Target gene expression of treated cells = $2^{(-\Delta\Delta CT)}$ [72].

Table 7. Master mix for quantitative real-time PCR

Component	Volume (µL)
SibirRoxHot MasterMix (Bioron, Ludwigshafen, Germany)	6,25
Forward primer (10 pmol/µL, Metabion, Planegg, Germany)	0.625
Reverse primer (10 pmol/µL, Metabion, Planegg, Germany)	0.625
Nuclease free water (EUR x, Gdansk, Poland)	3
Total volume	**10.5**

2.7 Western blot

In order to evaluate the accumulation of ERCC1 and the activity of ERK and p38, 5.0×10^5 HROC285 or HROC277 cells were seeded in a 6 well plate. After 24 hours, these cells were treated by Sham, 5 mg/ml PRIVIGEN® IgG, 6.25µM oxaliplatin, or the combination therapy, PRIVIGEN® IgG plus oxaliplatin. After 24 hours or 48 hours, the HROC285 and HROC277 cells were lysed by 150µL lysis buffer. Western blots were performed as previously described [73]. The cell lysates were separated on 12% SDS polyacrylamid gels and the proteins were transferred to polyvinyldifluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). In addition, 2.5% (wt/vol.) BSA was used to block the membranes and they were incubated overnight at 4°C with following primary antibodies: mouse anti-ERCC1 (Santa Cruz Biotechnology, Texas, USA, code sc-17809, dilution: 100×), mouse anti-ERK (R&D Systems, Wiesbaden, Germany, code MAB15761, dilution: 500×), rabbit anti-phospho-ERK (p-ERK, R&D Systems, code MAB1018,

dilution: 1,000×), rabbit anti-p38 MAPK (Cell Signaling, code 9212, dilution: 1,000×), rabbit anti-phospho-p38 MAPK (p-p38, Cell Signaling, code 9211, dilution: 1,000×), followed by the secondary antibodies: peroxidase-linked anti-rabbit-antibody (Cell Signaling, code 7074, dilution: 5,000-10,000×) or a peroxidase-linked anti-mouse antibody (Sigma-Aldrich, code A9044, dilution: 20,000-60,000×). In order to evaluate the accumulation of β-actin, all membranes were stripped and blocked by 2.5% (wt/vol.) BSA, followed by the incubation with mouse anti-β-actin antibody (Sigma-Aldrich, code A5441, dilution 1: 20,000×). Subsequently, the membranes were incubated by peroxidase-linked anti-mouse antibody (Sigma-Aldrich, code A9044, dilution 1: 60,000×). The accumulation of proteins was visualized by luminol-enhanced chemiluminescence (ECL plus; GE Healthcare, Munich, Germany) and was digitalized with Chemi-Doc XRS System (Bio-Rad).

2.8 Evaluation of anti-cancerous effect of IgG and oxaliplatin *in vivo*

The *in vivo* experiments were performed in accordance with the European Directive (2010/63/EU), and this procedure was approved by the local animal care committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, permit number: LALLF M-V/TSD/7221.3-1-005/17). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice were bred in a local animal facility (Rostock University Medical Center, Rostock, Germany) and kept on water and standard laboratory chow *ad libitum*. The 6-8-week-old mice were anesthetized by intraperitoneal injection of narcotics (xylazine/ketamine 90/60mg/kg body weight) and the eyes were protected by eye ointment. After absent of kicking response, a RFID transponder was injected into the nuchal fold of mice and the body temperature of mice was kept at 38°C by a heating plate. Subsequently, a 0.5cm incision was performed on the right flank side of the mice

and a 3 × 3 × 3mm piece of tumor tissue was implanted. The incision was closed by two surgical knots and was sterilized by povidone-iodine solution. Subsequently, the mice recovered in a fresh cage and were warmed by an infrared lamp. In order to protect the mice from potential bacterial contaminations, antibiotic water was supplied to these mice for 6 weeks.

After two weeks, the mice were started to be surveilled twice a week, and the diameter of tumors was determined, as indicated in Fig. 5, with the help of a caliper square. The volume of the tumor was calculated by the following formula: $\pi/6 \times [(\text{large diameter} + \text{small diameter})/2]^3$ and the volume change were calculated by using the volume of tumor on day 0 (Fig. 5) as a reference for the respective cohort. When the tumor grew to 33.3-50.6mm^3, the mice were injected with 0.9% Nacl (Sham), PRIVIGEN® IgG (sc), oxaliplatin (ip), or the combination therapy, PRIVIGEN® IgG plus oxaliplatin, as indicated in Fig. 5. Mice were euthanized when the tumor reached 1,426mm^3 or when they lost 20 % of the body weight. The tumor was cut into two pieces, one piece was preserved in 4% formafix and the other piece was sap-frozen and cryo-conserved.

2.9 Data presentation and statistical analysis

Data were presented as mean ± standard deviation (SD) or box plot. The distribution of data was evaluated by Shapiro-Wilk normality test, and the variance was determined by Levene median equal variance test. In order to determine the significance of differences, the data, which are normal distributed and equal in variance, were evaluated by two-way repeated measures analysis of variance with Holm-Sidak's post hoc test. Otherwise, the data were analyzed by Mann-Whitney U test and followed by the Bonferroni correction [74]. In addition, the inhibitory concentration (IC_{50}) of oxaliplatin was determined by GraphPad Prism 8 (GraphPad Software, San Diego, California, USA) and the correlation between cancer cell-derived IgG and IC_{50} was

determined by Pearson's correlation coefficient. All statistics were performed by Sigmaplot 12.0 (Systat Software, San Jose, CA, USA).

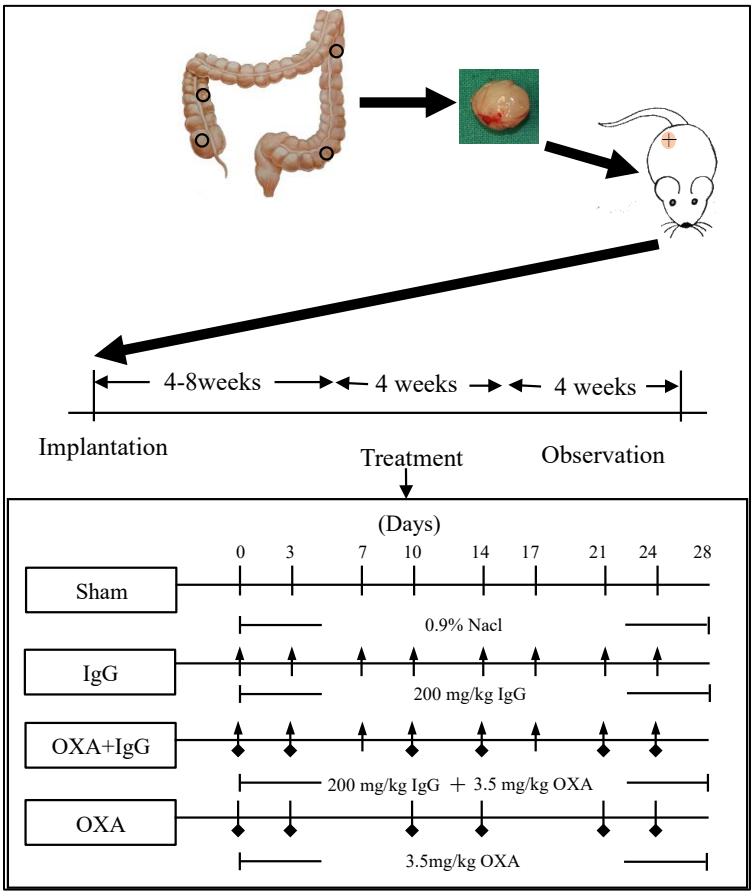

Fig. 5. Scheme of *in vivo* experiment. The treatments were started when the tumor grew to 33.3-50.6mm^3. The distinct mouse cohorts were either Sham treated with 0.9% Nacl (ip) for 8 times in 4 weeks, with oxaliplatin (3.5mg/kg, ip) for 6 times in 4 weeks, with IgG (200mg/kg, sc) for 8 times in 4 weeks or the combinational therapy, IgG plus oxaliplatin. The mice were followed up for 4 weeks.

3. Results

3.1 Clinical and pathological characteristics of patients

In order to evaluate whether or not PRIVIGEN® IgG application benefits colon cancer patients, low-passage individual cell lines from 11 colon cancer patients were used in this study, and all these patients underwent surgery in our clinic. The mean age of these patients was 66.00 years ± 14.04 years. Tumors were observed in cecum, ascending, descending, and sigmoid colon, and were all diagnosed as adenocarcinoma. In addition, the tumors of HROC18 and HROC370 were classified as UICC stage I; tumors of HROC50 and HROC87 were classified as UICC stage II; tumors of HROC131 and HROC374 were classified as UICC stage III; tumors of HROC46, HROC147, HROC277, HROC 278, HROC285 were classified as UICC stage IV.

3.2 PRIVIGEN® IgG does not significantly influent cell viability

In order to evaluate if PRIVIGEN® IgG has an effect on the viability of colon cancer cells, HROC18, HROC46, HROC50, HROC87, HROC131, HROC147, HROC277, HROC278, HROC285, HROC370, HROC374 cells were cultured in nutrition-rich (10% FBS) or starved (0% FBS) conditions (Fig. 6) and treated by Sham, 0.10mg/mL, 1mg/mL, and 10mg/mL PRIVIGEN® IgG for 5 days. This study proved that PRIVIGEN® IgG does not significantly (all $P > 0.05$) influent cell viability (Fig. 6).

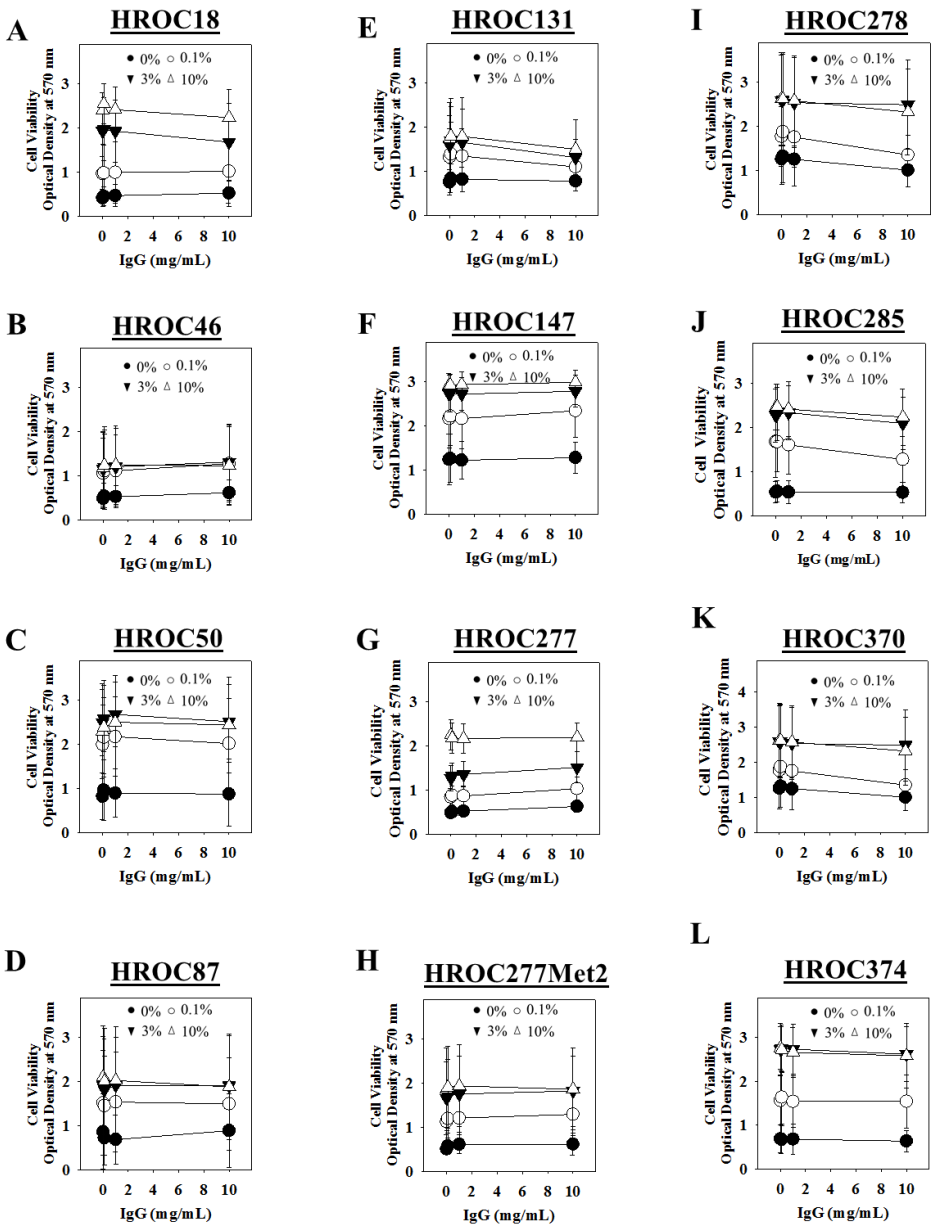

Fig. 6. PRIVIGEN® IgG fails to have a significant influence on the viability of colon cancers. The HROC18 (A), HROC46 (B), HROC50 (C), HROC87 (D), HROC131 (E), HROC147 (F), HROC277 (G), HROC277Met2 (H), HROC278 (I) HROC285 (J), HROC370 (K), HROC374 (L) cells were cultured in medium with different concentrations of FBS and treated by Sham, 0.10mg/mL; 1mg/mL, and 10mg/mL IgG for 5 days. PRIVIGEN® IgG did not significantly regulate the viability of these cells. n=5 for A and F; n=7 for B, G, H, I; n=6 for C, E, K, L; n=4 for D; n=8 for J.

3.3 PRIVIGEN® IgG does not significantly regulate the cell cycles

In order to evaluate if IgG regulates the cell cycle, HROC18, HROC46, HROC50, HROC87, HROC131, HROC147, HROC277, HROC277Met2, HROC278, HROC285, HROC370, HROC374 were cultured in 5% FBS medium and treated with 5mg/mL IgG for 2 days. IgG did not significantly (all $P > 0.05$) regulate the G_0/G_1, G_2/M and S phases, when compared to Sham-treated cells (Fig. 7).

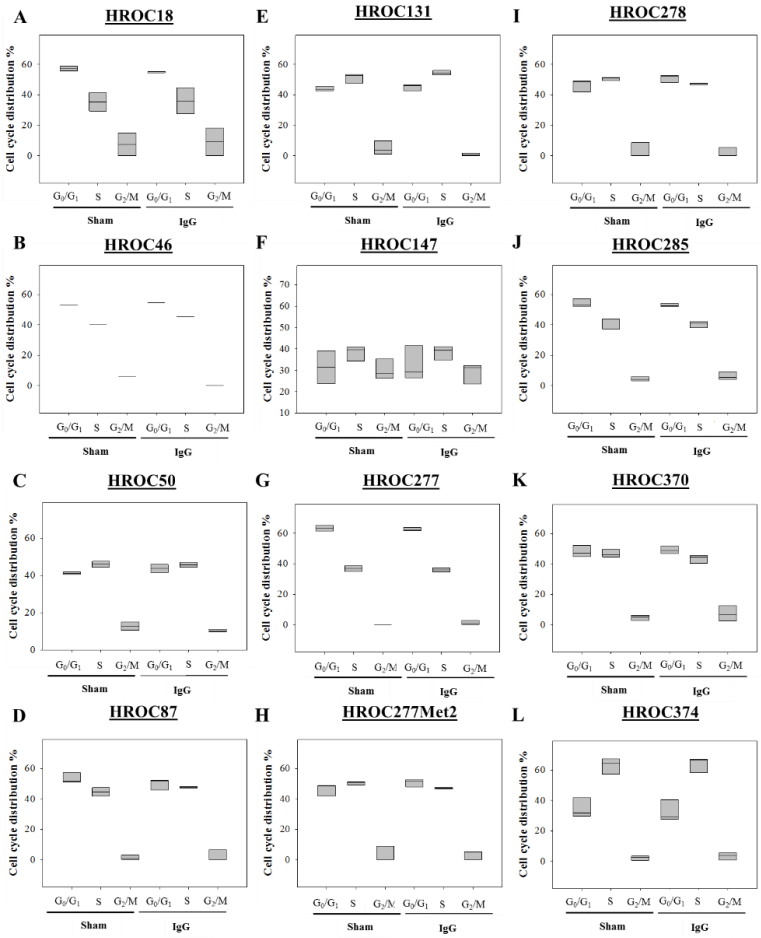

Fig. 7. PRIVIGEN® IgG fails to regulate the cell cycle. The HROC18 (A), HROC46 (B), HROC50 (C), HROC87 (D), HROC131 (E), HROC147 (F), HROC277 (G), HROC277Met2 (H), HROC278 (I) HROC285 (J), HROC370 (K), HROC374 (L) cells were cultured in 5% FBS medium and treated with 5mg/mL IgG for 2 days. n=2 for A and C; n=1 for B; n=3 for D-L.

3.4 PRIVIGEN® IgG cannot significantly regulate the anti-viability effect of 5-FU and irinotecan but significantly inhibits the anti-viability effect of oxaliplatin *in vitro*

In order to evaluate if IgG can influence the anti-cancerous effect of 5-FU or irinotecan, four cell lines, HROC277, HROC285, HROC370, and HROC374 cells were treated with Sham, PRIVIGEN® IgG, 5-FU, irinotecan, or the combinational therapies, IgG in combination with 5-FU or IgG in combination with irinotecan. The results demonstrated that 5-FU and irinotecan significantly ($P < 0.001$) inhibited the viability of HROC277 (Fig. 8), HROC285 (Fig. 9), HROC370 (Fig. 10), and HROC374 cells (Fig. 11), when compared with Sham-treated cells. However, 1.25mg/mL, 2.5mg/mL, and 5mg/mL IgG failed to significantly ($P > 0.05$) regulate the cell viability. In addition, IgG did not have significant ($P > 0.05$) influence on the anti-cancerous effect of 5-FU and irinotecan in HROC277 (Fig. 8), HROC285 (Fig. 9), HROC370 (Fig. 10), and HROC374 cells (Fig. 11).

In order to evaluate if IgG regulates the cytotoxcity of oxaliplatin, HROC277 (Fig. 12), HROC285 (Fig. 12), HROC370 (Fig. 13), and HROC374 (Fig. 13), were treated with PRIVIGEN® IgG and oxaliplatin. The results proved that 2.50μM and 6.25μM oxaliplatin significantly ($P < 0.001$) inhibited HROC277 cell viability, when compared to Sham-treated cells; however, 1.25mg/mL, 2.5mg/mL, and 5mg/mL IgG failed to significantly ($P > 0.05$) regulate the viability of HROC277 cells (Fig. 12). Interestingly, 2.5mg/mL, and 5mg/mL IgG significantly ($P < 0.001$) inhibited the anti-viability effect of oxaliplatin in HROC277 cells (Fig. 12). Very similar results were obtained with HROC285 (Fig. 12), HROC370 (Fig. 13), and HROC374 (Fig. 13) cells when they were treated with oxaliplatin and PRIVIGEN® IgG. This suggests that PRIVIGEN® IgG significantly inhibits the anti-viability effect of oxaliplatin.

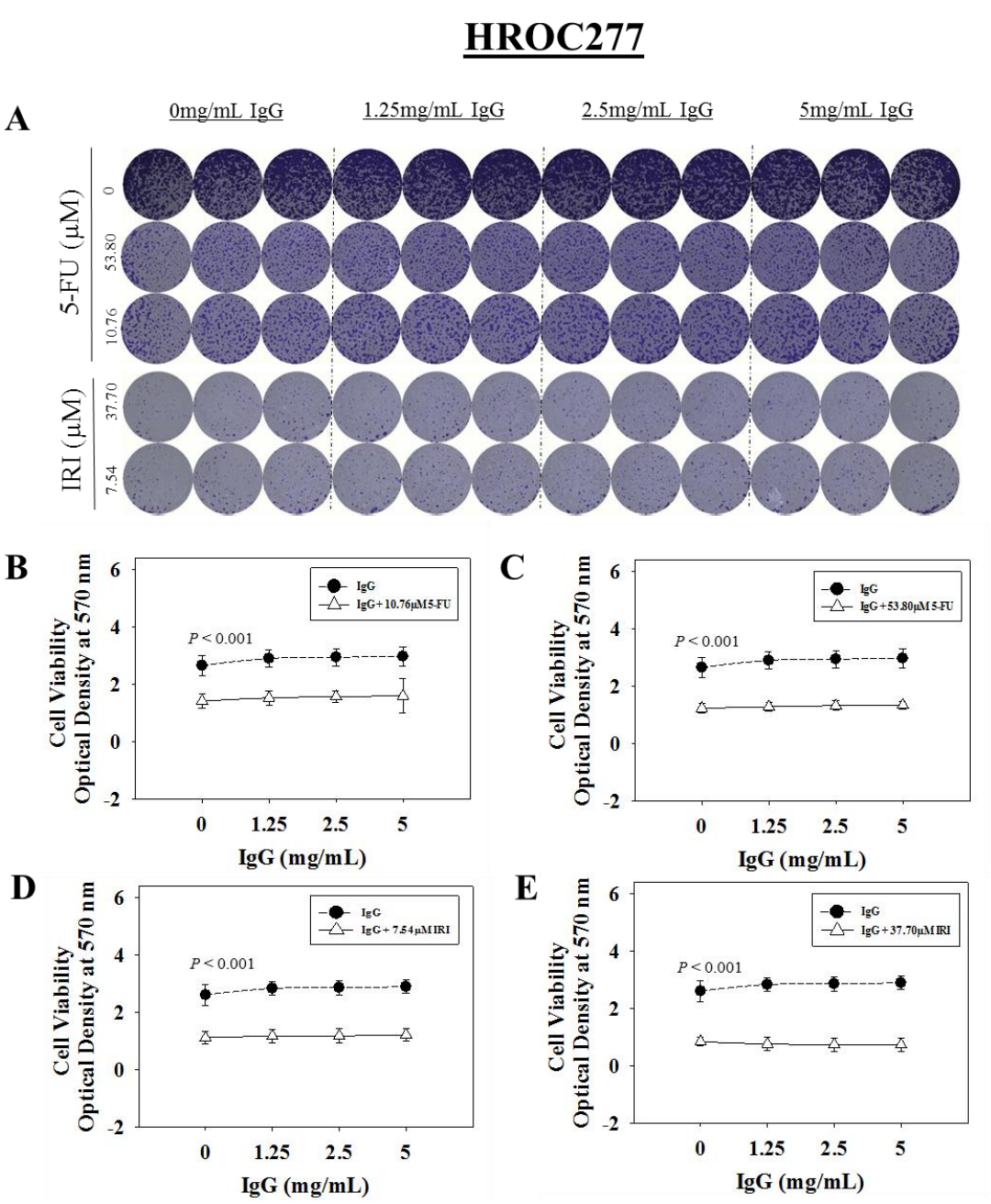

Fig. 8 PRIVIGEN® IgG does not significantly influence the anti-cancerous effects of 5-FU and irinotecan (IRI) in HROC277 cells. HROC277 cells were treated with Sham (A), PRIVIGEN® IgG (A), 10.76μM 5-FU (B), 53.80μM 5-FU (C), 7.54μM IRI (D), 37.70μM IRI (E), or the combinational therapy for 5 days. 5-FU and IRI significantly inhibited cell viability, when compared to Sham-treated cells; however, PRIVIGEN® IgG did not have significant influence on the toxic effects of 5-FU and IRI. n=4 for B-E.

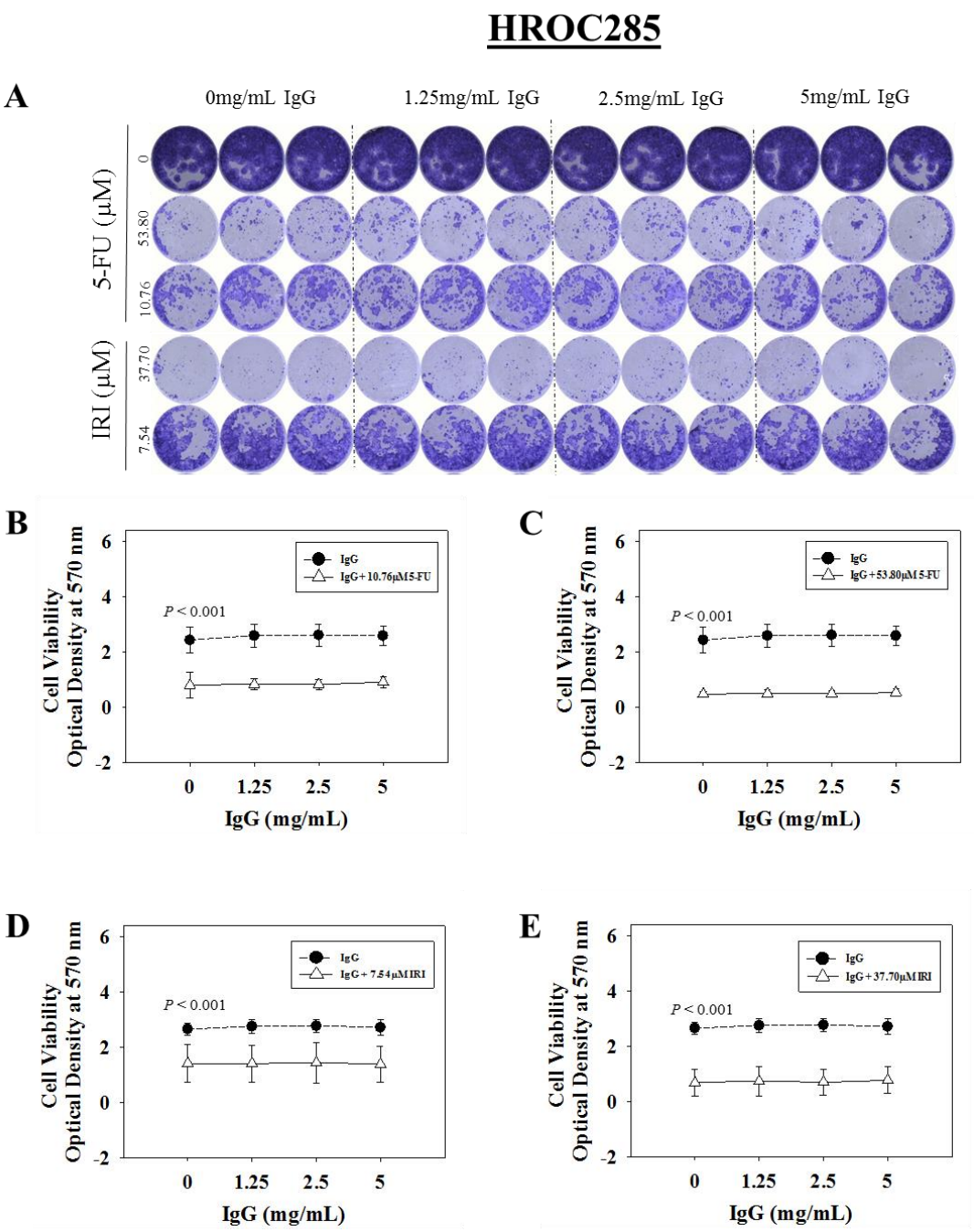

Fig. 9 PRIVIGEN® IgG does not have significant influence on the anti-cancerous effects of 5-FU and irinotecan (IRI) in HROC285 cells. HROC285 cells were treated with Sham (A), PRIVIGEN® IgG (A), 10.76μM 5-FU (B), 53.80μM 5-FU (C), 7.54μM IRI (D), 37.70μM IRI (E), or the combinational therapy for 5 days. 5-FU and IRI significantly inhibited cell viability, when compared to Sham-treated cells; however, PRIVIGEN® IgG did not significantly influence the toxic effects of 5-FU and IRI. n=5 for B-E

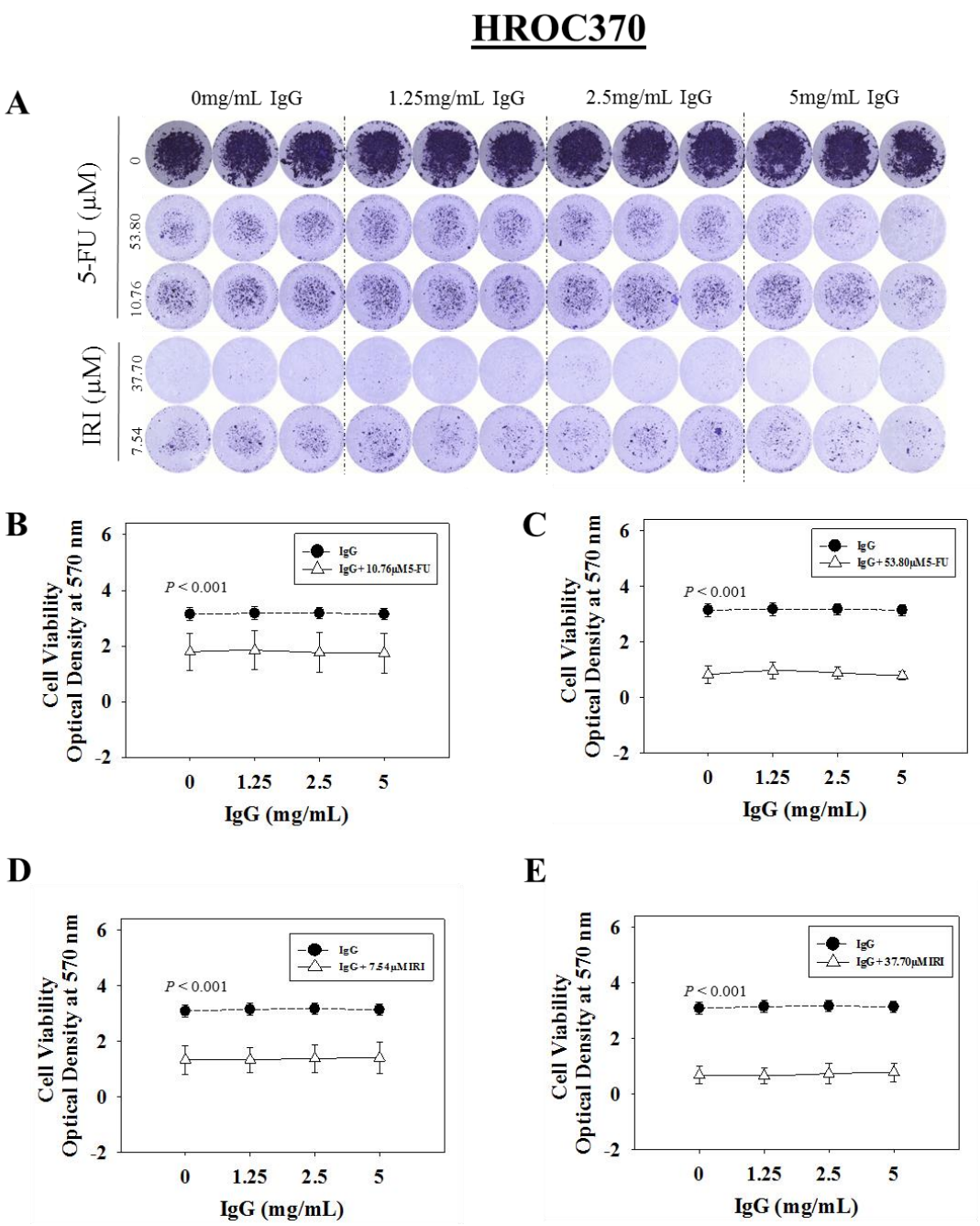

Fig. 10 PRIVIGEN® IgG does not have significant influence on the anti-cancerous effects of 5-FU and irinotecan (IRI) in HROC370 cells. HROC370 cells were treated with Sham (A), PRIVIGEN® IgG (A), 10.76μM 5-FU (B), 53.80μM 5-FU (C), 7.54μM IRI (D), 37.70μM IRI (E), or the combinational therapy for 5 days. 5-FU and IRI significantly inhibited cell viability, when compared to Sham-treated cells; however, PRIVIGEN® IgG did not significantly influence the toxic effects of 5-FU and IRI. n=9 for B-E

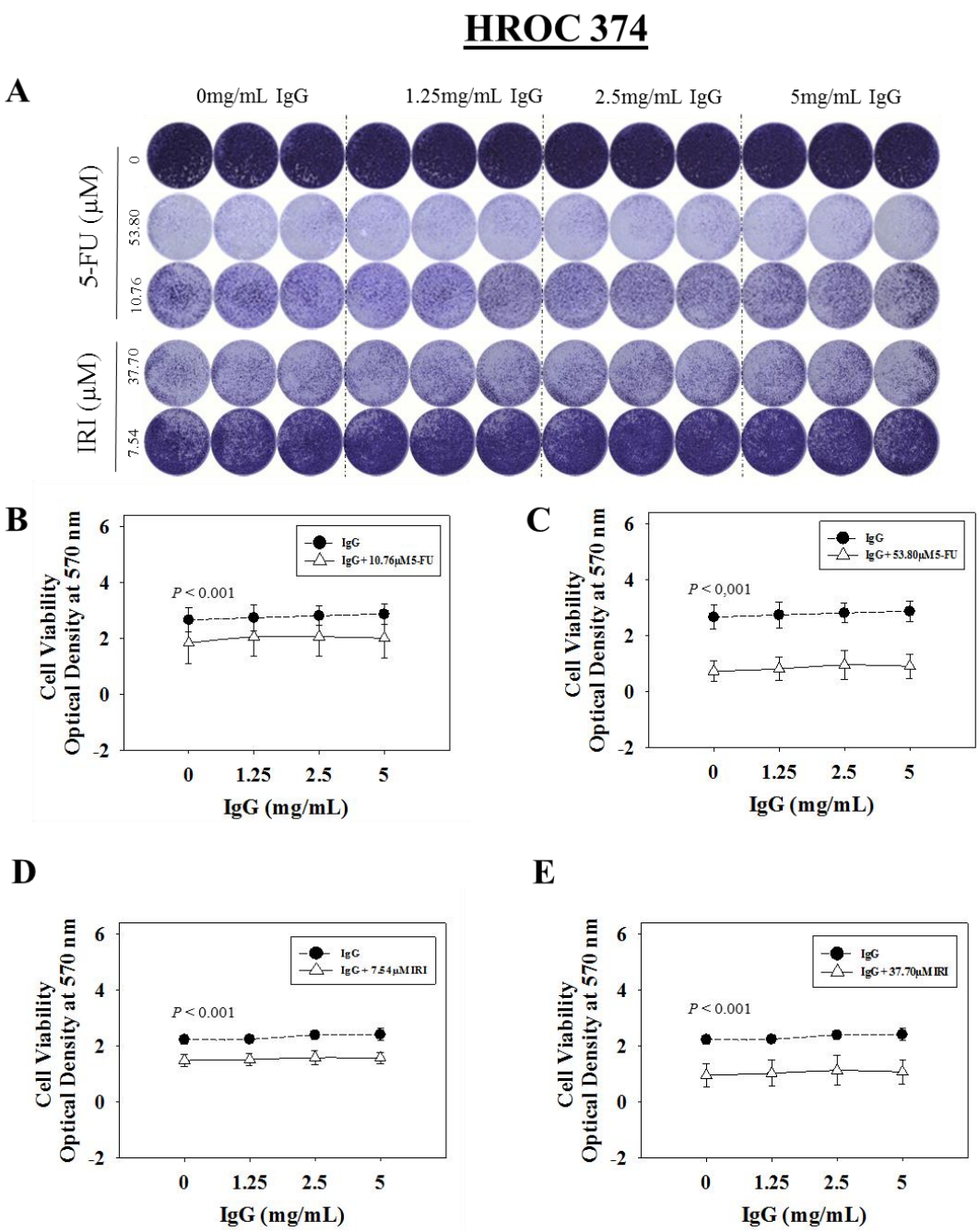

Fig. 11 PRIVIGEN® IgG does not have significant influence on the anti-cancerous effects of 5-FU and irinotecan (IRI) in HROC374 cells. HROC374 cells were treated with Sham (A), PRIVIGEN® IgG (A), 10.76μM 5-FU (B), 53.80μM 5-FU (C), 7.54μM IRI (D), 37.70μM IRI (E), or the combinational therapy for 5 days. 5-FU and IRI significantly inhibited cell viability, when compared to Sham-treated cells; however, PRIVIGEN® IgG did not have significant influence on the toxic effects of 5-FU and IRI. n=6 for B-E

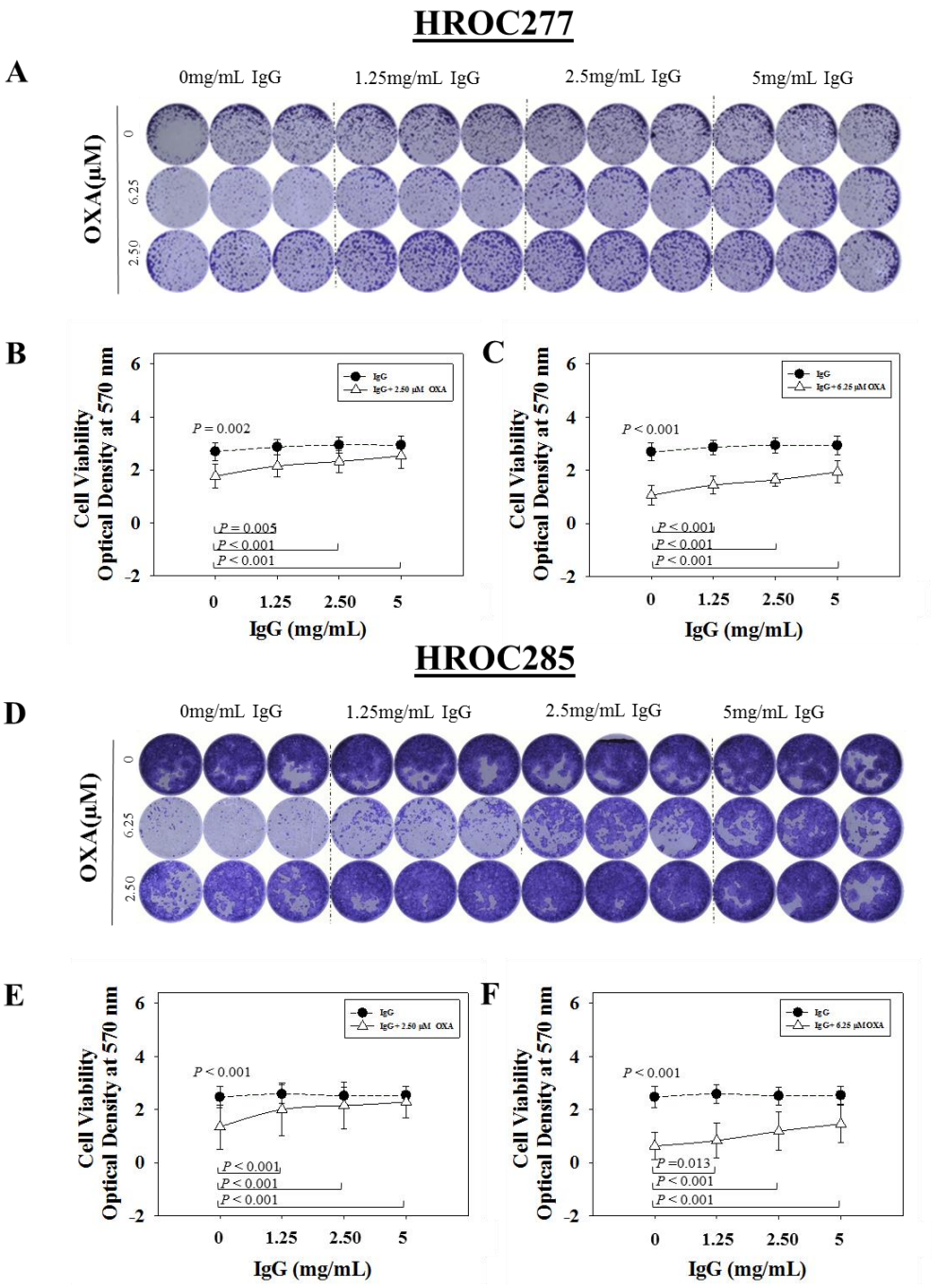

Fig. 12 PRIVIGEN® IgG impairs the cell-toxic effects of oxaliplatin (OXA) in HROC277 and HROC285. HROC277 (A-C) and HROC285 (D-F) cells were treated with Sham, PRIVIGEN® IgG, 2.50μM OXA (B and E), 6.25μM OXA (C and F), or the combinational therapy for 5 days. OXA significantly inhibited cell viability, when compared to Sham-treated cells. Interestingly, PRIVIGEN® IgG significantly impaired the toxic effects of OXA. n=6 for B, C, E, and F.

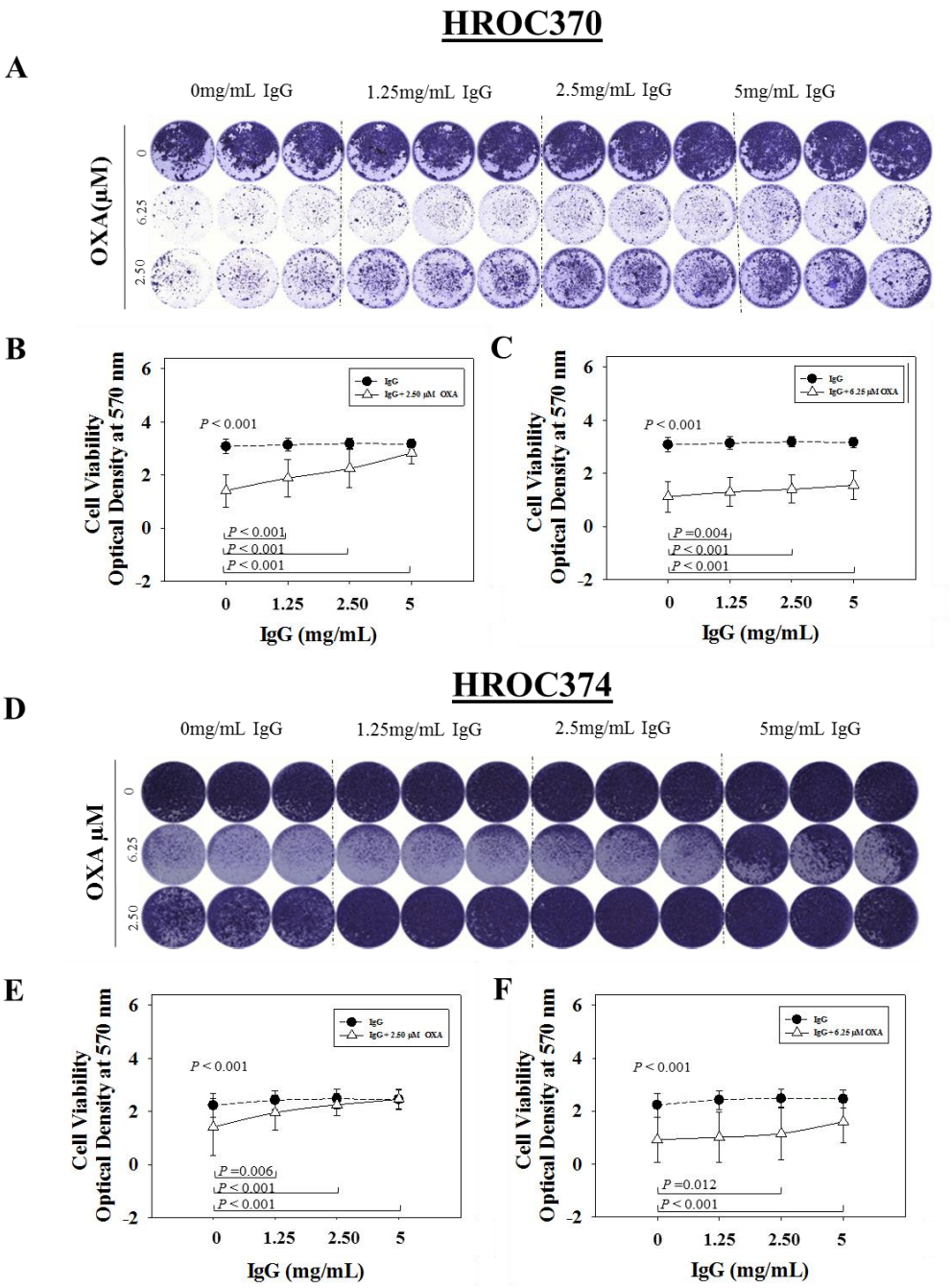

Fig. 13 PRIVIGEN® IgG impairs the cell-toxic effects of oxaliplatin (OXA) in HROC370 and HROC374. HROC370 (A-C) and HROC374 (D-F) cells were treated with Sham, PRIVIGEN® IgG, 2.50μM OXA (B and E), 6.25μM OXA (C and F), or the combinational therapy for 5 days. OXA significantly inhibited cell viability, when compared to Sham-treated cells. Interestingly, PRIVIGEN® IgG significantly impaired the toxic effects of OXA. n=7 for B and C; n=8 for E and F.

3.5 PRIVIGEN® IgG significantly reduces the oxaliplatin-induced cell death *in vitro*

To evaluate how PRIVIGEN® IgG regulates cell death, HROC277 (Fig. 14A), HROC285 (Fig. 14B), HROC370 (Fig. 15A), and HROC374 (Fig. 15B) cells were treated with 5mg/mL IgG and 6.25μM oxaliplatin. The results proved that oxaliplatin significantly (P=0.002) increased the percentage of dead cells, when compared to Sham-treated cells in HROC277. In addition, the combinational therapy, IgG in combination with oxaliplatin, significantly (P=0.002) reduced the percentage of cell death, when compared to oxaliplatin-treated cells. Very similar results were obtained with HROC285 (Fig. 14B), HROC370 (Fig. 15A), and HROC374 (Fig. 15B) cells when they were treated with oxaliplatin and PRIVIGEN® IgG. This suggests that PRIVIGEN® IgG significantly impaired the oxaliplatin-induced cell death and led to oxaliplatin resistance.

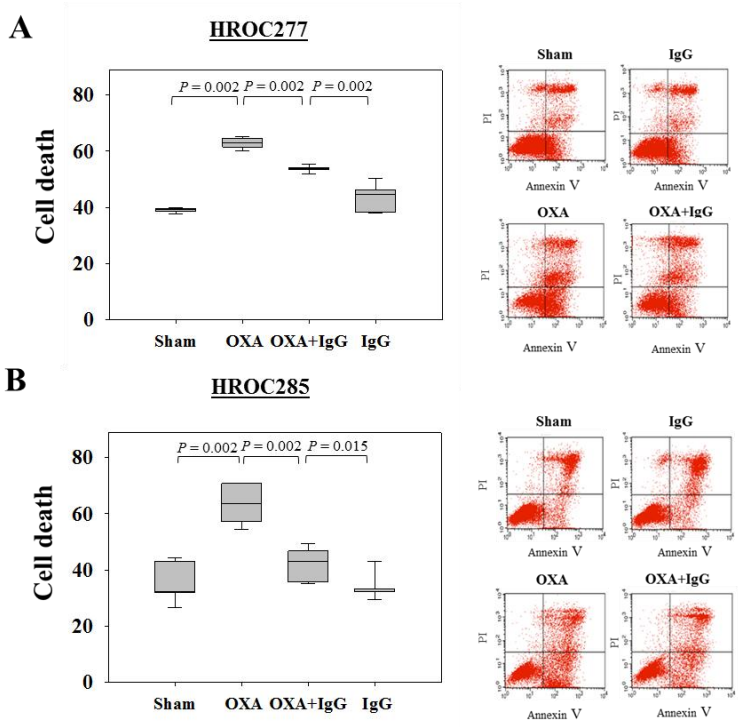

Fig. 14. PRIVIGEN® IgG decreases oxaliplatin-induced cell death in HROC277 and HROC285. HROC277 and HROC285 cells were incubated with Sham, 6.25μM oxaliplatin (OXA), 5mg/mL PRIVIGEN® IgG, or OXA in

combination with IgG for 48 hours. 6.25μM OXA significantly induced cell death, when compared to Sham-treated cells. In addition, IgG significantly inibited OXA cytotoxcity, when compared to OXA-treated cells. n=6 for A and B.

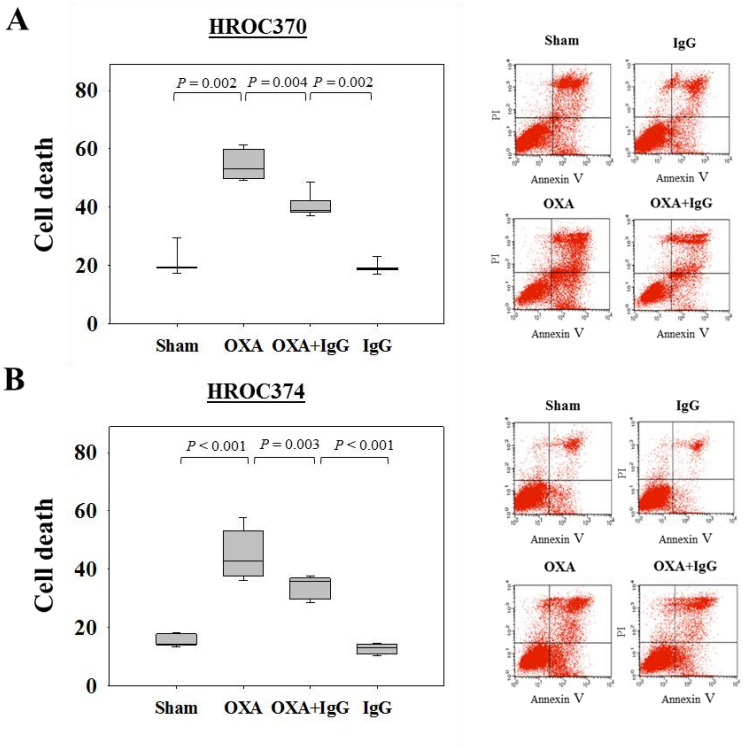

Fig. 15. PRIVIGEN® IgG decreases oxaliplatin-induced cell death in HROC370 and HROC374. HROC370 and HROC374 cells were incubated with Sham, 6.25μM oxaliplatin (OXA), 5mg/mL PRIVIGEN® IgG, or OXA in combination with IgG for 48 hours. 6.25μM OXA significantly induced cell death, when compared to Sham-treated cells. In the combinational therapy, OXA-triggered cell death significant decreased. n=6 for A; n=11 for B.

3.6 Tumor cell-derived IgG is positively correlated with intrinsic resistance to oxaliplatin

Subsequently, the study investigated if colon cancer cells express IgG and if this intrinsic IgG leads to oxaliplatin resistance. The study demonstrated that all four low passage colon cancer cell lines, HROC277, HROC285, HROC370 and HROC374 generated IgG. Interestingly, the

percentage of tumor cell-derived IgG-expressing cells in HROC277 or in HROC285 cells was significantly higher than that in HROC370 and HROC374 cells (Fig. 16).

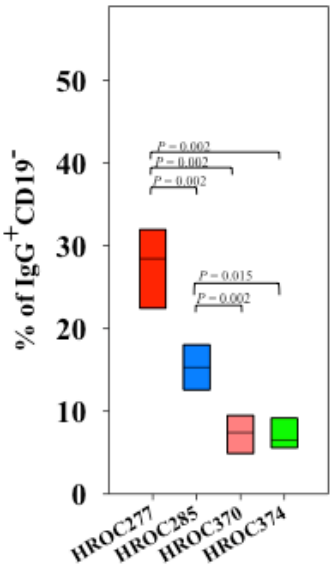

Fig. 16. The expression of tumor cell-derived IgG in colon cancer cells. The percentage of $IgG^{+}CD19^{-}$ in HROC277 and in HROC285 cells was significantly higher than that in HROC370 and HROC374 cells. n=6.

Next, we determined the IC_{50} of oxaliplatin for these HROC cell lines. After treating cells with 1μM oxaliplatin for 5 days, compared to Sham treatment, only 38.51% of HROC277 cells and 33.94% of HROC 285 cells were killed by 1μM oxaliplatin; however, 1μM oxaliplatin killed 64.44% of HROC370 and 63.00% of HROC374 cells (Table 8).

Table 8. Inhibition of cell viability (Compared to Sham-treated cells)

OXA (μM)	HROC277	HROC285	HROC370	HROC374
0.00	0.00%	0.00%	0.00%	0.00%
1.00	38.51%	33.94%	64.44%	63.00%
2.50	50.68%	58.37%	68.89%	76.56%
6.25	62.16%	84.16%	74.22%	80.22%
15.63	76.35%	83.26%	79.11%	80.95%

Subsequently, the IC_{50} values for oxaliplatin of HROC277, HROC285, HROC370, and HROC374 cells were caculated (Fig. 17). The IC_{50} value of HROC285 cells (1.79/1.55-2.11) was nearly 4.6-fold higher than that of HROC370 cells (0.35/0.23-0.41, Fig. 17) or of HROC374 cells (0.34/0.18-0.41, Fig. 17). The increase was more than 6-fold, when comparing HROC277 cells (2.40/2.17-2.76) to HROC370 (0.35/0.23-0.41) or HROC374 cells (0.34/0.18-0.41). This suggests that HROC277 and HROC285 cells are intrinsically resistant to oxaliplatin; at least in direct comparison to HROC370 or HROC374 cells. In addition, we evaluated if the level of tumor cell-derived IgG is correlated with the IC_{50} of oxaliplatin. The Pearson's correlation coefficient suggests that there is a strong positive correlation between the level of intrinsic IgG and oxaliplatin resistance (Pearson's correlation coefficient = 0.769; $P < 0.001$; Fig. 17).

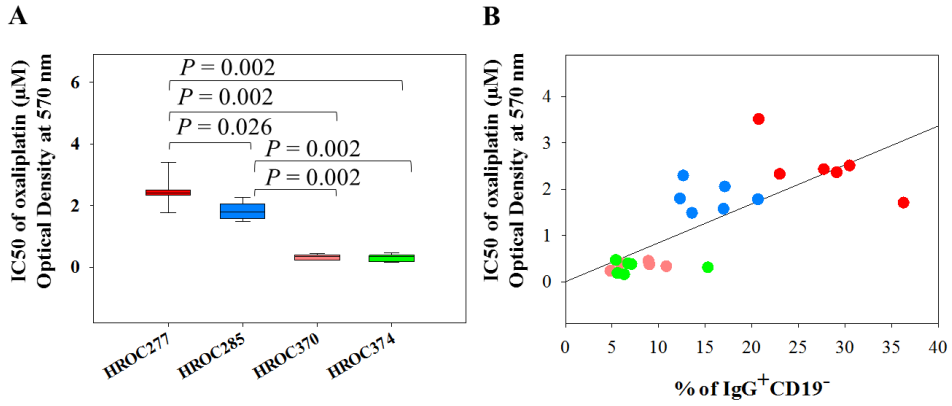

Fig. 17. Tumor cell-derived IgG is positively correlated with intrinsic resistance to oxaliplatin. The IC_{50} value for oxaliplatin (A) in HROC277 (red box or dots) and HROC285 cells (blue box or dots) is significantly higher than that of HROC370 cells (pink box or dots) or of HROC374 cells (green box or dots). The Pearson's correlation coefficient (B) demonstrated a strong positive correlation between the level of intrinsic IgG and oxaliplatin resistance (Pearson's correlation coefficient = 0.769; $P < 0.001$). n=6 for A and B.

3.7 PRIVIGEN® IgG fails to significantly impair the anti-cancerous effect of oxaliplatin *in vivo*

In order to verify the *in vitro* results, tumor tissues of patient HROC285 were implanted into the right flank of immunodeficient mice and were treated with Sham, PRIVIGEN® IgG, oxaliplatin, or IgG in combination with oxaliplatin as indicated in Fig. 5. The results proved that oxaliplatin inhibited the tumor growth, when it was compared to Sham-treated mice ($P = 0.032$, Fig. 18). However, IgG ($P = 0.841$) cannot significantly inhibit the tumor growth, when the increased tumor volume was compared to Sham-treated mice. IgG failed to significantly ($P = 0.429$) impair the anti-cancerous effect of oxaliplatin, when the increased tumor volume in combinational therapy was compared to oxalipatin-treated mice.

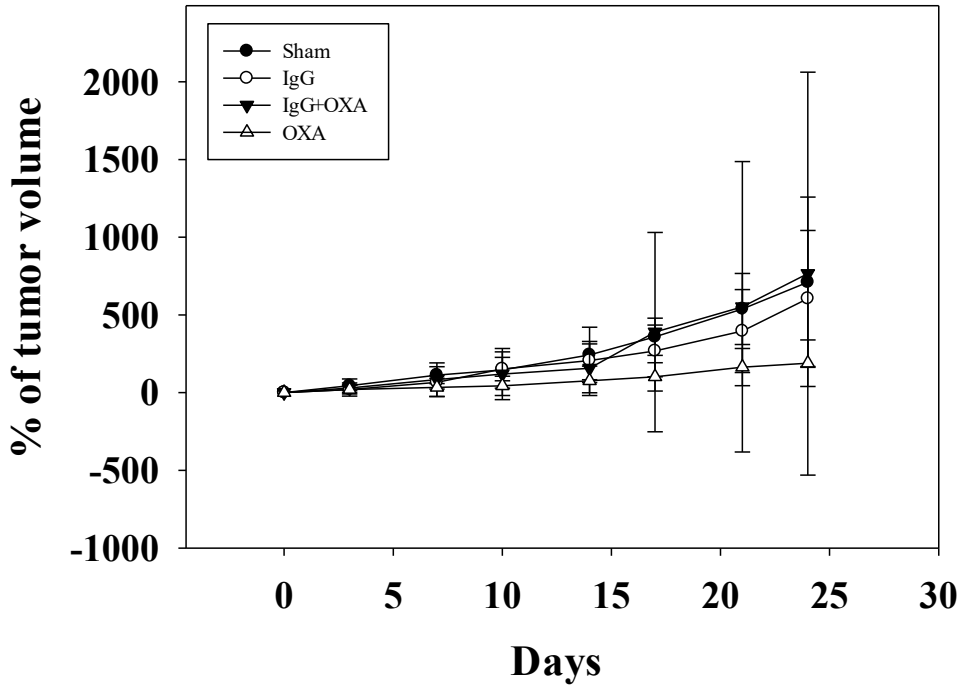

Fig. 18. PRIVIGEN® IgG fails to significantly impair the anti-cancerous effect of oxaliplatin *in vivo*. OXA inhibited tumor growth, when compared to Sham-treated mice (P = 0.032); IgG failed to significantly (P = 0.429) impair the anti-cancerous effect of oxaliplatin. n=5 for Sham; n=6 for IgG and OXA; n=7 for IgG+OXA.

3.8 PRIVIGEN® IgG fails to influence the accumulation of ERCC1 and p38 activity but inhibits the oxaliplatin-induced ERK activity

In order to evaluate if ERCC1 is involved in the IgG mediated resistance to oxaliplatin, HROC285 cells were treated by Sham, oxaliplatin, PRIVIGEN® IgG, and oxaliplatin in combination with IgG for 1 hour (Fig. 19A) or 24 hours (Fig. 19B) and the expression of *ERCC1* mRNA was measured. Oxaliplatin and IgG failed to significantly influence the accumulation of *ERCC1* mRNA. To investigate if IgG regulates the accumulation of ERCC1, the HROC285 cells were treated by Sham, oxaliplatin, PRIVIGEN® IgG, and oxaliplatin in combination with IgG for

24 hours (Fig. 19C) and 48 hours (Fig. 19D). Neither oxaliplatin nor IgG did have influence on the accumulation of ERCC1 on the protein level, when compared to Sham-treated cells.

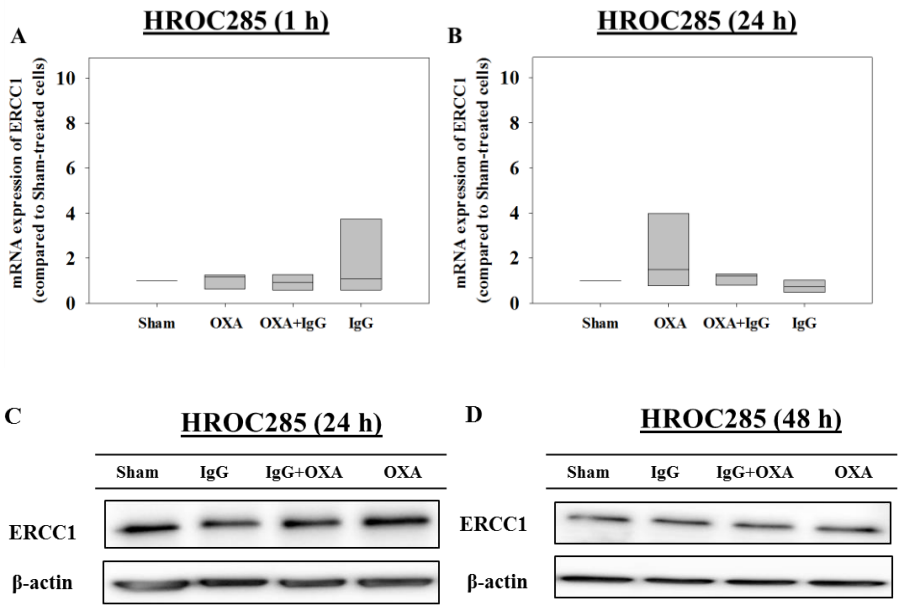

Fig. 19. Expression of ERCC1. HROC285 cells were treated with Sham, 5mg/ml IgG, 6.25µM oxaliplatin (OXA) and combinational therapy (IgG plus OXA). OXA and IgG failed to influence the accumulation of ERCC1 on both, RNA and protein levels. n=3 for A, B, C, and D.

In order to investigate if the MAPKs signaling pathway is involved in the IgG mediated resistance to oxaliplatin, we applied PRIVIGEN® IgG and oxaliplatin to HROC285 cells and measured the activity of p38 and p-ERK, two master proteins of the MAPK pathway. Both IgG and oxaliplatin failed to activate p38 in HROC285 cells (Fig. 20A and B), while oxaliplatin markedly induced ERK activity and IgG inhibited the activity of ERK, when compared to Sham-treated cells (Fig. 20A and B). In addition, IgG inhibited the oxaliplatin-induced ERK activity in HROC285 cells (Fig. 20A and B). To verify these findings, we applied IgG and oxaliplatin to

another colon cancer cell line, HROC277. Again, oxaliplatin induced the activity of ERK in HROC277 cells; however, IgG reduced the oxaliplatin-induced ERK (Fig. 20C and D).

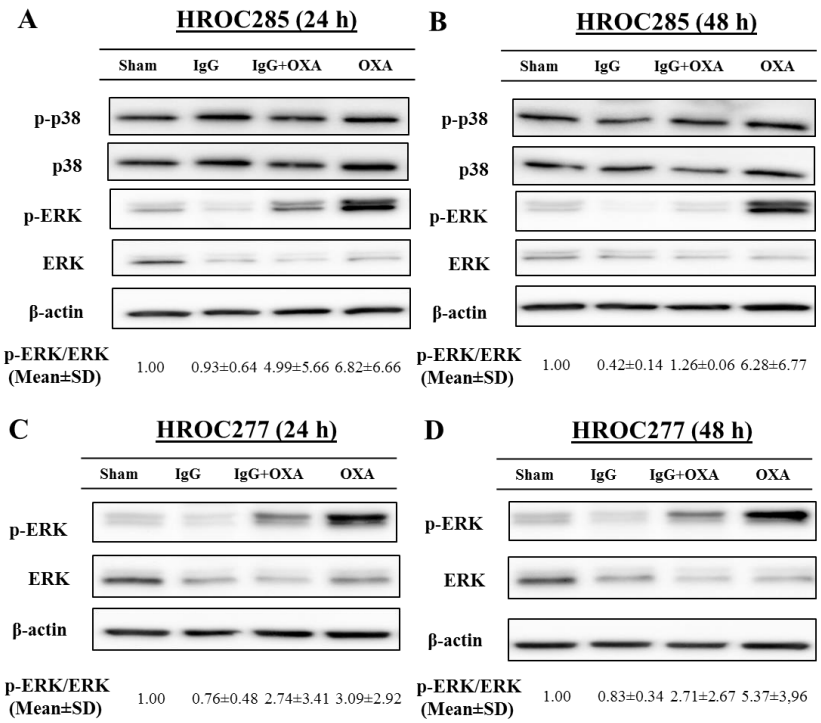

Fig. 20. IgG reduces the oxaliplatin-induced ERK activity. HROC285 (A and B) and HROC 277 (C and D) cells were treated with Sham, 5mg/ml IgG, 6.25μM oxaliplatin (OXA) and combination therapy (IgG plus OXA) for 24h and 48h. OXA activated ERK in HROC285 cells and HROC277 cells; however, IgG inhibited the oxliplatin-induced ERK activity. n=2 for A and B; n=3 for C and D.

3.9 Inhibition of ERK activity reduces oxaliplatin-induced cell death

In order to evaluate whether or not oxaliplatin cytotoxicity is dependent on ERK activity, a traditional inhibitor of ERK signaling, PD98059 was applied. PD98059 had no significant influence on the percentage of dead cells; however, oxaliplatin significantly induced cell death, when compared to Sham-treated cells in HROC285 (Fig. 21A) and HROC 277 (Fig. 21B).

Notably, PD98059 reduced the oxaliplatin-induced cell death in both cell lines (Fig. 21A and B). To verify these findings, HROC285 and HROC277 cells were treated with another inhibitor, U0126, of the ERK pathway and similar results were obtained (Fig. 21C and D). This suggests that the MAPKs signaling pathway is involved in the cytotoxicity effect of oxaliplatin.

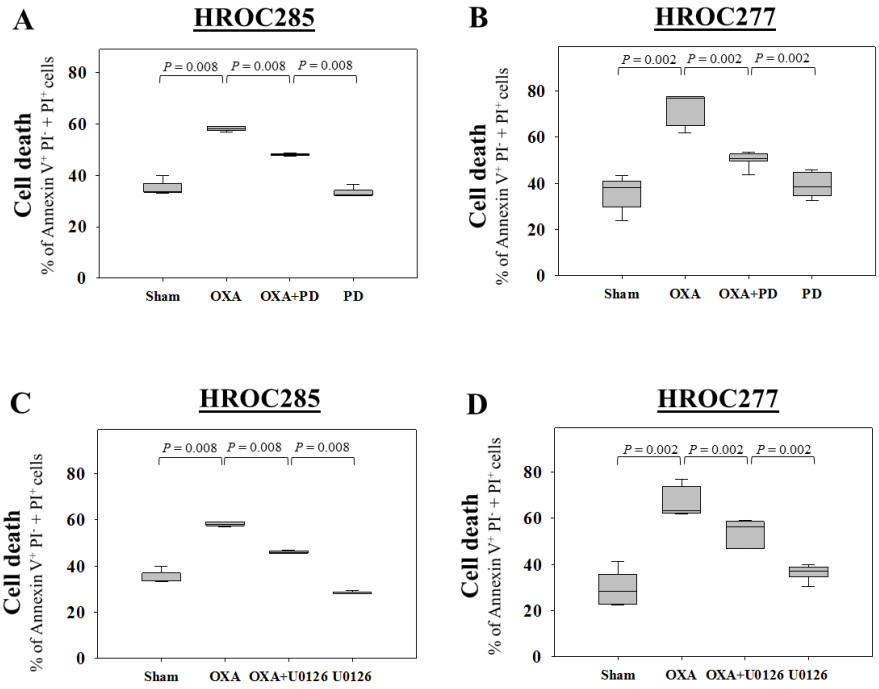

Fig. 21. Inhibition of ERK activity leads to oxaliplatin resistance. HROC285 and HROC277 cells were pretreated with 50μM PD98059 (PD) and 10μM U0126. Subsequently, these cells were treated by oxaliplatin (OXA) for 48 hours. OXA significantly increased the percentage of cell death, when compared to Sham-treated cells (A-D). However, PD98059 (PD) (A and B) and U0126 (C and D) significantly decreased the OXA-induced cell death. n=5 for A, C, AND D; n=7 for B.

4. Discussion

4.1 Patient derived xenograft model is a valuable platform for translational cancer research

For drug development, evaluating efficacy and toxicity of new compounds in preclinical is essential. Thus, development of an animal model, which appropriately mimics human tumor biology, is a core necessity in successful future drug development.

The traditional tumor model is to develop a cell line and to inject the cells into mice. A limitation of this strategy is that such models cannot sufficiently mimic the tumor biology of humans. For example, some factors of the tumor microenvironment, such as cancer associated fibroblasts and extracellular matrix are missing in such models. Thus, numerous chemical compounds have shown promising anti-cancerous activity using this strategy; but most of them failed to inhibit tumor growth and development of metastases in humans [75].

In recent years, PDX have become a preferred preclinical tool for drug development [76]. In order to appropriately mimic human tumor biology, the human tumor tissues are directly transplantation into immunodeficient mice. This preserves the genetic, histological and phenotypic characteristics of the tumor. In addition, this model also preserves the microenvironment of tumors. Thus, the PDX model is a valuable platform for translational cancer research, especially for drug development [75].

However, there are still some limitations of this model. Firstly, the PDX model needs to use immunodeficient mice for tumor engraftment and propagation. Thus, this model cannot be used to evaluate the anti-cancerous effect of novel immunotherapeutics like for example checkpoint inhibitors [75]. Secondly, some researchers recommend using individual PDX models to

determine the optimal chemotherapeutic strategies for a given cancer patient and some studies have proven that PDX are a promising tool for personalized medicine. However, it usually takes 4-8 months to develop a PDX model for a preclinical study, and this time delay limits the applicability of PDX models [76].

4.2 Oxaliplatin-based chemotherapy regimens for colon cancer

Consistent with previous studies, we also observed that oxaliplatin, 5-FU and irinotecan can significantly impair cell viability. Currently, there are 31 kinds of drugs which have been approved by the U.S. Food and Drug Administration for treating colon cancers [77] and the NCCN Clinical Practice Guideline recommends the following 5 combinational chemotherapy regimens for colon cancer: 5-FU plus leucovorin (5-FU/LV), CAPOX, FOLFOX, FOLFIRI, and FOLFOXIRI [78].

4.3 Tumor cell-derived IgG is positively correlated with oxaliplatin resistance

Traditionally, B-lymphocytes are thought to be the only source of IgG. However, recent evidence proves that tumor cells can generate and secret IgG [45, 62, 79, 80]. For example, Kimoto has detected the constant region of heavy chains in human colon, esophagus and breast carcinoma [81]. Another study detected the light chain of IgG in colorectal and breast carcinoma [82, 83]. Interestingly, some studies proved that cancer-derived IgG decreases the programmed cell death and promotes tumor growth [84]. Additionally, Liao et al. reported that high expression of IgG in

epithelial stem cells might lead to tumor metastasis [85]. Of note, no study evaluated if IgG can regulate the antitumor effect of chemotherapy.

Consistent with previous studies [62, 80], this study also proved that tumor cells could generate IgG. In addition, this study for the first time proved that the tumor cell-derived IgG is positively correlated with intrinsic resistance to oxaliplatin. However, the present study did not evaluate if stimulation or inhibition of the tumor cell-derived IgG can influence oxaliplatin resistance. In addition, this study only evaluated the accumulation of tumor cell-derived IgG in four human cell lines. Thus, further studies need to verify if tumor cell-derived IgG leads to intrinsic resistance to oxaliplatin.

4.4 Human IgG fails to influence cell viability but significantly induced oxaliplatin resistance *in vitro*

Contradictory to previous studies [67], the results of the present study suggest that application of human IgG to treat cancer patients might fail to benefit patients' outcome. This hypothesis is supported by two observations of our study: First, IgG could not significantly inhibit cell viability. Second, simultaneously treating cells with IgG and oxaliplatin reduced the cytotoxic effect of oxaliplatin. However, previous studies suggested that treating cancer patients with intravenous IgG might benefit patients. For example, Merimsky et al. proved in a preclinical analysis that intravenous IgG induced a dose-dependent inhibition of cell proliferation in Nb2-11C lymphoma cells, MCA-105 sarcoma cells and HCT-116 colon cancer cells [68].

Interestingly, the present study proved that PRIVIGEN® IgG significantly inhibited the cytotoxic effect of oxaliplatin *in vitro*; however, IgG failed to significantly impair the anti-cancerous effect of oxaliplatin *in vivo* (Fig. 18). This can be explained by a limitation of this study: I administrated

IgG subcutaneously; however, most animal studies on IgG injected via the tail veins [86]. The subcutaneous injection might result in weak IgG absorption by vascular endothelial cells, and therefore IgG failing to reach an effective concentration in the blood.

4.5 The interaction of ERK activity and oxalipatin resistance

ERK is an important member of the MAPKs signaling pathway, which regulates a wide range of cellular activities and physiological processes [87]. Traditionally, activation of ERK can induce cell proliferation and differentiation [87]. In addition, Xu et al. observed that the accumulation of phosphorylated ERK in oxaliplatin-sensitive cells is lower than that in cells resistant to oxaliplatin [88]. Depletion of oxaliplatin-induced ERK activity reduced oxaliplatin resistance [88]. These observations suggest that activation of ERK leads to oxaliplatin resistance. Surprisingly, the present study proved that IgG as well as PD98059 and U0126, two traditional and specific inhibitors of the ERK signaling pathway, impaired ERK signaling and that this triggered oxaliplatin resistance. Interestingly, although IgG, PD98059 and U0126 inhibited the ERK activity, all of them could not significantly regulate the cells' viability and death. This suggests that the function of ERK is context-dependent and inhibition of ERK signal pathway cannot always benefit patients. Thus, even though a number of preclinical studies proved that inactivation of ERK is a promising treatment strategy in several cancers [87, 88] and with even a clinical trial ongoing (ClinicalTrials.gov Identifier: NCT02420795), the data of this study suggest that mode of action and potential benefits of ERK inhibitors should be fully investigated before clinical application in cancer patients' treatment.

Interestingly, a recent study reported that the clinical sequences of cetuximab, an inhibitor of EGFR, and oxaliplatin administration could lead to different responses of colon cancer cells to oxaliplatin [89]. The authors observed that cetuximab inhibited the cytotoxic effect of oxaliplatin when administered before oxaliplatin; however, cetuximab provided additive effects when administered after oxaliplatin [89]. In addition, administration of cetuximab before oxaliplatin induced the phosphorylation of ERK when compared to the inverse treatment strategy with administration of cetuximab after oxaliplatin therapy [89]. However, the present study only evaluated the strategy of simultaneously treating colon cancer cells by IgG and oxaliplatin. If different sequences of IgG and oxaliplatin cause different responses of colon cancer cells to oxaliplatin, this needs to be evaluated in a future study

4.6 The interaction of ERCC1 or p38 activity and oxalipatin resistance

During DNA damage, cells can activate several DNA repair systems, such as the nucleotide excision repair pathway [90]. This pathway can eliminate the lesions of DNA by the activity of ERCC1, and enhance the re-synthesis and ligation of DNA (Fig. 22) [91]. A body of evidence demonstrates that ERCC1 is involved in the resistance to platinum compounds [91]. For example, Youn et al. proved that ERCC1 could protect embryonic fibroblasts and breast cancer cells from platinum-based anticancer drugs [92]. In addition, Olaussen et al. reported that cisplatin-based therapy improved survival only in patients with ERCC1 negative tumors [93]. However, in the present study, ERCC1 was not involved in IgG-mediated oxaliplatin resistance. This suggests that the level of ERCC1 may not be a stable prognosis factor of patients undergoing oxaliplatin-based chemotherapy.

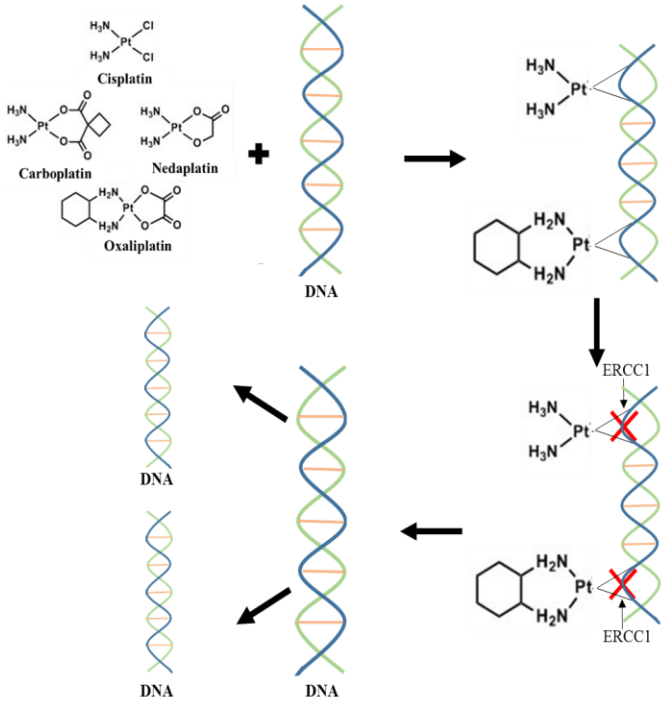

Fig. 22. ERCC1 impairs platinum-mediated DNA damage. ERCC1 can eliminate the platinum-DNA adducts and thus restore the DNA replication.

P38 is another member of the MAPKs signaling pathway, and it is known as "stress-activated protein kinases". Totally, there are four genes, MAPK14, MAPK11, MAPK12, MAPK13, encoding of p38α, p38β, p38γ, and p38δ, respectively [94]. Although p38α and p38β have the same function; p38β is highly rare in most cell types and its contribution to p38 MAPK signaling is not clear [94]. In addition, p38γ and p38δ are not involved in the signal transmission of the p38 MAPK pathway [94].

Similar to ERK, p38 MAPK is also involved in several cellular activities and physiological processes, such as inflammation, differentiation and cell death [95], and it is activated by MAPK kinase kinases (MAPKKKs/MAP3Ks)-MAPK kinases (MAPKKs/MAP2Ks) pathway. Two

MAP2Ks, MKK3 and MKK6 can directly activate p38 MAPK, and these MAP2Ks can be activated by MAP3K4 (also known as MEKK4) via targeting AVRB operation1 and 2 [96].

A body of evidence demonstrates that p38 is a tumor suppressor, which inhibits the proliferation and induces cell death [97]. For example, Zuluaga et al. proved that p38 induces cardiomyocytes' death by up-regulation of the pro-apoptotic proteins, such as Fas and Bax, and down-regulation of the activity of the ERK and Akt survival pathways [98]. Additionally, Maria et al. proved that α-cyano-4-hydroxycinnamate could active the p38 MAPKs pathway. Inhibition of the p38 signaling pathway by PH-797804 impaired the α-cyano-4-hydroxycinnamate-induced apoptosis [99]. However, the present study proved that p38 was not involved in the oxaliplatin-mediated cell death. This suggests that the tumor suppressive activity of p38 MAPK may be context-dependent.

4.7 The potential mechanisms of IgG-mediated oxaliplatin resistance

The present study demonstrated that IgG induces oxaliplatin resistance by inhibiting ERK activity. Notably, some other mechanisms may be also involved in the IgG-mediated oxaliplatin resistance.

4.7.1 Nucleotide excision repair system

Several pieces of evidence proved that the nucleotide excision repair system is a master regulator of cell sensitivity to platinum-based drugs [100]. The nucleotide excision repair system can excise the platinum-DNA adducts and repair the damaged DNA. Indeed, both cisplatin- and oxaliplatin-mediated DNA damage can be repaired by the nucleotide excision repair system [101]. Interestingly, the present study proved that IgG caused colon cancer cells resistance to

oxaliplatin (Fig. 12 and 13), but not to 5-FU and irinotecan (Fig. 8-11). Thus, we assume that IgG causes oxaliplatin resistance by impairing oxaliplatin-DNA adducts.

4.7.2 Drug accumulation and detoxification proteins

It has been proven that the intracellular accumulation of drugs and the activity of detoxification proteins are also two mechanisms causing cell resistance to platinum derivatives [102]. For example, a previous study proved that the major copper influx transporter CTR1 is involved in the transport of oxaliplatin, and that decreasing the accumulation of CTR1 can trigger oxaliplatin resistance [103]. In addition, depletion of glutathione (GSH), an antioxidant protein, by β-phenylethyl isothiocyanate could enhance the cytotoxic effects of oxaliplatin [104]. This suggests that GSH might also cause oxaliplatin resistance. However, other studies demonstrated that GSH was not correlated with oxaliplatin resistance [105, 106].

4.7.3 Hippo pathway

Recent evidence suggests that the Hippo pathway might be another mechnism of IgG-induced oxaliplatin resistance [107, 108]. In mammals, several proteins are involved in the Hippo signalling pathway [108], such as the mammalian sterile 20-like kinases (MST1 and MST2, MST1/2), the large tumor suppressor kinases (LATS1 and LATS2, LATS1/2), the yes-associated protein (YAP) and the TEA domain family (TEAD1-4) transcription factors (Fig. 23)[108].

When the Hippo pathway is swithced “ON”, the mammalian sterile 20-like kinases (MST1 and MST2, MST1/2) and Salvador homolog 1 (SAV1) activate the large tumor suppressor kinases (LATS1 and LATS2, LATS1/2) and phosphorylate yes-associated protein1 (YAP1). This leads to cytoplasmic retention and degradation of YAP1. However, when the signaling pathway is

switched “OFF”, YAP translocates to the nucleus and binds to some transcription factors such as TEA domain (TEAD) family transcription factors. These complexes activate the transcription of various genes.

Initially, the Hippo signal pathway has been reported to control organ size [108]. However, recent studies suggest that YAP1 is also involved in chemoresistance to cisplatin [109] and oxaliplatin [110]. Interestingly, other studies reported that YAP1 interacts with p73 and enhanced the p73-dependent apoptosis in response to DNA damage [108, 111]. These conflicting functions suggest that YAP1-induced oxaliplatin resistance may be context-dependent. Thus, a further study is needed to evaluated if and how IgG regulates YAP1 activity, and if the interaction of IgG and YAP1 – or better the simultaneous administration of both to colon cancer cells - leads to oxaliplatin resistance.

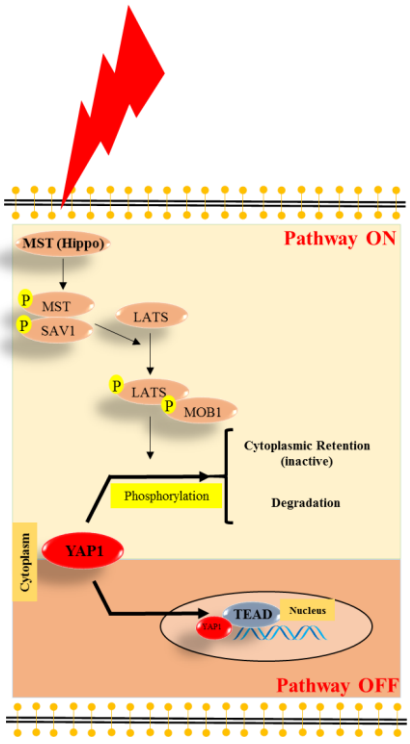

Fig. 23. Hippo signaling pathway. Several signals, such as G-protein-coupled receptor signaling, protein kinase A and cyclic adenosine monophosphate, can stimulate and switch "ON" the Hippo pathway and impair YAP1 nuclear localization **[108]**.

4.7.4 Autophay

Autophagy is a natural process controlling the recycling of unnecessary or dysfunctional components in the cell (Fig. 24) [74]. The damaged cell orgenelles and proteins are enclosed by isolation membranes and form the so-called autophagosomes. Subsequently, lysosomes fuse with autophagosomes and form autolysosomes. This leads to the degradatiuon and recycling of dysfunctional components [74].

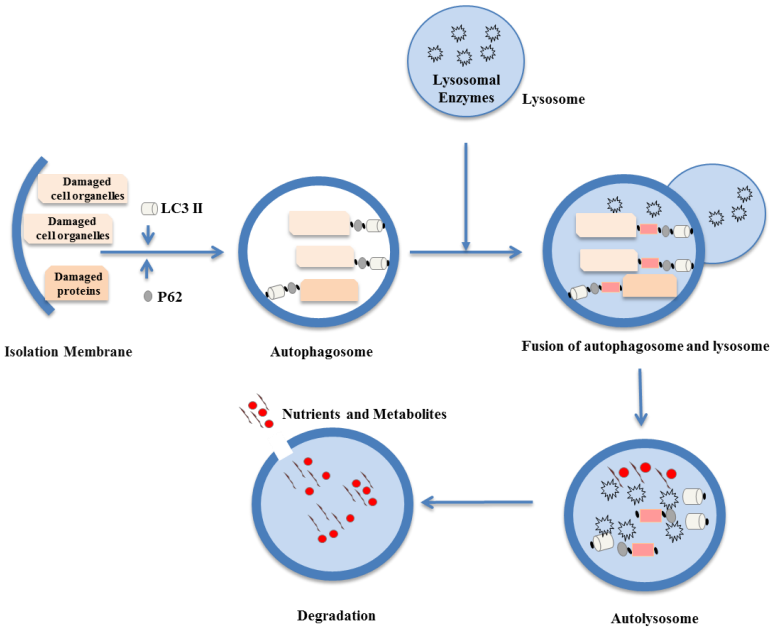

Fig. 24. The schema of autophagy. The intracellular or extracellular stimulators, such as hypoxia, starvation and infection can induce the formation of isolation membranes. The isolation membranes engulf the damaged cell organelles and proteins and form the autophagosomes with the help of LC3II and p62. Subequently, the organelles and proteins are transported to lysosomes by the fusion of autophagosomes and lysosomes, and degraded in the presence of lysosomal enzymes [74].

Interestingly, multiple studies reported that, in cancer cells, autophagy not only recycles dysfunctional components, but also impairs the anticancerous effect of oxaliplatin [112-114]. For example, Xiong et al. proved that inhibition of autophagy by chloroquine (CQ), a traditional inhibitor of autophagy, could increase oxaliplatin-induced cell death [113]. In addtion, two clinical trials (ClinicalTrials.gov Identifier: NCT01006369 and NCT01206530), which evaluate the benefit of oxaliplatin-based chemotherapeutics in combination with CQ, have been completed and the results may be available later in 2019. However, Soyeon et al. proved that induction of autophagy increased the oxaliplatin-induced cell death [113]. This suggests that inhibition of autophagy might not always benefit patients receving oxaliplatin. Thus, a further study is recommended to evaluate if and how autophagy is involved in the IgG-mediated oxaliplatin-resistance.

5. Conclusions and outlook

The present study demonstrated that supplementation of human IgG impairs the anti-viability effect of oxaliplatin and reduces the oxaliplatin-mediated cell death. Interestingly, tumor cells also can genereate IgG and this intrinsic IgG is positively correlated with oxaliplatin resistance. In addition, this study proved that oxaliplatin activates ERK signaling pathway and inhibition of ERK activity by IgG or PD98059 and U0126, two tranditional inhibitors of ERK significantly reduced the oxaliplatin-mediated cell death. This suggests that IgG leads to oxaliplatin resistance might by inhibiting the ERK signal pathway.

I think it would be interesting for future research to investigate how downstream proteins of the ERK transduction pathway are involved in the interaction of IgG and oxaliplatin. In addition, the

oxaliplatin-based regimens, FOLFOX and FOLFOXIRI, are currently evaluated for treatment of other cancer types such as pancreatic, rectal and esophageal cancer (https://clinicaltrials.gov/). Thus futher studies should investigate if IgG also leads to oxaliplatin resistance in other cancer types.

6. References

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7. Thesis statement

- Recent evidence proves that intravenous immunoglobulin G (IgG) can impair cancer cells.
- Cancer cells can produce and secreat IgG, and that tumor cell-derived IgG promotes tumor initiation
- No study evaluated whether or not IgG application benefits cancer patient who is receiving chemotherapeutics.
- Human normal IgG (PRIVIGEN®) cannot significantly influence tumor cell viability and cell cycle.
- Human normal IgG (PRIVIGEN®) significantly impairs the anti-cancerous effects of oxaliplatin.
- Tumor cell-derived IgG were positively correlated with oxaliplatin resistance.
- Inhibition of oxaliplatin-induced ERK activity significantly reduced oxaliplatin-mediated cell death.

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9. Eidesstattliche Versicherung

Ich versichere hiermit, dass ich die vorliegende Arbeit mit dem Thema:
“Immunoglobulin G mediates chemoresistance to oxaliplatin in colon cancer cells by inhibiting the ERK signal transduction pathway“

Ich erkläre an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel erstellt habe. Die aus anderer Literatur verwendeten Inhalte sind als solche kenntlich gemacht. Ich versichere, dass ich für die inhaltliche Erstellung der vorliegenden Arbeit nicht die entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen habe.

Ich erkläre hiermit weiterhin, dass ich meine wissenschaftlichen Arbeiten nach den Prinzipien der guten wissenschaftlichen Praxis gemäß der gültigen „Satzung der Universität Rostock zur Sicherung guter wissenschaftlicher Praxis“ angefertigt habe.

Shenzhen, 31/05/2021 ……Yuru Shang……

Ort, Abgabedatum Vollständige Unterschrift

10. Curriculum vitae

Personal Details

Name:	Yuru Shang
Gender:	Female
Data of birth:	04.March.1988
Marital Status:	Married
Telephone:	015908405634
Email:	yuru.shang@uni-rostock.de
Address:	Molekulare Onkologie und Immuntherapie (M.O.I) Schillingalle 69 18057 Rostock

Education

12/2016-	Doctor of Medicine in Molekulare Onkologie und Immuntherapie, University medicine Rostock, Germany
09/2011-07/2014	Master of Medicine in Burn Surgery, Chinese People's Liberation Army General Hospital, China
09/2006-07/2011	Bachelor of Clinical Medicine, Binzhou Medical University, China

Work Experience

09/2014-11/2016	Department of Burn Surgery, Chinese People's Liberation Army General Hospital, China

Grants

Chinese Government Graduate Student Overseas Study Program. Ministry of Education of the People's Republic of China (NO. 201708080032).

Honors and Awards

2017

Beijing Science and Technology Progress Award (First Class, 2017-1-002-12)

Publication

First Author Publication

1. **Shang Y**, Zhang X, Lu L, Krohn M, Matschos S, Mullins C et al. Immunoglobulin G mediates chemoresistance to oxaliplatin in colon cancer cells by inhibiting the ERK signal transduction pathway. Br J Cancer. 2021 Apr;124(8):1411-20.
2. **Shang Y**, Li D, Shen C, Yin K, Ma L, Li L et al. The benefit of microskin in combination with autologous keratinocyte suspension to treat full skin loss in vivo. J Burn Care Res. 2017 Nov 1;38(6):348-53.
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Co-author Publication

1. Cheng W, Shen C, Zhao D, Zhang H, Tu J, Yuan Z, Song G, Liu M, Li D, **Shang Y**, Qin B. The epidemiology and prognosis of patients with massive burns: A multicenter study of 2483 cases. Burns. 2019 May 1;45(3):705-16.
2. Lu L, Zhang X, Tang G, **Shang Y**, Liu P, Wei Y, Gong P, Ma L. Pancreaticoduodenectomy is justified in a subset of elderly patients with pancreatic ductal adenocarcinoma: A population-based retrospective cohort study of 4,283 patients. Int J Surg. 2018 May 1;53:262-8.
3. Song M, Li N, Zhang X, **Shang Y**, Yan L, Chu J, Sun R, Xu Y. Music for reducing the anxiety and pain of patients undergoing a biopsy: A meta-analysis. J Adv Nurs. 2018 May;74(5):1016-29.
4. Zhao D, Ma L, Shen C, Li D, Cheng W, **Shang Y**, Liu Z, Wang X, Yin K. Long-lasting glucagon-like peptide 1 analogue exendin-4 ameliorates the secretory and synthetic function of islets isolated from severely scalded rats. J Burn Care Res. 2018 Mar 20;39(4):545-54.

5. Zhang X, Song J, Liu P, Mazid MA, Lu L, **Shang Y**, Wei Y, Gong P, Ma L. A modified M-stage classification based on the metastatic patterns of pancreatic neuroendocrine neoplasms: a population-based study. BMC Endocr Disord. 2018 Dec;18(1):73.
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Shenzhen, 31/05/2021 Yuru Shang

City, Date Signature