Original article

Linsitinib and aspirin as the IGF1-R antagonists, inhibit regorafenib-resistant chemotherapy in colon cancer

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ABSTRACT

Colorectal cancer is one of the most common cancers. Regorafenib is used in patients with metastatic colorectal cancer and sometimes, the cancer cells become resistant to the drug. However, increased IGF-1R activity is associated with the invasion of cancer cells. Therefore, it is thought that inhibiting IGF-1R by Linsitinib and Aspirin, the resistance of colorectal cancer cells to Regorafenib can be reduced.

SW48 colon cancer cell line was cultured, resistance to the regorafenib and exposed to Linsitinib and Aspirin. The treatment cytotoxicity, Flow cytometry for determine cancer stem cell markers, and the mRNA expression of CD133, CD44, CD24, IGF1-R, CDX2 and PTEN were done. Then C57BL/6J mice tumor model was produced and treated with regorafenib, aspirin, and linsitinib. At least, Clinical symptoms, the levels of IL-6, and IL-1β, TNF-α and MCP-1 in the colon tissues and sera were assessed.

The linsitinib and aspirin as the IGF1-R antagonists inhibited colon cancer resistance against regorafenib, stem-cell like colon cancer cells growth, decreased expression of CD133, CD44, CD24, and also increased CDX2, PTEN gene expression. In the canceroous mice, linsitinib, aspirin and regorafenib treatment enhanced Body weight and survival, and also decreased fecal blood, number of tumors in colon and Inflammatory cytokines levels in serum and colon tissues.

In this study, we obtained the best in-vitro and in-vivo result of colon cancer treatment when combinitation therapy Linsitinib, Aspirin, and Regorafenib was used, and could prevent tumor resistance, stem cell producing, pathological interaction and disease activity index.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world. Surgery is the mainstay of treatment for colorectal cancer; however, sometimes adjuvant therapy with chemotherapy or radiotherapy is prescribed before or after surgery, depending on the stage of the disease. However, this type of treatment is not enough to control CRC, and about 30% of patients with Stage I-III and about 65% of patients with Stage IV progress to worsening disease (van der Stok et al., 2017; Schöning et al., 2017). This is a disease originating from the epithelial cells of the colon or rectum, most frequently as a result of mutations in the Wnt signaling pathway and increases signaling activity. Regorafenib is an inhibitor of multinuclear receptors that is currently used in patients with metastatic colorectal cancer who have previously received chemotherapy. However, after a while, the cancer cells become resistant to the drug and do not respond. Resistance to chemotherapy is the most important factor in poor therapeutic responses and relapse. Modified signaling pathways that prevent cell death are an important feature of drug-resistant cancer cells (Schöning et al., 2017; Tsuchihashi et al., 2017).

Many cancers, including colorectal cancer, have been shown to be associated with inappropriate IGF signaling. Many studies have shown that increased IGF-1R activity is associated with the proliferation, migration, and invasion of cancer cells. Many cancers,

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including colorectal cancer, have been linked to IGF mis-signaling. Many studies have shown that increased IGF-1R activity is associated with cancer cell proliferation, migration and invasion (Denduluri et al., 2015; Yavari et al., 2010). Linsitinib as an inhibitor of the insulin receptor and the insulin-like growth factor 1 receptor (IGF-1R) is candidate for the treatment of various types of cancer. It can prevent tumor cell proliferation and induce apoptosis of the tumor cell (Mulvihill et al., 2009; Fassnacht et al., 2015).

Regorafenib is a multi-kinase receptor blocker that currently used for patients with metastatic colorectal cancer who have previously undergone chemotherapy and targeted therapy (anti-VEGF and anti-EGFR monoclonal antibodies). Resistance to chemotherapy is the most important factor in poor therapeutic responses and relapse.

Modified signaling pathways that prevent cell death are an important feature of drug-resistant cancer cells. It inhibits several important angiogenic and tumorigenic kinases including AbL, FGFR-2, FGFR-1, PDGFR-b, KIT, VEGFR-3, VEGFR-2, VEGFR-1, RET, RAf (de la Fouchardière, 2018).

Therefore, it is thought that by inhibiting IGF-1R, the resistance of colorectal cancer cells to Regorafenib can be reduced. In the present study, after SW48 cancer cell culturing, SW48 cells became resistant to Regorafenib, then the inhibitory effect of small molecule Linsitinib and Aspirin on IGF-1R on the change of the resistance rate of cancer cell was investigated. Also, this combination therapy was tested in animal model of colon cancer as new chemotherapy protocol.

2. Material and methods

2.1. Treatment and drug-resistance of the SW48 cells

SW48 colon cancer cell line was cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2 in a cell culture incubator. Four sub-cultures were provided that seeded in 24-well plates. One cancer cell lines culture was treated with regorafenib (5 μmol/L for 96 h). One other cancer cell lines culture was exposed to Linsitinib (1.0 or 10.0 μmol/L for 48 h) and then was treated with regorafenib (5 μmol/L for 96 h). One other cancer cell lines culture was exposed to aspirin (10 mM for 48 h) and then was treated with regorafenib (5 μmol/L for 96 h). One other cancer cell lines culture was exposed to Linsitinib (1.0 or 10.0 μmol/L) and aspirin (10 mM) for 48 h then was treated with regorafenib (5 μmol/L for 96 h). The treated cells with regorafenib may initiate drug resistant.

2.2. Cytotoxicity assay to the cell line survival

The cytotoxicity of treatment against colon cancer cells was determined by MTT assay. Generally, the cells were seeded into 96-well plates and then treated with drugs. After 24 h incubation, MTT (10 μL) was added into each well and incubated (4 h). Then 100 μL/well of DMSO was added and the absorbance was determined using Spectrophotometer at 490 nm.

2.3. Flow cytometry for determine cancer stem cell markers

The 4 treated cell loines were rinsed with PBS, incubated with PE labeled anti-human CD133 antibody and FITC labeled anti-human CD44 antibody and isotype control at room temperature for 15 min and analyzed by a flow cytometer.

2.4. Real Time-polymerase chain reaction (RT-PCR)

The mRNA expression of CD133, CD44, CD24, IGF1-R, CDX2 and PTEN in the cells was determined by Real-time PCR. The total RNA was extracted and reverse transcribed into cDNA using a kit. The genes were amplified by PCR. The primer sequences for these experiments were CD133; forward: 5′-CGGGATGGTGCCTT GAGTGA-3′ and reverse: 5′-GGAAGGCAAGCGTGTTCCTG-3′, CD44; forward: 5′-AATGGTCGCTACAGCATCTC-3′ and reverse: 5′-GCCCTT CTATGAACCCATACC-3′, CD24; forward: 5′-GGCTCGGGCTGGGGCT GCTG-3′ and reverse: 5′-GGCACCACCAGCCGCCTTG-3′, IGF-1R; forward: 5′-CTCCTGTTTCTCTCCGCCG-′3 and reverse: 5′-ATAGTCGTT GCGGATGTCGAT-3′, CDX2; forward: 5′-GAGCTGGAGAAGGAGTTT-3′ and reverse: 5′-GGTGACGGTGGGGTTTAG-3′, PTEN; forward: 5′-ACCAGGACCAGAGGGAAACCT-3′ and reverse: 5′-GCTAGCCTCTG GATTTGACG-3′.

2.5. In vivo-induced colon cancer and treatment

Male C57BL/6J mice (4-week-old) were purchased and acclimated for 1 week with free access to water and a pelleted diet and were housed under controlled conditions of humidity, light, and temperature. The mice were divided in six groups (each group contain 7 mouse) that include; healthy mice and 5 colon cancer induced groups that were treated with 1) no treatment, 2) regorafenib, 3) regorafenib and aspirin, 4) regorafenib and linsitinib, 5) regorafenib and linsitinib and aspirin. Induction of colon cancer was described previously (Kimura et al., 2020). All 5 colon cancer mice received a single intraperitoneal injection (ip) of Azoxymethane (AOM) (10 mg/kg body weight) on day 0. The drinking water containing Dextran Sodium Sulfate (DSS; 1.5% w/v) was administered on day 5 for 4 days ad libitum and was repeated with 15-day and 11-day interval. After the first DSS treatment, the mice were treated with regorafenib, linsitinib and aspirin according to divided groups and the control group was intraperitoneally injected saline and given distilled water.

2.6. Clinical symptoms

Clinical symptoms were evaluated using the disease activity index (DAI) twice a week after DSS treatment that includes body weight loss, stool blood status and survival. The treated and non-treated mice were euthanized by CO_2 asphyxiation at 16 after AOM injection and then, blood and tissue samples were taken. Their large bowels (from the ileocecal junction to the anal verge) were flushed with saline, and excised, then cut open longitudinally along the main axis, and tumor lesions (polypoid lesions with diameter ≤ 2 or > 2 mm) were counted (Song et al., 2020).

2.7. Inflammatory cytokines

The levels of IL-6, and IL-1β, TNF-α and MCP-1 in the colon tissues supernatant were measured using mouse ELISA kit, according to the manufacturer's instructions. Also, the IL-6, and IL-1β, TNF-α and MCP-1 levels in sera were assessed using ELISA kits.

2.8. Data analysis

First, the normal distribution of data was determined by Kolmogorov-Smirnov test.

Normal and parametric data were analyzed using SPSS software version 20 by t-test and one-way ANOVA test.

3. Result

3.1. IGF1-R inhibition has protective effects against colon cancer cells

The inhibitory effects of linsitinib and aspirin as the IGF1-R antagonists against colon cancer cells were assessed in SW48 colon cancer cell lines. The cells without linsitinib and aspirin treatment had resistance against regorafenib as chemotherapy and after 2 weeks had 88 ± 5% survival. After the cells were cultured and treated with aspirin, linsitinib, linsitinib and aspirin together, as was shown in Fig. 1, treatments had significant inhibitory effects against the colon cancer cells and survival of SW48 cell were decreased (71 ± 3, 42 ± 7 and 26 ± 4 respectively in 2nd week).

Fig. 2. Flow cytometry assay for CD133 and CD44 in treated SW48 colon cancer cell were assessed and presented.

3.2. IGF1-R antagonists inhibit the stem-cell like characteristics of colon cancer cells

Our results showed that treatment with linsitinib and aspirin could reduce the CD133 and CD44 as cancer stem cell markers compared with non-treated cell line (Fig. 2. The cells that were treated with linsitinib and aspirin together has the lowest percentage of CD133 and CD44 on surface and both CD 133 and CD 44 null cells were in the highest percentage (CD44-CD133-: 96.2 ± 2.1, CD44 + CD133-: 2.3 ± 1.3, CD44-CD133+: 1.3 ± 1.0, and CD44 + CD133+: 1.2 ± 1.1 in linsitinib and aspirin treated group).

3.3. IGF1-R antagonists change gene expression of the chemotherapy received SW48

Meanwhile, three colon cancer stem cell markers (CD133, CD44, CD24), two anti-cancer genes (CDX2, PTEN) and also one chemotherapy resistance gene (IGF-1R) were selected and their expression were analyzed after cultured the cells with regorafenib to produce resistance to the chemotherapy drug, were treated with linsitinib and aspirin. The results showed that the regorafenib resistanced SW48 had increased expression of CD133 (11.9 ± 1.3) , CD44 (14.7 ± 2.2), CD24 (4.3 ± 1.7) and IFG1-R (1 ± 0.2) compared to treated groups and treatment with linsitinib and aspirin together (CD133: 1.9 ± 1.3, CD44: 2.0 ± 1.6, CD24: 2.0 ± 1.1 and IFG1-R: 0.2 ± 0.1) had strong effect in decreasing of these markers and decreasing was significant ($p < 0.05$) compared with other treated groups (Fig. 3). Treatment with linsitinib and aspirin could increase CDX2 and PTEN gene expression (9.7 ± 1 and 2.1 ± 0.2 respectively) significantly ($p < 0.05$) in regorafenib received SW48 cells compared to non-treated regorafenib received SW48 cells (0.5 ± 0.6 and 0.7 ± 0.1 respectively).
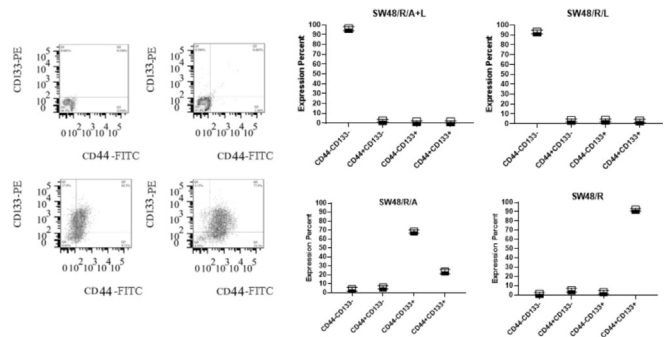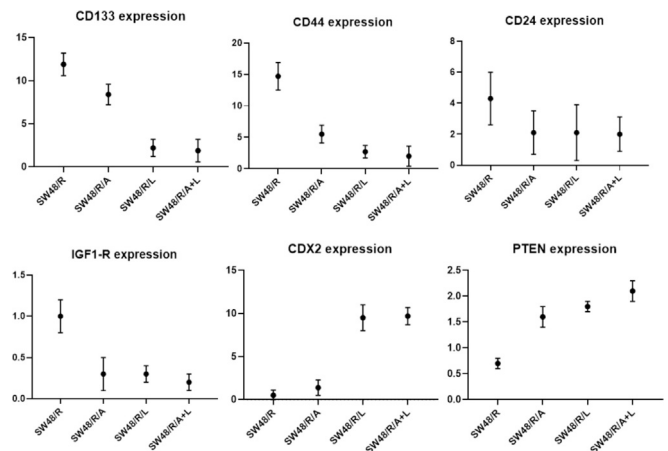

Fig. 3. The gene expression of the CD133, CD44, CD24, IGF1-R, CDX2 and PTEN in the SW48 colon cancer cell lines were studied by real time-PCR.

3.4. Body weight

Variations across of the body weight in all groups during the 15 weeks experiment were presented in Fig. 4. When the live weights of mice in the cancer group were compared with those of the healthy control group (21 ± 0.7, 21 ± 1 gr respectively), a decrease in weight was observed after during 15 weeks (15 ± 0.7, 28 ± 0.5 gr respectively) ($P < 0.05$). Treatment with the IGF1-R antagonists (linsitinib and aspirin) could prevent body weight losing in mice (week1: 21 ± 0.5, week15: 27 ± 0.5 gr).
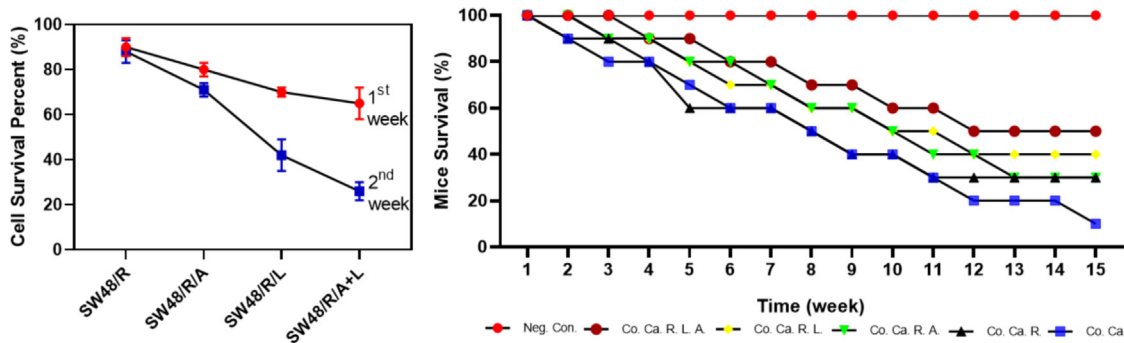

Fig. 1. The percentage of SW48 colon cancer cell line survival in the in-vitro study (in-vitro) and the survival study of the mice in 15 weeks was done and showed with survival percentage (in-vivo).
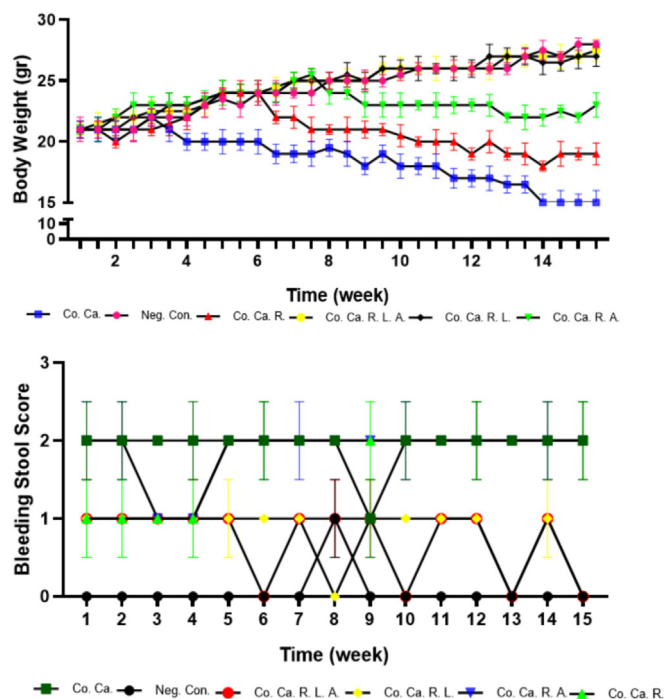
Fig. 4. Variations across of the body weight in all groups of the mice during the 15 weeks experiment and the fecal blood score was determined in mice during the treatment.

3.5. Fecal blood

Mice in the healthy group had no fecal blood during the 15 weeks experiment (score = 0 at all-time points). All non-treated cancerous mice showed gross bleeding (score = 2) in feces and the fecal blood score decreased during the treatment. The decreasing was notable in regorafenib-linsitinib treated group and regorafenib-linsitinib-aspirin treated group (Fig. 4).

3.6. Survival

According to survival study in 15 weeks, healthy group had one hundred percent survival but cancer group without any treatment had<10% in week 15. Treatment with regorafenib and regorafenib-aspirin had 30% survival in week 15 and also, treatment with regorafenib-linsitinib and regorafenib-linsitinib-aspirin had 40% and 50% survival in week 15 respectively (Fig. 1).

3.7. Number of tumors

The maximum number of tumors in colon was recorded in the non-treated cancer group (9 ± 1). Treatment could decrease tumor number with > 2 mm and < 2 mm diameter and total number. In the treated groups; total number of tumors was minimum in regorafenib, linsitinib, and aspirin treated group (1 ± 1) and the maximum regorafenib treated group (6 ± 1) that was shown in Fig. 5.

3.8. Inflammatory cytokines

The levels of IL-1β, IL-6, TNF-α and MCP-1 were increased in non-treated cancerous group (179.4 ± 21.4, 38.7 ± 0.8, 168.7 ± 8.4, 294.5 ± 28.4 pg/ml respectively) significantly (p < 0.05) compared healthy group (25.1 ± 3.4, 23.4 ± 0.5, 52.1 ± 2.1, 67.4 ± 9.6 pg/ml respectively) in serum. Treatment with regorafenib-aspirin, regorafenib-linsitinib and regorafenib-linsitinib-aspirin could reduce IL-1β (84.3 ± 9.2, 74.3 ± 11.4, 58.3
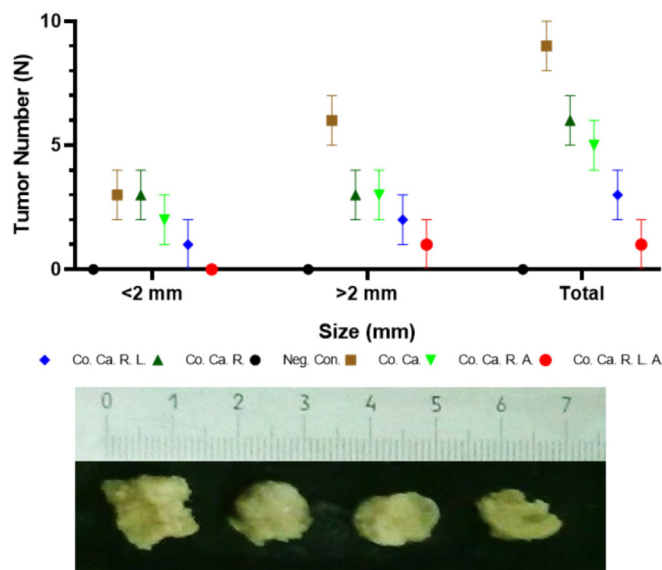

Fig. 5. The colon tissue tumors were excluded and counted. The maximum number of tumors and also with > 2 mm and < 2 mm diameter in colon were recorded in all groups. The four notable tumor were showed with their sizes.

± 21.7 pg/ml respectively), TNF-α (94.8 ± 5.9, 108.9 ± 14.6, 73.0 ± 7.3 pg/ml respectively) and MCP-1 (167.3 ± 27.3, 112.4 ± 20.0, 107. 3 ± 23.1 pg/ml respectively) levels significantly (p < 0.05).

The levels of IL-1β, IL-6, TNF-α and MCP-1 in the colon tissues were significantly increased in non-treated cancerous group compared healthy group (p < 0.05). Treatments significantly decreased IL-1β level compared non-treated cancer group (p < 0.05). Treatment with regorafenib-linsitinib-aspirin had the strongest effect in reduction of IL-1β, IL-6, TNF-α and MCP-1 in the colon tissues of the cancerous mice compared non-treated cancerous mice (Fig. 6).

4. Discussion

Many Invivo and Invitro studies and clinical studies have shown that increased IGF-1R activity is associated with proliferation, migration and invasion of colon cancer cells. Cell proliferation and differentiation is enhanced by IGF-1R signaling via the Ras/MAPK pathway. The IGF signaling system is composed of two ligands, IGF-1 and IGF-2, which act by binding to IGF-1R (primarily) and IGF-2R and the insulin receptor (IR), that all of them belong to the tyrosine kinase receptor family. By binding the ligand to IGF-R, the IGF-R receptor is activated by autophosphorylation and subsequently phosphorylates the insulin receptor substrate (IRS-1). Then activation of phosphoinositide 3-kinase (PI3K) resulted in an increase in phosphatidylinositol 3,4,5-triphosphate, which also
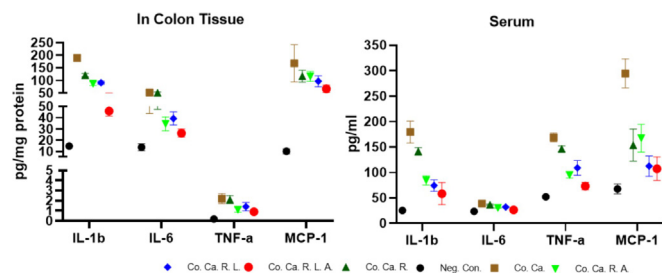

Fig. 6. The levels of IL-1β, IL-6, TNF-α and MCP-1 were measured by ELISA method in serum and the colon tissues of the studied mice.

activates the vital protein AKT/PKB (AKT) through phosphorylation. AKT is responsible for many functions, including the release of the anti-apoptotic Bcl2 protein from the Bad inhibitor, activation of protein synthesis by mTOR, and increased glucose metabolism by inhibiting GSK-3β, which is ultimately responsible for preventing cell death (Denduluri et al., 2015; Yavari et al., 2010). On the other hand, IGF-1R activates the SHC protein, which stimulates Raf protein via GTPase Ras. Raf protein triggers a kinase cascade that ultimately activates ERK-1 and ERK-2 mitogen-activating protein kinases. These MAPKs proteins cause phosphorylation and activation of several targets, in particular the transcription factor ELK1, which enhances gene expression and thus promotes cell growth (Denduluri et al., 2015; Yavari et al., 2010). Our results showed that the regorafenib resistanced cells had increased expression of CD133, CD44, CD24 as cancer stem cell markers and IFG1-R and treatment with linsitinib and aspirin together could decrease these molecules expression. Also, the treatment could increase CDX2 and PTEN gene expression as anti-cancer genes. This effects maybe related with inhibition of the IGF-1R signaling and with decreasing of resistance, lead to control of the cancer cells.

The cancer stem cells contribute to the development of chemotherapy-resistance in tumor. CD44 is an important prognosis biomarker and has a main role in transforming of the cancer cells to a cancer-stem like cells. Higher expression of CD44 in cancer has correlation with bad prognosis of tumor (Song et al., 2020; Zhou et al., 2018; Wang et al., 2020) and results of this study showed decreasing of CD44 in colon cancer cells. Cellular and molecular study in this research presented that treatment of the regorafenib resistance colon cancer cells with linsitinib and aspirin (as antagonists of the IGF1-R), not only tumor growth can be inhibited, but also, production of cancer stem cells may be harnessed.

In a 2015 study, Esanchez-lopez et al. Targeted colorectal cancer cells by inhibiting IGF1-R and inhibiting STAT3 signaling. Finally, the inhibitor was found to significantly reduce the amount of tumor, cancer-associated fibroblasts (CAF), and myeloid cells. Decreased CAF reduces the proliferation of cancer cells and increases apoptosis in cells (Sanchez-Lopez et al., 2016). In 2015, CatiaLippolis et al. investigated the association between IGF1 and the development of resistance to Regorafenib in HCC (hepatocellular carcinoma) cancer cells. The results showed that IGF-1 antagonizes the inhibitory effects of Regorafenib on cell growth, migration and invasion (Lippolis et al., 2015). In 2017, Chuanzongzhao et al. examined the effect of IGF-1 on the epithelial-mesenchymal transition (EMT) process that is performed by the STAT5 signaling pathway. This study found that in human HCC cells, the expression of N-Cadherin, Vimentin, Snail1, Snail2 and Twist was directly related to the expression of IGF-1, while the expression of E-cadherin was inversely related to the expression of IGF-1. As a result, IGF-1 stimulates the transition from epithelial to mesenchymal in HCC cells via the STAT5 signaling pathway (Zhao et al., 2017). Treatment with the IGF1-R antagonists could prevent body weight losing, decrease fecal blood score and increase survival. Also, number of tumors and inflammatory cytokines in the colon tissues and serum were reduced.

Eicosanoids (prostaglandins and thromboxanes) increase colon tumorigenesis possibly through chronic inflammation. It was suggested that acetylsalicylic acid prevent colorectal cancer, possibly through COX-mediated suppression of eicosanoid and PGE2, formation (Heike Gottschall et al., 2018). A study reported that ameliorated antioxidant supplementation such as juniper oil defenses against colon cancer as a chemopreventive dietary agent, inhibits COX-2 expression and induces apoptosis in colon tumor cells (Yaman et al., 2021). Therefore, manipulation of some genes by external molecules may have curable effect against cancer.

The AOM/DSS treated mice that were received JK5G as therapeutic agent, had up-regulated weight and food intake, and JK5G could inhibit growth of the colon tumor and cytokines levels in serum. Also, CD3CD4 T cells, and CD3CD8 T cells in the spleen of the JK5G mice were significantly increased. Similarly, B, T, and NK-T cells in the intestinal tumors of the JK5G mice were increased. The JK5G was involved in the intestinal microbiota and metabolite bands on different pathways (Weinan et al., 2020), which may influence on immune cells and inhibit tumor growth with immuneactivation and signaling pathways and in our study, inhibition of IGF1-R signaling with immunoregulation, had significant effect on control of tumor in booth in-vitro and in-vivo.

It was found that combined therapy with linsitinib that targets IGF-1R and also, another agent for NF-κB may provide a novel strategy to overcome the tumor's resistance to Linsitinib. The resistance to Linsitinib may be mediated by NF-κB activation and combined therapy (targets both IGF-1R and NF-κB) provides a novel treatment regime (Junzhou et al., 2017). Recognized harnessing pathways of cancer can be used with CAR-T cell as immune-target-therapy (Esmaeilzadeh et al., 2020). In this study, we obtained the best in-vitro and in-vivo result of colon cancer treatment when combinitation therapy (Linsitinib and Aspirin as IGF1-R antagonists) was used with Regorafenib, and this regime could prevent tumor resistance, stem cell producing, pathological interaction and disease activity index.

There were some limitation in this research. We did not survey the effect of linsitinib and aspirin on SW48 colon cancer cell line without regorafenib treatment. Also, in-vivo tumorogenecity of these cell lines as xenograft was not studied. Also, in the in-vivo study, we did not survey the histopathological factors, colon tissue lipid metabolite and oxylipin levels.

5. Ethics approval and consent to participate

All methods and animal study have been approved by ethical committee of animal house of ix.med.vet.dep, 2021 (No. IX.MED.VET.DEP.REC.2021.300121.9).

6. Authors' contributions

YG, EMN, FH, SSA participated in the design, examination, analysis and drafting the manuscript. YG and SSA supervised the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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