Targeting EGFR sensitizes 5-Fu-resistant colon cancer cells through modification of the IncRNA-FGD5-AS1-miR-330-3p-Hexokinase 2 axis

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INTRODUCTION

Colorectal cancer (CRC), which is one of the most diagnosed malignancies, is a leading cause for cancer-related mortality worldwide.1 Although the therapeutic approaches for colon cancer have been improved, the overall 5-year survival rate of CRC patients is still under that expected.2 5-Fluorouracil (5-Fu), which has been applied for CRC therapy for more than four decades, is a palliative and adjuvant anti-cancer agent by interfering with the nucleotide synthesis of cancer cells.2,3 Despite the impressive clinical responses initially, gradual development of 5-Fu resistance severely limits its applications.4 Thus, investigating the underlying molecular mechanisms for the progress of 5-Fu resistance and developing novel chemotherapeutic agents are critical tasks for the clinical administration of 5-Fu-resistant CRC patients.

Long noncoding RNAs (lncRNAs), which have no protein coding capacity, are a family of RNAs with relatively larger size (~200 nucleotides in length).5 Accumulating evidence has revealed that lncRNAs are dysregulated in diverse cancers and play essentially oncogenic or oncogenic roles during the oncogenesis, development, chemoresistance, prognosis, and survival of CRC patients.6 lnc-FGD5-AS1 (IncRNA FYVE, RhoGEF, and PH domain containing 5 antisense RNA 1) is a noncoding transcript that has been reported to be upregulated in multiple malignancies to facilitate tumor progression such as proliferation, metastasis, and chemoresistance.7,8 In colon cancer, FGD5-AS1 was known to promote the carcinogenesis, migration, and metastasis of tumor cells,8 suggesting that targeting FGD5-AS1 would contribute to development of anti-colon cancer therapeutic strategies. Currently, the precise molecular mechanisms of IncRNA-FGD5-AS1 in 5-Fu resistance of CRC are still under investigation.

Epidermal growth factor receptor (EGFR; ErbB-1/HER1), which belongs to the ErbB family, is a receptor tyrosine kinase.7 Activation of EGFR signaling is critical for various biological events.1,7 Importantly, most of the colorectal cancers associated with EGFR overexpression are diagnosed with high risk of metastasis and recurrence.9,10 Moreover, studies have unveiled that EGFR promoted tumorigenicity and 5-Fu resistance in human CRC.11 Thus, EGFR is a potential therapeutic target against chemoresistance. Cancer cells, distinct from normal cells, display particular metabolic features in that they demand higher aerobic glycolysis for rapid proliferations.12 This phenomenon is known as the “Warburg effect” and widely recognized as a hallmark.
of new therapeutic approaches for treatment of 5-Fu-resistant CRC will be examined by metabolism of colon cancer cells. The molecular pathway of the demonstrated that EGFR and FGD5-AS1 promoted the cellular glucose metabolism of colon cancer cells.

In this study, we aimed to investigate the biological roles and molecular mechanisms of EGFR and FGD5-AS1 in regulating 5-Fu resistance in colon cancer cells. Interestingly, dynamic bioenergetic results from in vitro and in vivo xenograft experiments. Our study will benefit the development of new therapeutic approaches for treatment of 5-Fu-resistant CRC patients.

**RESULTS**

**EGFR and IncRNA-FGD5-AS1 are positively associated with 5-Fu resistance in colon cancer**

As a well-characterized oncogene, EGFR was known to promote drug resistance, leading to impaired chemotherapy outcomes in cancer patients. To evaluate the roles of EGFR in 5-Fu-resistant CRC, we compared the expression levels of EGFR in 5-Fu-sensitive and -resistant CRC patients (Table 1). qRT-PCR results showed that the expressions of EGFR were significantly elevated in colon tumors and 5-Fu-resistant CRC tumors compared with normal colon tissues and 5-Fu-sensitive colon tumors, respectively (Figures 1A and 1B). Consistent results from immunohistochemical (IHC) staining of EGFR demonstrated that EGFR was remarkably elevated in 5-Fu-resistant CRC tumors (Figure 1C). In addition, we detected that IncRNA-FGD5-AS1, which has been reported as an oncogenic noncoding RNA in diverse cancers, was apparently elevated in CRC tissues and cell lines compared with normal colon tissues and cells (Figure 1D; Figure S1). Expectedly, FGD5-AS1 was significantly upregulated in 5-Fu-resistant colon cancer specimens (Figure 1E). These results suggested that targeting EGFR and FGD5-AS1 in colon cancer may facilitate the efficiency of 5-Fu treatment. To elucidate the regulatory roles between EGFR and FGD5-AS1 in colon cancer, we analyzed the co-expression pattern between them from starBase. A positivity correlation between EGFR and FGD5-AS1 was observed (Figure 1F). Silencing EGFR by small interfering RNA (siRNA) obviously downregulated FGD5-AS1 expression in HCT-116 and DLD-1 colon cancer cells (Figures 1G and 1H). Meanwhile, overexpression of EGFR significantly stimulated FGD5-AS1 expression (Figures 1I and 1J), indicating that EGFR positively regulates FGD5-AS1 in colon cancer cells.

To evaluate the functions of EGFR and FGD5-AS1 in the 5-Fu-mediated anti-cancer effects, EGFR or FGD5-AS1 was silenced by siRNA in DLD-1 cells. As we expected, CRC cells with knockdown of EGFR exhibited increased 5-Fu sensitivity under multiple concentrations of 5-Fu treatments (Figure 2A). The IC50 (Half-maximal inhibitory concentration) of DLD-1 cells with lower EGFR was dropped from 7.58 µM (control cells) to 2.2 µM (Figure 2A). Consistently, blocking FGD5-AS1 significantly sensitized DLD-1 cells to 5-Fu (Figures 2B and 2C), suggesting that EGFR and FGD5-AS1 positively correlated with 5-Fu resistance in CRC. To further investigate the mechanisms underlying the EGFR- and FGD5-AS1-mediated 5-Fu resistance, we established a 5-Fu-resistant CRC cell line (DLD-1 5-Fu Res) by continuous treatment of cells with concentrations of 5-Fu gradually elevated from 2 µM to 20 µM. After 3 months, resistant cell clones were selected and pooled. Consistent results from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic assay, and flow cytometry assay showed that 5-Fu-resistant cells could survive under higher concentrations of 5-Fu (Figures 2D–2F). Furthermore, expressions of EGFR and FGD5-AS1 were significantly elevated in 5-Fu-resistant cells (Figures 2G–2I). In summary, these results demonstrated that EGFR and FGD5-AS1 contribute to 5-Fu resistance in colon cancer cells.

**5-Fu-resistant CRC cells display increased glucose metabolism**

Accumulating evidence has revealed that cancer cells display dysregulated glucose metabolism. Moreover, drug-resistant cancer cells exhibited an elevated glycolysis rate. To gain an in-depth understanding of the mechanisms behind the EGFR-mediated 5-Fu resistance, we compared the glycolysis rates in 5-Fu-resistant and parental colon cancer cells. Interestingly, dynamic bioenergetic results from

| Characteristic | Cases | Percentages |
|---------------|------|-------------|
| **Age**       |      |             |
| <20           | 11   | 13.75%      |
| 20–50         | 47   | 58.75%      |
| >50           | 22   | 27.5%       |
| **Gender**    |      |             |
| male          | 37   | 46.25%      |
| female        | 43   | 53.75%      |
| **Differentiate** |    |             |
| well          | 19   | 23.75%      |
| moderately    | 37   | 46.25%      |
| poorly        | 20   | 25%         |
| undifferentiated | 4  | 5%          |
| **Stage**     |      |             |
| I             | 15   | 18.75%      |
| II            | 36   | 45%         |
| III           | 22   | 27.5%       |
| IV            | 7    | 8.75%       |
| **Metastasis**|      |             |
| no            | 51   | 63.75%      |
| yes           | 29   | 36.25%      |
| **5-Fu response** |    |             |
| sensitive     | 40   | 50%         |
| resistant     | 40   | 50%         |

The results demonstrated that EGFR and FGD5-AS1 contribute to 5-Fu resistance in colon cancer cells.
the Seahorse analyzer demonstrated that the extracellular acidification rate (ECAR) was significantly increased in DLD-1 5-Fu Res cells compared with that in DLD-1 parental cells (Figure 3A). In addition, the glucose consumption rate, which reflects the overall utilization of glucose, was apparently increased in 5-Fu-resistant cells (Figure 3B). Consistently, the expressions of HK2 and lactate dehydrogenase A (LDHA), which are key metabolic enzymes to catalyze the speed-limiting steps of glucose metabolism, were significantly upregulated in 5-Fu-resistant cells (Figure 3C).

**EGFR and FGD5-AS1 promote glycolysis rate of colon cancer cells**

We next asked whether glycolysis enzymes were regulated by EGFR and FGD5-AS1. Figures 3D and 3E show that blocking EGFR significantly attenuated glucose uptake (Figure 3D) and lactate product (Figure 3E) of DLD-1 and HCT-116 cells. Consistently, knockdown of FGD5-AS1 significantly decreased glucose uptake and lactate product (Figures 3F and 3G). To further investigate whether the upregulated glycolysis accounted for the 5-Fu resistance, we examined the 5-Fu sensitivity of DLD-1 5-Fu Res cells without or with silencing HK2. Results revealed that blocking glycolysis significantly resensitized 5-Fu-resistant cells to 5-Fu (Figure 3H), suggesting that targeting the upregulated glycolysis of 5-Fu-resistant cells might contribute to the development of therapeutic agents against drug resistance.

**miR-330-3p is downregulated by FGD5-AS1 and negatively associates with 5-Fu resistance**

A number of studies have demonstrated that lncRNAs function as competing endogenous RNA (ceRNA) of microRNAs (miRNAs) via sponging with them. Thus, we investigated the downstream miRNAs of FGD5-AS1 in colon cancer cells. A miRNA microarray was performed to identify differentially expressed miRNAs by FGD5-AS1 (Figure 4A). Intriguingly, we detected that miR-330-3p, which acts as a tumor suppressor by suppressing diverse oncogenes, was significantly upregulated by FGD5-AS1 silencing (Figure 4A). Results from microarray were further validated in four colon cancer cell lines (Figure 4B). qRT-PCR results showed that miR-330-3p was remarkably suppressed in colon tumor tissues as well as CRC cell lines compared with normal colon tissues and cells (Figure 4C). miR-330-3p was significantly downregulated in 5-Fu-resistant colon tumor specimens as well as 5-Fu-resistant DLD-1 cells (Figures 4D and 4E), suggesting that miR-330-3p plays essential roles in 5-Fu-resistant CRC. We thus hypothesized that exogenous overexpression of miR-330-3p could facilitate the
anti-tumor effects of 5-Fu. Results from cell viability assay clearly demonstrated that 5-Fu-resistant cells with miR-330-3p overexpression were more sensitive to 5-Fu (Figure 4F). Taken together, the above results elucidated that FGD5-AS1 downregulates miR-330-3p, which functions in suppressive roles in 5-Fu-resistant colon cancer cells.

miR-330-3p is sponged by FGD5-AS1 in colon cancer cells

We next tested whether FGD5-AS1 could bind to the seeding region of miR-330-3p as a ceRNA. Bioinformatics analysis suggested that one fragment of FGD5-AS1 potentially binds to miR-330-3p (Figure 5A). Overexpression of FGD5-AS1 significantly suppressed miR-330-3p expression in CRC cells (Figure 5B). As we expected, a significantly negative correlation between FGD5-AS1 and miR-330-3p was observed in CRC tumors (Figure 5C). To validate the association between FGD5-AS1 and miR-330-3p, RNA pull-down assay was performed and the results demonstrated that miR-330-3p specifically associated with FGD5-AS1 antisense probe (Figure 5D). In addition, luciferase reporter vector containing original (WT) or miR-330-3p binding site mutated (Mut) FGD5-AS1 was co-transfected with negative control or miR-330-3p into DLD-1 and HCT-116 cells. Results illustrated that overexpression of miR-330-3p blocked the luciferase activity of vector containing FGD5-AS1-WT (Figures 5E and 5F), but transfection of miR-330-3p failed to suppress luciferase activity of vector containing binding site mutant FGD5-AS1 (Figures 5E and 5F). In summary, these results demonstrated that FGD5-AS1 downregulates miR-330-3p through functioning as a ceRNA of miR-330-3p.

miR-330-3p suppresses glycolysis through direct targeting of HK2

The above results revealed that miR-330-3p was involved in the EGFR/FGD5-AS1-mediated 5-Fu resistance in colon cancer. We next explored the potential targets of miR-330-3p. By searching the miRNA databases seeking the putative miR-330-3p targets that may possibly regulate glycolysis, we found that the mRNA analysis database TargetScan predicted that the 3’ UTR of key glycolysis enzyme HK2 contains miR-330-3p binding sites (Figure 6A). To examine whether miR-330-3p could suppress HK2 expression in colon cancer cells, we transfected the control group (pre-miR-330-3p) or anti-miR-330-3p into DLD-1 cells. We detected that overexpression of miR-330-3p significantly downregulated miR-330-3p through functioning as a ceRNA of miR-330-3p.
the HK2 expression (Figure 6B, bottom). We then assessed whether miR-330-3p could directly bind on the 3' UTR of HK2. Luciferase assay was performed by co-transfecting a pMIR reporter-luciferase vector containing original sequence (WT) or predicted binding site mutant (Mut) sequence of HK2 3' UTR with miR-330-3p or negative control miRNA. Overexpression of miR-330-3p significantly decreased the luciferase activity of the vector containing WT 3' UTR of HK2 (Figure 6C). Meanwhile, no suppressive effects of miR-330-3p on the luciferase activity of vector containing binding site mutant HK2 3' UTR were detected (Figure 6C), indicating that miR-330-3p directly targets HK2 in colon cancer cells.

Expressions of HK2 were detected to be significantly elevated in CRC specimens compared with normal colon tissues (Figure 6D), suggesting that HK2 plays tumor-favoring roles in colon cancer. Consistently, significantly negative correlations between miR-330-3p and HK2 or EGFR mRNA were detected from clinical colon tumors (Figure 6E; Figure S3). To validate whether miR-330-3p inhibits glycolysis through direct targeting of HK2, we performed rescue experiments by transfection of control miRNA, miR-330-3p alone, or miR-330-3p plus HK2 overexpression plasmid into DLD-1 cells. Western blot results illustrated successful recovery of HK2 expression in miR-330-3p-overexpressing cells (Figure 6F). Importantly, recovering HK2 in miR-330-3p-overexpressed colon cancer cells successfully rescued the glucose uptake and ECAR (Figures 6G and 6H). We exposed the above transfected DLD-1 cells with miR-330-3p overexpression or HK2 recovery to multiple concentrations of 5-Fu. Cell viability assay and apoptosis assay in Figures 6I and 6J clearly demonstrated that DLD-1 cells with restoration of HK2 re-acquired 5-Fu resistance compared with miR-330-3p-transfected cells. Consistent results were observed in another colon cancer cell line, HCT-116 (Figures S4A and S4B). Taken together, these results demonstrated that miR-330-3p promoted 5-Fu sensitivity of colon cancer cells through direct targeting of HK2.

Targeting the EGFR-FGD5-AS1-miR-330-3p-HK2 axis overrides 5-Fu resistance of CRC cells in vitro and in vivo

Given that EGFR and miR-330-3p inversely modulate 5-Fu sensitivity of colon cancer, we examined whether EGFR accelerated 5-Fu resistance through suppressing the miR-330-3p-HK2 axis. We transfected DLD-1 cells with control or FGD5-AS1 alone or plus miR-330-3p. Cells were then treated with control or the EGFR inhibitor erlotinib. DLD-1 cells with erlotinib treatment displayed significantly upregulated miR-330-3p and downregulated FGD5-AS1 and HK2 expression (Figures 7A and 7B; Figure S5). Overexpression of FGD5-AS1 blocked the erlotinib-induced miR-330-3p and recovered the HK2 expression (Figures 7A and 7B). Moreover, these results were further blocked by restoration of miR-330-3p in FGD5-AS1-overexpressing cells (Figures 7A and 7B). Consistent results from the rescue experiments were observed that glucose uptake and lactate product of DLD-1 cells were significantly affected by the above transfection and treatments (Figures 7C and 7D). To evaluate the synergistic anti-colon cancer effect of EGFR inhibitor plus 5-Fu, the above transfected DLD-1 cells were subjected to elevated concentrations of 5-Fu treatment. Overexpression of FGD5-AS1 protected CRC cells under the combined treatment of erlotinib plus 5-Fu, whereas recovery of miR-330-3p effectively reversed the FGD5-AS1-protected CRC cell death by erlotinib plus 5-Fu treatments (Figures 7E and 7F). As we expected, the DLD-1 5-Fu-resistant cells displayed similar synergistic anti-cancer effects of the combined treatment of erlotinib plus 5-Fu, a phenotype that
was overcome by FGD5-AS1 overexpression (Figures 7G and 7H). Consequently, the above phenotypes from DLD-1 5-Fu-resistant cells were reversed by restoration of miR-330-3p in FGD5-AS1-overexpressing cells (Figures 7G and 7H). In summary, the above results consistently demonstrated that the EGFR-promoted 5-Fu resistance was through the FGD5-AS1-miR-330-3p-HK2 axis.

To validate the above proposed molecular pathway in vivo, a subcutaneous xenograft model was established. DLD-1 5-Fu-resistant cells were subcutaneously injected into the right flank of BALB/c nude mice. Once xenograft tumors were established, mice were separated into four groups with the following treatments: control, 5-Fu alone, erlotinib alone, and the combination of 5-Fu with erlotinib via intraperitoneal (i.p.) injection twice a week. Most of the saline- or 5-Fu-treated mice died within 2 months (Figure 8A). Although erlotinib-treated mice exhibited slightly improved survival rates, the combination of 5-Fu with erlotinib achieved a significantly improved survival rate (Figure 8A). Accordingly, the results in Figure 8B showed that mice undergoing the combined treatment grew much smaller tumors than control, 5-Fu, or erlotinib alone treated mice (Figures 8B and 8C). The body weights of mice with control, 5-Fu, or erlotinib alone treatment were slightly increased because of the development of tumors, whereas the body weights of mice with combined treatments showed not significant changes (Figure 8D), suggesting that the combined treatments effectively inhibited xenograft tumor growth. Furthermore, mice tumors were collected and subjected to qRT-PCR analysis. Results clearly demonstrated that the expressions of FGD5-AS1, HK2, and LDHA were suppressed in xenograft tumors with 5-Fu plus EGFR inhibitor treatments (Figures 8E–8G; Figure S6). Consistent with in vitro results, miR-330-3p was apparently upregulated in xenograft tumors with erlotinib plus 5-Fu treatment (Figure 8H). In summary, these xenograft experiments validated that mice bearing xenograft tumors from 5-Fu-resistant colon cancer cells were more sensitive to the combined treatment of EGFR inhibition plus 5-Fu through blocking the FGD5-AS1-miR-330-3p-HK2 axis.

DISCUSSION
This study demonstrated a molecular pathway for the EGFR-promoted 5-Fu resistance in colon cancer. We demonstrated EGFR positively regulated FGD5-AS1 expression in CRC tumors and cells. Moreover, 5-Fu-resistant cells exhibited enhanced glycolysis rate by EGFR through the upregulation of HK2. Further mechanistic investigations highlighted the associations of lncRNA-FGD5-AS1, miR-330-3p, and glycolysis enzyme HK2 in 5-Fu-resistant CRC. The Warburg effect is defined as cancer cells exhibiting more dependence on anaerobic glycolysis, glutaminolysis, and fatty acid synthesis for abnormal progression than normal cells.15,18 Moreover, a study revealed that glycolysis was increased in 5-Fu-resistant cancer cells,15 suggesting that the upregulated glycolysis by EGFR is one of the mechanisms of 5-Fu resistance in cancer. As an oncogene, EGFR is upregulated in 5-Fu-resistant cancer cells and CRC patient specimens, indicating that inhibition of the EGFR-promoted glycolysis might be a potent target for overcoming
5-Fu resistance. As such, our results are consistent with the idea that chemoresistance may be a metabolic phenomenon that can be overcome by inhibition of glycolysis.

Accumulating evidence has revealed that lncRNA-FGD5-AS1 plays important roles in the tumorigenesis and progression of diverse malignancies including colon cancer. Among them, FGD5-AS1 was upregulated in lung cancer cells and promoted cell proliferation through sponging hsa-miR-107 to upregulate FGFRL1. In addition, FGD5-AS1 enhanced the malignant phenotypes of esophageal squamous cell carcinoma by functioning as a ceRNA on microRNA-383. Furthermore, FGD5-AS1 promoted osteosarcoma cell proliferation and migration by targeting the miR-506-3p-RAB3D axis. Another study consistently uncovered oncogenic roles of FGD5-AS1 in accelerating cell proliferation of pancreatic cancer through regulating the miR-520a-3p-KIAA1522 axis. In colon cancer, FGD5-AS1 could induce cancer development, suggesting that targeting FGD5-AS1 could be a potential therapeutic strategy against colon cancer.

Recent studies elucidated that lncRNAs acted as a ceRNA of miRNAs through direct binding to the seed region of miRNAs, leading to de-repression of miRNA targets. In this study, bioinformatics analysis indicated a potential association between FGD5-AS1 and miR-330-3p, which was validated by luciferase assay, suggesting that FGD5-AS1 promotes CRC progressions by acting as a ceRNA of miR-330-3p, a tumor suppressor that is downregulated in multiple cancers such as liver cancer, gastric cancer, colon cancer, and pancreatic cancer. In accordance with numerous reports that miR-330-3p functioned as a tumor suppressor in cancers, we observed a significantly downregulated expression of miR-330-3p in 5-Fu-resistant cancer cells. miR-330-3p was shown to be significantly downregulated by EGFR. Moreover, our data illustrated that miR-330-3p is a downstream effector of FGD5-AS1: silencing FGD5-AS1 promoted miR-330-3p expression in colon cancer cells. Since miR-330-3p drives tumor suppressive phenotypes, its upregulation by an EGFR inhibitor uncovers an important therapeutic approach for overcoming 5-Fu resistance in colon cancer.

HK2 is known to be overexpressed in various cancers and involved in maintenance of tumor malignancies. Overexpression of HK2 in 5-Fu-resistant colon cancer cells provided cancer cells with growth advantages via elevated glycolysis. Here, we identified that HK2 was a direct target of miR-330-3p in CRC cells. Interestingly, our study integrates signal pathways to describe a novel molecular axis, EGFR-FGD5-AS1-miR-330-3p-HK2, to overcome 5-Fu resistance by glycolysis inhibition from in vitro and in vivo models.

Currently, the molecular mechanisms underlying the EGFR-promoted FGD5-AS1 are not evaluated yet. Studies have demonstrated that the promoters and the transcription factor (TF) binding sites within their promoters of lncRNAs are highly conserved, indicating a strictly regulatory mechanism that controls lncRNA transcription. In addition, accumulating evidence has revealed that lncRNAs are regulated by multiple TFs that directly bind on the promoter region of lncRNAs, such as p53 and c-MYC. We have analyzed the promoter region of FGD5-AS1 from the UCSC database and PROMO and observed
multiple oncogenic TFs such as c-MYC, HIF1-α, and c-Jun that were known EGFR downstream effectors in cancers, suggesting that EGFR might upregulate downstream oncogenic TFs to directly bind on the promoter region of FGD5-AS1, leading to promotion of its transcription. However, the precise TFs and the specific binding site on FGD-AS1 promoter are still under investigation. Our ongoing project will continue to investigate the EGFR-mediated lncRNA/miRNA signal pathways for enhancing the therapeutic effects of traditional anti-cancer drugs. Our results will contribute to improving the current understanding of the regulatory network of oncogene, lncRNA, mRNAs, glucose metabolism, and drug resistance in colon cancer.

MATERIALS AND METHODS

Patient sample collections

The current study was approved by the Institutional Review Board of the China-Japan Union Hospital of Jilin University. All participants gave written informed consent. A total of 40 5-Fu-resistant colon tumor specimens and 40 5-Fu-sensitive colon tumors were collected from CRC patients who underwent surgery at the Department of General Surgery, China-Japan Union Hospital of Jilin University from July 2014 to June 2017. The 5-Fu-sensitive and -resistant groups were classified according to the following standards: After diagnosis, patients were suggested for surgery. After surgery, patients were continually given regular 5-Fu treatment for 3 periods (3 weeks for each period). After the total 9-week 5-Fu treatment, all patients were subjected to a “whole abdominal enhanced computed tomography (CT)” inspection to evaluate the effects of chemotherapy. No tumor found was defined as “sensitive.” “Resistant” was defined as positive recurrence or metastatic tumor that was found in the gastrointestinal tract after the 9-week 5-Fu treatment. Patient samples from surgery were frozen immediately by liquid nitrogen and then transferred to /C0-80/C. No radio- or/and chemotherapies were received before surgery. All participants gave written informed consent. IHC assessment of EGFR protein expressions was conducted according to previous reports.30

Cells and cell culture

The human colon cancer cell lines LoVo, HCT-116, HT-29, and DLD-1 were obtained from the American Type Culture Collection (ATCC). DLD-1 resistant cells were selected by stepwise increases in 5-Fu concentrations from 2 to 40 μM. 5-Fu-resistant clones were collected and pooled for downstream analysis. Resistance of colon cancer cells was monitored by treating cells with 5-Fu at the described concentrations for 48 h.; cell viability was determined by MTT assay (I) and Annexin V apoptosis assay (J). Columns, mean of three independent experiments; bars, SD. *p < 0.05; **p < 0.01; ***p < 0.001.

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(A) Prediction from TargetScan.org indicated the 3' UTR of HK2 contains putative binding sites of miR-330-3p. (B) DLD-1 cells were transfected with control miRNA, pre-miR-330-3p (top), or anti-miR-330-3p (bottom). Protein expressions of HK2 were examined by western blot. (C) DLD-1 cells were co-transfected with luciferase vector containing WT or binding site mutant 3' UTR of HK2 plus control miRNA or pre-miR-330-3p for 48 h. The luciferase activities were measured. (D) miRNA expressions of HK2 were examined in normal colon tissues and CRC tumor tissues. (E) A significant inverse correlation between HK2 mRNA and miR-330-3p was detected in human colon tumors. (F) DLD-1 cells were transfected with pre-miR-330-3p alone or co-transfected with pre-miR-330-3p plus HK2 for 48 h. HK2 protein expressions were detected. (G and H) Glucose uptake (G) and ECAR (H) were examined from the above transfected cells. (I and J) The above transfected cells were treated with 5-Fu at the described concentrations for 48 h.; cell viability was determined by MTT assay (I) and Annexin V apoptosis assay (J). Columns, mean of three independent experiments; bars, SD. *p < 0.05; **p < 0.01; ***p < 0.001.

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serum (FBS) (Sigma-Aldrich, Shanghai, China) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) at 37°C with 5% CO2.

**Antibodies and reagents**

Rabbit anti-EGFR antibody (#2232), rabbit anti-LDHA monoclonal antibody (#3582), rabbit anti-Hexokinase2 monoclonal antibody (#2867), and rabbit anti-β-actin (#4967) antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Overexpression plasmid containing open reading frame (ORF) of EGFR was obtained from OriGene.com (RC217384). siEGFR, siFGD5-AS1, siHK2, and control siRNA were obtained from Ambion (Austin, TX, USA); shFGD5-AS1 and control shRNA were constructed by Hanbio (Shanghai, China); pre-miR-330-3p, anti-miR-330-3p, and negative control were purchased from GenePharma (Shanghai, China). 5-Fu was purchased from Sigma-Aldrich (F8423, Carlsbad, CA, USA).

**Cell viability assay**

CRC cells were treated with 5-Fu at various concentrations for 48 h. Cells (1 × 10⁴ cells/well) were seeded in a 48-well plate in 0.2 mL of RPMI-1640 containing 10% FBS overnight. Cells were refreshed with serum-free medium and cultured for 24 h. Cells were then treated with 5-Fu at the indicated concentrations. Cell viability was examined by MTT assay (Sigma-Aldrich, Shanghai, China). Briefly, MTT was added to each well and incubated at 37°C for 2 h. Cell culture medium was refreshed, and DMSO was added to each well to solubilize the crystals. Absorbance was recorded at 540 nm by a microplate reader. The relative viability was calculated by the absorbance at 540 nm of 5-Fu-treated cells/that of untreated cells. Experiments were performed in triplicate and repeated three times.

**Flow cytometry analysis of cell death**

Cells stained with Annexin V-propidium iodine (PI) were analyzed to assess the cell apoptosis. Colon cancer cells were collected and washed with cold PBS, followed by incubation with 5 μL of PI and 5 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin V (Bio-Legend, San Diego, CA, USA) at room temperature for 15 min with light protection. Bind buffer (400 μL) from the Annexin V-PI apoptosis kit was added into cells, followed by fluorescence-activated cell sorting (FACS) analysis using CellQuest Research Software (Largo, FL, USA). Experiments were performed in triplicate and repeated three times.

**Prediction of lncRNA-microRNA and microRNA-mRNA interactions**

The predictions of IncRNA-FGD5-AS1 with miR-330-3p and miR-330-3p with HK2 interactions were performed from starBase of ENCORI and TargetScan, respectively. The correlation between EGFR and FGD5-AS1 expression in colon cancer was analyzed from starBase of ENCORI.31

Figure 7. EGFR promotes 5-Fu resistance through the FGD5-AS1-miR-330-3p-HK2 axis

(A) DLD-1 cells were transfected with control or FGD5-AS1 alone or plus miR-330-3p. Cells were treated with control or erlotinib for 48 h. The expression of miR-330-3p was measured by qRT-PCR. (B) HK2 expressions from the above cells were analyzed by western blot. (C and D) The glucose uptake (C) and lactate product (D) from the above transfected cells were measured. (E and F) The above cells were treated without or with 5-Fu at the indicated concentrations for 48 h; cell viability and apoptosis were determined by MTT assay and Annexin V assay (F), respectively. (G and H) DLD-1 5-Fu-resistant cells were transfected with control or FGD5-AS1 alone or plus miR-330-3p for 48 h; cells were treated with control or erlotinib alone or plus 5-Fu at the indicated concentrations, and cell viability and apoptosis were determined by MTT assay (G) and Annexin V assay (H), respectively. Columns, mean of three independent experiments; bars, SD. **p < 0.01; ***p < 0.001.
siRNA, shRNA, plasmid DNA, and miRNA transfections

siRNA, vector containing wild-type EGFR, or HK2 was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In brief, 8 × 10^5 cells were plated in 6-well plates for 24 h. siRNA (100 nM) or plasmid DNA (2 μg) was diluted in Opti-MEM I Reduced Serum Medium, followed by mixing with Lipofectamine 2000 reagent at a total volume of 250 μL. After 15 min incubation at room temperature, DNA- or siRNA-lipid complex was formed, and the mixture was added into cell culture medium. After 48 h, cells were collected for downstream experiments. miRNA precursors and antisense miRNAs were purchased from Applied Biosystems. Transfection of negative control miRNA, pre-miRNAs, precursors and antisense miRNAs were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). qRT-PCR reactions were conducted with the TaqMan Universal PCR Master Mix reagent (Invitrogen, Shanghai, China). The quantity and quality of RNA samples were examined by a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qRT-PCR reactions were conducted with the TaqMan Universal PCR Master Mix reagent (Invitrogen, Carlsbad, CA, USA) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers for qRT-PCR were designed according to the sequences of FGD5-AS1-WT and FGD5-AS1-Mut. Mature miR-330-3p expression was detected with the TaqMan MicroRNA Assay kit (Invitrogen, Carlsbad, CA, USA). Luciferase assays were performed with the pMIR-REPORT dual-luciferase vector (Invitrogen, Carlsbad, CA, USA). U6 small nuclear RNA was used as an internal control. The RT-PCR reaction settings were 25 cycles at 95°C for 30 s, 56°C for 1 min. The relative expressions were analyzed by the 2^-ΔΔCt method. Experiments were performed in triplicate and repeated three times.

RNA isolation and real-time PCR

Total RNA was isolated from CRC cells or patient samples with TRIzol reagent (Invitrogen, Shanghai, China). The quantity and quality of RNA samples were examined by a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qRT-PCR reactions were conducted with the TaqMan Universal PCR Master Mix reagent (Invitrogen, Carlsbad, CA, USA) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers for qRT-PCR were designed according to the sequences of FGD5-AS1-WT and FGD5-AS1-Mut. Mature miR-330-3p expression was detected with the TaqMan MicroRNA Assay kit (Invitrogen, Carlsbad, CA, USA). U6 small nuclear RNA was used as an internal control. The RT-PCR reaction settings were 25 cycles at 95°C for 30 s, 56°C for 1 min. The relative expressions were analyzed by the 2^-ΔΔCt method. Experiments were performed in triplicate and repeated three times.

Luciferase reporter assay

The sequences of FGD5-AS1-WT and FGD5-AS1-Mut were synthesized according to wild-type and miR-330-3p binding site mutant sequences of FGD5-AS1. FGD5-AS1-WT or -Mut was inserted into the pMIR-REPORT dual-luciferase vector (Invitrogen, Carlsbad, CA, USA). Wild-type or binding site mutant 3' UTR of HK2 was synthesized and inserted into the pMIR-REPORT dual-luciferase vector (Invitrogen, Carlsbad, CA, USA). U6 small nuclear RNA was used as an internal control. The RT-PCR reaction settings were 25 cycles at 95°C for 30 s, 56°C for 1 min. The relative expressions were analyzed by the 2^-ΔΔCt method. Experiments were performed in triplicate and repeated three times.
with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was examined with the Dual-Glo luciferase kit (#E2940, Promega). Experiments were performed in triplicate and repeated three times.

Measurement of glucose metabolism
Cells were plated onto a 12-well plate at a density of $1 \times 10^5$ per well. The ECAR was measured with a glycolysis stress kit (Agilent, Santa Clara, CA, USA) on a Seahorse XFp Analyzer (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. Equal numbers of cells from each well were analyzed. The glucose uptake assay were performed with a glucose test kit (Applygen Technologies, Beijing, China) according to the manufacturer’s instructions. Results were normalized by the ratio of results from the experimental group to those from the control group. Experiments were performed in triplicate and repeated three times.

Western blotting
Colon cancer cells were collected and lysed at 4°C in radioimmuno-precipitation assay buffer buffer plus Protease Inhibitor Cocktail (Sigma-Aldrich, Shanghai, China) for 20 min on ice. Proteins were extracted by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentrations were determined with the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were separated by a 10% SDS-poly-acrylamide gel followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk in phosphate-buffered saline with Tween (PBST) for 1 h. Membranes were incubated with primary antibodies at 4°C overnight. After extensive washing, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:3,000) for 1 h at room temperature. After additional washes with PBST, proteins were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Shanghai, China). β-actin was an internal control.

In vivo xenograft experiments
Mouse experiments were approved by the Institutional Animal Care and Use Committee of the China-Japan Union Hospital of Jilin University. A total of forty 8-week-old nude mice were maintained in regular 12/12-h light-dark cycle cages. Mice were subcutaneously injected with DLD-1 5-Fu-resistant cells ($5 \times 10^6$) to establish xenograft tumors, which were monitored every 3 days until sizes of tumors were $>100 \text{ mm}^3$. Mice were randomly separated into 4 groups (10 mice per group). Mice from each group were treated with (1) PBS control; (2) 5-Fu alone (40 mg/kg i.p., 2 times/week); (3) erlotinib alone (40 mg/kg i.p., 2 times/week) or (4) 5-Fu plus erlotinib. Mortality was monitored daily. Tumor volumes were determined by Vernier caliper and calculated by the formula (width)$^2 \times$length/2 every 3 days. After 50 days, mice were euthanized by the CO2 method. Xenograft tumors were dissected and frozen in liquid nitrogen immediately. The xenograft mouse protocol was performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Statistical analysis
Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Analysis of the difference between two groups was performed by unpaired Student’s t test. Significance among three or more groups was analyzed by two-way ANOVA followed by post hoc analysis. All experiments were performed in triplicate and repeated three times. All data are shown as mean ± standard deviation (SD). p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.06.012.

ACKNOWLEDGMENTS
The authors sincerely thank all the doctors, research faculties, and staffs from the Department of General Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin. The authors also thank the reviewers and editors for critical and thoughtful comments for this paper.

AUTHOR CONTRIBUTIONS
S.-J.G., S.-N.R., and Y.-T.L. analyzed and interpreted the results. S.-J.G. wrote the manuscript. X.-B.C. designed the study. S.-J.G., S.-N.R., Y.-T.L., and H.-W.Y. carried out the experiments. S.-J.G., S.-N.R., Y.-T.L., and H.-W.Y. performed the statistical analysis.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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