Published in final edited form as:

J Genet Genomics. 2017 August 20; 44(8): 375–383. doi:10.1016/j.jgg.2017.04.008.

The role of *GLI2-ABCG2* signaling axis for 5Fu resistance in gastric cancer

Beiqin Yu^{1,2}, Dongsheng Gu², Xiaoli Zhang², Bingya Liu^{1, #}, and Jingwu Xie^{2, #}

¹Shanghai Key Laboratory of Gastric Neoplasms, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

²Departments of Pediatrics, Biochemistry and Molecular Biology, Pharmacology and Toxicology, The Wells Center for Pediatrics Research and IU Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, USA

Abstract

Gastric cancer is a leading cause of cancer-related mortality worldwide, and options to treat gastric cancer are limited. Fluorouracil (5Fu)-based chemotherapy is frequently used as a neoadjuvant or an adjuvant agent for gastric cancer therapy. Most patients with advanced gastric cancer eventually succumb to the disease despite the fact that some patients respond initially to chemotherapy. Thus, identifying molecular mechanisms responsible for chemotherapy resistance will help design novel strategies to treat gastric cancer. In this study, we discovered that residual cancer cells following 5Fu treatment have elevated expression of hedgehog target genes *GLI1* and *GLI2*, suggestive of hedgehog (Hh) signaling activation. Hh signaling, a pathway essential for embryonic development, is an important regulator for putative cancer stem cells/ residual cancer cells. We found that high *GLI1/GLI2* expression is associated with some features of putative cancer stem cells, such as increased side population. We demonstrated that *GLI2* knockdown sensitized gastric cancer cells to 5Fu treatment, decreased *ABCG2* expression, and reduced side population. Elevated *GLI2* expression is also associated with an increase in tumor sphere size, another marker for putative cancer stem cells. We believe that *GLI2* regulates putative cancer stem cells through direct regulation of *ABCG2*. *ABCG2* can rescue the *Gli2* shRNA effects in 5Fu response, tumor sphere formation and side population changes, suggesting that *ABCG2* is an important mediator for *GLI2*-associated 5Fu resistance. The relevance of our studies to gastric cancer patient care is reflected by our discovery that high *GLI1/GLI2/ABCG2* expression is associated with a high incidence of cancer relapse in two cohorts of gastric cancer patients who underwent chemotherapy (containing 5Fu). Taken together, we have identified a molecular mechanism by which gastric cancer cells gain 5Fu resistance.

[#]Corresponding authors. jinxie@iu.edu (J. Xie); liubingya@sjtu.edu.cn (B. Liu).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

5Fu; Gastric cancer; ABCG2; Hedgehog; Gli2

1. INTRODUCTION

Gastric cancer is the third leading cause of cancer-related deaths, with nearly 1 million cases a year and about 700,000 deaths worldwide (McLean and El-Omar, 2014; Razzak, 2014; Ferlay et al., 2015; Torre et al., 2015). The incidence of gastric cancer varies significantly in different geographic regions, with about 70% of the incidence in Eastern Asia. As a major solid tumor, gastric cancer development involves complex genetic and environment interactions.

Chemotherapeutic intervention is commonly used in the neoadjuvant, adjuvant, or primary treatment options of advanced gastric cancer whereas surgical resection is the curable therapy for early cancer (Cunningham et al., 2006). Despite the decline in gastric cancer incidence in the last few decades, the percentage of gastric cancer with metastasis has been on the rising between 1990 and 2011 (Bernards et al., 2013). The median survival of advanced gastric cancer remains 15–17 months (Orditura et al., 2014; Proserpio et al., 2014). While some patients respond initially to chemotherapy, almost all advanced gastric cancer patients eventually develop relapsed disease. Therefore, drug resistance is a major barrier to achieve effective gastric cancer treatment.

The first-line chemotherapy of gastric cancer includes 5Fu, cisplatin, and epirubicin according to NCCN guidelines (Ajani et al., 2013; Yuan et al., 2014). Furthermore, improved chemotherapeutic agents, including taxanes (docetaxel and paclitaxel), oral fluoropyrimidines (capecitabine and S-1), oxaliplatin and irinotecan are emerging.

Fluorouracil (5Fu), an analog of uracil with a fluorine atom substituted at the carbon-5 position of the pyrimidine ring in place of hydrogen, fulfills the expectations of biochemical, pharmacologic, and clinical activities of anticancer drugs. The 5-fluorinated pyrimidines have been widely used in the treatment of breast, gastric, colorectal, pancreatic cancers, and squamous cell carcinomas arising in the head and neck (Papanastasopoulos and Stebbing, 2014). The primary mechanisms of action for 5Fu include 1) incorporation of fluorouridine triphosphate into RNA to interfere with RNA synthesis and function, 2) inhibition of thymidylate synthase, 3) incorporation of fluorodeoxyuridine triphosphate and deoxyuridine triphosphate into DNA, and 4) genotoxic stress to trigger programmed cell death pathways.

Resistance to 5Fu in gastric cancer is a major clinical problem. While there are a number of mechanisms reported to be responsible for drug resistance (Domingo-Domenech et al., 2012; Keysar et al., 2013; Zahreddine et al., 2014; Della Corte et al., 2015; Alonso et al., 2016), activation of hedgehog (Hh), wnt and notch signaling pathways is particularly appealing as it is consistent with the “cancer stem cell (CSC)” theory (Yang et al., 2010; Nozawa et al., 2013; Basset-Seguín et al., 2015). Like wnt and notch signalings, Hh signaling is an important regulator for embryonic development, tissue polarity, cell differentiation and cancer development (Feldmann et al., 2007; Domingo-Domenech et al.,

2012; Steg et al., 2012a, b; Zahreddine et al., 2014). Thus, specific inhibitors for these signaling pathways may be used to sensitize cancer cells to 5Fu treatment. However, the significance of Hh signaling (or wnt and notch signalings) for 5Fu resistance in gastric cancer has not been well established.

To elucidate the underlying mechanism for 5Fu resistance in gastric cancer, we evaluated target gene expression of the Hh pathway in the residual cells following 5Fu treatment. We discovered the activation of the Hh-ABCG2 signaling axis in 5Fu-treated cells. We further demonstrated the significance of Hh signaling for regulation of side population. Similar studies were also conducted using acquired 5Fu resistant cell line N87/FR. We demonstrated the significance of *GLI2* for 5Fu resistance. This mechanistic study may help develop effective strategies against 5Fu resistance in gastric cancer.

2. RESULTS

2.1. 5Fu treatment influences Hh signaling and CSC properties in gastric cancer

Based on cancer stem cell theory (Wang and Dick, 2005; Cho and Clarke, 2008), we predicted that residual cancer cells after chemotherapy may contain high levels of drug resistant genes. We first measured the half maximal inhibitory concentration (IC₅₀) for 5Fu in gastric cancer N87 cells based on the cell viability in the presence of different concentrations of 5Fu (Fig. 1A). We then treated N87 cells with 5 μ M of 5Fu (about the IC₅₀ dose) for 48 h, and examined expressions of *GLI1* and *GLI2*, two important transcriptional factors for the Hh signaling pathway. We found elevated expressions of *GLI1* and *GLI2* in 5Fu-treated cells. In particular, the relative level of *GLI2* expression in the treated cells was over 15 times higher than that of the untreated control cells (Fig. 1B), suggesting that cells with activated Hh signaling survive better after 5Fu treatment. The elevated expressions of *GLI1* and *GLI2* appear to be specific to 5Fu treatment because we did not see significant changes in many other genes including *JAG2* and *TGF β 2* (data not shown here).

Because Hh signaling is critical for the maintenance of putative cancer stem cells or residual cancer cells (Reya et al., 2001; Taipale and Beachy, 2001; Takebe et al., 2015), we first examined several features of putative cancer stem cells (Fukamachi et al., 2011; Ishimoto et al., 2011; Wang et al., 2011; Chen et al., 2012; Jiang et al., 2012a; Chen et al., 2013; Dong et al., 2013; Rassouli et al., 2016) following 5Fu treatment in N87 cells. The side population is often enriched in cancer stem cells, and *ABCG2* is the major gene regulating side population (Jiang et al., 2012b). We found that 5Fu treatment for N87 cells caused an increase of side population (2.42% for untreated cells and 8.60% for treated cells) (Fig. 1C), indicating that residual cancer cells (or putative cancer stem cells) are enriched after 5Fu treatment. This result is consistent with that the elevated expressions of *GLI1* and *GLI2* following 5Fu treatment.

Tumor sphere formation is a known biological readout of cancer stem cells (Weiswald et al., 2015). We found that the tumor spheres (secondary) formed from the 5Fu-treated N87 cells are much bigger than that of the control cells (Fig. 1D), implying the high cancer stem cell activity after 5Fu treatment.

Both side population and tumor sphere size were increased following 5Fu treatment in N87 cells, indicating that elevated Hh signaling may be responsible for the maintenance of residual cancer cells (or putative cancer stem cells) following 5Fu treatment in gastric cancer cells.

2.2. 5-Fu resistant N87 cell keeps high level of Hh signaling and CSC properties

Elevated Hh signaling in cells after 5Fu treatment indicates the role of Hh signaling for intrinsic drug resistance in N87 cells. In the meantime, we established an acquired 5Fu resistant cell line, N87/FR, from the parental N87 cells through many rounds of 5Fu treatment with increased concentrations of 5Fu. N87/FR has an IC₅₀ of more than 15 μ M, whereas the parental cells have an IC₅₀ of about 5 μ M (Fig. 2A). We also examined gene expressions of *GLI1* and *GLI2*, and found elevated levels of *GLI1* and *GLI2* in N87/FR in comparison with the parental N87 cells, indicating activated Hh signaling in 5Fu resistant cells (Fig. 2B). Similarly, we found that N87/FR has a higher percentage of side population, and significantly larger tumor spheres in comparison with N87 cells (Fig. 2C and D).

Thus, elevated Hh signaling appears to be associated with an increased side population and bigger tumor spheres in both situations: after 5Fu treatment or in acquired resistant cells. The former represents intrinsic resistance to 5Fu, whereas the latter represents acquired resistance to 5Fu.

2.3. Knockdown of *GLI2* affects the CSC properties

To evaluate the functional relevance of Hh signaling for drug resistance in N87 cells, we first knocked down *GLI2* expression by expressing *GLI2* shRNAs (sh*GLI2*) in the 5Fu resistant N87/FR cells. We discovered that knocking down *GLI2* not only reduced *GLI2* expression by 80%, but also decreased *GLI1* transcript (>70%) (Fig. 3A), which is consistent with the notion that *GLI2* regulates *GLI1* expression in a number of biological systems (Motoyama et al., 1998; Ikram et al., 2004; Li et al., 2012). Down-regulation of *GLI2* in N87/FR cells reduced the IC₅₀ from 15 μ M to about 6 μ M (Fig. 3B), which is similar to the IC₅₀ of the parental N87 cells.

In consistent with reduced expressions of *GLI1* and *GLI2* after *GLI2* shRNA expression, we found that *GLI2* shRNA also reduced the side population (6.11% in N87/FR and 3.23% in N87/FR/sh*GLI2*) (Fig. 3C). We also found that *GLI1* knockdown significantly reduced the size of tumor spheres (Fig. 3D).

These data indicate that *GLI2* is a major trigger for the 5Fu resistance in gastric cancer cells, probably through regulation of residual cancer cells/cancer stem cells.

2.4. Regulation of *ABCG2* by Hh signaling in 5Fu resistance cells in gastric cancer

It is known that *ABCG2* is responsible for exclusion of Hoechst 33342 dye and the subsequent formation of side population. We examined *ABCG2* expression following 5Fu treatment, and found that 5Fu treatment caused an increase of *ABCG2* expression in parental N87 cells (both transcript and protein) (Fig. 4A and B). Similarly, acquired resistant N87/FR cells have elevated *ABCG2* expression (Fig. 4C and D).

Conversely, sh*GLI2* significantly reduced *ABCG2* expression, at both the RNA level and the protein level in N87/FR cells (Fig. 4E and F), indicating that *ABCG2* is regulated by *GLI2* in gastric cancer cells.

We believe that *GLI1/2* transcription factors can direct *ABCG2* expression through transcriptional regulation. First, a GLI-binding consensus site (Yang et al., 2010) appears in the *ABCG2* promoter (data not shown), suggesting that *ABCG2* may be a transcriptional target of *GLI* molecules. Furthermore, previous studies in lymphomas showed that *GLI* proteins may directly regulate *ABCG2* expression by transcriptional regulation (Singh et al., 2011). We have obtained similar results in gastric cancer cells (unpublished data).

From these data, we conclude that *GLI2* can directly regulate *ABCG2* expression in gastric cancer cells, leading to an increase in side population.

2.5. Overexpression of *ABCG2* rescues the CSC properties

We predict that if *ABCG2* is the major mediator for *GLI2*-associated 5Fu resistance, ectopic expression of *ABCG2* in sh*GLI2*-expressing N87/FR cells should increase drug resistance. As shown in Fig. 5, overexpression of *ABCG2* (Fig. 5A) increased the IC₅₀ of 5Fu by 4 folds (Fig. 5B), which is associated with an increase in side population (Fig. 5C) and sphere formation (Fig. 5D).

The effect of ectopic *ABCG2* expression on the IC₅₀ of 5Fu was very similar to that from elevated *GLI2* expression, suggesting that *ABCG2* appears to be the major mediator for regulating drug sensitivity in gastric cancer. In consistent with our hypothesis, ectopic expression of *ABCG2* had no significant effects on expression of *GLI1*, *GLI2* and *PTCH1* in N87 cells (unpublished data), further confirming that Hh signaling is upstream of *ABCG2* in our experiment system.

2.6 Relevance of the *GLI2-ABCG2* signaling axis in human gastric cancer

The relevance of our data in N87 and N87/FR cells to gastric cancer patients was reflected by analysis of cancer relapse in two cohorts of patients with gastric cancer (<http://www.cbioportal.org>). We correlated the high or low expressions of *GLI1/GLI2/ABCG2* in the tumor with cancer relapse in patients with chemotherapy (all patients had 5Fu treatment), which is the standard care for gastric cancer patients. We found that the patients with high *GLI1/GLI2/ABCG2* levels in the tumor had 40.5% tumor recurrence. In contrast, those patients with low *GLI1/GLI2/ABCG2* expressions had only 32.9% with relapse (Table 1). The odd ratio for cancer relapse in high *GLI1/GLI2/ABCG2* expression group is 1.7308. In another cohort, we found that those patients with high *GLI1/GLI2/ABCG2* had 30.8% with cancer relapse, while the patients with low expression of *GLI1/GLI2/ABCG2* had 17.8% with relapsed cancer. The odd ratio of cancer relapse with high *GLI1/GLI2/ABCG2* group in the second cohort is 1.2319. These data indicate that high expressions of *GLI1/GLI2/ABCG2* increase the risk of cancer relapse in gastric cancer patients with chemotherapy.

Taken all the data together, our study reveals a new mechanism responsible for 5Fu resistance in gastric cancer. We found the activation of the *GLI2-ABCG2* signaling axis in

residual cancer cells following 5Fu treatment and in 5Fu resistant cell line N87/FR. Down-regulation of *GLI2* sensitized cancer cells to 5Fu treatment. We believe that *GLI2* mediates cancer cell resistance to 5Fu through direct regulation of *ABCG2*, resulting in changes in side population and tumor sphere formation. The relevance of our data to gastric cancer patients is confirmed further by the increasing risk of cancer relapse in patients with high *GLI1/GLI2/ABCG2* expressions in the primary tumor.

3. DISCUSSION

As a major contributor for cancer-related mortality, most patients are diagnosed with advanced disease that the five-year survival rate is very low (<5%) (Ferlay et al., 2015). Chemotherapy with cisplatin and 5-FU has been the first-line treatment option for advanced gastric cancer, but most patients develop cancer relapse after initial treatment. Although the regulatory mechanisms for chemotherapy resistance in gastric cancer have been reported in the last ten years (Yu and Xie, 2016), little data have been linked with the mechanisms to cancer relapse. Our results indicate that the *GLI2-ABCG2* signaling axis is an important mechanism regulating side population and 5Fu resistance in gastric cancer cells. Our data show that knocking down *GLI2* will sensitize cancer cells to 5Fu treatment. More importantly, we have shown that high *GLI1/GLI2/ABCG2* expressions are associated with an increasing risk of developing cancer relapse in gastric cancer patients who underwent 5Fu based chemotherapy (Table 1). In addition, we have investigated the expression of *ABCG2* in patients who underwent chemotherapy (with cisplatin and 5Fu), and found that high *ABCG2* expression is associated with poor survival of the patients (data not shown). Since inhibitors for *GLI1* and *ABCG2* are already available, we predict that these novel reagents, together with chemotherapy, will improve the overall survival of gastric cancer patients.

As a critical regulator for embryonic development and cancer cell stemness maintenance in a number of cancer types (Reya et al., 2001; Taipale and Beachy, 2001; Yang et al., 2010; Liu et al., 2014; Takebe et al., 2015), Hh signaling has been associated with drug resistance in previous studies (Liu et al., 2014; Della Corte et al., 2015; Xu et al., 2015) although the underlying molecular mechanisms are not known. Our studies link *GLI2* to *ABCG2* to side population, and thus further promote our understanding of *GLI2*-mediated drug resistance. We found that the ligands, Shh and Ihh, were not significantly altered by 5Fu in N87 cells (data not shown), suggesting that up-regulation of *GLI1/2* was not caused by canonical Hh signaling. This implies that the SMO antagonists, such as vismodegib (Rimkus et al., 2016), will not be effective in sensitizing gastric cancer cells to chemotherapy. Targeting *ABCG2*, on the other hand, will be a more feasible strategy to improve the sensitivity of gastric cancer cells to 5Fu. Although *ABCG2* functions to transport several types of small molecules, such as dyes and some chemotherapeutic drugs, 5Fu transport is not affected by *ABCG2* (Sarkadi et al., 2004; Yoon et al., 2016), confirming that high *ABCG2* expression may represent cancer stem cell population (side population).

4. MATERIALS AND METHODS

4.1. Cell lines

The human gastric cancer cell line NCI-N87 (subsequently referred to as N87) was purchased from the American Type Culture Collection (ATCC, USA) and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. N87 cells were then maintained in 20 µM 5-Fluorouracil (5Fu, Sigma, USA) for approximately three months to obtain the stable resistant cell line named N87/FR.

4.2. Cell viability

Cells (4000/well) were seeded into 96-well plates the day before, culture medium was replaced by fresh medium with or without various concentrations of 5Fu (5, 10, 25, 50, 100 µM) for incubation 48 h, then stained using alamar Blue (Thermo scientific, USA) as an indicator of cell viability according to the manufacturer's instructions. Six wells were counted for each drug concentration and at least three independent experiments were done. The IC₅₀ value was defined as the concentration that resulted in a 50% reduction in cell growth compared with growth of the control.

4.3. RNA extraction, RT-PCR and quantitative PCR (qPCR)

Total RNA isolation from cells was extracted using Trizol reagent (Invitrogen, USA) and reverse transcribed into cDNA using the First-Strand Synthesis Kit (Roche, USA) according to the manufacturer's instructions. qPCR analyses were conducted according to 40 cycles of PCR amplification (95°C for 10 s, 55°C for 15 s and 72°C for 20 s) in an ABI7500 detection system (Applied Biosystems, USA) using Master mix (Roche) with the following probes ordered from Applied Biosystems: *GLI1* (Hs01110766), *GLI2* (Hs00257977), *ABCG2* (Hs01053790) and *GAPDH* (Hs03929097).

4.4. Lentiviral infection

GLI2 knockdown plasmid of pLKO.1/sh*GLI2* was stored at our lab, and *ABCG2* overexpression plasmid pSIN4/*ABCG2* was purchased from Addgene (#25983, USA). For viral package, plasmids PRRE, RSV/REV, CMVG and pLKO.1/sh*GLI2* or pSIN4/*ABCG2* were cotransfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). After 48 h, lentiviral supernatant was filtered through a 0.45-µm filter, supplemented with 10 µg/mL polybrene, and used to infect N87/FR or N87/FR sh*GLI2* cells. Twenty-four hours after infection, cells were selected with 1 µg/mL puromycin (Sigma) for two weeks.

4.5. Flow cytometry analyses

Single cells were trypsinized using Accutase (Gibco, USA), re-suspended in PBS containing 10% FBS, incubated with PE-conjugated *ABCG2* (1:100 dilution, Biolegend, USA) for 30 min at 4°C and analyzed on a FACS Canto II (Beckton Dickinson, USA) using Flowjo software after washes.

4.6. Side population assay

For side population, single cells were re-suspended at a concentration of 1×10^6 /mL in RPMI-1640 with 2% FBS and 10 mM HEPES, stained with 5 μ g/mL Hoechst 33342 (Invitrogen) and incubated at 37°C for 90 min with shaking. After washing with ice-cold HBSS containing 2% FBS and 10 mM HEPES, cells were dispersed in ice-cold HBSS containing 2 μ g/mL propidium iodide (PI, Invitrogen). Negative control was conducted by adding ABC transporter inhibitor fumitremorgin C (FTC, 10 μ M, Calbiochem, USA) before addition of Hoechst 33342.

4.7. Sphere formation

Cells were dissociated using Accutase, re-suspended in sphere formation medium which was made of Neural Basal medium with 1×10^{-8} M B-27, 20 ng/mL of EGF, 10 ng/mL of bFGF and 5 μ g/mL of heparin, and seeded on ultralow attachment 24-well plates in a concentration of 2000 cells each well (Corning, USA). Sphere formation medium was changed every other day and the cells in suspension culture were observed and counted under microscope. The sphere forming efficiency was calculated by counting the number of spheres formed from 2000 cells.

4.8. Statistics

Experimental data were expressed as the mean $\pm$ SD. Unpaired *t* test was used to determine the differences between two groups. All statistical analyses were performed using the SPSS 15.0 software. A two-tailed value of *P* less than 0.05 was considered to be statistically significant. Odd ratio was calculated according to a previously described formula (Persoskie and Ferrer, 2017).

Acknowledgments

This work was supported by National Cancer Institute (R01CA155086), The Wells Center for Pediatric Research, Riley Children Foundation, Jeff Gordon Children's Foundation and IU Simon Cancer Center. This work was also funded by grants from the National Natural Science foundation of China (Nos. 91529302 and 81472641), Key Projects in the National Science & Technology Pillar Program of China (No. 2014BAI09B03).

References

- Ajani JA, Bentrem DJ, Besh S, D'Amico TA, Das P, Denlinger C, Fakih MG, Fuchs CS, Gerdes H, Glasgow RE, Hayman JA, Hofstetter WL, Ilson DH, Keswani RN, Kleinberg LR, Korn WM, Lockhart AC, Meredith K, Mulcahy MF, Orringer MB, Posey JA, Sasson AR, Scott WJ, Strong VE, Varghese TK Jr, Warren G, Washington MK, Willett C, Wright CD, McMillian NR, Sundar H. National Comprehensive Cancer, Network. Gastric cancer, version 2.2013: featured updates to the NCCN Guidelines. *J. Natl. Compr. Canc. Netw.* 2013; 11:531–546. [PubMed: 23667204]
- Alonso S, Hernandez D, Chang YT, Gocke CD, McCray M, Varadhan R, Matsui WH, Jones RJ, Ghiaur G. Hedgehog and retinoid signaling alters multiple myeloma microenvironment and generates bortezomib resistance. *J. Clin. Invest.* 2016; 126:4460–4468. [PubMed: 27775549]
- Basset-Seguín N, Sharpe HJ, de Sauvage FJ. Efficacy of Hedgehog pathway inhibitors in Basal cell carcinoma. *Mol. Cancer Ther.* 2015; 14:633–641. [PubMed: 25585509]
- Bernards N, Creemers GJ, Nieuwenhuijzen GA, Bosscha K, Pruijt JF, Lemmens VE. No improvement in median survival for patients with metastatic gastric cancer despite increased use of chemotherapy. *Ann. Oncol.* 2013; 24:3056–3060. [PubMed: 24121120]

- Chen T, Yang K, Yu J, Meng W, Yuan D, Bi F, Liu F, Liu J, Dai B, Chen X, Wang F, Zeng F, Xu H, Hu J, Mo X. Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. *Cell Res.* 2012; 22:248–258. [PubMed: 21727908]
- Chen W, Zhang X, Chu C, Cheung WL, Ng L, Lam S, Chow A, Lau T, Chen M, Li Y, Nie Y, Wong BC, Pang R. Identification of CD44+ cancer stem cells in human gastric cancer. *Hepatogastroenterology.* 2013; 60:949–954. [PubMed: 23478146]
- Cho RW, Clarke MF. Recent advances in cancer stem cells. *Curr. Opin. Genet. Dev.* 2008; 18:48–53. [PubMed: 18356041]
- Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ, Participants MT. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N. Engl. J. Med.* 2006; 355:11–20. [PubMed: 16822992]
- Della Corte CM, Bellevicine C, Vicidomini G, Vitagliano D, Malapelle U, Accardo M, Fabozzi A, Fiorelli A, Fasano M, Papaccio F, Martinelli E, Troiani T, Troncone G, Santini M, Bianco R, Ciardiello F, Morgillo F. *SMO* gene amplification and activation of the Hedgehog pathway as novel mechanisms of resistance to anti-epidermal growth factor receptor drugs in human lung cancer. *Clin. Cancer Res.* 2015; 21:4686–4697. [PubMed: 26124204]
- Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V, Castillo-Martin M, Quinn SA, Rodriguez-Barrueco R, Bonal DM, Charytonowicz E, Gladoun N, de la Iglesia-Vicente J, Petrylak DP, Benson MC, Silva JM, Cordon-Cardo C. Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell.* 2012; 22:373–388. [PubMed: 22975379]
- Dong J, Li J, Liu SM, Feng XY, Chen S, Chen YB, Zhang XS. CD33(+)/p-STAT1(+) double-positive cell as a prognostic factor for stage IIIa gastric cancer. *Med. Oncol.* 2013; 30:442. [PubMed: 23307253]
- Feldmann G, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, Karikari C, Alvarez H, Iacobuzio-Donahue C, Jimeno A, Gabrielson KL, Matsui W, Maitra A. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer Res.* 2007; 67:2187–2196. [PubMed: 17332349]
- Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer.* 2015; 136:E359–386. [PubMed: 25220842]
- Fukamachi H, Shimada S, Ito K, Ito Y, Yuasa Y. CD133 is a marker of gland-forming cells in gastric tumors and Sox17 is involved in its regulation. *Cancer Sci.* 2011; 102:1313–1321. [PubMed: 21457403]
- Ikram MS, Neill GW, Regl G, Eichberger T, Frischauf AM, Aberger F, Quinn A, Philpott M. GLI2 is 17 expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *J. Invest. Dermatol.* 2004; 122:1503–1509. [PubMed: 15175043]
- Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, Oshima M, Ikeda T, Asaba R, Yagi H, Masuko T, Shimizu T, Ishikawa T, Kai K, Takahashi E, Imamura Y, Baba Y, Ohmura M, Suematsu M, Baba H, Saya H. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. *Cancer Cell.* 2011; 19:387–400. [PubMed: 21397861]
- Jiang J, Zhang Y, Chuai S, Wang Z, Zheng D, Xu F, Zhang Y, Li C, Liang Y, Chen Z. Trastuzumab (herceptin) targets gastric cancer stem cells characterized by CD90 phenotype. *Oncogene.* 2012a; 31:671–682. [PubMed: 21743497]
- Jiang Y, He Y, Li H, Li HN, Zhang L, Hu W, Sun YM, Chen FL, Jin XM. Expressions of putative cancer stem cell markers ABCB1, ABCG2, and CD133 are correlated with the degree of differentiation of gastric cancer. *Gastric Cancer.* 2012b; 15:440–450. [PubMed: 22395309]
- Keysar SB, Le PN, Anderson RT, Morton JJ, Bowles DW, Paylor JJ, Vogler BW, Thorburn J, Fernandez P, Glogowska MJ, Takimoto SM, Sehr DB, Gan GN, Eagles-Soukup JR, Serracino H, Hirsch FR, Lucia MS, Thorburn A, Song JI, Wang XJ, Jimeno A. Hedgehog signaling alters reliance on EGF receptor signaling and mediates anti-EGFR therapeutic resistance in head and neck cancer. *Cancer Res.* 2013; 73:3381–3392. [PubMed: 23576557]

- Li ZJ, Nieuwenhuis E, Nien W, Zhang X, Zhang J, Puviindran V, Wainwright BJ, Kim PC, Hui CC. Kif7 regulates Gli2 through Sufu-dependent and -independent functions during skin development and tumorigenesis. *Development*. 2012; 139:4152–4161. [PubMed: 23034632]
- Liu Z, Xu J, He J, Zheng Y, Li H, Lu Y, Qian J, Lin P, Weber DM, Yang J, Yi Q. A critical role of autocrine sonic hedgehog signaling in human CD138+ myeloma cell survival and drug resistance. *Blood*. 2014; 124:2061–2071. [PubMed: 25049282]
- McLean MH, El-Omar EM. Genetics of gastric cancer. *Nat. Rev. Gastroenterol. Hepatol*. 2014; 11:664–674. [PubMed: 25134511]
- Motoyama J, Liu J, Mo R, Ding Q, Post M, Hui CC. Essential function of *Gli2* and *Gli3* in the formation of lung, trachea and oesophagus. *Nat. Genet*. 1998; 20:54–57. [PubMed: 9731531]
- Nozawa YI, Lin C, Chuang PT. Hedgehog signaling from the primary cilium to the nucleus: an emerging picture of ciliary localization, trafficking and transduction. *Curr. Opin. Genet. Dev*. 2013; 23:429–437. [PubMed: 23725801]
- Oritura M, Galizia G, Sforza V, Gambardella V, Fabozzi A, Laterza MM, Andreozzi F, Ventriglia J, Savastano B, Mabilia A, Lieto E, Ciardiello F, De Vita F. Treatment of gastric cancer. *World J. Gastroenterol*. 2014; 20:1635–1649. [PubMed: 24587643]
- Papanastopoulos P, Stebbing J. Molecular basis of 5-fluorouracil-related toxicity: lessons from clinical practice. *Anticancer Res*. 2014; 34:1531–1535. [PubMed: 24692679]
- Persoskie A, Ferrer RA. A most odd ratio::interpreting and describing odds ratios. *Am. J. Prev. Med*. 2017; 52:224–228. [PubMed: 27639787]
- Proserpio I, Rauseri S, Barzaghi S, Frattini F, Galli F, Iovino D, Rovera F, Boni L, Dionigi G, Pinotti G. Multimodal treatment of gastric cancer. *World J. Gastrointest. Surg*. 2014; 6:55–58. [PubMed: 24829622]
- Rassouli FB, Matin MM, Saeinasab M. Cancer stem cells in human digestive tract malignancies. *Tumour Biol*. 2016; 37:7–21. [PubMed: 26446457]
- Razzak M. Genetics: new molecular classification of gastric adenocarcinoma proposed by The Cancer Genome Atlas. *Nat. Rev. Clin. Oncol*. 2014; 11:499. [PubMed: 25113841]
- Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–111. [PubMed: 11689955]
- Rimkus TK, Carpenter RL, Qasem S, Chan M, Lo HW. Targeting the sonic Hedgehog signaling pathway: review of smoothened and GLI inhibitors. *Cancers (Basel)*. 2016; 8:pii, E22.
- Sarkadi B, Ozvegy-Laczka C, Nemet K, Varadi A. ABCG2 -- a transporter for all seasons. *FEBS Lett*. 2004; 567:116–120. [PubMed: 15165903]
- Singh RR, Kunkalla K, Qu C, Schlette E, Neelapu SS, Samaniego F, Vega F. ABCG2 is a direct transcriptional target of hedgehog signaling and involved in stroma-induced drug tolerance in diffuse large B-cell lymphoma. *Oncogene*. 2011; 30:4874–4886. [PubMed: 21625222]
- Steg AD, Bevis KS, Katre AA, Ziebarth A, Dobbin ZC, Alvarez RD, Zhang K, Conner M, Landen CN. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clin. Cancer Res*. 2012a; 18:869–881. [PubMed: 22142828]
- Steg AD, Katre AA, Bevis KS, Ziebarth A, Dobbin ZC, Shah MM, Alvarez RD, Landen CN. Smoothened antagonists reverse taxane resistance in ovarian cancer. *Mol. Cancer Ther*. 2012b; 11:1587–1597. [PubMed: 22553355]
- Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. *Nature*. 2001; 411:349–354. [PubMed: 11357142]
- Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat. Rev. Clin. Oncol*. 2015; 12:445–464. [PubMed: 25850553]
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J. Clin*. 2015; 65:87–108. [PubMed: 25651787]
- Wang JC, Dick JE. Cancer stem cells: lessons from leukemia. *Trends Cell Biol*. 2005; 15:494–501. [PubMed: 16084092]
- Wang T, Ong CW, Shi J, Srivastava S, Yan B, Cheng CL, Yong WP, Chan SL, Yeoh KG, Iacopetta B, Salto-Tellez M. Sequential expression of putative stem cell markers in gastric carcinogenesis. *Br. J. Cancer*. 2011; 105:658–665. [PubMed: 21829201]

- Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. *Neoplasia*. 2015; 17:1–15. [PubMed: 25622895]
- Xu M, Gong A, Yang H, George SK, Jiao Z, Huang H, Jiang X, Zhang Y. Sonic hedgehog-glioma associated oncogene homolog 1 signaling enhances drug resistance in CD44(+)/Musashi-1(+) gastric cancer stem cells. *Cancer Lett*. 2015; 369:124–133. [PubMed: 26276718]
- Yang L, Xie G, Fan Q, Xie J. Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. *Oncogene*. 2010; 29:469–481. [PubMed: 19935712]
- Yoon C, Cho SJ, Aksoy BA, Park do J, Schultz N, Ryeom SW, Yoon SS. Chemotherapy resistance in diffuse-type gastric adenocarcinoma is mediated by RhoA activation in cancer stem-like cells. *Clin. Cancer Res*. 2016; 22:971–983. [PubMed: 26482039]
- Yu B, Xie J. Identifying therapeutic targets in gastric cancer: the current status and future direction. *Acta Biochim. Biophys. Sin. (Shanghai)*. 2016; 48:90–96. [PubMed: 26373844]
- Yuan M, Yang Y, Lv W, Song Z, Zhong H. Paclitaxel combined with capecitabine as first-line chemotherapy for advanced or recurrent gastric cancer. *Oncol. Lett*. 2014; 8:351–354. [PubMed: 24959275]
- Zahreddine HA, Culjkovic-Kraljacic B, Assouline S, Gendron P, Romeo AA, Morris SJ, Cormack G, Jaquith JB, Cerchietti L, Cocolakis E, Amri A, Bergeron J, Leber B, Becker MW, Pei S, Jordan CT, Miller WH, Borden KL. The sonic hedgehog factor GLI1 imparts drug resistance through inducible glucuronidation. *Nature*. 2014; 511:90–93. [PubMed: 24870236]

Fig. 1. Intrinsic response of gastric cancer cells to 5Fu

A: The IC₅₀ dose of 5Fu was calculated from measurement of the inhibition of cell viability by different concentrations of 5Fu (48 h). The X-axis indicates 5Fu concentration (μ M), and the Y-axis indicates the reduction of cell viability. **B:** qPCR analysis of *GLI1* and *GLI2* in N87 cells with or without 5Fu (5 μ M) treatment. **C:** Side population detected by flow cytometry after staining with Hoechst 33342. The average value from three independent experiments on side population was shown on the right. **D:** The average diameter of tumor spheres was measured from over 50 tumor spheres, and the typical morphology was shown

on the left. Significant difference was indicated by * ($P < 0.05$), ** ($P < 0.005$), or *** ($P < 0.0005$).

Fig. 2. Analysis of acquired 5Fu resistant cells

A: The IC₅₀ of 5Fu in parental and acquired resistant N87/FR cells was measured following 5Fu treatment at different concentrations. **B:** *GLI1* and *GLI2* expressions were measured by qPCR. **C:** Side population was detected after flow cytometry, and the average value was shown on the right (from three independent experiments). **D:** Tumor sphere morphology was shown on the left, and the diameter were calculated from over 50 tumor spheres formed from the corresponding cells. Significant difference was indicated by * ($P < 0.05$), ** ($P < 0.005$), or *** ($P < 0.0005$).

Fig. 3. The effect of *GLI2* knockdown on 5Fu response in N87/FR cells

A: The detection of *GLI1* and *GLI2* expressions after sh*GLI2* knockdown by qPCR. **B:** The effect of sh*GLI2* on the IC₅₀ of 5Fu was measured using the same method shown in Fig.1B. **C:** Change of side population by sh*GLI2* after 5Fu treatment, and the average change was shown on the right. **D:** Change of tumor sphere size by sh*GLI2*. The average value was shown on the right (from three independent experiments). Significant difference was indicated by * ($P < 0.05$), ** ($P < 0.005$), or *** ($P < 0.0005$).

Fig. 4. The effect of shGLI2 and 5Fu on ABCG2 expression

A: qPCR analysis of *ABCG2* after 5Fu treatment in N87 cells. **B:** Flow cytometry analysis of surface ABCG2 protein expression. The average positivity from three independent experiments was shown on the right. **C and D:** Comparison of 5Fu resistant N87/FR cells with the parental N87 cells on ABCG2 expression (transcript level shown on the left; and cell surface expression shown on the right). **E and F:** The effect of *GLI2* knockdown on *ABCG2* transcript (left) and on cell surface expression (right). Significant difference was indicated by * ($P < 0.05$), ** ($P < 0.005$), or *** ($P < 0.0005$).

Fig. 5. Rescuing effects of ectopic expression of ABCG2 on shGLI2

A: ABCG2 was ectopically expressed in acquired resistant N87/FR cells with shGLI2. **B:** IC50 detection after ABCG2 overexpression in N87/FR/shGLI2 cells. **C:** Comparison of the side population in ABCG2 ectopic expressed cells and the parental N87/FR/shGLI2 cells. **D:** Tumor sphere formation after ABCG2 overexpression. Significant difference was indicated by * ($P < 0.05$), ** ($P < 0.005$), or *** ($P < 0.0005$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1
Cancer relapse in patients with chemotherapy in high and low expression of *GLI1/GLI2/ABCG2*

Total patient number	Patient number with high <i>GLI1/GLI2/ABCG2</i>		Patient number with low <i>GLI1/GLI2/ABCG2</i>		Odd ratio for cancer relapse in patients with high <i>GLI1/GLI2/ABCG2</i>
	Relapse	No relapse	Relapse	No relapse	
295	4	13	24	135	1.2319
478	17	42	92	280	1.7308