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Influence of miRNAs in the chemoresistance of esophageal cancer

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*The greatest award the excellent people pursue
is
not what you acquire, but what you become
on the way.*

Declaration

I hereby declare that the thesis is my original work.

All the work and results presented in the thesis were performed independently.

Only establishment of 5-FU resistant esophageal cancer cells and custom PCR profiles parts of work were performed in the thesis in collaboration with Dr. Yue Zhao.

The results have been included in the manuscript:

miR-221 regulates the chemoresistance of esophageal cancer cell by targeting DKK2.

No unauthorized data were included.

Information from the literature was cited and listed in the reference.

All the data presented in the thesis will not be used in any other thesis for scientific degree application.

The work for the thesis began from Oct. 2012 with the supervision from Prof. Dr. med. Christiane J. Bruns in Chirurgische Klinik, Klinikum Großhadern, Ludwig-Maximilians University Munich, Germany.

08.2015, Munich

Yan Wang

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Abstract

Background: Esophageal cancer (EC) is the eighth most common incident cancer and the sixth most common cause of cancer death. Chemoresistance is the main obstacle of EC at present. Increasing evidence has shown that microRNAs (miRNAs) play an important role in carcinogenesis and chemotherapy resistance of various cancers. However, it is unknown how miRNAs regulate chemoresistance of EC. We performed a series of assays to investigate the mechanisms of miRNAs in chemoresistance of EC.

Methods: For this study, first 5-FU resistant cell models were established in four different esophageal cancer cell lines, then miRNAs levels were detected in 5-FU resistant esophageal cancer cells (5-FU_{res} EC cells) as well as the original sensitive cancer cell lines by qPCR. After transfecting miR-221 mimics (miR-221), inhibitors (anti221) or negative control siRNA (siRCtrl) into parental EC cells and 5-FU_{res} EC cells respectively, in vitro and in vivo experiments were performed in this study. In vitro, the cell proliferation and cytotoxicity of EC cells towards 5-FU were assessed by CCK-8. In vivo, OE33 and OE33-5FU_{res} cells were implanted subcutaneously in nude mice. The tumor sizes were measured at appropriate time. Ki67 and CD31 expression for assessing proliferation and angiogenesis were detected in tumor by immunohistochemistry staining (IHC) and immunofluorescence staining (IF). To further investigate the molecular mechanisms of miR-221 in 5-FU resistance of EC cells, target genes of miR-221 were predicted using bioinformatics analysis, and were further analyzed by qPCR and Western blot. For identification of direct target relationship of miR-221 and DKK2, the luciferase reporter vectors pGL3-DKK2-wt and pGL3-DKK2-mut with wild type or mutant 3'UTR sequence of DKK2 were constructed, and luciferase reporter assay was conducted after co-transfecting miR-221 mimics and luciferase reporter vectors. The distribution of β -catenin and activity of Wnt/ β -catenin pathway were assessed using IF and TOP/FOP Flash reporter assay. For understanding why 5-FU_{res} EC cells acquire mesenchymal cell-like morphology, EMT related markers E-cadherin and vimentin expression were detected by qPCR and Western blot. Published EMT related Wnt/ β -catenin target genes were detected by customer RT-PCR profile.

Results: In this study, miR-221 was significantly up-regulated in all 5-FU_{res} EC cells compared to the respective parental EC cells. *In vitro*, knockdown of miR-221 suppressed cell proliferation and sensitized esophageal cancer cells to 5-FU at 2.5 µg/ml and 20 µg/ml. *In vivo*, deregulation of miR-221 by its inhibitors suppressed xenograft tumor growth and also inhibited angiogenesis of tumors in BALB/c nu-nu mice. 120 common genes in four microRNA target prediction databases were predicted as candidate targets of miR-221. Here we focus on DKK2 an antagonist of Wnt/β-catenin pathway. The mRNA and protein expression of DKK2 was reversely associated with miR-221 expression in 5-FU_{res} EC cells. Luciferase reporter analysis revealed that DKK2 was a direct target gene of miR-221 in EC cells. IF staining results showed that β-catenin was located in the cell membrane and cytoplasm of the original EC cells whereas in 5-FU_{res} EC cells β-catenin was translocated into the nucleus. After transfection of miR-221 inhibitors in 5-FU_{res} EC cells, β-catenin moved back to the cytoplasm and cell membrane. Moreover, TOP/FOP Flash reporter analysis indicated a dramatic decrease of luciferase activity by β-catenin siRNA and miR-221 inhibitors. These results suggested Wnt/β-catenin pathway was inactivated by β-catenin siRNA and miR-221 inhibitors. In this study, acquisition of mesenchymal-like phenotype in 5-FU_{res} EC cells is associated with EMT pathway, and some EMT related Wnt/β-catenin target genes including MYC, CDH1, CD44 and chemoresistance marker ABCG2 were found to be regulated by miR-221 in 5-FU_{res} EC cells.

Conclusion: miR-221 plays an important role in chemoresistance of EC via modulating Wnt-EMT pathway. miR-221 may serve as a prognosis marker and therapeutic target for patient with 5-FU resistant EC.

Introduction

1. Esophageal cancer

Esophageal cancer is the eighth most common incident cancer and the sixth most common cause of cancer death^[1]. There are two main subtypes of esophageal cancer: squamous cell carcinoma and adenocarcinoma, which have quite distinct etiology and epidemiology^[3,4]. Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer which occurs mainly in China, India, Iran and the south of Africa^[1,7]. However esophageal adenocarcinoma (EAC) is more prevalent in European countries and the United States, which increases dramatically in incidence observed in the last twenty years^[3,8]. Nowadays clinical treatment for esophageal cancer consists of surgery, chemotherapy and radiotherapy. The prognosis of patients who receive only surgery is poor with a 5-year survival rate ranging from 15% to 39%^[9,10]. To improve the survival rate of patients with esophageal cancer, multimodal treatment, including chemotherapy plus surgery and chemoradiotherapy plus surgery, has been developed. Both cisplatin and 5-fluorouracil (5-FU) are the most common chemotherapy reagents used in esophageal cancer. However, the reported response rate to chemotherapy including 5-FU is very low which is only 19% to 40% and about half of these patients do not achieve a good response to chemotherapy^[10,11]. **Therefore, chemoresistance has become a major obstacle in the treatment of esophageal cancer.**

1.1 Definition of esophageal cancer

Esophageal cancer is cancer arising from the esophagus which runs between throat and stomach. The cancer cells start in the inner layer mucosa and grow outward through the submucosa and the muscle layer. It is divided into two main types: squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC): the esophagus is normally lined with squamous cells. Cancer arising from these cells is called esophageal squamous cell carcinoma which is mainly present in the upper two-thirds of esophagus^[12] (Fig I). Esophageal squamous cell carcinoma is similar to head and neck cancer in their appearance and association with tobacco and alcohol consumption^[13]. Esophageal adenocarcinoma (EAC): adenocarcinoma starts in gland cells which replace an area of squamous cells as what happens in Barrett's esophagus. It occurs mainly in the lower third of the esophagus (Fig I). Rare cancers: other types of cancer can also start in the esophagus, including lymphomas, melanomas, and sarcomas. But these cancers are rare and are not discussed further in this document.

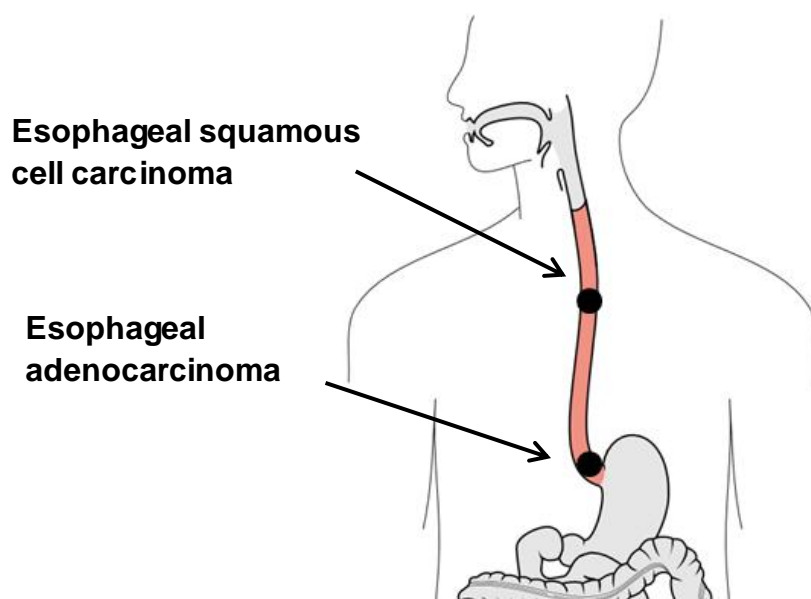


Figure I. Esophageal cancer. It is divided into two main types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is mainly present in the upper two-thirds of esophagus, while EAC occurs mainly in the lower third of the esophagus.

1.2 Chemotherapy and resistance of esophageal cancer

Esophageal cancer, a malignant carcinoma, has high invasiveness and metastasis, as well as the poor prognosis. Currently the combination therapy with 5-FU and cisplatin is the standard and active regimen for esophageal cancer. Chemotherapy has been proved effective for improving survival and life quality of esophageal cancer patients. However chemotherapy fails to kill all cancer cells because of intrinsic or acquired drug resistance. Here we mainly investigate the mechanism of 5-FU therapy and resistance in cancer.

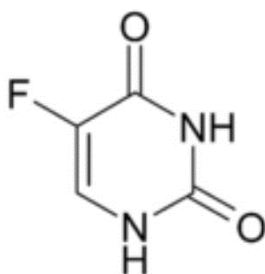


Figure II. Structure of 5-FU.

5-Fluorouracil (5-FU) is an important anticancer drug, which has been used as the first-line drug in treatment of various types of malignances. However, nowadays 5-FU resistance during the courses of treatment has been common, which is an important cause of failure for esophageal cancer therapy. 5-FU is a heterocyclic aromatic organic compound which has a similar structure with that of the pyrimidine molecules of DNA and RNA^[14]. It leads to cytotoxicity and cell death by interfering with nucleoside metabolism and is incorporated into RNA and DNA^[15,16]. 5-FU exerts its anticancer function through several ways: (1) Inhibition of thymidylate synthase (TS). TS is an important enzyme for synthesis of the pyrimidine thymidine and DNA replication. It methylates deoxyuridine monophosphate (dUMP) to form thymidine monophosphate (dTMP). 5-FU blocks synthesis of the pyrimidine thymidine and DNA replication by interrupting the action of TS and then results into cell apoptosis and cell death. (2) Incorporation of its metabolites into RNA and DNA. The 5-FU metabolite FUTP is extensively incorporated into RNA, disrupting RNA processing and leading to profound effects on cellular metabolism and viability.

Based on the above functional mechanism, dysregulation of TS, as an important cause of 5-FU resistance, has been reported in varieties of cancer. Spears CP et al^[17] found that TS level in 5-FU resistant colon tumors is higher than in nonresistant tumors; moreover the non-resistant tumors show a decrease in total TS after pretreatment with 5-FU, in contrast the resistant tumors do not exhibit such decrease of pretreatment levels. Shibata J et al^[18] found that an increase of TS transcription is related with the 5-FU resistance of colon cancer. Wang et al^[19] reported that ectopic expression of TS protein is a key cause of 5-FU resistance of cancers.

There are some other explanations of the mechanisms of 5-FU resistance, such as alterations in drug influx/efflux, enhancement of drug inactivation and activation of signal transduction pathways including Wnt/ β -catenin pathway and EMT pathway.

The superfamily of ATP-binding cassette (ABC) transporters, promiscuous transporters of both hydrophobic and hydrophilic compounds, function as active drug efflux pumps in cancer cells. It is reported that the overexpression of ABC transporters result in the reduction of cellular accumulation of drugs in cancer cells and lead to poor response of patients to chemotherapy^[20]. Members of ABC transporters ABCB1 (P-glycoprotein, MDR1), ABCC1

(multidrug resistance associated protein 1, MRP1) and ABCG2 (breast cancer resistance protein, BCRP) are well known to be involved in the active extrusion of anticancer drugs from cells^[21]. The emerging evidence indicates that the expression of ABCG2 is associated with a poor clinical response to chemotherapy^[22–25]. Inhibition or down-regulation of ABCG2 may be a valid approach to reverse ABCG2-mediated drug resistance and to improve the clinical efficacy of cancer chemotherapy.

Wnt/ β -catenin signaling pathway is also called the canonical Wnt pathway which plays a key role in controlling multiple tumor relevant aspects, such as cell fate specification, cell proliferation, and cell migration of cells^[26]. Activation of this pathway is a major factor in carcinogenesis and in various cancers. Of interest is the observation that Wnt/ β -catenin pathway is involved in the maintenance and self-renewal of cancer stem cells, which is a key cause of resistance in cancer therapy^[27]. In Wnt/ β -catenin signaling pathway, Wnt binding to cell surface receptors of the Frizzled family (FZD), the adenomatous polyposis coli (APC)/Axin/CK1/GSK3 β destruction complex function becomes disrupted^[26]. β -catenin is stabilized and accumulated in the cytoplasm, and then translocated to the nucleus to interact with the TCF/LEF transcription factors^[28]. Moreover, Wnt binding to a second receptor low-density lipoprotein receptor-related proteins (LRP-5/6), also induces the activation of the Wnt/ β -catenin signaling pathway^[29]. The Dickkopf families bind to and repress LRP, inactivating this pathway as shown in Figure III^[30].

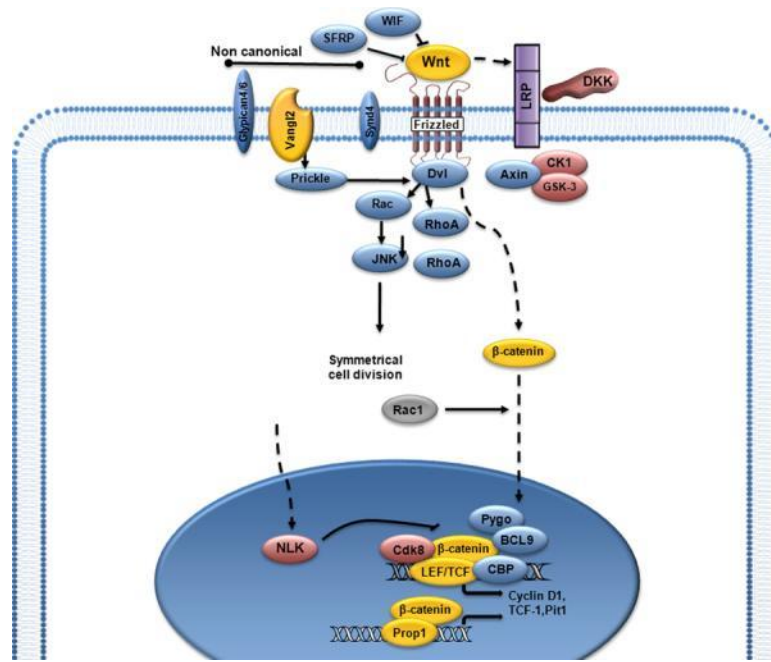


Figure III. Wnt/ β -catenin signaling pathway. In this canonical Wnt pathway, Wnt binding to cell surface receptors FZD and LRP leads to a release of DVL. The APC/Axin/CK1/GSK3 β destruction complex function becomes disrupted. β -catenin is stabilized and accumulated in the cytoplasm, and then translocated to the nucleus to interact with the TCF/LEF transcription factors. However, the Dickkopf families bind to LRP and repress the interaction between LRP and Wnt, further inactivating the Wnt/ β -catenin signaling pathway.

In preliminary work, our group compared Wnt pathway related gene expression in side population cells of 5-FU sensitive and resistant esophageal cancer cells. We found a significant increase of β -catenin mRNA expression as well as ectopic expression of other related genes such as WNT5a, WNT11, CTNNB1, GSK-3 and TCF4 in side population cells^[31]. It was found that the WNT signaling pathway participates in chemoresistance of several cancers like osteosarcoma^[32], prostate cancer^[33], ovarian cancers^[34], neuroblastoma^[27], hepatocellular carcinoma^[35], pancreatic cancer^[36] and colorectal cancer stem cells^[37]. If the Wnt/ β -catenin signaling pathway is involved in chemoresistance of esophageal cancer cells will be evaluated in this work.

Epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype^[38].

This process decreases intercellular contact and increases cellular mobility, which has also been recently implicated to provide cancer cells their migratory and invasiveness properties. More and more evidence suggests a direct link between EMT and self-renewal characteristics of stem cells. This finding indicates that EMT plays a critical role in resistance to chemotherapy by controlling the self-renewal of cancer stem cells. A majority of studies suggest the association of EMT and chemoresistance in a variety of cancer cells^[39]. Johji Hara et al. reported decreased expression of epithelial cell marker E-cadherin and increased expression of mesenchymal cell markers Snail, ZEB1 and N-cadherin in residual tumors after chemotherapy in human esophageal cancers, compared with chemo-naïve tumors. Moreover, in patients with preoperative chemotherapy, the decreased expression of E-cadherin and increased expression of Snail in residual tumors are correlated significantly with poor response to chemotherapy and poor prognosis^[39]. Grygielewicz P et al. found the resistant cell lines (SNU-16R) demonstrated changes characteristic of epithelial-to-mesenchymal transition (EMT)^[40]. In the present study, the 5-FU resistant esophageal cancer cell lines acquired EMT characteristics. These findings suggest that cancer cells after chemotherapy switch to mesenchymal phenotype, resulting in chemoresistance and poor clinical outcome.

2. MiRNAs and esophageal cancer

MicroRNAs (miRNA) are a family of endogenous noncoding single strand small RNA which is approximately 19 nt-21 nt in length. Mature miRNAs could degrade mRNA or suppress the protein translation by directing RISC complex and binding to the 3' untranslated region (3'UTR) of target mRNA^[41]. miRNAs have been found to play an important regulatory role in cell proliferation, differentiation and apoptosis, as well as carcinogenesis^[41]. Dysregulation of miRNAs has been found in a variety of cancers including esophageal cancer. Moreover, accumulating evidence indicates that miRNAs could also be involved in decreased sensitivity of cancer cells to chemotherapy, which has been an obstacle of anticancer treatment. Therefore, to better understand the function of miRNAs in the mechanisms of chemoresistance of esophageal cancer and target specific miRNAs will be helpful for anticancer therapy.

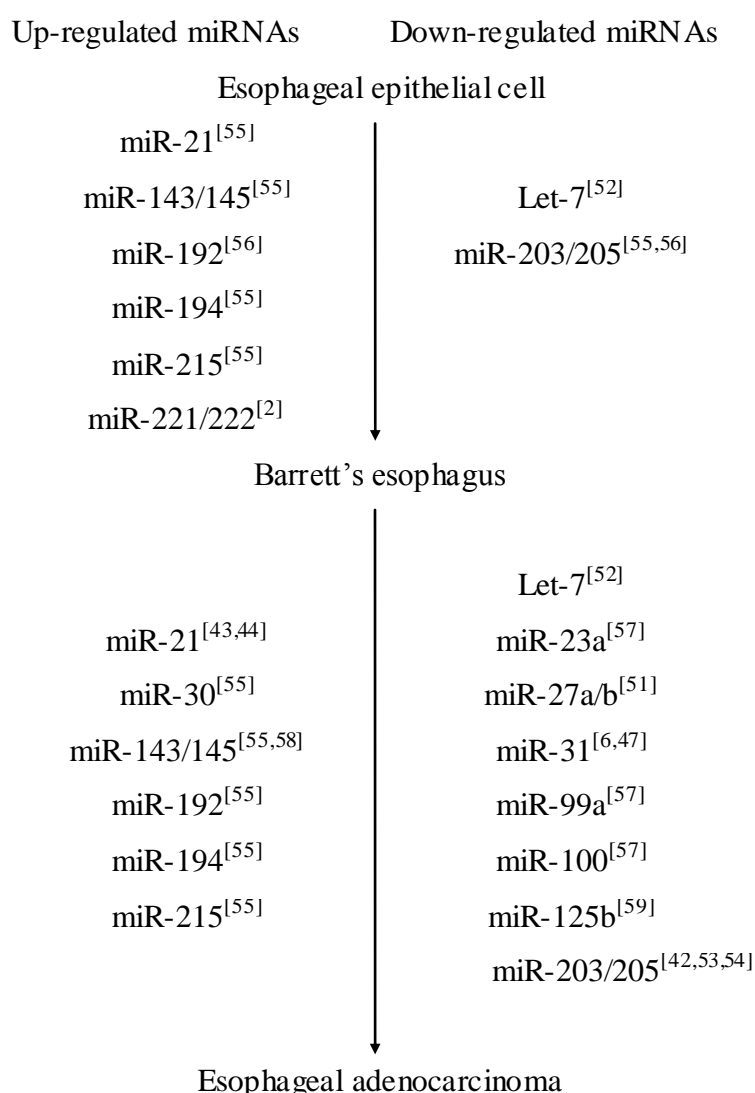
2.1 MiRNAs and carcinogenesis of esophageal cancer

To date, more and more studies have been reported that miRNAs are important for the development of esophageal cancer. Here we reviewed the characteristics of miRNAs which are relevant in carcinogenesis of esophageal cancer (Chart 1).

Some miRNAs play a role as oncomirs in the development of esophageal cancer. The level of miR-221 and miR-222 increases in esophageal cells exposed in bile acid and in EAC tissues versus BE tissues. And inhibitors of miR-221 and miR-222 can reduce EAC tumor growth by increasing the level of p27Kip1 and CDX2^[2]. MiR-21 expression in serum and tumor tissue of patients with esophageal cancer is higher, and it can induce cell proliferation and invasion in esophageal cancer^[42–45]. miR-192 can promote the progress of esophageal cancer^[46]. The microRNAs, miR-31 and miR-375, are candidate markers in Barrett's esophageal carcinogenesis^[47]. MicroRNA-100 promotes migration and invasion through mammalian target of rapamycin in esophageal squamous cell carcinoma^[48].

Some other miRNAs play a role as antagomirs in the development of esophageal cancer. The level of miR-27a decreases in esophageal squamous cell carcinoma and it functions as a tumor suppressor in esophageal squamous cell carcinoma by targeting KRAS^[49,50]. Down-regulation of miR-27a/b might reverse multidrug resistance of esophageal squamous cell carcinoma^[51]. Compared to the normal adjacent tissues, let-7 expression is lower in ESCC tissues, and the lower expression is correlated with lymph-node metastasis of ESCC. In addition, let-7 can inhibit cell proliferation in ESCC cells^[52]. MiR-203 and miR-205 expression is lower in BE and EAC compared with the normal esophageal epithelium. Overexpression of miR-203 and miR-205 can suppress cell proliferation, cell invasion and migration in esophageal cancer cells by regulating different target genes expression^[42,53,54]. The above findings suggest miRNAs play an important role in carcinogenesis of esophageal cancer by regulating oncogenes or suppressor genes.

Chart 1. Summary of miRNAs associated with esophageal cancer



2.2 MiRNAs and chemoresistance of esophageal cancer

Accumulating evidence suggests that dysregulation of miRNAs in chemoresistant esophageal cancer is common. Furthermore several studies report that miRNAs modulate cytotoxicity and sensitivity of esophageal cancer to chemotherapy by regulating chemoresistance-related genes expression or signal transduction pathways. In table 1, we reviewed the miRNAs which are deregulated and are related to chemoresistance of esophageal cancer.

Hsa-miR-141 is located on chromosome 12 and specifically expressed in human epithelial cancers. Imanaka Y et al. have found that miR-141 has an important regulatory function in the development of cisplatin resistance in cisplatin-resistant esophageal squamous cell carcinoma (ESCC) by directly targeting the 3'-untranslated region of YAP1^[60]. MiR-141 is highly expressed in cisplatin-resistant esophageal cancer cell lines, and when expressed ectopically

in cisplatin-sensitive cell lines, cell viability after cisplatin treatment is increased significantly. Wu Y et al. have found that miR-200c is up-regulated in endometrial and esophageal cancers, and its overexpression correlates with resistance to cisplatin treatment. miR-200c may recognize the miRNA response element (MRE) in the 3'UTR of the AP-2 gene^[61]. miR-200c expression correlates significantly with response to chemotherapy in esophageal cancers. Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the Akt signaling pathway^[62]. Taken together, miR-200c may induce cisplatin resistance by regulating AP-2 expression and the Akt signaling pathway. In EAC, miR-148a expression levels are inversely associated with cancer differentiation^[63]. miR-148a improves response to chemotherapy in sensitive and resistant esophageal cancer cells. Up-regulation of miR-148a may significantly increase sensitivity to chemotherapy in esophageal cancer cell lines, represented by a decrease in cell viability after 5-FU treatment. In addition miR-148a can sensitize chemotherapy-sensitive oesophageal cancer cell lines to cisplatin and attenuate resistance in chemotherapy-resistant variants. There is a trend toward a better response to 5-FU in 5-FU sensitive and resistant cells following miR-148a transfection. miR-296 is involved in many physiological and pathological processes, such as tumorigenesis, fetal alcohol syndrome, insulin production as well as insulin secretion^[64]. Down-regulation of miR-296 may inhibit growth of esophageal cancer cells in vitro and in vivo through regulation of cyclin D1 and p27. Down-regulation of miR-296 can confer sensitivity of drugs on esophageal cancer cells, and may promote adriamycin(ADR)-induced apoptosis, accompanied by increased accumulation and decreased release of ADR, through regulation of P-glycoprotein, Bcl-2 and Bax^[65]. miR-27a is widely expressed in cancer cells and may function as an oncogene through regulating cell survival and angiogenesis^[51]. Down-regulation of miR-27a may confer sensitivity toward drugs representing targets for P-glycoprotein or not to esophageal cancer cells, and also promotes ADR-induced apoptosis, accompanied by increased accumulation and decreased releasing amount of ADR. Down-regulation of miR-27a can significantly decrease the expression of P-glycoprotein, Bcl-2, and the transcription of the multidrug resistance gene 1 (MDR1), but upregulate the expression of Bax. In addition, downregulation of miR-483 and miR-214 may reverse drug resistance of esophageal cancer cells to ADR, 5-FU and cisplatin through regulation of intracellular drug accumulation and releasing index^[66].

Table 1. Summary of miRNAs associated with chemoresistance of esophageal cancer.

miRNA	Resistance	Drugs	Cancer
miR-301a	Down	Radiation	ESCC ^[67]
miR-483/214	Up	Adriamycin, 5-FU, Cisplatin	ESCC ^[66]
miR-21-3p	Up	5-FU	EAC ^[68]
MiR-222-3p	Up	5-FU	EAC ^[69]
miR-193b-3p/5p	Up	5-FU	EAC ^[69]
miR-378 a-3p	Down	5-FU	EAC ^[69]
miR-192-5p	Down	5-FU	EAC ^{[69][46]}
miR-22	Down	Radiation	ESCC ^[70]
Let-7	Down	Cisplatin	ESCC ^[52]
miR-31	Down	Radiation	EAC ^[71]
miR-31-5p	Down	Cisplatin	EAC ^[47]
miR-31-5p	Up	5-FU	ESCC ^[47]
miR-125a-5p	Down	Cisplatin/5-FU	EAC, ESCC ^[72]
miR-141	Up	Cisplatin	ESCC ^[66]
miR-200b/c/429	Up	Cisplatin	ESCC ^[61,62]
Mir-148a	Down	Cisplatin	ESCC ^[63]
miR-27a	Up	Adriamycin	ESCC ^[51]

In our preliminary study, we successfully developed cisplatin or 5-FU resistant oesophageal cancer cells and identified stem-like side population (SP) and non-side population (NSP) cells. Several miRNAs (miR-21, miR-200c, miR-221, miR-34a, miR-155 and miR-92a) were upregulated in resistant cancer cells or SP cells compared with the sensitive cell or NSP cells^[31]. These results suggested that miRNAs might play a role in the chemoresistance of oesophageal cancer. In this project, we further investigate the functional mechanism of miRNAs in chemotherapy resistance in oesophageal cancer cells.

Aim of study

Chemotherapeutic resistance of esophageal cancer is a main obstacle in the treatment of esophageal cancer. But the mechanisms of chemoresistance of esophageal cancer are still not clear. In this study, we focus on the analysis of miR-221 with respect to chemoresistance of esophageal cancer. miRNAs play an important role in chemoresistance of various cancers. In our preliminary work, we found dysregulation of several miRNAs in 5-FU resistant esophageal cancer cell lines.

Chemoresistance of cancer is a complicated and multistep process occurring during long term anticancer therapy. A variety of signal transduction pathways participate in that process including the Wnt pathway and EMT. In this study, we investigated whether Wnt pathway and EMT are involved in the 5-FU resistance of esophageal cancer. Previously published studies demonstrated that miR-221 can regulate cell proliferation of varieties of cancers by targeting the Wnt pathway or EMT ^[73-77]. Our preliminary data investigates that 5-FU resistance of esophageal cancer in our experimental model is regulated by miR-221 targeting Wnt pathways. We believe that miR-221 might be a good therapeutic target for esophageal cancer.

Materials and Methods

1. Materials

1.1 Cell lines

Human esophageal cancer cell lines JROECL19 (OE19), JROECL33 (OE33), PT1590 and LN1590 were used in this study. The first two cell lines were obtained from Sigma company (ECACC number: 96071721 and 96070808). The other two cell lines were kindly donated by the University Medical Center of Hamburg-Eppendorf. Origin and characteristics of these four cell lines are summarized below:

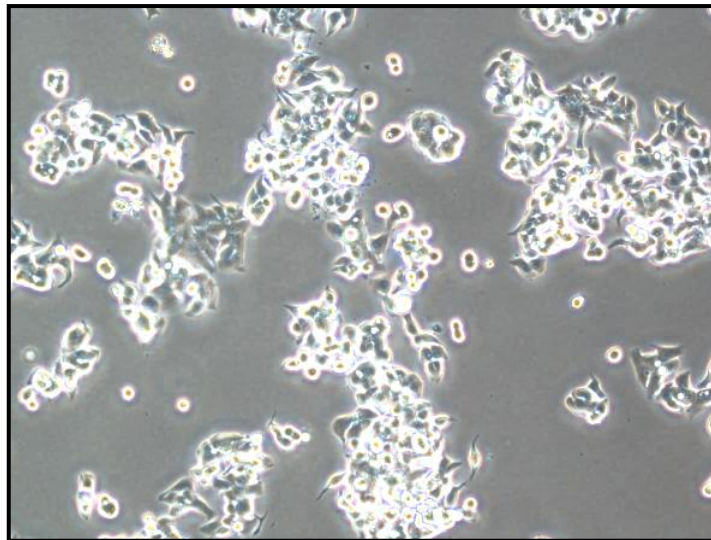


Figure IV. OE19 cell: this cell line was established in 1993 from an adenocarcinoma of gastric cardia/esophageal gastric junction of a 72 year old male patient. The tumor was identified as pathological stage III (UICC) and showed moderate differentiation. Details can be found at

(<http://www.sigmaaldrich.com/catalog/product/sigma/96071721?lang=en®ion=CA>).

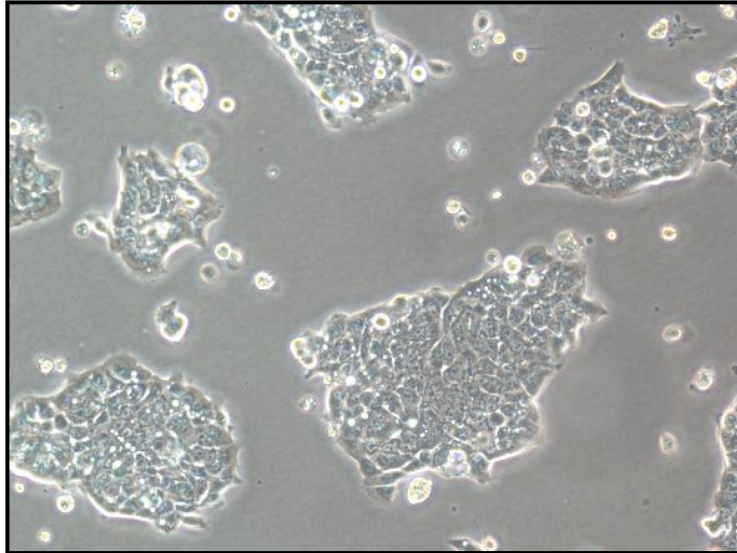


Figure V. OE33: this cell line was established from the adenocarcinoma of the lower esophagus (Barrett's metaplasia) of a 73 year old female patient. The tumor was identified as pathological stage IIA (UICC) and showed poor differentiation. Details can be found at (<http://www.sigmaaldrich.com/catalog/product/sigma/96070808?lang=en®ion=CA>)

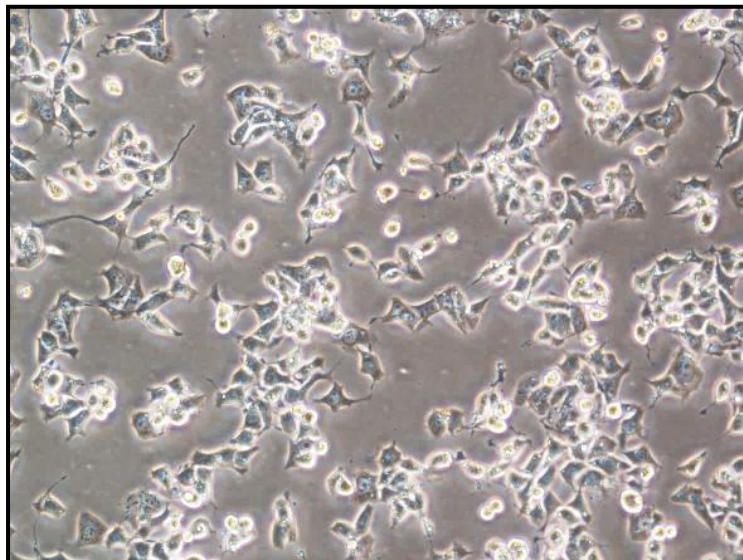


Figure VI. PT1590: this cell line was established from a primary tumor (PT) from a patient who was identified as stage IIB and had undergone radical esophagectomy for a poorly differentiated adenocarcinoma of the esophagus at the University Medical Center of Hamburg-Eppendorf.

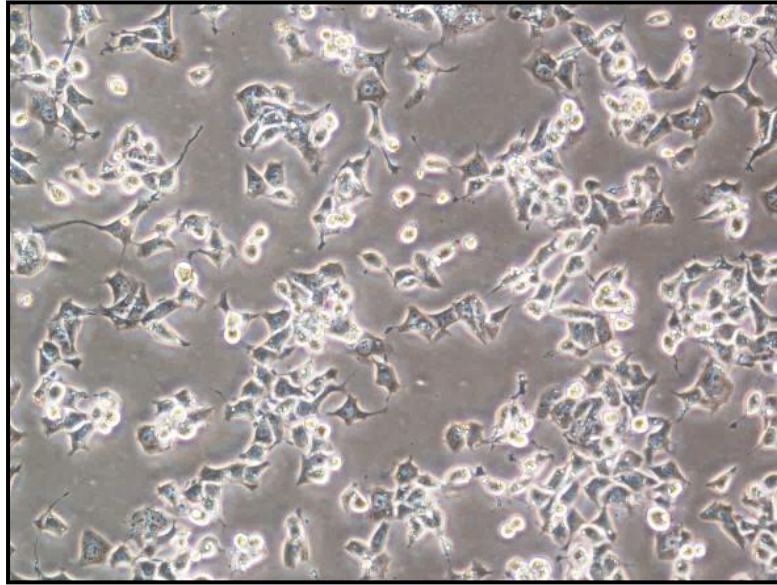


Figure VII. LN1590: this cell line was established from a lymph node with micro-metastasis (LN) from a patient who was identified as stage IIB and had undergone radical esophagectomy for a poorly differentiated adenocarcinoma of the esophagus at the University Medical Center of Hamburg-Eppendorf. It was generated from one of Ber-EP4-positive nodes (later known as EpCAM).

1.2 Animals

The 6-8 week old, 20-22 g Bagg-albino/c (BALB/c) nu/nu male mice were bought from Charles River, Sulzfeld, Germany.

1.3 Vectors

- 1) TOP/FOP Flash luciferase reporter gene vectors: The TOP/FOP flash luciferase reporter gene vectors were a kind gift from Dr. Andreas Herbst, University of Munich.
- 2) PCH110 β -galactosidase encoding plasmid: this plasmid was a kind gift from Dr. Andreas Herbst, University of Munich.
- 3) The pGL3 Luciferase Reporter Vectors: The pGL3 Luciferase Reporter Vectors, used in this study, provide a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. These may be cis- or trans-acting factors. The backbone of the pGL2 Luciferase Reporter Vectors was re-designed for the pGL3 Vectors for increased expression, with a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The assay of this genetic reporter is rapid, sensitive and quantitative.

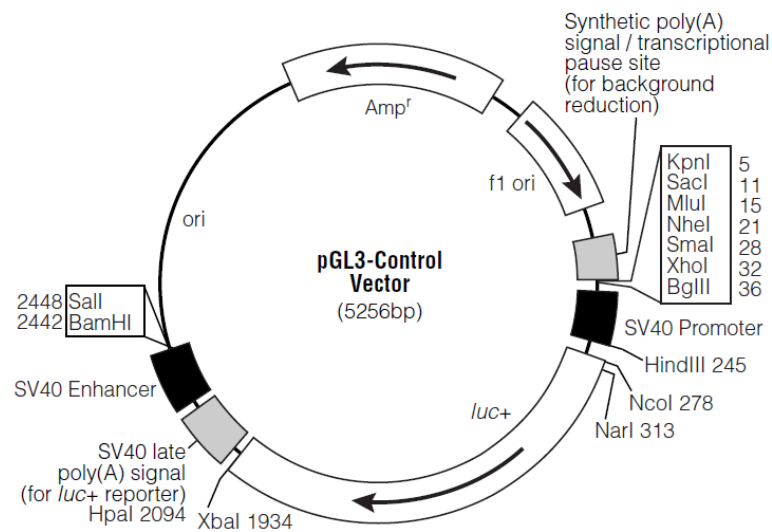


Figure VIII. pGL3 control vector: it contains SV40 promoter and enhancer sequences, resulting in strong expression of *luc+* in many types of mammalian cells. The open reading frame (ORF) allows interchange of DNA inserts at upstream positions relative to the luciferase reporter gene. Thus, positional effects of a putative genetic element may be readily tested.

1.4 Materials for qRT-PCR

All stars negative control siRNA with /without Alexa fluor 488 modification	Qiagen, USA
HiperFect transfection reagent	Qiagen, USA
Hsa_Mir-221 miscript primer assay	Qiagen, USA
Hsa_RNU6B miscript primer assay	Qiagen, USA
miRNeasy mini kit	Qiagen, USA
miScript reverse transcription kit	Qiagen, USA
miScript SYBR Green PCR kit	Qiagen, USA
SuperScript III Platinum SYBR Green One-Step qRT-PCR kit	Invitrogen, USA
QuantFast SYBR Green PCR kit	Qiagen, USA
RT2 First start kit	SuperArray Bioscience
RT ² Profiler PCR array system	SuperArray Bioscience

Table 2. Primers

Gene symbol	Sequence	
TS	Forward	5'-ACCAACCCTGACGACAGAAG-3'
	Reverse	5'-CATGTCTCCCGATCTCTGGT-3'
DKK2	Forward	5'-CCAGTACCCGCTGCAATAAT-3'
	Reverse	5'-ATGACCGTGGTTTCGATCTC-3'
CTNNB1	Forward	5'-GAGGGTACGAGCTGCTATGT-3'
	Reverse	5'-AACGCTGGACATTAGTGGGA-3'
CDH1	Forward	5'-GAAGCTGGCTGACATGTACG-3'
	Reverse	5'-CTCAAGGGAAGGGAGCTGAA-3'
VIM	Forward	5'-AGGAACCAATGAGTCCCTGG-3'
	Reverse	5'-GAAGGTGACGAGCCATTTCC-3'
	Reverse	5'-TTTCTAGACTGGGCATCGCA-3'
DKK2 3'UTR	Forward	5'-CGGGTACCCAGGTTGCTATAAGCTTGGTGA-3' (KpnI)
repoter construct	Reverse	5'-GCAGATCTCCTAAATTACAAAGTCAATAGCTG-3' (BglII)
DKK2 Mut 3'UTR	Forward	5'-GCGGGTACCTTTCTTATGTCCACCCTT-3' (KpnI)
reporter construct	Reverse	5'-GCAGATCTCGCTGTAAGCTTTCTGTAAG-3' (BglII)

Mut, mutated

Table 3. Sequence of miRNAs

Name	Sequence
hsa-miR-21-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'
hsa-miR-221-3p	5'-AGCUACAUUGUCUGCUGGGUUUC-3'
hsa-miR-34a-5p	5'-UGGCAGUGUCUUAGCUGGUUGU-3'
hsa-miR-200c-3p	5'-UAAUACUGCCGGGUAAUGAUGGA-3'
hsa-miR-92a	5'-UAUUGCACUUGUCCCGGCCUGU-3'
hsa-let-7b-5p	5'-UGAGGUAGUAGGUUGUGUGGUU-3'
hsa-let-7g-5p	5'-UGAGGUAGUAGUUUGUACAGUU-3'

1.5 Chemical reagents

DMSO (Dimethylsulphoxide)

Sigma-Aldrich, Steinheim, Germany

DPBS buffer

Biochrom AG, Berlin, Germany

EGF (Recombinant human EGF)	PeproTech EC, UK
Fetal bovine serum	Biochrom AG, Berlin, Germany
FGF (Recombinant human basic FGF)	PeproTech EC, UK
Insulin solution (human)	Sigma-Aldrich, Steinheim, Germany
Normocin	InvivoGen, San Diego, USA
Penicillin/Streptomycin 100 ml (10.000 Units Penicillin/ml, 10 mg Streptomycin/ml)	PAN Biotech, Aidenbach, Germany
RPMI 1640 + Glutamax-1 Gibco	Invitrogen, Germany
Trypsin 0.05%/EDTA 0.02 % in PBS without Ca ²⁺ and Mg ²⁺	PAN Biotech, Aidenbach, Germany
Trypan blue (0.4%)	Sigma-Aldrich, Steinheim, Germany
Transferin	Sigma-Aldrich, Steinheim, Germany

1.6 Materials for Western blot, flow cytometry, immunofluorescence, and immunohistochemistry

4% paraformaldehyde	Pathology LMU, Germany
Albumin from bovine serum (BSA)	Sigma-Aldrich, Steinheim, Germany
Avidin/Biotin blocking kit	Vector Laboratories, CA, USA
BCA protein assay reagent kit	Pierce Rockford, USA
Biotinylated secondary antibody	Vector Laboratories, CA, USA
DAPI in mounting medium	Vector Laboratories, CA, USA
ECL Western blotting detection system	Amersham Biosciences, Germany
Ethanol 70%, 80%, 96%, 100%	CLN GmbH, Niederhummel, Germany
FCR blocking reagent (human)	Miltenyi, Biotec GmbH, Germany
Neo-Clear (Xylene substitute)	Merck, Darmstadt, Germany
Hydrogen peroxide 30% (H ₂ O ₂)	Merck, Darmstadt, Germany
Kaiser's glycerol gelatin	Merck, Darmstadt, Germany
Liquid DAB+ substrate chromogen system	Dako, CA, USA
Mayer's hemalum solution	Merck, Darmstadt, Germany
Normal rabbit serum	Vector Laboratories, CA, USA

Normal goat serum	Vector Laboratories, CA, USA
Propidium iodide	BD phamingen, USA
Restore Western blot stripping buffer	Pierce, Rockford, USA
Sodium chloride	Merck, Darmstadt, Germany
Target retrieval solution 10×	Dako, CA, USA
TRIZMA base	Sigma-Aldrich, Steinheim, Germany
TRIZMA hydrochloride	Sigma-Aldrich, Steinheim, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Vectastain ABC kit	Vector Laboratories, CA, USA
Verapamil hydrochloride (verapamil)	Sigma-Aldrich, Steinheim, Germany
Flow cytometry tubes Belgium	BD Bioscience Europe
BD Falcon 5 ml polystyrene round-bottom tubes (REF 352052)	
BD Falcon 5 ml polystyrene round-bottom tubes with cell strainer cap (REF 352235)	
BD Falcon 5 ml polypropylene round-bottom tubes with cap (REF 352063)	

Table 4. Antibodies

Antibody	Company	Specificity	Host	Catalog	Molecular weight	Isotype
DKK2	Abcam	Human	Rabbit	ab38594	28kDa	IgG
E-cadherin	Santa Cruz	Human	Mouse	SC8426	120 kDa	IgG1
CD31	Abcam	Mouse	Rabbit	ab28364		
Ki67	Abcam	Human	Rabbit	ab16667		
β-actin	Sigma	Human	Mouse	A5316	42 kDa	IgG2a
β-catenin	BD bioscience	Human	Mouse	610154	92 kDa	IgG1
TS	Abcam	Human	Mouse	ab3145	35(36 kDa)	IgG1
Vimentin	Santa Cruz	Human	Rabbit	SC7557-R	57 kDa	IgG
Cy TM 3	Jackson ImmunoResearch	Mouse	Donkey	715-165-150		IgG
Cy TM 2	Jackson ImmunoResearch	Rabbit	Goat	111-225- 144		IgG

1.7 Technical equipments

ABI Prism 7500	Applied Biosystems, Foster City, USA
Automatic Tissue Processors Model 2065/2	MDS Group GmbH, Buseck, Germany
Automatic pipettes	Gilson, Middleton, WI, USA
Axioskop 40, AxioCam MRc5 Digital fluorescence	Carl Zeiss AG, Oberkochen, Germany
Centrifuges	Eppendorf, Germany
CO ₂ incubators	Heraeus, Rodenbach, Germany
Digital precision scale	K ERN & Sohn GmbH, Germany
FACS Calibur	BD Biosciences, USA
Freezer -20 °C	Siemens AG, Germany
Freezer -80 °C	Heraeus, Hanau, Germany
Fridge 4 °C	Siemens AG, Germany
Hand tally counter	Carl Roth GmbH, Karlsruhe, Germany
Herasafe EN12469 2000 Class II safety cabinet	Thermo Fisher Scientific Inc, Germany
Leica RM2255, Fully Motorized Rotary Microtome	Leica Microsystems, Germany
LSR II flow cytometry	BD Biosciences, USA
Liquid nitrogen tank	MVE, New Prague, MN, USA
Phase contrast microscope	Carl Zeiss GmbH, Germany
Microwave oven	Siemens, Germany
MoFlo high speed sorter	DAKO Cytomation, Glostrup, Denmark
Thermo Scientific Heraeus incubator	Thermo Fisher Scientific Inc, Germany
TECAN GENios Plus ELISA reader	TECAN, Salzburg, Austria
RNA/DNA calculator	GeneQuant Pro, GE, USA
Vortex	IKA Works, Wilmington, NC, USA
Water bath	GFL, Burgwedel, Germany

1.8 Cell culture materials

5 ml coster stripette	Corning Inc, New York, USA
10 ml coster stripette	Corning Inc, New York, USA
25 ml coster stripette	Corning Inc, New York, USA
25 cm ² nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark
75 cm ² nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark
150 cm ² nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark
15 ml centrifuge tubes	TPP, Switzerland
50 ml polypropylene conical tubes	BD Bioscience Europe, Belgium
6, 12, 24 and 96-well nunc delta surface culture Plates	Thermo Fisher Scientific Inc, Denmark
12 and 24-well companion plate notched for use with cell culture insert	BD Dicknson Labware, USA
Cell culture insert 8.0 µm	BD Bioscience, NJ, USA
Lab-Tek™ chamber slides	Thermo Fisher Scientific Inc, USA
Nunc cryotube (2.0 ml)	Thermo Fisher Scientific Inc, Germany
Eppendorf safe-lock tubes (0.6 ml, 1.5 ml, 2.0 ml)	Eppendorf AG, Hamburg, Germany
Hemacytometer and cover slip (Cell counting chambers)	Bürker-Türk, Germany

1.9 Materials for cell proliferation and cytotoxicity assay

5-FU (Fluorouracil-GRY)	GRY-Pharma GmbH, Germany
Cell counting kit-8 (CCK-8)	Dojindo Laboratories, Japan
AnnexinV-FITC Apoptosis Kit	R&D systems, Minneapolis, USA

1.10 Materials for transfection

Hiperfect transfection reagent	Qiagen, USA
X-tremeGENE HP DNA Transfection Reagent	Roche, USA
Lipofectamine-2000	Invitrogen, USA
SiRNA-CTNNB1	Gel Health Care, Germany
SiRNA Control	Gel Health Care, Germany
Hsa- miR-221 mimic	Qiagen, USA
Hsa- miR-221 inhibitor	Qiagen, USA
Control siRNA	Qiagen, USA
miScript Inhibitor Negative Control	Qiagen, USA

Table 5. Target sequence of CTNNB1 siRNA pool

Name	Target Sequence
siGENOME SMARTpool siRNA-CTNNB1-1	GCUGAAACAUGCAGUUGUA
siGENOME SMARTpool siRNA-CTNNB1-2	GAUAAAGGCUACUGUUGGA
siGENOME SMARTpool siRNA-CTNNB1-3	CCACUAAUGUCCAGCGUUU
siGENOME SMARTpool siRNA-CTNNB1-4	ACAAGUAGCUGAUUAUUGAU

1.11 Surgical materials

BODE Cutasept F	Bode Chemie, Hamburg, Germany
Disposable scalpels	Feather Safety Razor Co., Japan
Forceps	Dosch GmbH, Heidelberg, Germany
Hypodermic needle (30 G)	B-Braun, Melsungen, Germany
Needle holder	Dosch GmbH, Heidelberg, Germany
Isotonic saline	B-Braun, Melsungen, Germany
Q-tips (cotton applicator)	NOBA, Wetter, Germany
Rotilabo-embedding cassettes	Carl Roth GmbH, Karlsruhe, Germany
Scissors, sharp / blunt	Dosch GmbH, Heidelberg, Germany
Syringe (1 ml, 5 ml)	BD Plastipak™, Madrid, Spain

1.12 Medicine

Growth Factor Reduced (GFR)	BD
Matrigel™ Matrix, 10 ml	BD Biosciences, USA
Ketamin hydrochlorid (Ketavet) 100 mg/ml	Pfizer Pharmacia GmbH, Germany
Xylazin hydrochlorid, Xylazin (Rompun) 2% 25 ml	Bayer Healthcare, Leverkusen, Germany

1.13 Software

Adobe Acrobat 7.0 Professional Adobe	Systems Inc., USA
Axio Vision 4.4	Carl Zeiss GmbH, Germany
EndNote X5 (Windows Version X5)	Thomson Reuter, CA, USA
Graphpad Prism 6.0	GraphPad Software, Inc., USA
Image-Pro Plus 5.0	Media Cybernetics, Inc., USA
Microsoft Office 2007 (Word, Excel, PowerPoint) Microsoft	Corporation, USA
SoftMax Pro Molecular Devices	Corp., USA
Summit 4.3 software	Beckmann coulter GmbH, Germany
Windows XP Professional Microsoft	Corporation, USA
Venn	online

2. Methods

2.1 Establishment of chemoresistant esophageal cancer cell lines and cell culture

- 1) **Cell culture:** Human esophageal cancer cell lines OE19, OE33, PT1590 and LN1590 were used in this study. The first two cell lines were cultured in RPMI1640 supplemented with 2 mM glutamine, 10% fetal bovine serum, 1% penicillin and 1% streptomycin. The other two cell lines were cultured in RPMI1640 medium with 2 mM glutamine, 10% fetal bovine serum, 1% penicillin and 1% streptomycin, 10 ng/ml EGF, 10 ng/ml FGF, 10ng/ml insulin and 4 µg/ml transferrin. All cell lines were kept at 37 °C in a humidified incubator containing 5% CO₂.
- 2) **Establishment of chemotherapy resistant cell lines:** IC₅₀ of 5-FU was determined by cell cytotoxicity assay. The 5-FU-resistant esophageal cancer cell lines (OE19-5FU_{res}, OE33-5FU_{res}, PT-5FU_{res} and LN-5FU_{res}) were developed through a stepwise incremental treatment with 5-FU as follows: the initial concentration of 5-FU used was set at 5 µg/ml based on IC₅₀ values ranging between 3 and 6.5 µg/ml for the parental cell lines. After 24 hours, the cells were passaged with 5-FU free medium. Upon reaching confluent growth, the cells were treated with increased levels of 5-FU (1.5 to 2-fold). Over five subsequent increases in 5-FU exposure using the same procedure, 5-FU-resistant cell lines (OE19-5FU_{res}, OE33-5FU_{res}, LN-5FU_{res} and PT-5FU_{res}) were eventually established. The 5-FU resistant cells cultured in complete medium were continuously exposed to 5-FU with stepwise increased concentrations according to the IC₅₀ value (from 10% IC₅₀ up to 5 fold IC₅₀) respectively. Medium was changed twice a week.

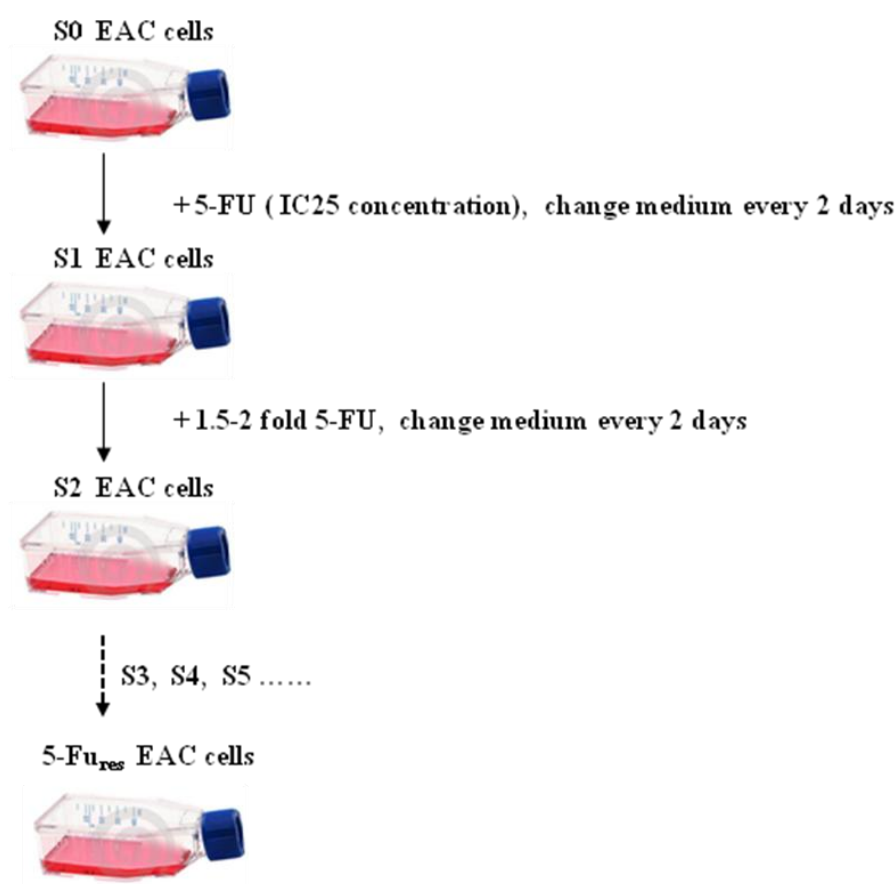


Figure IX. Establishment of 5-FU resistant esophageal cancer cell lines. The above human esophageal cancer cell lines were developed through a stepwise increment of 5-FU concentration as follows: the initial concentration of 5-FU was around 5 $\mu\text{g/ml}$ and, after 3 days, the cells were passed through a 5-FU free medium. Upon reaching confluence, the cells were treated with a higher concentration of 5-FU (1.5 to 2-fold). Over five subsequent increases in 5-FU exposure using the same procedure, 5-FU-resistant cell lines (OE19-5FU_{res}, OE33-5FU_{res}, LN-5FU_{res} and PT-5FU_{res}) were eventually established. Medium was changed twice a week.

2.2 RNA isolation and quantification of miRNA or target gene expression

- 1) **RNA isolation:** Total RNA was isolated from 5-FU sensitive and resistant esophageal cancer cells according to the manufacturer of miRNeasy mini kit as shown in Figure X.

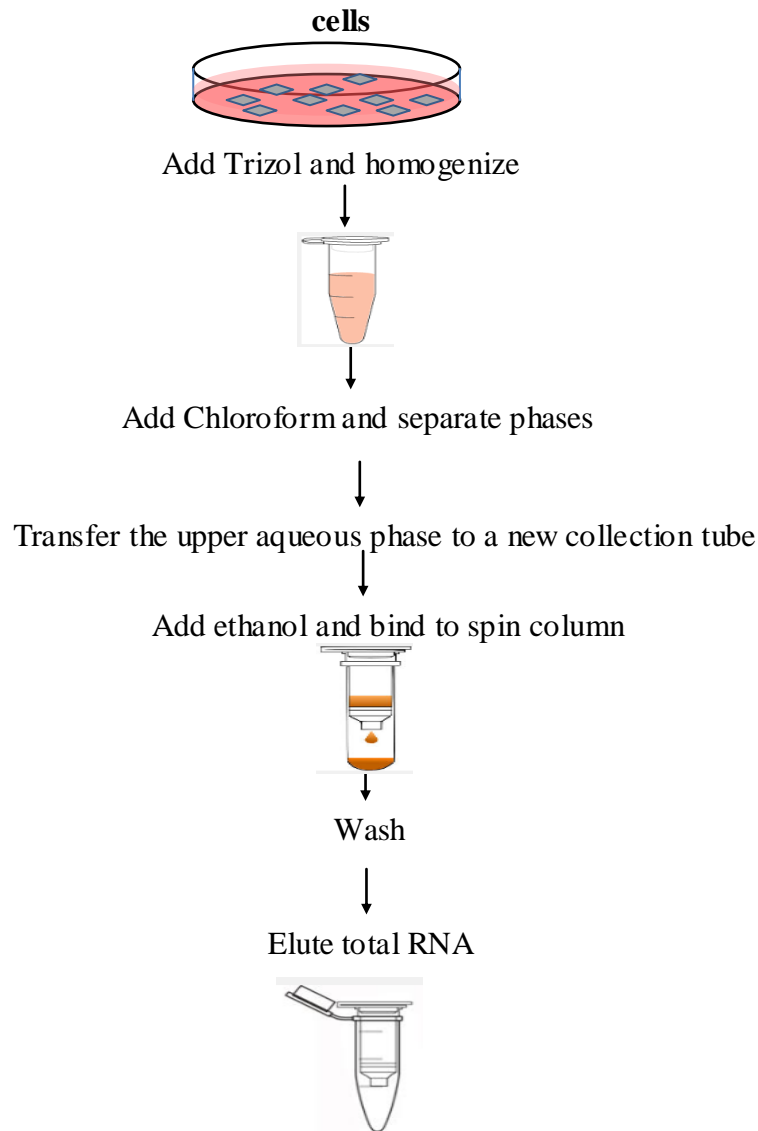


Figure XI. Isolation of total RNA. Total RNA was isolated from 5-FU sensitive and resistant esophageal cancer cells as recommended by the manufacturer of the miRNeasy kit. 100 ng of total RNA was used to prepare cDNA fragments for further quantification.

- 2) **RT-qPCR for miRNAs:** The cDNA was synthesized with miScript II RT Kit according to the manufacturer's instructions. Then the expression of miRNAs was quantified by using the miScript SYBR® Green PCR Kit according to the manufacturer's instructions. MiRNAs expression was related to the RNU6B internal control. Data was calculated using the $2^{-\Delta\Delta CT}$ method.

3) RT-qPCR for target genes

The expression of miRNA target genes were detected by Quanti Fast SYBR Green PCR Kit and normalized by GAPDH mRNA. Data was calculated using the $2^{-\Delta\Delta CT}$ method.

2.3 Trypan blue staining and CCK-8 assay

The cell viability was assessed by trypan blue staining. 10-100 μ l of single cell suspension were gently mixed with an equal volume of 0.4% trypan blue. The calculation is as below:

$$\text{Cell viability} = [\text{unstained cells}/(\text{unstained} + \text{trypan blue stained cells})] \times 100\%$$

CCK-8 assay was performed for cell proliferation and cytotoxicity according to the manufacturer's instructions. For cell proliferation measurement, 1×10^4 cells per well with or without transfection of miR-221 mimics or inhibitors, were seeded in a 96-well plate, grown over night, and cell viability was measured after 0 h, 24 h, 48 h, and 72 h according to the manufacture's instruction. For cell cytotoxicity assay, cells were seeded in a 96-well plate as before, further treated with 5-FU for 48 h, and analyzed as before using VersaMax tunable microplate reader and Softmaxpro 6.2 for data analysis.

2.4 Prediction of miR-221 target genes

Target genes of miR-221 were predicted using 4 microRNA-target predict tools:

TargetScan: <http://www.targetscan.org/>,

PITA: http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html,

miRTarBase: <http://mirtarbase.mbc.nctu.edu.tw/>

miRanda: <http://www.microrna.org/microrna/home.do>

The intersection of miR-221 target genes was analyzed by Venn online. The secondary structure of miR-221 and its target gene was predicted by RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>).

2.5 Luciferase reporter assay

Construction of luciferase reporter vectors: The 3'-UTR segments of DKK2, containing the putative binding sites for miR-221, were synthesized by PCR. The primers are shown in Table 1. After appropriate digestion and purification, the products were separately cloned into the KpnI and BglII sites on the pGL3-control vector (Promega, Madison, WI) downstream of the luciferase gene to generate wild type or mutant pGL3-DKK2 constructs.

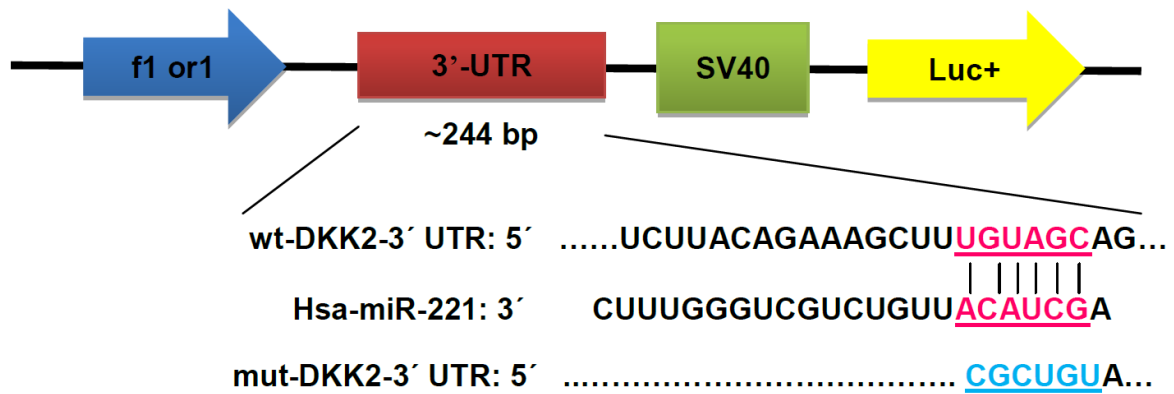


Figure XII. Design of luciferase reporter vectors. The 3'-UTR segments (244 bp) of DKK2, containing the putative binding sites for miR-221, were cloned into the restriction sites KpnI and BglII sites upstream of the luciferase reporter gene.

Luciferase reporter assay: 1×10^5 OE33 cells were seeded per well (12-well plate). Cells were transfected with miR-221 mimics and wt-pGL3-DKK2, mut-pGL3-DKK2 or pGL3-control using Lipofectamine-2000 (Invitrogen). The samples were also co-transfected with 50 ng of pRL-TK plasmid expressing *Renilla* luciferase as normalisation controls. Then, cells were harvested 24 h after transfection, and firefly and *Renilla* luciferase activity levels were measured by using the Dual-Luciferase Reporter Assay (Promega, USA). Each transfection was performed in triplicate.

2.6 TOP/FOP flash luciferase report assay

To assess TCF/ β -catenin-mediated transcriptional activity, TOP-flash luciferase reporter gene assays were performed using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. 1×10^5 OE33 and OE33-5FU_{res} cells were seeded per well (12 well plate). Cells were transfected with 5 nM hsa-miR-221 mimics (miR-221), 50 nM hsa-miR-221 inhibitors (anti221) or 5 nM siR-CTNNB1 (siR- β) using 3 μ l the Hiperfect transfect reagent. 24 h later, 1 μ g the 8 x TOP-flash reporter gene construct or 1 μ g 8 x FOP-flash reporter gene construct (a generous gift from PD Dr. Andreas Herbst, University of Munich, Germany) as well as 1 μ g the plasmid pCH110 encoding β -galactosidase (a generous gift from PD Dr. Andreas Herbst, University of Munich, Germany) were transfected into these cells using 3 μ l X-treme GENE HP DNA transfection reagent. 72 h after siRNA transfection, cells were harvested and lysed with reporter lysis buffer (Promega, USA), and luciferase activities were measured using a luciferase assay reagent (Promega, USA) and a luminometer (Orion II, Berthold Detection Systems, Wildbad, Germany). β -

galactosidase activity was determined by standard methods using o-nitrophenyl β -D-galactopyranoside (ONPG, Sigma-Aldrich, Taufkirchen, Germany) as a normalisation control.

2.7 Western blot analysis

Cell lysate preparation: After washing twice with 1x PBS, cells in a 10 cm dish were scraped off the plate and transferred to a 1.5 ml tube. Lysis buffer was added directly into the tube and the tube was incubated at room temperature for 15 min. The cell lysate was finally centrifuged at 16,000 g at 4 °C for 15 min. The supernatant was aliquoted and stored at -80 °C.

Protein concentration measurement: Protein concentrations were measured with the BCA protein assay. A serially diluted bovine serum albumin solution (2 mg/ml) was used as a standard. 5 μ l of protein lysate were diluted with 95 μ l ddH₂O and 100 μ l BCA reagent were added in duplicate into a 96-well plate at a final volume 200 μ l per well. The plate was incubated at 37 °C for 30 min before the absorbance was measured at 562 nm with a microplate reader.

Gel electrophoresis: The cell lysates were mixed with 5x loading buffer (4 volumes of sample + 1 volume of loading buffer), heated at 95 °C for 5-10 min and centrifuged at 16,000g at 4 °C for 5 min. The samples were then applied to 8-16% polyacrylamide gels. 6 μ l marker was loaded as molecular weight standard. The running condition was 65 V for 20 min and 100 V for 1 h-1.5 h at RT.

Transfer: After separation, proteins were transferred to a PVDF membrane in a semi-dry transfer chamber. Before transfer, the membrane was soaked in methanol for 1 min. The transfer took place in a semidry transfer unit at a constant current of 200 mA for 1 h at RT.

Blotting: After transfer, the PVDF membrane was washed in 1x TBST for 5 min on a shaker and then blocked in blocking buffer (5% milk in 1 x TBST) for 2 h at RT. Subsequently, the membrane was incubated with the primary antibodies (diluted in 1 x TBST with 5% milk) overnight at 4 °C on a rotating platform. The membrane was washed three times in 1 x TBST for 5 min and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Finally, the membrane was washed again three times for 5 min with 1 x TBST buffer and once with 1 x PBST. The detection was performed using the enhanced chemiluminescence system. β -actin detection was used to ensure equal protein loading.

2.8 Immunofluorescence staining

48 h after transfection with miR-221 mimics or inhibitors, 5-FU sensitive or resistant esophageal cancer cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with 1 x PBS for 5 min, permeabilized with 0.1% Triton X-100 in blocking serum for 30 min at room temperature, and finally incubated with anti- β -catenin antibody (dilution 1:200) at 4 °C overnight. After the cells were stained with a matched Cy3-conjugated secondary antibody (dilution 1:200) for 1 h at room temperature, the chamber slides were counterstained with DAPI in mounting medium and visualized by digital fluorescence microscopy using the AxioVision Rel. 4.6 software. For further image processing and analysis the software Image-pro was used.

2.9 Immunohistochemistry and immunofluorescence analysis for Ki67 and CD31

Tissue sections (4 μ m) were deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide followed by antigen retrieval using antigen retrieval solution. Endogenous avidin and biotin were blocked with the Avidin/Biotin blocking kit. The slides were treated for 20 min with blocking solution followed by overnight application of anti-Ki67 antibody as primary antibody. Then biotinylated secondary antibody as well as the ABC reagent for signal amplification was applied. Slides were washed in TBS buffer, stained with DAB and counterstained with hematoxylin and then mounted in Kaiser's glycerol gelatin.

Frozen tissue sections were fixed with 1% paraformaldehyde for 20 min and then washed 2 times with 1 x PBS. 10% goat serum was added for blocking the non-specific binding sites. Frozen tissue sections were stained with 1:100 diluted CD31 antibody at 4 °C overnight. After washed 2 times with 1x PBS, all sections were incubated with a DAPI nuclear counterstaining. Finally slides were analyzed at 200x magnification with a fluorescence microscope. Areas presenting the highest density of CD31 positive cells were chosen and captured as photographs. These photographs were analyzed by Image-J program. The Ki67 or CD31 index were evaluated in a blinded manner and calculated as Ki67 or CD31 positive cells in one field. Necrotic tumor cells were excluded from the cell count. MVD (Micro vascular density) was evaluated by CD31 positive structures that were identified for vessel counts.

2.10 *In vivo* subcutaneous esophageal cancer animal model

BALB/c nu-nu male mice were obtained from Charles River Deutschland (Sulzfeld, Germany) at 6-8 weeks of age and housed in the animal facility of the University Medical Center Magdeburg. These mice were divided into six groups (5 mice per group):

Group 1: OE33 + PBS (OE33 Ctrl) (n=5)

Group 2: OE33 + negative control siRNA (OE33 + siRCtrl) (n=5)

Group 3: OE33 + miR-221 mimics (OE33 + miR-221) (n=5)

Group 4: OE33-5FU_{res} + PBS (OE33-5FU_{res} Ctrl) (n=5)

Group 5: OE33-5FU_{res} + negative control siRNA (OE33-5FU_{res} + siRCtrl) (n=5)

Group 6: OE33-5FU_{res} + anti221 inhibitors (OE33-5FU_{res} + anti221) (n=5)

- 1) All cells were harvested after trypsinization and washed 3 times with medium. A total of 1×10^6 cells in a 200 μ l suspension were subcutaneously injected into the flanks of mice with a 1ml syringe (BD Plastipak, Becton Dickinson S.A., Spain) with a 27-gauge hypodermic needle (Sterican, Braun, Germany) within 40 min of harvest.

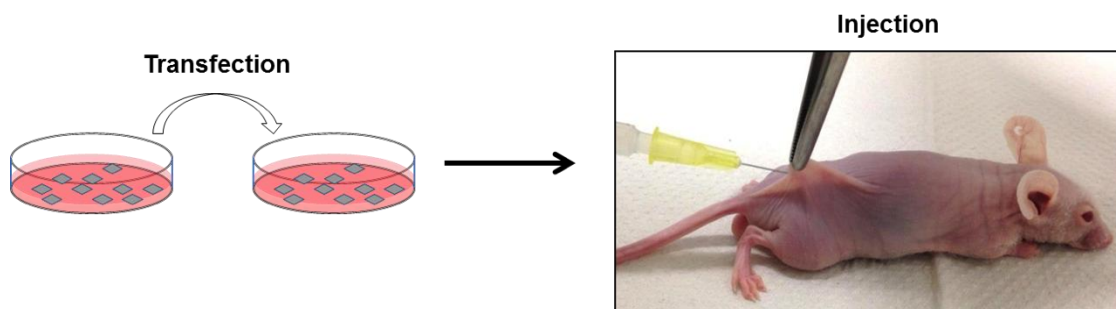


Figure XIII. Subcutaneous transplantation model of esophageal cancer. First, OE33 and OE33-5FU_{res} cells were seeded on 10 mm dishes and transfected with hsa-miR-221 mimics, hsa-miR-221 inhibitors and negative control siRNA, respectively. The next day cells were collected and resuspended in PBS; 1×10^6 cells in a 200 μ l suspension were subcutaneously injected into the flanks of mice with a 1 ml syringe with a 27-gauge hypodermic needle (Sterican, Braun, Germany) within 40 min of harvest.

- 2) Mice were weighed and tumor sizes were measured for tumor development every three days. When the biggest tumor size reached around 10 mm in diameter, all tumors were collected and the data was analyzed.

2.11 Identification of validated β -catenin target genes related with EMT pathway

Considering 5-FU resistance, Wnt/ β -catenin pathway and EMT pathway in this study, we collected and summarized common cancer relevant genes among Wnt/ β -catenin pathway and its targets, as well as EMT. We were particularly interested in β -catenin target genes that are associated with EMT. For this reason, the PubMed database was searched using the phrase “(beta-catenin AND target) AND emt”. 135 articles were identified (as of 12 1st, 2014) and publications were screened for the identification and characterization of β -catenin target genes related to EMT. We were able to identify 30 published β -catenin target genes related to the EMT pathway that matched our criteria (Table 5) and 3 chemoresistance related markers: ABCG2, ABCB1 and ABCC2.

Table 6. Candidate genes of EMT-related Wnt/ β -catenin target genes

AXIN2	BIRC5	BMP4	CCND1	CD44	CDH1	DKK1
EGFR	EPCAM	FN1	FOS	FZD7	GSK3B	MMP2
MMP7	MMP9	MSX2	MYC	NANOG	SNAI2	TCF4
VIM	WNT3A	WNT5A	ZEB1	EPHA2	SOX2	SNAI1
TGFB3	JUN	ABCG2	ABCB1	ABCC2		

2.12 RT² Profiler PCR Array System

The Custom Human RT² Profile TM PCR array (RT Profiler TM PCR Array: CAPH12950) was performed for examining genes of interest including targets of Wnt pathway, which are associated with EMT and chemoresistance. Total RNA of 5×10^5 OE33 and OE33-5FU_{res} cells with or without miR-221 mimics, inhibitors and siR-CTNNB1 transfection was isolated using the miRNeasy kit (Method 2.2). cDNA was synthesized from 500 ng of RNA using the RT² First Stand kit. PCR was performed with the RT² Profile PCR array system according to the manufacturer's instructions using ABI 7500. The expression levels of different mRNAs were normalized using housekeeping genes expression of ACTB, B2M, GAPDH, HPRT1 and RPLP0. The fold change of each gene from treatment group to control group was calculated as $2^{(-\Delta\Delta CT)}$.

2.13 Statistical analysis

All data are expressed as mean \pm SD. The correlation of β -catenin or DKK2 and each clinical pathologic variable was comparatively analyzed by the Fisher exact test and Chi-square test. A p value of less than 0.05 indicated the presence of statistically significant difference between groups. All statistical analyses were carried out with Graphpad Prism 6. For RT² profiler data analysis, we compared genes differently expressed in esophageal cancer cell lines OE33, OE33-5Fu_{res} cells after transfection with miR-221 mimic, inhibitors and siRNA-CTNNB1 by a web-based service of Qiagen data analysis center. Genes were called “differentially expressed” if the corrected P value was less than 0.05.

Results

1. Establishment of esophageal cancer cell lines resistant to 5-FU

At first we successfully established 5-FU resistant EC cell models from four different EC cells lines (OE19, OE33, PT1590 and LN1590). We compared the sensitivity of these 4 pairs of esophageal cancer cell lines to 5-FU by CCK-8 assays. The IC₅₀ of 5-FU resistant EC cells was approximately 10 to 60 fold higher than that of parental cells as shown in Figure 1-1. The IC₅₀ of the four 5-FU resistant esophageal cancer cells OE19-5FU_{res}, OE33-5FU_{res}, PT-5FU_{res} and LN-5FU_{res} were 78.65 ± 6.56 $\mu\text{g/ml}$, 102.80 ± 8.94 $\mu\text{g/ml}$, 81.91 ± 6.22 $\mu\text{g/ml}$ and 186.20 ± 6.98 $\mu\text{g/ml}$, respectively. The IC₅₀ of the 4 corresponding parental esophageal cancer cells were 4.29 ± 0.84 $\mu\text{g/ml}$, 4.30 ± 0.85 $\mu\text{g/ml}$, 6.53 ± 0.70 $\mu\text{g/ml}$ and 3.30 ± 0.50 $\mu\text{g/ml}$, respectively.

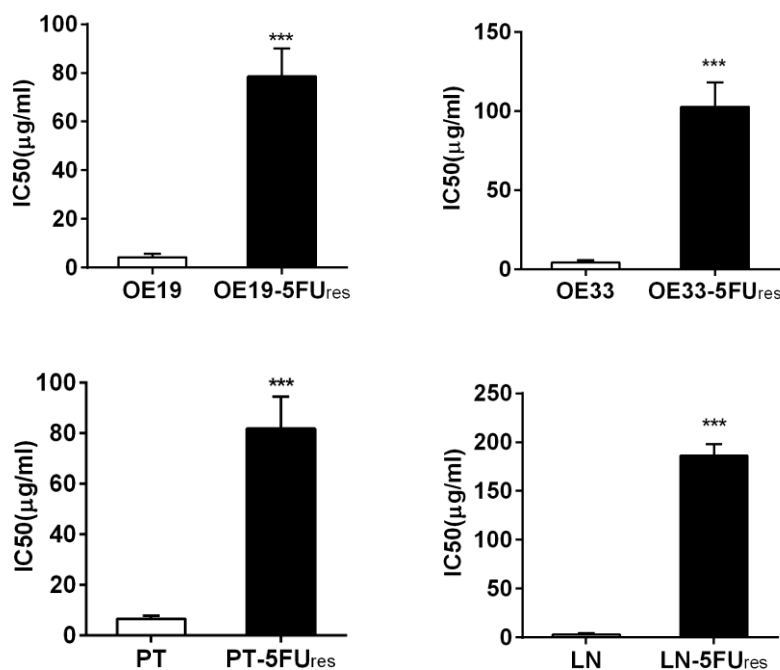


Figure 1-1. IC₅₀ of 5-FU sensitive and resistant esophageal cancer cells. The original IC₅₀ values for OE19, OE33, PT1590 and LN1590 were 4.3, 4.29, 6.53 and 3.3 $\mu\text{g/ml}$. The IC₅₀ values for OE19-5FU_{res}, OE33-5FU_{res}, LN-5FU_{res} and PT-5FU_{res} lines were 78.6, 102.8, 81.9, and 186.2 $\mu\text{g/ml}$ respectively.

2. MiR-221 is upregulated in 5-FU resistant esophageal cancer cells

In preliminary work on the function of miRNAs in 5-FU resistance of esophageal cancer cells we measured the level of several chemoresistance-related miRNAs (let-7b, let-7g, miR-21, miR-34a, miR-92, miR-200c and miR-221) in 5-FU sensitive and resistant esophageal cancer cell lines PT/PT-5FU_{res} and LN/LN-5FU_{res}. We found that miR-221 level in PT-5FU_{res} and LN-5FU_{res} was higher than that in parental cancer cell lines. The fold change was 6.69 ± 0.43 and 1.51 ± 0.04 , respectively (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) (Figure 2-1). Then we identified miR-221 expression in the other two pairs of esophageal cancer cell lines OE19-5FU_{res} and OE33-5FU_{res} as well as their parental cells. Compared to OE19 and OE33 cells, miR-221 expression was higher in both OE19-5FU_{res} and OE33-5FU_{res} (** $p < 0.01$, *** $p < 0.001$) (Figure 2-2).

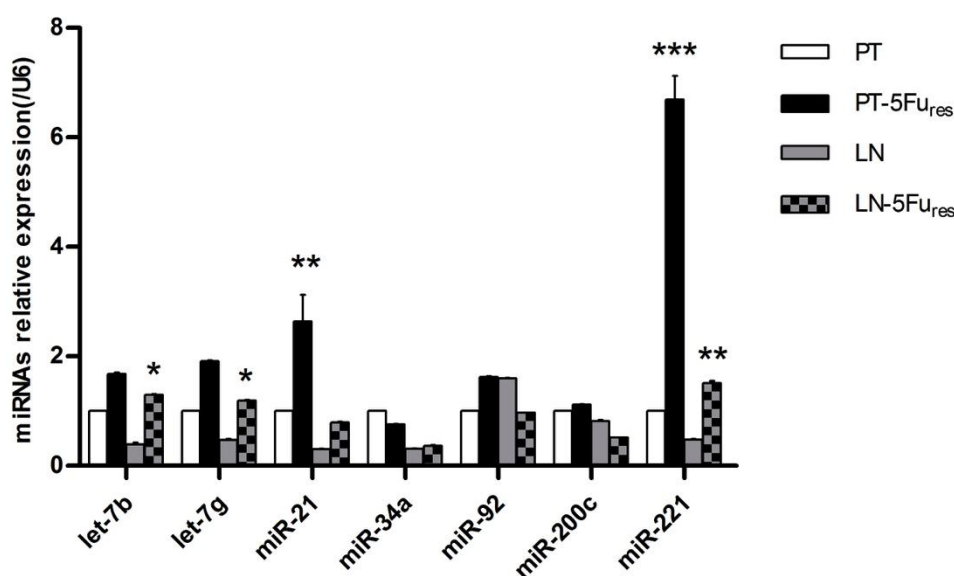


Figure 2-1. MiRNAs expression in 5-FU sensitive and resistant PT1590 and LN1590 cells. Compared to PT1590 and LN1590 cells, miR-221 expression in PT-5FU_{res} and LN-5FU_{res} was increased 6.69 ± 0.43 and 1.51 ± 0.04 fold respectively. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, t-test.

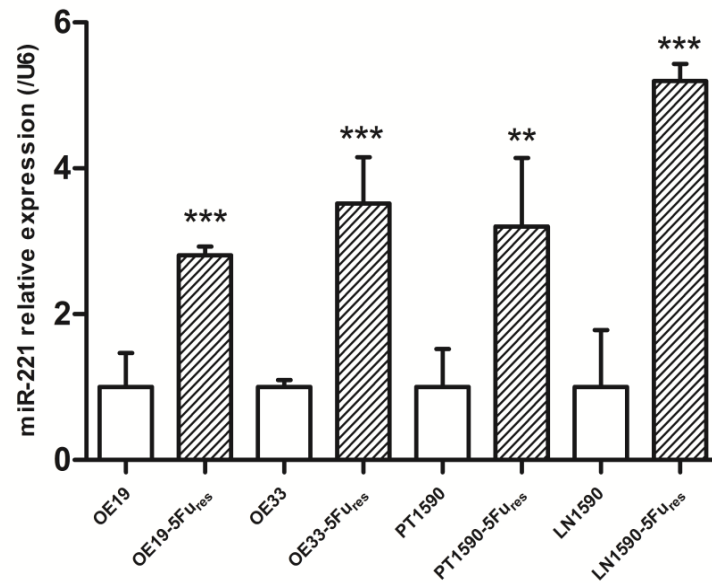


Figure 2-2. MiR-221 expression in four pairs of 5-FU sensitive and resistant esophageal cancer cell lines. miR-221 was upregulated in all four 5-FU resistant esophageal cancer cell lines compared to their corresponding parental cancer cells. ** $p < 0.01$ and *** $p < 0.001$, t-test.

For further study, we separately increased and decreased miR-221 expression by transfecting miR-221 mimics into OE33 cells and the miR-221 inhibitors into OE33-5FU_{res}; we conducted microRNA qPCR for determination of miR-221 expression to evaluate the transfection efficiency and the efficacy of miR-221 mimics and inhibitors. It was observed a dramatic increase of miR-221 expression in OE33 cells following transfection of miR-221 mimics and a decrease of miR-221 expression in OE33-5FU_{res} following transfection of miR-221 inhibitors. (** $p < 0.01$, *** $p < 0.001$) (Figure 2-3).

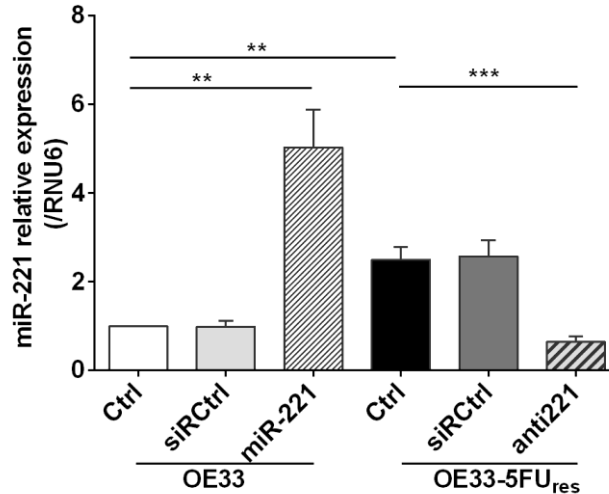


Figure 2-3. The efficacy of miR-221 mimics and inhibitors in EC cells. Compared to the corresponding control groups, the level of miR-221 in OE33 and OE33-5FU_{res} cells was increased and decreased by miR-221 mimics and miR-221 inhibitors, respectively. While there was no significant difference of miR-221 expression in OE33 and OE33-5FU_{res} cells with or without miRNA negative control and inhibitors negative control treatment. ** $p < 0.01$ and *** $p < 0.001$, t-test.

3. Dysregulation of miR-221 regulates cell proliferation and 5-FU sensitivity in 5-FU sensitive and resistant EAC cells

After transfection with miR-221 mimics or inhibitors in 5-FU sensitive or resistant esophageal cancer cells, we measured cell growth of esophageal cancer cells at 0 h, 24 h, 48 h and 72 h. We found miR-221 mimics can promote cell growth of 5-FU sensitive esophageal cancer cells, which was most significant at 48 h; and miR-221 inhibitors can suppress cell growth of 5-FU resistant esophageal cancer cells at 48 h. (Figure 3-1)

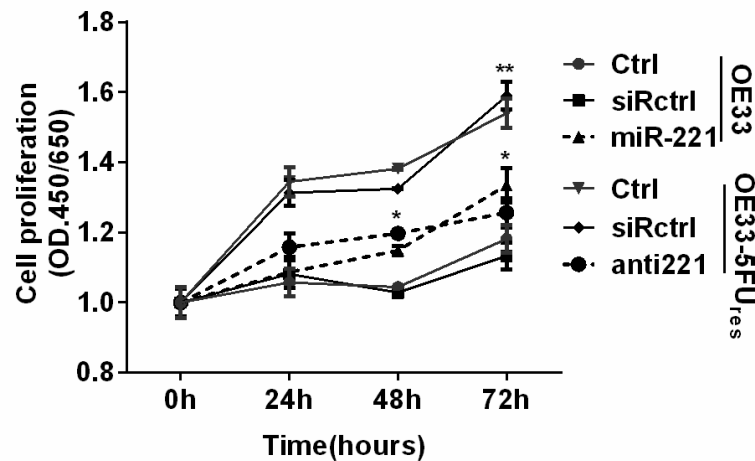


Figure 3-1. Cell proliferation of 5-FU sensitive and resistant OE33 cells with or without miR-221 mimics and inhibitors transfection. At 48 h after transfection, miR-221 mimics and inhibitors significantly promoted and inhibited cell growth of OE33 and OE33-5FU_{res} cells, respectively. * $p < 0.05$, ** $p < 0.01$, t-test.

For assessing the cytotoxicity of esophageal cancer cells to 5-FU with or without miR-221 mimics or inhibitors transfection, CCK-8 assay was performed to measure cell inhibition ratio. 37.23 ± 6.32 % OE33 cells and 6.28 ± 0.67 % OE33-5FU cells were inhibited at 48 h after 2.5 $\mu\text{g/ml}$ 5-FU treatment. MiR-221 mimics did not significantly increase the resistance of OE33 cells to 5-FU [33.43 ± 1.47 % dead cells]; however, miR-221 inhibitors did significantly lift the 5-FU sensitivity of OE33-5FU_{res} [29.85 ± 4.86 % dead cells] (** $p < 0.01$, Figure 3-2). When we treated these cells with 20 $\mu\text{g/ml}$ 5-FU, we got the same results. There were more dead OE33-5FU_{res} cells with low level of miR-221 [36.94 ± 6.17 % dead cells] (** $p < 0.01$) (Figure 3-3). Moreover, we confirmed the effect of miR-221 inhibitors to suppress resistance by measuring the expression of the 5-FU target and resistance relevant marker thymidylate synthase (TS). The Western blot result showed that miR-221 mimics slightly increased TS protein expression in OE33 cells, while miR-221 inhibitors decreased TS protein expression in OE33-5FU_{res} (Figure 3-4).

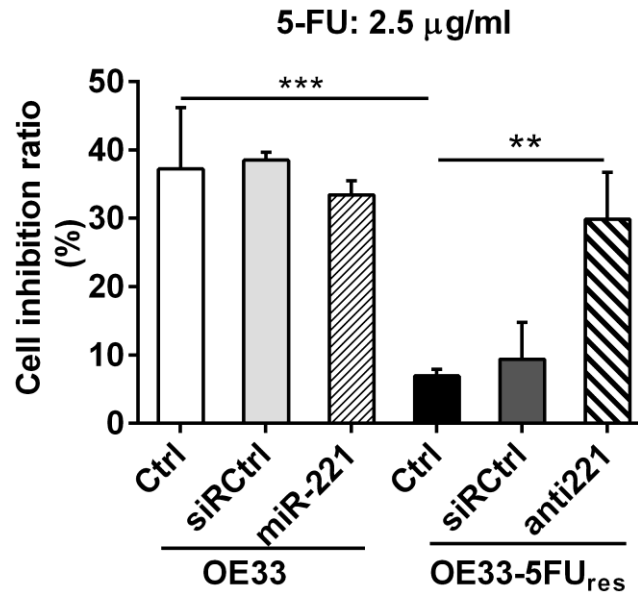


Figure 3-2. Sensitivity of OE33 and OE33-5FU_{res} cells with or without miR-221 mimics and inhibitors treatment to 2.5 μ g/ml 5-FU. miR-221 mimics did not significantly decrease the sensitivity of OE33 cells to 5-FU, but miR-221 inhibitors significantly increased the sensitivity of OE33-5FU_{res} to 5FU. ** $p < 0.01$ and *** $p < 0.001$, t-test.

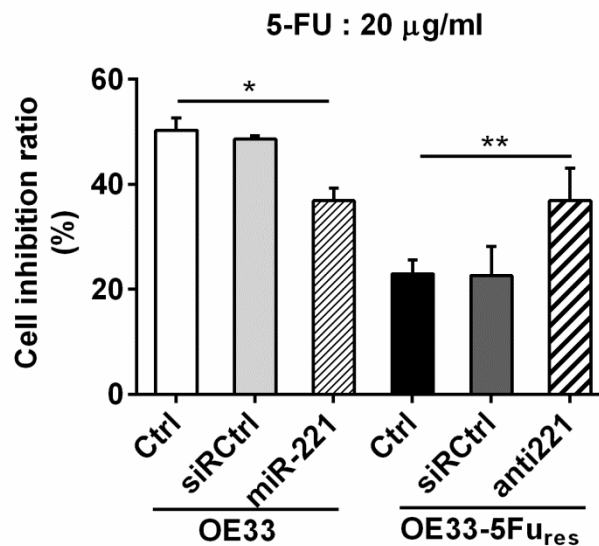


Figure 3-3. Sensitivity of OE33 and OE33-5FU_{res} cells with or without miR-221 mimics and inhibitors after 20 μ g/ml 5-FU treatment. miR-221 mimics did not decrease the sensitivity of OE33 cells to 5-FU, but miR-221 inhibitors significantly increased the sensitivity of OE33-5FU_{res} to 5-FU. ** $p < 0.01$ and *** $p < 0.001$, t-test.

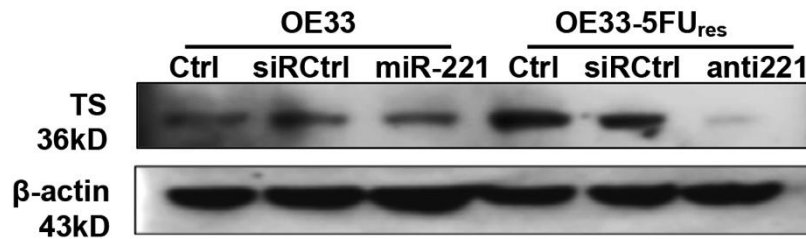


Figure 3-4. The protein level of TS in OE33 and OE33-5FU_{res}. miR-221 mimics increased TS protein expression slightly, while miR-221 inhibitors decreased TS protein level dramatically.

4. Dysregulation of miR-221 regulates esophageal cancer growth *in vivo*

OE33 tumor xenografts were established by subcutaneous injection of 1×10^6 OE33 and OE33-5FU_{res} cells with or without miR-221 mimics/inhibitors transfection in the flanks of mice. At 66 days after injection, all mice were sacrificed. The tumor sizes are shown in Figure 4-1. The tumors of the OE33-5FU_{res} control group and the OE33+miR-221 group were significantly bigger than that of the OE33 control group. While the tumor of the OE33-5FU_{res}+anti221 group was smaller than that of the OE33-5FU_{res} control group. The tumor growth curve analysis revealed that overexpression of miR-221 significantly promoted tumor growth of 5-FU sensitive esophageal cancer, while the loss of miR-221 by its inhibitors dramatically suppressed tumor growth of 5-FU resistant esophageal cancer (Figure 4-2). To assess tumor cell proliferation and angiogenesis, Ki67 and CD31 expression was detected by IHC staining and IF staining. The results were shown as Figure 4-3. In the OE33+miR-221 group, a clear increase of Ki67 and CD31 expression was observed compared to the OE33 control group. While there was a dramatic decrease of Ki67 and CD31 expression in the OE33-5FU_{res}+anti221-group compared to OE33-5FU_{res} control group. These results indicated that miR-221 could influence tumor cell proliferation and angiogenesis in both 5-FU sensitive and resistant esophageal cancer.

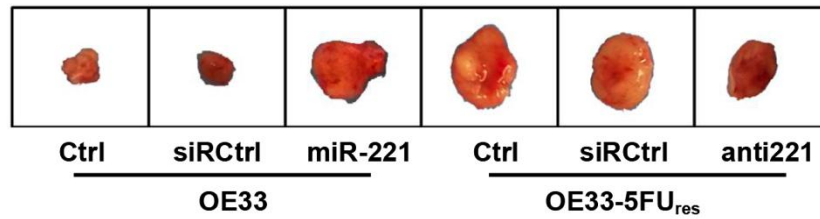


Figure 4-1. Comparison of tumor sizes among 6 groups (5 mice/group). The tumors of the OE33-5FU_{res}-control group and the OE33+miR-221-group were dramatically bigger than those of the OE33-control group, while tumors of the OE33-5FU_{res}+anti221-group were smaller than those of the OE33-5FU_{res} control group.

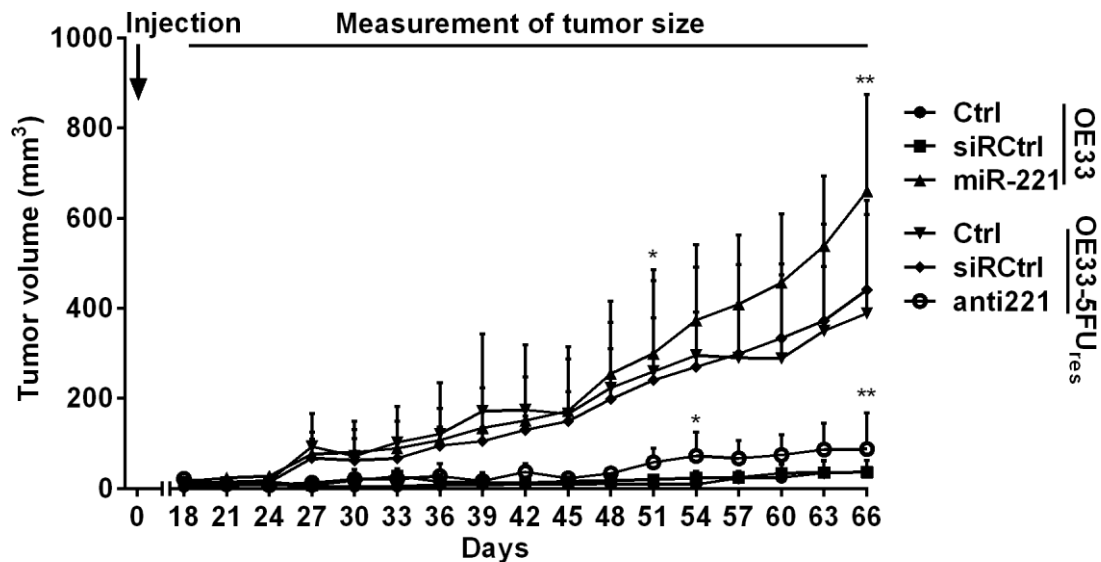


Figure 4-2. *In vivo* tumorigenesis of esophageal cancer. Enhanced expression of miR-221 promoted OE33 tumor growth in a xenograft nude mouse model and down-regulation of miR-221 expression inhibited OE33-5FU_{res} tumor growth. * $p < 0.05$, ** $p < 0.01$, t-test.

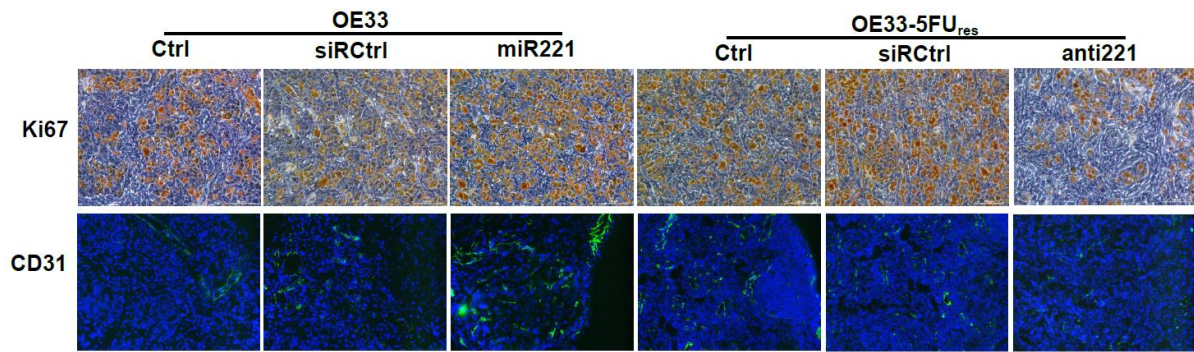


Figure 4-3. Analysis of cell proliferation and angiogenesis in tumor. The proliferation marker *Ki67* was substantially weaker expressed in the OE33-5FU_{res} +anti221 group, as compared to the control group. The angiogenesis marker CD31 expression in OE33+miR-221 group was dramatically increased as compared with that in control group, while a reduction in CD31 expression was seen in OE33-5FU_{res} +anti221 group.

5. MiR-221 acts pro-tumorigenically by directly targeting DKK2 in EC

To further investigate the molecular mechanism of miR-221 regarding its pro-tumorigenic ability, we determined its target genes in esophageal cancer by interrogating the interaction between miR-221 and its target mRNA transcripts. Candidate target genes were determined via four microRNA target prediction tools including TargetScan, PITA, miRTarBase and miRanda (Figure 5-1). 120 common candidate target genes were predicted by these 4 prediction tools. In these candidate target genes, we chose the gene DKK2 for further studies. DKK2 is an antagonist of the WNT pathway, which is associated to the development of esophageal cancer. The sequence of 3'UTR of DKK2 is conserved in wide variety of mammalian species such as human, chimpanzee, rat, mouse, rabbit, etc. The interactive capacity of miR-221 and 3'-UTR of DKK2 was analyzed using the miRNA target prediction tool RNAhybrid (Figure 5-2).

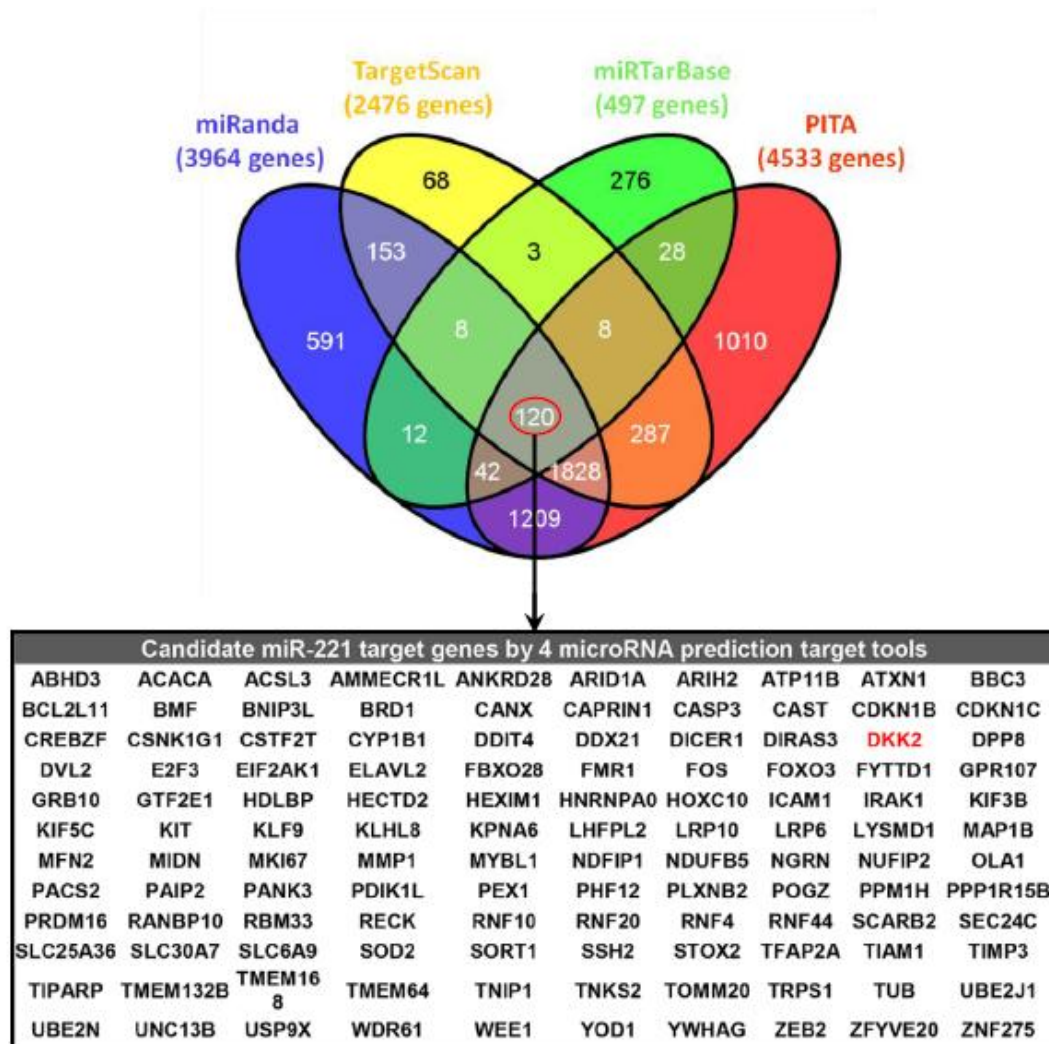


Figure 5-1. Prediction of miR-221 target genes by microRNA target prediction tools. 120 candidate target genes with miR-221 seed sites were predicted via four different miRNA target prediction tools (TargetScan, PITA, miRTarBase and miRanda). DKK2 was chosen for further study.

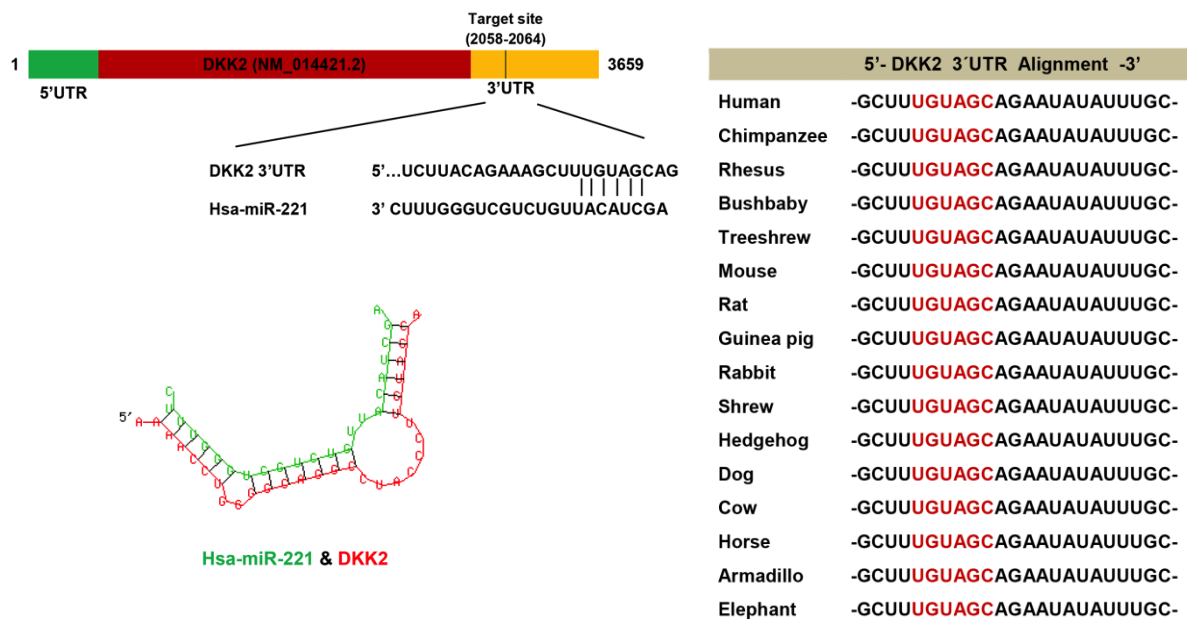


Figure 5-2. Interaction of miR-221 and 3'UTR of DKK2. miR-221 could bind the bases from 2058 to 2064 in the 3'-UTR of DKK2 predicted by TargetScan. The sequence of the DKK2 3'UTR is conserved in different species: human, chimpanzee, rat, mouse, rabbit, etc. The interactive capacity of miR-221 and 3'UTR of DKK2 was analyzed using the miRNA target prediction tool RNAhybrid.

To confirm the interaction of miR-221 and DKK2 analyzed by the miRNA targets prediction engine, we performed a series of assays to determine the direct target relationship between miR-221 and DKK2 in esophageal cancer cell lines. Overexpression of miR-221 by introducing miR-221 mimics in OE33 cells resulted in a significant reduction of DKK2 mRNA transcription as well as protein expression (Figure5-3). Reversely, down-regulation of miR-221 by its inhibitors enhanced DKK2 expression at both the mRNA and protein level in OE33-5FU_{res} cells (Figure 5-3).

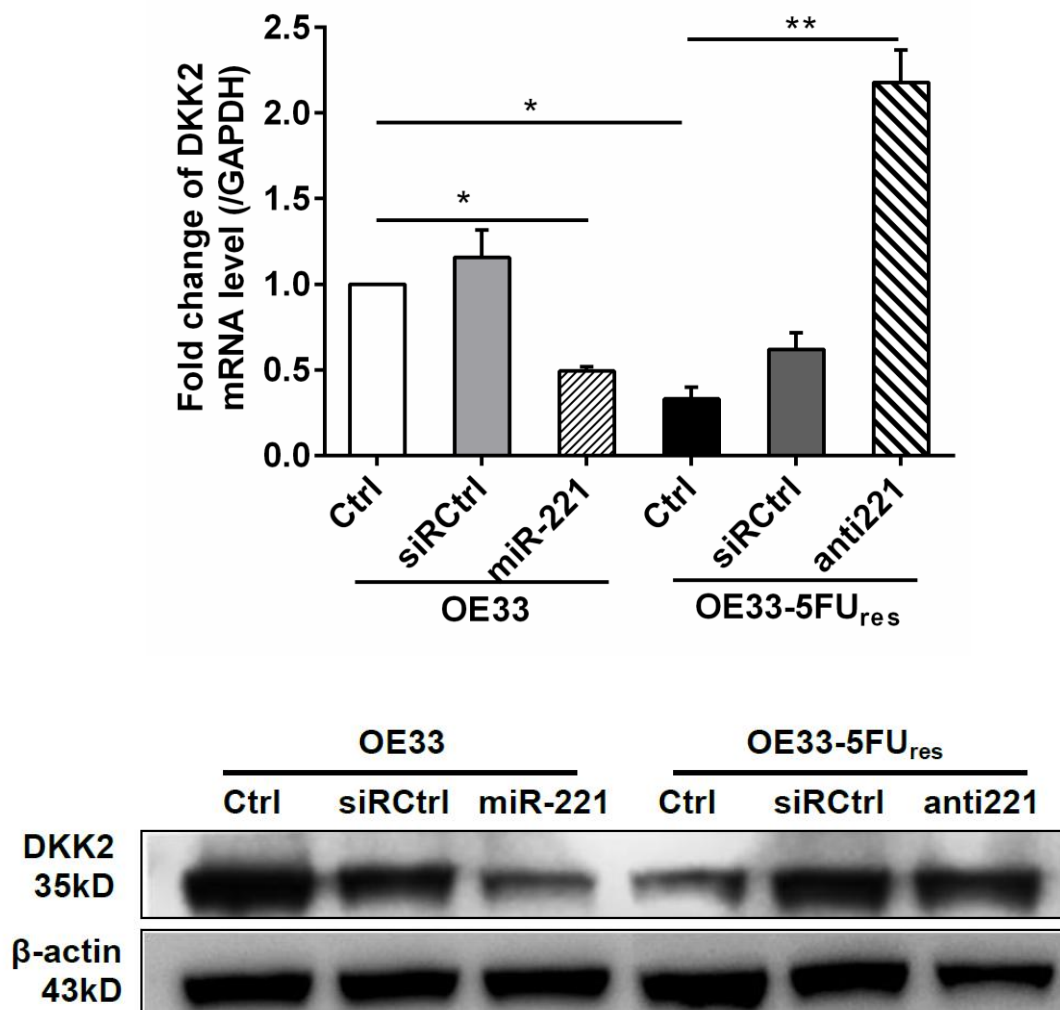


Figure 5-3. DKK2 expression in OE33 and OE33-5FU_{res} cells. qPCR analysis showed that DKK2 mRNA levels were reduced in 5-FU resistant esophageal cancer cells, as compared to the 5-FU sensitive esophageal cancer lines. MiR-221 knockdown was shown to result in a significant increase in DKK2 mRNA and protein expression. * $p < 0.05$, ** $p < 0.01$, t-test.

Even though miRNAs are known to affect the translation of gene transcripts, we performed luciferase reporter assays to identify whether miR-221 directly targets the 3'-UTR of DKK2. Esophageal cancer cells OE33 were co-transfected with various combinations of pGL3 luciferase reporter vectors. The plasmids used in the luciferase reporter assay included pGL3 control vector, luciferase reporter vector with wild type or mutant type 3'-UTR of DKK2 and miR-221 mimics or negative control (Figure 5-4). Compared with wild type 3'-UTR of DKK2 and negative control, a significantly decreased luciferase activity was observed in cells transfected with wild type 3'-UTR of DKK2 and miR-221 mimics. However, there was no difference of luciferase activity observed in cells transfected with mutant type 3'-UTR of

DKK2 and miR-221 mimics. These results indicated that miR-221 directly targets the 3'-UTR sequences of DKK2 to mediate its tumor promoting function.

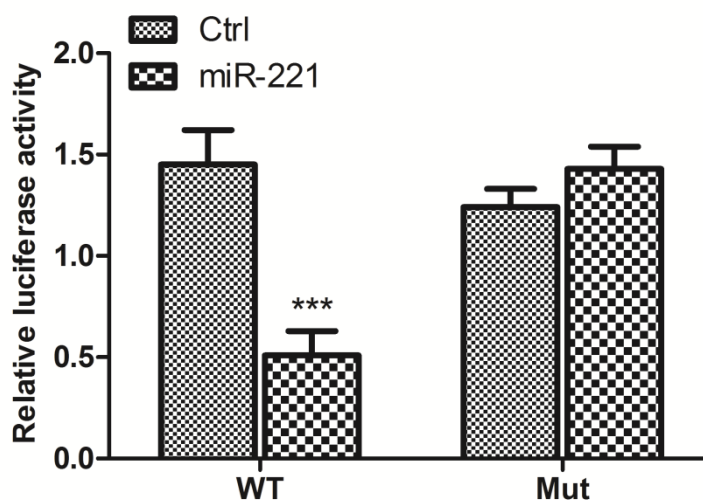


Figure 5-4. Luciferase reporter analysis. The results revealed direct binding of miR-221 to the wild type (WT), but not the mutant (Mut) sequences within the 3'UTR regions of DKK2. Ctrl: transfection of negative control; miR-221: transfection of miR-221 mimics. *** $p < 0.001$, t-test.

6. MiR-221 regulates Wnt/ β -catenin pathway in EC cells

As mentioned above, we have identified that DKK2 (an inhibitor of Wnt pathway) is a direct target of miR-221 in esophageal cancer cells. Next we investigated whether miR-221 modulated Wnt/ β -catenin pathway by targeting DKK2.

Firstly we performed IF staining for distribution of β -catenin protein in 5-FU sensitive and resistant esophageal cancer cells after transfection with miR-221 mimics, miR-221 inhibitors and negative control siRNA. Immunofluorescence analysis revealed that β -catenin is expressed at cell membrane of OE33, and in cell plasma and cell nuclei of OE33-5Fu_{res}. Up-regulation of miR-221 promoted the translocation of β -catenin into cell plasma and cell nuclei in OE33 cells, while down-regulation of miR-221 enhanced β -catenin returning back to cell membrane of OE33-5Fu_{res} cells (Figure 6-1).

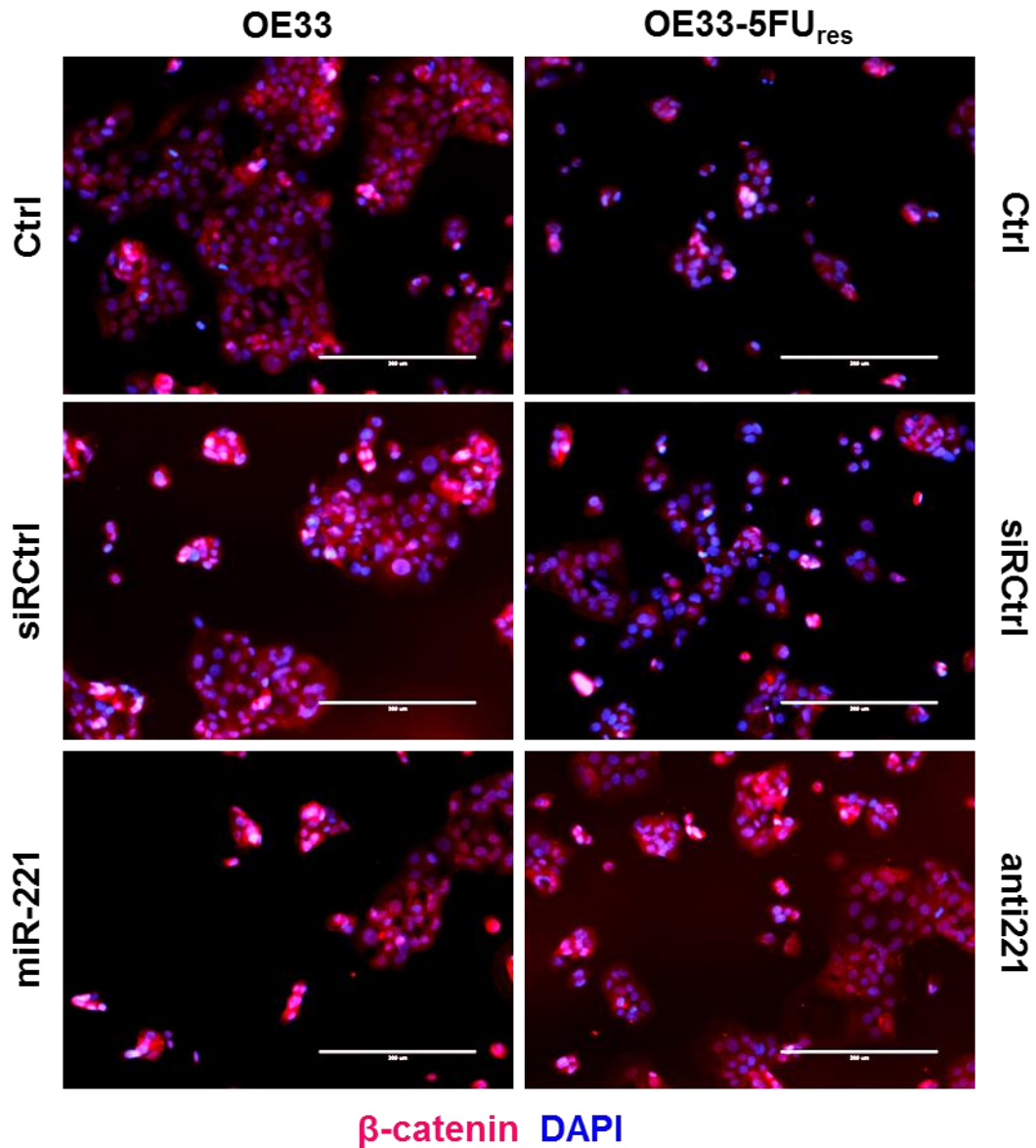


Figure 6-1. Distribution of β -catenin in OE33 and OE33-5FU_{res} with hsa-miR-221 mimics or inhibitors transfection. β -catenin is expressed at cell membrane of OE33, and in cell plasma and cell nuclei of OE33-5FU_{res}. Restoration of miR-221 promoted the translocation of β -catenin into cell plasma and cell nuclear in OE33 cells. While loss of miR-221 promoted β -catenin returning back to the cell membrane of OE33-5FU_{res} cells.

In addition, we used TOP/FOP Flash luciferase reporter assay for further verification to the activity of β -catenin after transfection with miR-221 mimics, miR-221 inhibitors, negative control siRNA and positive control β -catenin siRNA (siR- β). The Western blot result in Figure 6-2 showed the efficacy of β -catenin siRNA, which can strongly knockdown β -catenin in 5-FU resistant EC cells.

TOP/FOP Flash luciferase reporter analysis revealed an significantly increased luciferase activity following transfection with miR-221 mimics and a dramatic decreased luciferase activity following transfection with miR-221 inhibitors or siRNA-CTNNB1(siR- β) (** p <0.01, Figure 6-2). These results indicated that miR-221 mediated Wnt/ β -catenin pathway modulation in esophageal cancer cells via interaction with DKK2 mRNA.

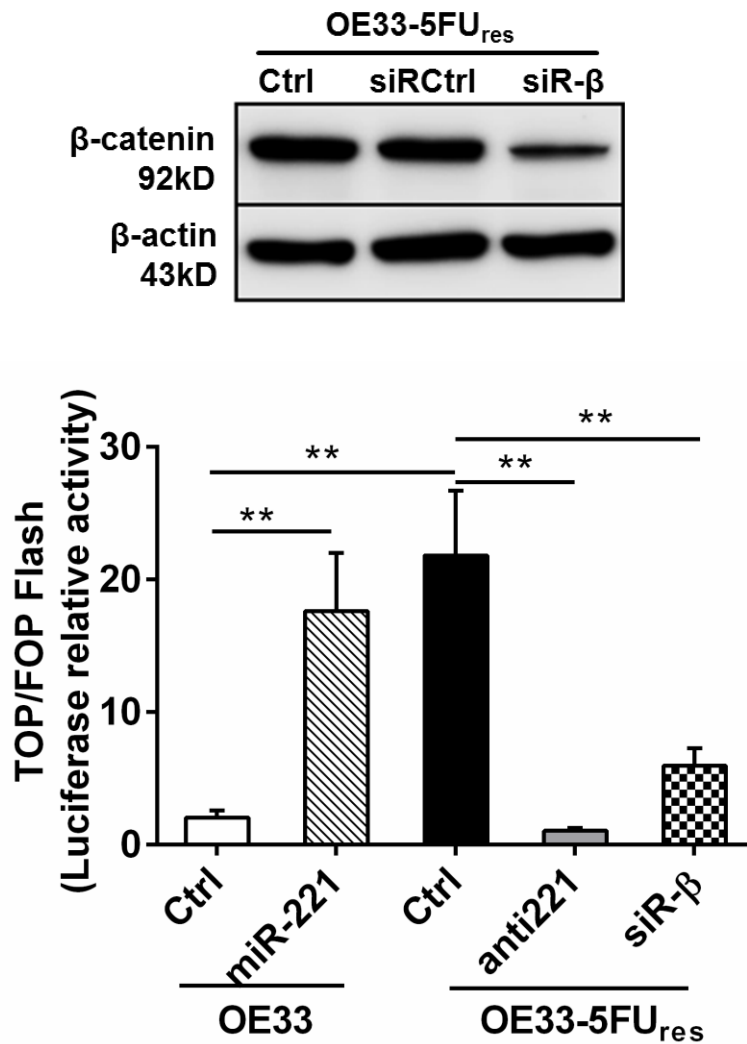


Figure 6-2. Top/Fop Flash luciferase reporter analysis. Overexpression of miR-221 in OE33 cells increased the luciferase activity compared to the control group, while loss of miR-221 by its inhibitors in OE33-5FU_{res} cells decreased the luciferase activity. Moreover, knockdown of β -catenin by siRNA-CTNNB1 in OE33-5FU_{res} cells significantly inhibited the luciferase activity. ** p <0.01, t-test.

7. EMT related genes are regulated by miR-221 and β -catenin in EC cells

In this study, 5-FU resistant EC cells acquired a mesenchymal cell like phenotype as shown in Figure 7-1. This indicated that epithelial-mesenchymal transition (EMT) might be involved in the 5-FU resistance of esophageal cancer cells. Moreover, in our preliminary work, we found dysregulation of EMT related genes in side population cells of 5-FU sensitive and resistant esophageal cancer cells^[31]. Therefore, here we investigated whether miR-221 regulates epithelial-mesenchymal transition in 5-FU resistant esophageal cancer cells.

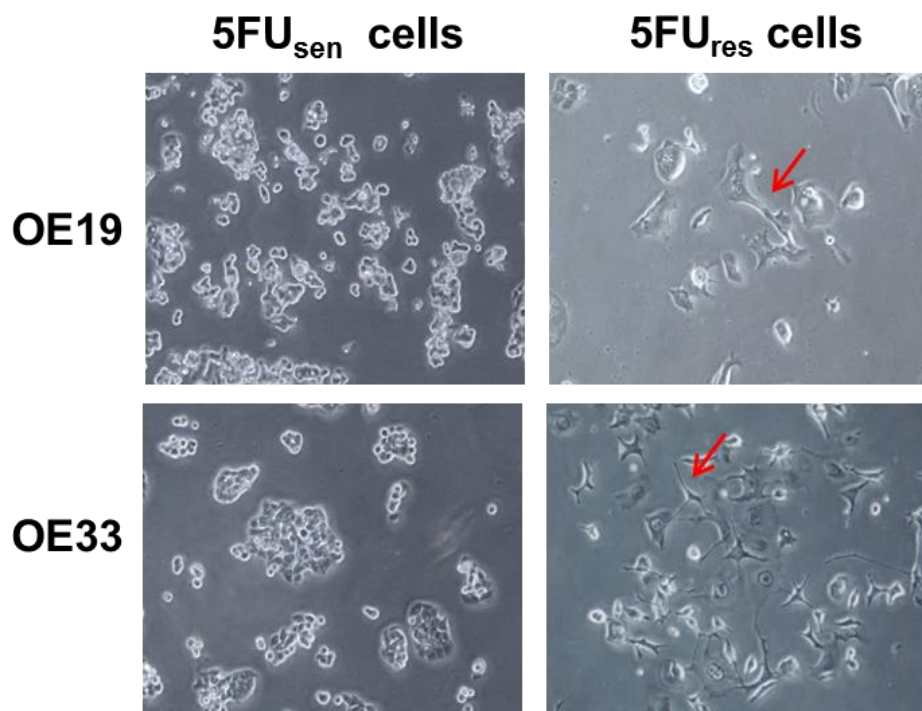


Figure 7-1. Morphology of 5-FU sensitive and resistant esophageal cancer cells. Mesenchymal cell-like morphology changes were observed in 5-FU resistant OE19 and OE33 cell lines as compared with 5-FU sensitive esophageal cancer cells (red arrows).

We analyzed the protein level of the epithelial cell marker E-cadherin and the mesenchymal cell marker vimentin in OE33 and OE33-5FU_{res} cells after treatment with miR-221 mimics, inhibitors and siRNA-CTNNB1. Western blot analysis (Figure 7-2) revealed a dramatic increase of vimentin and a noticeable decrease of E-catenin in OE33-5FU_{res} cells compared with OE33 cells. Overexpression of miR-221 dramatically increased vimentin expression in OE33 cells, while loss of miR-221 and β -catenin resulted in an increased E-cadherin and decreased vimentin expression in OE33-5FU_{res} cells. These results indicated that miR-221 could modulate the epithelial-mesenchymal transition in esophageal cancer cells.

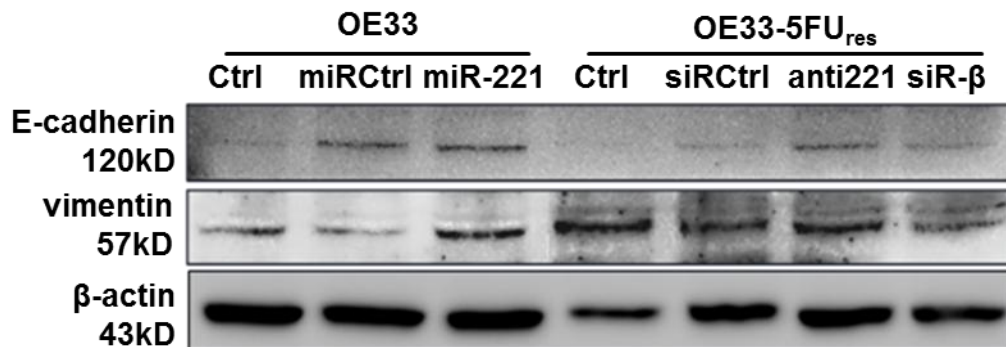


Figure 7-2. Analysis of EMT related genes in 5-FU sensitive and resistant esophageal cancer cells. Western blot analysis showed that the epithelial cell marker E-cadherin was expressed at a lower level in OE33-5FU_{res} cells as compared to the parental OE33 cells; by contrast, the mesenchymal cell marker vimentin was increased in the OE33-5FU_{res} cells as compared to the OE33 cells. An effect of miR-221 on this process was established by knockdown of miR-221 expression in OE33-5FU_{res} cells which resulted in a significant increase in E-cadherin and a decrease in protein expression of vimentin.

It has been reported that the Wnt pathway can mediate tumorigenesis and chemoresistance of various cancers via activation of the EMT [74,78–83]. We found Wnt pathway-mediated 5-FU resistance in esophageal cancer cells regulated by miR-221. To analyze this interaction we performed a series of assays to determine the relationship between miR-221/Wnt/β-catenin and EMT.

To identify EMT associated target genes of Wnt/β-catenin in esophageal cancer cells, we analyzed the expression of selected β-catenin target genes in OE33 and OE33-5FU_{res} cells after transfection with miR-221 mimics, inhibitors or siRNA-CTNNB1 by using customer RT-PCR profiles. The overexpression or loss of miR-221 and knockdown of CTNNB1 in OE33-5FU_{res} resulted in differential gene expression (Figure 7-3). CD44, ABCG2, MYC and CDH1 were found to be expressed significantly different in OE33-5FU_{res} cells versus OE33 cells and to be regulated by miR-221 mimics, miR-221 inhibitors and siR-CTNNB1.

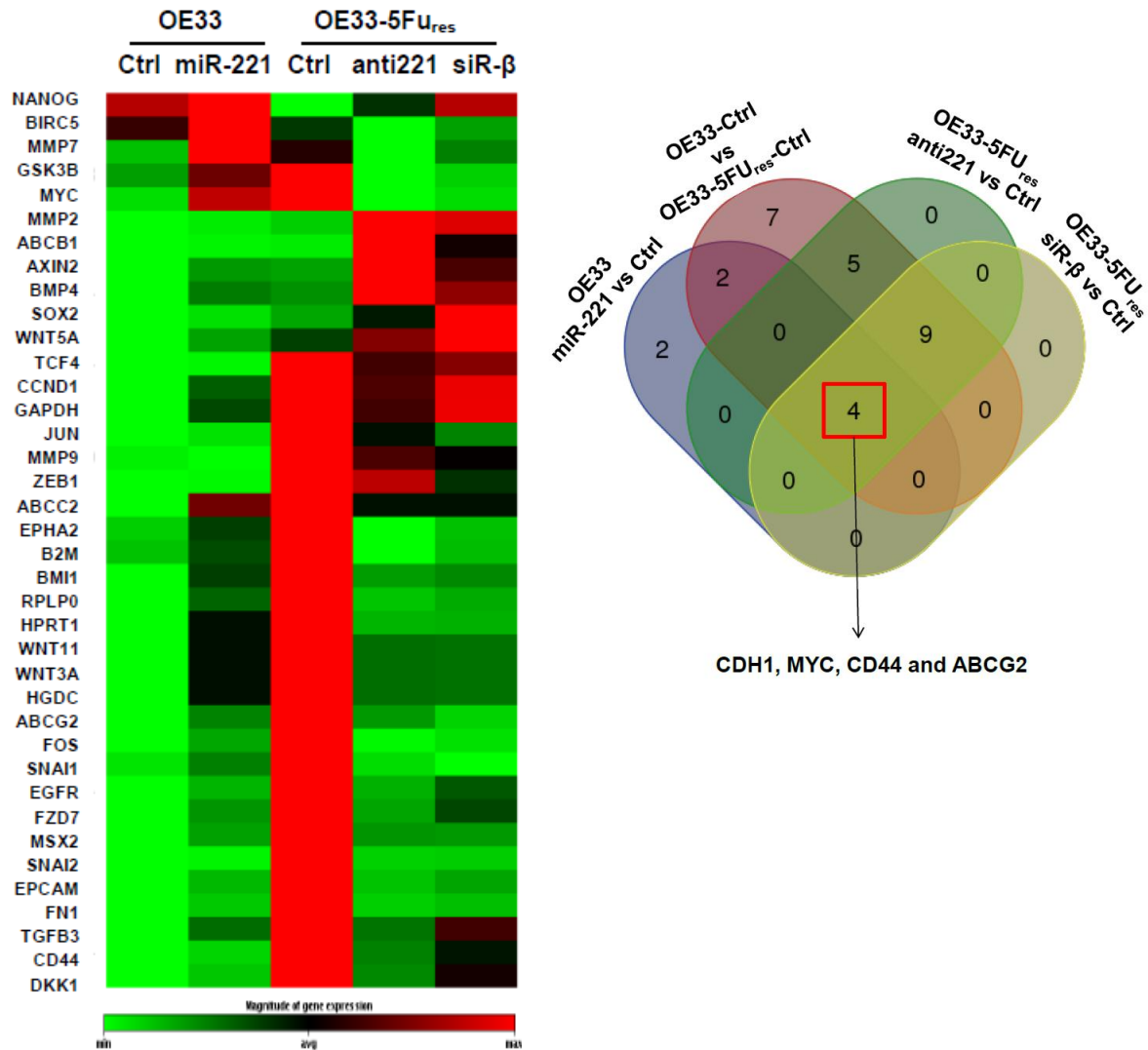


Figure 7-3. Customer RT-PCR array analysis. EMT related Wnt/ β -catenin target genes are expressed differently in OE33 and OE33-5FU_{res} cells after treatment with miR-221 mimics, inhibitors or siR-CTNNB1. The expression of seven genes in OE33 cells were increased by treatment with miR-221 mimics, while most of the 33 selected genes were regulated by miR-221 inhibitors in OE33-5FU_{res} cells. Four genes MYC, CD44, ABCG2 and CDH1 were found to be dysregulated between OE33 and OE33-5Fures cells, and to be influenced by miR-221 up or down regulation. Four genes CDH1, MYC, CD44 and ABCG2 were regulated by both miR-221 and β -catenin.

Discussion

The development of chemoresistance is one of the major challenges in the treatment of patients with cancer including esophageal cancer. In the present study, we focused on miRNAs that are known to be associated with the sensitivity to anticancer therapies. We have 3 new findings in this study. First, in esophageal cancer, overexpression of miR-221 was closely associated with resistance to anticancer therapy with 5-FU. Second, miR-221 could regulate Wnt/ β -catenin pathway by directly targeting a Wnt inhibitor DKK2. Third, miR-221 could mediate epithelial mesenchymal transition through regulating Wnt/ β -catenin pathway.

Many miRNAs are known to be associated with chemotherapeutic efficacy. For example, it is reported that overexpression of miR-214 can induce cisplatin resistance by targeting PTEN in ovarian cancer^[84] and high expression of miR-21 is involved in gemcitabine resistance in pancreatic cancer and chemosensitivity is regained by downregulation of miR-21 by indole-3-carbinol^[85]. On the other hand, let-7a can repress chemoresistance and tumorigenicity in head and neck cancer through stem-like properties ablation^[86]. Low expression of miR-345, miR-7, miR-181a and miR-630 are associated with chemoresistance of breast, lung and ovarian cancer^[87,88]. To our knowledge, the relationship between miR-221 expression and chemoresistance in cancers has not been analyzed previously. In the present study, we showed that miR-221 expression is significantly associated with chemosensitivity of esophageal cancer to 5-FU.

The results showed that up-regulation of miR-221 is critical for 5-FU resistance and tumor growth of esophageal cancer cells both *in vitro* (Figure 3-1~Figure3-4) and *in vivo* (Figure 4-1~Figure 4-3). MiR-221 is located on chromosome X and has been shown to be over expressed in colorectal cancer, ovarian cancer, breast cancer, osteosarcoma, and pancreas cancer. In colorectal cancer, miR-221 promotes cell proliferation and metastasis both *in vivo* and *in vitro*^[89,90]. In addition, it could mediate the epithelial-mesenchymal transition in pancreas cancer cells^[76]. Moreover, miR-221 plays an important role in mediating radio-chemo resistance by targeting multi-pathways. MiR-221 targeting PI3K/Akt signaling axis induces cell proliferation and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) resistance in human glioblastoma and down-regulation of miR-221 regulates radiation sensitivity by targeting the PTEN pathway. Overexpression of miR-221 is associated with tamoxifen resistance in breast cancer by negatively regulating estrogen receptor alpha^[91-93]. MiR-221

induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma and up-regulation of miR-221 can regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN^[94,95]. MiR-221 confers breast cancer fulvestrant resistance by regulating multiple signaling pathways^[96].

Besides the role of miR-221 in esophageal cancer chemotherapy resistance, we identified DKK2 as the functional target of miR-221 regulating cell proliferation and 5-FU resistance (Figure 5-1~Figure 5-4). DKK2, one member of the Dickkopf protein family, is generally thought to be a direct inhibitor of the Wnt pathway. It can suppress the activity of Wnt pathway by binding to the LRP5/LRP6 co-receptors of frizzled (FZD). It is reported that DKK2 expression is remarkably decreased in melanoma and gastrointestinal cancer^[97-99]. In glioma, DKK2 was identified as a direct target of miR-222^[100]. MiR-221 and miR-222 belong to the same family and are highly similar. Here we found that miR-221 can bind the 3'UTR of DKK2 and inhibit the expression of DKK2 possibly at both mRNA and protein level (Figure 5-4).

The Wnt/ β -catenin signaling pathway is the canonical Wnt pathway, which plays an important role in cell self-renewal, cell proliferation, cell migration and carcinogenesis as well as chemoresistance of cancer. It has been reported to be activated by 5-FU^[101-103]. In this study, we found that the Wnt/ β -catenin signaling pathway was activated in 5-FU resistant EC cells and knockdown of β -catenin could decrease the activity of the TCF/LEF transcriptional complex identified by TOP/FOP Flash luciferase reporter analysis (Figure 6-2). We also found overexpression and loss of miR-221 could regulate the activity of TCF/LEF transcriptional complex. We speculated that miR-221 might promote 5-FU resistance by regulating Wnt/ β -catenin signaling pathway via DKK2. To elucidate the underlying mechanism, we measured the activity of the Wnt/ β -catenin signaling pathway. The results (Figure 5-3) showed that miR-221 overexpression inhibited the expression of DKK2 proteins, which abrogated the inhibitory effect of DKK2 on Wnt/ β -catenin signaling. This phenomenon then led to the disruption of the Axin/APC/GSK3 complex, thereby stabilizing β -catenin and permitting its subsequent activation. The activated β -catenin was then translocated into the nucleus, where it in turn activated its downstream effectors and functionally contributed to tumorigenesis. In the present study, we demonstrated for the first time that miR-221 functions as an oncomiR in esophageal cancer via targeting DKK2 and activating the Wnt/ β -catenin signaling pathway.

In this study, the phenomenon of EMT was shown as a result of continuously exposing esophageal cancer cell lines to 5-FU (Figure 7-1) and EMT related markers E-cadherin and vimentin were expressed differently between 5-FU sensitive and resistant EC cells (Figure 7-2). It was reported before that EMT is closely associated with the resistance of cancer to chemotherapy. In MCF-7 breast cancer cells, Zhang W et al found that chemoresistance of 5-FU induces epithelial-mesenchymal transition via up-regulation of Snail^[104]. Arumugam T et al found that epithelial-mesenchymal transition contributes to drug resistance in pancreatic cancer^[105]. Findlay VJ et al found that Snail2 modulates colorectal cancer 5-FU sensitivity^[106]. In addition, EMT is reported to be regulated by the Wnt/ β -catenin signaling pathway^[79,107,108]. We hypothesized that miR-221 induced 5-FU resistance through the cross talk between Wnt/ β -catenin pathway and EMT in esophageal cancer. To elucidate this hypothesis, we analyzed the Wnt/ β -catenin pathway genes, which also might be related with EMT. We found that MYC, CD44, CDH1 and ABCG2 were expressed differently between 5-FU sensitive and resistant EC cells and were regulated by miR-221 (Figure 7-3). This is the first report to connect the Wnt/ β -catenin pathway and EMT in 5-FU resistance of esophageal cancer.

Chemoresistance is a major issue of treatment in the majority of human tumors, including esophageal cancer. Thus, detecting rationale biomarkers to predict chemotherapy sensitivity and screening for targets to overcome resistance are significant for cancer therapy. A specific miRNA can affect simultaneously the expression of proteins involved in multiple cellular pathways, potentially serving as better therapeutic target or biomarker for clinical outcome than single proteins. In fact, several miRNAs, including miR-21, miR-200c and miR-125b, have been used as predictors of chemoresistance in cancers^[62,68,109]. Herein, our observation that increased miR-221 expression is associated with chemotherapy resistance, and poor patient prognosis may provide a surrogate marker to predict chemotherapeutic sensitivity for esophageal cancer.

Summary

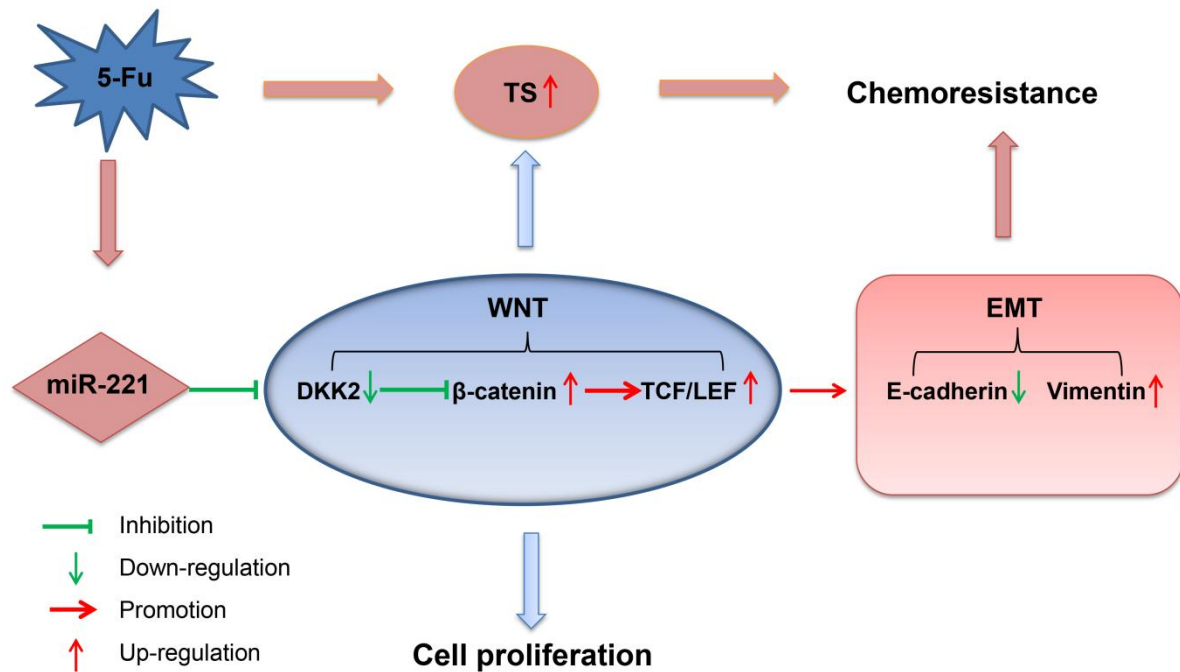


Figure XIV. Summary of this study. In conclusion, our results propose miR-221 as a promoter of 5-FU resistance of esophageal cancer. We defined a signaling axis of miR-221-DKK2-Wnt/β-catenin-EMT in esophageal cancer. In esophageal cancer cells, miR-221 activates the Wnt/β-catenin pathway by directly targeting DKK2 (an inhibitor of the Wnt/β-catenin pathway), which might promote cell proliferation. The activation of the Wnt/β-catenin pathway leads to accumulation of TS (a 5-FU target and resistance marker) and epithelial-mesenchymal transition by regulating its downstream targets (c-myc, E-cadherin, CD44 and ABCG2), which might induce 5-FU resistance of esophageal cancer cells.

Zusammenfassung

Einleitung

Das Ösophaguskarzinom ist der achthäufigste Krebs und die sechsthäufigste krebsbedingte Todesursache. Chemotherapie-Resistenz stellt in der Behandlung des Ösophaguskarzinoms ein sehr großes Problem dar. Es haben sich zunehmend mehr Hinweise in der Literatur ergeben, dass microRNAs (miRNAs) eine wichtige Rolle bei der Tumorentstehung und Chemotherapie-Resistenz verschiedener Tumorerkrankungen spielen. Es ist jedoch nicht bekannt, wie miRNAs Chemotherapie-Resistenz beim Ösophaguskarzinom regulieren. Ziel dieser Arbeit war es, zu analysieren inwieweit beim Ösophaguskarzinom miRNAs Chemotherapie-Resistenz und damit möglicherweise gesteigerte Metastasierungsfähigkeit regulieren.

Methodik

Für diese Studie wurden zuerst 5-FU-resistente Zelllinien aus vier verschiedenen Ösophaguskarzinomzelllinien hergestellt. Anschließend wurde das miRNA-Profil in 5-FU resistenten Ösophaguskarzinomzelllinien sowie den ursprünglichen sensitiven Tumorzelllinien mittels qPCR erfasst. Nach Transfektion von miR-221, Antagomirs als Inhibitoren von miR221 (anti221) oder entsprechenden Negativkontrollen (antiCtrl oder siRCtrl) in resistente sowie sensitive Ösophaguskarzinomzelllinien erfolgten sowohl *In-vitro*- als auch *In-vivo*-Experimente zur Analyse der zell- und molekularbiologischen Veränderungen. *In vitro* wurden die Zell-Proliferation und Zytotoxizität der Ösophaguskarzinomzelllinien gegenüber 5-FU durch CCK-8 untersucht. *In vivo* wurden OE33 und OE33-5FU_{res} Zellen subkutan in Nacktmäusen implantiert und die Tumorgroße gemessen. Ki67- und CD31-Expression zur Bewertung der Proliferation und Angiogenese in Tumoren wurde durch immunhistochemische Färbung (IHC) und Immunfluoreszenz (IF) analysiert. Zur weiteren Untersuchung der molekularen Mechanismen von miR-221 auf 5-FU-Behandlung von Ösophaguskarzinomzellen, wurden miR-221-Zielgene bioinformatisch detektiert und durch qPCR und Western-Blot analysiert. Die Identifizierung der Interaktion von miR-221 und DKK2 erfolgte durch Luciferase-Reporter-Assays mittels Luciferase-Reporter-Vektorkonstrukten pGL3-DKK2-wt und pGL3-DKK2-mut (mit Wildtyp oder mutierter 3'-UTR-Sequenz). Die Lokalisation von β -Catenin und die Aktivität des WNT/ β -Catenin-Signalwegs wurden durch Immunocytofluoreszenz-Färbung (IF) und TOP/FOP Flash-Reporter-Assays beurteilt. Zur Analyse einer eher mesenchymalen Zellmorphologie von 5-

FU_{res}-Ösophaguskarzinomzellen wurden EMT-Marker E-Cadherin und Vimentin durch qPCR und Western-Blot nachgewiesen. EMT-assoziierte relevante WNT/ β -Catenin-Zielgene wurden mittels der "Customized Array"-Technologie analysiert.

Ergebnisse

In dieser Studie war miR-221 deutlich in allen 5-FU_{res}-Ösophaguskarzinomzelllinien im Vergleich zu den jeweiligen sensitiven Zellen hochreguliert. *In vitro*-Knockdown von miR-221 führte zur Reduktion der Zellproliferation und sensibilisierte die Ösophaguskarzinomzellen gegenüber 5-FU bei 2.5 μ g/ml und 20 μ g/ml. *In vivo* führt eine miR-221-Inhibition zu deutlich geringerem Tumorwachstum subkutan in BALB/c nu-nu Mäusen und inhibiert Tumorangiogenese. 120 gemeinsame Gene in vier microRNA-Zielbanken wurden als Kandidatengenen von miR-221 vorhergesagt. Wir konzentrierten uns auf DKK2, einem Antagonisten des Wnt/ β -Catenin-Signalwegs. Die mRNA und Proteinexpression von DKK2 war umgekehrt proportional zur miR-221-Expression in 5-FU_{res} Ösophaguskarzinomzellen exprimiert. Luciferase-Reporter-Analysen ergaben, dass DKK2 ein direktes Zielgen von miR-221 in Ösophaguskarzinomzellen ist. Nach Transfektion von miR-221-Inhibitoren in 5-FU_{res}-Ösophaguskarzinomzellen, war β -Cateninlokalisierung im Zytoplasma und an der Zellmembran. Durch Immunfluoreszenz-cytochemische-Färbungen zeigte sich, dass β -Catenin in der Zellmembran und im Zytoplasma von sensitiven Ösophaguskarzinomzellen lokalisiert ist, während in 5-FU_{res}-Ösophaguskarzinomzellen β -Catenin in den Zellkern transloziert war. Darüber hinaus zeigten TOP/FOP-Flash-Reporter-Analysen eine dramatische Abnahme der Luciferase-Aktivität von β -Catenin-siRNA und miR-221-Hemmern. Diese Ergebnisse zeigten, dass der Wnt/ β -Catenin-Signalweg durch β -Catenin-siRNA und miR-221-Inhibitoren inaktiviert war. 5-FU_{res} Ösophaguskarzinomzellen weisen eine mesenchymale Zellmorphologie auf mit aktivierter EMT. Einige EMT relevante WNT/ β -Catenin-Zielgene MYC, CDH1 und CD44 sowie der Chemotherapie-Resistenz-Marker ABCG2 wurden in 5-FU_{res} -Ösophaguskarzinomzellen durch miR-221 reguliert.

Schlussfolgerung

miR-221 spielt eine wichtige Rolle bei der Chemotherapie-Resistenz beim Ösophaguskarzinom und moduliert über den Wnt-Signalweg EMT. miR-221 könnte einen Prognosemarker und therapeutisches Ziel für Patienten mit 5-FU_{res} Ösophaguskarzinom darstellen.

Abbreviation

3'-UTR, the three prime untranslated region

5-FU, fluorouracil

5-FU_{res}, 5-FU resistant esophageal cancer cells

ABC, avidin biotin complex

Anti-221, Hsa-miR-221 inhibitor

BALB/C nu/nu, bagg-albino/c nude/nude

BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea

BE, Barrett's esophagus.

CCK-8, cell counting kit-8

CD, cluster of differentiation

CDK, cyclin-dependent kinase

cDNA, complementary deoxyribonucleic acid

CT, cycle threshold

DAPI, 4', 6-Diamidin-2-phenyl-indol

DMEM, dulbecco's modified eagle medium

DMSO, dimethyl sulfoxide

DNA, desoxyribonucleic acid

FACS, fluorescence activated cell scan

FCS, fetal calf serum

FZD, frizzled

EC, esophageal cancer

EAC, esophageal adenocarcinoma

EGF, epithelial growth factor

EMT, epithelial to mesenchymal transition

ESCC, esophageal squamous cell carcinoma

FGF, fibroblast growth factor

HE, hematoxylin and eosin

HLA-DR, human leukocyte antigens-DR (major histocompatibility complex, MHC class II)

HRP, horseradish peroxidase

IC50, the half maximal inhibitory concentration

IHC, immunohistochemistry

IF, immunocytofluorescence

miRNA, micro ribonucleic acid

siRCtrl, negative control siRNA

Mut, luciferase reporter vector with mutant 3'-UTR of DKK2

PECAM-1, platelet-endothelial-cell-adhesion-molecule-1 (CD31)

PT, PT1590 cell line

qRT-PCR, real time quantitative reverse transcriptase polymerase chain reaction

LN, LN1590 cell lines

MDR, multidrug resistance gene 1

RNA, ribonucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

s.c., subcutaneous

siRCtrl, miRNA inhibitor negative control

siR- β , β -catenin siRNA

TS, thymidylate synthase

SP, side population

VEGF, vascular endothelial growth factor

Wnt, wingless-type MMTV integration site family

WT, luciferase reporter vector with wild type 3'-UTR of DKK2

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Curriculum Vitae

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Publications

1. Camaj, Peter*; Primo, Stefano*; **Wang, Yan***; Heinemann, Volker; Zhao, Yue; Laubender, Ruediger; Stintzing, Sebastian; Giessen-Jung, Clemens; Jung, Andreas; Gamba, Sebastian; Bruns, Christiane; Modest, Dominik. KRAS exon 2 mutations influence activity of regorafenib in an SW48-based disease model of colorectal cancer. *Future Oncology*. 11. 2014. (*Co-first author)
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Manuscripts in preparation

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Patents

1. The application of miR-34c in the manufacture of a medicament for treatment or prevention of liver cancer

Yugang Liu, **Yan Wang**, Chunmei Wang, Chunguang Fan, Wensheng Sun.

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2. Small molecule RNA Hsa-miR-29c in the manufacture of a medicament for treatment of liver cancer

Yugang Liu, Chunmei Wang, **Yan Wang**, Chunguang Fan, Wensheng Sun

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