miR-221 Mediates Chemoresistance of Esophageal Adenocarcinoma by Direct Targeting of DKK2 Expression

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Background: Chemoresistance is a main obstacle to effective esophageal cancer (EC) therapy. We hypothesize that altered expression of microRNAs (miRNAs) play a role in EC cancer progression and resistance to 5-fluorouracil (5-FU) based chemotherapeutic strategies.

Methods: Four pairs of esophageal adenocarcinoma (EAC) cell lines and corresponding 5-FU resistant variants were established. The expression levels of miRNAs previously shown to be involved in the general regulation of stem cell pathways were analyzed by qRT-PCR. The effects of selected miRNAs on proliferation, apoptosis, and chemosensitivity were evaluated both in vitro and in vivo. We identified a particular miRNA and analyzed its putative target genes in 14 pairs of human EC tumor specimens with surrounding normal tissue by qRT-PCR as well as Wnt pathway associated genes by immunohistochemistry in another 45 EAC tumor samples.

Results: MiR-221 was overexpressed in 5-FU resistant EC cell lines as well as in human EAC tissue. *DKK_2* was identified as a target gene for miR-221. Knockdown of miR-221 in 5-FU resistant cells resulted in reduced cell proliferation, increased apoptosis, restored chemosensitivity, and led to inactivation of the Wnt/β-catenin pathway mediated by alteration in DKK_2 expression. Moreover, miR-221 reduction resulted in alteration of EMT-associated genes such as E-cadherin and vimentin as well as significantly slower xenograft tumor growth in nude mice. RT^2 profiler analysis identified a substantial dysregulation of 4 Wnt/β-catenin signaling and chemoresistance target genes as a result of miR-221 modulation: *CDH1*, *CD44*, *MYC*, and *ABCG2*.

Conclusion: MiR-221 controls 5-FU resistance of EC partly via modulation of Wnt/β-catenin-EMT pathways by direct targeting of DKK_2 expression. MiR-221 may serve as a prognostic marker and therapeutic target for patients with 5-FU resistant EAC.

Keywords: 5-FU resistance, epithelial-mesenchymal transition, esophageal cancer, miR-221, Wnt/β-catenin signaling

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Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death.[1] The current clinical treatment options for EC are surgery, chemotherapy, and radiotherapy. The prognosis of patients receiving surgery alone is still poor.[2] Multimodal treatment improves the survival. Survival rates following esophagectomy with added neoadjuvant therapy are increasing to 30% to 45% for 5-year survival.[3] However, the response rate to chemotherapy, including 5-FU, is still lower than 50%.[4] Chemoresistance is thus seen as a major obstacle in the effective treatment of EC. The 2 main subtypes of the disease are esophageal squamous-cell carcinoma (often abbreviated to ESCC), which is more common in the developing world, and esophageal adenocarcinoma (EAC) is more common in the European and north American countries,[5] highly associated with smoking tobacco, obesity, and acid reflux.[6]

MicroRNAs (miRNAs) are small 19 to 22nt noncoding single-strand RNAs that mediate gene expression by interacting with the 3′UTR regions of their target gene mRNA and blocking its translation.[7] MiRNAs have been shown to regulate diverse cellular processes, including cell proliferation, stemness and differentiation, apoptosis, as well as therapy resistance.[8] Accumulating evidence suggests that miRNAs are dysregulated in a variety of cancers including EC. Cancer chemoresistance is a complicated process manifested through multiple mechanisms, including DNA damage repair, expression of ATP-binding cassette drug transporters, and activation of PI3K/AKT and Wnt pathways.[9–13] In addition, micro-environmental stimuli such as tissue hypoxia or signals that influence epithelial-mesenchymal transition can also impact chemoresistance.[9–13] Recent reports have described miR-141, miR-200c, miR-148a, miR-296, and miR-27a as functionally contributing to chemoresistance of EC.[14–19] Overexpression of miR-200c was shown to induce resistance in ECs through activation of the Akt signaling pathway.[15] However, the molecular mechanism underlying this observation remains unclear. In the present study, we show that miR-221 expression is significantly increased in 5-FU resistant EC cell lines and the underlying molecular mechanism was further elucidated in a series of in vitro and in vivo experiments.

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This study was conducted with the approval of the Otto-von-Guericke University Hospital Research ethics committee and informed consent was obtained according to the committee’s regulations and the Declaration of Helsinki.

The authors report no conflicts of interest.

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METHODS

Cell Culture and Establishment of Chemotherapy-Resistant EC Cell Lines

The OE19 and OE33 human EC cell lines were obtained from the Sigma Cell Line Bank (Sigma, 96071721 and 96070808). Two additional human EC cell lines, PT1590 and LN1590, were provided by the University Medical Center of Hamburg-Eppendorf. All cell lines were maintained in culture as described previously.[19] 5-FU resistant EC 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 on the basis of IC50 values ranging between 3 and 6.5 μg/mL for the original sensitive cell lines. After 24 hours, the cells were passaged with 5-FU free medium. Upon reaching confluency, the cells were treated with increasing levels of 5-FU (1.5- to 2-fold). After 5 subsequent steps with increasing 5-FU concentration, the resistant cell lines were eventually established.

Tissue Specimens

Tissue samples were obtained from patients with EAC between 2005 and 2015 at the Department of Surgery, University of Magdeburg, Germany (Ethic Committee approval 33/01, University of Magdeburg, Magdeburg, Germany). The description of these specimens is detailed in supplementary table S1, http://links.lww.com/SLA/B69.

MiRNA and Gene Expression Analysis

Total RNA from fresh-frozen esophageal tumor tissues or cell lines was isolated using QIAzol lysis reagent (Qiagen, USA). Expression of let-7b, let-7g, miR-21, miR-34a, miR-92, miR-200c, and miR-221 was determined using miScript SYBR Green PCR kits (Qiagen, USA). Expression of RNU6B (Qiagen, USA) was used as endogenous control. miRNAs sequences are provided in the supplementary table S2, http://links.lww.com/SLA/B69. For determining steady-state mRNA expression, total RNA was reverse transcribed to cDNA and qRT-PCR was performed as described previously.[20] Primer sequences are provided in supplementary table S3, http://links.lww.com/SLA/B69.

Transfection of miR-221 Mimics, miR-221 Inhibitor, and CTNNB1 siRNA

To transiently modulate miR-221 expression, hsa-miR-221 mimics (Qiagen, Cat. no: MSY0000278, USA), hsa-miR-221 inhibitor (Qiagen, Cat. no: MIN0000278, USA), or negative control siRNA (Qiagen, Cat. no: 1022076, USA) were transfected into 5-FU sensitive or resistant EC cells.[20] To suppress CTNNB1, expression cells were transfected with either CTNNB1 siRNA (GE Healthcare, Cat.no: M-003482-00-0005) or negative control siRNA using HiperFect transfection reagent (Qiagen, Cat.no:301705, USA). Sequences for the hsa-miR-221 mimics, hsa-miR-221 inhibitor, and CTNNB1 siRNA are provided in Table S4.

TOP Flash Luciferase Report Assay

To assess TCF-β-catenin mediated transcriptional activity, hsa-miR-221 mimics/inhibitor or siR-CTNNB1 and/or the 8×TOP-flash reporter gene construct were cotransfected into cells, and TOP-flash luciferase reporter gene assays were performed using a Luciferase Reporter Assay System (Promega, Madison, WI).[21]

Protein Analysis

Western blotting was performed as described previously.[22] Antibody information is provided in supplemental materials, http://links.lww.com/SLA/B69. The protein bands were visualized using a Leica DFC450D fluorescence microscope (Leica, Wetzlar, Germany).

Cell Proliferation, Apoptosis, and Chemosensitivity

Regarding cell proliferation, EC cells were analyzed at 0, 24, 48, and 72 hours after transfection with hsa-miR-221 mimics (miR-221), hsa-miR-221 inhibitor (anti221), or negative control siRNA (siRCtrl) using the Cell Counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (Miltenyi Biotec, Germany) were applied to determine the percentage of transfected cells undergoing apoptotic or necrotic cell death after 24 hours. To assess cytotoxicity of 5-FU, all transfected cells were analyzed at 48 hours following 2.5 or 20 μg/mL 5-FU treatment.

Esophageal Adenocarcinoma Xenograft Mouse Model

To determine tumor growth in vivo, EC cells transfected with miR-221, anti221, or siRCtrl were implanted into the flanks of 6 to 8-week-old male Balb/c nu-nu mice (Charles River Deutschland, Sulzfeld, Germany). Tumor size was measured in specified time intervals. Upon sacrifice of the animals, tumor volume was calculated and analyzed. All protocols were approved by the regional commission for animal experiments in the state of Sachsen-Anhalt of Germany (no.42502-2-1266 uniMD).

Histology and Immunostaining

For immunohistochemical staining samples from the in vivo animal experiments, patient-derived esophageal tumor or adjacent normal tissues were fixed, embedded and sectioned at 3 μm thickness. These sections were then stained with anti-*Ki*67 (1 : 100, Abcam, UK), anti-β-catenin (1 : 500, BD Bioscience, Cat.no: 610154, USA), and anti-DKK_2 (1 : 200, Abcam, UK).[20] Frozen tumor tissues embedded in O.C.T at −20°C and sectioned to 3 μm thickness were used for immunofluorescent staining with anti-CD31 (1 : 50, Abcam, UK).

RT² Profiler PCR Array System

The expression of selected Wnt/β-catenin-EMT related genes was examined using the Custom Human RT^2 Profiler TM PCR array (RT^2 Profiler TM PCR Array: CAPH12950), which includes targets of the Wnt/β-catenin signaling pathway as well as genes associated with the general process of EMT. Total RNA was isolated from 5×10^5 OE33 and OE33-$5Fu_{res}$ cells with or without hsa-miR-221 mimics, inhibitor, or siR-CTNNB1 using the miRNeasy kit. PCR was performed with the RT^2 profiler PCR array system and analyzed using an ABI 7000 PCR machine. The expression levels of different mRNAs were normalized using a series of housekeeping genes: *ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*. The fold change of gene expression from the different treatment as compared with control groups was calculated as $2^{(-\Delta\Delta CT)}$.

Statistical Analysis

All data are expressed as mean ± SD. The correlation of β-catenin or DKK_2 and each clinical pathologic variable was comparatively analyzed by χ^2 test and the Fisher exact 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 RT^2 profiler data analysis, we applied a web-based service of Qiagen data analysis center. Genes were called "differentially expressed" if the corrected P value was less than 0.05.

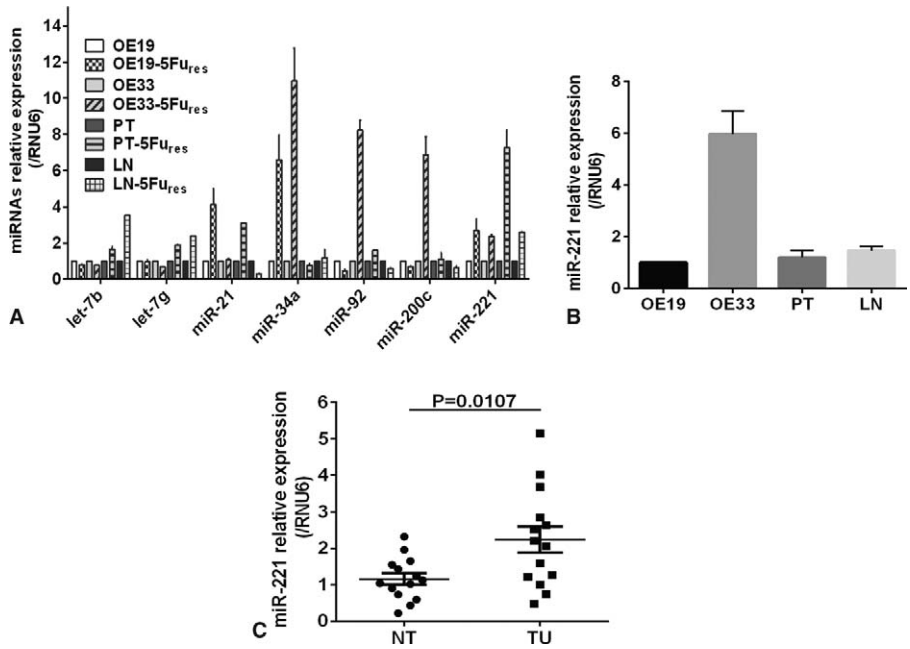

FIGURE 1. miRNA expression in esophageal cancer cell lines and patient tissues. A, Relative expression of selected miRNAs assessed in 4 pairs of 5-FU sensitive and resistant esophageal cancer cells by qPCR. B, miR-221 expression in 4 esophageal adenocarcinoma cell lines. C, miR-221 expression in 14 sets of corresponding tumor and nontumor specimens derived from esophageal cancer patients. NT indicates nontumor; TU, tumor.

RESULTS

miR-221 is Upregulated in 5-FU Resistant EC Cells Lines and Tumor Tissues

The biology underlying stemness has been linked to the biology of chemoresistance.[23–25] A series of miRNAs have been previously implicated in the regulation of ''stem cell'' function.[26,27]

To study the role of miRNAs in the context of chemotherapy resistance in EC, we established a series of 5-FU resistant EC cell lines (OE19-5Fu$_{res}$, OE33-5Fu$_{res}$, LN-5Fu$_{res}$, and PT-5Fu$_{res}$) (Fig. S1, http://links.lww.com/SLA/B69) and then analyzed them for their differential expression of miRNAs by RT-qPCR. In a panel of 5-FU resistant EAC cells (OE19, OE33, PT1590, and LN1590), miR-221 was overexpressed in all resistant variants (Fig. 1A). The highest expression of miR-221 was found in the OE33 cell line (Fig. 1B). However, we did not show all miRNA transfection data in the other 3 cell lines; OE19-5FU$_{res}$ did not display an ideal growth under transfection. Therefore, OE33 and OE33-5Fu$_{res}$ cells were selected for the subsequent functional studies. MiR-221 was further validated in EC patient's samples. MiR-221 was significantly higher expressed in tumor than in adjacent normal tissue from 14 EC patients (Fig. 1C, $P = 0.0107$).

Inhibition of miR-221 in EC Cells Decreases Cell Proliferation, Induces Apoptotic Cell Death, and Restores 5-FU Sensitivity

To investigate the role of miR-221 on proliferation, cell death, and chemosensitivity for EC, OE33-5Fu$_{res}$ cells were transiently transfected with has-miR-221 mimics (miR-221), miR-221 inhibitor (anti-221), or negative control siRNA (siRCtrl) (Fig. 2A). Transfection of miR-221 inhibitor (anti-221) significantly reduced cell viability (Fig. 2B) and increased the proportion of apoptotic cells (Figs. 2C, D) as compared with negative control siRNA in OE33-5Fu$_{res}$ cells ($45.63 \pm 2.76\%$ vs $5.44 \pm 1.26\%$ at 24 hours, $P < 0.001$).

Transient knockdown of miR-221 expression was found to restore sensitivity of OE33-5Fu$_{res}$ cells to 5-FU leading to an increasing percentage of dead OE33-5Fu$_{res}$ cells after re-introduction of 2.5 and 20 μg/mL 5-FU treatment (4.7-fold and 1.5-fold, Fig. 2E).

In addition, the protein expression of thymidylate synthase (TS), a known 5-FU target and resistance marker, was reduced by transient miR-221 knockdown in OE33-5Fu$_{res}$ as evidenced by western blotting (Fig. 2F).

Knockdown of miR-221 Inhibits Tumor Growth In Vivo

BALB/c nu-nu male mice were obtained from Charles River Deutschland (Sulzfeld, Germany) at 6 to 8 weeks of age and housed in the animal facility of the University Medical Center Magdeburg. Thirty mice were randomized into 6 groups (5 mice per group). EC tumor xenografts were established by subcutaneous injection of 1 x 10^6 of OE33 or OE33-5Fu$_{res}$ following transfection with has-miR-221 mimics, miR-221 inhibitor, or negative control siRNA in the same position on the flanks of nude mice. Sixty-six days after injection, all mice were sacrificed and the harvested tumors were analyzed. Knockdown of miR-221 in OE33-5Fu$_{res}$ cells led to a significantly slower tumor growth in vivo than controls (anti-221 vs Ctrl or siRCtrl, $P < 0.001$, Fig. 3C). Immunohistochemical analysis revealed that *Ki*67 was significantly weaker expressed in the anti-221 transfected OE33-5Fu$_{res}$ cells than the controls (anti-221 vs Ctrl or siRCtrl, $P < 0.0001$) (Fig. 3E). CD31 was performed to analyze effects on tumor angiogenesis. OE33-5Fu$_{res}$ control tumors revealed a high number of CD31-positive cells, which was substantially reduced in anti-221 transfected OE33-5Fu$_{res}$ tumors ($P < 0.05$) (Fig. 3F).

DKK$_2$ is a Direct Target of miR-221 and miR-221 Induced Chemoresistance is Mediated Through the Wnt/β-catenin Signaling

To search for target genes of miR-221, we used 4 miRNA target prediction tools: TargetScan, PITA, miRTarBase, and miRanda

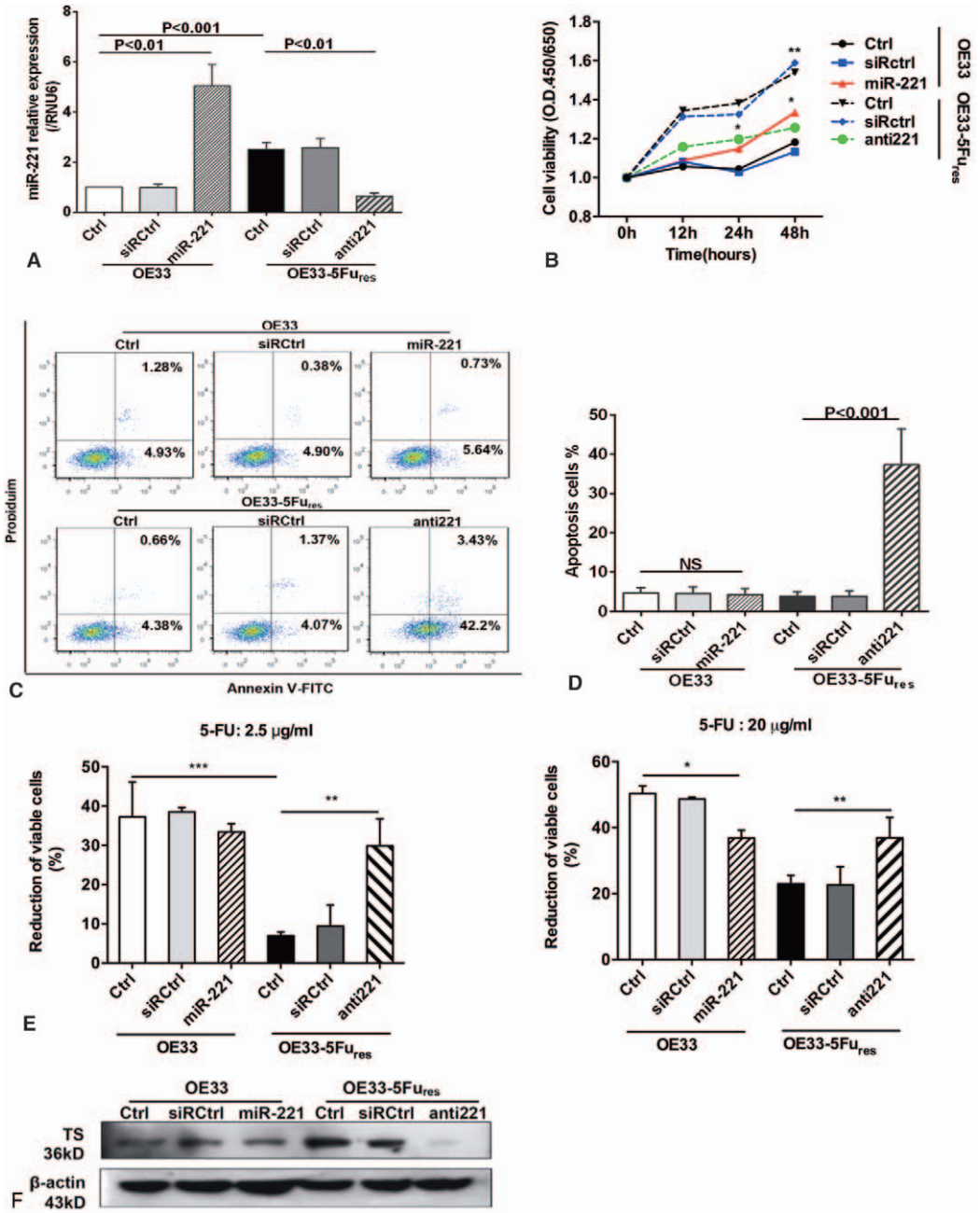

FIGURE 2. Functional analysis of miR-221 in 5-FU resistant EC cells. A, Efficacy of has-miR-221 mimics, has-miR-221 inhibitor in EC cells. Transfection with miR-221 mimics increases miR-221 expression in 5-FU sensitive EC cell (OE33), while transfection with miR-221 inhibitor decreases miR-221 expression in 5-FU resistant EC cell (OE33-5Fu$_{res}$). B, Cells were transfected and their viability determined by CCK-8 assay at 0, 24, 48, and 72 hours. The viability of control cells, cells transfected with miR-221 mimics (miR-221), inhibitor (anti221), and negative control siRNA (siRCtrl) was detected. Inhibition of miR-221 expression led to reduction of cell proliferation. $^{*}P < 0.05$, $\dagger P < 0.01$, 2-way ANOVA. C, Analysis of cell apoptosis of 5-FU sensitive and resistant OE33 cells at 24 hours after transfection. Overexpression of miR-221 had no impact on the cell apoptosis of 5-FU sensitive EC cells, while inhibition of miR-221 significantly promoted the cell apoptosis of 5-FU resistant EC cells. D, Annexin V-positive cells were quantified by flow cytometry. Data are presented as mean SD of 3 independent experiments. E, Cell response to 5-FU (2.5 and 20 μg/mL) after transfection. With 20 μg/mL of 5-FU, treatment-naive OE33 cells showed a 50.3% ± 4.1% reduction of viable cells of as compared with 36.9% ± 4.1% in miR-221 mimic transfected cells ($P < 0.05$), while in OE33-5FU$_{res}$ cells, reduction of viable cells was significantly increased from 23.0% ± 4.6% to 37.0% ± 8.7% ($P < 0.01$). F, Protein expression of 5-FU resistant marker TS in OE33 and OE33- 5Fu$_{res}$ cells. Transfection with miR-221 mimics slightly increased TS protein expression, while transfection with miR-221 inhibitor significantly decreased TS protein level.

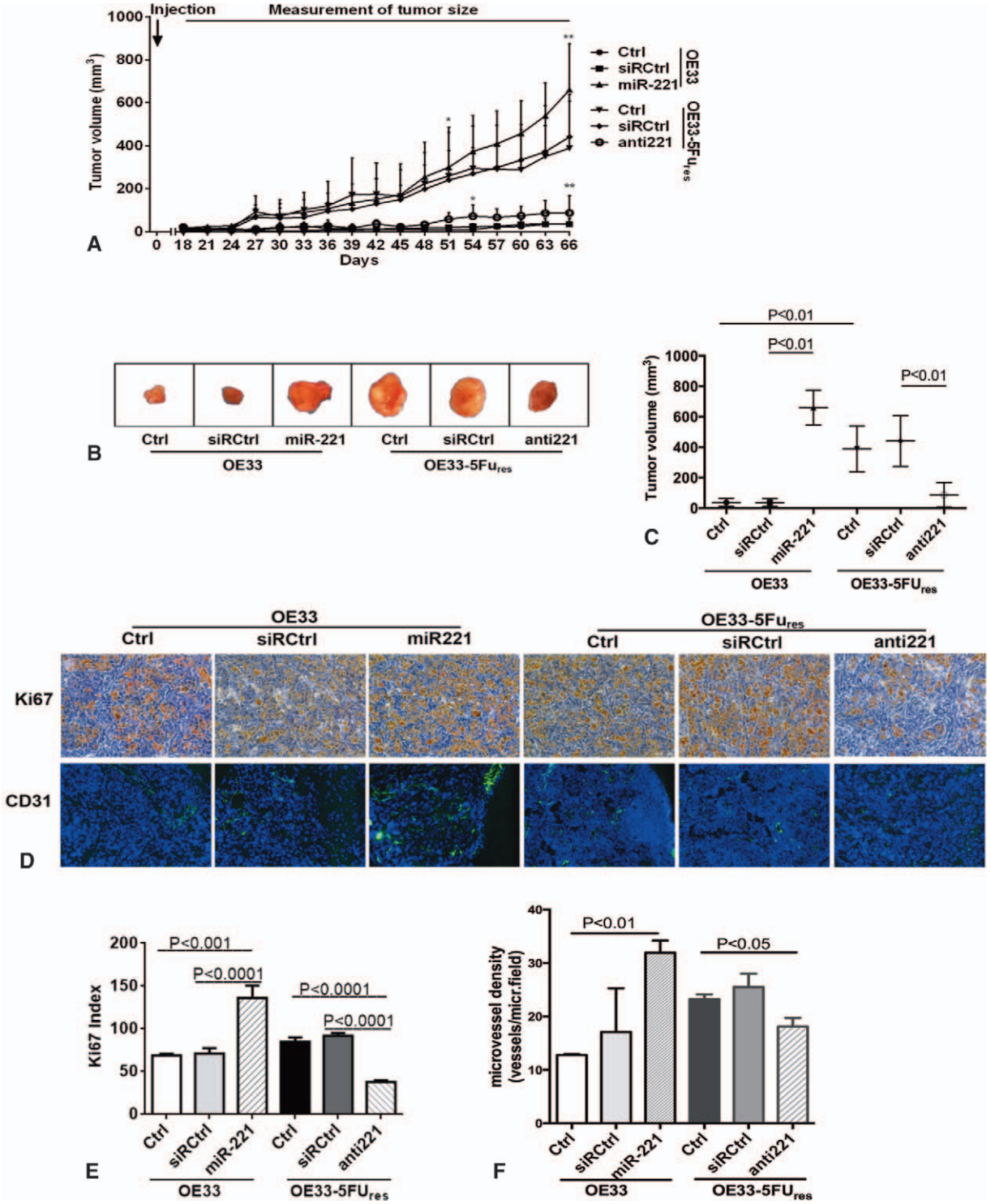

FIGURE 3. Analysis of in vivo EC tumorigenesis in a xenograft nude mouse model. A–C, Increased expression of miR-221 led to enhanced OE33 tumor growth in a xenograft nude mouse model, knockdown of miR-221 expression inhibited OE33-5FU$_{res}$ tumor growth. $^{*}P < 0.05$, $\dagger P < 0.01$, 2-way ANOVA. D–F, Immunohistochemical analysis of cell proliferation and immunofluorescence staining of angiogenesis. In tumors with miR-221 overexpression, *Ki67* expression was significantly higher than in control groups ($P < 0.0001$). In tumors with low levels of miR-221 of OE33-5FU$_{res}$ after antagomir therapy, the expression of *Ki67* was significantly reduced ($P < 0.0001$). CD31 expression was dramatically increased in miR-221 transfected OE33 ($P < 0.01$) and decreased in OE33-5FU$_{res}$ tumor with anti-miR-221 therapy ($P < 0.05$).

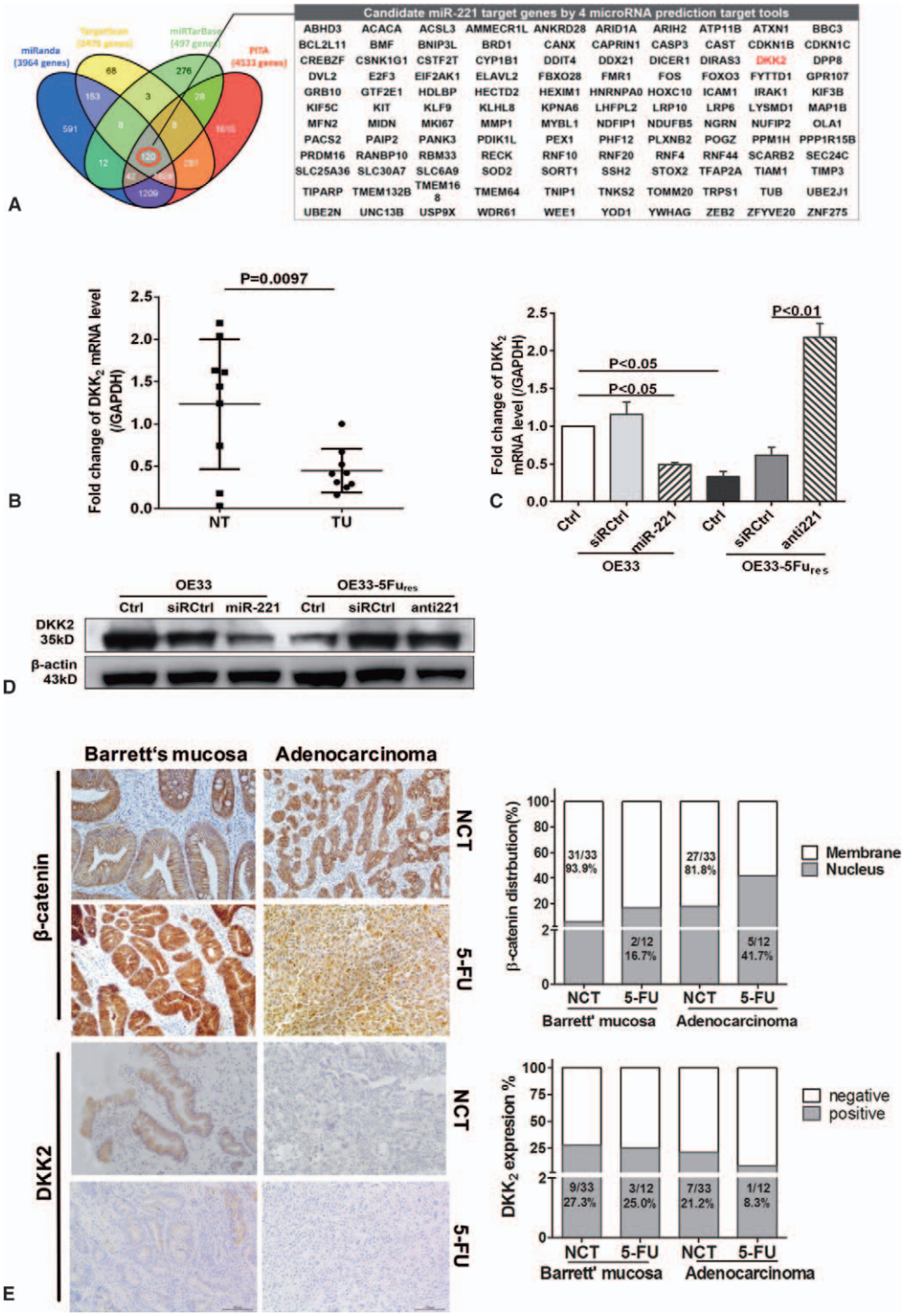

FIGURE 4. Identification of miR-221 target genes and its regulation of the Wnt/β-catenin signaling pathway (A) Prediction of miR-221 target genes. One hundred twenty target genes that have miR-221 seed sites are predicted via 4 different miRNA target prediction tools (TargetScan, PITA, miRTarBase, and miRanda). B–D, DKK_2 expression in esophageal tumor tissue and esophageal cancer cells. DKK_2 was substantially lower expressed in esophageal tumors than in adjacent nontumor tissues. Furthermore, DKK_2 was downregulated in 5-FU resistant EC cells compared with the respective sensitive cells. Overexpression of miR-221 suppressed mRNA (detected by qRT-PCR) and protein (detected by western blot) expression of DKK_2 while downregulation of miR-221 increased both DKK_2 mRNA and protein expression. E, Distribution of β-catenin in esophageal tumor tissues. Nuclear localization of β-catenin in 5-FU treated esophageal tumors. 5-FU indicates 5-fluoruracil; NCT, no chemotherapy.

(Fig. 4A). Among a large number of potential targets based on bioinformatic investigations, we identified DKK_2 for further functional analysis. A potential targeting of the 3′UTR of the human *DKK_2* gene by miR-221 was predicted by the TargetScan and RNAhybrid software and further supported by the high level of evolutionary conservation of the seed sequence between species (data were not shown).

qPCR analysis showed that DKK_2 mRNA levels were reduced in tumor samples as compared with nontumor tissue samples and in 5-FU resistant as compared with 5-FU sensitive EC cells. Thus, expression of DKK_2 is inversely correlated to expression of miR-221 (Figs. 1C, 4B). However, due to the sample size, the statistics of correlation analysis between miR-221 and DKK_2 mRNA expression is not significant ($P = 0.216$, $R = 0.339$; nonparametric spearman correlation). In addition, miR-221 knockdown resulted in a significant increase in DKK_2 mRNA and protein expression (Figs. 4C, D).

To verify that DKK_2 is a direct target of miR-221, luciferase reporter vectors were then applied. Overexpression of miR-221 dramatically decreased the luciferase reporter activity when the wild-type DKK_2 3′UTR was used, but did not influence reporter expression when the DKK_2 mutant control construct containing mutant seed sequences was used (data were not shown).

To investigate a potential association between miR-221 expression, chemoresistance, and activation of the Wnt/β-catenin signaling pathway, β-catenin distribution and DKK_2 expression were detected in tumor samples. We found no significant difference in DKK_2 expression in Barrett's mucosa (9/33, 27.3%) or tumor tissues (7/33, 21.2%) before chemotherapy. However, samples from esophageal tumor patients with prolonged exposure to chemotherapy showed a substantial decrease in DKK_2 expression (1/12, 8.3%) as compared with the corresponding expression level seen in adjacent Barrett's mucosa (3/12, 25.0%).

Tumor samples following chemotherapy also demonstrated evidence of enhanced canonical Wnt signaling. Although inactivated β-catenin was mainly localized to the cell membrane and cytoplasm in Barrett's mucosa (31/33, 93.9%) and EAC specimens without chemotherapy treatment (27/33, 81.8%), an accumulation of nuclear β-catenin was found in EAC after long-term exposure to 5-FU based chemotherapy (5/12, 41.7%) (Fig. 4E). Among all available clinical and pathological parameters, only DKK_2 expression was found to significantly correlate with the stage of tumor differentiation ($P = 0.024$, Table 1).

To validate the functional role of miR-221 regulating β-catenin activation, OE33-$5Fu_{res}$ cells were transfected with has-miR-221 mimics, inhibitor, siR-CTNNB1/siR-β-catenin, or negative control siRNA (Fig. 2A, S2B and C, http://links.lww.com/SLA/B69). TOP Flash luciferase reporter analysis showed decreased luciferase activity following transfection with miR-221 inhibitors equivalent to siR-CTNNB1/siR-β-catenin ($^{**}P < 0.01$, Fig. S3B, http://links.lww.com/SLA/B69). These results suggest that miR-221 influences chemoresistance at least in part through activation of the Wnt/β-catenin pathway via direct control of DKK_2 expression.

miR-221 Mediates EMT Through Activating Wnt/β-catenin Signaling

EMT is associated with chemoresistance in various cancers. We observed a morphological change toward a mesenchymal cell phenotype in 5-FU resistant EC cells (Fig. 5A). Western blot analysis showed that the epithelial cell marker E-cadherin was expressed at a lower level, whereas the mesenchymal cell marker Vimentin was expressed at a higher level in OE33-$5Fu_{res}$ cells than the respective sensitive EC cells. These results suggest that the process of EMT is associated with the development of 5-FU resistance in EC cells. Interestingly, a knockdown of miR-221 expression in OE33-$5Fu_{res}$ resulted in a significant increase in E-cadherin and a decrease in Vimentin protein expression (Fig. 5B).

To validate downstream effects of miR-221 expression on regulation of Wnt/β-catenin signaling and EMT, we identified 30 β-catenin target genes related to EMT by PCR array (Fig. 5C, Table S6, http://links.lww.com/SLA/B69). Four genes *MYC*, *CD44*, *ABCG2*, and *CDH1* were significantly dysregulated between OE33 and OE33-$5Fu_{res}$, following induction or inhibition of miR-221 (Fig. 5D).

DISCUSSION

MiR-221 is located on chromosome X and is overexpressed in osteosarcoma,[28] colorectal,[29] ovarian,[30] breast,[31] and pancreatic cancer.[20] One current study showed that miR-21, miR-143, miR-203, miR-205, and miR-221 were overexpressed in squamous-cell carcinoma cancer tissues compared with normal esophageal tissues.[32] In colorectal cancer, miR-221 promotes cell proliferation.[29] In addition, miR-221 plays a role in mediating radio-chemoresistance of various cancers by targeting various signal transduction pathways.[28,31,33,34] Targeting of the PI3K/Akt signaling axis by miR-221 can induce cell proliferation and BCNU resistance in human glioblastoma.[35] Downregulation of miR-221 also alters radiation sensitivity in these cells by targeting the PTEN pathway.[36] Overexpression of miR-221 is associated with tamoxifen resistance in breast cancer through its negative regulation of estrogen receptor alpha.[31] Furthermore, miR-221 has been shown to confer breast

TABLE 1. β-catenin Localization and DKK2 Expression in Association With Clinical and Pathological Parameters in Human Esophageal Tumor Samples

Feature	β-catenin (Cell Membrane) n = 34	β-catenin (Cytoplasm and Nucleus) n = 11	*P*	DKK_2 Negative n = 37	DKK_2 Positive n = 8	*P*
Age (mean ± SD), y	67.6 ± 11.4	70 ± 10.2	0.536	67.0 ± 10.4	72.8 ± 13.1	0.179
Differentiation			0.619			0.024
Well differentiated	7 (20.6%)	1 (9.1%)		8 (21.6%)	0 (0%)	
Moderately differentiated	14 (41.2%)	6 (54.5%)		13 (35.1%)	7 (87.5%)	
Poorly differentiated	13 (38.2%)	4 (36.4%)		16 (43.2%)	1 (12.5%)	
Stage (I+II/III+IV)	22/12	5/6	0.257	23/14	4/4	0.524
pT (T1/T2/T3/T4)	16/4/14/0	7/2/2/0	0.379	19/6/12/0	5/0/3/0	0.472
pN (N0/N1/N2/N3)	20/10/0/4	8/2/1/0	0.169	22/10/1/4	6/2/0/0	0.718
Chemotherapy (5-Fu based)			0.136			0.419
Yes	7 (20.6%)	5 (45.5%)		11 (29.7%)	1 (12.5%)	
No	27 (79.4%)	6 (54.5%)		26 (70.3%)	7 (87.5%)	

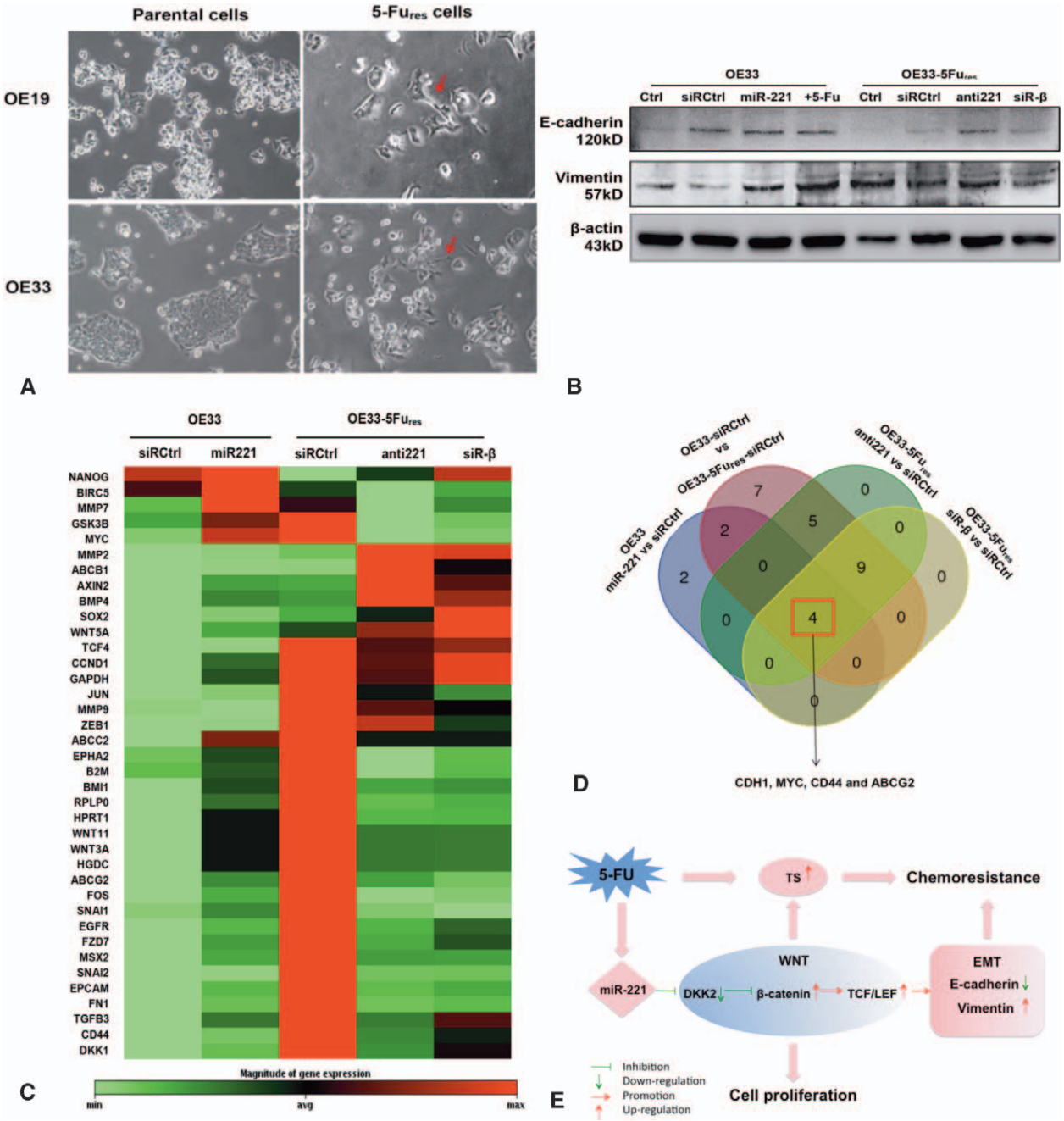

FIGURE 5. Effects of miR-221 on the interaction of WNT/β-catenin signaling and EMT in EC cells. A, Morphological comparison of 5-FU sensitive and resistant EC cells: spindle-like phenotype in 5-FU resistant OE19 and OE33 cells. B, EMT-related markers E-cadherin and Vimentin are regulated by miR-221 in OE33 and OE33-5Fu$_{res}$ cells. C, D, EMT-related Wnt/β-catenin target genes are expressed differently in OE33 and OE33-5Fu$_{res}$ cells following transfection with miR-221 mimics, inhibitor or siR-CTNNB1. Four genes *CDH1*, *MYC*, *CD44*, and *ABCG2* were regulated by both miR-221 and β-catenin. E, The above results suggest that miR-221 acts as a driver of 5-FU resistance in EC. A potential signaling axis between miR-221-DKK$_2$-WNT/β-catenin-EMT was identified in EC. MiR-221 activates the WNT/β-catenin pathway by direct targeting of DKK$_2$ (an antagonist of the WNT/β-catenin pathway), leading to enhanced cell proliferation. Activation of the WNT/β-catenin pathway leads to accumulation of TS with increase of chemoresistance and epithelial-mesenchymal transition by regulating its downstream targets (c-myc, E-cadherin, CD44, and ABCG2).

cancer resistance to fulvestrant by acting through multiple signaling pathways.[34] In a previous study, we showed that combined suppression of miR-21 and miR-221 sensitized pancreatic cancer cells to treatment with either gemcitabine or 5-FU. Accordingly, our finding that miR-221 is overexpressed in EC cells prompted us to speculate an association between miR-221 and chemotherapy resistance in EC.[20] In general, chemotherapy regimens for EC are 5-FU based. Hummel et al[37] used an in vitro model of acquired chemotherapy resistance in esophageal adeno- and squamous cell carcinoma cells showing miRNA expression profiles for cisplatin or 5-FU resistant variants versus chemotherapy-sensitive controls and found miR-27b-3p, miR-193b-3p, miR-192-5p, miR-378 a-3p, miR-125a-5p, and miR-18a-3p dysregulated.

In this study, we show that expression of miR-221 is associated with 5-FU resistance and EC tumor growth. Furthermore, we demonstrate that increased expression of miR-221 correlates with activation of Wnt/β-catenin signaling and EMT in EC cells. The Wnt/β-catenin signaling pathway is indeed linked to cancer progression and chemoresistance in various tumors via EMT.[38–40] An antagonist of the Wnt/β-catenin pathway is DKK_2,[41] a member of the Dickkopf family, which is activated by treatment with 5-FU.[42,43] We thus speculated that miR-221 might account for 5-FU resistance in EC by modulation of DKK_2 expression. To address this, we examined the expression of DKK_2 together with the distribution of β-catenin in EAC tumor specimens. Our immunohistochemistry analysis demonstrated no significant difference in DKK_2 expression in Barrett's mucosa or EC tumor tissue before chemotherapy. We speculate that the missing statistical significance of the correlation analysis between DKK_2, β-catenin and chemotherapy might be a consequence of limited sample size. Interestingly, esophageal tumor specimens following prolonged exposure to chemotherapy showed a substantial decrease in DKK_2 expression together with an accumulation of nuclear β-catenin (Fig. 4E), while inactivated β-catenin was mainly localized to the cell membrane and cytoplasm in Barrett's mucosa and EAC specimens without prior chemotherapy treatment. These results suggest that miR-221 influence chemoresistance at least in part through activation of the Wnt/β-catenin pathway via regulation of DKK_2 expression.

In correlation to changes of miR-221 expression, we further identified significant alterations in expression levels of β-catenin/Wnt- and EMT-associated genes such as *MYC*, *CD44*, *CDH1*, and *ABCG2*. We therefore propose that increased expression of miR-221 leads to reduced expression of DKK_2, further releasing an activation of the Wnt/β-catenin pathway resulting in nuclear translocation of β-catenin with corresponding expression of Wnt target genes.

Accordingly, our results suggest that miR-221 functions as an oncomiR in EAC. Moreover, the present study is the first to connect Wnt/β-catenin signaling and EMT-related gene expression in association to 5-FU resistance of EC. The potential mechanism for this general phenomenon is summarized in Fig. 5E.

Chemoresistance is a major issue for effective treatment of EC. On the basis of our results, increased miR-221 could either act as a surrogate marker to predict chemotherapy resistance or as a potential therapeutic target against chemotherapy resistance in EAC.

The results show a decrease in the expression of DKK_2 in esophageal tumor tissues, and nuclear accumulation of β-catenin in 5-FU resistant EC cells, and in tumor samples linked to a poor response to 5-FU therapy. The data suggest a tendency toward activation of the Wnt/β-catenin pathway in EAC that is associated with long time exposure to anticancer drugs especially 5-FU. However, due to the limitation of acquiring more EAC tissues from either local or independent clinical centers, we expected this finding could be further confirmed in other studies.

We further validated significant changes in the expression levels of β-catenin/Wnt- and chemotherapy-associated genes *MYC*, *CD44*, *CDH1*, and *ABCG2* in response to changes in miR-221 expression. On the basis of our results, we propose that increased expression of miR-221 leads to reduced expression of DKK_2, further releasing a DKK2-mediated blockade of the Wnt/β-catenin signaling pathway. Increased activation of this pathway results in disruption of the Axin/APC/GSK3 degradation complex leading to stabilization of β-catenin. β-catenin then translocates to the nucleus wherein it helps control expression of Wnt targets.

Our results suggest that miR-221 functions as an oncomiR in EAC by direct targeting of DKK_2, as evidenced by the associated effects observed on the Wnt/β-catenin signaling pathway. Processes linked to EMT resulted from the continuous 5-FU treatment of EAC cell lines. EMT has been previously associated with 5-FU resistance[44–46] and miR-221 in pancreatic cancer cells.[47]

The present study is the first to connect Wnt/β-catenin pathway and the process of EMT in 5-FU resistance of EAC. The potential mechanism for this general phenomenon is summarized in Fig. 5E.

Chemoresistance is a major issue in the effective treatment of EAC. Thus, detecting rational biomarkers to predict chemotherapy sensitivity and screening for targets to overcome resistance are significant for cancer therapy. On the basis of our results, increased miR-221 expression is associated with chemotherapy resistance and poor prognosis. MiR-221 may thus act as a surrogate marker to predict chemoresistance in EAC.

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DISCUSSANTS

L. Bonavina (Milano, Italy):

Tumor heterogeneity and chemoresistance remain indeed a main issue for an effective neo-adjuvant therapy in esophageal adenocarcinoma. 5-FU has been a mainstay of treatment for a long time, but most clinical studies have focused on speed of drug delivery (whether bolus or continuous) and on the enzimatic defects associated with 5-FU toxicity rather than on chemoresistance.

You have described a large set of experiments showing that miR-221 positive tumor cells have more malignant potential and are more resistant to 5-FU than naive tumor cells. You have also shown that miR-221 may serve as a prognostic marker and as a therapeutic target for patients with 5-FU resistant esophageal adenocarcinoma.

To be considered an efficient prognostic marker, miR-221 needs further validation in clinical trials. In addition, the possibility to sample blood or other body fluids should be investigated to assess specificity and sensitivity in esophageal adenocarcinoma.

My questions for you are the following: First, is there any possible relationship between speed of 5-FU delivery and induction of chemoresistance? Second, is there any association between the risk of 5-FU toxicity and 5-FU chemoresistance? Third, would you speculate that knockdown of miR-221 to restore chemosensitivity to 5-FU may be the winning strategy in the future or rather that it may be more convenient to focus on the more innovative targeted drugs?

Response From C. Bruns (Cologne, Germany):

With respect to the possible relationship of the speed of 5-FU delivery or continuous versus bolus 5-FU application, all analyzed specimen derived from patients who received continuous 5-FU injection within their chemotherapy protocol. However, I think that indeed long-term, continuous exposure to 5-FU—here in the experimental setting even with increasing doses—induces resistance mechanisms and select for cell populations that are highly resistant. Transferred to clinical situations, protocols with short-term exposure to chemotherapy should rather be conducted.

With respect to second question, we did not find any association between 5-FU toxicity and 5-FU resistance. 5-FU toxicity is based on a DPD mutation and we have not investigated the cell lines regarding their DPD mutational status.

With respect to the last question, we indeed speculate that possible new techniques to target resistant esophageal

adenocarcinoma would be specific miRNA aside of being markers for resistance. Another option would be to target downstream molecular markers or pathways associated with specific miR-RNAs. As miR-221 is activating the wnt-pathway, it would be interesting to think of wnt-inhibitors as therapeutic targets of resistant esophageal adenocarcinoma. A well-known wnt-inhibitor is aspirin, which is already been known to interfere in GI malignancies with the adenoma-carcinoma sequence.

J.V. Reynolds (Dublin, Ireland):

miR-221, in addition to being an oncogene, is also a known tumor-suppressor gene and low expression levels or downregulation are implicated, for example, in the development of oropharyngeal SCCs. It also downregulates the c-kit receptor, so it is implicated in the development of hemopoietic malignancies. Did you notice when you knocked down miR-221 expression that it induced development of SCCs in the oropharynx or did you check in your mice if there were any erthyropoietic maligancies as a consequence of that?

Finally, do you have plans to measure miR-221 in pre- and post-neoadjuvant chemoradiotherapy esophageal biopsies to see if expression levels change in response to effective treatment?

Response From C. Bruns (Cologne, Germany):

No, we did not check our animals for development of oropharyngeal SCCs.

However, we analyzed commercially available esophageal SCC cell lines and found the expression of miR-RNA 221 in these esophageal SCC cell lines not that impressive.

With respect to your second question, we have not yet analyzed pre- and post-neoadjuvant chemoradiotherapy esophageal biopsies. We only analyzed full histology samples after esophageal tumor resection. In the future, this would of course be the correct way to identify miR-RNA 221 expression in tumor biopsies to individualize chemotherapy for each patient.