Overexpression of ERα inhibits proliferation and invasion of MKN28 gastric cancer cells by suppressing β-catenin

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Received April 5, 2013; Accepted June 3, 2013

DOI: 10.3892/or.2013.2610

Abstract. The relationship between estrogen receptor (ER)α and patient prognosis has been identified in gastric cancer; however, the definite role of ERα in gastric cancer remains to be fully elucidated. The aim of the present in vitro study was to investigate the impact of ERα on cell proliferation, migration and invasion in gastric cancer cell lines. We investigated the biological effect of ERα overexpression on gastric carcinoma cells. An MKN28 gastric cancer cell line stably overexpressing ERα was established. The effect of ERα overexpression on cell growth was assessed by evaluating cell survival, colony formation, cell cycle progression and apoptosis. Cell migration and invasion were detected by Transwell migration/invasion assays. The protein levels of several potentially involved genes were determined by western blotting to elucidate the underlying molecular mechanisms. The Student’s t-test was used to determine the statistical differences between various experimental and control groups, and one-way ANOVA test was used to determine the difference between three or more groups. The results showed that ERα overexpression significantly inhibited cell growth and proliferation, blocked cell entry into the G1/G0 phase and promoted cell apoptosis. In addition, ERα reduced the motility and invasion of gastric cancer cells. These phenotypes may partly be explained by a decrease in β-catenin expression caused by ERα overexpression. ERα overexpression effectively inhibited cell growth and cancer progression by suppressing β-catenin in gastric cancer, identifying ERα as a promising target with therapeutic potential for development of new approaches to treat gastric cancer.

Introduction

Although the incidence of gastric cancer has been declining globally since World War II, and it is one of the least common cancers in North America, the incidence of gastric cancer is still high in many countries around the world. In 2012, an estimated 21,320 new cases were diagnosed, and 10,540 cases will eventually die of the disease in the United States (1). Gastric cancer is estimated to be the fourth most common cancer worldwide (2).

Despite a major decline in the incidence of gastric cancer and substantial breakthroughs in our understanding of gastric cancer both from a clinical as well as a preclinical perspective over the past decades, gastric cancer remains a significant public health burden worldwide, particularly in developing countries. Hence, it is urgent to develop novel therapeutics for gastric cancer. The pathogenesis of gastric carcinoma is still unclear, and increasing evidence shows that many factors contribute to this process.

In the past three decades, utilization of the estrogen receptor (ER) in breast carcinoma is well established. Women with ER-positive breast cancer benefit by substantial improvements in outcomes due to current endocrine therapies (3,4). Unfortunately, the role of the ER in other types of cancers is largely unknown. Estrogen receptor (ER)α expression in human gastric cancer was first reported by Tokunaga et al (5). Since that time, the relationship between ERα status and tumor progression was reported in a series of studies. Our previous study demonstrated that ERα status strongly influences patient survival in gastric cancer (37). It is tempting to postulate that ERα may play an essential role in the carcinogenesis of gastric cancer; however, its definitive role in the cell biological characteristics and related involved mechanisms have not yet been fully elucidated. Better understanding of the role of ERα as well as the related pathway will lead to more effective targeting of this pathway for cancer prevention and therapy.

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Key words: ERα, gastric cancer, MKN28, proliferation, metastasis
The present study aimed to investigate the involvement of ERα in cell growth and progression in gastric cancer. To accomplish this, we constructed an eukaryotic expression vector with the ERα gene to determine the effects of ERα overexpression on the cell biological characteristics of the gastric cancer cell line MKN28. The long-term goals of our research are to ascertain whether ERα may serve as a potential diagnostic and prognostic marker of gastric cancer and as a target for the development of therapeutic approaches to treat this disease.

Materials and methods

Cell culture. Human gastric adenocarcinoma cell lines, BGC823, KATOIII, MKN45, MKN28, AGS, N87 and SGC7901, were purchased from the Cell Bank, Chinese Academy of Science, Shanghai, China. They were cultured in an incubator at 37˚C under a humidified atmosphere of 5% CO₂ and 95% air in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Life Technologies, Gaithersburg, MD, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Life Technologies), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated for 24 h in Phenol red-free minimum essential medium (MEM; Invitrogen Life Technologies, Carlsbad, CA, USA) without FBS prior to all experiments (termed cell cycle synchronization).

Construction and transfection of the ERα plasmid expression vector. We used plasmid pcDNA3.1+ (Shanghai Cancer Institute, China) to construct the ERα expression vector. The methods of pcDNA3.1+ERα and transfection of MKN28 gastric cancer cells with pcDNA3.1+ (vector) or pcDNA3.1+ERα were conducted as follows. Briefly, an ERα cDNA PCR product and pcDNA3.1+ (vector) were digested with EcoRI. The digested PCR product was electrophoresed through and isolated from an agarose gel. After purification, it was ligated into the cut vector to form pcDNA3.1+ERα. After the ligation, the plasmid was transformed into Escherichia coli TOP10 cells, and then planted on solid LB medium. Ampicillin-resistant colonies were cultured at 37˚C overnight in a rocking bed. The recombinant plasmid was prepared, and the sequences were verified by electrophoresis of the digested product. MKN28 cells (1x10⁵) were inoculated into a 6-well plate and transfected with pcDNA3.1+ or pcDNA3.1+ERα recombination plasmids when the confluency achieved 90%. Forty-eight hours after transfection, cells were diluted to 1:10 for passage, and cultured for at least 2 weeks in medium containing G418 (Geneticin)² selection agent; Invitrogen Life Technologies, Carlsbad, CA, USA).

MTT assay. MKN28 cells with or without ERα overexpression (1x10⁵/well) were seeded into a 96-well plate and incubated in an incubator at 37˚C under a humidified atmosphere of 5% CO₂ and 95% air. Growth was measured by adding 20 µl of 5 mg/ml methyl-thiazolytetrazolium (MTT) to each well, and the plates were incubated at 37˚C for 4 h. Then, 200 µl dimethyl sulfoxide was added to each well after removal of the old medium, and absorbance was measured at 570 nm using a multi-well spectrophotometer (Bio-Rad, Hercules, CA, USA).

Colony formation assay. Cell suspensions from each group were diluted in DMEM supplemented with 10% FBS, and immediately re-plated (1,000 cells/well) in 6-well plates. The plates were incubated until the cells had formed sufficiently large colonies. The colonies were fixed with dehydrated ethanol and stained with 0.5% crystal violet. The plates were photographed and their digital images were manually analyzed to determine the colony number.

Flow cytometric analysis. For cell cycle analysis, cells (1x10⁵) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4˚C for at least 24 h, washed twice in PBS, and incubated in 25 µg/ml of RNase for 30 min at 37˚C. Before analysis, cells were stained with 50 µg/ml of propidium iodide (PI) (Cell Apoptosis PI Detection kit; Keygentec, China) at room temperature for 30 min. Analyses were performed using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA). Data obtained from the cell cycle distributions were analyzed using FlowJo v7.6 (Tree Star, Inc., Ashland, OR, USA).

Analysis of apoptosis. Enumeration of apoptotic cells was carried out using the Cell Apoptosis PI Detection kit (Keygentec). Cells were washed twice in cold PBS and re-suspended in 1X buffer A at a concentration of 100x10⁶ cells/ml. This suspension (95 µl) was stained with 5 µl of PI. The cells were gently vortexed and incubated for 5 min at room temperature in the dark. Cells were observed under a fluorescence microscope according to the protocol. The number of cells undergoing apoptotic cell death was analyzed by an inverted fluorescence microscope. Images were captured randomly from 5 fields of vision with x200 magnification. Independent experiments were performed in triplicate.

Transwell migration and invasion assays. For the migration studies, cells with or without ERα overexpression were dispersed using trypsin and adjusted to a density of 1x10⁵ cells/ml with serum-free DMEM. Then, 100 µl of the solution (1x10⁵ cells/ml) was placed in the upper chambers of Transwell plates (Millipore, Billerica, MA, USA), and 500 µl of DMEM with 10% FBS was added to the lower chambers. The plates were then placed in an incubator at 37˚C with 5% CO₂ for 24 h. After incubation, the cells remaining in the upper chamber were carefully removed, and the Transwell membrane was fixed with dehydrated ethanol and stained with 0.5% crystal violet. To count the fixed cells, images were captured randomly from 5 fields of vision with x200 magnification. Independent experiments were performed in triplicate.

For the cell invasion assay, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was thawed on ice at 4˚C overnight and diluted with serum-free medium at a ratio of 1:3. Then, the Transwell chambers were coated with 30 µl of diluted Matrigel in a 24-well plate and incubated at 37˚C for 2 h. Afterward, 1x10⁵ cells in serum-free DMEM were seeded into the prepared Transwell chambers. Then, 500 µl of DMEM with 10% FBS was added to the basal chamber. The 24-well plate was then incubated at 37˚C with 5% CO₂ for 24 h. Cells were stained and counted as in the migration assay.
Western blot analysis. Whole-cell proteins were isolated using a protein extraction buffer containing 150 mmol/l NaCl, 10 mmol/ml Tris (pH 7.2), 5 mmol/l ethylenediaminetetraacetic acid, 0.1% Triton X-100, 5% glycerol and 1% sodium dodecyl sulfate. Equal amounts (40 µg/lane) of proteins were fractionated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-ERα (Epitomics, Inc., Burlingame, CA, USA), -GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and -β-catenin (Epitomics, Inc.) primary antibodies. After being washed with TBS Tween-20 (0.1%), the membranes were incubated with peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and subjected to enhanced chemiluminescent staining using an ECL detection system (Bio-Rad). All experiments were conducted in triplicate.

Statistical analysis. Data are presented as means ± standard error of the mean (SEM) of 3 independent experiments. The Student's t-test was used to determine the statistical differences between various experimental and control groups, and one-way ANOVA test was used to determine the difference between three or more groups. P-values <0.05 were considered to indicate statistically significant differences.

Results

ERα expression in gastric cancer cell lines. Western blotting was performed using 7 gastric cancer cell lines. The protein level of ERα was not detectable in all 7 gastric cancer cell lines based on western blot analysis (Fig. 1A).

Construction and transfection with the pcDNA3.1+ERα recombinant vector. To examine the effect of ERα on gastric tumor cell progression in vitro, the plasmid pcDNA3.1 was used to construct the ERα expression vector, pcDNA3.1+ERα. The MKN28 cell line was engineered to stably express increased levels of ERα protein, and the engineered cell lines are referred to as MKN28-ERα(C6) and MKN28-ERα(C15), respectively. A control cell line was transfected with the empty vector and is referred to as MKN28-Vector. ERα protein was detectable in the MKN28-ERα(C6) and MKN28-ERα(C15) cells. In contrast, no ERα expression was observed in the MKN28-Vector cells (Fig. 1B). The data suggest that the pcDNA3.1+ERα recombinant vector was successfully constructed, and ERα was stably overexpressed in the MKN28-ERα(C6) and MKN28-ERα(C15) cells. Furthermore among the two cell lines expressing ERα, MKN28-ERα(C6) cells exhibited much higher expression than the other, which enabled us to examine how different
degrees of ERα expression influence the progression of MKN28 cell.

**ERα overexpression inhibits cell growth and proliferation in gastric cancer MKN28 cells.** To determine the effect of ERα expression on the growth and proliferation of MKN28, we determined the in vitro survival rates of the MKN28-ERα(C6) and MKN28-ERα(C15) cells. MKN28-ERα(C6) and MKN28-ERα(C15) cells exhibited significantly reduced cell survival, as assessed by the MTT assay (Fig. 2A). The mean proliferation rate was 1.2- to 1.5-fold higher in the MKN28-Parental and MKN28-Vector cells when compared to the rate in the MKN28-ERα(C6) and MKN28-ERα(C15) cells (P<0.05) (Fig. 2B). In addition, MKN28-ERα(C6) cells had a slower rate of growth than the MKN28-ERα(C15) cells, which was consistent with the elevated levels of ERα in MKN28-ERα(C6) cells.

We utilized a colony formation assay to further confirm the suppressive effect of ERα on the growth of MKN28 cells. The mean number of colonies formed by MKN28-ERα(C6) cells after 10 days of culture was 32.67±4.16, and it was 59.7 and 62.1% decreased, respectively, when compared with that of the MKN28-Vector and MKN28 cells (P<0.001) (Fig. 3). Furthermore, more colonies were observed in the MKN28-ERα(C15) cells when compared to the number of colonies in the MKN28-ERα(C6) cells. Taken together, these data suggest that ERα inhibits cell growth and proliferation in gastric cancer MKN28 cells.

**Effect of ERα overexpression on cell cycle control in gastric cancer MKN28 cells.** Flow cytometry was used to determine whether the inhibitory effect of ERα on MKN28 cell proliferation was mediated, at least partly, by the cell cycle. Both MKN28-ERα(C6) and MKN28-ERα(C15) cells showed an...
increase in the number of G2-M-arrested cells, when compared to this number in the parental cells (Fig. 4). Cell growth inhibition by ERα was associated with significant cell cycle arrest at the G2/M phase, implicating that ERα suppresses cell proliferation by controlling the G2 and M checkpoints and induces specific blockage of cell cycle progression.
ERα overexpression induces apoptosis of gastric cancer cells. PI staining was used to evaluate the degree of apoptosis in the different cell lines. There was a significantly increased number of apoptotic cells noted in the MKN28-ERα(C6) and MKN28-ERα(C15) cells, as compared with the MKN28-Parental and MKN28-Vector cells. Notably, the proportion of apoptotic cells in the MKN28-ERα cells was in line with the level of ERα expression (Fig. 5).

ERα overexpression inhibits the migration and invasion of gastric cancer cells. To determine whether ERα is involved in mediating the migration and invasion of gastric cancer cells, we performed in vitro migration and invasion assays using Transwell chambers. After the cells were incubated for 24 h in the Transwell assay system, the number of MKN28-Vector cells that had moved through the membrane of the chamber was ~3.7 and 1.7-fold higher than the number of the MKN28-ERα(C6) (P<0.001) and MKN28-ERα(C15) cells (P<0.05) (Fig. 6A and B), respectively. MKN28-ERα cells migrated at a significantly lower rate than the control cells after 24 h. The results indicate that ERα reduces the migratory ability of MKN28 cells.

Similarly, MKN28-ERα cells were observed to be less invasive. After the cells were incubated for 24 h in the Transwell assay system, the number of MKN28-Vector cells that had invaded through the membrane of the Matrigel chamber was ~3.8-fold higher than that of the MKN28-ERα(C6) cells (P<0.001) and 1.6-fold higher than that of the MKN28-ERα(C15) cells (P<0.05) (Fig. 6C and D).

The migration and invasion ability was 2.2-fold reduced in the MKN28-ERα(C6) cells when compared to the MKN28-ERα(C15) cells (Fig. 6B and D). Taken together, these results showed that overexpression of ERα may suppress the migration and invasion of MKN28 cells.

Overexpression of ERα inhibits progression by suppressing β-catenin in MKN28 cells. Next, to determine the potential molecular mechanism of the phenotypes gained by ERα overexpression in MKN28 cells, we examined the protein level of β-catenin, as β-catenin has been implicated in the initiation and progression of gastric cancer (6,7); interaction between ERα and β-catenin has been delineated (8). Our results showed that β-catenin was significantly reduced in MKN28-ERα(C6) and MKN28-ERα(C15) cells, compared with the control cells (Fig. 7).

Discussion
In the present study, we investigated the role of ERα expression on the cell growth and metastasis of gastric cancer cell line MKN28. We observed that ERα protein was not expressed in the 7 gastric cell lines studied. ERα transfection inhibited cell growth by G2/M arrest, induced apoptosis, and suppressed cell migration and invasion in gastric cancer cell line MKN28. In addition, these inhibitory effects by ERα were in line with the level of ERα expression in gastric cancer. Our study also observed that the biological changes induced by ERα may possibly be through the suppression of β-catenin expression.

Estrogen protects against gastric cancer through ERs. Gastric cancer has an unexplained strong and enigmatic male predominance (2:1) (9,10), which cannot entirely be explained on the basis of gender differences in the prevalence of known risk factors (11). Cumulative evidence suggests that the differences are rooted in basic biological differences between men and women, and this phenomenon would be explained by the hypothesis that estrogens are protective in this respect (reviewed in ref. 12).

This hypothesis has gained support from a number of studies based on different aspects. Epidemiological studies have confirmed that estrogen exposure is associated with a decreased risk of developing gastric cancer (13-16). Women with a longer fertility life and those on hormone replacement therapy appear to have a decreased risk of gastric cancer. Furthermore, men who have been treated with estrogen for prostate cancer also have a decreased risk. In a meta-analyses, risks for ever vs. never use of hormone treatment (HT) were significantly reduced for gastric cancer (RR 0.78, confidence interval (CI) 0.65-0.94; P=0.008) (17). A nested case-control study of hormone replacement therapy (HRT) demonstrated that the risk of stomach cancer increases among users of HRT compared to nonusers [odds ratio (OR), 0.48, 95% CI 0.29-0.79] (18). Furthermore, tamoxifen (TAM) an anti-estrogen, may increase the risk of gastric cancer starting with a decrease of the risk of adenocarcinoma was found among users of HRT compared to nonusers.

On the contrary, Harrison et al (21) demonstrated that estradiol caused a strong stimulation of gastric cell lines and physiologic concentrations, meanwhile, addition of the active metabolite of the estrogen-receptor blocker/partial-agonist 4-hydroxytamoxifen had a stimulating effect on the growth rate of the gastric cell lines. On the other hand, another study reported that estrogen did not affect the proliferation of gastric cancer cell lines (22). Despite these contradictory results, in animal studies, female and castrated rats have a lower incidence of chemically induced gastric cancer (23). This hypothesis was further validated by animal studies (23-26). These preclinical studies indicate that estrogen may offer protection against the development of gastric cancer, as for example, ovariectomized mice are at an increased risk, while administration of female sex hormones decreases the incidence of gastric cancer.

The biological means behind this hypothesis is still inconclusive but various mechanisms have been suggested.
Estrogen exerts its biological actions through the activation of two nuclear receptors, ERα and ERβ, with distinctive tissue distribution and a counteracting function (27-29). In addition, ERα has been proven to have a critical role in gastric cancer, which will be subsequently discussed. It is therefore reasonable to hypothesize that estrogen may protect women against gastric cancer through the ERα pathway.

ERα is involved in the development and progression of gastric cancer. The discovery of the ERα provided us not only with a powerful predictive and prognostic marker, but also an efficient target for the treatment of hormone-dependent breast cancer with anti-estrogens. The important role of ERα in the development, progression and treatment of breast cancer are well established, but the role of such an evaluation in other types of cancers is largely unknown.

ERα expression in human gastric cancer was first reported by Tokunaga et al (5) as far back as in 1986. Nonetheless, the role of ERα in human gastric cancer is not yet fully elucidated. It has been suggested that the ERα pathway may have a role in the progression of gastric cancer (5,22,30-32). Contradictory findings have emerged on the basis of publicly accessible in vivo and in vitro studies. We found that numerous investigators have reported the relationship of ERα status to carcinogenesis and tumor progression; even though, their findings are still controversial (33-36), including our previous study (37). Most recently, another study indicated that sex hormone receptors (including ERα) may be partly involved in gastric carcinogenesis yet their clinicopathological and prognostic significance in gastric cancer appears to be limited (38). The possibility exists that these discrepancies result from small numbers and inconsistencies in methodologies.

Based on the evidence that ERα is expressed in poorly differentiated adenocarcinomas more frequently than in well differentiated gastric cancer (39-43), several clinical trials using a partial estrogen antagonist, tamoxifen, have been conducted for the management of ERα-positive gastric cancer patients. However, the results have not been consistent, and the utility of ERα for the treatment of gastric cancer is still inconclusive (21,39,44,45).

Even more, the expression of ERα in gastric cancer has shown marked variability (0-62.5%) (12,46). Consistent with our result, several studies also found that ERα cannot be detected in gastric cancer cell lines (46,47).

Based on the currently available evidence, the clinical significance and implication of ERα expression in human gastric carcinoma are still not fully elucidated. Elucidation of the precise roles of estrogen and/or its receptors in gastric cancer will provide new insights that will contribute to diagnosis and treatment.

Role of β-catenin in gastric cancer. Wnt and estrogen signaling represent important regulatory pathways, each controlling a wide range of biological processes. Crosstalk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and ERα (8), can provide fine-tuned regulation of many cellular processes. In the present study, we aimed to ascertain whether ERα plays a suppressive role in the proliferation and metastasis of MKN28 cells through altering β-catenin expression.

The roles of β-catenin in mediating intercellular adhesion and regulation of cell growth, differentiation, invasion and metastasis have been well characterized (48,49). The β-catenin-TCF/LEF complex regulates and activates its downstream target transcription genes which are involved in the development and progression of cancer (50-52). The abnormal activation of β-catenin frequently occurs in gastric cancer and has been proven to promote tumor growth, invasion and metastasis (6,7). Furthermore, previous studies have confirmed that high β-catenin expression is an independent indicator of poor prognosis for these carcinomas and is closely correlated with enhanced tumor progression (53,54).

In the present study, expression of β-catenin was found to be notably decreased in ERα-overexpressing MKN28 cells (Fig. 7). Importantly, the degree of decrease was in line with the level of ERα expression in the two different MKN28-ERα cells.

Limitation of this present study and future perspectives. These preliminary findings will require further replication and in-depth investigation. In our present study, only one gastric cancer cell line MKN28 was studied. Thus, it would be sensible to reanalyze our findings using another gastric cancer cell line to confirm our results and to exclude a cell-specific phenomenon.

In addition, cell lines do not always accurately represent the phenotype of the tumors from which they were derived. Therefore, in vivo studies using xenografts should be embarked on in the near future.

It should also be noted that this study was primarily concerned with gain-of-function analysis of the biological effect of overexpression of ERα in MKN28 cells. Unfortunately as mentioned above, none of the 7 gastric cancer cell lines in our study had an inherent ERα protein level expression. Because of this reason, we could not provide evidence whether inhibition of ERα promotes the aggressive phenotype of gastric cancer cells.

Additionally, based on the present study, whether or not the effect of ERα is estrogen mediated was not directly determined. Nonetheless, estrogen-free Dulbecco's modified Eagle's medium (DMEM) was used in this study, which can exclude the influence of estrogen. An estrogen-containing condition should be further investigated, to delineate whether ERα can exhibit a further suppressive effect on the malignant phenotype of MKN28 cells in the context of the presence of estrogen.

Finally, the specific mechanism between ERα and β-catenin interaction needs to be further clarified.

In conclusion, despite its preliminary character, the research reported here indicates that overexpression of ERα inhibits tumor cell proliferation, migration and invasion. To the best of our knowledge, this is the first finding that demonstrates that ERα expression is a possible protective mechanism against the progression of gastric cancer and suggests that ERα may be a potential target for utilization in gastric cancer treatment.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant no. 81101659/H1609,
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Menopausal hormone: Prognostic role of estrogen-

Wnt/-nitro-nitrosoguanidine. N'


