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Anti-tumor efficacy of fulvestrant in estrogen receptor positive gastric cancer

SUBJECT AREAS:

GASTRIC CANCER
TUMOUR BIOMARKERSJun Ho Yi^{1,3*}, In-Gu Do^{2*}, Jiryeon Jang³, Seung Tae Kim³, Kyoung-Mee Kim⁴, Se Hoon Park³, Joon Oh Park³, Young Suk Park³, Ho Yeong Lim³, Won Ki Kang³ & Jeeyun Lee³Received
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Correspondence and requests for materials should be addressed to J.L. (jyunlee@skku.edu) or H.Y.L. (hoylim@skku.edu)

* These authors contributed equally to this work.

¹Division of Hematology-Oncology, Department of Medicine, Hanyang University Hospital, Seoul, Korea, ²Department of Pathology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Korea, ³Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea, ⁴Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.

To investigate the prognostic role of the estrogen receptor (ER) in gastric cancer (GC) patients, tumor tissues from 932 patients with advanced GC were assessed for ER expression using immunohistochemistry, and their clinicopathologic features were evaluated. Forty patients (4.3%) had ER expression and they were more frequently associated with diffuse type gastric cancer and shorter disease free survival. Furthermore, we carried out *in vitro* analysis to evaluate the effect of ER modulation on the proliferation of GC cell lines. Estradiol enhanced proliferation of ER positive GC cells while it did not show any effect on ER negative GC cells. When ER was inhibited by fulvestrant and ER siRNA, estradiol-induced proliferation of ER positive GC cell was suppressed. Paclitaxel showed synergistic anti-proliferative impacts with fulvestrant. Suppressing ER by fulvestrant, paclitaxel and ER siRNA showed increased expression of E-cadherin, which is a crucial factor in diffuse-type carcinogenesis.

Gastric cancer (GC) is the fourth most common cancer worldwide, with nearly one million new cases diagnosed every year¹, and it is one of the leading causes of cancer-related mortality, especially in Asia^{2,3}. Although surgical resection remains the primary treatment of choice, less than 50% of patients are eligible⁴. Thus, a substantial portion of patients receive palliative chemotherapy, but the expected survival duration barely exceeds 1 year, in spite of recent progress^{5–8}.

GC can be categorized into two distinct histologic subtypes, intestinal and diffuse, which are distinct in their microscopic and gross appearance, epidemiology, pathogenesis, and prognosis⁹. In diffuse-type GC, female and young patients predominate; they are usually diagnosed at an advanced stage and their prognosis is often very poor^{10,11}. Defective intercellular adhesion is a unique molecular feature of diffuse-type GC; loss of the cellular adhesion molecule, E-cadherin, is crucial to the pathogenesis of diffuse GC^{12–14}.

Several epidemiologic studies have suggested that the female sex hormone estrogen may play a role in gastric carcinogenesis^{15–17}. Furthermore, the estrogen receptor (ER) has been found to be expressed in GC tissue¹⁸, and its clinical implications have been investigated in several studies^{19–23}. In these studies, several consistent findings can be noted. First, approximately 20% of patients with GC were positive for ER- α in immunohistochemical (IHC) studies. Second, ER- α -positive GC is more common in poorly differentiated and signet ring cell carcinomas than in well or moderately differentiated carcinomas. Third, even after stage adjustment, patients with ER- α -positive GC demonstrate a poorer prognosis, while its counterpart, ER- β , implies a favorable prognosis. There are three isoforms of estrogen, and 17 β -estradiol (E2) is the most potent. In several *in vitro* studies, E2 has been shown to enhance proliferation of GC cells that harbor ER- α ^{24,25}, and there is also evidence that E2 down-regulates E-cadherin through ER- α ^{26–28}, which may initiate diffuse GC²⁹.

Fulvestrant (Faslodex®) is an analog of E2 that down-regulates and degrades ER- α without agonism. The efficacy of this agent has already been demonstrated in patients with ER-positive breast cancer³⁰, and it is regarded as a standard of care. In addition, it has been shown to exhibit excellent anti-proliferative effects in several *in vitro* studies dealing with ER- α -positive ovarian²⁶, non-small cell lung³¹, and GC cells²⁵.

In the current study, we have focused on demonstrating two hypotheses. First, that expression of ER- α implies a poor prognosis in GC patients. The other is that ER- α inhibition may show anti-neoplastic efficacy in ER- α -positive GC. To investigate the former, we have performed an IHC study in our GC patient cohort and analyzed their clinical outcomes. To investigate the latter, we have performed various *in vitro* analyses using GC cell lines.



Methods

The study has been approved by the institutional review board at Samsung Medical Center. All methods used in this study were carried out in accordance with the approved guidelines and all experimental protocols were approved by Samsung Biomedical Research Institute.

IHC studies of ER expression. We collected medical records of patients with GC who had undergone curative gastrectomy followed by 5-FU/leucovorin-based concurrent chemoradiation as an adjuvant aim from July 1995 to December 2005. Patients who met the following criteria were included in the analysis: histologically confirmed adenocarcinoma of the stomach; surgical resection of the tumor without macroscopic or microscopic residual disease; age ≥ 18 ; pathology stage IB (T2bN0 or T1N1 but not T2aN0) to IV (not TxNxM1), according to the 6th edition of the staging system published by the American Joint Committee on Cancer (AJCC); complete surgical records and treatment records, and the availability of FFPE (formalin-fixed paraffin-embedded) tissue suitable for IHC study.

For the IHC study, formalin-fixed, paraffin-embedded, 4 μm -thick tissue sections were deparaffinized 3 times in xylene for a total of 15 min and subsequently rehydrated. Immunostaining for ER was performed using a Bond-max autoimmunostainer (Leica Biosystem, Melbourne, Australia) with BondTM Polymer refined detection, DS9800 (Vision Biosystems, Melbourne, Australia). Briefly, antigen retrieval was performed at 97°C for 20 min in ER2 buffer. After blocking endogenous peroxidase activity with 3% hydrogen peroxidase for 10 min, slides were incubated with mouse monoclonal estrogen receptor antibody (NCL-L-ER-6F11, Novocastra, Newcastle, United Kingdom) for 15 min at room temperature, at a dilution of 1:200. Normal breast tissue was used as a positive control for ER expression.

Following the ASCO-CAP guidelines for breast cancer³², cancer cells with nuclear staining $> 1\%$ were interpreted as positive.

Statistical analysis. Disease-free survival (DFS) was defined as the time from the curative surgery to the time of first relapse, and it was calculated using the Kaplan–Meier method and compared using a log-rank test. Pearson's χ^2 test was used for comparison of clinical parameters, including gender, age, and histology of patients with and without ER expression. Multivariate analysis was performed using a logistic regression test for ER expression rate, and a Cox proportional hazards regression test was used for DFS. *P*-values > 0.05 were considered statistically significant, and all *P*-values corresponded to two-sided significance tests.

Cell culture and reagents. Human GC cells were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). All of the cell lines were grown in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin. All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were incubated for 24 h in phenol-red-free minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) without FBS before all experiments. Thereafter, cells were principally cultured in MEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS-MEM) in a humidified atmosphere containing 5% CO₂ at 37°C. 17- β -Estradiol was purchased from Sigma (Deisenhofen, Germany). Fulvestrant (ICI 182780 (ICI)), was purchased from Tocris Cookson, Ltd. (Ellisville, MO, USA). Paclitaxel was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA).

Western blot analysis and RT-PCR for ER in GC cell lines. For western blot analysis, total cell extracts were obtained using protein lysis buffer. The protein concentration was determined using a BCA Protein Assay (Thermo Scientific, Rockford, IL USA), and equal amounts (60 μg) of cell lysates were dissolved in 4–12% Bis-Tris gels with MOPS running buffer (Invitrogen, Carlsbad, CA, USA), transferred onto nitrocellulose membranes, and incubated with the following specific antibodies: ER- α antibody (Cat No. 2512, Cell Signaling Technology, Boston, MA, USA), E-cadherin (Cat No. 610181, BD, Franklin Lakes, NJ, USA), β -actin (Cat. No. sc-47778, Santa Cruz Biotechnology). Immune complexes were visualized using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

For RT-PCR, RNA was synthesized from first-strand cDNA using a Maxime RT premix kit (Intron Biotechnology, Korea), following the manufacturer's protocol (60 min reaction at 45°C). The sequence of the ER- α forward primer was 5'-CAG GGG TGA AGT GGG GTC-3', and the reverse primer was 5'-ATC TCG GTT CCG CAT-3'; these primers were predicted to produce a band of 483 bp. The sequence of the GAPDH forward primer was 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', and the reverse primer was 5'-GGT GGA CCT GAC CTG CCG TCT AGA-3', predicted to produce a 598-bp amplification band. The PCR conditions were as follows: 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension cycle for 10 min at 72°C.

Viability assay and colony forming assay. After estrogen starvation, SNU-216 (5×10^3 per well) and SNU-620 (5×10^3 per well) cells were seeded in 96-well plates in 5% DCC-FBS-MEM, then incubated overnight, treated with various concentrations of reagents, and treated with E2 (3 nM). After 72 h of incubation, they were assayed using WST-1 (Cat. No. 11644807001, Roche, Pleasanton, CA, USA).

SNU-216 (100 cells/well) cells were seeded in 6-well plates in 5% DCC-FBS-MEM and incubated overnight. They were treated with fulvestrant (1 μM) and paclitaxel (500 nM). After 10 days of incubation, cells were stained with 0.1% crystal violet in

methanol and the number of colonies was counted using a Cell Counting Kit-8 (Dojindo Laboratories, Japan).

Small interfering RNA (siRNA) against ER- α . SNU-216 cells (3.0×10^5 per well) were transfected with siRNA (12.5 pmol) against ER- α using Lipofectamine. After 72 h of incubation, cells were harvested by trypsinization, and then used for western blot analysis.

Results

Patient characteristics. A total of 932 patients were included in the analysis. The median age of the patients was 52 (range 23–74) and the M:F ratio was 611 (65.6%):321 (34.4%). Regarding the WHO histologic subtypes, 31 (3.3%) were well-differentiated tubular adenocarcinoma (TAC), 237 (25.4%) were moderately differentiated TAC, 422 (45.3%) were poorly differentiated TAC, and 192 (20.6%) were signet ring cell carcinomas. According to Lauren's classification, 279 (30.7%) were classified as intestinal type, 595 (65.4%) were classified as diffuse type, and 36 (3.9%) were classified as mixed type. Other details, including location, stage, and type of surgery, are described in TABLE 1.

ER immunohistochemical expression and survival analysis. Among the 932 patients, ER expression was found in 40 patients (4.3%). The results of the IHC study showed that cancer cells exhibited diffuse nuclear staining for ER (FIGURE 1). Most cases of positive staining showed intermediated intensity, and more than 50% of cancer cells were stained. Female patients (19/321, 5.9%) showed a higher incidence than male patients (21/590, 3.4%, *P* = 0.076) and the cases of diffuse-type cancer, as defined by Lauren's classification (34/595, 5.7%), were more frequently associated than cases of intestinal-type cancer (4/279, 1.4%, *P* = 0.015). Other variables, such as age, stage, and anatomical location, were not associated with ER expression rate (TABLE 2). The multivariate

Table 1 | Baseline characteristics of the patients (N = 932)

Characteristics	N (%)
Median age, (range)	52 (23–74)
Sex	
Male	611 (65.6)
Female	321 (34.4)
Type of surgery	
Subtotal gastrectomy	578 (62.0)
Total gastrectomy	349 (37.4)
Others	5 (0.6)
Location of tumor	
Gastroesophageal junction	95 (10.2)
Stomach	837 (89.8)
Histology	
Well differentiated TAC*	31 (3.3)
Moderately differentiated TAC*	237 (25.4)
Poorly differentiated TAC*	422 (45.3)
Signet ring cell carcinoma	192 (20.6)
Mucinous adenocarcinoma	29 (3.1)
Others	21 (2.3)
Lauren type (N = 910)	
Intestinal	279 (30.7)
Diffuse	595 (65.4)
Mixed	36 (3.9)
Lymphovascular invasion (N = 537)	
Present	462 (86.0)
Absent	75 (14.0)
Stage**	
IB	122 (13.1)
II	337 (36.2)
IIIA	246 (26.4)
IIIB	76 (8.2)
IV (not M1)	151 (16.2)

*TAC, tubular adenocarcinoma; **Categorized according to AJCC 6th edition.

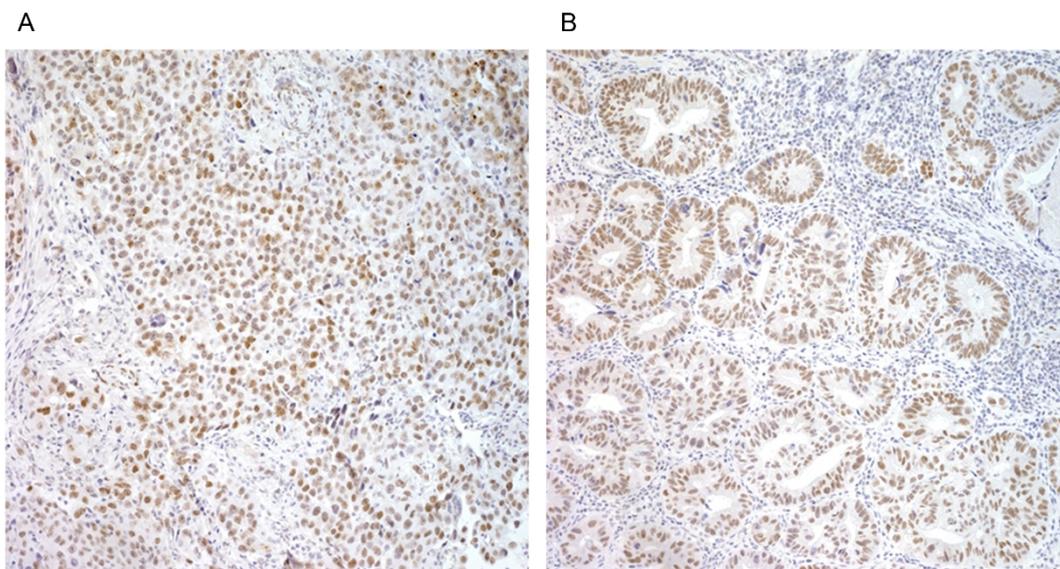


Figure 1 | Immunohistochemical analysis for ER- α . (A), Nuclear positivity in tumor cells of poorly differentiated tubular adenocarcinoma; (B), Nuclear positivity in tumor cells of moderately differentiated tubular adenocarcinoma. ($\times 200$)

analysis showed that diffuse-type cancer was significantly associated with ER expression (TABLE 3).

The median DFS for all patients was 143.2 months (95% CI, not available). The DFS of those who were ER-positive was 55.6 months (95% CI 23.8–87.4) and the DFS of those who were ER-negative was 143.2 months (95% CI, not available); these values were significantly different ($P = 0.044$) (FIGURE 2). Multivariate analysis showed that ER expression was associated with poor DFS (hazard ratio (HR) 1.62, 95% CI 1.04–2.52, $P = 0.034$), along with advanced-stage, (HR 5.10, 95% CI 4.46–5.91, $P < 0.001$) and diffuse-type cancers (HR 1.39, 95% CI 1.10–1.77, $P = 0.007$) (TABLE 4).

Table 2 Characteristics of ER positive patients		
	ER positive rate (N, %)	<i>p</i>
Age		.159
< 60 years	34/705 (4.8)	
≥ 60 years	6/227 (2.6)	
Gender		.076
Male	21/611 (3.4)	
Female	19/321 (5.9)	
Location of the primary disease		.622
Gastroesophageal junction	5/95 (5.3)	
Stomach	35/802 (4.2)	
Histology		.126
Well differentiated TAC*	1/31 (3.2)	
Moderately differentiated TAC*	3/237 (1.3)	
Poorly differentiated TAC*	21/422 (5.0)	
Signet ring cell carcinoma	15/195 (7.8)	
Mucinous adenocarcinoma	0/29 (0.0)	
Others	0/21 (0.0)	
Lauren's classification		.015
Intestinal	4/279 (1.4)	
Diffuse	34/595 (5.7)	
Mixed	2/36 (5.6)	
Lymphovascular invasion		.536
Present	26/462 (5.6)	
Absent	3/75 (4.0)	
Stage		.505
IB+II+III	32/781 (4.1)	
IV	8/151 (5.3)	

*tubular adenocarcinoma.

Estradiol and its antagonist, fulvestrant, on proliferation of GC cells. First, we examined ER- α expression in human GC cell lines. Seven cell lines showed ER- α expression by RT-PCR (FIGURE 3A), and three of these, KATOIII, NCI-N87, and SNU-216 showed ER- α expression in western blot analyses (FIGURE 3B). After several rounds of cell culture we found that SNU-216 (ER- α positive) and SNU-620 (ER- α negative) were suitable models for the current analysis.

We then examined whether E2 plays a role in the proliferation of GC cells according to ER- α status. E2 enhanced the proliferation of SNU-216, and fulvestrant, the antagonist of E2, produced an anti-proliferative effect on SNU-216 cells, in both E2-added and E2-depleted conditions (FIGURE 4A). However, we did not observe a pro-proliferative effect of E2 or an anti-proliferative effect of fulvestrant on SNU-620 cells (FIGURE 4B).

The colony-forming assay produced similar findings. Control SNU-216 cells formed an average of 37.3 colonies. When fulvestrant was administered, SNU-216 cells formed average of 28.7 colonies. Supplementation with E2 enhanced colony formation up to an average of 41.7 colonies. Again, fulvestrant inhibited colony forming, to an average of 22.3 colonies, when supplemented with E2 (FIGURE 4C).

Combination treatment with fulvestrant and paclitaxel. Next, we examined the synergistic anti-proliferative impacts of fulvestrant and paclitaxel on SNU-216 cells. Paclitaxel showed an anti-proliferative effect on SNU-216 cells, both alone and when combined with fulvestrant; the effect of combined administration was synergistic, comparing to their respective effects (FIGURE 5A). This synergism was not observed in SNU-620 cells (FIGURE 5B). In colony-forming assays, paclitaxel completely blocked cell proliferation; no colonies were formed (data not shown). Thus, it was not possible to determine whether the combination was synergistic using this assay.

Table 3 Multivariate analysis for ER- α expression			
	Hazard ratio	95% CI	<i>p</i>
Diffuse type cancer	3.61	1.25–10.41	.018
Stage IV	1.08	0.46–2.53	.854
Age < 60 years	1.55	0.59–4.11	.378
Female	1.69	0.87–3.28	.120

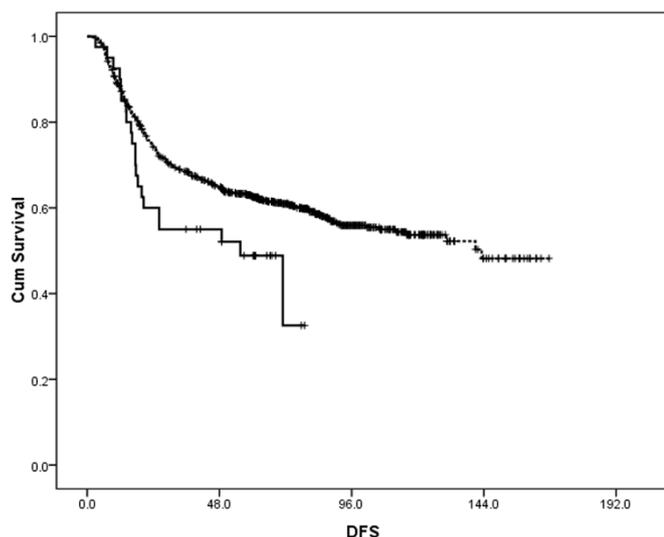


Figure 2 | Kaplan-Meier curve of disease-free survival of patients with (solid line) and without (dotted line) ER- α expression.

	Hazard ratio	95% CI	<i>p</i>
ER- α expression	1.62	1.04–2.52	.034
Stage IV	5.10	4.46–5.91	<.001
Diffuse type cancer	1.39	1.10–1.77	.007

ER- α inhibition and E-cadherin expression. As previously mentioned, several studies have demonstrated that ER plays an important role in regulating E-cadherin, which may induce diffuse-type cancers^{27,29}. Thus, we analyzed whether inhibition of ER- α affects levels of E-cadherin. SNU-216 cells alone expressed only small amounts of E-cadherin. When cells were treated with fulvestrant and paclitaxel, expression of E-cadherin increased (FIGURE 6A). To determine whether this phenomenon is ER- α -mediated, we knocked down ER- α with siRNA. As expected, compared to SNU-216 cells and control siRNA, knocking down ER- α with siRNA enhanced E-cadherin expression (FIGURE 6B).

Discussion

Our retrospective study showed that in a total of 932 patients with GC who had received curative resection followed by adjuvant chemoradiation, 40 patients (4.3%) were ER- α positive by IHC. ER- α expression was associated with diffuse-type cancer and a poorer clinical outcome. Our *in vitro* study demonstrated that E2 enhances proliferation of an ER- α -positive GC cell line and that both fulvestrant and paclitaxel inhibited its proliferation; this result was not observed in ER- α -negative GC cells. Combination of fulvestrant and paclitaxel may show synergism. Both fulvestrant and paclitaxel enhanced E-cadherin expression, a crucial factor in diffuse-type carcinogenesis; this effect was mediated via the ER- α pathway.

The carcinogenic role of estrogen in breast and ovarian cancers is well understood, and in breast cancer, estrogen-directed therapy is a mainstream treatment. It has been suggested that E2 may play a role in the carcinogenesis of tissues other than female reproductive organs, including in lung³³, thyroid³⁴, or gall bladder cancers³⁵. It has also been suggested that estrogen is involved in development of non-small cell lung cancer, especially in adenocarcinoma of non-smoking women, and that there is functional cross-signaling between EGFR-ER pathways. Several *in vitro* studies have shown that combination treatment with fulvestrant enhances the anti-tumor efficacy of gefitinib³⁶ and vandetanib³⁷.

Since the late 1980's, estrogen and ER have been suspected to play roles in GC. Owing to the male predominance of GC and the fact that males who were treated with estrogen for prostate cancer showed a reduced risk of GC, some investigators assumed that estrogen plays a preventive role against GC¹⁵. However, as older menopause and null parity are associated with an increased risk of development of GC in women, in the same way as breast cancer, some investigators have regarded estrogen as pro-carcinogenic for GC¹⁶. One population-based cohort study has reported that endogenous estrogen exposure was associated with a lower frequency of intestinal-type cancers and a higher frequency of diffuse-type cancers, giving rise to the idea that the role of estrogen may vary with GC histology³⁸.

In contrast to estrogen, the clinical implications of ER, especially the α subtype, have been relatively consistent for a long time^{19–23}. As described in the Introduction, approximately 20% of GC patients are positive for ER- α , and it is associated with poorly differentiated histology and a poor prognosis. In the present study, however, we found that less than 5% of patients were ER- α -positive. This may have resulted from our use of the usual ER- α IHC method for breast cancer, which differs in antibody concentration, incubation time,

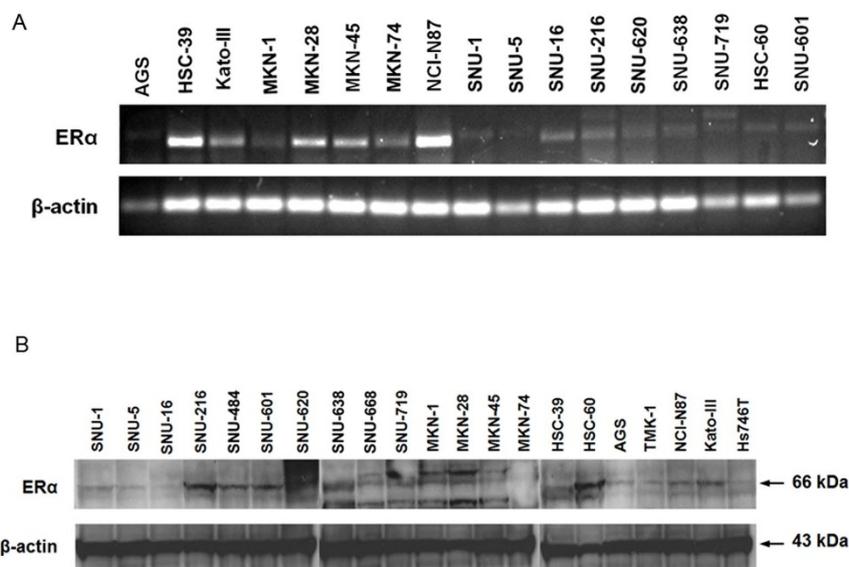


Figure 3 | Screening of gastric cancer cell lines (A, RT PCR; B, Western blot).

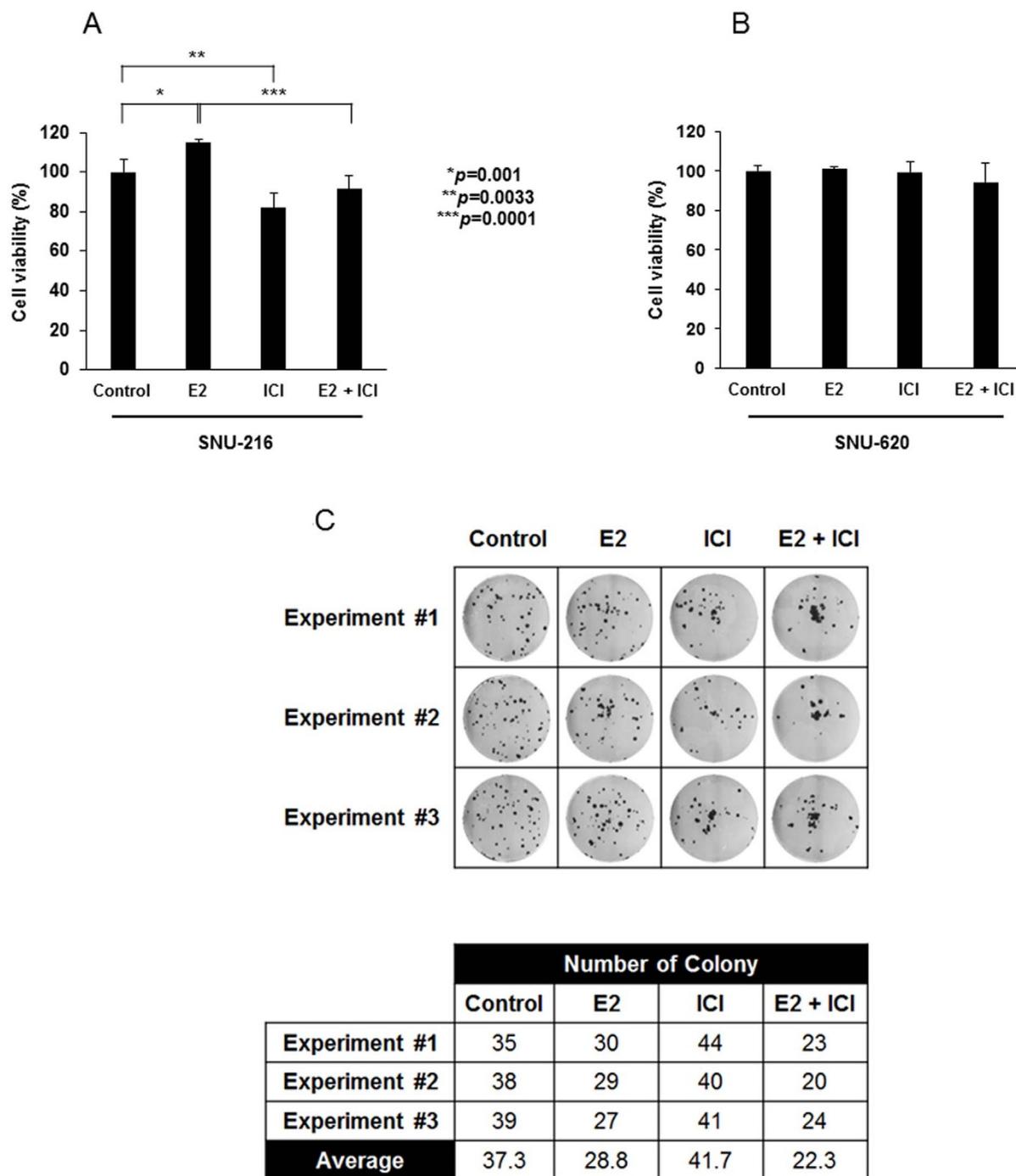


Figure 4 | Impact of estradiol (E2) and fulvestrant (ICI) on gastric cancer cell proliferation. (A), when SNU-216 cells were treated with E2 and ICI, E2 significantly enhanced proliferation while ICI significantly inhibited both E2-naïve and E2-enhanced proliferation; (B), The growth of SNU-620 cells was not affected by either E2 or ICI; (C), the colony-forming assay also showed that E2 promotes colony formation of SNU-216 cells, whereas it is hampered by ICI).

and temperature from the method employed by the former studies. As no validated ER- α IHC protocol or interpretation guidelines exist for GC, further study is needed. From a histological perspective, we also found that ER- α was significantly associated with diffuse-type GC. While 4 of 279 patients (1.4%) with intestinal-type cancer showed ER- α expression, 34 of 595 patients (5.7%) with diffuse-type cancer and 2 of 36 (5.6%) with mixed-type cancer showed ER- α expression; multivariate analysis of the results showed that the differences were significant. In regard to survival, patients with ER- α expression showed shorter median DFS than patients without ER- α expression (55.6 months vs. 143.2 months, $P = 0.044$); again, ER- α

expression was shown to be associated with poor DFS following multivariate analysis.

In the *in vitro* analysis, ER- α expression, examined by protein and mRNA expression, was found to be associated with E2-dependent growth and inhibition. In SNU-216 cells, an ER- α positive cell line, E2 led to cellular proliferation which was suppressed by fulvestrant; these results were not observed in SNU-620 cells, an ER- α negative cell line. Kameda et al. have also shown that E2 induces proliferation of KATO-III and NCI-N87 cells, which was suppressed by fulvestrant and ER- α siRNA²⁵. We further analyzed the impact of paclitaxel in this setting. Keeping further analysis, including clinical trials in

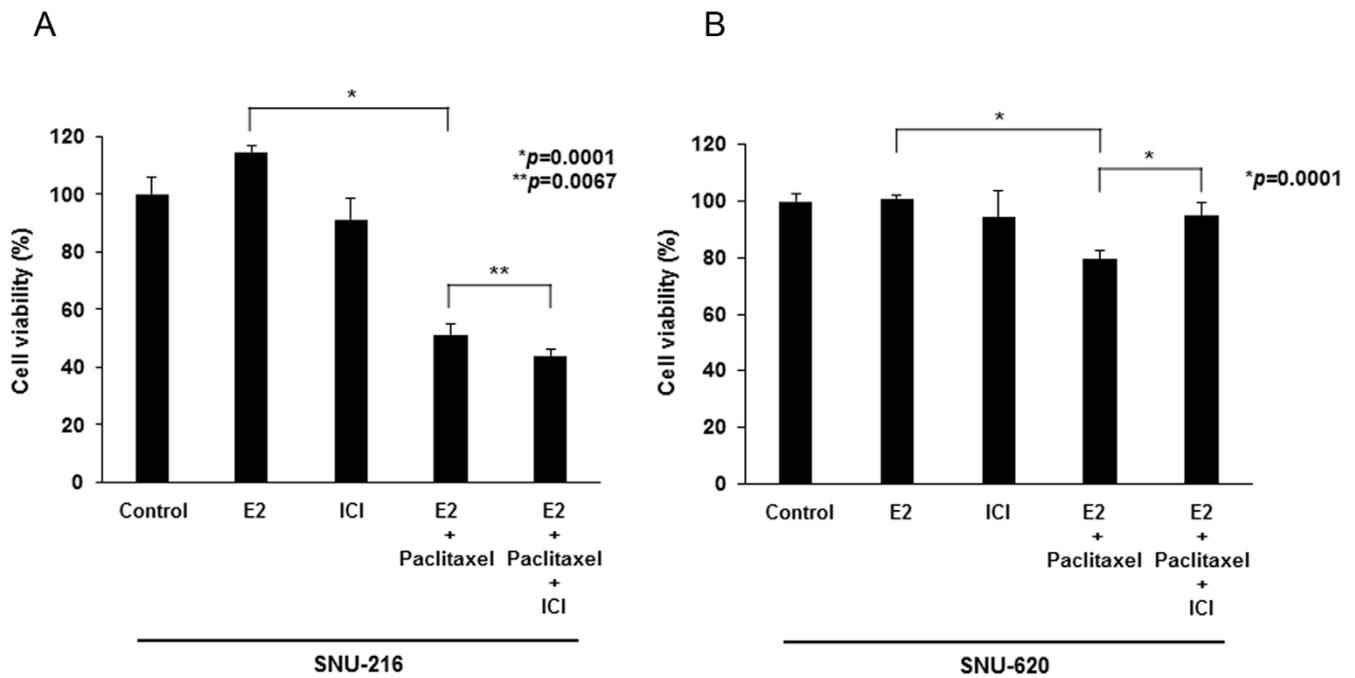


Figure 5 | Combination treatment of fulvestrant (ICI) and paclitaxel. (A), when SNU-216 cells were treated with ICI combined with paclitaxel, synergism was observed; (B), the combination treatment did not effect SNU-620 cells).

mind, we chose to use paclitaxel because it has been demonstrated to produce a synergistic impact with fulvestrant in breast cancer models³⁹, and because it is widely used as a standard treatment for metastatic GC patients, with a 4-week administration schedule that is compatible with that of fulvestrant. Although we could not draw any conclusions from the colony-forming assay, the combination of fulvestrant and paclitaxel showed synergistic effects in the viability assay. This synergism is concordant with their known modes of

action; paclitaxel is a microtubule-stabilizing agent, and E2 enhances cell motility by destabilizing microtubules via deacetylation of α -tubulin, thereby causing paclitaxel resistance⁴⁰.

Absent or aberrant expression of E-cadherin is pivotal in both familial and sporadic forms of diffuse gastric carcinogenesis, probably via methylation of the promoter of the E-cadherin gene⁴¹. Park et al. showed that ER- α regulates E-cadherin levels in ovarian cancer cell lines²⁶, and a study by Oesterreich found that ER- α and core-

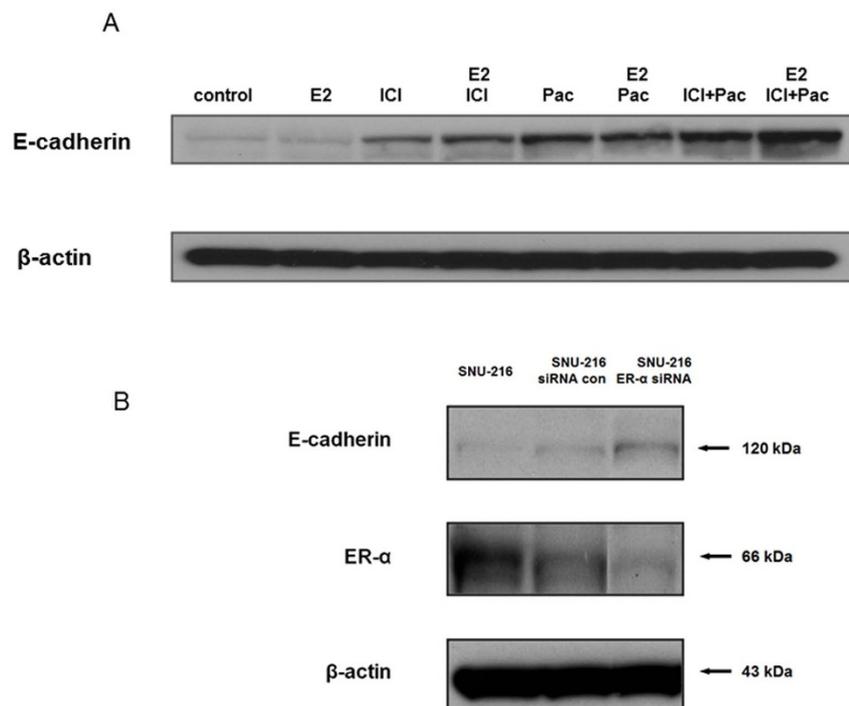


Figure 6 | E-cadherin expression following drug treatment or ER- α knockdown. (A), Drug treatment with ICI or paclitaxel or both enhanced E-cadherin expression; (B), transfection with ER- α siRNA caused increased expression of E-cadherin in SNU-216 cells, whereas transfection with control siRNA produced no observable effect).



pressors bind to the E-cadherin promoter and that overexpression of corepressors down-regulated E-cadherin in breast cancer cell lines²⁸. In the present study, suppressing ER- α with fulvestrant and siRNA resulted in increased E-cadherin levels. Because E-cadherin loss is not only involved in carcinogenesis but also in cancer invasion and metastasis, we hypothesize that restoring E-cadherin may have a beneficial influence on disease course. One of the motives that initiated this analysis was that we observed a considerable number of patients with diffuse-type GC. In our cohort, 65.4% were diffuse-type GC and in Asian trial, it is a quite common finding that diffuse-type GC being more predominant than intestinal-type^{42–44}. It becomes dramatically remarkable by an international AVAGAST trial, in which diffuse-type cancer predominated in Asian population while intestinal-type cancer predominates in European population⁴⁵. We think that there is an ethnic difference regarding the Lauren's classification and probably the incidence of ER expressing GC.

The present study has several limitations. Regarding the retrospective analysis, patients with metastatic disease were not included. If ER- α and its correlated E-cadherin are associated with tumor progression, patients with metastatic disease may show more frequent ER- α expression. The population of the current study is all Asians and interestingly, most studies dealing with this issue came from East Asia^{18,19,21–23}. Although there is one study dealing with this issue in Western population⁴⁶, ethnic difference should be investigated in the future. Also, as described above, our IHC method requires further validation. Although poorly differentiated histology was associated with more frequent ER- α expression (5.4%), the fact that the remaining 94.6% of the patients did not show ER- α expression warrants further investigation to elucidate the pathogenesis of this disease. For the *in vitro* analysis, we used the methods described in Kameda's study²⁵. Thus, fixed concentrations of E2 and fulvestrant were used throughout the analyses. However, E2 acts via 2 pathways: via the estrogen receptor (the genomic pathway) and via transcriptional cross-talk (the non-genomic pathway). We have only focused on the genomic pathway; the role of the non-genomic pathway should be clarified by further studies. At last, efficacy of paclitaxel in this subset should be investigated, as this agent demonstrated complete inhibition of cellular proliferation in colony-forming assay.

The present study implies that some portion of GC patients express ER- α and that they have distinct clinicopathologic features. Whether ER- α is simply another prognostic factor, or whether it may act as a therapeutic target, similar to HER-2, in GC patients require further investigation, including prospective clinical trials.

- Jemal, A. *et al.* Global cancer statistics. *CA Cancer J Clin* **61**, 69–90 (2011).
- Jung, K. W. *et al.* Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2008. *Cancer Res Treat* **43**, 1–11 (2011).
- Tanaka, M. *et al.* Trends of stomach cancer mortality in Eastern Asia in 1950–2004: comparative study of Japan, Hong Kong and Singapore using age, period and cohort analysis. *Int J Cancer* **130**, 930–936 (2012).
- Cunningham, D. *et al.* Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* **355**, 11–20 (2006).
- Van Cutsem, E. *et al.* Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* **24**, 4991–4997 (2006).
- Al-Batran, S. E. *et al.* Phase III trial in metastatic gastroesophageal adenocarcinoma with fluorouracil, leucovorin plus either oxaliplatin or cisplatin: a study of the Arbeitsgemeinschaft Internistische Onkologie. *J Clin Oncol* **26**, 1435–1442 (2008).
- Bang, Y. J. *et al.* Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* **376**, 687–697 (2010).
- Ohtsu, A. *et al.* Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study. *J Clin Oncol* **29**, 3968–3976 (2011).
- Lauren, P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* **64**, 31–49 (1965).
- Ribeiro, M. M., Sarmiento, J. A., Sobrinho Simoes, M. A. & Bastos, J. Prognostic significance of Lauren and Ming classifications and other pathologic parameters in gastric carcinoma. *Cancer* **47**, 780–784 (1981).
- Kunz, P. L. *et al.* Long-term survivors of gastric cancer: a California population-based study. *J Clin Oncol* **30**, 3507–3515 (2012).
- Guilford, P. *et al.* E-cadherin germline mutations in familial gastric cancer. *Nature* **392**, 402–405 (1998).
- Carneiro, F. *et al.* Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening. *J Pathol* **203**, 681–687 (2004).
- Barber, M. *et al.* Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer. *J Pathol* **216**, 295–306 (2008).
- Lindblad, M., Ye, W., Rubio, C. & Lagergren, J. Estrogen and risk of gastric cancer: a protective effect in a nationwide cohort study of patients with prostate cancer in Sweden. *Cancer Epidemiol Biomarkers Prev* **13**, 2203–2207 (2004).
- Freedman, N. D. *et al.* Menstrual and reproductive factors and gastric cancer risk in a large prospective study of women. *Gut* **56**, 1671–1677 (2007).
- Chandanos, E. *et al.* Tamoxifen exposure and risk of oesophageal and gastric adenocarcinoma: a population-based cohort study of breast cancer patients in Sweden. *Br J Cancer* **95**, 118–122 (2006).
- Tokunaga, A. *et al.* Hormone receptors in gastric cancer. *Eur J Cancer Clin Oncol* **19**, 687–689 (1983).
- Yokozaki, H. *et al.* Estrogen receptors in gastric adenocarcinoma: a retrospective immunohistochemical analysis. *Virchows Arch A Pathol Anat Histopathol* **413**, 297–302 (1988).
- Harrison, J. D., Jones, J. A., Ellis, I. O. & Morris, D. L. Oestrogen receptor D5 antibody is an independent negative prognostic factor in gastric cancer. *Br J Surg* **78**, 334–336 (1991).
- Matsui, M., Kojima, O., Kawakami, S., Uehara, Y. & Takahashi, T. The prognosis of patients with gastric cancer possessing sex hormone receptors. *Surg Today* **22**, 421–425 (1992).
- Xu, C. Y. *et al.* Prognostic role of estrogen receptor alpha and estrogen receptor beta in gastric cancer. *Ann Surg Oncol* **17**, 2503–2509 (2010).
- Ryu, W. S. *et al.* Expression of estrogen receptors in gastric cancer and their clinical significance. *J Surg Oncol* **106**, 456–461 (2012).
- Takano, N. *et al.* Expression of estrogen receptor-alpha and -beta mRNAs in human gastric cancer. *Cancer Lett* **176**, 129–135 (2002).
- Kameda, C. *et al.* Oestrogen receptor-alpha contributes to the regulation of the hedgehog signalling pathway in ERalpha-positive gastric cancer. *Br J Cancer* **102**, 738–747 (2010).
- Park, S. H., Cheung, L. W., Wong, A. S. & Leung, P. C. Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Mol Endocrinol* **22**, 2085–2098 (2008).
- Helguero, L. A. *et al.* Different roles of estrogen receptors alpha and beta in the regulation of E-cadherin protein levels in a mouse mammary epithelial cell line. *Cancer Res* **68**, 8695–8704 (2008).
- Oesterreich, S. *et al.* Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. *Cancer Res* **63**, 5203–5208 (2003).
- Humar, B. *et al.* E-cadherin deficiency initiates gastric signet-ring cell carcinoma in mice and man. *Cancer Res* **69**, 2050–2056 (2009).
- Di Leo, A. *et al.* Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. *J Clin Oncol* **28**, 4594–4600 (2010).
- Stabile, L. P. *et al.* Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen. *Cancer Res* **62**, 2141–2150 (2002).
- Pathologists' Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. *Breast Care (Basel)* **5**, 185–187 (2010).
- Dubey, S., Siegfried, J. M. & Traynor, A. M. Non-small-cell lung cancer and breast carcinoma: chemotherapy and beyond. *Lancet Oncol* **7**, 416–424 (2006).
- Hiasa, Y. *et al.* Immunohistochemical analysis of estrogen receptors in 313 paraffin section cases of human thyroid tissue. *Oncology* **50**, 132–136 (1993).
- Gupta, P. *et al.* Expression and clinicopathological significance of estrogen and progesterone receptors in gallbladder cancer. *Gastrointest Cancer Res* **5**, 41–47 (2012).
- Stabile, L. P. *et al.* Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced antiproliferative effects. *Cancer Res* **65**, 1459–1470 (2005).
- Siegfried, J. M., Gubish, C. T., Rothstein, M. E., Henry, C. & Stabile, L. P. Combining the multitargeted tyrosine kinase inhibitor vandetanib with the antiestrogen fulvestrant enhances its antitumor effect in non-small cell lung cancer. *J Thorac Oncol* **7**, 485–495 (2012).
- Chandanos, E. *et al.* Endogenous estrogen exposure in relation to distribution of histological type and estrogen receptors in gastric adenocarcinoma. *Gastric Cancer* **11**, 168–174 (2008).
- Ikeda, H. *et al.* Combination treatment with fulvestrant and various cytotoxic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) has a synergistic effect in estrogen receptor-positive breast cancer. *Cancer Sci* **102**, 2038–2042 (2011).



40. Tokuda, E. *et al.* Estrogen receptor- α directly regulates sensitivity to paclitaxel in neoadjuvant chemotherapy for breast cancer. *Breast Cancer Res Treat* **133**, 427–436 (2012).
41. Corso, G. *et al.* Somatic mutations and deletions of the E-cadherin gene predict poor survival of patients with gastric cancer. *J Clin Oncol* **31**, 868–875 (2013).
42. Sakuramoto, S. *et al.* Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* **357**, 1810–1820 (2007).
43. Koizumi, W. *et al.* S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol* **9**, 215–221 (2008).
44. Lee, J. *et al.* Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *J Clin Oncol* **30**, 268–273 (2012).
45. Van Cutsem, E. *et al.* Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. *J Clin Oncol* **30**, 2119–2127 (2012).
46. Harrison, J. D., Morris, D. L., Ellis, I. O., Jones, J. A. & Jackson, I. The effect of tamoxifen and estrogen receptor status on survival in gastric carcinoma. *Cancer* **64**, 1007–1010 (1989).

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Author contributions

J.Y. and J.J. wrote the main manuscript text. J.J. carried out *in vitro* analysis and prepared figure 3–6. I.D. and K.K. carried out immunohistochemical analysis, and prepared figure 1. S.K., S.P., J.P. and Y.P. carried out data collection, statistical analysis, and prepared figure 2. W.K. carried out interpretation of *in vitro* analysis. J.Y., J.L. and H.L. designed the study. All the authors reviewed the manuscript.

Additional information

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