



The endothelin A receptor and epidermal growth factor receptor signaling converge on β -catenin to promote ovarian cancer metastasis

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ARTICLE INFO

Article history:

Received 2 November 2011

Accepted 8 March 2012

Keywords:

Ovarian cancer

Beta-catenin

Metastasis

Endothelin A receptor

Epidermal growth factor receptor

ABSTRACT

Aims: Endothelin A receptor (ET_AR) and epidermal growth factor receptor (EGFR) cross-talk enhances the metastatic potential of epithelial ovarian cancer (EOC) cells activating different pathways, including β -catenin signalling. Here, we evaluated β -catenin as one of ET_AR/EGFR downstream pathway in the invasive behaviour of EOC cells and their therapeutic potential to co-target ET_AR and EGFR.

Main methods: The phosphorylation status and interactions of different proteins were analysed by immunoblotting and immunoprecipitation. Reporter activity and RT-PCR was used for evaluation of β -catenin transcriptional activity and gene expression. Functional effects were evaluated by gelatin zymography and cell invasion assays. An orthotopic model of metastatic human EOC in mice was used for in vivo studies.

Key findings: In EOC cell lines, ET-1 induced Src-dependent EGFR transactivation, causing tyrosine (Y) phosphorylation of β -catenin at the residue Y654, its dissociation from E-cadherin complexes and the accumulation as an active form. This pool of Tyr- β -catenin relocalised to the nucleus promoting its transcriptional activity, and the expression of its target genes, such as MMP-2. At functional level, ET-1 and EGFR circuits enhanced protease activity and cell invasion. All these effects were significantly inhibited by the ET_AR antagonist, zibotentan, or EGFR inhibitor, gefitinib, and are completely blocked by co-addition of both drugs. In vivo, zibotentan treatment significantly inhibited metastases, associated with reduced expression and activation of MMPs and active β -catenin, especially when combined with gefitinib.

Significance: Altogether these findings provide additional support to the potential use of ET_AR and EGFR blockade as a new therapeutic opportunity for EOC treatment.

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Introduction

Interference with endothelin-1 (ET-1) signalling pathway has emerged as a promising strategy for cancer therapy, as this pathway is frequently activated in a variety of human malignancies, including epithelial ovarian carcinoma (EOC) (Bagnato and Rosanò, 2008; Nelson et al., 2003). Actually, ET-1 and the highly specific endothelin A (ET_A) receptor, two members of the endothelin family, have been reported to induce pleiotropic effects in EOC cells, including cell proliferation, survival, angiogenesis, and invasive activity (Bagnato et al., 2005). Downstream ET_AR activation, ET-1 triggers the activation of various signaling pathways, including Src family tyrosine kinases, and epidermal growth factor receptor (EGFR) transactivation (Vacca et al., 2000; Rosanò et al., 2007a). In particular, sustained autocrine ET-1/ET_AR signalling contributes to induction of epithelial-to-mesenchymal transition (EMT) (Rosanò et al., 2005), a key process

in the development of an aggressive cell phenotype enabling tumour cells to invade and metastasise (Polyak and Weinberg, 2009). Usually, the β -catenin turnover is regulated in the cytosol by a large protein complex including axin, and glycogen synthase kinase-3 β (GSK3 β), that prevents its nuclear translocation and promotes its degradation (Nusse, 2005). In this context, ET-1 can drive inhibition of GSK-3 β to stabilise β -catenin proteins to engage transcriptional programs that control EMT and increased cell invasion (Rosanò et al., 2005).

Recent studies indicate that β -catenin is a target for various tyrosine kinases, including EGFR, and its tyrosine phosphorylation may disrupt the core complex with E-cadherin and engage β -catenin in its transcriptional activity (Lilien and Balsamo, 2005; Piedra et al., 2003; Roura et al., 1999). In particular, phosphorylation of tyrosine β -catenin residue Y654 results in its release from E-cadherin and an increase in TCF-mediated transcriptional activity (Zeng et al., 2006). As such, enhanced Y654 phosphorylation of β -catenin is believed to increase cell migration and induce an invasive behaviour of tumour cells (Muller et al., 1999), although functional evidence for the latter is still lacking.

Previous studies showed that in EOC cells ET_AR cooperate with EGFR to promote mitogenic and invasive signalling pathways (Rosanò et al., 2007a). Thus, in these cells, β -arrestin-1 is recruited to ET_AR to form a

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trimeric complex with Src upon ET-1 stimulation (Rosanò et al., 2009). This complex leads to EGFR transactivation and downstream activation of Akt and mitogen-activated protein kinase (MAPK), and β -catenin tyrosine phosphorylation, thereby mobilising the fraction of β -catenin that translocates to the nucleus. In parallel and coordinated manner, β -arrestin-1/ET_AR forms a complex with axin, contributing to inactivate GSK-3 β and to stabilise β -catenin (Rosanò et al., 2009). This is consistent with previous observations that demonstrate that ligand-dependent activation of different G-protein coupled receptor (GPCR), such as prostaglandin E(2) or obetastin, induces Akt phosphorylation via EGFR and MMP activity, through a mechanism involving the association with Src and β -arrestin-1 (Buchanan et al., 2006; Alvarez et al., 2009). The critical role of ET-1 axis in the nuclear translocation of β -catenin led us to investigate the functional connection between ET_AR/EGFR cross-talk and the β -catenin signalling in order to dissect the molecular mechanisms following ET_AR activation in invasion and metastasis, and to test in vivo the therapeutic efficacy by co-targeting ET_AR and EGFR in this tumour.

Materials and methods

Materials

Clinical grade zibotentan, N-(3-methoxy-5-methylpyrazin-2-yl)-2-(4-[1,3,4-oxadiazol-2-yl]phenyl)pyridine-3-sulfonamide, and, gefitinib, 4-quinazolinamine, N-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(4-morpholin propoxy)] were kindly provided by AstraZeneca (Macclesfield, UK). ET-1 was purchased from Peninsula Laboratories (Belmont, CA, USA).

Cell culture

Human ovarian carcinoma cell line HEY and OVCA 433 were generously provided by Prof. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). The SKOV-3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI containing 10% foetal calf serum (FCS) and 1% penicillin-streptomycin at 37 °C under 5% CO₂-95% air. Cells were serum-starved by incubation for 24 h in serum-free RPMI. All culture reagents were from Life Technologies Ltd (Paisley, UK).

Luciferase reporter gene assay

To measure the transcriptional activity of β -catenin, 3×10^5 cells/well were transiently cotransfected using LipofectAMINE reagent (Life Technologies Ltd) with 1 μ g pTOP/Flash (Millipore, Milan, Italy) and 100 ng pCMV- β -galactosidase (Promega Italia, Milan, Italy) vectors. Reporter activity was measured using the Luciferase assay system (Promega Italia) and normalised to β -galactosidase activity. The mean of five independent experiments performed in sextuplicate was reported.

Immunoblotting and immunoprecipitation

NE-PER nuclear and cytoplasmic extraction reagents Fisher Scientific (Illkirch Cedex, France) were used to separate cytoplasmic and nuclear fractions. For Western blotting analysis, whole cell lysates or nuclear fractions or homogenised HEY tumour specimens were resolved by SDS-PAGE, followed by immunoblotting (IB) using Ab to: anti-phosphotyrosine (PY-20), E-cadherin (BD Becton Dickinson Italia, Buccinasco (MI), Italy), EGFR, phospho-EGFR (Tyr845), phospho-GSK-3 β (pSer9), GSK-3 β , active- β -catenin (Millipore), β -catenin, matrix metalloproteinase-2 (MMP-2), MMP-9, β -actin (Oncogene Research Products, La Jolla, CA), PCNA (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti- β -catenin (Y86), anti- β -catenin (Y142), anti- β -catenin (Y654) (Abcam, Cambridge, UK). For immunoprecipitation (IP),

precleared whole cell lysates were immunoprecipitated with anti- β -catenin insolubilised on protein A-Sepharose CL-4B (GE Healthcare, Milan, Italy). Then, immunoprecipitates were loaded on a 7.5% or 10% SDS-PAGE and then analysed by SDS/PAGE and IB with different Abs as before. Blots were developed with the enhanced chemiluminescence detection system (ECL; GE Healthcare).

RT-PCR

The primers sets were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GTGAAGGTCGGAGTCAACG-3' and 5'-GGTGAAGACGCCAGTGGACTC-3'; MMP-2, 5'-GGATGATGCCTTTGCTCG-3' and 5'-ATAGGATGTGCCTGGAA-3'; Cyclin D1, 5'-CTAATGGAATGGT-TTGGGAATATCCATGTA-3' and 5'-AAAGGAACATATCATCCTGCGCAAT-3'. The cDNA was amplified for 28–35 cycles at 94 °C for 45 s for denaturation step, at specific melting temperature for 45 s for primer annealing step and for extension step at 72 °C for 45 s (Life Technologies Ltd.). The PCR was conducted in the automated DNA Thermal Cycler GeneAmp PCR System 2400 (Bio-Rad Laboratories S.r.l., Milan, Italy). The PCR products were analysed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Gelatin zymography and MMP activity

Conditioned media from HEY cells or homogenised HEY metastases specimens were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/ml gelatin, as previously described (Rosanò et al., 2007b). Briefly, the gels were washed for 30 min at 22 °C in 2.5% Triton X-100 and then incubated in 50 mM Tris-HCl (pH 7.6), 1 mM ZnCl₂, and 5 mM CaCl₂ for 18 h at 37 °C. After incubation the gels were stained with 0.2% Coomassie blue.

Gelatinase activities in conditioned media were also determined by a MMP gelatinase activity assay kit (Millipore) according to the manufacturer's instructions. The sensitivity of the assay is <5 ng/ml MMP in the range 10 to 200 ng/ml. The mean of six independent experiments performed in duplicate was reported.

Chemoinvasion assay

Chemoinvasion assay was performed as previously described (Rosanò et al., 2005). The filters were coated with an even layer of 10 mg/ml Cultrex Basement Membrane Extract Matrigel (Trevigen, Gaithersburg, MD). After 6 h of incubation at 37 °C, the filters were removed, stained with Diff-Quick (Dade Behring Inc., Newark, NJ, USA) and the migrated cells in 10 high-power fields were counted. The mean of six independent experiments performed in triplicate was reported.

Metastasis assay

HEY cells (1.8×10^6) were i.p. injected into female athymic nude mice (Charles River Laboratories, Milan, Italy), following the guidelines for animal experimentation of the Italian Ministry of Health. In all experiments, each group consisted of 10 mice. One week after injection of cancer cells, one group was treated i.p. for 21 days with zibotentan (diluted in PBS) (10 mg/kg/day), one group was treated for 21 days p.o. with gefitinib (125 mg/kg/day, diluted in polyethylene glycol 400), and one group was treated with zibotentan in combination with gefitinib. Control animals received the same volume of polyethylene glycol 400 p.o. as those receiving gefitinib and were injected with an equal volume of saline solution as animals receiving zibotentan. At the end of the treatment, mice were sacrificed; the number of metastases was counted and the removed tumours were weighed, carefully dissected and snap frozen for IB analysis. All metastases from three independent experiments were included for final calculation of number of metastatic nodules.

Statistical analysis

Data from each experimental group are expressed as the mean \pm standard deviation (SD). Multiple group comparisons were performed by one-way analysis of variance (ANOVA), followed by Bartlett's test and Kolmogorov and Smirnov Comparisons Test using GraphPad InStat version 3.0 for Macintosh (GraphPad Software, San Diego California, USA). *P*-values <0.05 were considered statistically significant.

Results

The ET_AR/EGFR cross-talk is an upstream event in ET-1-induced β -catenin tyrosine phosphorylation

In this study we analysed the role of ET-1 on β -catenin tyrosine phosphorylation in SKOV3, HEY, OVCA 433 ovarian cancer cells. In agreement with previous data (Vacca et al., 2000; Rosanò et al., 2007a, 2009), we found that, also in SKOV3 cells, exogenous ET-1 induced a rapid EGFR transactivation on tyrosine 845 (Fig. 1A). Zibotentan, a specific ET_AR antagonist, or gefitinib, an EGFR inhibitor, or PP2, a Src inhibitor, reduced the ET-1-induced EGFR activation, whereas combination of zibotentan plus gefitinib completely inhibited EGFR phosphorylation, confirming that a dual inhibition of EGFR and ET_AR pathways is more effective in controlling the activation of the EGFR pathway (Fig. 1A). Next, we investigated the putative role of EGFR in the ET-1-elicited tyrosine phosphorylation of β -catenin. The treatment with ET-1 for 15 min resulted in a dose-dependent tyrosine

phosphorylation of β -catenin with a maximum at the dose of 100 nM (Fig. 1B). The pattern of tyrosine residue phosphorylation of β -catenin has been shown to be kinase specific. Activation of tyrosine kinase FYN, FER and MET leads to phosphorylation at Y142, whereas Y654 or Y86 can be phosphorylated directly by EGFR (Lilien and Balsamo, 2005). Therefore, we investigated which tyrosine residues could be phosphorylated by ET-1 treatment. Since three different tyrosine residues, Y86, Y142 and Y654, play an important role in β -catenin phosphorylation-mediated stability and transcriptional activity, immunoblotting analysis was then performed using specific anti-phosphotyrosine Abs. Interestingly, we observed an enhanced level of tyrosine phosphorylation using anti-Y654 β -catenin Abs in all three cell lines (Fig. 1C), whereas no signal was observed using Abs recognising Y86 or Y142 (data not shown), indicating that Y654 tyrosine residue may be preferentially phosphorylated upon ET-1 treatment. Moreover, in all cell lines, ET-1 promoted the accumulation of active form of β -catenin, demonstrating a functional link between the increased Y-phospho β -catenin and the active β -catenin in ET-1-treated cells (Fig. 1D), thus representing a transcriptional active pool.

As shown in Fig. 1E, the specific tyrosine phosphorylation of β -catenin induced by ET-1 was inhibited by zibotentan, as well as gefitinib, treatment and a further inhibition was found in combined treatment, indicating that Y654 tyrosine residue may be preferentially phosphorylated by Src-dependent EGFR transactivation induced by ET-1. It is noteworthy that both tyrosine phosphorylation and inhibition of GSK-3 β -dependent serine/threonine phosphorylation are involved in β -catenin accumulation and transcriptional activation, whereas β -catenin serine/threonine phosphorylation is required for its degradation (Barker, 2008). According

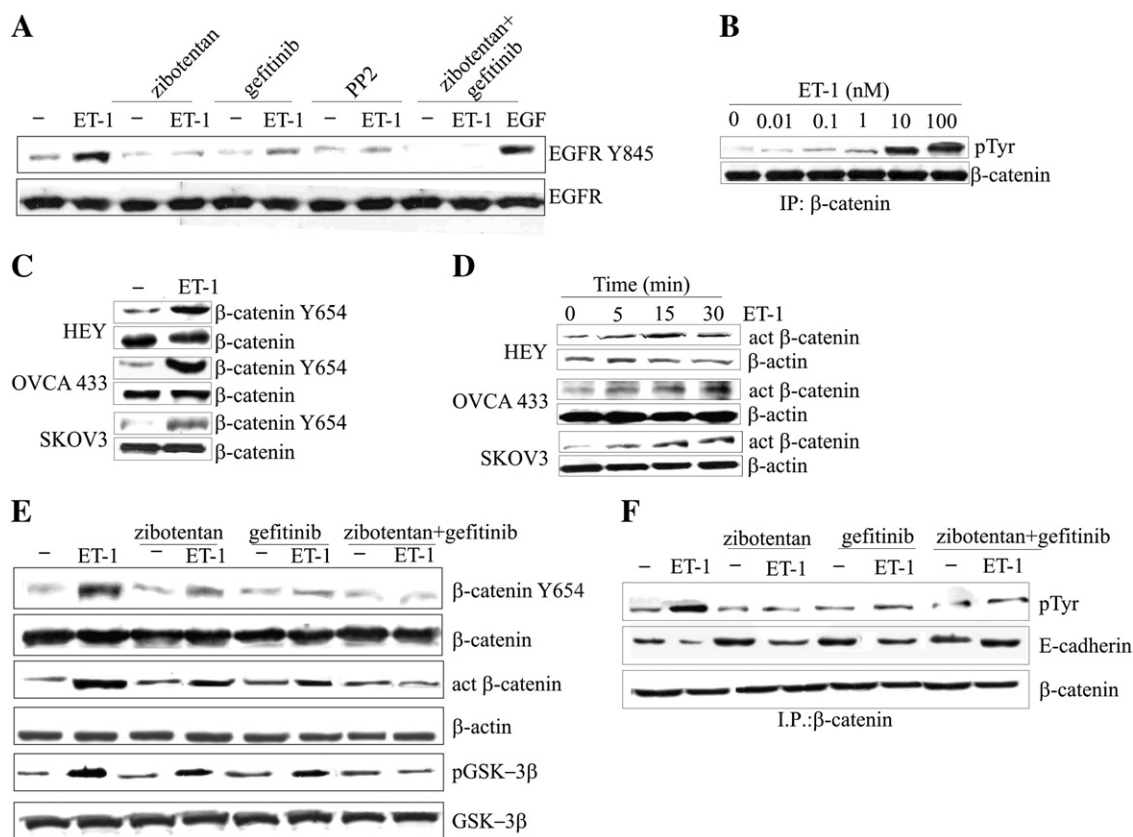


Fig. 1. ET-1 induces β -catenin tyrosine phosphorylation in EOC cells. **A.** Lysates of SKOV3 cells treated with 10 ng/ml EGF, or 100 nM ET-1, and/or 1 μ M zibotentan, or 1 μ M gefitinib, or 1 μ M PP2, or combination of zibotentan and gefitinib, were analysed by IB with anti-Y845-EGFR and anti-EGFR (*n* = 3). **B.** Lysates of SKOV3 cells treated for the indicated doses of ET-1 for 5 min were immunoprecipitated (IP) with anti- β -catenin and immunoblotted (IB) with anti-pTyr. The membranes were re-probed with anti- β -catenin (*n* = 3). **C.** Lysates of HEY, OVCA 433, and SKOV3 cells treated for 15 min with ET-1 (100 nM) were IB with anti-Y654- β -catenin and anti- β -catenin (*n* = 3). **D.** Lysates of HEY, OVCA 433 and SKOV3 cells treated with 100 nM ET-1 for indicated times were IB with an anti-non-serine-threonine phosphorylated, nonubiquitinated, active (act) β -catenin and with anti- β -actin (*n* = 3). **E.** Lysates of HEY cells treated for 5 min with 100 nM ET-1 and/or 1 μ M zibotentan, or 1 μ M gefitinib, or zibotentan and gefitinib were IB with anti-Y654- β -catenin, anti- β -catenin, with anti-act β -catenin, anti-pGSK-3 β (Ser 9), anti-GSK-3 β and with anti- β -actin (*n* = 3). **F.** Lysates of HEY cells treated as in C were IP with anti- β -catenin and IB with anti-pTyr and anti-E-cadherin. The membranes were re-probed with anti- β -catenin (*n* = 3).

with the above results, the ET-1-induced accumulation of active β -catenin was inhibited by zibotentan, as well as by gefitinib, and the combined treatment led to a significantly greater degree of inhibition than single drug (Fig. 1E). Moreover, the dual treatment completely inhibited the ET-1-induced GSK-3 β phosphorylation and inactivation, blocking the degradative pathway. Altogether these results confirmed that the activation of EGFR by ET-1 is sufficient to induce the accumulation of β -catenin by concomitant tyrosine phosphorylation and inhibition of its degradative pathway, and that a combined inhibition of ET_AR and EGFR pathways is more effective in controlling these effects.

Previous studies have documented that β -catenin tyrosine phosphorylation causes a lowering of its affinity for E-cadherin and changes in morphology and mobility (Hudson et al., 2008). By immunoprecipitation assay, we found that increased tyrosine phosphorylation of β -catenin in ET-1-treated cells was accompanied by reduced E-cadherin association with β -catenin compared with untreated cells, that was inhibited by zibotentan and gefitinib, and to a greater extent, by the combination of both (Fig. 1F). These results indicated the ability of ET-1 to induce the relocalisation of β -catenin from the E-cadherin-bound membrane by promoting its tyrosine phosphorylation and that EGFR activation is required for this event.

Effects of ET-1 on β -catenin transcriptional activity and cell invasion

Previous studies have described that β -catenin tyrosine phosphorylation may result in its relocalisation into the nucleus (Lilien and Balsamo, 2005). Therefore, we hypothesised that ET-1 could induce EGFR-dependent β -catenin nuclear distribution. As shown in Fig. 2A, tyrosine phosphorylated β -catenin is almost absent in the nucleus of untreated cells, while ET-1 stimulation promotes the accumulation of this form in the nuclear compartment. Moreover, the combined treatment with zibotentan and gefitinib completely inhibits this accumulation, suggesting that the ET-1-induced nuclear distribution of β -catenin is dependent of its EGFR-mediated tyrosine phosphorylation.

Next, we analysed whether nuclear Y-phospho β -catenin could exert a transcriptional function through its association with TCF4 transcription factor, regulating the transcription of important tumour-promoting genes, such as MMP-2, and cyclin D1 (Nusse, 2005). Therefore, we examined the effects of zibotentan and gefitinib on ET-1-induced β -catenin transcriptional activation. As shown in Fig. 2B, treatment with ET-1 specifically induced a significant increase in TCF/LEF transcriptional activity in both HEY

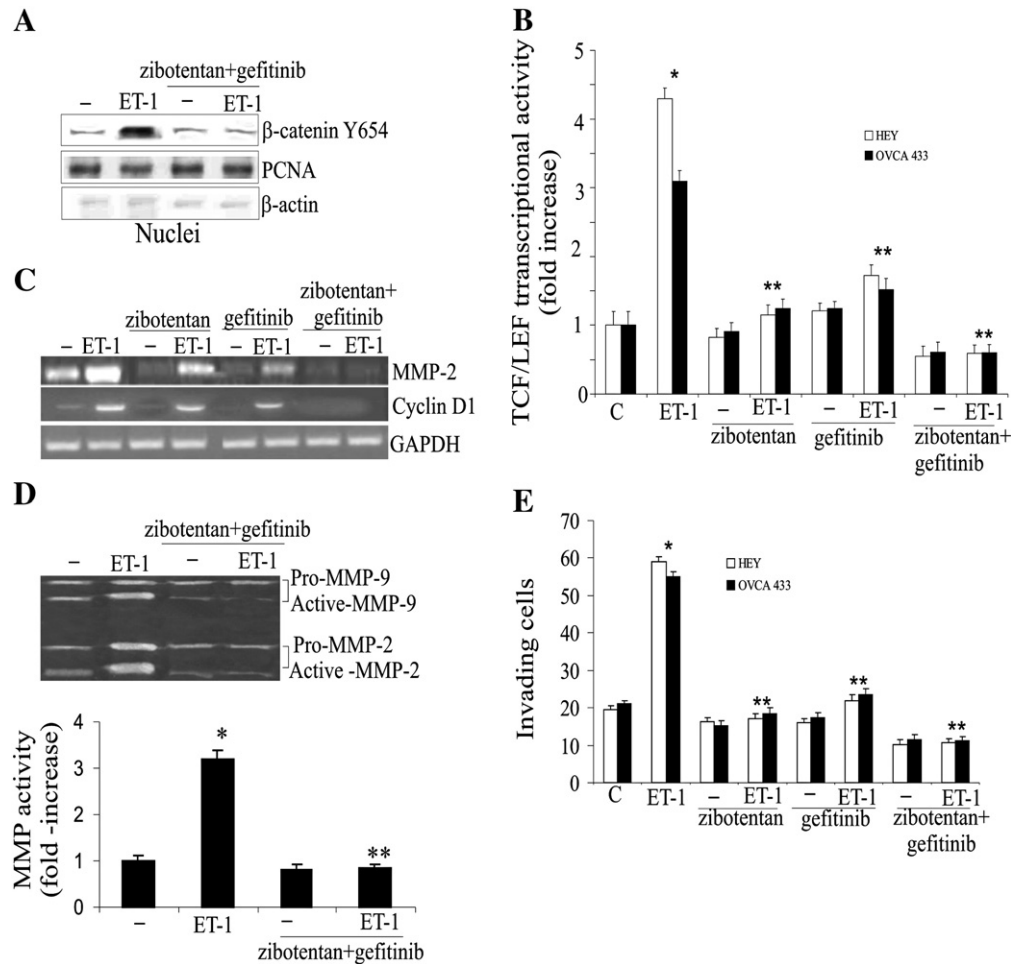


Fig. 2. ET_AR and EGFR cross-talk regulates β -catenin transcriptional activity and cell invasion. A. Nuclear extracts of HEY cells treated with ET-1 and/or gefitinib and zibotentan were IB with anti-Y-654- β -catenin, anti-PCNA, and anti- β -actin, used as nuclear and cytosolic controls, respectively ($n = 3$). B. β -catenin/TCF transcriptional activity was evaluated in EOC cells, transiently co-transfected with TOPFlash reporter construct and pCMV- β -galactosidase plasmid, incubated with ET-1 (100 nM), and/or 1 μ M zibotentan, and/or 1 μ M gefitinib, and/or zibotentan and gefitinib for 24 h. Data shown are means (SD) of 5 independent experiments; * $p < 0.001$ versus untreated cells (C); ** $p < 0.05$ versus ET-1-treated cells. C. MMP-2 and Cyclin D1 mRNA expression was analysed in HEY cells treated for 24 h as in B. by RT-PCR. GAPDH expression was used as loading control ($n = 3$). D. HEY cells were treated with ET-1 and/or zibotentan and gefitinib for 48 h. Gelatin zymography was used to determine the activities of MMP-2 and MMP-9. Lower. MMP activity was also measured by a MMP gelatinase activity assay kit. Data shown are means (SD) of 3 independent experiments; * $p < 0.001$ versus untreated cells; ** $p < 0.01$ versus ET-1-treated cells. E. HEY and OVCA 433 cells were treated with ET-1 (100 nM), and/or 1 μ M zibotentan, and/or 1 μ M gefitinib, and/or zibotentan and gefitinib and cell invasion was measured using an invasion assay. Data shown are means (SD) of 6 independent experiments, each done in triplicate; * $p < 0.001$ versus untreated cells (C); ** $p < 0.05$ versus ET-1-treated cells.

and OVCA 433 cells ($p < 0.001$ versus untreated cells). The stimulatory effect of ET-1 was significantly inhibited by pretreatment with zibotentan and gefitinib and further inhibitory effect was observed in combination treatment ($p < 0.05$ versus ET-1-treated cells). Moreover, RT-PCR confirmed that ET-1 treatment markedly increased the expression of MMP-2 and cyclin D1, which was partially blocked by pretreatment with zibotentan or gefitinib and at greater extent in combination of both, confirming the cross-talk between ET-1 and EGFR in induction of β -catenin signaling (Fig. 2C). Since the invasive activity of ET-1 in EOC cells is known to be exerted mainly through the expression and activation of proteolytic enzymes (Rosanò et al., 2001), including MMP-2 and MMP-9, which are target genes of β -catenin, we analysed whether zibotentan and gefitinib could exert anti-invasive effects through inhibition of MMP-2 and -9. As shown in Fig. 2D, both gelatin zymography and protease activity measurements demonstrated a significant increase in MMP-2 and MMP-9 activity in ET-1-stimulated HEY cells ($p < 0.001$ versus untreated cells), whereas pretreatment with zibotentan in combination with gefitinib reduced these effects ($p < 0.01$ versus ET-1-treated cells). Combined treatment significantly inhibited also ET-1 induced invasion of these cells (Fig. 2E), thus suggesting that concomitant blockade of the cross-talk between ET_A R and EGFR can control cellular motility by inhibiting β -catenin signalling.

Effects of combination treatment with zibotentan and gefitinib in metastatic potential of EOC cells

In order to investigate whether the combined regimen of zibotentan and gefitinib could inhibit metastatic dissemination, we used an orthotopic model of metastatic human ovarian carcinoma in athymic immunodeficient mice. This model corresponds to stage III ovarian cancer, which is the stage at which the majority of patients with ovarian cancer are first treated. We found a widespread dissemination of tumour cells in control mice, including multiple metastatic nodules throughout the peritoneum, stomach, duodenum, liver, spleen, and diaphragm (Fig. 3A).

Conversely, mean metastatic nodules were significantly reduced in the group treated with zibotentan or gefitinib ($p < 0.001$ versus mice treated with vehicle) (Fig. 3A). Moreover, the combined

regimen of zibotentan and gefitinib resulted in a further reduction in the number of metastatic nodules compared with single treatment ($p < 0.005$ versus mice treated with zibotentan or gefitinib) (Fig. 3A).

To analyse whether zibotentan and gefitinib could exert anti-metastatic effects also through inhibition of MMPs, we performed gelatin zymography analysis performed on HEY metastatic nodules of control and treated animals. According with reduction of the number of intra-abdominal metastases, the gelatinolytic activities of MMP-2 and -9 found in the control group was significantly reduced in the zibotentan-treated group and almost completely inhibited in the zibotentan plus gefitinib-treated group, (Fig. 3B), suggesting that the anti-invasive mechanism of zibotentan might mainly involve inhibition of MMP activation, that can be amplified through the combination with gefitinib. In parallel, Western blotting analyses on the same tissues confirmed a marked decrease of MMP expression in zibotentan-treated mice and especially in zibotentan plus gefitinib-treated mice compared with control (Fig. 3C).

Based on our previous results showing an ET_A R-dependent activation β -catenin pathway in promoting metastatic ability of ovarian (Rosanò et al., 2009), we also evaluated the pattern of expression of active β -catenin in the same metastatic nodules. We observed a strong decrease in the active β -catenin expression in the metastatic nodules derived from zibotentan-treated mice when compared to control, that reached almost complete loss of expression in the group of zibotentan plus gefitinib-treated mice (Fig. 3C). These results further support the role of ET_A R/EGFR cross-talk to promote the metastatic behaviour of ovarian cancer cells and harbour the efficacy of targeting both receptors to reduce cancer metastasis in vivo.

Discussion

Drug development strategies include the evaluation of new drug combinations of targeted agents, such as with small molecules, which may have improved efficacy compared with single agents. EOC is a leading cause of death from gynaecological malignancy (Jemal et al., 2007). Ovarian cancer metastases are frequently established by a unique microenvironmental niche comprised of tumour and inflammatory cells, along with a wide range of bioactive soluble factors, including ET-1 (Khan et al., 2010; Barbolina et al., 2009;

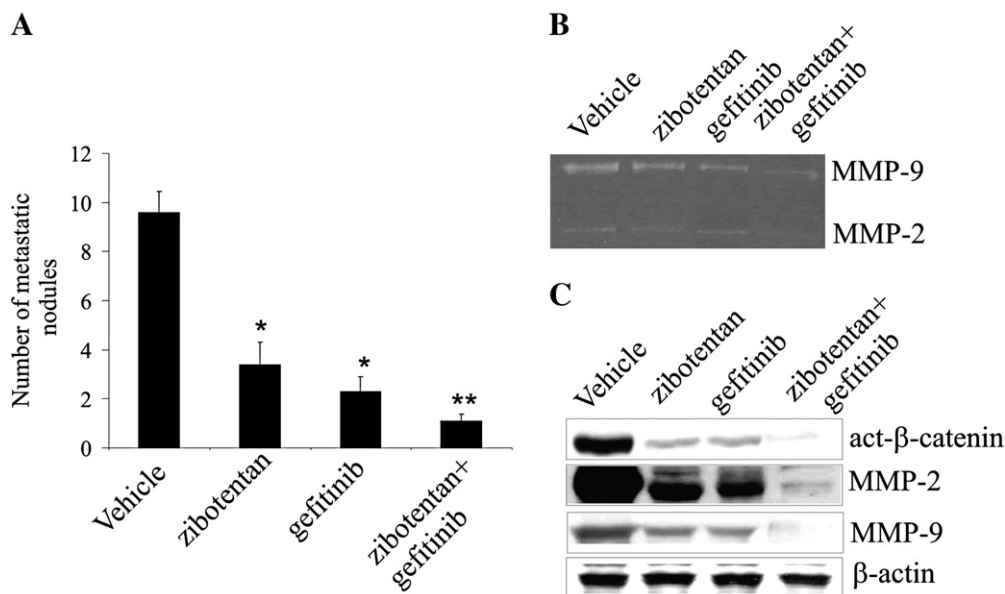


Fig. 3. Effect of zibotentan and gefitinib treatments on metastatic potential of ovarian cancer cells. A. HEY cells were injected i.p. into nude mice and one week after cell injection, mice were treated for 21 days with vehicle, or with zibotentan (10 mg/kg/day), or with gefitinib (125 mg/kg/day), or with a combination of zibotentan and gefitinib. Each group consists of 10 mice. Data shown are means (SD) of 5 independent experiments; * $p \leq 0.001$ versus mice treated with vehicle; ** $p < 0.005$ versus mice treated with zibotentan or gefitinib. B. Total lysates obtained from homogenised HEY tumour xenografts treated as in A were subjected to gelatin zymography ($n = 3$). C. Total lysates obtained from homogenised HEY tumour xenografts treated as in A were IB with anti-actin- β -catenin, anti-MMP-2, anti-MMP-9 and with anti- β -actin for internal control ($n = 3$).

Freedman et al., 2004; Salani et al., 2000). The available data about the role of ET-1/ET_AR axis in tumour progression and metastasis in preclinical studies suggests that modulating the activity of the ET_AR offers opportunity for targeted intervention in patients with metastatic disease (Rosanò et al., 2007a,b, 2009). In the present work, we describe a functional interaction between ET_AR and EGFR to promote the ET-1-elicited biological subroutines, involving β -catenin signaling through its tyrosine phosphorylation, and to sustain ovarian cancer invasive and metastatic growth, having significant implications for the control of metastatic ovarian cancer.

We provide evidences that, upon ET-1 stimulation, activated EGFR associates and phosphorylates β -catenin, which in turn initiates the dissociation from E-cadherin, its redistribution to the nucleus and, ultimately, the formation of the transcriptional active β -catenin/TCF4 complex. The activation of β -catenin signaling by the ET_AR/EGFR cross-talk is also responsible for the expression and activation of metastatic genes, such as MMP-2, and enhanced cell invasion. Finally, the concomitant blockade of ET_AR and EGFR *in vivo* completely reduces the number of metastasis, associated with downregulation of active β -catenin and MMP activity.

β -catenin is a pivotal molecule of the Wnt signalling pathway, involved in regulation of developmental and oncogenic processes as well as in intercellular adhesion. The oncogenic properties of β -catenin signaling stem from alteration in phosphorylation-dependent protein degradation and subcellular localisation of β -catenin from cell membrane to the nucleus, where it binds to TCF and facilitates transcription of target genes that encode effectors facilitating tumour progression. β -catenin complex with the cytoplasmic tail of E-cadherin is mostly regulated by phosphorylation of the Y654 tyrosine residue, which induces β -catenin dissociation from E-cadherin by several tyrosine kinase receptors, such as EGFR (Lilien and Balsamo, 2005; Brembeck et al., 2006). Most importantly, completely unrelated cell surface receptors, such as GPCRs, are able to activate tyrosine kinase receptors that, in turn, promote β -catenin tyrosine phosphorylation and subsequently translocation into the nucleus (Fischer et al., 2004), thereby influencing the invasive outcomes in cancer cells. These findings strongly suggest that EOC cells, as well as stromal cells, in the tumour microenvironment, by secreting ET-1, can activate EGFR expressed at the plasma membrane cells resulting in increased tyrosine phosphorylation of β -catenin, affecting its functions in cell adhesion and signalling. In particular, we found that ET-1-dependent phosphorylation of β -catenin residue Y654 by EGFR results in its release from E-cadherin and an increase in TCF-mediated transcriptional activity, suggesting that β -catenin Y654 phosphorylation balances its role between cell adhesion and Wnt signalling. Recently, data, obtained using a conditional knock-in mouse model in which the phospho-mimicking Y654E modification in the endogenous β -catenin gene was introduced, uncovered a new mechanism in which phosphorylation of β -catenin at Y654 introduces a conformational change affecting protein-protein interactions. This reduces its affinity for the adherens complex, while it is simultaneously more accessible for additional phosphorylation, which increases the recruitment of proteins involved in the transcription machinery (van Veelen et al., 2011). Moreover, we found that enhanced ET-1-induced Y654 phosphorylation of β -catenin is associated to increased invasive behaviour of tumour cells. Consistent with this, it has been demonstrated that MMP genes are under the influence of the transcriptional activity of β -catenin, as β -catenin responsive elements are reported to be present in the MMP genes (Munshi and Stack, 2006). Thus, ET_AR/EGFR related activation of β -catenin represents a central node of ET-1 axis where different signals converge to regulate metastatic process. Interestingly, our *in vitro* results showed that the inhibition of ET_AR and EGFR, with zibotentan or gefitinib, effectively blocked the ET-1-induced β -catenin tyrosine phosphorylation and transcriptional activity, and downstream cell invasion. Moreover, these activities were substantially abolished by simultaneous administration of zibotentan and gefitinib, further supporting the possible use of the

combination of targeted therapies in EOC, to block the interrelated ET_AR/EGFR pathway in the acquisition of invasive and metastatic phenotypes.

Previous preclinical studies focused to study ET_AR for targeted therapy in this tumour demonstrated that blockade of ET_AR with zibotentan impedes *in vitro* and *in vivo* the ET_AR-driven tumour cell proliferation, angiogenesis, invasion, and inhibition of apoptosis (Rosanò et al., 2003, 2007b). The coadministration of gefitinib enhanced the efficacy of zibotentan in a subcutaneous model of EOC, leading to partial or complete tumour regression, associated with decreased vascularisation, and expression of cancer progression related markers (Rosanò et al., 2007a). Here, to better define the antimetastatic ability of zibotentan in combination with gefitinib in human EOC, we conducted this study using the orthotopic model of metastatic human EOC. This is the first report showing that the concomitant blockade of ET_AR and EGFR inhibited tumour cells from disseminating to internal organs to a greater degree than single-agent therapy. Strikingly, this combination is also effective against the proteolytic activity and the invasiveness of ovarian tumours.

At translation level, a novel and promising cancer treatment strategy could be the incorporation of ET_AR blockade with other molecular-targeted drugs to therapeutically overcome compensatory mechanisms of escape, which could provide an advantage to metastatisation.

Conclusions

Clinical and experimental studies have identified the final step of ovarian cancer progression, metastatic colonisation of peritoneum, as a tractable therapeutic target (Barbolina et al., 2009; Khan et al., 2010). Molecular characterisation of ovarian cancer has led to the identification of potential molecular targets for new treatments, including ET_AR. Since the results of the clinical trials of zibotentan as a monotherapy and in combination with cytotoxic drugs to interrupt the ET-axis has met with mixed levels of success, in which the promising delay in disease progression did not translate into an overall-survival benefit (Bagnato et al., 2011), the discovery of new biologic co-targeted agents is an exciting new chapter in the treatment of gynecologic malignancies. Based on the cooperative antimetastatic activity of ET_AR and EGFR inhibitors in our preclinical model, we suggest that substances targeting these systems should be considered as a new tool for treatment of ovarian cancer.

Conflict of interest statement

None.

Acknowledgments

We gratefully acknowledge Valentina Caprara and Aldo Lupo for excellent study assistance, Maria Vincenza Sarcone for secretarial assistance. This study was funded in part by Associazione Italiana Ricerca sul Cancro and Italian Ministry of Health.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.lfs.2012.03.023>.

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