Acquisition of Chemoresistance and EMT Phenotype Is Linked with Activation of the Endothelin A Receptor Pathway in Ovarian Carcinoma Cells

Laura Rosanò1, Roberta Cianfrocca1, Francesca Spinella1, Valeriana Di Castro1, Maria Rita Nicotra2, Alessandro Lucidi3, Gabriella Ferrandina3, Pier Giorgio Natali1, and Anna Bagnato1

Abstract

Purpose: Emerging evidence suggests molecular and phenotypic association between chemoresistance and epithelial–mesenchymal transition (EMT) in cancer. Endothelin-1 (ET-1)/endothelin A receptor (ETAR) axis is implicated in the pathobiology of epithelial ovarian cancer (EOC) by driving tumor-promoting effects, including EMT. Here, we analyzed how ETAR regulates chemoresistance and EMT in EOC.

Experimental Design: The effects of ET-1 axis on cell proliferation, drug-induced apoptosis, invasiveness, and EMT were analyzed in cultured EOC cells sensitive and resistant to cisplatinum and taxol. Tumor growth in response to ETAR antagonist was examined in EOC xenografts. ETAR expression was examined in 60 human EOC tumors by immunohistochemistry and correlated with chemoresistance and EMT.

Results: In resistant EOC cells ET-1 and ETAR are upregulated, paralleled by enhanced mitogen activated protein kinase (MAPK) and Akt phosphorylation and cell proliferation. Moreover, in these cells the expression of E-cadherin transcriptional repressors, including Snail, Slug, and Twist, as well as of mesenchymal markers, such as vimentin and N-cadherin, were upregulated and linked with enhanced invasive behavior. Interestingly, ETAR blockade with zibotentan, a specific ETAR antagonist, or its silencing, downregulated Snail activity, restored drug sensitivity to cytotoxic-induced apoptosis, and inhibited the invasiveness of resistant cells. In vivo, zibotentan inhibited tumor growth of sensitive and resistant EOC xenografts, and sensitized to chemotherapy. Analysis of EOC human tissues revealed that ETAR is overexpressed in resistant tumors and is associated with EMT phenotype.

Conclusions: Our data provide the first evidence that blockade of ETAR-driven EMT can overcome chemoresistance and inhibit tumor progression, improving the outcome of EOC patients’ treatment.

Introduction

Ovarian cancer accounts for the highest tumor-related mortality in women with gynaecologic malignancy (1). The identification of the molecular mechanisms underlying chemoresistance is mandatory to achieve advancement in ovarian cancer therapy (2). Accumulating evidences demonstrated that epithelial–mesenchymal transition (EMT), which modulates cancer progression and metastasis, has also been implicated in the onset of drug resistance and tumor relapses, representing an escape mechanism from apoptosis (3). Therefore, the acquisition of mesenchymal phenotypes engenders tumor cells with a multifaceted capacity to proliferate, migrate, and avoid cell death and permanent arrest, as well as protection from extracellular signals and drug effect activities (4). The hallmark of EMT is loss of the epithelial molecule E-cadherin and gain of mesenchymal markers, such as N-cadherin and vimentin. The E-cadherin repressors, Snail and Slug, which interact with E-box elements located within the proximal region of the E-cadherin promoter, and the basic helix-loop-helix transcription factor Twist, are significant inducers of EMT in cancer cells by repressing E-cadherin expression (5). Several clinical studies have shown that increased expression of E-cadherin is associated with improved survival in several tumor types (6, 7), and silencing of E-cadherin transcriptional suppressors can increase cellular sensitivity to genotoxic stress (8). Since EMT development is driven by key modulators that are directly controlled by numerous extracellular signals and pathways (5), it is becoming clear that the blockade of these signaling pathways is critical for...
Overcoming chemoresistance is the major challenge in treating epithelial ovarian cancer (EOC). Our study well explains the effect of endothelin-1 (ET-1) axis on the acquisition of chemoresistance and epithelial–mesenchymal transition (EMT) phenotype and provides possible strategies in clinical oncotherapy. In a combined in vitro/in vivo model of EOC cells resistant to taxol and cisplatinum, we revealed that ET-1 triggers major tumorogenic signals, including the activation of the EMT-driver Snail. Endothelin A receptor (ETAR) blockade by a specific ET_{\alpha}R antagonist results into reverted EMT, restored drug sensitivity, and inhibited cell invasion. Analysis of human EOC tissues validated the preclinical results revealing that ET_{\alpha}R is overexpressed in the chemoresistant tumors and is associated with EMT marker expression. In summary, this study unravels opportunities to interfere with major signals involved in the chemoresistance onset and EMT by manipulating ET_{\alpha}R-mediated pathways, pinpoints that blockade of ET_{\alpha}R may provide appropriate choice in clinical EOC treatment.

Materials and Methods

Cells and cell culture conditions

The human ovarian carcinoma cell line A2780 WT was obtained from European Collection of Cell Cultures. To retain cisplatin (CIS) and paclitaxel (TAX) resistance, 1 \(\mu\)mol/L cisplatin and 60 nmol/L paclitaxel were added to the culture medium A2780 CIS and A2780 TAX, respectively, every 2 passages (17). The 2008 cell line and its cisplatinum resistant subclone 2008C13 (CIS) were kindly provided by Dr. S.B. Howell, University of San Diego, La Jolla, CA). Further details are described in the Supplementary Materials and Methods.

Thymidine incorporation assay

Serum-starved A2780 WT and its resistant sublines, or ET_{\alpha}R- or scrambled-siRNA transfected cells, were treated with ET-1 and/or zibotentan and after 24 hours, \(^{3}H\)-thymidine was used as previously described (13). Responses to all treatments were assayed in sextuplicate, and results were expressed as the means of 3 separate experiments.

Apoptosis assay

For detection of early apoptotic events, cells were double stained with fluorescein isothiocyanate–conjugated annexin-V and propidium iodide using the Vybrant apoptosis kit according to the manufacturer’s instructions (Invitrogen). Further details are described in the Supplementary Materials and Methods.

Western blot analysis

Cell lysates or conditioned media obtained from cell cultures were subjected to SDS-PAGE and revealed by Western blotting (WB). Further details are described in the Supplementary Materials and Methods.

Quantitative real-time PCR

Total RNA was isolated using the Trizol (Invitrogen) according to the manufacturer’s protocol. RNA (5 \(\mu\)g) was reverse transcribed using Superscript VILO cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed by using LightCycler rapid thermal cycler system (Roche Diagnostics) according to the manufacturer’s instructions. The primers sets used and further details were shown in the Supplementary Materials and Methods.

Reverse Transcriptase (RT)-PCR

RT-PCR was performed using a Superscript One-Step RT-PCR System (Invitrogen) according to the manufacturer’s
instructions. The primers sets used and further details were shown in the Supplementary Materials and Methods.

**Luciferase reporter gene assay**

To measure the transcriptional activity of Snail and E-cadherin promoter, 3 × 10^5 cells/well were transiently transfected with 0.5 μg of pGL3-SNA (−869/+59), or with 0.5 μg pGL2 Ecad3/luc construct, or with empty control vectors (Promega). Where indicated, cells were transfected with 100 nmol/L scrambled or ETαR siRNA duplexes against mRNA (SMART pool) or mock siRNA obtained commercially (Dharmacon). Further details were shown in the Supplementary Materials and Methods.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (18). Details were shown in the Supplementary Materials and Methods.

**Invasion assay**

Cell invasion assays were carried out using modified Boyden chamber. Details were shown in the Supplementary Materials and Methods.

**Xenografts in nude mice**

Female athymic (nu+/nu+) mice, 4 to 6 week of age (Charles River Laboratories, Milan, Italy) were treated following the guidelines for animal experimentation of the Italian Ministry of Health. Mice were injected s.c. into 1 flank with 4 × 10^6 viable A2780 WT, cisplatinum- and taxol-resistant cells. After 7 days, when tumors reached approximately 0.2 to 0.3 cm in diameter, mice were randomized in groups (n = 10) to receive different treatments. One group was treated i.p. for 21 days with zibotentan (diluted in PBS) at the daily dose of (10 mg/kg/day), 1 group was treated with paclitaxel (20 mg/kg per dose given i.v. 3 times a day every 4 days), 1 group with cisplatinum (5 mg/kg i.p. 1 time on day 1), 1 group with combination of zibotentan and cisplatinum or zibotentan and paclitaxel. Control mice were injected i.p. with vehicle. Three different experiments with a total of 30 mice for each group were performed. On day 45 after tumor injection, tumors were removed from control and treated mice and analyzed for WB. Tumor size was measured with caliper and was calculated using the formula π/6 × larger diameter × (smaller diameter)^3.

**Patient population**

The study included 60 primary untreated ovarian cancer patients admitted to the Gynecologic Oncology Unit, Catholic University of Campobasso and Rome treated with platinum-based chemotherapy, plus paclitaxel. Further details were shown in the Supplementary Materials and Methods.

**Immunohistochemistry**

Immunohistochemical analysis of ovarian cancers was performed on archival from 60 frozen tumors collected from patient population above described with informed consent, as indicated by Institutional Review Board, and classified according with WHO criteria. Further details were shown in the Supplementary Materials and Methods.

**Statistical analysis**

Statistical analysis was performed using Student’s t-test and Fisher’s exact test as appropriate. The time course of tumor growth was compared across the groups using 2-way ANOVA, with group and time as variables. All statistical tests were carried out using SPSS software (SPSS 11, SPSS Inc.). A 2-sided probability value of less than 0.05 was considered statistically significant.

**Results**

**Expression of ET-1/ETαR axis and activation of signaling pathways are upregulated in chemoresistant ovarian cancer cells**

In order to assess the potential involvement of ET-1 axis in chemoresistance, we used the A2780 (WT) human EOC cell line, the derivative cisplatinum-resistant (A2780 CIS) and taxol-resistant (A2780 TAX) sublines, and the 2008 cell line and its cisplatinum-resistant variant 2008 CIS. The different sensitivity to these drugs was evident after treatment of serum-starved cells with the cytotoxic drugs, cisplatinum and taxol (Supplementary Fig. S1A). In both A2780 and 2008 sensitive cells, cisplatinum or taxol treatment increased the number of early apoptotic cells, while in the resistant sublines the apoptotic pathway was compromised, confirming the acquisition of drug resistance in these cells.

The ET-1 axis is expressed in ovarian cancer cells acting as survival factor against chemotherapeutic drug treatment (12), suggesting that ET-1/ETαR axis would be upregulated in cisplatinum and taxol-resistant cells. As shown in Figure 1(A–C) and Supplementary Figure S2A, the cisplatinum- and taxol-resistant cells significantly expressed ETαR at higher levels compared to sensitive cells, and the concentrations of ET-1 released by resistant cells increased of about 8-fold compared to parental cells. All these data show that ET-1 axis is upregulated in EOC resistant cells correlating with the reduced sensitivity of these sublines to cytotoxic drugs.

We next determined the signaling pathways activated by ET-1/ETαR axis to modulate cell survival. ET-1 activated both MAPK and Akt in sensitive A2780 cells, but a significant higher activation was observed in the resistant sublines that was inhibited when the cells were pretreated with the specific ETαR antagonist zibotentan (Fig. 1D). Accordingly, ET-1 promoted A2780 cell proliferation, which was significantly inhibited in zibotentan-treated cells. To further verify the role of ETαR in regulating cell proliferation, we knockeddown ETαR with siRNA and we found that also in ETαR-silenced cells the ET-1-induced cell proliferation was inhibited (Fig. 1E). Specificity of siRNA oligos was confirmed by WB analysis, which showed a 90% knockdown of ETαR (Supplementary Fig. S2B).
These results suggest that in chemoresistant cells the higher activation of ET-1 axis lead to enhanced MAPK and Akt signaling pathways, which promote cell survival and proliferation.

**Chemoresistant cells display molecular changes consistent with ET-1-driven EMT and invasiveness**

Given the ability of ET-1 to promote EMT machinery in ovarian tumor cells and the association between chemoresistance and acquisition of EMT phenotype in different tumor cells (11, 19–21), we analyzed whether the chemoresistance in A2780 and 2008 cells was associated with molecular changes consistent with EMT and whether the ETAR pathway is involved in this process. To this end we examined the expression of E-cadherin and its transcriptional repressors, Snail, Slug, and Twist, and other mesenchymal markers, such as vimentin and N-cadherin. Enhanced mRNA expression levels for Snail, Slug, Twist, vimentin, and N-cadherin were observed in resistant sublines compared with parental cells, associated with a concomitant decrease in E-cadherin expression at level of mRNA and protein (Fig. 2A and C). Moreover, ET-1 enhanced the expression of Snail and Twist in both sensitive and resistant cells and the treatment with zibotentan inhibited the ET-1-induced effects, restoring E-cadherin expression (Fig. 2B and C). Remarkably, ETAR blockade, by both zibotentan or its silencing, reverted the ET-1-induced suppression of E-cadherin promoter activity and in resistant cells prevented that induced at higher level by endogenous ET-1/ETAR axis (Fig. 2D), suggesting that the transcriptional regulation of E-cadherin may be important to ETAR-driven EMT and acquisition of chemoresistance. In agreement with above results, ET-1 induced significant induction of Snail promoter activity in parental cells, reaching about 4-fold increase in resistant cells, which was significantly inhibited by zibotentan (Fig. 2E), indicating that ET-1/ETAR controls the transcriptional repression of E-cadherin through Snail in chemoresistant cells. Furthermore, we examined how Snail binds to the E-cadherin gene promoter. As shown in Fig 2F, the E-cadherin promoter sequences were detected bound to Snail upon treatment with ET-1 in a time-dependent manner. The association of Snail with E-cadherin promoter began 5 minutes after ET-1 exposure and declined rapidly in sensitive A2780 cells.
Interestingly, in resistant cells the recruitment, starting after 5 minutes, was long-lasting with a maximum after 30 minutes, and persisting for 60 minutes, suggesting that the ET-1-dependent and sustained binding of Snail in the E-cadherin promoter might account for the EMT and chemoresistant phenotype of these cells.

To assess whether the acquisition of EMT phenotype correlates with functional changes in resistant cells, we evaluated the expression of several matrix-metalloproteases (MMP) and the invasive potential of EOC cells. As shown in Figure 3A and B, a significant upregulation and activity of MMP-2 and -9 was observed in resistant cells, confirming the association between invasive phenotype and chemoresistant properties of these cells. As demonstrated by invasion assay, the A2780 WT cells showed a significant increase in the number of invading cells after treatment with ET-1 that was inhibited by zibotentan (Supplementary Fig. S3). The invasive capability of chemoresistant sublines was greater, with a 2-fold increase in the number of invading cells compared with sensitive cells that was blocked by pretreatment with zibotentan (Fig. 3C), demonstrating that ETAR blockade is associated with a reverted EMT phenotype and reduced invasiveness in chemoresistant cells.
and taxol-resistant tumors with zibotentan. This treatment was generally well tolerated with no detectable signs of acute or delayed toxicity, and produced a 55% inhibition of tumor growth on day 41 after tumor injection in A2780 WT bearing mice ($P \leq 0.005$; Fig. 5A). A similar growth inhibitory effect was observed when zibotentan was used in both taxol (58%) and cisplatinum (54%)-resistant ovarian carcinoma xenografts, causing significant inhibition in tumor growth at the end of the 4-week treatment period in all mice ($P < 0.002$ for A2780 CIS and $P < 0.005$ for A2780 TAX; Fig. 5B). Most importantly, a further significant growth inhibitory effect was observed when zibotentan was used in combination with cisplatinum (71%) or in combination with paclitaxel (81%), in resistant EOC xenografts. Remarkably, a marked increase of E-cadherin expression in zibotentan-treated mice, especially in xenografts from resistant cells, which was paralleled to the reduction of N-cadherin expression (Fig. 5C). These results indicate a specific therapeutic window in which blockade of ET$_{A}$R with zibotentan, in combination with chemotherapeutic drugs, controlling the EMT and aggressive phenotype of chemoresistant cells, is therapeutic effective in resistant EOC cells, increasing sensitivity to the chemotherapy.

**ET$_{A}$R is overexpressed in resistant human ovarian tumors**

Finally we analyzed the importance of ET$_{A}$R as potential marker of resistance in human EOC tissues from patients treated with platinum-based chemotherapy, plus paclitaxel. Clinicopathologic characteristics of the overall series are summarized in Table 1. Forty-seven cases (78.3%) were stage III to IV disease, and serous histotype was documented in the vast majority ($n = 44, 73.3\%$) of cases. Optimal cytoreduction (residual tumor $\leq 1$ cm) was achieved in 32 (53.3%) patients. Thirty-one (51.7%) cases were defined as platinum resistant, while 29 patients (48.3%) were platinum sensitive. Patients optimally cytoreduced at first surgery were more likely to be platinum sensitive compared to patients judged as unresectable (62.5% vs. 32.1%, $P = 0.017$). Patients with no ascites were more frequently platinum sensitive than patients with ascites at primary laparotomy (66.7% vs. 33.3%, $P = 0.018$). We analyzed the ET$_{A}$R levels by immunohistochemistry (IHC) in these human ovarian cancer specimens encompassing platinum sensitivity and platinum resistance. Overall, ET$_{A}$R was significantly upregulated in 66% ($P < 0.017$) of resistant ovarian cancers compared to sensitive tumors (Table 1 and Fig 5D). Consistent with our observations in EOC cell cultures and xenografts, overexpression of ET$_{A}$R in platinum-resistant human ovarian cancer tissues was associated with the downregulation of E-cadherin ($P < 0.002$) and with enhanced expression of mesenchymal N-cadherin ($P < 0.07$; Fig 5D). These results are consistent with our previous findings in primary ovarian tumor tissues (10), indicating the biological relevance of ET$_{A}$R in the regulation of EMT in the tumor context.

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**Figure 3. ET$_{A}$R-mediated MMP activity and cell invasion is upregulated in chemoresistant cells.** A, RT-PCR analysis of MMP-2, -9, and GAPDH expression in sensitive and resistant A2780 cells. B, conditioned media from A2780 cells analyzed for MMP-2 (latent form, 72 kDa; active form, 66 kDa) and for MMP-9 (latent form, 92 kDa; active form, 86 kDa) by WB. Hsp70 was used as loading control. C, invasion assay of A2780 WT, CIS- and TAX-resistant cells exposed to zibotentan. * $P < 0.02$ compared to untreated cells; ** $P < 0.002$ compared to control of A2780 WT cells.

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**Endothelin Axis Promotes Chemoresistance**
Development of drug resistance remains the major therapeutic barrier in EOC. Consequently an in depth understanding of the mechanisms underlying the development of chemoresistance is of utmost importance for improving the therapeutic regimens (2, 21). Since ET-1/ETAR axis is critically involved in EOC growth and progression, in the present study we investigated the pathobiological role of ET-1 and ETAR in developing drug resistance. Our results provide evidence that ET-1/ETAR overexpression, by regulating EMT and invasive behavior, endows EOC cells with an increased survival capacity and resistance to 2 presently employed chemotherapeutic agents, such as cisplatinum and taxol. The blockade of ETAR by zibotentan reverted EMT, restored drug sensitivity, and inhibited invasiveness and growth of ovarian tumor xenografts. Finally, through the analysis of primary advanced EOC patients, we reported that the overexpression of ETAR correlates with clinical chemoresistance and EMT phenotype.

Figure 4. ETAR blockade sensitizes chemoresistant cells to drug-induced apoptosis. Sensitive and cisplatinum-resistant A2780 and 2008 cells were maintained for 48 hours in absence or presence of cisplatinum and/or zibotentan. Apoptosis was measured by Annexin V and PI staining and expressed as percentage of apoptotic cells.
Drug resistance is a multifactorial phenomenon involving interrelated and/or independent pathways (22). The overall apoptotic balance within a given cell population relies on a number of signaling pathways differentially regulated between chemosensitive and chemoresistant cancer cells. In this context, it has been demonstrated that ET-1 acts as an antiapoptotic factor, modulating cell survival pathways through a PI3-K-mediated Akt activation, which is considered to be a molecular “crutch” to escape cell death (12). The onset of chemoresistance of EOC cells can also be related to the overexpression of the ET-1/ET_{α}R signaling pathway that, in turn, may control the apoptotic response in resistant cells through the activation of survival pathways, such as MAPK or the PI3K/AKT signaling. It is becoming clear that EMT may reflect an ultimate adaptation of cancer cells to survive to cytotoxic drug activity, thus being responsible for chemo sensitivity and that EMT inducing transcription factors such as Snail, Slug, and Twist which confer resistance to cell death (3, 23–29). In human EOC, changes in the expression of Snail, Slug, and Twist play an important role in ovarian tumorigenesis and progression, and are significantly higher in advanced stages and metastatic lesions (30–33). Moreover, it was reported that cellular morphology, motility, and molecular changes consistent with EMT, including enhanced Snail and Twist expression, were related to paclitaxel-resistance in EOC cells (34, 35). Recent work has suggested that may be a link between the cancer stem cell (CSC) phenotype and that induced by the process of EMT (36). The CSC isolated from EOC samples express markers associated with stem cells and EMT, including Snail and Slug, suggesting that EOC cells, by going through an EMT, acquire stemness.

Figure 5. Antitumor effects of zibotentan in A2780 sensitive and resistant xenografts is associated with reverted EMT phenotype. A2780 WT (A), CIS and TAX (B) cells were injected into nude mice. After 7 days, the mice were treated as indicated for 21 days. Each experiment was performed 3 times with a total of 30 mice per group. The comparison of the time course of tumor growth curves by 2-way ANOVA with group-by-time interaction for tumor growth was statistically significant in both sensitive and resistant A2780 xenografts. Data points, averages; bars, ± SD. C, E-cadherin and N-cadherin expression in tumors from A2780 xenografts treated as described in A and B. β-actin was used as loading control. D, the expression patterns of ET_{α}R and EMT markers in the 60 platinum sensitive and resistant human ovarian cancer samples were determined by IHC. Representative immunohistochemical staining in sensitive and resistant human primary ovarian carcinoma samples for ET-1, ET_{α}R, E-Cadherin, and N-cadherin expression (original magnification, ×200).
characteristics qualifying them to acquire chemoresistance by overcoming p53-mediated apoptosis (16, 37–41). In this context, recent data suggest that Twist1 may also be an important regulator of "stemness" in EOC cells (42), indicating that the initiation of the EMT programme may be critical for the acquisition of stem cell-like characteristics resulting in chemoresistance (43). In this regard, a recent pathway analysis revealed ET-1 signaling among the canonical pathways significantly ($P < 0.02$) associated with resistance to platinum-based chemotherapy, whose several are linked to EMT and stemness reinforcing the relationship of both processes with therapy resistance (16).

The present study reveals the molecular mechanisms of resistance activated by ET-1/ETAR axis through EMT transcriptional programs. Thus, the transcription activity of Snail, upon its recruitment on E-cadherin promoter, is essential in ETAR-mediated E-cadherin repression in resistant EOC cells. The upregulation of EMT transcription factors in chemoresistant cells is associated with enhanced MMP activity and invasiveness, suggesting that these factors might control acquisition of EMT in cancer cells to induce motility in response to adverse environmental changes facilitating cancer progression and therapeutic resistance (44, 45). Under this scenario EMT initiating signals would be an ideal target as they are the seeds for metastasis and recurrence. Of clinical relevance, knockdown of ETAR levels by siRNA, or blockade by zibotentan, reverted EMT phenotype, inhibited invasive behavior, and increased susceptibility to chemotherapeutic agents, suggesting that ETAR-mediated EMT signaling can represent a "salvage pathway" occurring during chemoresistance development. The significant association between ETAR overexpression and the resistant phenotype, identified for the first time ETAR as predictor of chemoresistance in human EOC tissues and its relationship with EMT marker expression in the resistant tumor context.

In summary, our study provides evidence that targeting ETAR with zibotentan, in combination with chemotherapy can sensitize tumors to chemotherapeutics by preventing EMT-associated escape signaling thus offering a rationale for the clinical evaluation of this target-based drug as modulator of both chemoresistance and tumor progression.

**Disclosure of Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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**Table 1. Clinicopathologic characteristics and platinum sensitivity in the current series of patient population**

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<th>Platinum sensitive no. (%)</th>
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Abbreviation: NA, not available.

$^*$Fisher’s exact test was used to analyze the distribution of cases between groups.
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References


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