Original Article

The inhibition of BRAF activity sensitizes chemoresistant human ovarian cancer cells to paclitaxel-induced cytotoxicity and tumor growth inhibition

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Abstract: Ovarian cancer is one of the most common cancers in women and the second most common cause of gynecologic cancer death in women worldwide. While ovarian cancer is highly heterogeneous in histological subtypes and molecular genetic makeup, epithelial ovarian cancer is the most common subtype. The clinical outcomes of ovarian cancer largely depend on early detection and access to appropriate surgery and systemic therapy. While combination therapy with platinum-based drugs and paclitaxel (PTX) remains the first-line systemic therapy for ovarian cancer, many patients experience recurrence and die of progressive chemoresistance. Thus, there is an unmet clinical need to overcome recurrent disease due to resistance to chemotherapies of ovarian cancer. Here, we investigated whether BRAF inhibitors (BRAFi) could sensitize PTX-resistant ovarian cancer cells to PTX, and thus would overcome the resistance to chemotherapies. We found that BRAF and several members of the RAS/MAPK pathways were upregulated upon PTX treatment in ovarian cancer cells, and that BRAF expression was significantly elevated in the PTX-resistant ovarian cancer cells. While the BRAFi vemurafenib (VEM) alone did not cause any significant cytotoxicity in PTX-resistant ovarian cancer cells, VEM significantly enhanced PTX-induced growth inhibition and apoptosis in a dose-dependent manner. Furthermore, VEM and PTX were shown to synergistically inhibit tumor growth and cell proliferation of PTX-resistant human ovarian cancer cells in vivo. Collectively, these findings strongly suggest that BRAFi may be exploited as synergistic sensitizers of paclitaxel in treating chemoresistant ovarian cancer.

Keywords: Ovarian cancer, paclitaxel (PTX), chemoresistance, paclitaxel resistance, BRAF inhibitor (BRAFi), vemurafenib, combination chemotherapy, targeted therapy

Introduction

Ovarian cancer is one of the most common cancers in women and ranks third after cervical and uterine cancer worldwide [1-6]. Even though ovarian cancer has a lower prevalence in comparison with breast cancer, it is three times more lethal than breast cancer, and is the second most common cause of gynecologic cancer death in women around the world [1, 4-8]. Due to the lack of effective early detection strategies, over 80% of ovarian cancers are diagnosed with potential metastatic lesions. Ovarian tumors usually originate from one of
three cell types: epithelial cells, stromal cells, and germ cells. Epithelial ovarian cancer (EOC) is the most common subtype while EOC itself is composed of a diverse group of tumors that can be further classified into five main histological subtypes on the basis of distinctive morphologic and genetic features including: high-grade serous (HGSOC; 70%), endometrioid (ENOC; 10%), clear cell (CCOC; 10%), mucinous (MOC; 3%), and low-grade serous (LGSOC; <5%) [1, 5, 6, 9-11]. It has been shown that noncoding RNAs and cancer stem cells may contribute to the progression and metastasis of ovarian cancers [12-15].

The clinical outcomes of ovarian cancer are rather complicated, largely depending on early diagnosis and access to appropriate surgery and systemic therapy [1]. Common clinical management of ovarian cancer includes debulking surgery, combination chemotherapy, radiation therapy, and/or antiangiogenic agents in patients with suboptimally debulked and stage IV disease, as well as currently evolving therapies such as folate receptor targeting and immunotherapy [1, 5, 6, 9-11]. Major progress in maintenance therapy has been made by incorporating recently approved poly (ADP-ribose) polymerase (PARP) inhibitors in a recurrent setting and in a first-line setting among women with BRCA1/BRCA2 mutations [16, 17]. Combination therapy with platinum-based drugs (e.g., cisplatin, carboplatin, or oxaliplatin) and paclitaxel (PTX) remains the first-line systemic therapy for ovarian cancer [1]. Nonetheless, many patients experience recurrence within 12-24 months and succumb to progressively chemotherapy-resistant disease [5, 18]. Even though the five-year survival rate for ovarian cancer patients has improved over the past two decades, the overall cure rate remains approximately 30% [5, 18]. Clinical management of ovarian cancer has met many challenges, which is in part because the origin and pathogenesis of ovarian cancer are poorly understood [6]. Thus, any significant improvement in long-term survival will hinge on translating our understanding of molecular and cellular characteristics of ovarian cancers into personalized treatment strategies, optimizing methods of screening or early detection, and developing novel therapeutics [5, 9-11, 19].

Here, we investigated whether BRAF inhibitors (BRAFi) can sensitize paclitaxel (PTX)-resistant ovarian cancer cells to PTX in order to overcome the recurrent disease due to resistance to chemotherapies. We found that BRAF was upregulated by PTX in ovarian cancer cells, as well as elevated in PTX-resistant ovarian cancer cells. While the BRAFi vemurafenib (VEM) alone did not cause any significant cytotoxicity in PTX-resistant ovarian cancer cells, VEM was shown to effectively enhance PTX-induced growth inhibition and apoptosis in a dose-dependent manner. Furthermore, VEM and PTX were shown to synergistically inhibit tumor growth and cell proliferation of PTX-resistant human ovarian cancer cells in vivo. Thus, these findings strongly suggest that BRAFi may be exploited as synergistic sensitizers of paclitaxel in treating chemoresistant ovarian cancer.

Materials and methods

Cell culture and chemicals

Human ovarian cancer cell lines OVCAR8, Hey-A8, and the paclitaxel (PTX)-resistant Hey-A8-MDR were kindly provided by Dr. Ernest Lengyel of The University of Chicago. The PTX-resistant OVCAR8-PR29A and OVCAR8-PR29B lines were independently established in the authors’ laboratory through PTX dose-escalating selection process. All cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO2 as described [20-24]. Chemicals paclitaxel (PTX) and vemurafenib (VEM) (aka, PLX4032, RG7204, or RO5185426; marketed as Zelboraf) were purchased from Selleckchem (Houston, TX). Unless indicated otherwise, all other chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Crystal violet staining

Crystal violet staining assay was conducted as described [25-28]. Briefly, subconfluent HeyA8-MDR, OVCAR8-PR29A and/or OVCAR8-PR29B cells were treated with varied concentrations of DMSO, PTX, and/or vemurafenib (VEM). At 72 h after treatment, the cells were washed with PBS and stained with 0.5% crystal violet/formalin solution at room temperature for 20-30 min. The stained cells were rinsed with tape water, air-dried, and documented by a photo
scanner. The scanned images were quantitatively analyzed by using the ImageJ software.

**WST-1 cell proliferation assay**

Cytotoxicity was assessed by using Premixed WST-1 Reagent (Clontech, Mountain View, CA) as described [22, 29-32]. Briefly, subconfluent HeyA8-MDR, OVCAR8-PR29A and/or OVCAR8-PR29B cells were seeded in 96-well plates, and were treated with different concentrations of PTX and/or VEM, or DMSO control. At the indicated time points, the freshly prepared WST-1 Working Mix was added to each well, followed by incubating at 37°C for 2 h and reading at 450 nm using a microplate reader (BioTek EL800, Winooski, VT). Each assay condition was done in triplicate.

**Chou-Talalay drug combination index analysis**

The combination effects between paclitaxel and vemurafenib were calculated with the Chou-Talalay method [20, 28, 33, 34]. Briefly, the dose-dependent WST-1 assay data obtained above were analyzed with the CompuSyn software (ComboSyn, Inc.). The calculated combination index (CI) theorem of Chou-Talalay analysis usually yields a quantitative definition for additive effect (CI = 1), synergism (CI<1), or antagonism (CI>1) in drug combinations.

**RNA isolation and quantitative PCR analysis**

Total RNA was extracted by using the TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and subjected to reverse transcription reactions using hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The resultant cDNA products were diluted 10- to 100-fold and used as PCR templates. PCR primers were designed by using the Primer3 Plus program [35]. All qPCR primer sequences are listed in Supplementary Table 1. The quantitative PCR analysis was carried out by using our previously optimized TqPCR protocol [23, 36-43]. Briefly, the 2x SYBR Green qPCR reactions (Bimake, Houston, TX) were set up according to manufacturer’s instructions. The cycling program was modified by incorporating 4 cycles of touchdown steps prior to the regular cycling program. GAPDH was used as a reference gene. All sample values were normalized to GAPDH expression by using the 2^(-ΔΔCt) method. Each qPCR assay condition was done in triplicate.

**Apoptosis analysis (Hoechst 33258 staining)**

The Hoechst 33258 apoptosis staining assay was carried out as previously described [44-49]. Briefly, exponentially growing OVCAR8-PR29B and HeyA8-MDR cells were treated with DMSO, PTX and/or VEM. At 48 h post treatment, cells were collected, fixed and stained with the Magic Solution (10× stock: 0.5% NP-40, 4% formaldehyde, 10 μg/ml Hoechst 33258 in PBS). Apoptotic cells were examined and recorded under a fluorescence microscope. Each assay condition was done in triplicate. The average numbers of apoptotic cells were calculated by counting apparent apoptotic cells in at least ten random fields at 100× magnification for each assay condition.

**Xenograft tumor model of human ovarian cancer cells**

The use and care of animals were approved by the Institutional Animal Care and Use Committee. The xenograft tumor model was established as previously described [49-54]. Briefly, exponentially growing HeyA8-MDR cells were collected, resuspended in PBS at 10^7 cells/ml, and injected subcutaneously into the flanks of athymic nude mice (Harlan Laboratories, 6-8 week old, female, 10^6 cells per injection, and 4 sites per mouse). At three days post injection, the mice were divided into four groups (n = 4 per group): PTX group, the animals were treated with PTX (10 mg/kg body weight); VEM group, the animals were treated with vemurafenib (10 mg/kg body weight); PTX/VEM group, the animals were treated with PTX and VEM (each at 10 mg/kg body weight); and DMSO control group, the animals were treated with DMSO. All drugs were given intraperitoneally daily. Tumor growth was monitored with caliper measurement at the indicated time points. The mice were sacrificed at the endpoint of day 18. The subcutaneous tumor masses were retrieved for histologic evaluation and immunohistochemical analysis.

**Hematoxylin and Eosin (H & E) and immunohistochemical (IHC) analysis**

H & E staining was carried out as described [55-59]. Briefly, the retrieved tissues were fixed
with 10% buffered formalin, decalcified and embedded in paraffin. Serial sections of the embedded specimens were deparaffinized and subjected to H & E staining. Results were documented under a bright field microscope. Representative images are shown.

The IHC analysis was carried out as described [60-63]. Experimentally, the tissue sections were deparaffinized, rehydrated, and subjected to IHC staining with the PCNA antibody (Santa Cruz Biotechnology). Minus primary antibody and control IgG were used as negative controls.

**Statistical analysis**

All quantitative assays were performed in triplicate and/or in three independent batches. Data were expressed as mean ± standard deviation. Statistical significances were determined by one-way analysis of variance and the Student t-test. Statistical analysis was performed using the SPSS software version 19. A p-value <0.05 was considered statistically significant.

**Results**

**BRAF/RAS/MAPK pathways are up-regulated in PTX-resistant human ovarian cancer cells**

We first analyzed the status of the RAS/MAPK signaling pathways in three PTX-resistant ovarian cancer cell lines. The HeyA8-MDR line is a previously established paclitaxel-resistant line [64, 65] and conferred a modest PTX resistance (up to 200 nM PTX) (Figure 1Aa). We also conducted dose-escalating selection experiments and established two independent PTX-resistant lines from the sensitive OVCAR8 cells, designated as OVCAR8-PR29A and OVCAR8-PR29B, in which OVCAR8-PR29A cells conferred a robust PTX resistant (up to 200 nM PTX), whereas OVCAR8-PR29B cells were the most resistant line among the three PTX resistant lines (up to 20 µM PTX) (Figure 1Ab and 1Ac). Thus, the three PTX-resistant lines may represent the diverse features of PTX resistance in human ovarian cancer cells.

We analyzed the expression of essential signaling mediators of the RAS/MAPK pathways. The qPCR analysis revealed that the expression of IGF1R, KRAS, BRAF, MEK1 and ERK1 was significantly upregulated upon PTX treatment, while the expression of NRAS and MEK2 was down-regulated by PTX in both parental OVCAR8 and PTX-resistant OVCAR8-PR29B cells, respectively (Figure 1Ba). Similar results were obtained in HeyA8 and HeyA8-MDR cells except that IGF1R, KRAS, MEK1 and ERK1 were not upregulated by PTX in the parental HeyA8 cells while MEK2 was upregulated in HeyA8-MDR cells (Figure 1Bb). Noticeably, BRAF was not only upregulated by PTX in the test ovarian cancer lines but also significantly elevated in both PTX-resistant lines, suggesting that BRAF activation may be associated with PTX treatment.

**BRAFi vemurafenib (VEM) sensitizes paclitaxel (PTX) in PTX-resistant human ovarian cancer cells**

We next tested whether human PTX-resistant ovarian cancer cells were sensitive to BRAFi VEM. In our preliminary studies, we found that the lethal concentrations for the parental ovarian cancer lines HeyA8 and OVCAR8 were 50 nM and 20 nM, respectively (data not shown). As shown in Figure 2, the HeyA8-MDR cells and OVCAR8-PR29A were resistant to PTX and survived well at 200 nM and 600 nM, respectively, whereas the OVCAR8-PR29B cells were resistant to PTX at up to 10 µM. Using these three PTX-resistant lines, we found that no significant cytotoxicity was observed in HeyA8MDR (Figure 2A), OVCAR8-PR29A (Figure 2B) and OVCAR8-PR29B (Figure 2C and 2D) treated with up to 10 µM of VEM alone. However, in the presence of 8 µM VEM, HeyA8-MDR cells exhibited significant cytotoxicity for PTX at as low as 50 nM, and were completely killed at 200 nM (Figure 2Aa and 2Ab). Significant cytotoxicity was observed in OVCAR8-PR29A cells in presence of 400 nM PTX and 2 µM VEM, or 200 nM PTX and 8 µM VEM (Figure 2Ba and 2Bb). Similarly, in the more resistant OVCAR8-PR29B cells, significant cytotoxicity was observed at 5 µM PTX and 10 µM VEM, both in crystal violet staining and colony formation assays (Figure 2Ca, 2Cb, 2Da and 2Db). Collectively, these results strongly suggest that the BRAFi VEM, while ineffective alone, may significantly sensitize PTX-resistant ovarian cancer cells to PTX.

**BRAFi vemurafenib (VEM) and paclitaxel (PTX) synergistically inhibit cell proliferation and induce apoptosis in PTX-resistant human ovarian cancer cells**

We sought to test whether VEM and PTX would act synergistically to inhibit cell proliferation of
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Figure 1. Multiple components of BRAF/KRAS/MAPK pathways are up-regulated in paclitaxel (PTX)-resistant human ovarian cancer cells. (A) Characterization of three PTX-resistant human ovarian cancer lines. Subconfluent HeyA8 and HeyA8-MDR (a), OVCAR8 and OVCAR8-PR29A (b), and OVCAR8 and OVCAR8-PR29B (c) cells were treated with DMSO or PTX at the indicated concentrations. At 72 h post treatment, cells were fixed and subjected to crystal violet staining. Representative results are shown. PA, parental line; MDR, HeyA8-MDR; PR29A, OVCAR8-PR29A; and PR29B, OVCAR8-PR29B. (B) qPCR analysis of the expression of essential signaling mediators of the RAS/MAPK pathways. Subconfluent OVCAR8-PR29B and its parental OVCAR8 (a), or HeyA8-MDR and its parental HeyA8 (b)
cells were treated with DMSO or 10 µM PTX and/or 10 µM VEM. At 48 h, total RNA was isolated from the cells and subjected to qPCR analysis using primers specific for the indicated genes. GAPDH was used as a reference gene. All assays were done in triplicate. * P<0.05; ** P<0.01 compared with that of the parental cells treated with DMSO group.

**Figure 2.** BRAFi Vemurafenib (VEM) sensitizes paclitaxel (PTX) in PTX-resistant human ovarian cancer cells. Subconfluent HeyA8MDR (A), OVCAR8-PR29A (B) and OVCAR8-PR29B (C and D) cells were seeded into 12 or 24-well cell culture plates, and treated with DMSO, paclitaxel (PTX) and/or vemurafenib (VEM) at the indicated concentrations for 72 h. The cells were fixed and subjected to crystal violet staining. For the OVCAR8-PR29B cells, in addition to conventional crystal staining assay (C), the colony formation assay was also carried out (D). All treatment conditions were done in triplicate. Representative results are shown. The staining results were further quantitatively analyzed with ImageJ software. *** P<0.01, compared with that of the DMSO group.

the PTX-resistant ovarian cancer cells through drug combination analysis. Using the quantitative WST-1 cell proliferation assay, we found that VEM significantly enhanced PTX-induced
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Figure 3. BRAFi Vemurafenib (VEM) and paclitaxel (PTX) synergistically inhibit cell proliferation of PTX-resistant human ovarian cancer cells. Subconfluent OVCAR8-PR29A (A and B) and HeyA8-MDR (C and D) cells were seeded into 96-well cell culture plates, and treated with DMSO, paclitaxel (PTX) and/or vemurafenib (VEM) at the indicated concentrations. At 72 h after treatment, WST-1 working mix was added to the cells and incubated for 2 h before subjected to absorbance reading at 450 nm (A and C). The WST-1 data were further subjected to Chou-Talalay drug combination index analysis (B and D). All assays were done in triplicate. **P<0.01 compared with that of the DMSO group at respective VEM concentrations.

BRAFi vemurafenib (VEM) and paclitaxel (PTX) synergistically inhibit tumor growth of PTX-resistant human ovarian cancer cells

Lastly, we tested whether VEM would enhance PTX cytotoxicity and inhibit tumor growth of PTX-resistant ovarian cancer cells. When HeyA8-MDR cells were subcutaneously injected into the nude mice and treated with PTX, and/or VEM, the tumor growth rate significantly decreased in the PTX and VEM combined therapy group, compared with that of the DMSO control group after two weeks (Figure 5A), which is confirmed by the macrographic images and average tumor weights of the retrieved tumor samples although the average tumor weight decreased in the PTX or VEM treatment alone group (Figure 5Ba and 5Bb). Histologic analysis revealed that the tumor masses retrieved from the PTX/VEM combined treatment group exhibited a significantly decrease in cellularity and an increase in necrotic tissue, compared with that of the DMSO control group, as well as the PTX or VEM alone group (Figure 5C). Immunohistochemical analysis of the cell proliferation marker PCNA further revealed sparsely positive staining in the tumor tissues retrieved from the PTX/VEM combined treatment group, while strong positive staining was
observed in the DMSO control group, to lesser extent in the PTX or VEM alone group (Figure 5D). Collectively, these in vivo results strongly suggest that VEM may synergistically enhance the PTX's cytotoxic activity in PTX-resistant ovarian cancer cells.

Discussion

PTX belongs to a class of taxanes with microtubule stabilizing ability, and together with platinum based therapy, remains as a part of the standard care for ovarian cancer management [5, 9-11, 19, 66]. In addition to stabilizing microtubules, PTX has been shown to activate multiple signaling pathways, which may be associated with proapoptotic signaling, includ-

In this study, we found that BRAF and several members of the RAS/MAPK pathways were upregulated upon PTX treatment in ovarian cancer cells, and that BRAF expression was significantly elevated in the PTX-resistant ovarian cancer cells, compared to that of the parental non-resistant cells. These results prompted us to investigate whether BRAFi can sensitize PTX-resistant ovarian cancer cells to PTX, and found that VEM effectively enhanced PTX-induced growth inhibition and apoptosis in a dose-dependent manner, and that VEM and PTX synergistically inhibited tumor growth and cell proliferation of PTX-resistant human ovarian cancer cells

BRAFi Vemurafenib (VEM) enhances paclitaxel (PTX)-induced apoptosis in PTX-resistant human ovarian cancer cells. Subconfluent OVCAR8-PR29B cells were seeded into 6-well cell culture plates, and treated with DMSO, 10 µM paclitaxel (PTX) and/or 10 µM vemurafenib (VEM). At 48 h, the cells were fixed and stained with the Magic Solution and examined under a fluorescence microscope (A). Representative apoptotic cells are indicated with arrows. Average % apoptotic cells (B) were calculated by counting % apoptotic cells in at least 10 random high power fields. Each assay condition was done in triplicate. Representative images are shown.

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BRAFi overcomes paclitaxel resistance in ovarian cancer

Figure 5. BRAFi Vemurafenib (VEM) and paclitaxel (PTX) synergistically inhibit tumor growth of PTX-resistant human ovarian cancer cells. Exponentially growing HeyA8MDR cells were collected and subcutaneously injected into the flanks of athymic nude mice, which were randomly divided into four groups. At 3 days post implantation, the mice were injected with DMSO, paclitaxel PTX, and/or VEM intraperitoneally daily till sacrificed at 18 days after cell injection. Tumor growth was monitored and average tumor growth rate was calculated (A). ** P<0.05; *** P<0.01 compared with that of the DMSO group at respective time points. The retrieved tumors were photographed (B, a) and weighed (B, b). Representative images are shown. ** P<0.05; *** P<0.01 compared with that of the DMSO
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pathway, which regulates growth, proliferation, differentiation, and apoptosis in human cells. Mutations in the BRAF oncogene are frequently found in 7% to 9% of advanced cancers, with the high incidence in melanoma (50%), papillary thyroid cancer (45%), colorectal cancer (11%-12%), and non-small cell lung cancer (3%-5%). Interestingly, BRAF mutation was found in approximately 35% of low-grade serous ovarian cancer (LGSC). A Phase 1 clinical trial has recently been conducted to investigate a combination therapy with vemurafenib, carboplatin, and paclitaxel in patients with BRAF-mutated melanoma and other advanced malignancies; and it has been shown that the combination therapy was well tolerated and demonstrated encouraging activity [73].

Interestingly, an early study examined the common mutations at codon 599 of BRAF and codons 12 and 13 of KRAS in human ovarian cancer samples and found that mutations in either codon 599 of BRAF or codons 12 and 13 of KRAS occurred in 68% of invasive micropapillary serous carcinomas and in 61% of serous borderline tumors, while none of the tumors contained a mutation in both BRAF and KRAS [74]. Another study revealed that activating KRAS mutations were more common in mucinous tumors (50%) than in all other histologic types combined (5%) [75]. A recent study revealed that KRAS mutations and the expression of EGFR and PKCα could be used as predictive biomarkers in patients with LGSC treated with MEKi, and that combination therapy using MEKi with EGFR inhibition may represent a promising new therapy for patients with MEKi-resistant LGSC [76]. Therefore, these reported findings are highly supportive of the outcomes of our studies and suggest that targeting RAS/MAPK pathways may overcome chemoresistance to PTX in ovarian cancer.

In summary, we demonstrated that BRAF was upregulated by PTX in ovarian cancer cells, and significantly elevated in PTX-resistant ovarian cancer cells. While the BRAFi vemurafenib (VEM) alone did not cause any significant cytotoxicity in PTX-resistant ovarian cancer cells, VEM was shown to effectively enhance PTX-induced growth inhibition and apoptosis in a dose-dependent manner. Furthermore, VEM and PTX were shown to synergistically inhibit tumor growth and cell proliferation of PTX-resistant human ovarian cancer cells in vivo. Collectively, these findings strongly suggest that BRAFi may be exploited as synergistic sensitizers of paclitaxel in treating chemoresistant ovarian cancer.

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Disclosure of conflict of interest

None.

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Pfannenstiel LW, Lam SS, Emens LA, Jaffee EM and Armstrong TD. Paclitaxel enhances
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**Supplementary Table 1.** List of qPCR Primers

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<th>GENE</th>
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<th>Reverse Primer</th>
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