Lapatinib Inhibits Amphiregulin-induced BeWo Choriocarcinoma Cell Proliferation by Reducing ERK1/2 and AKT Signaling Pathways

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Abstract. *Background:* Human choriocarcinoma is the most aggressive type of gestational trophoblastic neoplasia. The expression of epidermal growth factor receptor (EGFR) in choriocarcinomas is significantly higher than those of trophoblastic cells in healthy placentas. Lapatinib is a potent EGFR and human epidermal growth factor receptor 2 (HER2) inhibitor that inhibits cell proliferation and induces apoptosis in various human cancer cells. Amphiregulin (AREG) is the most abundant EGFR ligand in amniotic fluid during human pregnancy. *Aim:* To explore the role of AREG in human choriocarcinoma cell proliferation. *Materials and Methods:* The effect of lapatinib and AREG on cell proliferation was examined by the MTT assay. Western blots were used to investigate EGFR and HER2 expression, and the activation of caspase-3, extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase /protein kinase B (PI3K/AKT) signaling pathways. *Results:* Treatment with lapatinib reduced BeWo cell proliferation by inducing apoptosis. Moreover, AREG treatment stimulated BeWo cell proliferation by activating ERK1/2 and PI3K/AKT signaling pathways, which was blocked by lapatinib. *Conclusion:* Targeting EGFR/HER2 might be a useful therapeutic strategy for human choriocarcinoma.

The placenta is an important organ required for the successful development of the fetus during pregnancy.

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Key Words: Lapatinib, EGFR, HER2, choriocarcinoma.

Gestational trophoblastic disease is a group of rare diseases in which placental trophoblast cells grow abnormally inside the uterus after conception. Based on the histological and pathological characteristics, these can be broadly divided into two groups: i) Benign trophoblastic lesions that include hydatidiform moles (partial and complete); and ii) gestational trophoblastic neoplasia (GTN) (1-3). GTNs are uncommon malignancies which encompass a group of interrelated, but distinct tumors that include invasive hydatidiform mole, placental-site trophoblastic tumor, epithelioid trophoblastic tumor and choriocarcinoma (4, 5).

Although human choriocarcinoma is the most aggressive type of GTN, with abnormal invasive, migratory and proliferative properties, it is among the few types of human cancer that are highly responsive to chemotherapy and, unlike other types of malignant tumors, metastatic choriocarcinomas are potentially curable by combined chemotherapy and adjuvant surgical procedures (6, 7). However, in a small but significant proportion of patients, the disease exhibits resistance to traditional chemotherapy and patients fail to survive due to delayed diagnosis or a recurrent tumor (8-10). Therefore, it is important and necessary to develop new therapies or strategies for treating or preventing choriocarcinomas (11).

The Erb-b2 receptor tyrosine kinases (ERBB) receptor tyrosine kinase (RTK) family comprises four members: Epidermal growth factor receptor (EGFR; ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4). The expression level of EGFR in choriocarcinomas is significantly higher than that of trophoblastic cells in healthy placentas, whereas the expression levels of ERBB3 and ERBB4 do not vary significantly (12). In addition, the HER2 expression level in hydatidiform mole is increased with malignant transformation (13, 14). EGF, amphiregulin (AREG) and transforming growth factor α (TGF α) bind

specifically to EGFR (15, 16). Among these EGFR ligands, AREG is primarily expressed in human placenta throughout pregnancy and is thought to be the key molecule involved in the regulation of placental development and maturation. Importantly, the expression level of AREG is significantly higher than that of EGF and TGF α in human choriocarcinoma cells (17). Together, these results suggest that AREG/EGFR signaling may play important roles in the development and progression of human choriocarcinomas.

Despite having no direct identified ligand, HER2 can be activated *via* dimerization with other ligand-bound ERBB receptors and this heterodimerization can further potentiate signaling that is mediated by those ligand-bound ERBB receptors (18, 19). Using drugs that target EGFR or HER2 has emerged as effective treatments against various types of human cancers. However, to the best of our knowledge, the role of EGFR-mediated signaling in the regulation of human choriocarcinoma progression remains unclear. Lapatinib is a potent inhibitor of EGFR and HER2 tyrosine kinase catalytic activity. It has been shown that treatment with lapatinib inhibits proliferation and induces apoptosis of different types of human cancer cells (20). Given the high expression levels of AREG and EGFR in choriocarcinoma, the present study examined the effect of lapatinib on cell apoptosis and on AREG-induced cell proliferation in two human choriocarcinoma cell lines.

Materials and Methods

Antibodies and reagents. The polyclonal anti-EGFR (1:1,000) and monoclonal anti- α -tubulin (1:5,000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-HER2 (1:2,000), anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1,000) and polyclonal anti-caspase-3 (1:1,000), anti-ERK1/2 (1:1,000), anti-phospho-AKT (Ser473) (1:1,000) and anti-AKT (1:1,000) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Lapatinib was purchased from Selleckchem (Houston, TX, USA). PI3K inhibitor LY294002 was obtained from Sigma (St. Louis, MO, USA). Mitogen-activated protein kinase kinase enzymes (MEK) inhibitor U0126 was obtained from Calbiochem (San Diego, CA, USA). Recombinant human AREG was purchased from R&D Systems (Minneapolis, MN, USA). Lapatinib, LY294002, and U0126 solutions were prepared in dimethyl sulfoxide (DMSO; Sigma), and AREG was prepared in phosphate buffer solution. Equal volumes of vehicle control were loaded in all treatment groups.

Cell culture. The human choriocarcinoma cell lines BeWo and JEG-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in a 1:1 (v/v) mixture of Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) medium (Gibco, Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Figure 1. The expression levels of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) in BeWo and JEG-3 cells. A: Endogenous levels of EGFR were examined in BeWo and JEG-3 cells by western blot. B: Endogenous levels of HER2 were examined in BeWo and JEG-3 cells by western blot. The ovarian cancer cell line SKOV3 was used as a positive control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in a 24-well plate (2 $\times$ 10⁴/well) with 500 μ l of medium and treated with different concentrations (1 and 10 μ M) of lapatinib every 24 h for 96 h. In different experiments, cells treated with 20 ng/ml recombinant AREG every 24 h for 96 h. Effects of different compounds were also examined by pre-treating the cells with LY294002 (10 μ M), U0126 (10 μ M) or lapatinib (1 or 5 μ M) for 1 h prior to treatment with 20 ng/ml AREG every 24 h for 96 h. At least three independent experiments were performed. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added at different time points to a final concentration of 0.5 mg/ml and cells were then incubated for 4 h. After incubation, the media were removed and DMSO was added to each well to dissolve the crystals. The absorbance values were examined by spectrophotometric microplate reader.

Western blot. The protein levels of EGFR and HER2 in BeWo and JEG-3 cells were examined by western blot. To examine the effect of lapatinib on the protein levels of cleaved caspase-3, cells were treated with 10 μ M lapatinib for 24 h. To examine the effects of AREG on the phosphorylation of ERK1/2 and AKT, cells were treated with 20 ng/ml AREG for 10 min, 30 min, or 60 min. The effects of lapatinib was examined by pre-treating the cells with 1 μ M or 5 μ M lapatinib for 1 h prior to treatment with 20 ng/ml AREG for 60 min. At least three independent experiments were performed.

Briefly, cells were lysed in ice-cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After blocking for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), the membranes were

Figure 2. Lapatinib induces cell apoptosis in BeWo cells. A: BeWo and JEG-3 cells were treated with vehicle (dimethyl sulfoxide; DMSO) control, 1 or 10 μ M lapatinib every 24 h. Cell proliferation was examined by MTT assay. B: BeWo cells were treated with 10 μ M lapatinib for 24 h. The protein level of cleaved caspases-3 was examined by western blot. Cell proliferation results are presented as the mean $\pm$ SEM of at least three independent experiments. *Significantly different at $p < 0.05$ compared with the control.

incubated overnight at 4°C with primary antibodies diluted in TBS with 5% non-fat milk. Following primary antibody incubation, the membranes were incubated for 1 h with appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescent substrate or Super Signal West Femto chemiluminescent substrate (Pierce, Thermo Fisher Scientific) and X-ray film.

Statistical analysis. Results are presented as the mean $\pm$ SEM of at least three independent experiments performed with different passages of cells. Multiple group comparisons were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using PRISM software (GraphPad Software, San Diego, CA, USA). Differences in means were considered significant at $p < 0.05$.

Results

EGFR and HER2 are expressed in BeWo and JEG-3 cells. To the best of our knowledge, thus far, the expression levels of EGFR and HER2 in human choriocarcinoma cell lines have not been clearly demonstrated. To examine the expression levels of EGFR and HER2, the total protein extracted from two human choriocarcinoma cell lines, BeWo

and JEG-3, were analyzed by western blot. A human ovarian cancer cell line, SKOV3, which has been reported to express EGFR and high levels of HER2, was used a positive control (21, 22). As shown in Figure 1A, both BeWo and JEG-3 cells expressed EGFR and the protein level of EGFR was higher in JEG-3 cells than that in BeWo cells. Similarly, HER2 protein was detected in both cell lines. Interestingly, BeWo cells expressed a higher level of HER2 when compared with JEG-3 cells (Figure 1B).

Lapatinib inhibits cell proliferation in BeWo, but not in JEG-3 cells. To examine the effect of lapatinib on cell proliferation in human choriocarcinoma cells, BeWo and JEG-3 cells were treated with 1 μ M and 10 μ M of lapatinib every 24 h for 96 h. As shown in Figure 2A, treatment with 1 μ M of lapatinib did not affect proliferation of BeWo cells, while cell proliferation was significantly inhibited by treatment with 10 μ M lapatinib for 48 h, with the greatest inhibitory effect being observed after 96 h of treatment. To our surprise, neither treatment with 1 μ M nor 10 μ M of lapatinib affected proliferation of JEG-3 cells. Treatment with 10 μ M lapatinib

Figure 3. Amphiregulin (AREG) stimulates BeWo cell proliferation and activates extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathways. A: Cells were treated without or with 20 ng/ml AREG every 24 h. The cell proliferation was examined by the MTT assay. B: Cells were treated with 20 ng/ml AREG for the indicated durations. Phosphorylation of ERK1/2 and AKT was determined by western blot using antibodies specific for the phosphorylated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT). Membranes were stripped and re-probed with antibodies to total ERK1/2 and AKT. C: Cells were pre-treated with mitogen-activated protein kinase enzyme (MEK) inhibitor U0126 (10 μ M) or PI3K inhibitor LY294002 (10 μ M) for 1 h and then treated with 20 ng/ml AREG every 24 h for 96 h. Cell proliferation was examined by MTT assay. DMSO: Dimethyl sulfoxide. The cell proliferation results are presented as the mean $\pm$ SEM of at least three independent experiments. *Significantly different at $p < 0.05$ compared to the control (Ctrl).

reduced the basal number of BeWo cells, suggesting its pro-apoptotic effect. To further confirm this, BeWo cells were treated with 10 μ M lapatinib and the expression level of cleaved caspase-3, an important apoptosis marker, was examined by western blot. As shown in Figure 2B, treatment for 24 h with 10 μ M lapatinib significantly induced the expression of cleaved caspase-3. These results agree with a previous study demonstrating the pro-apoptotic effect of lapatinib on other human cancer cells (20).

AREG stimulates cell proliferation by activating ERK1/2 and PI3K/AKT signaling pathways. To date, the effect of AREG on human choriocarcinoma cell proliferation remains

unclear. It has been shown that the concentration of AREG in human amniotic fluid can reach 20 ng/ml (17). Therefore, BeWo cells were treated with 20 ng/ml AREG and the effect of AREG on cell proliferation was examined by MTT assay. As shown in Figure 3A, cell proliferation significantly increased after 72 h and 96 h of AREG treatment. The ERK1/2 and PI3K/AKT signaling pathways are well-established mediators of EGFR. Our results showed that in BeWo cells, treatment with AREG for 10, 30 and 60 min induced the phosphorylation of ERK1/2 and AKT, indicating the activation of these two pathways (Figure 3B). In addition, inhibition of ERK1/2 by pre-treating cells with MEK inhibitor U0126 reduced basal cell proliferation and

abolished AREG-stimulated cell proliferation. Similarly, the basal level of cell proliferation and AREG-stimulated cell proliferation were blocked by pre-treatment with PI3K inhibitor LY294002 (Figure 3C). Together, these results indicate that AREG can stimulate human choriocarcinoma cell proliferation *via* activating ERK1/2 and PI3K/AKT signaling pathways.

Lapatinib blocks AREG-stimulated cell proliferation by attenuating ERK1/2 and PI3K/AKT signaling pathways. Our results showed that treatment with low concentration (<10 μ M) of lapatinib alone did not induce apoptosis of BeWo cells. Therefore, we examined whether low concentrations (1 μ M and 5 μ M) of lapatinib affected AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways as well as AREG-stimulated cell proliferation. As shown in Figure 4A, pre-treatment of BeWo cells with 1 μ M of lapatinib partially attenuated AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways. The AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways was totally blocked by pre-treatment with 5 μ M of lapatinib. MTT assay results further showed that treatment with 1 μ M or 5 μ M of lapatinib blocked AREG-stimulated BeWo cell proliferation (Figure 4B).

Discussion

Lapatinib has been shown to inhibit cell proliferation and induce cell apoptosis of different types of human cancer cells. However, its effects on human choriocarcinoma cells remain unknown. The present study, for the first time, showed that treatment with lapatinib alone reduced cell proliferation by inducing apoptosis of BeWo cells. In addition, we demonstrated that AREG stimulated BeWo cell proliferation by activating ERK1/2 and PI3K/AKT signaling pathways. Inhibition of EGFR/HER2 by treatment with lapatinib blocked the AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways as well as AREG-stimulated cell proliferation.

It is interesting to note that treatment with lapatinib did not affect proliferation of JEG-3 cells. It has been shown that the PI3K/AKT signaling pathway appears to be more dependent upon HER2 than EGFR signaling (23). Our results showed that the expression of HER2 was higher in BeWo cells than that in JEG-3 cells. In addition, compared to MEK inhibitor, treatment of cells with PI3K inhibitor induced a greater decrease in basal cell proliferation. Therefore, the low expression of HER2 and low activation of HER2-mediated PI3K/AKT signaling pathway in JEG-3 cells may be why lapatinib was unable to induce apoptosis in this cell line. The induction of apoptosis by lapatinib might, in part, be dependent upon the degree of HER2 expression and activation of the AKT signaling pathway.

Figure 4. Lapatinib (Lap) blocks amphiregulin (AREG)-induced activations of extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathways and AREG-stimulated cell proliferation in BeWo cells. A: Cells were pre-treated with 1 or 5 μ M lapatinib for 1 h and then treated with 20 ng/ml AREG for 60 min. Phosphorylation of ERK1/2 and AKT was determined by western blot using antibodies specific for the phosphorylated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT). Membranes were stripped and reprobed with antibodies to total ERK1/2 and AKT. B: Cells were pre-treated with 1 or 5 μ M lapatinib for 1 h and then treated with 20 ng/ml AREG every 24 h for 96 h. Cell proliferation was examined by the MTT assay. DMSO: Dimethyl sulfoxide. Cell proliferation results are presented as the mean $\pm$ SEM of at least three independent experiments. *Significantly different at $p < 0.05$ compared with the control (Ctrl).

EGFR signaling is known to regulate cell proliferation, motility, and invasion of many types of cancer (24, 25). EGFR is up-regulated in human gestational trophoblastic diseases including choriocarcinoma (12, 26, 27). Among EGFR ligands, AREG is the most highly expressed in amniotic fluid during human pregnancy. In addition, AREG treatment was found to stimulate human chorionic gonadotropin production in BeWo cells (17). Our study demonstrated that treatment with recombinant human AREG stimulated BeWo cell proliferation.

Similarly to our previous studies on other cell types (28, 29), AREG treatment activated ERK1/2 and PI3K/AKT signaling pathways in human choriocarcinoma cells. We also reported that AREG-stimulated cell proliferation required the activation of ERK1/2 and PI3K/AKT signaling pathways. ERK1/2 is well known as a signaling pathway that regulates cancer cell proliferation and survival. In many types of human cancers, ERK1/2 signaling pathway has been found to be activated (30). The activation PI3K/AKT signaling pathway is involved in protecting cells from apoptosis. In cancer cells, constitutive activation of AKT signaling pathway has been implicated as a mechanism of resistance to cytotoxic chemotherapies (31). Our results showed that lapatinib blocked AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways in BeWo cells. These findings indicate that lapatinib acts as a potent EGFR/HER2 inhibitor, which can lead to cell growth arrest and apoptosis of human choriocarcinoma cells. Therefore, targeting EGFR might be a useful therapeutic strategy for the prevention of human choriocarcinoma development and progression

In summary, we demonstrated that treatment with lapatinib alone induced apoptosis of human choriocarcinoma cells with high HER2 expression. In addition, we showed the stimulatory effect of AREG on cell proliferation. Moreover, AREG-stimulated cell proliferation was found to require the activation of ERK1/2 and PI3K/AKT signaling pathways. Furthermore, blocking EGFR/HER2 by lapatinib inhibited AREG-induced activation of ERK1/2 and PI3K/AKT signaling pathways as well as AREG-stimulated cell proliferation. The present study provides important insight into the molecular mechanisms mediating AREG-stimulated cell proliferation.

Conflicts of Interests

The Authors declare that they have no competing interests in regard to this study.

Authors' Contributions

LVC, JCC, PCKL and ISB conceived and designed the experiments; LVC, YY, LS and EC performed the experiments and analyzed the data; LVC, PCKL and ISB wrote the article. All Authors read and approved the final article.

Acknowledgements

This work was supported by a Foundation Grant from the Canadian Institutes of Health Research to P.C.K.L (FDN-143317).

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Received January 31, 2019

Revised April 10, 2019

Accepted April 11, 2019