

# The receptor tyrosine kinase inhibitor vandetanib activates Akt and increases side population in a salivary gland tumor cell line (A253)

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Received January 5, 2012; Accepted March 7, 2012

DOI: 10.3892/ijo.2012.1434

**Abstract.** We and others have reported that cancer side population (SP) cells have self-renewal and multidrug resistance capabilities. These phenotypes are similar to those of cancer stem cells (CSCs), cancer stem-like cells and tumor-initiating cells (TICs). It has also been reported that upregulation of the epidermal growth factor receptor (EGFR) significantly increases the number of cancer SP cells, conversely, molecular targeting of EGFR tyrosine kinases using specific kinase inhibitors downregulates CSCs. Thus, we used flow cytometric analysis and cell sorting to examine cancer SP cells in the SCA9.cl-15, WR21 and A253 cell lines that originate from a salivary gland tumor (SGT). We successfully isolated cancer SP cells from all of these cell lines. SP cells were detected following treatment of these cell lines with the receptor tyrosine kinase inhibitors (RTKIs) lapatinib, erlotinib and vandetanib. Several studies reported that RTKIs mostly reduced the SP population in cancer cells. We did not observe any detectable morphological differences between SP cells and non-SP cells. We found that the EGF RTKI lapatinib decreased the number of cancer SP cells in all cell lines investigated; however, the EGF RTKI erlotinib did not cause significant differences in the frequency of cancer SP cells in these cell lines. Addition of vandetanib significantly increased the number of cancer SP cells and upregulated the phosphorylated Akt. As far as we know, this is the first report to show that one of the RTKIs, vandetanib, can activate Akt and increase the number of cancer SP cells. It has been reported that RTKIs could competitively

inhibit ABC transporters and subsequently reduced the number of SP cells. However, our observation indicated that signaling changes induced by RTKIs could even activate Akt and induce the SP population. Investigation of the SP phenotype of SGTs is important for the establishment of optimal cancer therapy.

## Introduction

Many recent studies have suggested that tumor initiating cells (TICs), as well as cancer stem cells (CSCs) or cancer stem-like cells, can be isolated from tumor tissues (1-5). A few TICs are considered to have CSC-characteristics. These cells reside long-term in tissues and would have to self-renew and clonally expand in order to initiate and drive tumor growth. The presence of CSCs in tissues is supported by many reports and many cancers contain a small but significant proportion (~1%) of CSCs (6). Moreover, there is increasing evidence to suggest that metastases develop when distant organs are seeded with CSCs. This result implicates CSCs in the initiation and growth of metastatic lesions. CSC-mediated metastases may account for the failure of current anti-tumor treatments to eradicate advanced tumors. Thus, current systematic cancer therapies fail due to their inability to effectively eliminate CSCs (7). There are two reasons for the resistance of CSCs to current anti-tumor therapies. First, CSCs proliferate only infrequently, which would make them relatively resistant to anti-proliferative agents because most current anti-cancer agents rapidly kill proliferating cells. Second, CSCs express multidrug resistant and anti-apoptotic proteins, which further contribute to their resistance to anti-cancer agents. Therefore current chemotherapeutic approaches are not sufficient for elimination of CSCs and an ideal anti-cancer agent should selectively target CSCs. Molecular targeted therapy is a recent development in cancer therapy, and this approach shows great promise for the design of anti-tumor treatments. Receptor tyrosine kinases are attractive targets for drug development since they have been extensively studied and have well described oncogenic properties (8-12). There are currently two major effective anti-tyrosine kinase receptor treatment strategies. One strategy is antibody therapy that targets the extracellular domain of the receptor and another

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**Key words:** cancer stem cells, side population cell, tyrosine kinase inhibitor, epidermal growth factor receptor, vascular endothelial growth factor receptor, PI3K

strategy is the use of small molecules that compete with ATP for tyrosine kinase activation (13,14). There are extensive clinical trial data regarding the use of small-molecule inhibitors of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) for many cancers, which have shown several advantages in the use of these molecules for chemotherapy (15-18).

We previously reported that a cancer cell line derived from a head and neck cancer contained side population (SP) cells (19). These cancer SP cells showed self-renewal capacity and generated both SP and non-SP cells. The SP cells derived from cancer cell line also expressed high levels of ABC transporter proteins, which are related to multidrug-resistance (MDR) proteins. The SP cells were quite resistant to anticancer agents, including 5-fluorouracil and carboplatin, and upregulated expression of anti-apoptosis-related genes. We also reported that transforming growth factor- $\beta$  dramatically increases the cancer SP cell population and that this increase is accompanied by upregulation of EGFR expression (20).

Hence, we show that inhibitors of EGFR and VEGFR, molecular targeting agent, possibly regulate cancer SP phenotype, one of the candidates for CSCs or TICs, by regulating signal transductions initiated by EGF or VEGF. Malignant salivary gland tumors (SGT) are relatively rare cancers, accounting for less than 1% of all cancers and 6 to 7% of head and neck tumors. SGTs display a heterogeneous histology, are aggressive, and show varied responsiveness to current therapies.

We considered that CSCs should be targeted for anti-tumor therapy of SGTs, since the presence of CSCs in different kind of cancers, regardless of their origin, is believed to be critical for tumor development. Since receptor tyrosine kinase inhibitors are potential candidate tools for effective elimination of CSCs, this study focused on the effects of receptor tyrosine kinase inhibitors on CSCs of SGT.

## Materials and methods

**Tyrosine kinase inhibitors (TKI).** Vandetanib (Zactima, ZD6474), erlotinib (Tarceva, OSI-774) and Lapatinib (Tykerb, GW572016) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Each TKI was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM.

**Cell lines and cell culture.** The A253 (human salivary gland epidermoid carcinoma), WR21 (salivary gland tumor type in was-rap transgenic mice) and SCA9.cl-15 (SCA, mouse submandibular gland undifferentiated carcinoma) cell lines were purchased from the American Type Culture Collection (ATCC) and were cultured on 100 mm dishes in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% penicillin and streptomycin (Invitrogen) and 10 mM (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES buffer) (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Flow cytometry.** The cells were removed from the culture dishes with 0.25% trypsin-EDTA (Invitrogen), were pelleted by centrifugation at 1,200 rpm, washed with phosphate-buffered saline (PBS), and resuspended in 37°C Hank's balanced salt solution (HBSS) (Sigma) containing 2% FBS and 10 mM

Table I. FACS analysis of SP cells population.

	SP cell population (%) $\pm$ SD
A253	0.4 $\pm$ 0.3
WR21	1.5 $\pm$ 0.4
SCA	4.3 $\pm$ 0.8

The percentage of SP cells in the total population of each cell line (% SP). Data are the means  $\pm$  SD of at least three independent experiments each performed in triplicate. Cancer SP abundance varied between each cell line examined from 0.4 $\pm$ 0.3% (A253) to 4.3 $\pm$ 0.8% (SCA9.cl-15).

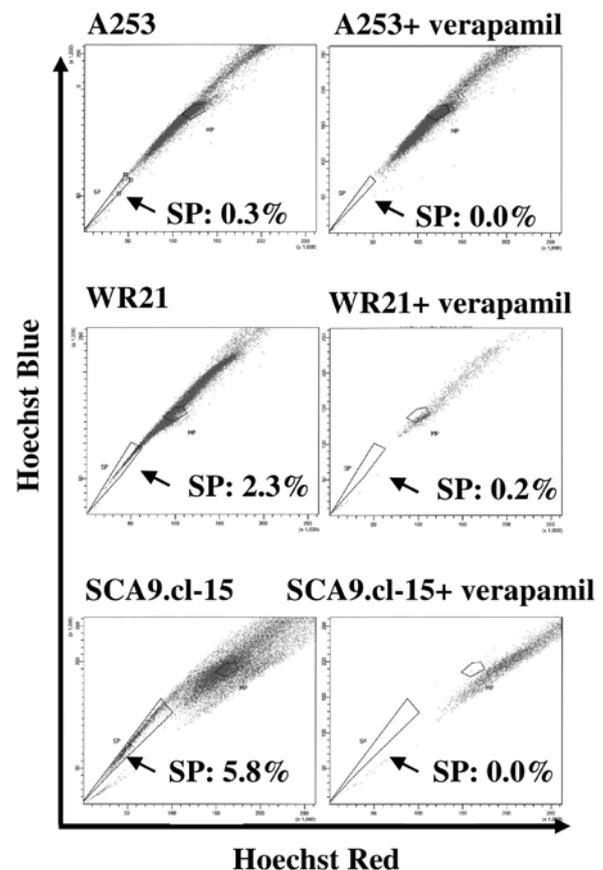


Figure 1. Salivary gland tumors contain SP cells. The salivary gland tumor cell lines, SCA9.cl-15 (SCA), A253 and WR21, were stained with Hoechst 33342 and analyzed by dual-wave flow cytometry. SP cells with low blue/red fluorescence were detected in all cell lines. (A) The outlined area indicates the SP fraction. The percentage of the total cell population that is composed of SP cells is shown. (B) Verapamil treatment significantly reduced the number of SP cells, indicating their dependence on ABC transporter activity. Each sample is representative of 9 samples assayed.

HEPES buffer. The cells (1 $\times$ 10<sup>6</sup> cells/ml) were stained with various concentrations of Hoechst 33342 (Sigma). The stained cells were incubated for 90 min with or without 50  $\mu$ M verapamil. The cells were then washed with PBS, resuspended in HBSS containing 2% FBS and 10 mM HEPES, passed through a 40- $\mu$ m mesh filter, and maintained at 4°C until analysis. Dead cells were excluded on the basis of propidium iodide staining

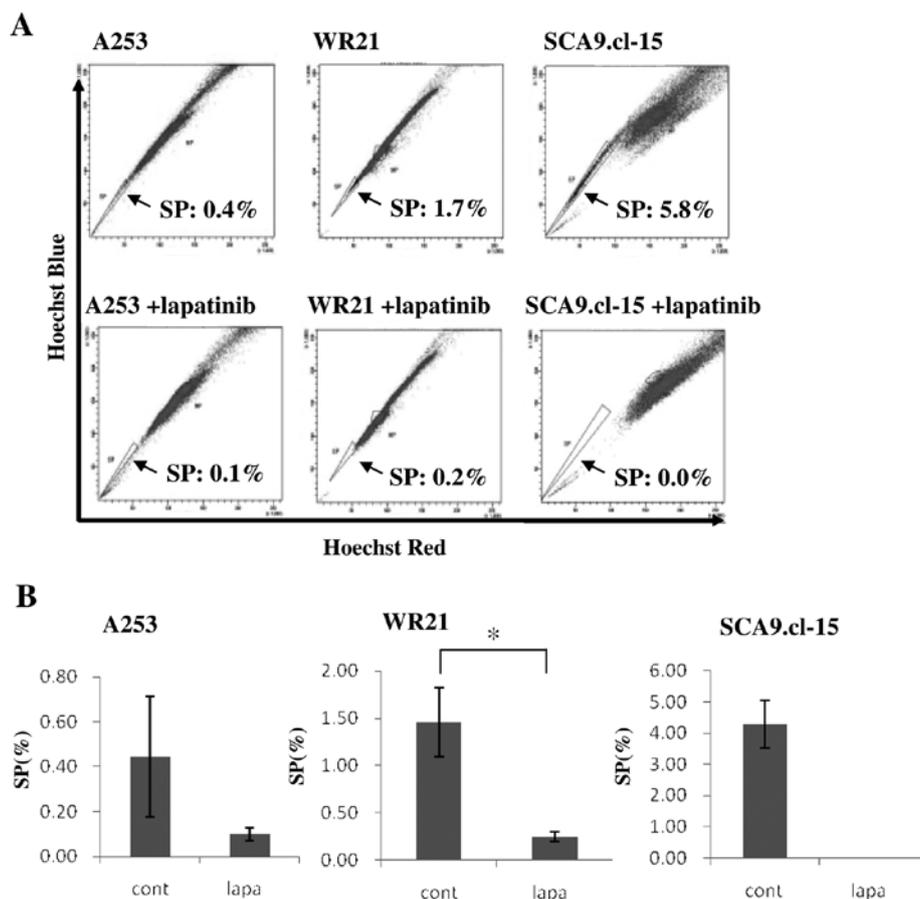


Figure 2. FACS analysis of the effect of lapatinib ( $5 \mu\text{M}$ ) on the SP cell-population. The effect of lapatinib on SP populations of SGT cell lines was determined by FACS analysis of cells treated with or without  $5 \mu\text{M}$  lapatinib for 48 h. Lapatinib treatment drastically reduced the number of cancer SP cells in all SGT cell lines. (A) Representative examples of the  $5 \mu\text{M}$  lapatinib-induced decrease in the percentage of SP cells within the total population of each cell line. Each sample is representative of 9 samples assayed. (B) Quantification of the SP cell population following treatment of the cell lines with DMSO (cont) or  $5 \mu\text{M}$  lapatinib (lapa) for 48 h. At least three independent experiments were performed in triplicate for each cell line. Error bars are means  $\pm$  SD. \* $P < 0.05$ . Lapatinib essentially abolished SP cells in the SCA cell line.

( $2 \mu\text{g/ml}$ ) and forward wave scatter. Flow cytometric analysis and cell sorting (FACS) were performed using a dual-laser FACS Aria (Beckton-Dickinson, San Jose, CA, USA). The Hoechst dye was excited at 350 nm and the fluorescence was measured at two wavelengths, Hoechst blue and red, using 450/50 nm (Hoechst blue) and 530/30 nm (Hoechst red) band pass filters. Hoechst 33342 blue and red fluorescence values are shown on a linear scale and a low value indicates the side population cells.

**Tyrosine kinase inhibitor treatment.** The cells were seeded at a density of  $2.5 \times 10^6$  cells in 100-mm dishes in culture medium as described above at  $37^\circ\text{C}$  in an atmosphere containing 5%  $\text{CO}_2$ . After 24 h culture, when the cells were confirmed to be 70% confluent, the TKIs (erlotinib, lapatinib or vandetanib) were added at a final concentration of  $5 \mu\text{M}$  into each culture dish, and the culture was continued as described above for 48 h. The cells were then removed from the dishes and were analyzed by FACS or by western blotting. Cells cultured with DMSO were used as a control.

**Protein extraction and immunoblotting.** The cells were cultured in 60-mm dishes until 70% confluent. The culture medium was

then changed to medium without serum. After 24 h culture, each TKI, or control DMSO, was added at a final concentration of  $5 \mu\text{M}$  and the cells were cultured for a further 24 h. The cells were then washed once with ice-cold PBS, and immediately lysed with lysis buffer [20 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate and complete protease inhibitor mixture (Roche, Basal, Switzerland)]. The protein extracts were collected by centrifugation at  $12,000 \times g$  for 15 min at  $4^\circ\text{C}$ . Equivalent protein amounts were resolved on NuPAGE 5% Tris-Acetate gels (Invitrogen) and were then electrophoretically transferred to PVDF membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in TBST buffer (0.1 M Tris pH 7.6, 0.5 M NaCl, 0.1% Tween-20) for 1 h at room temperature. The membranes were probed with the appropriately diluted primary antibody in TBST (anti-Akt rabbit monoclonal antibody, anti-phosphorylated (P)-Akt rabbit monoclonal antibody, anti-ERK1, anti-ERK2 or anti-P-ERK1/2 rabbit monoclonal antibody; all purchased from Cell Signaling Technology Inc., Danvers, MA, USA) followed by alkaline phosphatase (AP)-conjugated secondary antibody diluted in TBST containing 5% BSA. Bound antibodies were visualized using 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt/nitro blue tetrazolium chloride BCIP/NBT (Roche).

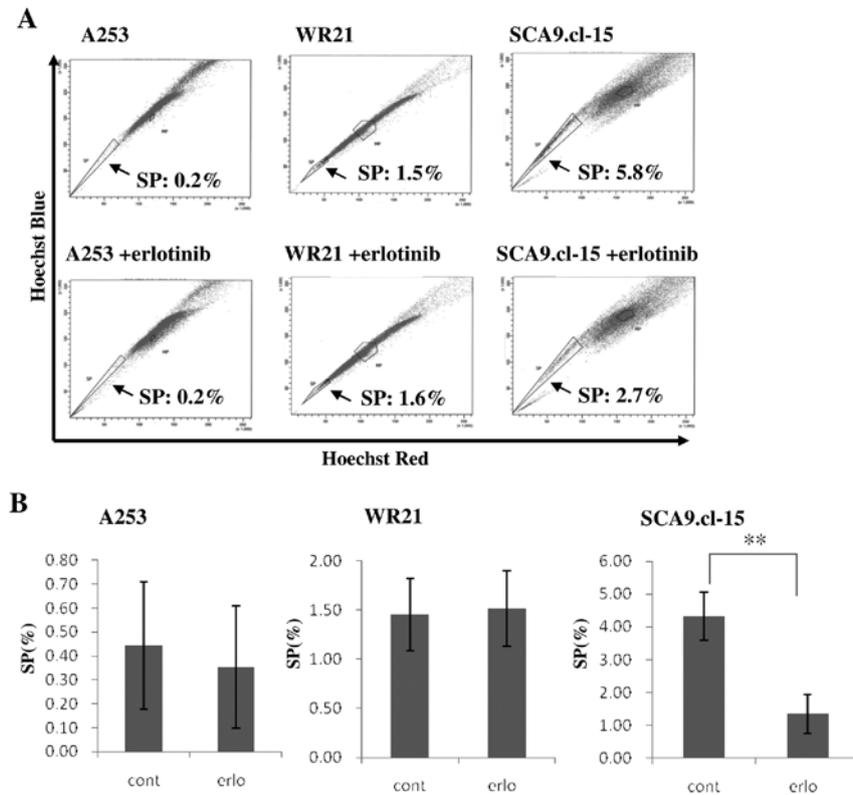


Figure 3. FACS analysis of the effect of erlotinib (5  $\mu$ M) on the SP cell-population. The effect of erlotinib on SP cells was assayed as for lapatinib. (A) Representative examples of the change in the percentage of SP cells within the total population of each cell line following treatment with 5  $\mu$ M erlotinib. Each sample is representative of 9 samples assayed. Erlotinib only dramatically reduced the % of SP cells in the SCA cell line. (B) Quantification of the SP cell population following treatment of the cell lines with DMSO (cont) or 5  $\mu$ M erlotinib (erlo) for 48 h. At least three independent experiments were performed in triplicate for each cell line. Error bars are means  $\pm$  SD. \*\* $P < 0.01$ .

**Statistical analysis.** Results are reported as means  $\pm$  SD. Student's t-test was used to analyze statistical differences between groups. Statistical significance was defined as  $p < 0.05$ .

## Results

**Isolation of SP cells.** We used the Hoechst 33342 dye exclusion technique to identify SP cells in SGT cell lines. FACS analysis clearly defined a cancer SP that constituted  $0.4 \pm 0.2\%$  of A253 cells,  $1.5 \pm 0.4\%$  of WR21 cells and  $4.3 \pm 0.8\%$  of SCA cells (Table I). We verified the SP identity of these cells by treatment of the cells with the calcium-channel blocker verapamil hydrochloride followed by FACS analysis (Fig. 1). Verapamil hydrochloride blocks the membrane pump responsible for the SP phenotype. Each experiment was performed at least in triplicate unless otherwise specified. These observations suggested that the cell lines studied do contain SP cells. We could detect no obvious morphological difference between SP and non-SP cells. We also could not identify any cell morphological changes in freshly sorted SP and non-SP cells after seeding and culture for 24 or 48 h, or for 8 days.

**Effect of the EGF receptor tyrosine kinase inhibitors, lapatinib and erlotinib on cancer SP cells.** Since several reports have shown that EGFR inhibition remarkably reduces the size of cancer SP cell populations, we evaluated the effect of two different selective EGF-receptor tyrosine kinase inhibitors,

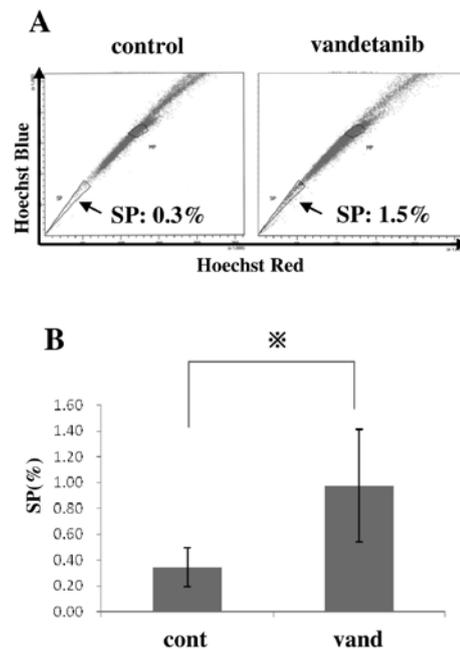


Figure 4. Vandetanib (5  $\mu$ M) increases the percentage of SP cells in the A253 cell line. The effect of vandetanib on SP cells was assayed as for lapatinib. (A) Representative examples of the change in the percentage of SP cells within the total population of the A253 cell line following treatment with 5  $\mu$ M vandetanib. Each sample is representative of 9 samples assayed. (B) Quantification of the SP cell population following treatment of A253 cells with DMSO (cont) or 5  $\mu$ M vandetanib (vand) for 48 h. Three independent experiments were performed in triplicate. Error bar are means  $\pm$  SD. \* $P < 0.05$ .

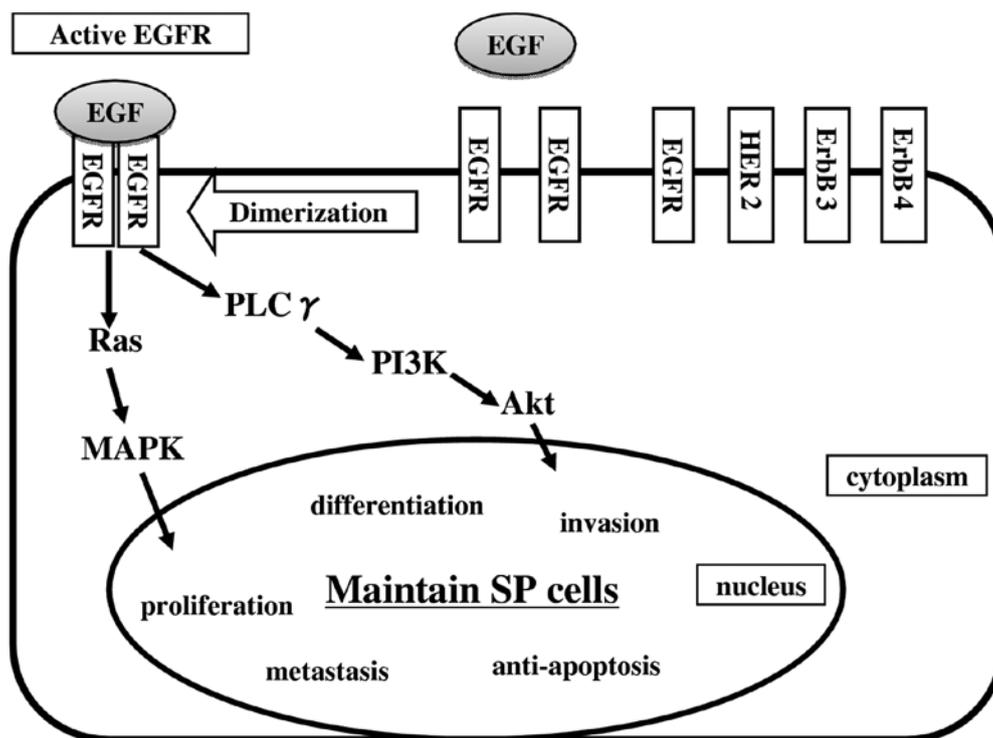


Figure 5. Schematic representation of EGF-receptor family signaling involved in SP cell maintenance. The two key EGFR-family induced signaling pathways believed to be involved in SP cell maintenance are illustrated. These pathways are the MAPK cascade and the PI3K pathway. These pathways are induced by all of the EGF receptor family members shown; EGFR, HER2, ErbB3 and ErbB4. Our experiments using A253 cells indicate that the PI3K pathway plays an important role in maintenance of the SP population.

lapatinib and erlotinib, on SGT cancer cell lines using side population analysis. Lapatinib ( $5 \mu\text{M}$ ) treatment drastically reduced the number of cancer SP cells in all cell lines. Thus, the number of cancer SP cells was significantly lower in A253 cells treated with  $5 \mu\text{M}$  lapatinib than in the DMSO-treated control ( $0.4 \pm 0.3\%$  versus  $0.1 \pm 0.0\%$ ). SCA cells treated with DMSO had  $5.8 \pm 0.8\%$  cancer SP cells whereas SCA cells treated with  $5 \mu\text{M}$  lapatinib had essentially no cancer SP cells. WR21 cells treated with DMSO had  $1.5 \pm 0.4\%$  cancer SP cells, while after treatment with  $5 \mu\text{M}$  lapatinib, only  $0.2 \pm 0.0\%$  cancer SP cells were detected (Fig. 2). Treatment with  $5 \mu\text{M}$  erlotinib induced little, if any, reduction of SP cells in either A253 or WR21, but did decrease the number of SP cells in the SCA cell line compared to DMSO-treated control cells (Fig. 3).

*Effect of the EGF receptor tyrosine kinase inhibitor, vandetanib on cancer SP cells.* Vandetanib treatment had no significant effect on the level of SP cells in WR21 cells or in SCA cells. However, we found significantly more SP cells in A253 that were treated with  $5 \mu\text{M}$  vandetanib than in A253 that were treated with DMSO (Fig. 4 and Table II).

*Western blot analysis of P-Akt and P-ERK1/2.* Signal transduction pathways are initiated when the EGF receptor tyrosine kinase is activated. One of the major EGF receptor-induced signal transduction pathways involves the activation of phospholipase C $\gamma$  (PLC $\gamma$ ), Src, PI3K and Akt (Fig. 5). Several reports have indicated that the PI3K and Akt pathway regulates the activity of ABCG2, which is the key ABC transporter for

Table II. FACS analysis was performed in the control (DMSO) or vandetanib in three cell lines.

	SP cell population (%) $\pm$ SD	
	Control	Vandetanib
A253	$0.3 \pm 0.2$	$1.0 \pm 0.4$
WR21	$3.7 \pm 1.5$	$3.7 \pm 2.2$
SCA	$1.0 \pm 0.4$	$1.3 \pm 1.3$

Vandetanib increases the SP cell population in A253 cells. The effect of  $5 \mu\text{M}$  Vandetanib or control DMSO on the SP population of the three tumor cell lines was analyzed by FACS analysis. Data are the means  $\pm$  SD of at least three independent experiments each performed in triplicate.

Hoechst 33342 and the SP phenotype. To investigate PI3K activation in TKI-treated cells, we performed western blot analysis of P-Akt. We found that vandetanib induced significant activation of PI3K as judged by a significant increase in P-Akt in A253 cells compared to the DMSO-treated cells. However, treatment with  $5 \mu\text{M}$  vandetanib reduced the level of P-Akt in WR21 (Fig. 6). A second major pathway that is modulated by EGFR activation is the MEK-ERK pathway. We also determined whether vandetanib treatment modulated this pathway. We detected a slight reduction in P-ERK1/2 in all cells that were treated with vandetanib compared to DMSO controls, but this decrease was very small in all cases.

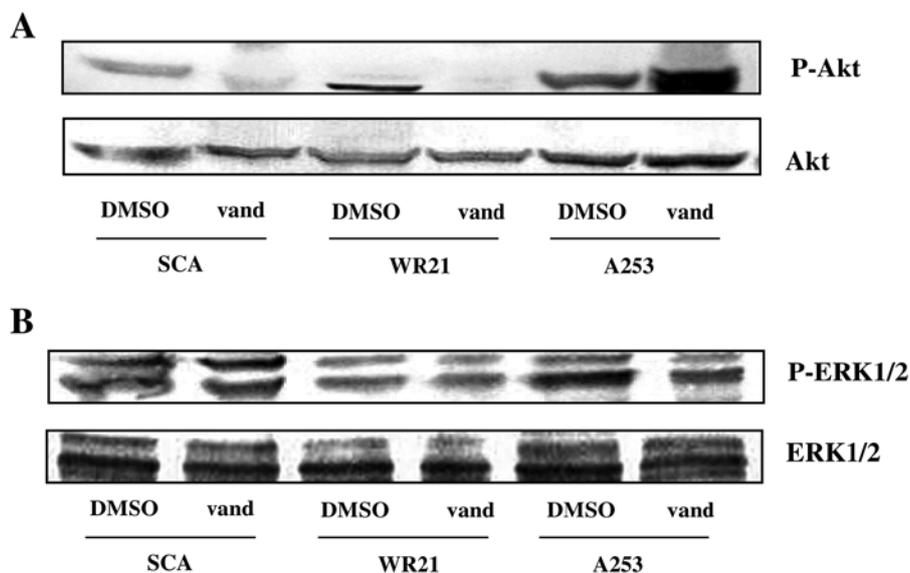


Figure 6. Effect of vandetanib treatment on the phosphorylation of Akt and ERK1/2 in the three tumor cell lines. The effect of 5  $\mu$ M vandetanib (vand), or control DMSO, treatment on the level of phosphorylated (P-)Akt (A) and P-ERK1/2 (B) in the three tumor cell lines was analyzed by western blotting. The total level of Akt and ERK1/2 protein is shown at the bottom. Vandetanib only upregulated P-Akt in A253 cells and had no discernable effect on P-ERK1/2 in any cell line.

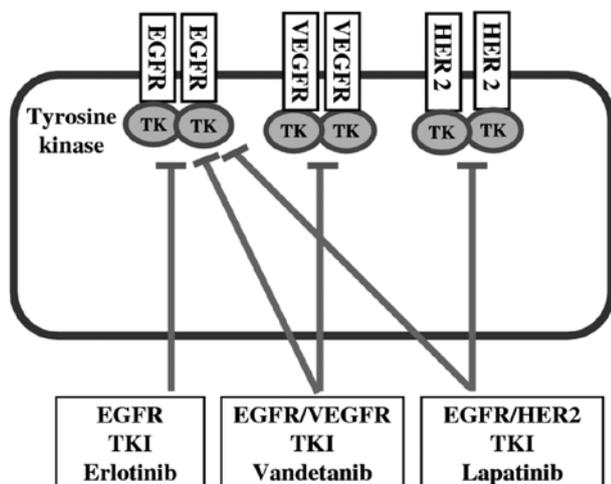


Figure 7. Schematic representation of the dependence of receptor tyrosine kinase inhibition on the affinity of the RTKI for each receptor. Erlotinib, lapatinib and vandetanib have similar molecular structures and they competitively block receptor tyrosine kinases. However, despite this fact, their specificity and affinity for receptors are different as outlined in the scheme.

## Discussion

In this study we found that the effect of TKIs on SP cells may depend on the cellular origin of the side population. These data suggest that the use of TKI for targeted therapy of SP cells requires careful selection of target SP cells.

Molecular targeted therapy is designed to inhibit key signaling pathways in various malignant tumors that are involved in tumor growth and metastasis. The TKIs used in this study inhibit one of the two key molecular targets: EGFR and VEGFR tyrosine kinases (21-24). Several reports have shown that EGFR tyro-

sine kinase inhibitors (gefitinib, erlotinib and lapatinib) reverse chemotherapy resistance by blocking ABC transporters (25-29). These kinase inhibitors block ATP-binding by competitively binding to its binding sites. Because the molecular mechanisms of ATP-binding sites of different molecules are similar, one of the major reasons why EGFR kinase inhibitors inhibit ABC transporter effects may be due to direct binding of the EGFR kinase inhibitors to the ATP-binding sites of ABC transporters. However, we found that not all of these EGF receptor tyrosine kinase inhibitors reduced the number of cancer SP cells. This result indicated that some, but not all, EGF receptor tyrosine kinase inhibitors can inhibit ABC transporters and reduce the cancer SP cell population. Our data support the possibility that TKIs function through a mechanism other than through direct inhibition of ABC transporters. One such potential mechanism is through inhibition of signal transduction initiated by the EGFR or the VEGFR. As shown in Fig. 7, two key signaling pathways are involved. One pathway is the MAPK cascade (30) and the second is the PI3K pathway (31,32). We found that vandetanib treatment induced the upregulation of PI3K activity in A253 cells, as assessed by an increase in P-Akt (Fig. 7). Bleau *et al* reported that the PI3K/Akt pathway regulates the cancer SP phenotype and ABC transporter activity (32). This increased ABC transporter activity caused an increase in the cancer SP cell population that was accompanied by an increase in P-Akt. Our results suggested that vandetanib induced P-Akt upregulation in A253 resulting in cancer SP phenotypes. Erlotinib, lapatinib and vandetanib have similar molecular structures and they all probably competitively block the EGF-receptor tyrosine kinase, despite the fact that their receptor specificity and affinity are different. Erlotinib only binds to the EGFR (ErbB1). Lapatinib binds to both the EGFR and HER2 (ErbB2), another major receptor for EGF. Vandetanib binds to the EGFR (ErbB1) and the VEGFR (22). All these molecular targeted agents were reported to show clinical promise for the elimination of CSCs.

In particular, Choi *et al* showed that vandetanib inhibited the growth of adenoid cystic carcinomas derived from the salivary gland (16). However, our results suggested that vandetanib was the least effective of all of the TKIs tested in reducing cancer SP populations, especially in A253 salivary gland epidermoid carcinoma, and vandetanib may even upregulate these SP populations. These results indicated that vandetanib may not always be effective for eradication of CSCs. As far as we can ascertain, this is the first report that shows that RTKIs can upregulate P-Akt and cancer SP phenotypes. We described above that vandetanib binds and inhibits both EGFR and VEGFR, but, it could activate Akt and increase SP population in A253 cells. These results suggest that EGF receptor-mediated signal transduction plays an important role in the regulation of cancer SP phenotypes that have high chemoresistance.

In conclusion, vandetanib actually increased the number of cancer SP cells in A253 derived from salivary gland and this increase was accompanied by upregulation of PI3K. Therefore, vandetanib has the potential to promote chemoresistance in certain cell types. Investigation of the cancer SP phenotype of SGT may therefore be important for determination of the optimum chemotherapy of these tumors.

#### Acknowledgements

We thank Ms. A. Yokoyama for her excellent technical assistance. This research was supported by an HRC7 Oral Health Science Center grant from Tokyo Dental College, by the High-Tech Research Center Project for Private Universities, and by a matching subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2007-2010 (No. 19592414).

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