Generation of osimertinib-resistant cells from epidermal growth factor receptor L858R/T790M mutant non-small cell lung carcinoma cell line

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Abstract

Background: Lung cancer contributes to high cancer mortality worldwide with 80% of total cases diagnosed as non-small cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) tyrosine kinase (TK) domain serves as a druggable target in NSCLC patients with exon 19 deletion and L858R mutation. However, patients eventually succumbed to resistance to first- and second-generation EGFR-TK inhibitors through activation of T790M mutation. Third-generation EGFR-TKI, Osimertinib exhibits high efficacy in patients with exon 19 deletion/L858R/T790M mutation but they experienced acquired resistance thereafter. Available treatment options in NSCLC patients remains a challenge due to unknown molecular heterogeneity responsible for acquired resistance to EGFR-TKI. In this study, we aim to generate Osimertinib-resistant (OR) cells from H1975 carrying L858R/T790M double mutation which can be used as a model to elucidate mechanism of resistance.

Methods: OR cells were established via stepwise-dose escalation and limiting single-cell dilution method. We then evaluated Osimertinib resistance potential via cell viability assay. Proteins expression related to EGFR-signalling, epithelial to mesenchymal transition (EMT), and autophagy were analyzed via western blot.

Results: OR cell lines exhibited increased drug resistance potential compared to H1975. Distinguishable mesenchymal-like features were observed in OR cells. Protein expression analysis revealed EGFR-independent signaling involved in the derived OR cells as well as EMT and autophagy activity.

Conclusion: We generated OR cell lines *in-vitro* as evidenced by increased drug resistance potential, increased mesenchymal features, and enhanced autophagy activity. Development of Osimertinib resistance cells may serve as *in-vitro* model facilitating discovery of molecular aberration present during acquired mechanism of resistance.

Keywords: Acquired mechanism of resistance; Epidermal growth factor receptor; Non-small cell lung cancer; Osimertinib; Tyrosine kinase inhibitors

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide in both men and women accounting for 18.4% out of 9.6 million total cancer deaths reported in 2018. In fact, mortality rate of lung cancer reported is greater than those from any other types of malignancy, followed by stomach cancer (8.2%), liver cancer (8.2%), and colon cancer (5.8%). Small cell lung cancer and non-small cell lung cancer (NSCLC) are two major types of lung cancer diagnosed in patients.[1] Approximately, 85% of total lung cancer cases documented are NSCLC and histologically sub-classified into adenocarcinoma (40%) and squamous cell carcinoma (30%), and large cell carcinoma (15%).[1,2] Most NSCLC patients are often diagnosed with lung adenocarcinoma at an advanced stage.[3–5]

Multiple genetic mutations (KRAS, ALK, MET, ROS1, HER2, BRAF, MEK, PIK3CA, and NTRK1)[6] were identified in NSCLC and among these mutations, the second most common mutation contributed by activation of epidermal growth factor receptor

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Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2021) 84: 248-254.

Received August 3, 2020; accepted September 18, 2020.

doi: 10.1097/JCMA.0000000000000438.

(EGFR) in the tyrosine kinase domain (TKD).[7,8] EGFR-TKD confer sensitivity to tyrosine kinase inhibitors (TKI) and therefore serves as a druggable target in NSCLC patients.[9,10] Exon 19 deletion and L858R point mutation (Exon 21) are predominant EGFR mutation subtypes detected in NSCLC patients which grant higher survival rate as they respond well to gefitinib and erlotinib (first generation) and afatinib (second generation) EGFR-TKI.[11] However, due to mechanism of resistance, patients failed to respond to first- and second-generation EGFR-TKI following activation of secondary mutation, T790M in TKD.[12] Third-generation EGFR-TKI (Osimertinib/AZD9291), possess high efficacy in patients with T790M mutation[13] but again these patients acquired resistance and showed insufficient response to Osimertinib after 10–12 months of receiving treatment.[14–16] To date, increasing frequency of tertiary EGFR mutations (C797S, L718/G719, G796/C797, L792, and L798) had been reported as Osimertinib resistance was demonstrated in NSCLC patients.[17–19]

At present, treatment options in NSCLC patients have proven to be ineffective due to unknown molecular mechanism of acquired resistance.[20,21] Although there are a number of EGFR-TKI resistant cell lines being developed, yet the exact mechanism of resistance in NSCLC is not well defined.[21] Here, in our study, we derived Osimertinib-resistant (OR) cell lines from H1975 harboring double mutation (EGFR L858R/T790M). The resistant cell lines developed in this study exhibited higher OR potential and epithelial to mesenchymal transition (EMT) morphological features. Nevertheless, our resistant cell lines exhibit pro-survival function via EGFR-independent signaling pathways. Further characterization of the OR cell lines and its extensive evaluation before being employed in our future studies for elucidation of mechanism of Osimertinib-resistance in NSCLC are currently being undertaken.

2. METHODS

2.1. Cell culture maintenance of non-small cell lung cancer cell line (H1975)

H1975 cell line was maintained in Roswell Park Memorial Institute Medium (RPMI-1640) growth medium supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY), 4 mM L-glutamine (Gibco/Invitrogen), and 1% penicillin-streptomycin (Gibco/Invitrogen) at 37°C in the presence of 5% CO_2. The cells were sub-cultured every 3 days upon reaching 80% confluency.

2.2. Generation of Osimertinib-resistant cell lines

Approximately, 1 × 10^6 H1975 cells were seeded in 10 cm2 cell culture dish. Osimertinib concentration starting from 500 nM was used to treat the cells. Medium changed every 2 days and exposure dose was increased by 500 nM every 15 days until the final concentration of 1.5 μM was achieved. Osimertinib treated cells were maintained for 2 months, able to resume normal growth and proliferate without major cell death. Heterogenous H1975 Osimertinib treated cells were further seeded in 96-well plate using limiting dilution. We selected three single clones from respective single well of 96-well plate (OR3, OR4, and OR6 clones). All clones were maintained RPMI complete medium supplemented with 1.5 μM Osimertinib (4 months).

2.3. Drug sensitivity assay

Resistant cell lines seeded at a density of 3 × 10^3 cells/well and Osimertinib concentrations ranging from 0.001 to 10 μM and DMSO were added a day after cell seeding. Osimertinib treated cells incubated at 37°C in the presence of 5% CO_2. AlamarBlue (Thermo Fisher Scientific, Waltham, MA) was added to the culture medium with further incubation for 3 hours. Absorbance was measured at 560 nM (excitation) wavelength and 590 (emission) wavelength, respectively.

2.4. Western blot analysis

Total protein was harvested using Ripa lysis buffer (according to manufacturer's protocol) (Merck, Burlington, MA) and protein concentrations were measured via Bradford assay (according to manufacturer's protocol) (BioRad, Hercules, CA). A total of 50 μg protein/well was resolved on 10% SDS-PAGE and transferred onto a 0.45-μm nitrocellulose membrane. Membrane was blocked in 5% milk and hybridized with primary antibodies (Supplementary Table 1, http://links.lww.com/JCMA/A63) overnight. Blots were then incubated in secondary antibodies for 1 hour to detect protein of interest. GAPDH was used as the housekeeping gene. Protein of interest on blots were detected by UVP Chemi-doc system (Thermo Fisher Scientific).

2.5. Statistical analysis

Statistical data analysis was carried out with Paired *t*-tests. All tests were conducted at 95% confidence level and all data were presented as mean ± SEM.

3. RESULTS

3.1. Generation of Osimertinib-resistant cells from H1975 (epidermal growth factor receptor mutant *L858R*/T790M) cell line

H1975-OR cell lines were derived via stepwise dose-escalation method. In-vitro dosage was selected based on FDA recommendation (480 nM–1.8 μM).[22] Parental H1975 was exposed to increasing Osimertinib concentrations from 500 nM to 1.5 μM until stable cell growth without major cell death in culture condition was observed. We then performed limiting dilution in 96-well format to establish monoclonal cell growth. Each clones was expanded in 1.5 μM Osimertinib concentration and we further selected three clones that survived over 6 months during Osimertinib selective pressure (Fig. 1A). Morphological changes between parental H1975 cell line and OR cells are apparent. OR cells developed elongated and spindle-shaped cells similar to fibroblast-like cells (Fig. 1B).

3.2. Drug resistance properties in Osimertinib-resistant cells

Osimertinib sensitivity in parental H1975 and OR cells were determined by AlamarBlue assay to quantify cell viability. This assay uses cell-permeable and non-toxic Resazurin fluorescence dye to measure cell viability. Both H1975 and OR cells were treated with Osimertinib ranging from 0.001 to 10 μM and DMSO as control for 48 hours. Relatively, established resistant clones showed higher IC_{50} value from that of the parental counterpart. The IC_{50} values for OR3, OR4, and OR6 were 6.67, 6.81, and 6.09 μM compared to that of parental H1975 (4.95 μM) (Fig. 2A).

3.3. Epidermal growth factor receptor signaling, epithelial and mesenchymal transition, and autophagy associated protein expression

We evaluated protein expression level of EGFR signaling and its downstream signal pathways AKT and ERK. We examined EMT-related protein expression as we observed distinguishable morphological changes in OR cell lines. The cells were treated with Osimertinib (1.5 μM) and DMSO (control) for 24 hours before western blot analysis. OR cells potentially acquired resistance via EGFR-independent signaling as phosphorylation of EGFR expression was absent in all resistant cells, including in both Osimertinib treated and DMSO (control) (Fig. 3A). Osimertinib

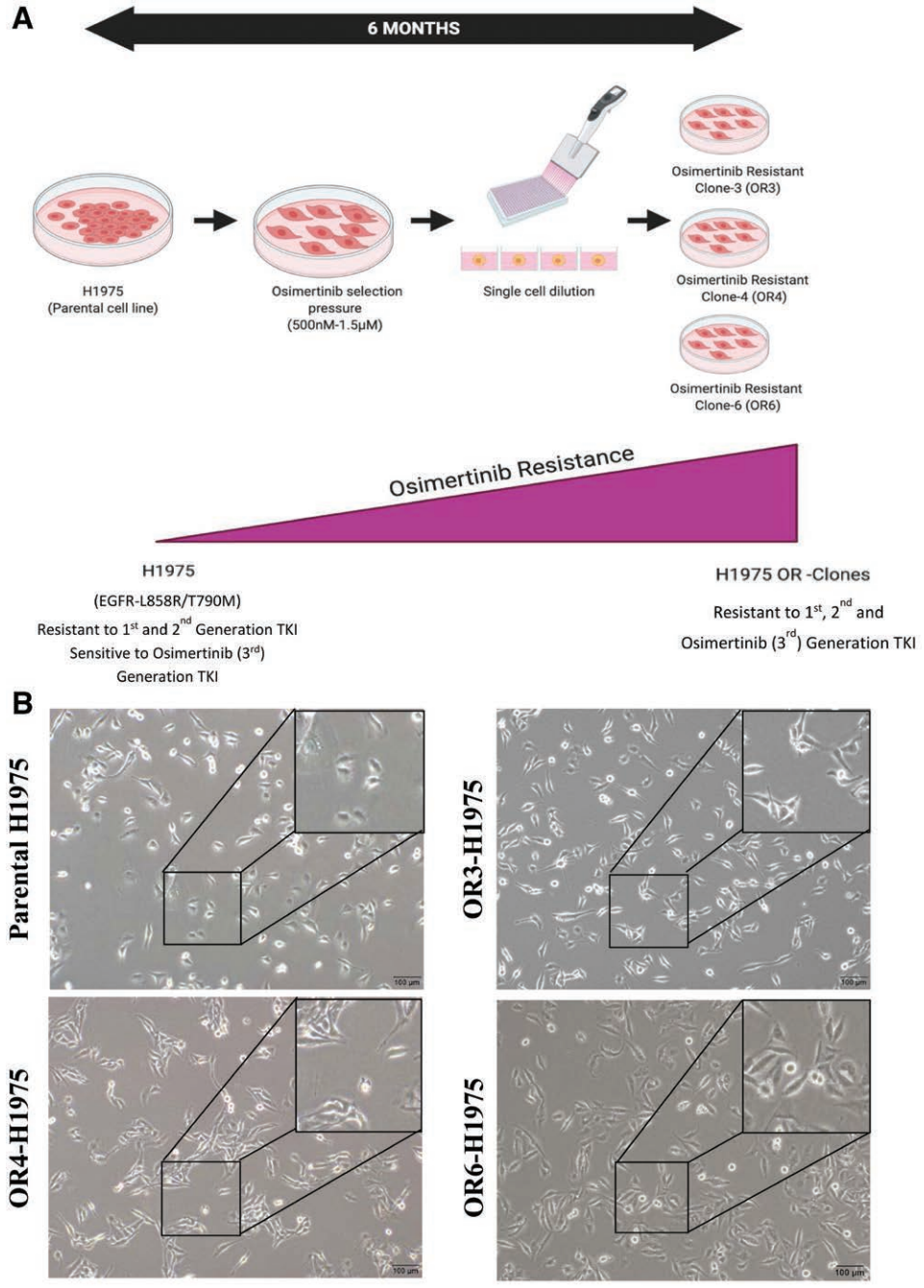

Fig. 1 Establishment of osimertinib-resistant clones. A, Resistant clones were selected based on stepwise-dose exposure of the H1975 cell line to Osimertinib (500 nM–1.5 μM). B, Morphology of isolated resistant clones resembles an elongated fibroblast-like cells and distinguishable to H1975 parental cell line.

treatment inhibited phospho-ERK and phospho-AKT expression in parental H1975 but not in resistant cell lines (Fig. 3A). Our results indicated, Osimertinib-resistant derived cells bypasses EGFR signaling by retaining ERK and AKT signaling.

On another note, we observed loss of epithelial protein expression (E-Cadherin and EpCAM) and increase of mesenchymal markers in resistant cells (Vimentin and CD44) (Fig. 3B). These observations corroborated with morphological changes noted in our OR cells. Overall, we discovered higher autophagic activity in OR cells. Sequestosome (SQSTM1) or p62 protein expression usually degraded when autophagy is induced. Our OR cells under DMSO (control) treatment tend to have lower SQSTM1 expression than that of Osimertinib treated group indicating Osimertinib exerted some inhibition effect. Microtubule-associated protein light chain 3 (LC3) is an autophagosome marker essential in autophagy monitoring and during autophagy LC3-1 gets lipidated to LC3-II.[23] We noticed, Osimertinib treatment was unable to inhibit total autophagic activity in resistant cells since we observed increased LC3-II expression but not so in parental H1975 (Fig. 3B).

4. DISCUSSION

In this study, we derived OR cells which can be employed to study the molecular heterogeneity and cellular signaling involved in acquired resistance of NSCLC. We used stepwise

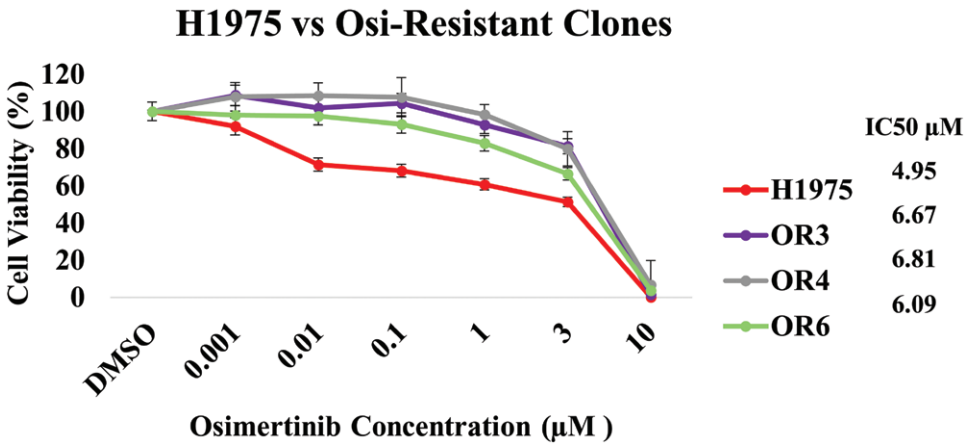

Fig. 2 Osimertinib sensitivity assay. A, Alamarblue assay (colorimetric dye) were conducted in the osimertinib-resistant clones and H1975 cell lines at 48 hours. Data are presented as the mean ± SEM (n = 3).

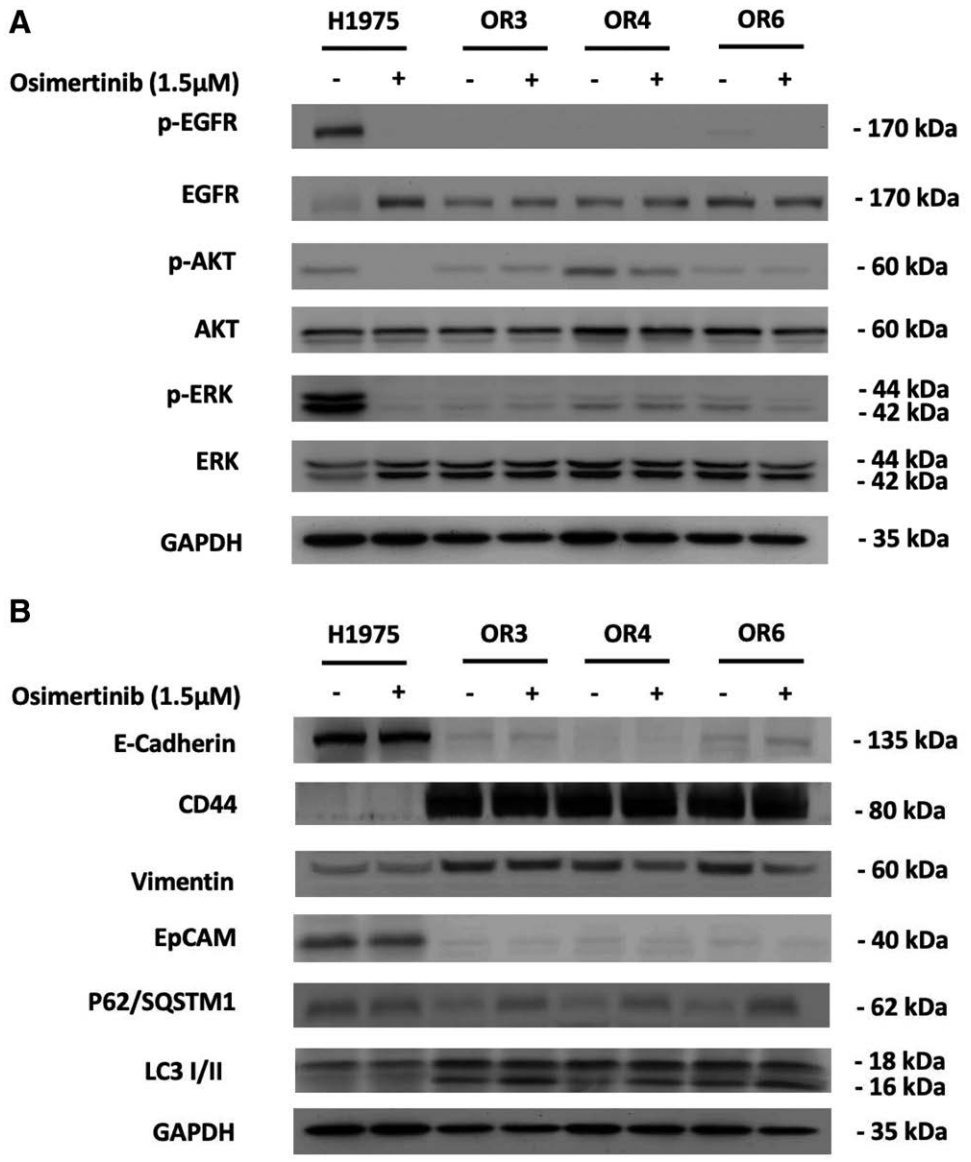

Fig. 3 Western blot analysis. A, EGFR signaling pathways, (B) EMT and autophagy related protein expressions were evaluated in both parental cell line and resistant clones prior to osimertinib and DMSO (control) treatments for 24 h.

dose-escalation method to derive the resistant cell lines followed by limiting dilution to further select homogenous resistant clones (OR3, OR4, and OR6) (Fig. 1A). Stepwise dose-escalation method involves prolonged increasing Osimertinib concentrations to select drug-tolerant cells via induced selective pressure. Previous studies reported that stepwise dose-escalation method is reproducible, reliable, economical compared to high drug concentration exposure and in-vivo resistance methods.[24,25] Here, we generated drug resistance cells from parental H1975 cell line with higher IC_{50} values achieved in the resistant group (Fig. 2A). H1975 cell line was used to induce Osimertinib resistance owing to the fact that this cell line harbors double mutations, L858R and secondary mutation in EGFR exon 20 T790M ("gatekeeper mutation"). T790M was the most frequent secondary mutation discovered in NSCLC patients who acquired resistance to EGFR-TKI and therefore our resistant cell lines may represent acquired Osimertinib resistance model in-vitro.[26,27]

Drug resistance indeed an important cancer hallmark contributing to cancer progression affecting cancer patients globally.[28] Relatively, mechanism of drug resistance can be divided into intrinsic and acquired resistance. Patients who encountered drug resistance before the treatment have intrinsic resistance while those who developed resistance after treatment are said to acquire drug resistance.[29] A major clinical setback in NSCLC patients is caused by acquired resistance to EGFR-TKI. Even though, significant survival advantage in patients with exon 19 deletion and L858R mutations were observed before EGFR-TKI in some cases, patients acquired resistance (T790M mutation) after 9–14 months of treatment onset with average progression-free survival.[30–32] Studies showed prolonged treatment with first- and second-generation EGFR-TKI (gefitinib, erlotinib, or afatinib) exert TKI-induced selection pressure in NSCLC patients.[33] Presence of T790M mutation inhibited bindings of first and second-generation EGFR-TKI to ATP-binding site of EGFR (exon 19 deletion and L858R point mutation) leading to treatment failure in patients.[34] Thereafter, Osimertinib/AZD9291 (third-generation EGFR-TKI) later approved by FDA and demonstrated high efficacy against T790M mutation in in-vitro and clinical studies (AURA 3 clinical trial)[35,36] but again inadequate responses were observed in patients subsequently.[37] Subsequent onset of tertiary mutations at multiple codons (C797S, L718/G719, G796/C797, L792, and L798) due to Osimertinib resistance resurfaced in NSCLC patients warrants further development of EGFR-TKI and new therapeutic approach.[18]

Mechanisms of resistance are multifactorial and available treatments to delay onset of resistance remain elusive. Not only T790M mutation but also bypass signaling pathways,[38] EMT,[39,40] and autophagy[41,42] have been strongly associated with mechanisms of acquired resistance in NSCLC. In our study, we observed possible involvement of ligand-independent EGFR /non-canonical EGFR signalling pathways (Fig. 3A). Phosphorylated-EGFR was not activated in our resistant cells being under control treatment (DMSO) or Osimertinib treatment but activation of ERK and AKT were observed. Typically, ligand-dependent EGFR signaling activation during NSCLC progression transduces its downstream pathways (RAS-RAF-MEK-ERK-MAPK and AKT-PI3K-mTOR) that promotes tumor cells survival, cellular differentiation, proliferation, enhanced motility, and migration.[43–45] Studies by Zhang et al[46] reported on activation of HGF/MET pathways, a ligand-independent EGFR signaling pathway activates ERK and AKT signaling pathways contributing to EGFR-TKI resistance in NSCLC. Another study by Jafarnejad et al[47] also described the role of HGF/MET pathways contributing to activation of AKT and ERK in hepatocellular carcinoma. Further investigations are required to elucidate non-canonical pathways involved in our Osimertinib resistance cell lines.

Acquired EGFR-TKI resistance facilitates phenotypic transformation and our data showed, all resistance cell lines (OR3, OR4, and OR6) exhibited morphological changes upon Osimertinib-resistance induction. Morphologically, H1975 cell line has an epithelial-like morphology, however exposure to increasing Osimertinib in-vitro induced a morphological transformation from its epithelial into mesenchymal like-cells (elongated cell shapes and loss of tight junction) (Fig. 1B). EMT is a well-known phenomenon in drug resistance, often associated with poor prognosis and NSCLC progression.[48,49] Once the cancer cells acquired resistance to drug and mesenchymal-like phenotype, these cells gain higher invasion and migration ability which eventually transform into metastatic cancer cells.[50,51] Earlier studies have indicated EGFR-TKIs treatment elevates cell adhesion molecules (EpCAM and E-Cadherin) substantially prolongs survival rate with favorable prognosis in NSCLC patients.[52,53] Our protein expression data showed, loss of EpCAM and E-Cadherin protein expression in resistance cell lines. Besides, increased Vimentin expression supported our morphologic changes observed in our resistant cell lines (Fig. 3B). Vimentin is an intermediate filament protein highly expressed in cells of mesenchymal origin and promotes cell motility and metastasis. High Vimentin expression in NSCLC patients has been correlated to poor prognosis.[54] Expression of CD44 was previously denoted as cancer stem cells marker and expressed in tumor of epithelial origin.[55] However, recent study by Suda et al demonstrated CD44 expression in lung adenocarcinoma patient samples who developed acquired resistance to gefitinib or afatinib. The authors indicated CD44 can be used as a EMT predictor and mesenchymal marker.[56]

The role of autophagy in cancer remained ambiguous as it may promote tumorigenesis or induce cell death.[57] Yet, available studies indicated autophagy activity was increased through cellular stress including hoisted drug pressure.[58] During autophagy, formation of double-membraned autophagosome engulf damaged organelles, pathogens, cellular proteins, and macromolecules for delivery to the lysosome.[23] Translocation of lipidated microtubule-associated protein 1A/1B-light chain 3 (LC3) from LC3-I (cytosol) to LC3-II (autophagosome membrane) was used to assess autophagy flux activity. LC3-II exhibits higher mobility than LC3-I on western blot (SDS-Page).[59,60] As such, we detected LC3-II protein expression lipidated from LC3-I indicating presence of autophagic flux in our resistant cell line (Fig. 3B). Recent study by Li et al reported that autophagy was detected in lung cancer patients resistance to Osimertinib. They have also demonstrated autophagy maintained cancer stem-like properties induced by Osimertinib with high CD44 cell population in OR cells.[61] On a side note, Chen et al[62] outlined the fact that Osimertinib induced pro-survival properties in NSCLC cell lines and they demonstrated Metformin inhibited autophagy and further enhanced sensitivity of H1975 and PC-9GR cells to Osimertinib. Sequestosome (SQSTM1) or p62 an autophagy receptor encoding cargo adaptor protein is another marker widely used as autophagy indicator. During autophagy, SQSTM1 binds to autophagic substrates LC3 further transports them for degradation in autophagosome. Therefore reduction in SQSTM1 expression associated with increased of autophagic flux.[63] We observed SQSTM1 expression was slightly increased in our resistant cell lines under Osimertinib treatment compared to DMSO (control) (Fig. 3B). We postulated that SQSTM1 protein expression was being rescued in the presence of Osimertinib.

In conclusion, our results confirmed, OR cell lines (OR3, OR4, and OR6) were established from EGFR L858R/T790M Mutant NSCLC Cell Line exhibiting criterions of fundamental hallmark of cancer. The drug-resistant cells derived achieved higher resistance potential upon stepwise Osimertinib exposure mediated by non-canonical EGFR signaling. Morphological

switch from epithelial-like cells to elongated mesenchymal-like cells were observed in conjunction with acquired EMT properties and activation of autophagy. Yet, additional investigations would be required to investigate the migration and invasion properties of the resistant cell lines. Altogether, our OR cell lines may serve as a model in understanding mechanisms of acquired resistance of the third-generation EGFR-TKI (Osimertinib) and for exploring tumor evolution during prolonged drug selective pressure as well as to investigate the signaling pathways regulated during acquired resistance. Furthermore, application of genetics and molecular screening of these OR cell lines[64,65] facilitate discovery of new generation of EGFR-TKIs and strategized tailored drug design for NSCLC patients.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Science and Technology (MOST) (106-2320-B-075-002 and 109-2320-B-075-001), Taipei Veterans General Hospital (V107B-017, V108D46-004-MY2-2, V108E-006-4, and V109C-123), Yen-Tjing-Ling Medical Foundation (CI-108-11), Far Eastern Memorial Hospital National Yang-Ming University Joint Research Program (108DN13 and 107DN09), and the Cheng-Hsing General Hospital (CHGH108-11, CHGH107-14, and CHGH107-08).

We would like to thank Dr. Ting-Fang Lee from the Institute of Clinical Medicine (National Yang-Ming University) for her assistance and advice on derivation of drug-resistance cell lines.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://doi.org/10.1097/JCMA.0000000000000264.

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