Development of Stemness in Cancer Cell Lines Resistant to the Anticancer Effects of Zoledronic Acid

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Abstract. *Background: Drug resistance is closely related to cancer cell stemness, that is acquired along with resistance to various anticancer agents. However, this has not been investigated as a potential mechanism underlying cancer cell resistance to zoledronate, that is used to suppress bone metastasis. Materials and Methods: Zoledronate-resistant A549 lung cancer and MG63 osteosarcoma cell lines were established by repeated treatment with sub-lethal concentrations of zoledronate. Expression levels of the stem cell marker NANOG, cMYC, octamer-binding transcription factor 4, and sex-determining region Y-box 2 were evaluated and sphere formation was compared between parental and resistant cell lines. Tumourigenicity was assessed in vivo. Results: Stem cell marker expression was up-regulated and sphere formation was enhanced in resistant compared to parental cells and showed greater tumour formation capacity in mice. Conclusion: Repeated treatment of malignant tumour cell lines with zoledronate, induces the development of drug resistance and stemness.*

Zoledronate, a nitrogen-containing *bis*phosphonate, has anti-osteoclastic, as well as direct antitumourigenic activity, which is exerted *via* induction of apoptosis in various types of malignant tumour cells including myeloma, breast, prostate and lung cancer, and sarcoma (1-5). Zoledronate is frequently prescribed for the treatment of bone metastases in cancer and hypercalcaemia (1). However, prolonged administration of the drug can lead to development of resistance in cancer cells. We previously investigated the anticancer effect of zoledronate and the underlying molecular mechanisms in drug-resistance models and found that resistance was at least partly due to the modulation of the apoptosis pathway (6, 7).

Cancer cell stemness refers to the capacity of cells to divide and thereby expand the stem cell pool and also differentiate into the heterogeneous non-tumourigenic cancer cell types that constitute the bulk of cancer cells (8). Cancer stem cells (CSCs) were first identified in acute myeloid leukaemia (AML) as a small population of cluster of differentiation (CD)34^+/$CD38^-$ cells that are able to initiate human AML in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, as well as to differentiate and proliferate (9). Since this discovery, various types of CSC have been identified in other tumour types, including lung cancer and osteosarcoma (10-12). CSCs are characterized by their sphere-forming capacity under serum-free, non-adherent conditions (11, 13-17), while a subset of cells exhibit enhanced efflux (18-21) and specific cell surface markers such as B cell-specific Moloney murine leukemia virus integration site 1 (22), sex-determining region Y-box (SOX)-2 (23, 24), CD133 (25-28), chemokine C-X-C motif receptor 4 (12) and overexpress aldehyde dehydrogenase (ALDH) 1 (11, 29). It has also been reported that CSCs possessing such properties exhibited increased resistance to anticancer drugs such as cisplatin (13, 15, 16, 18, 19, 22, 25, 29), adriamycin (15, 16, 18, 29), methotrexiate (18), and gefitinib (12, 30), that are used to treat osteosarcoma and lung cancer. However, the relationship between cancer cell resistance to zoledronate and cancer stemness has not been previously investigated, to our knowledge.

In the present study, we hypothesized that zoledronate-resistant osteosarcoma and non-small cell lung cancer (NSCLC) cell lines acquire stemness during the development of drug resistance. Resistant cell lines were established in our laboratory and were evaluated for stemness properties, including the expression of stem cell markers, sphere-forming capacity, and *in vivo* tumourigenic potential.

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Key Words: Osteosarcoma, lung cancer, drug resistance, zoledronic acid, cancer stem cells.

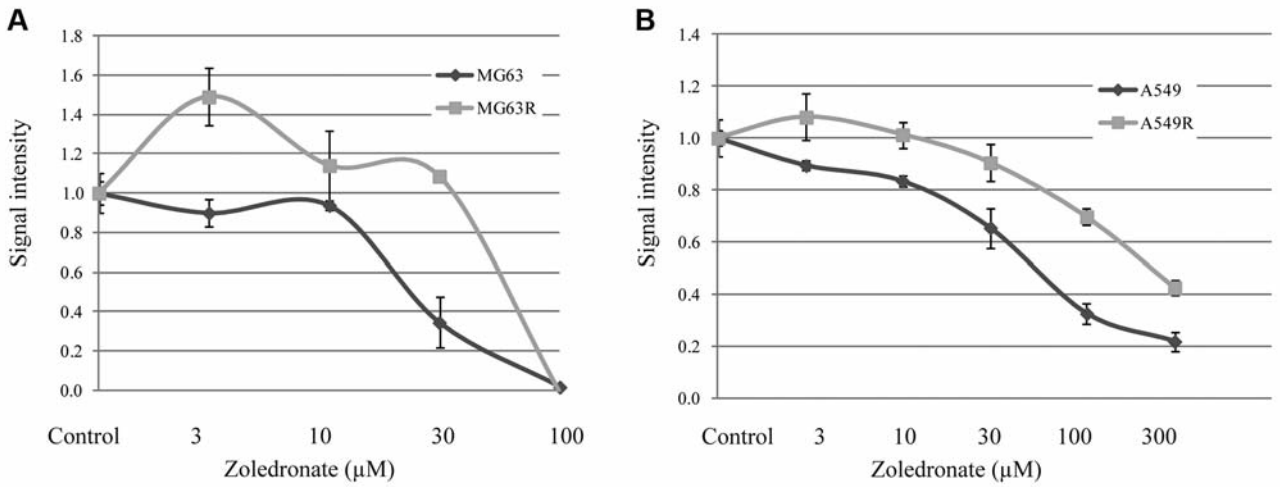

Figure 1. *Development of resistance against zoledronate, an anticancer drug. MG63 osteosarcoma (A) and A549 lung cancer (B) cell lines were treated with sub-lethal concentrations of zoledronate to induce development of drug resistance. Cell viability was determined with the cell proliferation assay and expressed as absorbance to confirm the resistance of established cell lines (MG63R and A549R). Data are the mean±standard deviation.*

Materials and Methods

Cell culture. A549 human lung cancer cells [CCL-185; wild-type, epidermal growth factor receptor (EGFR)-expressing] and MG63 human osteosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in Roswell Park Memorial Institute-1640 medium (A549 cells) or in Eagle's minimum essential (complete) medium (MG63 cells) supplemented with 10% foetal calf serum.

Induction of drug resistance. Zoledronate was provided as a hydrated disodium salt (MW 401.6) by Novartis International AG (Basel, Switzerland). The zoledronate-resistant MG63R cell line was derived from the parental MG63 cell line by repeated exposure to sublethal concentrations of zoledronate (30-80 μM) for 96 to 120 h, followed by incubation in complete medium lacking the drug for an additional 10 days. The protocol for establishing the zoledronate-resistant A549R cell line was described in our previous report (6); the cells were used in this study after confirming their drug resistance.

Cell proliferation assay. Cell proliferation was monitored as previously described (7) with some modifications. Resistant cells (MG63R and A549R) and parent cell lines as control were seeded in 96-well plates at a density of 3×10^3 cells/well. After incubation for 24 h, cells were incubated with medium containing zoledronate (0-300 μM), and 96 h later, viable cells were detected with the spectrophotometric CellTiter 96 aqueous cell proliferation assay (Promega, Madison, WI, USA).

Immunoblot analysis. Cell lysates (40 μg of protein/sample) were resolved by 5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using antibodies against NANOG (reproCELL, Kanagawa, Japan), c-MYC (551101; BD Pharmingen/Becton Dickinson, Tokyo Japan), octamer-binding transcription factor (OCT)-4 (NB100-91901; Novus Biologicals US, Littleton, CO, USA), sex-determining region Y-box (SOX)-2 (561469; BD Pharmingen/Becton Dickinson), and β-actin (M177-3; Medical and Biological Laboratories, Woburn, MA, USA). The blot was incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G and HRP-linked secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and fluorescence was detected using LumiGLO peroxidase chemiluminescent substrate kit (KPL, Gaithersburg, MD, USA).

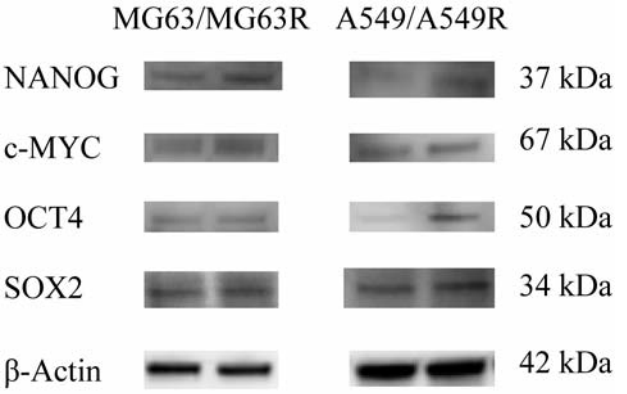

Figure 2. *Expression of stem cell markers in parental and zoledronate-resistant cell lines. Expression of a panel of stem cell markers including NANOG, c-MYC, octamer-binding transcription factor (OCT)-4, and sex-determining region Y-box (SOX)-2 were determined by western blotting. β-Actin served as a loading control.*

Sphere-forming assay. The sphere-forming assay was performed as previously described (16). Briefly, cells were plated at a density of 6×10^4 cells/well in 6-well ultra-low attachment plates (Dow Corning, Corning, NY, USA) in N2 medium with 1% methylcellulose. Dulbecco's modified Eagle's medium/F12 containing 20 nM progesterone, 100 mM putrescine, 30 nM sodium selenite, 25 mg/ml transferrin, 20 mg/ml insulin (Sigma, St. Louis, MO, USA), 10 ng/ml

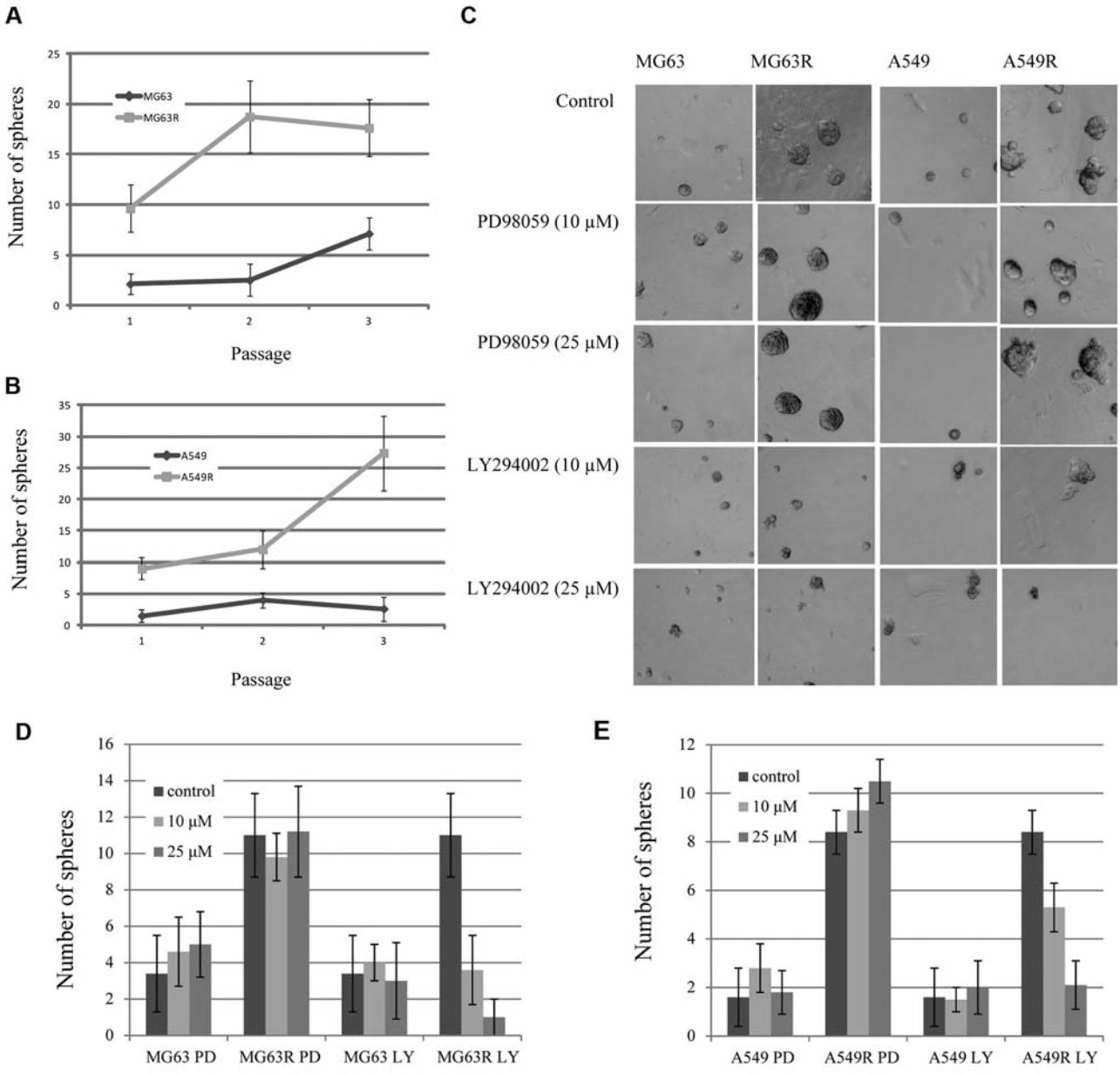

Figure 3. *Sphere-forming assay. A, B: Number of spheres as a function of passage number. The number of spheres in eight high-power microscopic fields was counted in primary, second-, and third-passage MG63 and MG63R cells (A) and A549 and A549R cells (B). C: Representative micrographs of spheres formed by parental and resistant cells. Cells were treated with dimethylsulfoxide as a control, or with LY294002 (phosphoinositide 3-kinase inhibitor) or PD98059 (extracellular signal-regulated kinase 1/2 inhibitor) at the indicated concentrations or were untreated. D, E: Number of spheres formed by MG63 and MG63R (D) and A549 and A549R (E) cultures as a function of inhibitor (LY294002 or PD98059) concentration. Data are the mean±standard deviation in all graphs.*

human EGF, and 10 ng/ml human basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA) was mixed with an equal volume of 2% methylcellulose (Sigma) and added to the wells. In addition, fresh aliquots of EGF and bFGF were added every other day. Cells that formed spheres were subcultured three times. After 7 days of culturing, the number of spheres in eight different high-power microscopic fields was counted in order to compare sphere-forming capacity of parental and resistant cell lines. Extracellular signal-regulated kinase (ERK)1/2 inhibitor PD98059 (Tocris Bioscience, Bristol, UK) or phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (Tocris Bioscience) were added to cells in order to determine the signalling pathway associated with sphere formation.

In vivo tumourigenicity study. Animal protocols and studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyorin University. NOD/SCID

mice (male, 4 weeks old) were purchased from the CLEA Japan (Tokyo, Japan). A549R and A549 (control) cells (1×10^5, 1×10^4, 1×10^3, and 1×10^2) were mixed with Matrigel and injected subcutaneously into NOD/SCID mice. Tumour formation was examined 2-10 weeks later and the number of mice with tumours in each group was recorded. Tumour volume was calculated as follows: length × width^2 × $\pi/6$ (31).

Statistical analysis. Data were analyzed with the Mann–Whitney *U*-test. Differences were considered statistically significant at $p<0.05$.

Results

Development of zoledronate resistance. MG63R and A549R zoledronate-resistant cell lines were obtained by exposing the parental cell lines to increasing concentrations of drug over several months (6). A subpopulation of cells was obtained that was insensitive to zoledronate at concentrations that were lethal to the parental cell lines (Figure 1). Half-maximal inhibitory concentration values were 25 and 83 μM against MG63 and MG63R cells, respectively, and 48 and 240 μM against A549 and A549R cells, respectively. Microscopic examination revealed no differences between resistant and parental cell populations in terms of morphology (data not shown).

Zoledronate-resistant cells express stem cell markers. Recent studies on the development of drug resistance in several cancer cell lines have suggested a close relationship between the acquisition of resistance and stemness properties. We, therefore, examined MG63R and A549R cell lines for the expression of stem cell markers. The levels of NANOG, c-MYC, OCT4, and SOX2 were up-regulated in resistant compared to the respective parental cell lines, suggesting a link between the development of zoledronate resistance and the acquisition of stemness in osteosarcoma and lung cancer cells (Figure 2).

Zoledronate resistance is associated with sphere-forming capacity. An increase in sphere formation under non-adhesive culture conditions is reported to be a feature of stemness (11, 12, 16). In order to determine whether a larger sub-population of stem-like cells exists in resistant compared to non-resistant cell populations, we evaluated parental and resistant MG63 and A549 cells regarding their ability to generate clonal spheres by self-renewal in a neurosphere culture system. More stem-like cells with sphere-forming capacity were observed in both primary cultures and sub-cultures of resistant compared to parental cell lines, with sphere number increasing with the number of passages (Figure 3A and B).

Zoledronate resistance is associated with increased PI3K signaling. Sphere formation by NSCLC cells resistant to gefitinib was reported as being closely related to PI3K but not mitogen-associated protein kinase (MAPK) signaling (12). To assess whether this is true in zoledronate-resistant cells, we used the PI3K inhibitor LY294002. We found that inhibitor treatment suppressed sphere formation and self-renewal in MG63R and A549R cells (Figure 3C-E). In contrast, treatment with the ERK1/2 inhibitor PD98059 had no effect on sphere formation.

Zoledronate-resistant cells have higher tumour-forming capacity in vivo. An increase in tumourigenic capacity is a defining characteristic of CSCs. To assess *in vivo* tumour-forming capacity, NOD/SCID mice were inoculated with A549, or A549R cells. A significantly higher number of tumours were formed by resistant than by parental cells (Figure 4A), and tumour volumes were higher in mice inoculated with A549R compared to A549 cells (Figure 4B). At a dosage of 1×10^5 cells, the rate of tumour formation was similar (6 out of 6 mice for parental and resistant cells) (Figure 4C). At 1×10^4 cells, tumour incidence was also similar (6 out of 6 mice for both conditions), however, tumours were observed at an earlier time point for mice injected with resistant cells (Figure 4D), and at 1×10^3 cells, tumour incidence was higher in inoculation with resistant (5 out of 5 mice) compared to parental (3 out of 5 mice) cells (Figure 4E), suggesting that tumourigenic potential increases as a function of stemness. There were no tumours formed when mice were inoculated with 1×10^2 parental or resistant cells (data not shown).

Discussion

One way in which cancer cells develop drug resistance is through selection of a specific cell population that exhibits prosurvival characteristics, which include stemness properties. We previously demonstrated that a subpopulation of HOS osteosarcoma cells resistant to zoledronate exhibited anti-apoptotic features, including up-regulation of heat-shock protein 27 (7). Similarly, in MCF-7 breast cancer cells resistant to zoledronate, expression of the anti-apoptotic factor B-cell lymphoma 2 was up-regulated along with that of the breast cancer resistance protein ATP-binding cassette transporter G2 and lung cancer resistance protein (3). Based on these observations, we hypothesized that cell lines resistant to zoledronate would exhibit stemness properties, as previously shown for cells resistant to other conventional anticancer drugs (12, 30, 32-34).

NANOG, c-MYC, and SOX2 levels were found to be up-regulated in zoledronate-resistant lung cancer and osteosarcoma cell lines in the present study. These are not only stem cell markers but also confer anti-apoptotic/pro-survival functions and drug resistance, implying a close relationship between drug resistance and cancer cell stemness. Inhibiting NANOG expression enhanced sensitivity to apoptosis-inducing BH3 mimetics in colorectal carcinoma (35) and increased chemosensitivity to doxorubicin *via* reduction of multidrug resistance (MDR) 1 in hepatic cell carcinoma (36). Meanwhile, c-MYC expression was up-regulated in $CD34^+/CD38^-$ stem cells, that was associated with drug resistance in acute myeloid

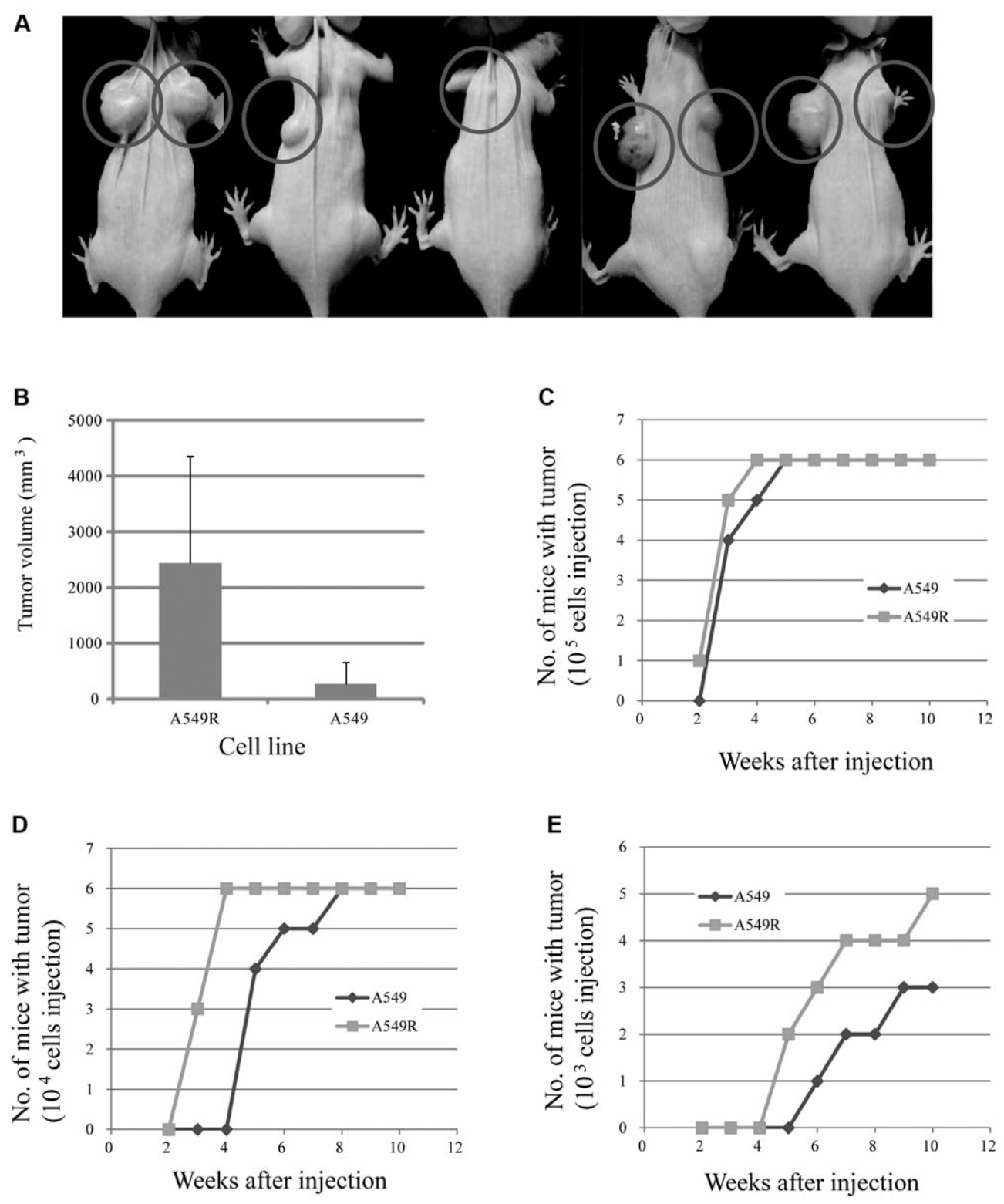

Figure 4. *Inoculation of parental and resistant MG63 and A549 cells in mice. A549 and A549R cells were subcutaneously injected into mice; tumour formation was confirmed 1-10 weeks later. A: Representative micrographs of tumour formation 10 weeks after injection of 1×10^3 tumour cells. A549 and A549R cells were injected into the right and the left shoulders, respectively. Tumours are circled. B: Tumour volumes in mice 10 weeks after injection with 1×10^3 A549 and A549R cells. Volumes were greater for resistant than for parental cells (p=0.04). Data are the mean±standard deviation. C-E; Number of mice with tumour formation as a function of time after injection of 1×10^5 cells (C), 1×10^4 cells (D), and 1×10^3 cells (E).*

leukaemia cells (37). Conversely, inhibiting c-MYC expression in gastric cancer cells reversed MDR (38). SOX2 overexpression was linked to paclitaxel resistance *via* the PI3K/protein kinase B pathway in ovarian cancer (39) and was observed in sphere-forming A549 lung cancer cells that showed increased resistance to anticancer drugs such as cisplatin and gemcitabine (40). These results indicate that cancer cells overexpressing stem cell markers also exhibit increased drug resistance, which was supported by our data showing that zoledronate-resistant MG63 and A549 cell lines overexpressed stem cell markers.

Sphere-forming capacity was also used as an indicator of stemness in the present study. Sphere formation in a serum-free, non-adherent culture represents a cellular survival mechanism under conditions of stress. The fact that drug treatment is also a form of stress for cancer cells may explain the close association between drug resistance and stemness. Indeed, increased resistance to doxorubicin and cisplatin along with an up-regulation in stem cell marker expression was detected in an isolated population of sphere-forming sarcoma cells (11). Another group reported sphere formation in NSCLC cell lines that were resistant to gefitinib and exhibited various stemness properties, which was associated with activation of PIK3 but not MAPK signalling (12). Increased PI3K signalling is associated with cancer progression (41); in lung cancer, inhibiting this pathway suppressed stem cell proliferation (42). Our results provide evidence that inhibiting PI3K signalling suppressed drug resistance in MG63 and A549 cells, suggesting a possible therapeutic strategy for targeting CSCs. Further studies are needed in order to clarify the detailed mechanism by which this signalling pathway abrogates drug resistance and stemness properties.

Importantly, our results indicate that stemness may be enhanced by first inducing drug resistance. CSCs in previous reports have been identified based on the presence of specific stem cell subpopulations (18, 19), expression of ALDH1 (11, 29) or other stem cell markers (13, 22, 25), or sphere-forming capacity (11, 14, 15). On the other hand, some CSC models have been established by confirming stemness in drug-resistant cell lines (12, 30, 32-34). The advantage of this type of model is its similarity to the development of drug resistance *in vivo* through repeated exposure to anticancer drugs, as opposed to selection based on markers or cell properties. Our model can, therefore, provide insight into the process by which stemness and drug resistance is established in cancer cells.

A limitation of the present study is the fact that the differentiation potential of MG63 and A549 cells into various lineages was not confirmed. In addition, analysis of stem cell marker expression using arrays or proteomics approaches and *in vivo* confirmation of the role of PI3K signaling in drug resistance are important considerations for future investigations.

In conclusion, repeated treatment of malignant tumour cell lines with zoledronate induce the development of drug resistance and stemness.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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