

Nrf2/p-Fyn/ABCB1 axis accompanied by p-Fyn nuclear accumulation plays pivotal roles in vinorelbine resistance in non-small cell lung cancer

SHIGEYUKI TAMARI, TOSHI MENJU, TOSHIYA TOYAZAKI, HIDEAKI MIYAMOTO, NAOHISA CHIBA, MISA NOGUCHI, HIROYUKI ISHIKAWA, RYO MIYATA, HIDENAO KAYAWAKE, SATONA TANAKA, YOSHITO YAMADA, YOJIRO YUTAKA, DAISUKE NAKAJIMA, AKIHIRO OHSUMI, MASATSUGU HAMAJI and HIROSHI DATE

Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

Received April 12, 2022; Accepted July 19, 2022

DOI: 10.3892/or.2022.8386

Abstract. Adjuvant cisplatin-vinorelbine is a standard therapy for stage II/III lung cancer. However, a poor survival rate of patients with lung cancer is attributed to vinorelbine resistance arising from ATP-binding cassette (ABC) sub-family B member 1 (ABCB1) and phosphorylated Fyn (p-Fyn) overexpression. However, the underlying mechanisms remain unclear. NF-E2-related factor 2 (Nrf2) regulates the ABC family and activates the nuclear transport of Fyn. The present study evaluated the roles of the Nrf2/p-Fyn/ABCB1 axis in vinorelbine-resistant (VR) cells and clinical samples. To establish VR cells, H1299 cells were exposed to vinorelbine, and the intracellular reactive oxygen species (ROS) level in the H1299 cells was determined using a DCFH-DA assay. The total and subcellular expression of Nrf2, ABCB1 and p-Fyn in VR cells was evaluated. Immunofluorescence was used to detect the subcellular localization of p-Fyn in VR cells. A cell viability assay was used to examine whether the sensitivity of VR cells to vinorelbine is dependent on Nrf2 activity. Immunohistochemistry was performed on 104 tissue samples from patients with lung cancer who underwent surgery followed by cisplatin-vinorelbine treatment. The results revealed that persistent exposure to vinorelbine induced intracellular ROS

formation in H1299 cells. p-Fyn was localized in the nucleus, and ABCB1 and Nrf2 were overexpressed in VR cells. ABCB1 expression was dependent on Nrf2 downstream activation. The decreased expression of Nrf2 restored the sensitivity of VR cells to vinorelbine. In the surgical samples, Nrf2 and ABCB1 were associated with disease-free survival, and p-Fyn was associated with overall survival ($P < 0.05$). On the whole, the present study demonstrates that Nrf2 upregulates ABCB1 and, accompanied by the nuclear accumulation of p-Fyn, induces vinorelbine resistance. These findings may facilitate the development of drug resistance prevention strategies or new drug targets against non-small cell lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Cisplatin-vinorelbine is a standard adjuvant chemotherapy for patients with resected stage II and III non-small cell lung cancer (2). Adjuvant cisplatin-vinorelbine treatment has been shown to improve the overall survival rate by 8.9% and the disease-free survival rate by 9.2% at 5 years (3). The 5-year survival rate is, however, unsatisfactory at 40.6% for patients with p-stage IIA, 41.1% for IIB and 28.3% for IIIA (4). Chemotherapeutic failure occurs owing to resistance to chemotherapeutic agents, which remains a challenge in cancer treatment (5). The development of resistance is associated with the overexpression of energy-dependent drug efflux pumps, known as the ATP-binding cassette (ABC) family of proteins, which eject anticancer drugs from cells (6). Activated ABC sub-family B member 1 (ABCB1) is commonly associated with drug resistance (7,8).

The overexpression of ABCB1 and the activation of phosphorylated Fyn (p-Fyn) play a crucial role in vinorelbine resistance, and integrin $\beta 3$ functions as an upstream regulator of Src family kinases (SFKs), including Fyn, in vinorelbine-resistant (VR) cells (9). Fyn, a non-receptor tyrosine kinase, belongs to the Src family of kinases and contributes to the development and progression of cancer by regulating cell growth, death, morphogenic transformation and motility in several types of cancer, including glioblastoma,

Correspondence to: Dr Toshi Menju, Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
E-mail: toshimnj@kuhp.kyoto-u.ac.jp

Abbreviations: ABC, ATP-binding cassette; ABCB1, ABC subfamily B member 1; ARE, antioxidant response element; DMSO, dimethyl sulfoxide; Keap1, kelch-Like ECH associated protein 1; Nrf2, NF-E2-related factor 2; p-Fyn, phosphorylated Fyn; ROS, reactive oxygen species; VR, vinorelbine-resistant

Key words: non-small cell lung cancer, vinorelbine resistance, phosphorylated Fyn, ABC sub-family B member 1

chronic myelogenous leukemia, prostate cancer and breast cancer (10-13). However, the mechanisms through which ABCB1 and p-Fyn contribute to vinorelbine resistance in lung cancer remain unknown.

Reactive oxygen species (ROS) are products of oxygen metabolism and play a critical role in cellular proliferation and homeostasis. An imbalance between ROS generation and elimination results in intracellular damage. Chemotherapeutic agents, including vinorelbine, induce ROS generation to kill or inhibit the antioxidant mechanism of cancer cells (14). The cancer cells that succeed in controlling elevated ROS levels by upregulating cellular antioxidant systems become more resistant to exogenous stimuli, such as chemotherapy (15).

Previous studies have focused on the development of NF-E2-related factor 2 (Nrf2) inhibitors to overcome cancer drug resistance (16,17). Nrf2, a key transcription factor, neutralizes cellular ROS and maintains cellular redox homeostasis to protect cells against toxic xenobiotics (15). It also regulates the expression of antioxidants, metabolism and detoxification, and the expression of transporter genes by combining with the antioxidant response element (ARE) to confer cytoprotection against various harmful stimuli (18). Notably, Nrf2 promotes the survival of normal cells and creates an environment that protects cancer cells against chemotherapy and radiotherapy (19). Nrf2 is negatively regulated by Kelch-like ECH-associated protein (Keap1). The loss of Keap1 function leads to the constitutive activation of Nrf2-mediated gene expression and induces resistance to chemotherapeutic drugs (20). However, only a limited number of studies have demonstrated the role of Nrf2 in ABCB1-mediated vinorelbine resistance (8,21).

The present study evaluated the significance of Nrf2, ABCB1 and p-Fyn in VR cells and clinical lung cancer tissue samples. In addition, the possible association between protein expression and the clinicopathological features of patients who underwent adjuvant cisplatin-vinorelbine treatment was examined. Furthermore, the functional consequences of the increased Nrf2 expression in vinorelbine resistance was determined.

Materials and methods

Cells and cell culture. NCI-H1299 cells (CRL-5803, 70026320, human lung carcinoma) purchased from the American Type Culture Collection (ATCC) were cultured in RPMI-1640 medium (MilliporeSigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biosera), 100 units/ml penicillin and 100 mg/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation) at 37°C in a humidified atmosphere containing 5% CO₂. A549 cells (RCB-0098, 010, human lung carcinoma, ATCC cat. no. CCL-185) purchased from RIKEN Cell Bank were cultured in the same manner as H1299 cells, except in DMEM (D5796, MilliporeSigma) instead of RPMI-1640 medium (MilliporeSigma). The authenticity of the cell lines was confirmed by ATCC or RCB. Mycoplasma negativity was confirmed using PCR for parental cells and VR cells prior to use. All cells were preserved with CELLBANKER 1 (Nippon Zenyaku Kogyo Co., Ltd.) in liquid nitrogen.

Determination of ROS generation. The cells were seeded into 6-well plates at 4x10⁴ cells per well and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ with or without 500 nmol/l vinorelbine. ROS Assay Kit-Highly sensitive DCFH-DA (Dojindo Laboratories, Inc.) was used to detect ROS according to the manufacturer's protocol. Fluorescent images were obtained using a BZ-X810 fluorescence microscope (Keyence Corporation). The fluorescence intensity was determined using ImageJ 1.53 software (National Institute of Health).

Reagents. Vinorelbine (FUJIFILM Wako Pure Chemical Corporation) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc.). VR cells were cultured with the indicated concentrations from 5 to 500 nmol/l of vinorelbine for >4 months. Bardoxolone (Selleck Chemicals), a Keap1 inhibitor, was dissolved in DMSO. Bardoxolone prevents the ubiquitination and degradation of Nrf2 and, therefore, functions as an Nrf2 activator. For the inhibition of Keap1, the cells were exposed to the indicated concentrations from 0.1 to 2.0 µmol/l of bardoxolone for 24 h until they were harvested for lysate preparation using the same procedure as described below in 'Western blot analysis'.

Subcellular fractionation. The passage number of each cell line was <50. The H1299 cells were seeded at 1x10⁶ cells per 10-cm dish and cultured at 37°C for 96 h until just before confluency. Whole-cell lysates were then fractionated into cytoplasmic, membrane and nuclear protein extracts using the Subcellular Protein Fractionation kit for Cultured Cells (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To confirm that the nuclear and cytoplasmic fractions predominantly consisted of proteins derived from the nuclear and cytoplasmic compartments, respectively, the extracts were probed with nucleus-specific anti-lamin B1 antibody (cat. no. 12586, Cell Signaling Technology, Inc.) and cytoplasm-specific anti-α-tubulin antibody (017-25031, FUJIFILM Wako Pure Chemical Corporation).

Western blot analysis. To prepare total cell lysates, the cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet p-40, 1% deoxycholic acid and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 ng/ml aprotinin, 1 ng/ml leupeptin and 1 ng/ml pepstatin A). Protein concentrations were determined using Bio-Rad Protein Assay Dye Reagent Concentrate (#5000006JA; Bio-Rad Laboratories, Inc.). The primary antibodies used were as follows: Anti-ABCB1 (1:1,000; cat. no. 13978; Cell Signaling Technology), anti-Fyn (1:1,000; cat. no. A0086, ABclonal Biotech Co., Ltd.), anti-phosphorylated Fyn (p-Fyn) (1:1,000; cat. no. AP0510, ABclonal Biotech Co., Ltd.), anti-Nrf2 (1:1,000; cat. no. sc-722, Santa Cruz Biotechnology, Inc.), anti-caspase-3 (1:1,000; cat. no. 9665, Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (1:500; cat. no. 9664, Cell Signaling Technology, Inc.), anti-Bcl-2 (1:1,000; sc-7382, Santa Cruz Biotechnology, Inc.), anti-MRP1/ABCC1 (1:1,000; cat. no. 72202, Cell Signaling Technology, Inc.), anti-MRP2/ABCC2 (1:1,000; cat. no. 12559, Cell Signaling Technology, Inc.),

anti-MRP3/ABCC3 (1:1,000; cat. no. 14182, Cell Signaling Technology, Inc.) and anti- β -actin (1:1,000; cat. no. A5441, MilliporeSigma). Gel electrophoresis was performed using PowerPac™ HC High-Current Power Supply (1645052; Bio-Rad), and 10% gel was used for all antibodies except for cleaved caspase-3 and 15% gel was used for cleaved caspase-3. The polyvinylidene difluoride membranes (IPVH00010; Merck Millipore Ltd.) were incubated with 2% bovine serum albumin (A9647-100G; MilliporeSigma) for 60 min at room temperature (20–22°C) for blocking. Following incubation with the primary antibodies overnight at 4°C, the polyvinylidene difluoride membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; 711-035-152; donkey anti-rabbit IgG, 715-035-150; donkey anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc.) for 60 min at room temperature (20–22°C). The protein bands were visualized using an Ez West Lumi Plus detection kit (ATTO Corporation) and the LuminoGraph II imaging system (ATTO Corporation). The protein expression was quantified using ImageJ 1.53 software (National Institute of Health).

Immunofluorescence assay. The cells were seeded at 2×10^4 cells/well of a 2-chamber slide and were then fixed in 4% paraformaldehyde for 10 min at 37°C, permeabilized with 0.1% Triton X-100 for 15 min at room temperature (20–22°C) and blocked in 2% bovine serum albumin (Biosera) in phosphate-buffered saline for 60 min at room temperature (20–22°C). Antigen recognition was performed by incubation with primary antibodies against p-Fyn (1:400; cat. no. AP0510, ABclonal Biotech Co., Ltd.) and integrin β 3 (1:200; cat. no. 336402, BioLegend, Inc.) overnight at 4°C, followed by incubation with Alexa Fluor 568- (1:600; cat. no. ab175471, Abcam) and 488- (1:600; A-21441, Invitrogen; Thermo Fisher Scientific, Inc.) conjugated secondary antibodies. Nuclei were counterstained with Invitrogen Prolong Gold Antifade Mountant with DAPI (P36931; Invitrogen; Thermo Fisher Scientific, Inc.). Fluorescent images were obtained using a TCS SP8 microscope (Leica Microsystems GmbH). Co-localization analysis was performed using ImageJ 1.53 software (National Institute of Health). Pearson's R value was determined as follows:

$$R = \sum_i (X_i - \bar{X}) \times (Y_i - \bar{Y}) / \sqrt{\sum_i (X_i - \bar{X})^2 \times \sum_i (Y_i - \bar{Y})^2}$$

where X_i or Y_i is the individual intensity in the pixel indexed with i , and $\bar{X}$ or $\bar{Y}$ is the mean intensity. R value ranges between -1 and 1 continuously, and $0 < R < 1$ and $-1 < R < 0$ suggest correlation and anti-correlation, respectively.

Drug sensitivity assay. The cells were seeded in 96-well microplates at 5×10^3 cells/well and cultured at 37°C for 48 h. The cells were then incubated with increasing concentrations of vinorelbine from 0.1 nmol/l to 50 μ mol/l for 96 h. The cells were additionally incubated with 10 μ l Cell Counting Kit-8 reagent (Dojindo Laboratories, Inc.) for 2 h, and the absorbance at the 450 nm wavelength was measured using Microplate Manager 6 (Bio-Rad Laboratories, Inc.). The half-maximal inhibitory concentration (IC_{50}) was calculated using Prism 7 software (GraphPad Software, Inc.) with a three-parameter sigmoidal curve fit.

Cell proliferation assay. The cells were seeded in 96-well microplates at 5×10^3 cells/well and cultured at 37°C for 48 h. The cells were additionally incubated with 10 μ l Cell Counting Kit-8 reagent (Dojindo Laboratories, Inc.) for 1 h, and the absorbance at 450 nm wavelength was measured using Microplate Manager 6 (Bio-Rad Laboratories, Inc.). The ratio of cell proliferation was determined by comparing the measured absorbance.

Small interfering (si)RNA-mediated gene silencing. Ambion's Silencer™ Select Pre-Designed siRNAs (ID nos. s9491, 9492 and 9493) targeting Nrf2 were purchased from Thermo Fisher Scientific, Inc. The sequences of the siRNAs used are presented in Table SI. The cells were seeded at ~60% confluency in 6-cm dishes with antibiotic-free medium containing 12.5 μ l Lipofectamine 2000® (cat. no. 11668019; Thermo Fisher Scientific, Inc.) and 1,000 μ l Opti-MEM (cat. no. 31985062; Thermo Fisher Scientific, Inc.), which gave a final siRNA concentration of 20 nmol/l, transfected for 48 h, and then harvested for lysate preparation.

Patients and sample collection. In total, 104 surgical specimens were obtained from patients who underwent lung cancer resection followed by adjuvant cisplatin-vinorelbine treatment for pathological stage II to IIIA (UICC, 7th edition) (22) between December, 2006 and June, 2018 at the Department of Thoracic Surgery, Kyoto University Hospital (Kyoto, Japan). The follow-up period ranged from 3 to 159 months (median, 40 months). The characteristics of the 104 patients are presented in Table SII. Disease-free survival and overall survival were available for all patients. The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University (approval no. G0028-7, R1706, R1486). Informed consent was obtained from all patients.

Immunohistochemistry. Immunohistochemistry was performed to evaluate the expression of Nrf2, ABCB1 and p-Fyn in formalin-fixed, paraffin-embedded tissue sections from 3 to 4 μ m in thickness of the 104 surgical samples mentioned above. The slides were stained using the VECTASTAIN Elite ABC HRP kit (Vector Laboratories, Inc.) according to the manufacturer's protocol. Diaminobenzidine (049-22831, FUJIFILM Wako Pure Chemical Corporation) was used as the chromogen.

Statistical analysis. The log-rank test was used to compare two Kaplan-Meier survival curves, and statistical analyses were performed using Prism 7 (GraphPad) and JMP12 software (SAS Institute Inc.). Fisher's exact test was used to compare the patient clinicopathological characteristics. An unpaired t-test with Welch's correction was used to compare the fluorescence strength. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of VR lung cancer cells. VR cell lines were established by exposing H1299 cells to increasing concentrations of vinorelbine from 5 to 500 nmol/l. These cells were

classified into three groups according to the resistance level as follows: VR5, resistant cells cultured at 5 nmol/l; VR50, resistant cells cultured at 50 nmol/l; and VR500, resistant cells cultured at 500 nmol/l (Fig. 1A). According to the datasheet of Navelbine® Injection (Kyowa Kirin Co., Ltd.), 5 nmol/l corresponds to the plasma concentration of vinorelbine at 24–48 h following the intravenous injection of 20 mg/m² vinorelbine in humans. The vinorelbine IC₅₀ values for the H1299 parental, VR5, VR50 and VR500 cells were 11.3, 43.9, 99.9 and 137.3 nmol/l, respectively (Fig. 1B). In Fig. S1, the ratio of H1299 VR500 over H1299 parental cells in number were plotted at 2, 3, 4 and 5 days after seeding in 96-well microplates at 5x10³ cells/well using cell proliferation assay. The ratio of cells declined as the time passed to day 5, and this result suggested that the H1299 VR500 cells were less proliferative than the H1299 parental cells. To evaluate the apoptosis of the H1299 cells treated with vinorelbine, lysates obtained from H1299 parental cells treated with or without 500 nmol/l vinorelbine and the VR500 cells were immunoblotted with anti-caspase-3, anti-cleaved caspase-3 and anti-Bcl-2 antibodies. Western blot analysis revealed that the expression of cleaved caspase-3 was augmented in the H1299 parental cells treated with 500 nmol/l vinorelbine, and conversely, Bcl-2 expression was increased in the VR500 cells (Fig. S2). The A549 VR500 cells were established in the same manner as the H1299 VR500 cells.

ROS formation in lung cancer cells induced by vinorelbine. The H1299 parental cells and VR500 cells were uniformly seeded into 6-well plates at 4x10⁴ cells/well. The parental cells were incubated for 24 h with or without 500 nmol/l vinorelbine, and the VR500 cells were incubated for 24 h with 500 nmol/l vinorelbine as above. Intracellular ROS formation was detected in both parental cells and VR500 cells treated with 500 nmol/l vinorelbine (Fig. 1C). To quantify the strength of ROS formation, the area of fluorescence per cell and the mean intensity of fluorescence in the parental cells and VR500 cells were calculated. The area of fluorescence per cell in the parental and VR500 cells was 449.5±480.8 and 1,474±1,731 μm² (P<0.0001). The mean fluorescence intensity in the parental cells and VR500 cells was 51.47±10.84 and 71.83±4.674 μm² (P<0.0001; Fig. 1D).

ABCB1 overexpression, and p-Fyn and Nrf2 nuclear accumulation in VR cells. The total expression of ABCB1, Nrf2 and p-Fyn was augmented in the crude lysate of the VR cells (Fig. 2A), indicating that Nrf2 contributes to vinorelbine resistance, in addition to ABCB1 and p-Fyn. In the MRP family, MRP3/ABCC3 expression was augmented in a manner similar to ABCB1 (Fig. S3). In the A549 VR500 cells, ABCB1 expression was augmented, whereas Nrf2 and p-Fyn expression was not upregulated (Fig. S4). Given that the diverse activities of SFKs, including Fyn, are dependent on their subcellular localization (23,24), lysates obtained from the subcellular extracts of H1299 parental, VR5, VR50 and VR500 cells were immunoblotted with anti-ABCB1, anti-Fyn, anti-p-Fyn and anti-Nrf2 antibodies to investigate the subcellular localization and expression of ABCB1, p-Fyn and Nrf2 in the VR cell lines. ABCB1 expression was observed in the membrane extracts and compared with that in the nuclear and cytoplasmic extracts

(Fig. 2B). The VR cells exhibited the nuclear and cytoplasmic localization of p-Fyn.

Immunofluorescence was used to confirm the precise subcellular localization of p-Fyn downstream of integrin β3. The VR5 cells were incubated with primary antibodies against p-Fyn and integrin β3. The fluorescence images revealed that p-Fyn was located in the nucleus, as well as the cytoplasm adjacent to integrin β3 in the membrane (Fig. 2C). Co-localization analysis demonstrated a positive linear association between integrin β3 and p-Fyn (Fig. 2D). These findings suggest that p-Fyn functions in both the cytoplasm downstream of integrin β3 and the nucleus.

Dependence of ABCB1 expression on Nrf2 in VR cells. To elucidate whether the decreased activity of Nrf2 downregulates ABCB1 expression in VR cells, knockdown experiments were performed using siRNA. The VR500 cells in which Nrf2 was overexpressed, as described above in the paragraph entitled ‘ABCB1 overexpression, and p-Fyn and Nrf2 nuclear accumulation in VR cells’ were transfected with siRNAs targeting Nrf2 and incubated for 48 h. Scrambled RNA was used as a negative control. In the VR500 cells, siRNAs with different sequences targeting Nrf2 similarly decreased Nrf2 expression and downregulated ABCB1 expression (Fig. 3A).

To examine whether activated Nrf2 upregulates ABCB1 expression, Keap1 was inhibited using bardoxolone. The VR5 cells in which the Nrf2 level was lower than that in the VR500 cells were treated with the indicated concentrations of bardoxolone for 24 h. Bardoxolone treatment increased the expression of both Nrf2 and ABCB1 in a concentration-dependent manner up to 0.8 μmol/l (Figs. 3B and S5).

Effect of Nrf2 knockdown and Keap1 inhibition on the sensitivity and resistance to vinorelbine. As Nrf2 regulates the expression and functions of ABCB1 (21), the present study analyzed the drug resistance profile of VR cells to examine whether Nrf2 contributes to vinorelbine resistance. Nrf2 knockdown lowered the vinorelbine IC₅₀ value in VR50 cells from 119.8 to 36.6 nmol/l, and restored vinorelbine sensitivity (P<0.001; Fig. 3C). By contrast, bardoxolone exposure for 96 h at 0.8 μmol/l increased the vinorelbine IC₅₀ value in the VR5 cells from 43.9 to 111.8 nmol/l and contributed to vinorelbine resistance (P<0.001; Fig. 3D).

Immunohistochemical analysis of clinical lung cancer tissue samples. To elucidate whether the expression of Nrf2, ABCB1 and p-Fyn is associated with the prognosis of patients with completely resected lung cancer, 104 tissue samples obtained from patients who underwent adjuvant cisplatin-vinorelbine treatment were stained immunohistochemically. The survival curves and clinicopathological data of the patients are presented in Fig. 4 and Table SII, respectively. The clinical samples were stained for Nrf2, ABCB1 and p-Fyn; representative staining patterns are illustrated in Fig. 5A–F. Nrf2, ABCB1 and p-Fyn were predominantly expressed in the nucleus, cytoplasm and nucleus, respectively. This finding is compatible with the subcellular localization of each protein shown in Fig. 2B. Of the 104 patients, 47 (45%) were positive for Nrf2, 53 (50%) for ABCB1, and 48 (46%) for p-Fyn (Tables I and SII). Nrf2 and ABCB1 were significantly associated with disease-free survival

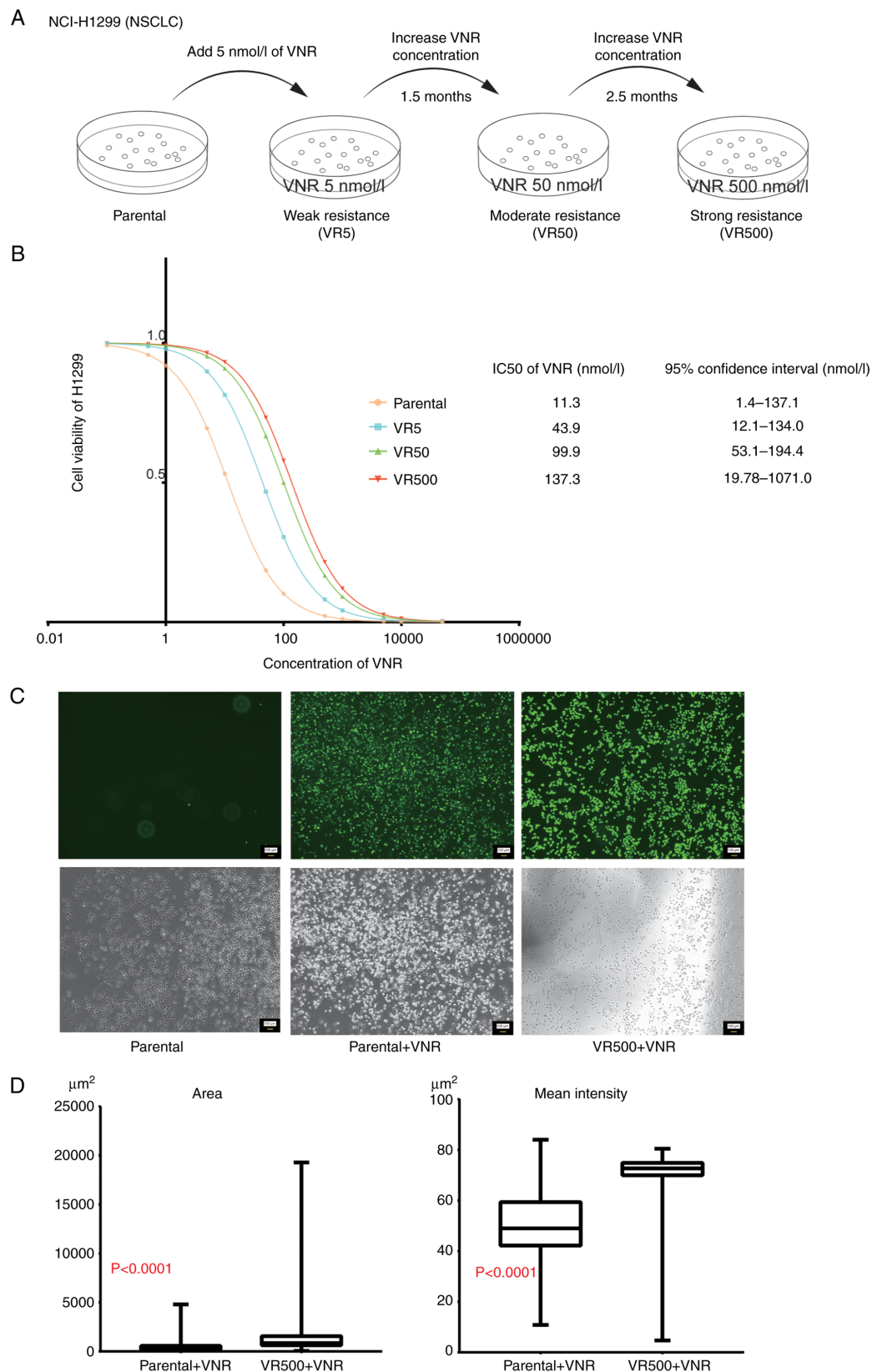


Figure 1. Establishment of VR cell lines. (A) VR cells were obtained following the repetitive subculture of H1299 cells in graded concentrations of vinorelbine from 5 to 500 nmol/l. VR5 cells were cultured in 5 nmol/l vinorelbine, the VR50 cells in 50 nmol/l vinorelbine and the VR500 cells in 500 nmol/l vinorelbine. (B) Viability of parental and VR cell lines incubated with increasing concentrations of vinorelbine for 96 h. The IC₅₀ values were calculated using Prism 7. VR500. The cells exhibited a >10-fold increase in vinorelbine resistance compared with the parental cells. (C) Parental cells were incubated for 24 h with or without 500 nmol/l vinorelbine, and the VR500 cells were incubated for 24 h with 500 nmol/l vinorelbine. Cells were observed under a fluorescence (GFP) microscope (upper) and an optical microscope (lower) in the same field of vision. Scale bars were 100 μ m. Fluorescence images were obtained using a BZ-X810 fluorescence microscope. (D) Area of fluorescence per cell and mean intensity of fluorescence in parental cells and VR500 cells. VR500 cells exhibited a larger area of fluorescence ($P<0.0001$) and a higher mean fluorescence intensity ($P<0.0001$) than the parental cells. Fluorescence strength was determined using ImageJ software. The data are presented as the mean $\pm$ SEM. An unpaired t-test with Welch's correction was used to compare fluorescence strength. VR, vinorelbine-resistant; VNR, vinorelbine.

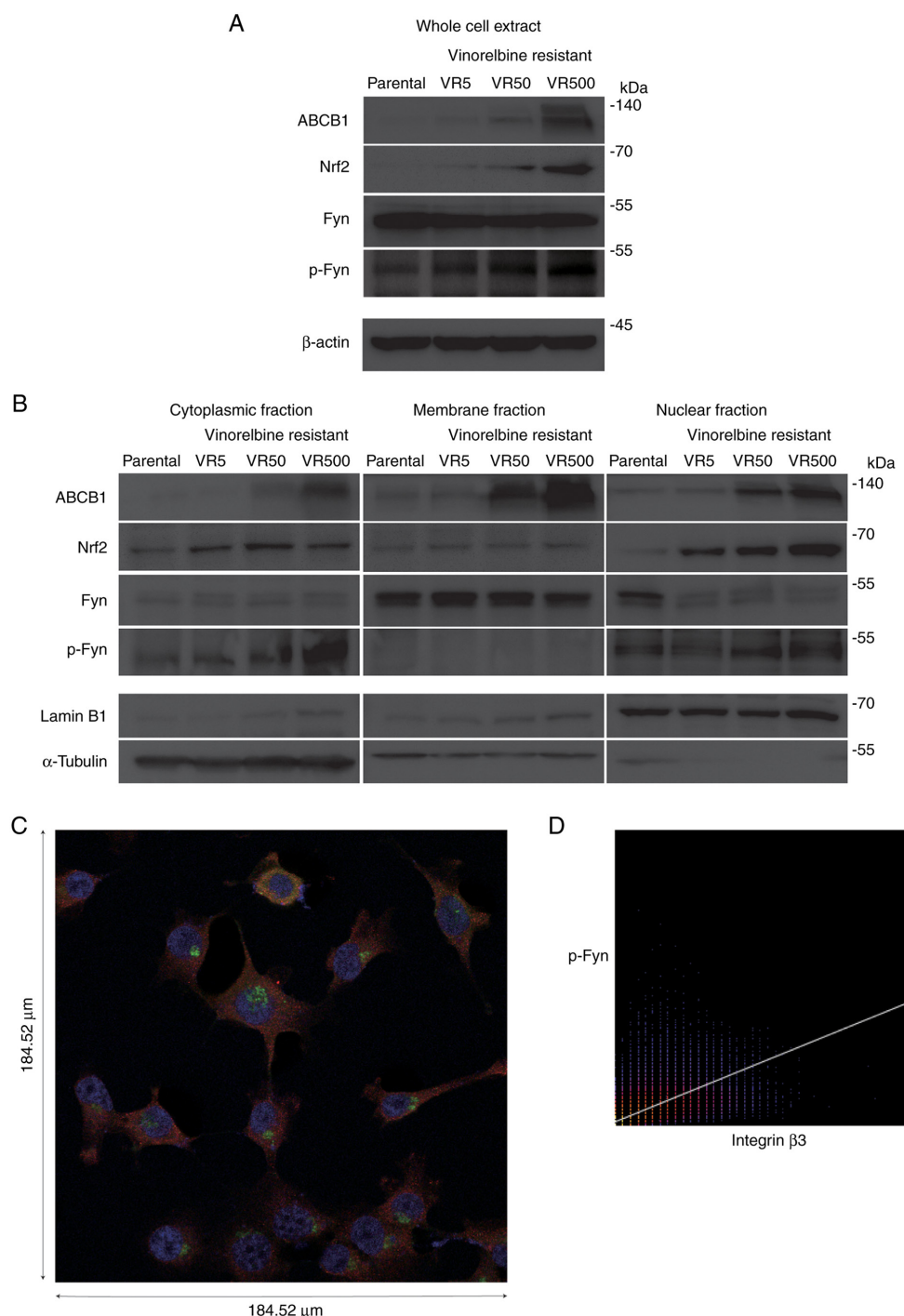


Figure 2. Subcellular localization of ABCB1, Nrf2 and p-Fyn in VR cells. (A and B) Parental and VR cell lines were harvested, and cytoplasmic, membrane, nuclear, and whole-cell extracts were prepared. All lysates were immunoblotted with anti-ABCB1, anti-Nrf2, and anti-Fyn, anti-p-Fyn antibodies. Lamin B1, α-tubulin and β-actin were probed as loading controls for nuclear, cytoplasmic and whole-cell extracts, respectively. (C) Immunofluorescence image illustrating p-Fyn localization in the nucleus, as well as on the membrane adjacent to integrin β3. Alexa 568 (red) staining indicates integrin β3, Alexa 488 (green) indicates p-Fyn, and DAPI (blue) indicates the nucleus. Scale bars were 184.52 μm. (D) Co-localization analysis for integrin β3 and p-Fyn using ImageJ software based on the immunofluorescence image. Pearson's R value was 0.65, and this indicated a positive linear relationship between integrin β3 and p-Fyn. VR, vinorelbine-resistant; VNR, vinorelbine; ABCB1, ABC subfamily B member 1; Nrf2, NF-E2-related factor 2; p-Fyn, phosphorylated Fyn.

($P=0.029$ and 0.035 , respectively), and p-Fyn was associated with overall survival ($P=0.040$) (Fig. 4). The expression of Nrf2 was significantly associated with that of p-Fyn ($P=0.005$), but not with that of ABCB1 ($P=0.244$). The frequency of positive Nrf2 expression was significantly associated with squamous cell carcinoma ($P=0.022$), but not with adenocarcinoma ($P=0.097$) or other cancers ($P=0.726$) (Table I).

Discussion

Vinorelbine induces intracellular ROS. In the present study, Nrf2 expression was augmented in the nuclei, as well as the whole cell of VR cells, and intracellular ROS formation was detected in both parental and VR cells exposed to vinorelbine. Notably, VR cells, which had been cultured with vinorelbine for

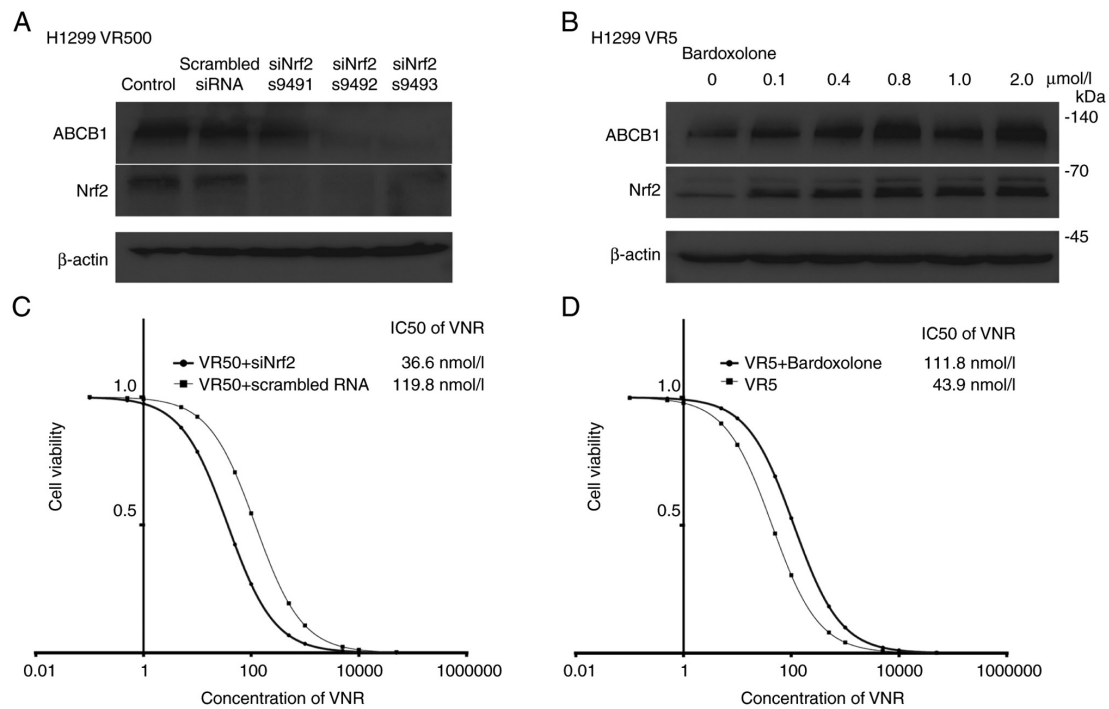


Figure 3. Dependence of ABCB1 on Nrf2. (A and B) VR500 cells were treated with lipofectamine and Opti-MEM (control) or transfected with scrambled siRNA or siRNAs with sequences targeting Nrf2 (s9491, s9492 and s9493). VR5 cells were treated with DMSO or the indicated concentrations of bardoxolone for 24 h. Lysates were immunoblotted with anti-ABCB1 and anti-Nrf2 antibodies. β-actin was probed as a loading control. (C and D) Viability of VR50 cells transfected with siRNA targeting Nrf2 (s9492) and VR5 cells treated with 0.8 μmol/l bardoxolone and incubated with increasing concentrations of vinorelbine for 96 h. VR, vinorelbine-resistant; VNR, vinorelbine; ABCB1, ABC subfamily B member 1; Nrf2, NF-E2-related factor 2.

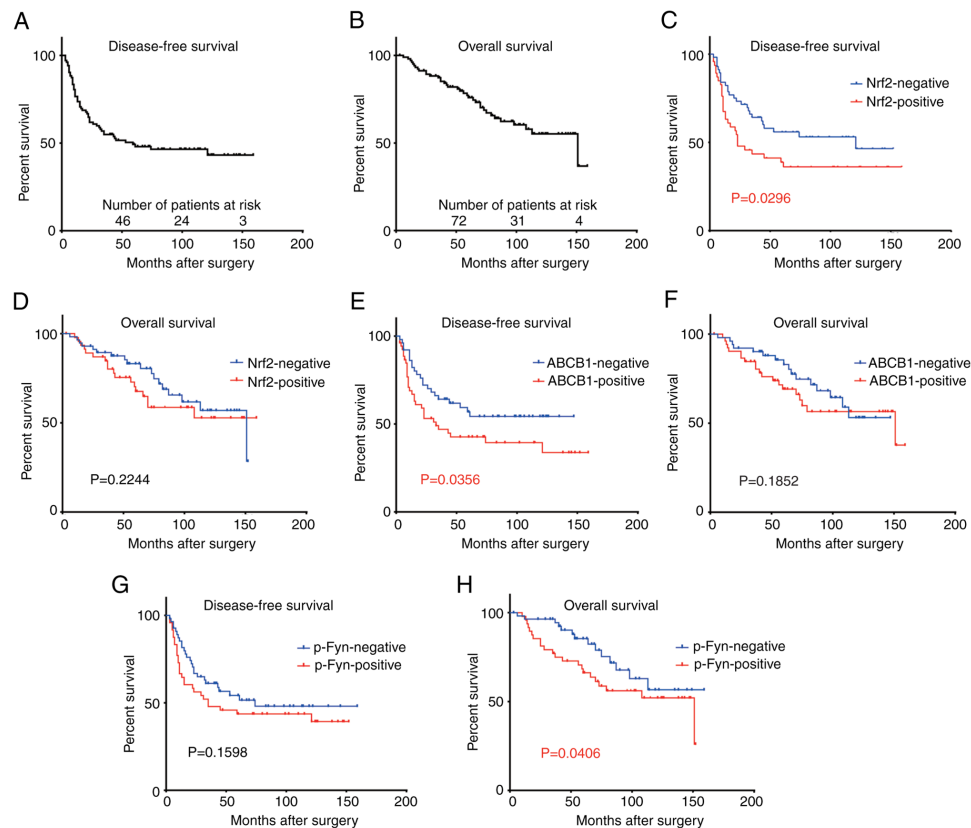


Figure 4. Survival curves of the 104 patients. (A) Disease-free survival (49.2% at 5 years) (A) and (B) overall survival (75.9% at 5 years) of all patients. (C-H) Disease-free survival and overall survival rates based on the expression of (C and D) Nrf2, (E and F) ABCB1, and (G and H) p-Fyn. Significant differences were found in disease-free survival rates for Nrf2 or ABCB1 and overall survival for p-Fyn. The log-rank test was used to compare two Kaplan-Meier survival curves, and statistical analyses were performed using Prism 7 (GraphPad) and JMP12 software. ABCB1, ABC subfamily B member 1; Nrf2, NF-E2-related factor 2; p-Fyn, phosphorylated Fyn.

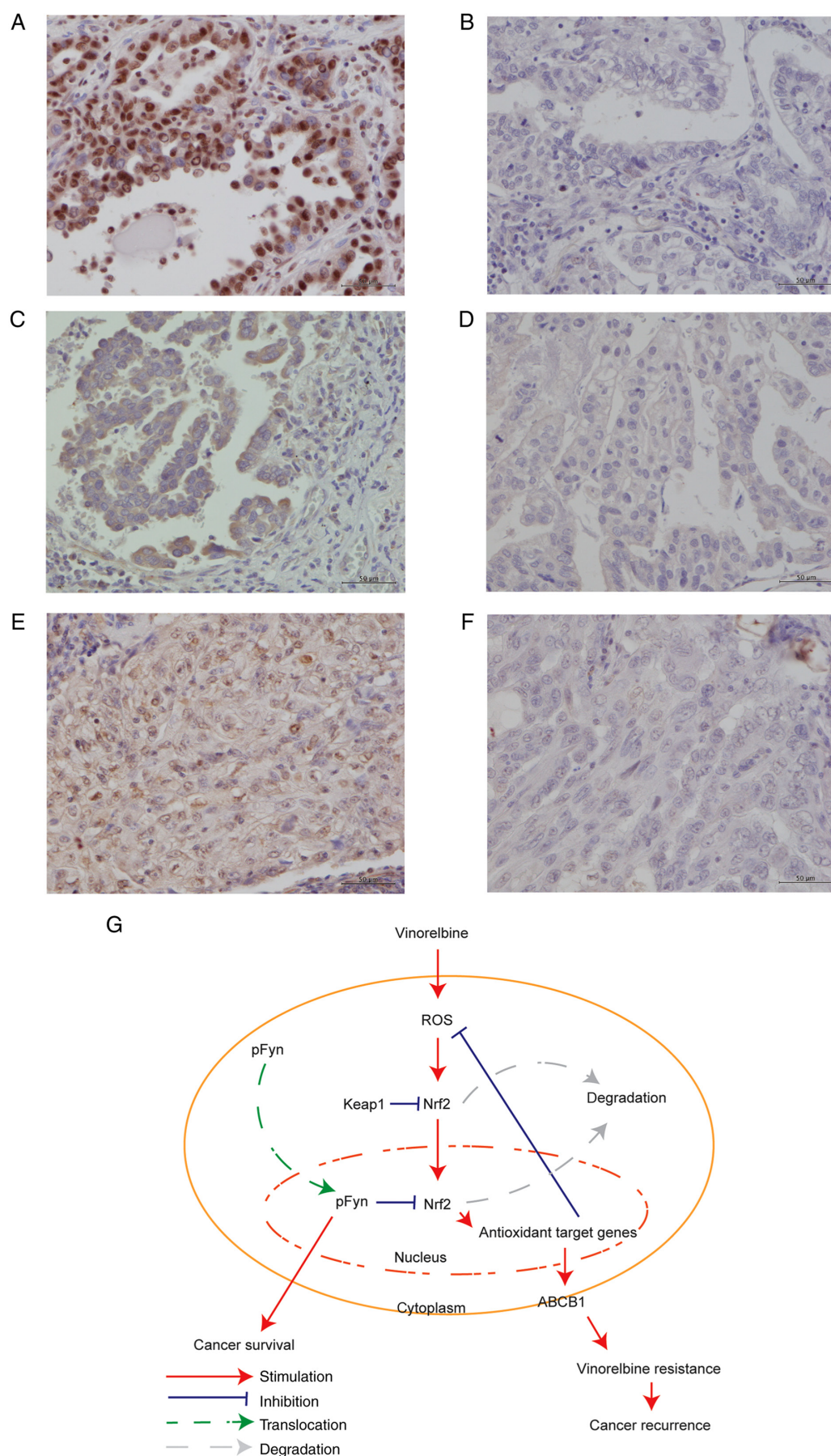


Figure 5. Examples of immunohistochemistry staining and proposed model of the signaling pathway. (A-F) Representative images of Nrf2, ABCB1 and p-Fyn immunohistochemical staining showing (A) Nrf2-positive, (B) Nrf2-negative, (C) ABCB1-positive, (D) ABCB1-negative, (E) p-Fyn-positive, and (F) p-Fyn-negative cells. Original magnification, x400; scale bars, 50 μ m. (G) Proposed model of the signaling pathway for vinorelbine resistance. ABCB1, ABC subfamily B member 1; Nrf2, NF-E2-related factor 2; p-Fyn, phosphorylated Fyn.

Table I. Association of Nrf2 expression with the expression of ABCB1 and p-Fyn, and histological types of lung cancer in the 104 patients.

	Nrf2 expression			Odds ratio (95% CI)	P-value
	Total n=104 (100%)	Positive n=47 (45.2%)	Negative n=57 (54.8%)		
ABCB1 expression				1.609 (0.738-3.505)	0.244
Positive	53 (51.0)	27 (26.0)	26 (25.0)		
Negative	51 (49.0)	20 (19.2)	31 (29.8)		
p-Fyn expression				3.222 (1.439-7.212)	0.005
Positive	48 (46.2)	29 (27.8)	19 (18.2)		
Negative	56 (53.8)	18 (17.3)	38 (36.5)		
Histology					
Squamous cell carcinoma				3.022 (1.195-7.643)	0.022
Yes	26 (25.0)	17 (16.4)	9 (8.6)		
No	78 (75.1)	30 (28.9)	48 (46.1)		
Adenocarcinoma				0.482 (0.211-1.101)	0.097
Yes	69 (66.4)	27 (26.0)	42 (40.4)		
No	35 (33.6)	20 (19.2)	15 (14.4)		
Other types				0.709 (0.160-3.136)	0.726
Yes	8 (7.7)	3 (2.9)	5 (4.8)		
No	96 (92.3)	44 (42.3)	52 (50)		

ABCB1, ABC subfamily B member 1; Nrf2, NF-E2-related factor 2; p-Fyn, phosphorylated Fyn. P-values were all determined using Fisher's test.

months and acquired resistance to vinorelbine, exhibited higher levels of ROS than the parental cells treated with vinorelbine for 24 h. The formation of excessive ROS is essential for the induction of apoptosis by commonly used chemotherapeutic agents such as cisplatin, bleomycin, paclitaxel, adriamycin, etoposide and vinca alkaloids (15). Vinorelbine, a semi-synthetic vinca alkaloid, arrests cell growth at the prometaphase by inhibiting microtubule polymerization and induces the accumulation of mitochondrial ROS followed by prolonged JNK activation, DNA damage, a decrease in Mcl-1 expression, mitochondrial dysfunction and caspase-mediated apoptosis (14). Under conditions of oxidative stress, increased levels of intracellular ROS promote the dissociation of Nrf2 and Keap1. Free Nrf2 translocates to the nucleus, where it binds to ARE and transactivates downstream cytoprotective genes to induce cell defense processes and enhance cell resistance (15,25). Oxidants, xenobiotics and electrophiles, including chemotherapeutic agents hamper the Keap1-mediated proteasomal degradation of Nrf2 and induce the transcription of target genes (26). The results of the present study suggested that persistent exposure to vinorelbine induced intracellular ROS in VR cells, which may subsequently stimulate Nrf2.

Activated Nrf2 upregulates ABCB1 in VR cells. Whole-cell extracts obtained from VR cells exhibited an increased expression level of ABCB1 and Nrf2. The level of MRP3/ABCC3,

which belongs to ABC sub-family C, was also augmented in VR cells, indicating that MRPs, as well as ABCB1 were associated with vinorelbine resistance. In a previous study, the authors compared the gene and protein expression of H1299 parental cells with that of VR cells using DNA microarray, and this microarray-based comparison did not reveal any specific change in MRP expression, but revealed a 10-fold or more change in ABCB1 expression (9). Therefore, the present study focused on a mechanism involving ABCB1 in vinorelbine resistance. Nrf2, a key transcription factor, protects cells from oxidative damage and harmful xenobiotics (27). Under unstimulated conditions, Nrf2 is retained in the cytoplasm by the anchor protein, Keap1, and is constantly ubiquitinated and degraded in the proteasome (20). Upon exposure to oxidative or xenobiotic stress, the Keap1-mediated proteasomal degradation of Nrf2 is inhibited, and Nrf2 translocates to the nucleus, where it forms a heterodimer with the small Maf protein and binds to ARE (28). The Nrf2 effector genes bearing ARE include those that encode the majority of antioxidant and phase II detoxifying enzymes (29). In addition to these enzyme-coding genes, Nrf2 transactivates a wide variety of other genes, including several ATP-dependent drug efflux pumps (30,31). This suggests that activated Nrf2 in cancer cells provides advantages in terms of survival and drug resistance (32). Nrf2 expression enhances the resistance of myelogenous leukemia and colorectal, breast, pancreatic,

and gallbladder cancers to chemotherapeutic agents, such as imatinib, oxaliplatin, tamoxifen, gemcitabine and 5-fluorouracil, respectively (33-37). The results of the present study indicated that ABCB1 and Nrf2 are both involved in vinorelbine resistance, and that activated Nrf2 upregulates ABCB1 expression. These findings suggest that ABCB1 is dependent on Nrf2 and its downstream activity in vinorelbine resistance.

Decrease in Nrf2 expression enhances sensitivity to vinorelbine. The results of the present study revealed that the suppression of Nrf2 potentiated the cytotoxicity of vinorelbine, and the upregulation of Nrf2 conferred resistance to vinorelbine. These findings suggested that Nrf2 plays a crucial role in regulating the susceptibility to vinorelbine, and that the upregulation of Nrf2 in VR cells confers vinorelbine resistance. Thus, lowering the expression of Nrf2 may present a novel therapeutic approach for VR lung cancer.

p-Fyn accumulates in VR cell nuclei. p-Fyn accumulated in the nuclei of VR cells and was located in the cytoplasm adjacent to integrin $\beta 3$ in the membrane. In the focal adhesion pathway, Fyn functions downstream of several important cell surface receptors, including integrin $\beta 3$, and upstream of several cellular signals important for cancer progression. Although the integrin cytoplasmic domains are short and do not have any known catalytic activity, the engagement of integrins by extracellular matrix ligands triggers outside-in signals that collaborate with growth factor-initiated signals to determine cell fate and function (38,39). Deregulated integrin signaling empowers cancer cells with the ability to proliferate without restraint, invade through tissue boundaries, and survive in foreign microenvironments (40). SFKs bind to multiple integrin β cytoplasmic domains to transmit integrin-dependent signals pivotal for cell movement and proliferation; in particular, Fyn selectively binds to the integrin $\beta 3$ domain (38). Similar to other Src family members, Fyn regulates cell shape and migration (12) and is located primarily in the cytoplasm, although it is also observed in other cellular compartments, including the nucleus (41). Fyn activity is regulated in different subcellular compartments, and different equilibrium states between Fyn and the corresponding kinase are maintained in the cytoplasm and nucleus (23). Resting-state Fyn is localized near the perinuclear region in endosomes, whereas activated Fyn is trafficked to the plasma membrane via the actin cytoskeleton (42,43). The nuclear expression of p-Fyn is highly associated with the poor prognosis of patients with resected lung adenocarcinoma (44). Here, immunofluorescence images revealed the nuclear accumulation of p-Fyn in VR cells. These results are consistent with those of previous studies by the authors (9,44). The nuclear or cytoplasmic localization of Fyn in breast cancer cells has been shown to be associated with early recurrence in patients treated with endocrine therapy (13,41). However, the role of nuclear p-Fyn in vinorelbine resistance remains unclear.

Nuclear accumulation of p-Fyn follows Nrf2 activation. In the present study, subcellular fractionation and immunofluorescence demonstrated that p-Fyn accumulated in the nucleus, as well as the cytoplasm. The regulation of Nrf2, particularly its abundance in the nucleus, is important for controlling the

expression of cell-protective genes in response to oxidative stress. The continuous accumulation of Nrf2 in the nucleus can cause disease conditions (45). For example, in Keap1-deficient mice, the persistent accumulation of Nrf2 in the nucleus causes hyperkeratosis in the esophagus and forestomach, leading to neonatal death (45). As a persistent increase in the expression of cell-protective genes threatens cell survival (46), aggregated Nrf2 should be subsequently exported out of the nucleus and degraded.

The abundance of Nrf2 inside the nucleus is tightly controlled by positive and negative regulators that affect nuclear import, ARE binding, nuclear export, and degradation of Nrf2 under normal and stressful conditions (47). The nuclear export of Nrf2 is activated after Fyn accumulates in the nucleus (48). Fyn is responsive to oxidative stress (47). It is exported out of the nucleus soon after exposure to oxidative stress, which allows Nrf2 to bind to ARE and activate NAD(P)H:quinone oxidoreductase 1. The nuclear export of Fyn is an integral part of the ARE/Nrf2-mediated activation of cytoprotective genes (47). Chemical stress induces activated GSK-3 β , which phosphorylates Fyn and accumulates p-Fyn in the nucleus, resulting in the ubiquitination and degradation of nuclear Nrf2 (48). The results of the present study suggested that the nuclear accumulation of p-Fyn following Nrf2 activation in VR cells triggers the nuclear export and degradation of Nrf2.

Expression of Nrf2 and ABCB1 predicts a poor disease-free survival. The immunohistochemical findings demonstrated that Nrf2 and ABCB1 were significantly associated with disease-free survival. Previous studies have demonstrated an association between ABCB1 expression and chemotherapy resistance in colorectal cancer (49), breast cancer (50) and chronic myelogenous leukemia (51). Nrf2 expression significantly promotes tumor size, histological grade, distant metastasis and lymph node metastasis, and reduces sensitivity to chemotherapy or radiotherapy (8,17,52). A positive Nrf2 expression in the nucleus is associated with a poor prognosis of patients with esophageal squamous cell carcinoma following chemoradiotherapy (53). The nuclear Nrf2 expression in malignant lung cancer cells is related to resistance to chemotherapy in squamous cell carcinoma (54). Consistent with these reports, the results of the present study revealed that Nrf2 expression was significantly associated with squamous cell carcinoma, implying that squamous cell carcinoma is a predictor of vinorelbine resistance. It was also found that Nrf2 and ABCB1 were closely associated with a poor susceptibility to vinorelbine and subsequent tumor relapse, and p-Fyn was not associated with disease-free survival, but with overall survival. These findings reveal that vinorelbine resistance is not a direct cause of the nuclear accumulation of p-Fyn, but rather the overall prognosis, which emerges from Nrf2 activation in the nucleus.

Proposed model for signaling pathway for vinorelbine resistance. The results of the present study support the following model depicting the role of Nrf2 and ABCB1 in vinorelbine resistance (Fig. 5G). In the absence of stress, Nrf2 is bound to Keap1 and degraded through a proteasome-dependent pathway, whereas the presence of ROS induced by vinorelbine

hinders the Keap1-mediated proteasomal degradation of Nrf2. Nrf2 released from Keap1 is translocated into the nucleus and activates the transcription of a broad spectrum of defensive genes, including *ABCB1*. The increase in the expression of chemoprotective genes neutralizes chemical stress and confers vinorelbine resistance to the cells, which consequently promotes tumor recurrence. As a persistent increase in defensive gene expression threatens cell survival, Nrf2 is exported out of the nucleus and degraded only after p-Fyn accumulates in the nucleus. The nuclear accumulation of p-Fyn itself is a predictor of a poor overall survival of patients with lung cancer.

The present study has the following limitations: The sample size was small, as only datasets of patients with resected stage II or IIIA lung cancer and adjuvant cisplatin-vinorelbine treatment at a single institute were analyzed. In addition, the present study was retrospective in nature. Further studies are thus required to develop improved strategies for VR non-small cell lung cancer.

In conclusion, the Nrf2-pFyn-ABCB1 axis plays a pivotal role in vinorelbine resistance in non-small cell lung cancer. Nrf2 upregulates ABCB1 and induces vinorelbine resistance, and Nrf2 activation causes the nuclear accumulation of p-Fyn, which triggers Nrf2 export out of the nucleus and subsequent degradation. The present study revealed the possible pathway underlying vinorelbine resistance in non-small cell lung cancer. It is hoped that these findings will help researchers to develop strategies with which to avoid this resistance or develop novel drug targets.

Acknowledgements

The authors would like to thank the Medical Research Support Center, Graduate School of Medicine, Kyoto University for TCS SP8 and the Center for Anatomical Pathological and Forensic Medical Researches, Graduate School of Medicine, Kyoto University for the formalin-fixed, paraffin-embedded tissue sections.

Funding

The present study supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 20H03770).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors (ShT, TM, TT, HM, NC, MN, HI, RM, HK, SaT, YYa, YYu, DN, AO, MH and HD) participated in the study design and data interpretation, revised the manuscript, approved the manuscript for publication, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. ShT, TM, TT, HM and NC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University (approval no. G0028-7, R1706, R1486). Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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