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SHORT COMMUNICATION

Enhancing effect of connexin 32 gene on vinorelbine-induced cytotoxicity in A549 lung adenocarcinoma cells

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Abstract
Purpose Connexin (Cx) genes exert negative growth effects on tumor cells with certain cell specificity, and tumor-suppressive effects of the Cx genes contribute to enhancement of chemotherapeutical agents-induced cytotoxicity in some cancer cells. Since we and others have been reported that Cx32 acts as a tumor suppressor gene in lung adenocarcinomas, this study was undertaken to estimate if the combination of Cx32-dependent tumor-suppressive effect and vinorelbine (VBN), a chemotherapeutic agent which has been utilized for clinical lung adenocarcinoma treatment, could be effective in enhancing the sensitivity of the lung cancer to VBN treatment.
Methods We established the A549 cells (a human lung adenocarcinoma cell line) which had stable expression of Cx32 and estimated effect of Cx32 on VBN-induced cytotoxicity in the established cells.
Results Cx32 expression in A549 cells significantly potentiated VBN-induced cytotoxicity on the cells due to enhancement of apoptosis induction. The enhancing cytotoxicity in A549 cells by Cx32 mainly depended on a decrease in expression of multi-drug resistance-1 (MDR-1) gene responsible for reduction of VBN accumulation into the cells. We also observed that silencing of Cx32 by siRNA treatment elevated the expression level of MDR-1 mRNA in A549 cells and that inhibition of MDR-1 gene product-dependent function enhanced VBN-induced cytotoxicity in the cells.
Conclusion These results suggest that Cx32 contributes to the enhancement of VBN-induced cytotoxicity in A549 cells via the reduction of MDR-1 expression.

Keywords Connexin 32 · Lung adenocarcinoma · Vinorelbine · MDR-1 · Tumor suppressor gene

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Introduction

Lung cancer, particularly non-small cell lung cancer (NSCLC), is one of the most common cancers and the leading cause of cancer death in Western countries, as well as Japan [24, 48]. Of NSCLC, adenocarcinoma type is one of the major histological types and relatively drug-resistant [5]. Previous trials at cancer therapy of lung adenocarcinoma have met with failure to improve survival, largely due to older and relatively ineffective chemotherapy agents [14, 18, 32]. Vinorelbine (VBN), a relatively new semisynthetic *Vinca* alkaloid with significant activity in lung adenocarcinoma and a relatively low toxicity profile even in the elderly patients [10, 11]. VBN has a substitution on the catharanthine rather than the vindole moiety of the molecule, which imparts increased lipophilicity, presumably allowing the agent to more readily diffuse into the cell. This modification might account for the higher

therapeutic index and different spectrum of anti-tumor activity comparing other *Vinca* alkaloids [31, 34, 40]. Due to these positive properties, clinical benefit of VBN as a chemotherapy agent against lung adenocarcinoma is clear. However, the tumors have VBN resistance based on multiple molecular factors [26], so establishment of a procedure to abrogate the VBN resistance is required for optimizing lung adenocarcinoma therapy using the agent.

Of multiple molecular determinants related to VBN resistance, it is expected that multidrug resistance predominates [13]. The expression of multidrug resistance-1 (MDR-1) gene product, P-glycoprotein (Pgp), and multidrug resistance-related protein-1 (MRP-1) are correlated with multidrug resistance associated with VBN [1, 38], and *Vinca* alkaloids are good substrates for these transporters [14, 43]. For instances, Pgp functions as an energy-dependent transport pump capable of decreasing the intracellular concentration of a wide range of anticancer agents including *Vinca* alkaloids, which confers a chemoresistant phenotype in cancer cells [2, 8]. In addition to these pumps (ATP binding cassette transporter), it has been recently reported that a different type pump named RLIP76 (non-ATP binding cassette transporter) also catalyzes energy-dependent efflux of *Vinca* alkaloids and contributes to transportation of VBN in NSCLC [3, 37]. Thus, in order to improve VBN resistance in lung adenocarcinoma, it is an effective procedure to reduce the activity or expression of these pumps.

In general, it is considered that disruption of functions to maintain homeostasis in cellular society leads to the appearance of malignancies in the cells [29]. That is, the down-regulation of tumor suppressor genes keeping the homeostatis directly relates to the development of cancers. Among the tumor suppressor genes contributing to tissue homeostatis, down-regulation of connexin (Cx) genes, a member of gap junction (GJ) is associated with the development of cancers [15, 28, 41, 42]. GJ is comprised of two hemichannels (connexons), which are in turn formed oligomerization of six Cx proteins [15]. It has been established that Cx acts as a tumor suppressor gene by keeping electrical and metabolic cell homeostatis, via GJ-dependent transfer of small molecules less than 1,500 Da among neighboring cells which is termed GJ intercellular communication (GJIC) [28]. In addition to the GJ-dependent mechanism, the Cx gene exerts a tumor-suppressive effect in a GJ-independent manner [7, 12]. In fact, we have recently demonstrated that Cx32 acts as a tumor suppressor gene against lung adenocarcinoma cells in both a GJ-dependent and an-independent manner [19]. In a recent study, we have shown that one of the GJ-independent mechanisms is associated with reduction of chemoresistance in cancer cells [37]. Based on these reports, we hypothesized that Cx32 could enhance VBN-induced cytotoxicity against lung adenocarcinoma cells via the reduction in the level of VBN transporter(s). Furthermore, it is well established that the Cx gene can potentiate cytotoxicity of anti-cancer agents on cancer cells through a GJ-dependent mechanism [30], so it is likely that Cx32 also reinforces the VBN-induced cytotoxicity on lung adenocarcinoma cells, due to the same mechanism. Therefore, the present study was conducted to investigate the possibility.

Materials and methods

Reagents

All cultures and chemical reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Antibodies were obtained from BD Biosciences (San Jose, CA, USA) and Upstate (Charlottesville, VA, USA).

Cell culture, construct, transfection and treatment

A549 cells, a representative human lung adenocarcinoma cell line, was obtained from Riken cell bank (saitama, Japan), and maintained in media and culture conditions as described in the following: the cells were cultured in DMEM medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin on standard plastic tissue culture dishes and incubated in an atmosphere of 95% air/5% CO_2 at 37°C. The human Cx32 cDNA insert containing the entire coding region [16], was subcloned into the expression vector, pcDNA3 (Invitrogen), at the *KpnI–BamHI* site, and the sequence of construct pcDNA3-Cx32 was confirmed by DNA sequencing. Parental cells were transfected with either 3 μg of Cx32 cDNA or empty pcDNA3 vector as a control using Gene Jamar Transfect Reagent (Stratagene, La Jolla, CA, USA). After 48 h, the transfected cells were selected in culture medium containing 0.6 mg/ml G418 for 2 weeks. In order to avoid clonal variations by selection, we combined all of the surviving clones and used the combined clones to estimate tumor-suppressive effects of Cx32 [16]. For treatment experiments, exponentially growing cells were used. Cells were plated on culture plates and cultured for 24 h to permit the cells to adhere. After attachment, the cells were cultured for a further 0–48 h in culture medium containing VBN at each indicated dose or vehicle, and

each assay was performed. Verapamil hydrochloride (VPL), a Pgp inhibitor, at each indicated dose was added to culture medium and incubated for 48 h for the experiments as indicated in the results. PP1 (Biomol, Plymouth Meeting, PA, USA), a Src inhibitor, at 0.1 μM was added to culture medium and incubated for 24 h for the experiments. The cells were treated with 18-glycyrrhetinic acid (GA), a specific inhibitor against GJIC, at 1 μM for 24 h and then with VBN (5 nM) for 72 h.

Assessment of cell viability

The cells were plated at 1×10^4 cells/plate in microtiter plates, cultured in culture medium for 24 h, and treated with VBN and/or VPL for 48 h at indicated doses. Following treatment, cell proliferation assay was undertaken with WST-1 assay kit (Quick Cell Proliferation Assay Kit, MBL, Nagoya, Japan), according to the manufacturer's instruction. In brief, 10 μl of WST-1 reagent were added to each well, and the plate was incubated in an atmosphere of 95% air/5% CO_2 at 37°C for 10 min. After incubation, optical density at 450 nm in each well was determined using a microplate reader (Atto, Osaka, Japan).

Cell cycle and apoptosis analyses

The cells were plated at 2×10^5 cells/plate in plate, grown in culture medium for 24 h, and treated with VBN for 24 h to analyze cell cycle progress and 48 h to estimate apoptotic frequency at 5 nM. After that, the cells were fixed in 70% ethanol for 30 min at 4°C and incubated in PBS containing 0.05 mg/ml propidium iodide, 1 mM EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A for 30 min at room temperature. The cell suspension was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACScan.

Immunoblot analysis

The level of each molecule was estimated by immunoblot analysis, using a specific antibody against each protein. In brief, the cells were lyzed in 1 ml of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM dithiothreitol). The lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and subjected to immunoblotting with anti-β-actin, anti-total Src, and anti-phosphorylated Src. Detection was accomplished using the ECL system (Amersham, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver System (Atto, Tokyo, Japan). A two-dimensional densitometric evaluation of each band was performed using ATTO Image Analysis Software (ATTO). Molecular sizing was done using Rainbow Molecular Weight Marker (Amersham, Piscataway, NJ, USA). Protein concentration was determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA).

Determination of each mRNA level

Total RNA was isolated from cultured cells as described previously [47]. cDNA synthesis for each gene was carried out according to a previous method [46]. PCR reactions were carried out by using primers MDR-1 (NCBI reference number 42741658): sense primer (nucleotides 2,779–2,798); antisense primer (nucleotides 2,905–2,924), GAPDH (NCBI 7669491): sense primer (nucleotides 174–193), antisense primer (nucleotides 313–332), MRP-1 (NCBI reference number 9955961): sense primer (nucleotides 291–310); antisense primer (nucleotides 428–447), RLIP76 (NCBI reference number 974142): sense primer (nucleotides 610–630); antisense primer (nucleotides 737–756) and SYBR Green dye. The reactions were performed at 95°C for 10 s, followed by 35 cycles of 95°C for 5 s, then 60°C for 1 min. The products were detected with the ABI Prism 7000 Sequence Detector (Perkin Elmer Biosystems). The abundances of amplified DNA were determined from the threshold cycle values and were normalized to the values for the control gene GAPDH to yield the relative abundance. The value of each gene/GAPDH was normalized to that of control.

siRNA treatment

Cx32 gene was down-regulated by siRNA for Cx32 (Qiagen, MD, USA) as previously described [16]. At 48 h after siRNA treatment in Cx32-expressed A549 cells (A549-Cx32), the expression level of Cx32 protein was determined by Western blot analysis, and each mRNA level was estimated by real-time PCR as mentioned above.

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple-range test or Student's *t*-test. A P value of 0.05 or less was considered significant.

Results

Effect of Cx32 on cell viability, cell cycle progress and apoptosis induction in A549 cells treated with VBN

In our previous study, we have already established A549-Cx32 and mock-transfected A549 cells (A549-mock) [19]. The present study was carried out, using the two established cell clones. In order to examine an enhancing effect of Cx32 on VBN-induced cytotoxicity in A549 cells, we compared the cytotoxic potential of VBN on A549-mock and A549-Cx32. As shown in Fig. 1a, the cytotoxic effect of VBN on A549-Cx32 was higher than that on A549-mock in examined doses (0.1–5 nM) with statistical significances. Next, in order to investigate why Cx32 enhanced VBN-induced cytotoxicity against A549 cells, we compared the cell cycle progress and induction of apoptosis between A549-mock and A549-Cx32. As shown in Fig. 1b, VBN induced accumulation of the cells to G2/M phase in both A549-mock and A549-Cx32, and there was no difference on cell cycle distribution between the two groups. On the other hand, VBN caused apoptosis in A549-Cx32 more severely than that in A549-mock, and the difference showed a statistical significance (Fig. 1c). Finally, in order to estimate the involvement of GJIC in the VBN-mediated cytotoxicity on A549-Cx32, we examined if GA, a specific inhibitor of GJIC, could reduce the cytotoxic effect of VBN on the cells. As shown in Fig. 2, GA treatment partially abrogated VBN-mediated cytotoxicity in A549-Cx32, but the treatment did not affect the cytotoxicity in A549-mock treated with VBN. We have already confirmed that GJIC in GA-treated A549-Cx32 was almost inhibited under the GA treatment condition [19]. These results suggest that Cx32 potentiates VBN-induced cytotoxicity in A549 cells, due to enhancement of apoptosis induction, and that Cx32-mediated GJIC partly contributes to the potentiation of the VBN-induced cytotoxicity.

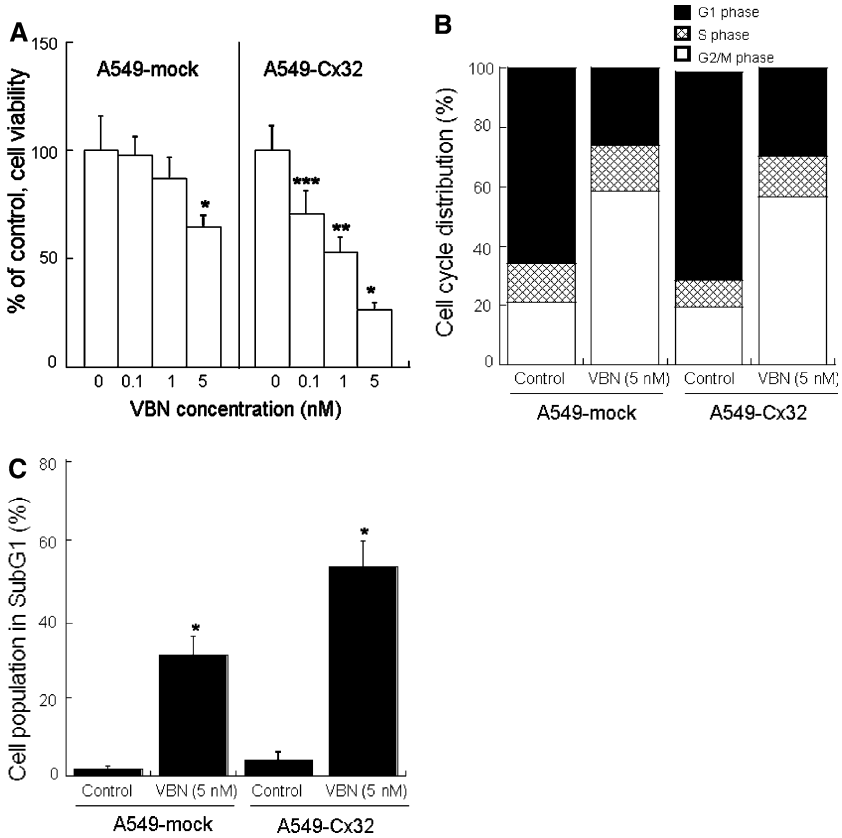

Fig. 1 Effects of VBN on cell viability (**a**), cell cycle progress (**b**) and induction of apoptosis (**c**) in A549-mock and A549-Cx32. **a** The cells were treated with indicated concentrations of VBN for 48 h, and subsequently cell viability was determined, using WST-1 reagent. Each value indicates the mean from six determinants; *vertical lines* indicate SD. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus treatment dose (0 nM). **b** The cells were treated with VBN (5 nM) for 24 h. After the treatment, cell cycle analysis was performed by FACS. Each value indicates the mean from three determinants. **c** The cells were treated with VBN (5 nM) for 48 h. After the treatment, the induction of apoptosis was estimated as cell population in SubG1 phase by FACS. The cell population in subG1 phase is expressed as the ratio to total cell population in cell cycle. Each value indicates the mean from three determinants; *vertical lines* indicate SD. $*P < 0.001$ versus control

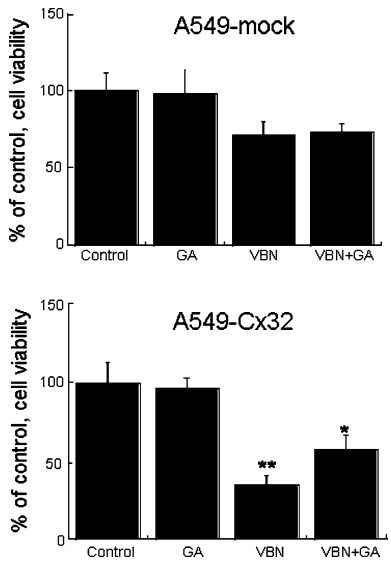

Fig. 2 Effect of GA (a GJIC inhibitor) on VBN-induced cytotoxicity in A549 cells. The cells (A549-mock and A549-Cx32) were treated with GA (1 μM) and then treated with 24 h later with VBN (5 nM) for 72 h. Cell viability was determined, using WST-1 reagent. Each value indicates the mean from three determinants; *vertical lines* indicate SD. $*P < 0.05$, $**P < 0.01$ versus control. *Control* vehicle treatment; *GA* treatment with 18-glycyrrhetinic acid; *VBN* treatment with VBN; *VBN + GA* co-treatment with VBN and GA

Effect of Cx32 on MDR-1 expression in A549 cells

As mentioned in introduction, three ATP- and non-ATP binding cassette transporters mainly contribute to VBN resistance in cancer cells [13, 37, 38], so we examined which transporter could be important for the enhancement of VBN-induced cytotoxicity in A549-Cx32. Of three transporters, only expression of MDR-1 gene in A549 cells was suppressed by Cx32 expression with a statistical significance (Fig. 3a). Also, expression of MDR-1 gene in A549-Cx32 was elevated under silencing of Cx32 gene by siRNA treatment, and the elevation showed a statistical significance (Fig. 3b). Furthermore, VPL (an inhibitor against Pgp) attenuated VBN-induced cytototxicity in A549-mock and brought the cytotoxic level down to the level in A549-Cx32 (Fig. 3c). From these results, it is indicated that the down-regulation of MDR-1 gene by Cx32 is responsible for enhancement of VBN-induced cytotoxicity in A549 cells.

The relation of Cx32 expression, Src activation and MDR-1 expression

In our previous study, we have suggested that Cx32 reduces MDR-1 expression, partly due to the inactivation of Src in cancer cells [35]. Thus, we investigated if the reduction of MDR-1 expression in A549-Cx32 was associated with the inactivation of Src. As shown in Fig. 4a, the activated level of Src in A549-Cx32 was much lower than that in A549-mock, and the difference showed a statistical significance. Additionally, the inhibition of Src activity by a Src inhibitor (PP1) induced the reduction of MDR-1 expression in A549-mock (Fig. 4b). These results suggest that Cx32-induced decrease of MDR-1 expression in A549 cells is associated with the inactivation of Src.

Discussion

It has been well known that human lung adenocarcinoma has severe chemoresistance against several types of chemotherapeutic agents and has a poor prognosis in most cases. Therefore, it is required to establish a new therapy against lung adenocarcinoma. In our previous study, we have reported that Cx32, a member of GJ protein family, reduces several malignant phenotypes of lung adenocarcinoma cells, mainly due to the regulation of Src signaling [19]. Also, it has been shown that the specific activity of Src is increased five- to eightfold compared to normal colon tissues in pre-malignant lesions as well as in the majority of colorectal adenocarcinomas, and that the difference in Src activity is correlated with tumor progression [6]. Furthermore, a further increase of specific Src activity is seen in cells derived from metastases versus cells from primary tumors [20]. These reports indicate a stepwise increase in Src activity during various stages of tumor progression and its possible contributions to tumor development. In other reports, increase of Src activity during tumor progression in some cancers including lung adenocarcinoma contributes to acquisition of resistance against apoptosis induction [21, 27]. Based on these reports, we hypothesized that the Cx32-dependent tumor-suppressive effect could lead to abrogation of chemoresistance observed in lung adenocarcinoma cells. In this context, the present study was undertaken to clarify this possibility. Herein, we reported that Cx32 enhanced VBN-induced cytotoxicty in A549 cells via the inactivation of Src.

As mentioned above, it has been well established that MDR-1 gene is a critical factor to determine chemoresistance of cancer cells against *Vinca* alkaloid including VBN [1]. Also, MRP-1 and RLIP76 have been determined as chemoresistant factors against *Vinca* alkaloid [37, 38]. This study showed that of the three chemoresistant factors, MDR-1 gene expression was significantly reduced by Cx32 expression and that

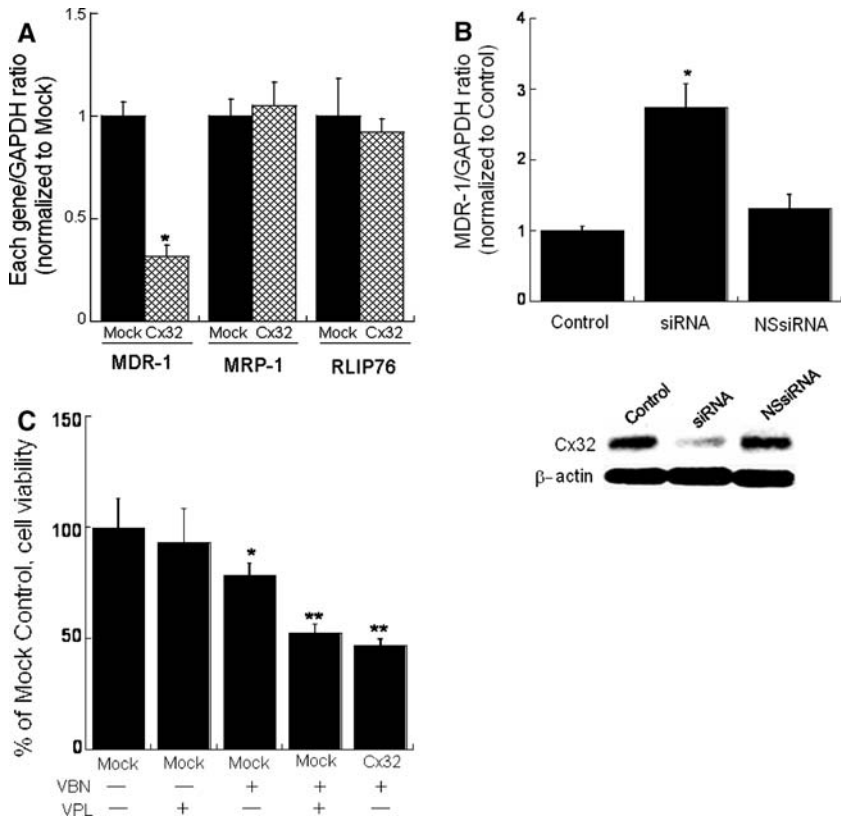

Fig. 3 Reduction of MDR-1 mRNA (**a**) and restoration of MDR-1 mRNA under silencing of Cx32 gene (**b**) in A549-Cx32, and enhancing effect of VPL on VBN-induced cytotoxicty in A549-mock (**c**). **a** Each mRNA level was determined by real-time PCR and quantified as the ratio of Each gene/GAPDH. Each value indicates the mean from three samples; *vertical lines* indicate SD. $*P < 0.001$ versus Mock. **b** Cx32 siRNA treatment in A549-Cx32 was performed as described in "Materials and methods". MDR-1 mRNA and Cx32 protein levels were also determined and quantified as described in "Materials and methods". Each value is the mean from three samples, and *vertical lines* indicate SD. $*P < 0.001$ versus control. *Control* non-treatment in A549-Cx32; *NSsiRNA* non-specific control siRNA treatment in A549-Cx32; *siRNA* Cx32 siRNA treatment in A549-Cx32. **c** The cells were treated with VBN (5 nM) and/or VPL (10 μM) for 48 h, and cell viability was determined, using WST-1 reagent. Each value indicates the mean from six determinants; *vertical lines* indicate SD. $*P < 0.05$ and $**P < 0.01$ versus non-treated group

knockout of Cx32 by siRNA restored the MDR-1 expression. These results mean that Cx32 increases the accumulation of VBN into A549 cells due to the reduction of the MDR-1 gene expression, because MDR-1 gene product (Pgp), an adenosine triphosphate-driven efflux pump for VBN [1], stimulates excretion of VBN from the cells. Additionally, we observed that VPL-dependent inhibition of Pgp function enhanced VBN-induced cytotoxicity in A549 cells. This observation further supports that Cx32 potentiates the VBN-induced cytotoxicity in A549 cells via the suppression of MDR-1 gene expression. As a possible mechanism on Cx32-dependent suppression of MDR-1 gene expression, we speculated that Src inactivation by Cx32 plays a role in reducing the expression level. It has been reported that Pgp is up-regulated by hypoxia-inducible factor 1α, which is stabilized by Src [9, 17], suggesting that Cx32-dependent inhibition of Src causes reduction of chemoresistance against VBN in A549 cells, due to a decrease of Pgp level. In this study, we confirmed that Cx32 induced the inactivation of Src and that inhibition of Src activity by PP1 reduced the expression level of MDR-1 gene in A549 cells. These results support the above speculation.

On the other hand, another Cx subtype, Cx43, is well known to be phosphorylated by Src, and it induces disruption of Cx43-mediated GJIC. But any phosphorylation sites by Src have not been identified in Cx32, nor any sites which are phosphorylated by cAMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase, and so on, have not been found to be directly relevant to Cx32 function before now [23, 39]. So we could say that at least Src would not inhibit Cx32-expected functions by phosphorylation.

In addition to the Cx32-dependent enhancing effect, the Cx has an advantage to establish a new cancer therapy for lung adenocarcinoma. In general, it is well established that Cx genes acts as tumor suppressor

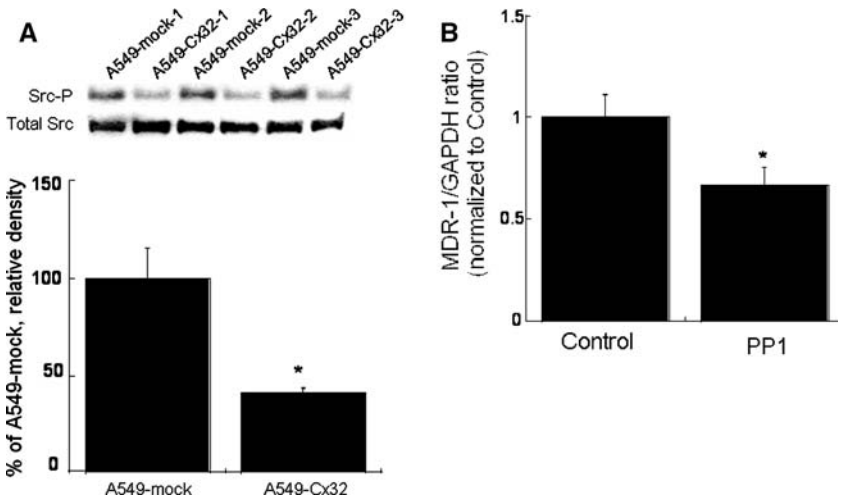

Fig. 4 Effects of Cx32 on the activation of Src (**a**) and of PP1 on MDR-1 mRNA level (**b**) in A549 cells. **a** Src activation was estimated as the ratio of phosphorylated Src (Src-P) to total Src. Each protein level was determined by immunoblot analysis as described in "Materials and methods". Densitometric evaluation was performed as mentioned in "Materials and methods". Each intensity shown is the mean of three samples; *vertical lines* indicate SD. $*P < 0.001$ versus A549-mock. **b** The cells (A549-mock) were treated with PP1 (0.1 μM) for 24 h, and MDR-1 mRNA level was determined by real-time PCR and quantified as the ratio of MDR-1/GAPDH. Each value indicates the mean from three samples; *vertical lines* indicate SD. $*P < 0.05$ versus control

genes via GJ allowing direct transfer of small cytoplasmic hydrophilic metabolites (Mr < 1,500) between neighboring cells to maintain cellular homeostasis [28]. A recent study has suggested that GJIC acts as an important determinant to induce the apoptosis [44]. In a more recent report, it has been shown that due to GJ-dependent cell coupling, individual dying bladder cancer cells can spread cell death signals into adjacent cells which then also die by apoptosis, and that the messenger molecules which pass through gap junctions to kill the cell are very likely calcium ions [22]. In another study, glioma cells that resisted apoptosis due to the overexpression of Bcl-2 could, nevertheless, be killed when they were coupled via GJ with vulnerable non-transfected counterparts [25]. From this report, it is assumed that the effective dose of anti-cancer agent against cancer cells is reduced due to the propagation of cell death signals from dying cells to surrounding living cells via GJ. We also observed that inhibition of GJ-dependent functions by a specific inhibitor against the functions (GA) partly abrogated VBN-induced cytotoxicity in A549 cells, so the above GJ-dependent functions might be effective in A549 cells. Overall, the combination of the tumor-suppressive effect of Cx32 and VBN is a promising strategy to establish a new cancer therapy for lung adenocarcinoma.

In order to establish the combination strategy for clinical usage, an effective procedure to induce expression of Cx32 is necessary. Methylation of cytosine residues in CpG islands of the gene promoter region is the most common mechanism of gene inactivation in cancers [4]. We and other group have reported that loss of Cx function in cancers depends on methylation of the promoter regions [45]. Additionally, loss of Cx function based on mutations or deletion of DNA has been found to be a rare event in cancers [33]. These reports mean that demethylation of the promoter regions by DNA methyltransferase inhibitors is effective to gain Cx function in cancers. In fact, we have observed that two DNA methyltransferase inhibitors (decitabine and zebularine) can restore the expression of Cx32 gene in cancer cells in vitro [36]. Taken together, it seems that the epigenetic approach leads to establishment of the combination strategy for clinical usage in lung adenocarcinoma therapy.

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References

1. Adams DJ, Knick VC (1995) P-glycoprotein mediated resistance to 5′-nor-anhydro-vinblastine (Navelbine). Invest New Drugs 13:13–21
2. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39:361–398
3. Awasthi S, Singhal SS, Singhal J, Cheng J, Zimniak P, Awasthi YC (2003) Role of RLIP76 in lung cancer doxorubicin resistance II. Doxorubicin transport in lung cancer by RLIP76. Int J Oncol 22:713–720

4. Baylin SB, Herman JG, Garff JR, Vertino PM, Issa JP (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196
5. Bos JL (1989) *ras* oncogenes in human cancer: a review. Cancer Res 49:4682–4689
6. Cartwright CA, Meisler AI, Eckhart W (1990) Activation of the pp60c-src protein kinase is an early event in colonic carcinogenesis. Proc Natl Acad Sci USA 87:558–562
7. Chipman JK, Mally A, Edwards GO (2003) Disruption of gap junctions in toxicology and carcinogenesis. Toxicol Sci 71:146–153
8. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654
9. Comerford KM, Cummins EP, Taylor CT (2004) c-Jun NH_2-terminal kinase activation contributes to hypoxia-inducible factor 1α-dependent P-glycoprotein expression in hypoxia. Cancer Res 64:9057–9061
10. Cros S, Wright M, Morimoto M, Lataste H, Couzinier JP, Krikorian A (1989) Experimental antitumor activity of Navelbine. Semin Oncol 16:15–20
11. Curran M, Plosker G (2002) Vinorelbine a review of its use in elderly patients with advanced non-small cell lung cancer. Drugs Aging 19:695–721
12. Duflot-Dancer A, Mesnil M, Yamasaki H (1997) Dominant-negative abrogation of connexin-mediated cell growth control by mutant connexin genes. Oncogene 15:2151–2158
13. Dumontet C, Branimir S (1999) Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. J Clin Oncol 17:1061–1070
14. Einhorn L (1997) Non-small cell lung cancer: improved chemotherapy. Lung Cancer 18:111
15. Evan WH, Martin PE (2002) Gap junctions: structure and function. Mol Membr Biol 19:121–136
16. Fujimoto E, Sato H, Shirai S, Nagashima Y, Fukumoto K, Hagiwara H, Negishi E, Ueno K, Omori Y, Yamasaki H, Hagiwara K, Yano T (2005) Connexin 32 as a tumor suppressor gene in a metastatic renal cell carcinoma cell line. Oncogene 24:3684–3690
17. Gary MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE (2005) HIF-1α, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene 24:3110–3120
18. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2:48–58
19. Hada S, Sato H, Fukumoto K, Virgona N, Hagiwara H, Saito T, Suzuki K, Asano R, Yamasaki H (2007) Reduction of malignant phenotype of lung adenocarcinoma cell by connexin 32: possible involvement of Src. Oncol Rep (in press)
20. Irby RB, Yeatman TJ (2000) Role of Src expression and activation in human cancer. Oncogene 19:5636–5642
21. Karni R, Jove R, Levitzki A (1999) Inhibition of pp60c-Src reduces Bcl-xL expression and reverses the transformed phenotype of cells overexpressing EGF and Her-2 receptors. Oncogene 18:4654–4662
22. Krutovskikh VA, Piccoli C, Yamasaki H (2002) Gap junction intercellular communication propagates cell death in cancerous cells. Oncogene 21:1989–1999
23. Lampe P, Lau A (2000) Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 3842:205–215
24. Landis SH, Murry T, Bolden S, Wingo P (1998) Cancer statistics, 1998. CA Cancer J Clin 48:6–29
25. Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergard M (1998) Gap-junction-mediated propagation and amplification of cell injury. Nat Neurosci 1:494–500
26. Liscovitch M, Lavie Y (2002) Cancer multidrug resistance: a review of recent discovery research. IDrugs 5:349–355
27. Masaki T, Igarashi K, Tokuda M, Yukimasa S, Han F, Jin YJ, Li JQ, Yoneyama H, Uchida N, Fujita J, Yoshiji H, Watanabe S, Kurokohchi K, Kuriyama S (2003) pp60c-src activation in lung adenocarcinoma. Eur J Cancer 39:1447–1455
28. Mesnil M (2002) Connexins and cancer. Biol Cell 94:493–500
29. Mesnil M, Yamasaki H (1993) Cell-cell communication and growth control of normal and cancers: evidence and hypothesis. Mol Carcinog 7:14–17
30. Mesnil M, Yamasaki H (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. Cancer Res 60:3989–3999
31. Ngan VK, Bellman K, Panda D, Hill BT, Jordan MA, Wilson L (2000) Novel actions of the antitumor drugs vinflunine and vinorelbine on microtubules. Cancer Res 60:5045–5051
32. Nishio K, Nakamura T, Koh Y, Suzuki Y, Fukumoto H, Saijo N (1999) Drug resistance in lung cancer. Curr Opin Oncol 11:109–115
33. Omori Y, Krutovskikh V, Mironov N, Tsuda H, Yamasaki H (1996) Cx32 gene mutation in a chemically induced rat liver tumour. Carcinogenesis 17:2077–2080
34. Potier P (1989) The synthesis of Navelbine prototype of a new series of vinblastine derivatives. Semin Oncol 16:2–4
35. Sato H, Senba H, Virgona N, Fukumoto K, Ishida T, Hagiwara H, Negishi E, Ueno K, Yamasaki H, Yano T (2007) Connexin 32 potentiates vinblastine-induced cytotoxicity in renal cell carcinoma cells. Mol Carcinog (in press)
36. Shirai S, Hagiwara H, Sato H, Fukumoto K, Kobayashi S, Seki T, Ariga T, Yamasaki H, Hagiwara K, Yano T (2005) Prevention of renal cell carcinoma from hemodialysis patients by regulating epigenetic factors. Kidney Int 67:2506–2507
37. Singhal SS, Singhal J, Sharma R, Singh SV, Zimniak P, Awasthi YC, Awasthi S (2003) Role of RLIP76 in lung cancer doxorubicin resistance. I. The ATPase activity of RLIP76 correlates with doxorubicin and 4-hydroxynonenal resistance in lung cancer cells. Int J Oncol 22:365–375
38. Stavrovskaya AA (2000) Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc) 65:95–106
39. Swenson K, Piwnica-Worms H, McNamee H, Paul D (1990) Tyrosine phosphorylation of the gap junction protein connexin43 is required for the $pp60^{v\text{-}src}$-induced inhibition of communication. Cell Regul 1:989–1002
40. Toso C, Lindley C (1995) Vinorelbine: a novel *Vinca* alkaloid. Am J Health Syst Pharm 52:1287–1304
41. Trosko JE, Ruch RJ (1998) Cell-cell communication in carcinogenesis. Front Biosci 3:208–236
42. Trosko JE, Ruch RJ (2002) Gap junctions as targets for cancer chemoprevention and chemotherapy. Curr Drug Target 3:465–482
43. Ueda K, Cardarelli C, Gottesman MM, Pastan I (1987) Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicines, doxorubicin, and vinblastine. Proc Natl Acad Sci USA 84:3004–3008
44. Wilson MR, Close TW, Trosko JE (2000) Cell population dynamics (apoptosis, mitosis, and cell-cell communication) during disruption of homeostasis. Exp Cell Res 254:257–268

45. Yano T, Ito F, Kobayashi K, Yonezawa Y, Suzuki K, Asano R, Hagiwara K, Nakazawa H, Toma H, Yamasaki H (2004) Hypermethylation of the CpG island of connexin 32, a candidate tumor suppressor gene in renal cell carcinomas from hemodialysis patients. Cancer Lett 208:137–142
46. Yano T, Yamasaki H (2001) Regulation of cellular invasion and matrix metalloproteinase activity in HepG2 cell by connexin 26 transfection. Mol Carcinog 31:101–109
47. Yano T, Zissel G, Muller-Qernheim J, Shin SJ, Satoh H, Ichikawa T (2002) Prostaglandin E2 reinforces the activation of Ras signal pathway in lung adenocarcinoma cells via EP3. FEBS Lett 518:154–158
48. Yoshimura K, Yamashita N, Ishikawa H (1980) Clinical statistics for lung neoplasms in Japan according to their histological types. Nippon-Rinsho 38:2581–2591