

Short Communication

Cytotoxic Effect of the Her-2/Her-1 Inhibitor PKI-166 on Renal Cancer Cells Expressing the Connexin 32 Gene

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Abstract. We have reported that connexin (Cx) 32 acts as a tumor suppressor gene in renal cancer cells partly due to Her-2 inactivation. Here, we determined if a Her-2/Her-1 inhibitor (PKI-166) can enhance the tumor-suppressive effect of Cx32 in Caki-2 cells from human renal cell carcinoma. The expression of Cx32 in Caki-2 cells was required for PKI-166-induced cytotoxic effect at lower doses. The cytotoxicity was dependent on the occurrence of apoptosis and partly mediated by Cx32-driven gap junction intercellular communications. These results suggest that PKI-166 further supports the tumor-suppressive effect of the Cx32 gene in renal cancer cells through the induction of apoptosis.

Keywords: connexin 32, PKI-166, renal cancer cell

Gap junctions are aqueous channels that connect the cytoplasm of contiguous cells, enabling cells to share directly small metabolites by the process called gap junction intercellular communication (GJIC) or cell coupling. It has been shown that GJIC is implicated in the regulation of cell growth, mostly by negative growth control of cell proliferation (1). Correspondingly, disorders of GJIC are strongly associated with aberrant cell growth diseases, including cancer (2). Due to their frequent functional alteration in tumors, the genes of the gap junction, connexin (Cx) genes, have been classified as tumor suppressor genes (3). Previous reports have found that due to cell coupling, individual cancer cells can propagate cell death signals into adjacent cells which then also die by apoptosis and that the effect called bystander effect can enhance cytotoxicity of anti-cancer agents on the cancer cells (4, 5). In addition to this beneficial effect, each Cx gene has a negative growth effect on the cancer with certain specificity, and the effect is independent of GJIC (6, 7). That is, the Cx

gene preferentially exerts the tumor-suppressive effect on the tumor from normal progenitor cells in which the particular Cx gene is naturally expressed. These reports suggest the idea of using specific Cx gene to achieve efficient cancer control by direct cell growth control and by exerting a bystander effect on several cancer therapies.

Renal cell carcinoma (RCC) has a very poor prognosis, due in large part to the fact that 40% of patients ultimately develop distant metastases following removal of the primary tumor, and available chemotherapeutic agents are ineffective against RCC (8). Therefore, management of RCC may rely on the establishment of new potential therapies for the cancer. In our recent studies, we have shown that Cx32 is prominently expressed in normal human renal epithelial cells, a progenitor cell of RCC, while the down-regulation of Cx32 occurs in human RCC cell lines as well as cancerous regions of human kidneys (9). Also, we have found that Cx32 has negative growth effects on Caki-2 cells from human RCC (10). These reports suggest that Cx32 acts as a tumor suppressor gene in RCC. In our

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previous study, we suggested that Cx32 suppresses cell growth control of the RCC cells partly through the inactivation of Her-2 (10). Menard et al. has been reported that the expression of Her-2 is a negative prognostic factor and is associated with increased resistance to cancer chemotherapy in breast cancers (11). Another report suggested that Her-2 forms a heterodimer with Her-1 in the cancers and that the formation of the heterodimer contributes to the resistance to the cancer chemotherapy in the cancers (12). As the activation of Her-1 as well as Her-2 is required for the development of RCC (13), the heterodimer of Her-2/Her-1 may stimulate the development of the RCC. Collectively, it seems that the combination of the Cx32-dependent tumor-suppressive effect and inactivation of Her-2/Her-1 is an effective procedure to regulate the development of the RCC. In this context, the present study was undertaken to clarify the above hypothesis.

The cell lines we used in this study, Caki-2 cells transfected with Cx32 gene (CAKI-2T) or void vector (CAKI-2M), have been established in our previous study (10). The cells were routinely grown in McCoy's 5A medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. The cells were plated and cultured for 24 h to permit them to adhere. After the attachment, the medium was changed and the cells were cultured for 24–72 h in the culture medium containing PKI-166 (Novartis Pharma AG, Basel, Switzerland) and each assay was performed. The cells were pretreated with 18 β -glycyrrhetic acid (GA) (Aldrich Chemicals, Milwaukee, WI, USA) for 24 h before the start of PKI-166 treatment. Vehicle treatment was used as the control.

The cytotoxicity of PKI-166 was examined by WST-1 reagent (MBL, Nagoya) according to the manufacturer's instructions. In order to estimate the induction of apoptosis quantitatively, a sandwich enzyme immunoassay was conducted by using the "Cellular DNA fragmentation ELISA Kit" (Roche Diagnostics, Tokyo), and caspase 3 activity was determined by using the "Caspase-3 Colorimetric Assay Kit" (MBL), according to the manufacturer's instructions. For cell cycle analysis, 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.

To measure GJIC, 5% neurobiotin and 0.4% rhodamine dextran in phosphate-buffered saline (PBS) was microinjected into a single cell (10). Rhodamine dextran was used to identify the injected cells. At 15 min

after the injection, the cells were fixed with 4% paraformaldehyde, and permeabilized with 2% bovine serum albumin and 0.25% Triton X-100 in PBS. The cells were reacted with streptavidin-FITC conjugate (Sigma), and subsequently dye transfer was quantified by counting the number of fluorescent cells surrounding an injected cell under a phase contrast and fluorescence microscope (Olympus, Tokyo). The average number of communicating cells was determined from 20 injections.

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.

First, in order to estimate the advantage of using the combination of Cx32 and PKI-166 as a cancer therapy for RCC, we compared the cytotoxic potential of PKI-166 on CAKI-2M and CAKI-2T cells. As shown in Fig. 1, the cytotoxicity of PKI-166 on CAKI-2T cells was higher than that on CAKI-2M cells at lower doses. Especially, the differences showed statistical significances at PKI-166 doses from 0.05 μ M to 0.5 μ M. Next, to distinguish whether the observed difference at lower doses in the PP1-induced cytotoxicity resulted from the inhibition of cell cycle and/or the induction of apoptosis, we examined the effects of PKI-166 at a lower dose (treatment doses, 0.1 μ M) on cell cycle regulation and the induction of apoptosis in CAKI-2M and CAKI-2T cells. PKI-166 treatment induced G1 arrest in the cell cycle but not apoptosis on CAKI-2M cells (Fig. 2: A–C). On the other hand, in CAKI-2T cells, the expression of Cx32 induced G1 arrest (Fig. 2A), and this observation was in agreement with

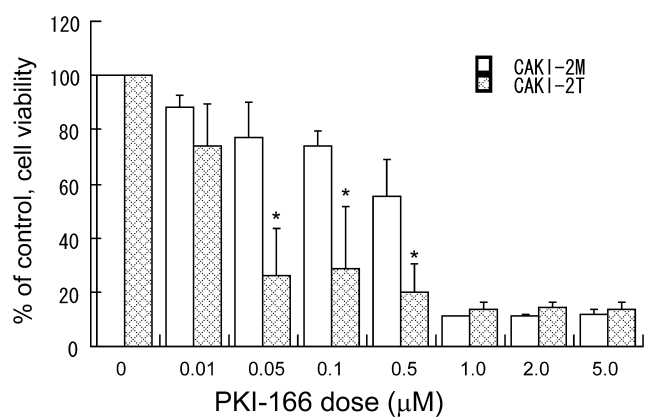


Fig. 1. Dose-dependent effect of PKI-166 on cell viability in CAKI-2M and CAKI-2T. The cells were treated with the indicated concentrations of PKI-166 for 72 h, and subsequently cell viability was determined using WST-1 reagent. Each value indicates the mean from six determinants; vertical lines indicate S.E.M. *Significant difference from CAKI-2M at each treatment dose. CAKI-2M, mock-transfected Caki-2 cells; CAKI-2T, Cx32-transfected Caki-2 cells.

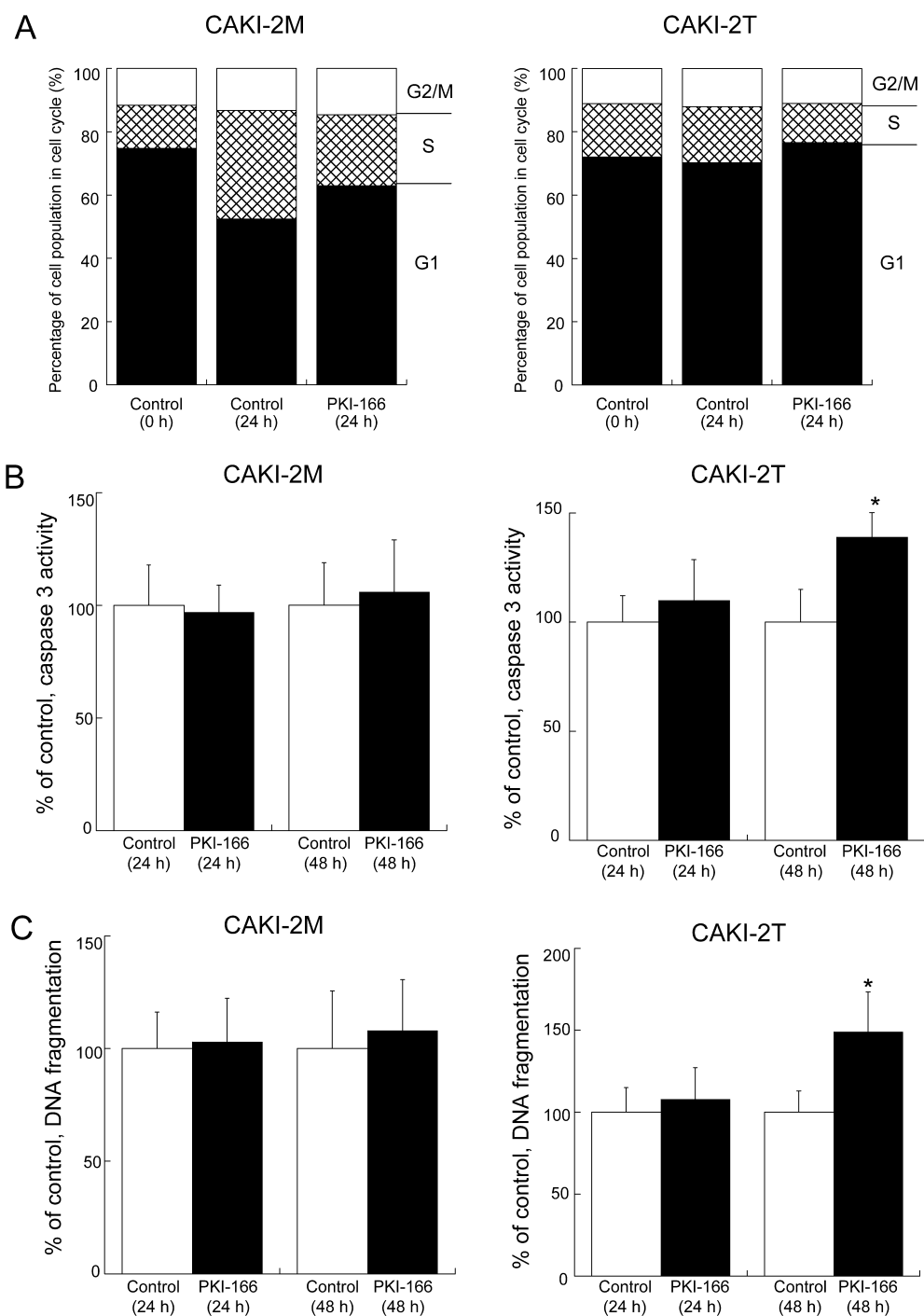


Fig. 2. Cell cycle progression (A), caspase 3 activity (B), and DNA fragmentation (C) in CAKI-2M and CAKI-2T. A: The cells were treated with PKI-166 (0.1 μ M) for 24 h. Each value indicates the mean from three determinants. B and C: The cells were treated with PKI-166 (0.1 μ M) for 24 and 48 h. Each value indicates the mean from five determinants; vertical lines indicate S.E.M. * P <0.05, significant difference from the control in each clone. Control, vehicle treatment; PKI-166, PKI-166 treatment. The values parentheses indicate treatment time.

that in our previous report (10). PKI-166 treatment slightly increased G1 arrest in CAKI-2T cells, but the treatment caused the induction of apoptosis in CAKI-2T cells with a significant difference compared to CAKI-

2M cells (Fig. 2: A – C). These results suggest that the induction of cytotoxicity of PKI-166 at lower doses against CAKI-2T cells was dependent on the occurrence of apoptosis rather than cell cycle regulation. Finally,

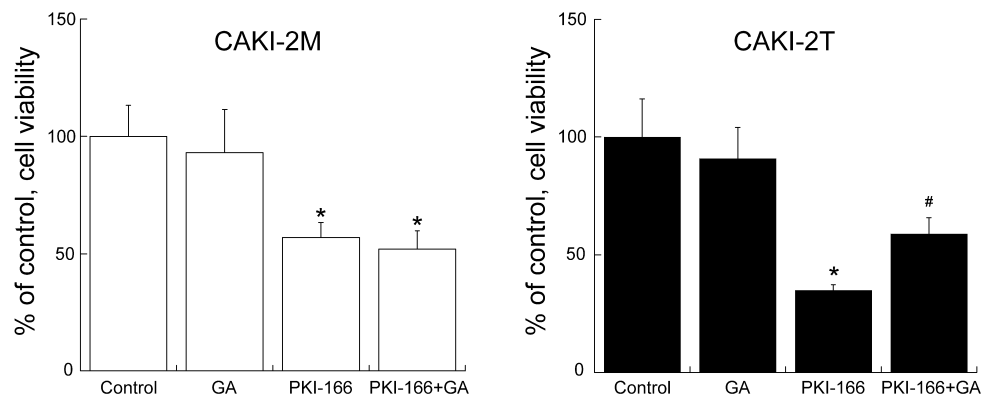


Fig. 3. Effect of GA (a GJIC inhibitor) on PKI-166-induced cytotoxicity in Caki-2 cells. The cells (CAKI-2M and CAKI-2T cells) were treated with GA (1 μ M) and then treated 24 h later with PKI-166 (0.1 μ M) for 72 h. Cell viability was determined using WST-1 reagent. Each value indicates the mean from six determinants; vertical lines indicate S.E.M. * P <0.05, significant difference from the Control and GA-treated group; # P <0.05, significant difference from the Control, GA-treated group, and PKI-166-treated group. Control, vehicle treatment; GA, treatment with 18 β -glycyrrhetic acid; PKI-166, PKI-166 treatment; PKI-166+GA, co-treatment with PKI-166 and GA.

in order to estimate the involvement of GJIC in the PKI-166-mediated cytotoxicity on CAKI-2T cells, we examined if GA, a specific inhibitor of GJIC, can reduce the cytotoxic effect of PKI-166 on the cells. As shown in Fig. 3, GA treatment partially abrogated PKI-166-mediated cytotoxicity in CAKI-2T cells, but the treatment did not affect the cytotoxicity in CAKI-2 M cells treated with PKI-166. Under this GA treatment condition, GJIC in GA-treated CAKI-2T cells was inhibited by about 80% comparing to that in non-treated cells (data not shown). This result indicates that the occurrence of PKI-166-dependent cytotoxicity at lower doses in CAKI-2T cells was partly mediated by Cx32-driven GJIC.

In our previous study, we showed that Cx32 exerted negative growth control of Caki-2 cells partly due to the inactivation of Her-2. However, the Cx32-dependent effect is cytostatic but not cytotoxic. On the other hand, Cx32 reduces the level of representative anti-apoptotic molecules such as Bcl-2 and Bcl-xL in Caki-2 cells (10). Since apoptosis is generally modulated by a changing balance in anti-apoptotic molecules and pro-apoptotic molecules (14), the induction of apoptosis in CAKI-2T cells may be easier than that in CAKI-2M cells due to the reduction of Bcl-2 and Bcl-xL levels. Furthermore, a previous study showed that the activation of multi-signaling related to cell survival and growth via the formation of Her-2/Her-1 heterodimer is required for the appearance of malignant phenotypes in several cancer cells and that the inhibition of Her-2/Her-1 activation by its specific inhibitor effectively induces apoptosis in the cells (12). From these reports and our present results, it is assumed that inhibition of

Her-2/Her-1 activation by PKI-166 could induce apoptosis in CAKI-2T. As expected, PKI-166 at lower doses effectively induced apoptosis in CAKI-2T compared to CAKI-2M.

A recent report showed that due to GJIC, 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 cells are very likely calcium ions (15). From that report, it is likely 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 GJIC. Since we also observed that GA partly abrogated PKI-166-induced cytotoxicity in CAKI-2T, the above effect via GJIC may be effective in Caki-2 cells.

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References

- 1 Mesnil M. Connexin and cancer. *Biol Cell*. 2002;94:493–500.
- 2 Mesnil M, Yamasaki H. Cell-cell communication and growth control of normal and cancer cells: evidence and hypothesis. *Mol Carcinog*. 1993;7:14–17.
- 3 Yamasaki H, Naus CCG. Role of connexin genes in growth control. *Carcinogenesis*. 1996;17:1199–1213.
- 4 Yamasaki H, Katoh F. Novel method for selective killing of

- transformed rodent cells through intercellular communication with possible therapeutic application. *Cancer Res.* 1998;48:3202–3207.
- 5 Mesnil M, Piccoli C, Yamasaki H. A tumor suppressor gene, Cx26, also mediated the bystander effect in HeLa cells. *Cancer Res.* 1997;57:2929–2932.
 - 6 Yano T, Hernandez-Blazquez FJ, Omori Y, Yamasaki H. Reduction of malignant phenotype of HepG2 is associated with the expression of connexin 26 but not connexin 32. *Carcinogenesis.* 2001;22:1593–1600.
 - 7 Duflot-Dancer A, Mesnil M, Yamasaki H. Dominant-negative abrogation of connexin-mediated cell growth control by mutant connexin genes. *Oncogene.* 1997;15:2151–2158.
 - 8 Motzer RJ, Bander N, Nanus DM. Renal-cell carcinoma. *N Engl J Med.* 1996;335:865–875.
 - 9 Yano T, Ito F, Satoh H, Hagiwara K, Nakazawa H, Toma H, et al. Tumor-suppressive effect of connexin 32 in renal cell carcinoma from hemodialysis patients. *Kidney Int.* 2003;63:381.
 - 10 Fujimoto E, Satoh H, Negishi E, Ueno K, Nagashima Y, Yano T, et al. Negative growth control of renal cell carcinoma cell by connexin 32: possible involvement of Her-2. *Mol Carcinog.* 2004;40:135–142.
 - 11 Menard S, Tagliabue E, Campiglio M, Pupa SM. Role of HER2 gene overexpression in breast carcinoma. *J Cell Physiol.* 2000;182:150–162.
 - 12 Chen X, Yeung TK, Wang Z. Enhanced drug resistance in cells coexpressing ErbB2 with EGF receptor or ErbB3. *Biochem Biophys Res Commun.* 2000;277:757–763.
 - 13 Lager DJ, Slagel DD, Palechek PL. The expression of epidermal growth factor receptor and transforming growth factor- α in renal cell carcinoma. *Mod Pathol.* 1994;7:544–548.
 - 14 Chao DT, Korsmeyer SJ. Bcl-2 family: regulators of cell death. *Annu Rev Immunol.* 1998;16:395–419.
 - 15 Krutovskikh VA, Piccoli C, Yamasaki H. Gap junction intercellular communication propagates cell death in cancerous cells. *Oncogene.* 2002;21:1989–1999.