Nicotine Induces Tumor Growth and Chemoresistance through Activation of the PI3K/Akt/mTOR Pathway in Bladder Cancer

Kazuyuki Yuge, Eiji Kikuchi, Masayuki Hagiwara, Yota Yasumizu, Nobuyuki Tanaka, Takeo Kosaka, Akira Miyajima, and Mototsugu Oya

Abstract

Continued smoking is highly associated with not only a higher incidence but also greater risk of tumor recurrence, progression, and acquired chemoresistance of urothelial carcinoma. We investigated whether nicotine affects urothelial carcinoma, and the detailed mechanism by which nicotine could induce tumor growth and any associated chemoresistance. Cell viability was evaluated in the human bladder cancer cell line T24 exposed to nicotine with or without cisplatin (CDDP) and NVP-BEZ235 as a PI3K/mTOR dual inhibitor by the WST-1 assay. Protein expression of the PI3K/Akt/mTOR pathway was investigated by Western blotting or immunohistochemical analysis. The influence of nicotine on tumor growth was also evaluated with or without CDDP and/or NVP-BEZ235 in a subcutaneous bladder tumor model. The result demonstrated that cell proliferation was increased in T24 cells after exposure to nicotine. Phospho-specific Akt (pAkt) and phospho-specific p70 S6 kinase (pS6) were significantly upregulated by nicotine exposure. Tumor growth in vivo was significantly induced by nicotine exposure in accordance with increased pS6 expression. Nicotine attenuated inhibition of T24 cell growth by CDDP and further upregulated pS6 expression in vitro and in vivo. NVP-BEZ235 inhibited T24 cell proliferation and pAkt and pS6 expression induced after exposure to nicotine and/or CDDP. In conclusion, nicotine increases tumor growth and induces acquired chemoresistance through activation of the PI3K/Akt/mTOR pathway in bladder cancer. Mol Cancer Ther; 14(9): 2112–20. © 2015 AACR.

Introduction

Urothelial carcinoma occurs in the urinary tract, which includes the bladder, upper urinary tract, and urethra, and approximately 90% of bladder cancers are urothelial carcinoma. Cigarette smoking has been considered to be one of the most important risk factors for bladder cancer for almost 60 years (1). Moreover, a recent study showed that the mean relative risk of bladder cancer in current smokers, relative to nonsmokers, was 4.0 (2). Recent studies have demonstrated that smoking status is associated not only with tumor incidence but also with the subsequent clinical outcome of bladder cancer and upper tract urothelial carcinoma (UTUC; refs. 3–6). Lammers and colleagues showed that ex-smokers and current smokers had a significantly shorter recurrence-free survival than nonsmokers after transurethral resection of non–muscle-invasive bladder cancer (7). Hagiwara and colleagues reported that smoking status is associated with tumor recurrence after nephroureterectomy in UTUC (8). These studies suggest that cigarette smoking may enhance the tumor aggressiveness of urothelial carcinoma, such as by promoting tumor proliferation, angiogenesis, and survival. However, little is known about the mechanism by which cigarette smoking enhances tumor aggressiveness in bladder cancer and UTUC. Moreover in other cancers, retrospective studies have showed that continued smoking during therapy was associated with lower response rates to chemotherapy or radiation (9, 10). In urothelial carcinoma treatment, cisplatin (CDDP)-based chemotherapy is the most effective therapeutic regimen for metastatic urothelial carcinoma. However, acquired chemoresistance in some patients with urothelial carcinoma is a critical problem in the management of advanced urothelial carcinoma. So far, no study has investigated the association between cigarette smoking and chemoresistance of CDDP in urothelial carcinoma.

Nicotine is an addictive component in cigarettes but is a noncarcinogen. Nicotine exerts its biologic function mainly through nicotinic acetylcholine receptors (nAChR), β-adrenoreceptors (β-AR), and/or EGF receptors (EGFR) in the motor endplates of muscle and the nervous system (11–13). The nAChR is also expressed in various cancer cells (14, 15). Recent studies have demonstrated that nicotine regulates multiple signaling proteins, especially phospho-specific Akt (pAkt) in lung, oral, and breast cancer cells (16–19). The PI3K/Akt pathway is a critical pathway in cancer and activation of the pathway is associated with tumorigenesis, tumor growth, and therapeutic resistance (20, 21). However, in the field of bladder cancer research, few studies have investigated the direct effect of nicotine on tumor viability or the associated mechanism of nicotine with acquired chemoresistance (22).

In this study, we investigated (i) whether nicotine affects the PI3K/Akt pathway in a bladder cancer cell line, (ii) whether nicotine induces bladder tumor growth in vitro and in vivo, and (iii) the association of chemoresistance of CDDP and nicotine exposure.
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Materials and Methods

Materials

We used rabbit polyclonal or monoclonal antibodies for pAkt at Ser473 and phospho-specific p70 S6 kinase (pS6; Cell Signaling Technology) and mouse monoclonal antibody for β-actin (Sigma). All cell culture reagents were purchased from Invitrogen. Nicotine and methyllycaconitine (MLA) as an α7-nAChR subunit (α7-nAChR) antagonist were obtained from Sigma-Aldrich. CDDP was purchased from Nippon Kayaku Co. NVP-BEZ235 as a dual PI3K and mTORC1/2 inhibitor was provided by Novartis. Water soluble tetrazolium salt (WST) reagents (Takara Bio) were also used.

Cell line and culture

T24, a human bladder cancer cell line, was obtained from the ATCC. T24 cells were obtained more than 1 year ago from each experiment. T24 cells were initially grown and multiple aliquots were frozen and stored at –80°C for future use. T24 cells were used at low passage in our laboratory and authenticated morphologically. T24 cells were routinely maintained in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a 5.0% CO2 atmosphere incubator.

WST cell viability assay

T24 cells were seeded on 96-well plates, allowed to attach for 24 hours, and then treated with MLA (100 μmol/L), CDDP (0.5–20 μmol/L), or NVP-BEZ235 (30–1,000 nmol/L) for 24 hours in the absence or presence of nicotine (0.3–10 μmol/L). At the end of the incubation period, WST reagents were added to each well and the cells incubated for 1 hour. Cell viability was estimated by colorimetry by reading color intensity in a plate reader at 570 nm.

Cell extracts and immunoblotting

T24 cells were treated with MLA (100 μmol/L), CDDP (10 μmol/L), or NVP-BEZ235 (500 nmol/L) for 24 hours in the absence or presence of nicotine (10 μmol/L). Whole-cell extracts were obtained using radioimmunoprecipitation assay buffer (Cell Signaling Technology) containing protease inhibitors, according to the manufacturer’s protocol. The extracted whole protein (50 μg) with sample buffer containing 2-mercaptoethanol was separated on 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked of the stained cancer cells was assessed in at least 10 representative areas with original magnification x400.

Murine xenograft model of bladder cancer

Nude athymic BALB/c mice, 6 weeks of age with an average body weight of 20 g, were obtained from Sankyo Laboratory Service Co. T24 cells (2 × 106 cells), suspended in 100 μL of Matrigel (Recom Dickinson Labware), were implanted s.c. in the flank of each nude mouse. The animals were carefully monitored, and the tumor was measured twice a week. Tumor volume was calculated using the following formula: tumor volume (mm3) = length × width × height × 0.52.

Results

Nicotine promotes cell proliferation and increases pAkt and pS6 in bladder cancer

To determine whether nicotine exposure could influence bladder cancer cell viability, we examined the human bladder cancer
cell line T24 exposed to various concentrations of nicotine for 24 hours (Fig. 1A). The relative viability of T24 cells exposed to 5 and 10 μmol/L of nicotine was significantly greater than that of unexposed cells (P < 0.05). T24 cell proliferation (mean ± SE) in cells exposed to nicotine (5 and 10 μmol/L) was 127% ± 18% and 143% ± 11%, respectively, relative to nicotine-untreated control.

We focused on the α7-nAChR, which is highly associated with tumor growth and cancer progression in other types of cancer (23–25), and found that at least α7-nAChR was expressed in T24 cells (data not shown). As shown in Fig. 1B, cell viability relative to control T24 cells (mean ± SE) exposed to 10 μmol/L nicotine and treated with 100 μmol/L MLA, which is α7-nAChR antagonist, (117.2% ± 2.2%) was significantly lower than that of the cells exposed to nicotine alone (129.9% ± 1.7%, P < 0.01). On the contrary, MLA could not completely inhibit the cell proliferation increased by nicotine exposure.

Western blot analysis was used to investigate whether nicotine leads to the activation of the PI3K/Akt/mTOR pathways in bladder cancer cells. As shown in Fig. 1C and D, 10 μmol/L nicotine increased pAkt and pS6 in T24 cells. This concentration might be attainable in smokers because average steady-state serum nicotine concentrations of approximately 200 nmol/L have been reported and increases to 10 to 100 μmol/L in serum after smoking has also

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been reported (26, 27). Moreover, tumor growth in mice treated with or without nicotine and/or NVP-BEZ235 was monitored. Nicotine was administered intraperitoneally (i.p.) 3 times a week. The mice were administered NVP-BEZ235 by gavage every day. A, time course changes in tumor volume in mice treated with or without nicotine and/or NVP-BEZ235. Nicotine was administered BEZ235 alone, 1 mg/kg nicotine and NVP-BEZ235 combined, or vehicle control. Nicotine was administered intraperitoneally (i.p.) 3 times a week. The mice were administered NVP-BEZ235 by gavage every day. A, time course changes in tumor volume in mice treated with or without nicotine and/or NVP-BEZ235. Nicotine was administered BEZ235 alone, 1 mg/kg nicotine and NVP-BEZ235 combination group. B, immunohistochemistry of pS6 expression in xenograft tumors treated with or without nicotine and/or NVP-BEZ235. Magnification, ×400. Scale bars, 100 μm.

PI3K/mTOR dual inhibitor reduces cell proliferation through inhibition of induced pAkt and pS6 in T24 cells exposed to nicotine

As shown in Fig. 1E, T24 cell growth inhibition by NVP-BEZ235 was observed without exposure to nicotine in a dose-dependent manner. The relative cell viability of T24 cells treated with 30 nmol/L or higher NVP-BEZ235 was significantly lower than that of the untreated vehicle control cells. In T24 cells treated without exposure to nicotine, the cell viability (mean ± SE) after treatment with 500 nmol/L of NVP-BEZ235 was 38.1% ± 4.5% relative to untreated control (Fig. 1E). A dose-dependent cytotoxic effect of NVP-BEZ235 was also observed in T24 cells exposed to 10 μmol/L of nicotine. In T24 cells exposed to nicotine, the mean cell viability after treatment with 500 nmol/L of NVP-BEZ235 was 39.2% ± 2.0% relative to untreated vehicle control (Fig. 1F).

Figure 1G presents the relative cell viability of T24 cells treated with and without NVP-BEZ235 in the absence or presence of nicotine. In the cells treated without NVP-BEZ235, the cell viability (mean ± SE) after exposure to nicotine was 131.0% ± 10.4% (P < 0.05) relative to vehicle control. In the cells treated with NVP-BEZ235, cell viability (mean ± SE) with and without exposure to nicotine was 49.4% ± 1.4% (P < 0.01) and 46.1% ± 2.2% (P < 0.01), respectively, relative to vehicle control.

Western blot analysis demonstrated that the protein levels of pAkt and pS6 in cells with or without exposure to nicotine were inhibited by 500 nmol/L of NVP-BEZ235 for 24 hours when compared with vehicle control cells (Fig. 1H and I).

Figure 2.
T24 mouse xenograft model was used to evaluate whether nicotine and/or NVP-BEZ235 could affect tumor growth and the regulation of pS6 expression in the tumor tissue. Ten mice per group were treated with 1 mg/kg nicotine alone, 40 mg/kg NVP-BEZ235 alone, 1 mg/kg nicotine and 40 mg/kg NVP-BEZ235 combined, or vehicle control. Nicotine was administered intraperitoneally (i.p.) 3 times a week. The mice were administered NVP-BEZ235 by gavage every day. A, time course changes in tumor volume in mice treated with or without nicotine and/or NVP-BEZ235. Nicotine was administered BEZ235 alone, 1 mg/kg nicotine and NVP-BEZ235 combination group. B, immunohistochemistry of pS6 expression in xenograft tumors treated with or without nicotine and/or NVP-BEZ235. Magnification, ×400. Scale bars, 100 μm.

PI3K/mTOR dual inhibitor has antitumor effect in mice exposed to nicotine in vivo

We evaluated whether the injection of nicotine could affect bladder tumor growth and whether NVP-BEZ235 could inhibit tumor growth in a subcutaneous bladder tumor xenograft model. As shown in Fig. 2A, on day 21, tumor volume (mean ± SE) in mice injected with nicotine was 929.1 ± 180.2 mm³, which was significantly larger than that in mice injected only with vehicle control (470.3 ± 73.4 mm³, P = 0.039). Tumor volume in mice treated with NVP-BEZ235 without nicotine injection was 282.0 ± 22.6 mm³ that was significantly smaller than that in control mice injected only with vehicle control (P = 0.034). Moreover, tumor volume in mice treated with nicotine and NVP-BEZ235 (295.4 ± 101.4 mm³) was significantly smaller than that in mice injected with nicotine (P = 0.019). Immunohistochemical analysis demonstrated that expression of pS6 was increased in the tumors of mice injected with nicotine (Fig. 2B). In the tumors of mice treated with NVP-BEZ235, no pS6 immunostaining was observed regardless of whether or not nicotine was administered (intensity: 0.4 ± 0.2 and 0.3 ± 0.2, respectively).

Nicotine attenuated the cytotoxic efficacy of CDDP through activation of pAkt and pS6 in T24 cells

We examined the cytotoxic effect of CDDP with or without nicotine exposure in T24 cells (Fig. 3). Regardless of any exposure to nicotine, CDDP had a dose-dependent cytotoxic effect. However, tumor cell inhibition was 91.8% ± 0.4% (mean ± SE) in cells exposed to 10 μmol/L CDDP without nicotine (Fig. 3A) but only 49.8% ± 1.2% in cells exposed to 10 μmol/L CDDP with nicotine when compared with vehicle control (Fig. 3B). The doses that suppressed cell growth by 50% (IC₅₀) in T24 cells exposed and not exposed to nicotine

www.aacrjournals.org  Mol Cancer Ther; 14(9) September 2015 2156

Published OnlineFirst July 16, 2015; DOI: 10.1158/1535-7163.MCT-15-0140

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were 10.04 and 5.50 mmol/L, respectively. Western blot analysis demonstrated that the expressions of pAkt and pS6 were upregulated after CDDP treatment for 24 hours in T24 cells not exposed to nicotine (Fig. 3C and D). Further upregulation of these protein expressions after the CDDP treatment was observed in cells exposed to nicotine.

Nicotine attenuated the antitumor effect of CDDP in a subcutaneous bladder tumor model

We investigated whether nicotine administration to mice had an effect on the antitumor effect of CDDP treatment in a T24 bladder tumor xenograft model. As shown in Fig. 4A, on day 21, tumor volume (mean ± SE) in mice treated with CDDP but without nicotine was 231.5 ± 53.8 mm³, which was significantly smaller than that in mice treated only with vehicle control (489.1 ± 73.4 mm³, P < 0.05, compared with vehicle control). *, P < 0.01, compared with vehicle control. T24 cells were exposed to 10 μmol/L nicotine and/or 10 μmol/L CDDP for 24 hours, and protein expression of pAkt (C) and pS6 (D) in cells treated with nicotine and/or CDDP was evaluated using Western blot analysis. †, P < 0.01, compared with cells treated with vehicle control or nicotine.

PI3K/mTOR dual inhibitor reduces cell proliferation and expression of pAkt/pS6 activated by nicotine and CDDP

We examined the combination effects of 10 μmol/L of CDDP and 500 nmol/L of NVP-BEZ235 in T24 cells exposed to 10 μmol/L of nicotine (Fig. 5). Figure 5A presents the relative cell viability of T24 cells treated with CDDP and/or NVP-BEZ235 in nicotine-exposed T24 cells. The WST assay demonstrated that in T24 cells exposed to nicotine, cell viability relative to vehicle control in the combination use of CDDP and NVP-BEZ235 (10.9% ± 1.0%) was significantly lower than that in the cells exposed to CDDP alone or NVP-BEZ235 alone (58.0% ± 1.8%, P < 0.01 and 44.6% ± 2.9%, P < 0.01, respectively; Fig. 5A). Western blot analysis revealed that pAkt and pS6, which were upregulated by nicotine and CDDP, were significantly inhibited by NVP-BEZ235 (Fig. 5B and C).
The combination therapy with PI3K/mTOR dual inhibitor and CDDP has strong antitumor effect in mice exposed to nicotine in vivo

As shown in Figs. 2A and 4A, we found that the inhibitory effect of 5 mg/kg of CDDP or 40 mg/kg of NVP-BEZ235 was significant (28–30) and then selected doses of CDDP and NVP-BEZ235 of 2 and 12.5 mg/kg, respectively, for the experiment of their combination study in nicotine-exposed T24 tumor-bearing mice. In nicotine-exposed T24 tumor-bearing mice, on day 21, tumor volume in mice treated with 2 mg/kg of CDDP (1,218.4 ± 116.0 mm³) was not significantly different from that treated with vehicle control (1,299.5 ± 126.5 mm³, \( P = 0.643, \) Fig. 6A). Meanwhile, tumor volume in mice treated with 12.5 mg/kg of NVP-BEZ235 (849.1 ± 110.7 mm³) was significantly smaller than that treated with vehicle control (\( P = 0.017 \)). Moreover, tumor volume in mice treated with the combination of CDDP (2 mg/kg) and NVP-BEZ235 (12.5 mg/kg, 509.9 ± 69.0 mm³) was significantly smaller than that in mice treated with CDDP alone (\( P < 0.01 \)) or NVP-BEZ235 alone (\( P = 0.021 \)). In nicotine-exposed T24 tumor-bearing mice, immunohistochemical analysis demonstrated that expression of pS6 increased in tumors treated with CDDP and NVP-BEZ235.
was suppressed in tumors treated with the combination of CDDP and NVP-BEZ235 (Fig. 6B).

Discussion

A history of cigarette smoking is the strongest risk factor associated with tumor initiation and even tumor progression in bladder cancer. Several recent retrospective studies reported that tumor progression and cancer-specific survival were significantly associated with the duration and intensity of cigarette smoking in patients with urothelial carcinoma (7, 8, 31). Rink and colleagues reported that cumulative smoking exposure based upon duration of smoking and quantity smoked was independently associated with worse prognosis in patients treated with radical cystectomy and that smoking cessation for more than 10 years mitigated the risk of tumor recurrence (32). To date, however, little is known about the basic mechanism by which cigarette smoking induces carcinogenesis and promotes cell viability in bladder cancer cells (33). We investigated whether nicotine, the main chemical ingredient of cigarettes, affects bladder cancer and attempted to ascertain the mechanism by which nicotine induces bladder tumor aggressiveness.

In our in vitro study, exposure to 5 μmol/L or more nicotine induced an increase in cell viability in T24 cells, a human bladder cancer cell line. The in vivo study results demonstrated that tumor volume in mice injected with nicotine was significantly larger than that in those injected with vehicle control. However, inhibition of the PI3K/Akt/mTOR pathway with NVP-BEZ235 as a PI3K/mTOR dual inhibitor was able to suppress T24 cell viability in vitro and inhibited the tumor growth induced by nicotine. Meanwhile, α7-nAChR is one of the main nAChR subunits in T24 cells and the modulation of α7-nAChR could affect the cell viability through the PI3K/mTOR pathway. Although the exact roles of all types of nAChR subunits in T24 cells have not been evaluated and are beyond the scope of our study, we believe that detailed evaluation of downstream pathways of the receptors such as the PI3K/Akt/mTOR pathway is important for exploring the mechanism by which nicotine could have cell growth activity in bladder cancer cells.

The present results demonstrated that Akt and S6 were activated by exposure to nicotine. Immunohistochemical analysis in vivo revealed the protein expression of pS6 was increased in the nicotine-injected tumors. Our results showing the role of nicotine in the activated PI3K/Akt/mTOR pathway in bladder cancer were consistent with previous studies that showed inhibition of the PI3K/mTOR pathway suppressed the tumor growth induced by nicotine.
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The same as previous studies on other types of cancer such as lung, breast, and pancreatic cancers [17, 23, 35]. Moreover, our study found that increased expression of pAkt was observed within 30 minutes in a time-dependent manner at a dose of 10 μmol/L of nicotine (data not shown). These results suggest that nicotine at doses that could be physiologically relevant may activate the PI3K/Akt/mTOR pathway within minutes. Meanwhile, in the present study, CDDP treatment increased the expression of pAkt and pS6. Belyanskaya and colleagues also reported that CDDP induced activation of the PI3K/Akt pathway and survivin in patients with small cell lung cancer (36).

It was reported that the PI3K/Akt/mTOR pathway played a role in increased cellular proliferation and conferred protection against different cellular stresses in various cells (19, 20, 37, 38). Thus, activation of the pathway by nicotine in bladder cancer cells suggests that cellular proliferation is increased and the cells acquire chemoresistance toward anticancer drugs through the pathway. In the present study, we demonstrated that the effect of CDDP-induced cytotoxicity was inhibited by exposure to nicotine in vitro and in vivo that tumors treated with nicotine and CDDP were significantly larger than those treated with CDDP alone. These findings suggest that exposure to nicotine enhances the chemoresistance of CDDP through increased activation of the PI3K/Akt/mTOR pathway. Recent studies reported that various cancer cells acquired chemoresistance toward anticancer drugs by cigarette smoke condensate (CSC) or nicotine. An and colleagues reported that Akt activated by CSC induced doxorubicin resistance in lung cancer cells (39). Furthermore, in lung cancer, it was reported that nicotine induced antiapoptotic effects and cellular proliferative effects through the PI3K/Akt pathway (16, 18). The findings of other studies of various cancers suggested that nicotine inhibited apoptosis induced by TNF, UV light, and chemotherapeutic drugs (19, 37, 38, 40). These studies suggested that nicotine has the potential to regulate cell proliferation and is associated with therapeutic resistance in various cancers.

Finally, we investigated the effects of combination use with CDDP and PI3K/mTOR dual inhibitor on T24 cells exposed to nicotine. PI3K/Akt/mTOR pathway activation by nicotine exposure and/or CDDP was significantly inhibited by NVP-BEZ235 in vitro and in vivo studies. The results of the present study suggest that the combination of CDDP and NVP-BEZ2335 could have a durable cytotoxic effect against bladder cancer and chemoresistance of CDDP induced by nicotine exposure might be overcome by treatment with NVP-BEZ2335 through inhibiting the activated PI3K/Akt/mTOR signal.

To the best of our knowledge, this is the first report demonstrating that nicotine increases tumor growth and was related to chemoresistance through activation of the PI3K/Akt/mTOR pathway in bladder cancer both in vitro and in vivo. The campaign against smoking must be supported by not only publicizing epidemiologic information but also basic clinical evidence, including our data. Further clinical studies are warranted to establish novel therapeutic strategies, such as using a PI3K/Akt/mTOR inhibitor alone or in combination with conventional chemotherapy, to treat highly aggressive bladder cancer associated with cigarette smoking.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K. Yuge, E. Kikuchi, M. Oya
Development of methodology: K. Yuge, E. Kikuchi, M. Hagiwara, Y. Yasumizu, T. Kosaka
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Yuge, T. Kosaka
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Yuge, E. Kikuchi, N. Tanaka, T. Kosaka
Writing, review, and/or revision of the manuscript: K. Yuge, E. Kikuchi, N. Tanaka, T. Kosaka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Kikuchi, M. Hagiwara, Y. Yasumizu, T. Kosaka, M. Oya
Study supervision: E. Kikuchi, A. Miyajima, M. Oya

Grant Support

This work was supported, in part, by Grants-in-Aid for Young Scientists (to K. Yuge; grant 26861295) and Grants-in-Aid for Scientific Research (to E. Kikuchi; grant 26462428) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

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Received February 17, 2015; revised June 26, 2015; accepted July 6, 2015; published OnlineFirst July 16, 2015.

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