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The effect of low dosage of procaine on lung cancer cell proliferation

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is the most common type of human lung cancer leading cause of cancer death worldwide. However, first-line drugs such as gefitinib and erlotinib showed great drug resistance in the clinical.

MATERIALS AND METHODS: The cell proliferation was evaluated by MTT assay; gene expression was detected by qPCR assay. The protein expression was analyzed by Western blotting.

RESULTS: Our results showed that in mouse models of lung cancer by A549 or NCI-H1975 xenograft, the local anesthetic drug Procaine (PCA) with 50 mg/kg specifically attenuated tumors compared with the vehicle-treated group. In vitro, PCA suppressed both two human NSCLC cell lines A549 and NCI-H1975 proliferation in a lower dose (at nM grade). The cell proliferation marker PCNA was also downregulated after PCA treatment *in vivo*. Furthermore, low-dose of PCA could inhibit the mRNA expression of the key NSCLC target EGFR selectively in the A549 cells, however, it was not observed in another cell line NCI-H1975, implying a specific signaling by PCA in the cell type.

CONCLUSIONS: Taken together, our data indicate that PCA treatment leads to suppression of tumor growth and proliferation in A549 and NCI-H1975, and there is an EGFR transcription pathway by PCA in A549 cells.

Key Words:
NSCLC, Procaine, A549, EGFR, NCI-H1975.

Introduction

Kinase inhibitors have played an important role in tumor therapy, while the severe drug resistance limits their functions. Drug resistance mechanisms have been associated with genome regulation in the target kinase that changes biochemical properties of the receptor and result in drug resistance. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are the central signaling and drugs in non-small cell lung cancer (NSCLC) in clinical[1-3]. The representative drugs such as gefitinib and erlotinib are proved effective for advanced NSCLC patients with activated EGFR mutations[4,5]. However, although EGFR-TKIs demonstrated significant treatment in this genetically modified EGFR mutation, most of the patients finally display drug resistance. Two major resistance mechanisms have been investigated in patients. EGFR T790M mutation is conserved in 50% of resistance cases, and amplified MET oncogene constitutes 20% of resistance cases[6-8]. Therefore, effective therapy of EGFR mutant lung tumor by EGFR-TKIs is confined by the frequent occurrence of drug resistance, novel drugs different from conventional EGFR-TKIs with a fewer drug resistance have yet to be developed. In the present study, we modeled human lung cancer in the NSCLC cell line A549 and NCI-H1975. We analyzed the potential of Procaine (PCA) with different doses on the xenograft models and the expression of EGFR *in vivo*. In addition, we evaluated the potency of PCA on cell proliferation *in vitro*.

Materials and Methods

Cell Culture and MTT Assay

The lung cancer cell lines NCI-H1975 and A549 were purchased from the China Center for Type Culture Collection (CCTCC, China). A549 cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/ml penicillin, 100 mg/ml streptomycin. NCI-H1975 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. 80% confluent lung cancer cells were re-suspended in DMEM medium in 96-well plate after trypsinized. Cell proliferation of NCI-H1975 and A549 was evaluated

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by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) using the CCK-8 kit (DOJINDO, Kumamoto, Japan) according to the manufacturer's instructions. Absorbency value was determined by a spectrophotometer at 450 nm, and a reference wavelength was at 650 nm. Percent cell viability (%) was calculated by viable cell number compared to untreated cell number (A450 of treated cells/A450 of untreated cells).

Murine Models of Lung Cancer

All animal experiments were carried out according to approved protocols of the Animal Ethics Committee of Institution. Mice were bred and maintained under a specific pathogen-free environment. Nude mice received NCI-H1975 cells or A549 (2.5×10^6) subcutaneous injection. Animals with certain tumor size were randomly divided into 3 groups (n = 8 for each group) and treated with high dose PCA (500 mg/kg/day), low-dose PCA (50 mg/kg/day) or vehicle control (PBS) for 3 weeks. Animals were sacrificed when tumors reached 2 cm. At the time of the animals' death, tumors were collected for a subsequent test. Procaine hydrochloride (PCA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

RNA Isolation and Real-Time RT-qPCR

Total RNA was extracted by Trizol and subjected to RT-qPCR in triplicates, using SYBR Green chemical dye. Comparative CT method normalized to internal control of beta-actin was applied for relative mRNA expression calculation. Primer sequences are available on request.

Western Blotting Assay

Cells were suspended in lysis buffer. Whole-cell lysates were obtained by centrifugation. Protein samples with equal amount were separated by SDS-PAGE, and then transferred to nitrocellulose for immunoblotting with the antibody.

Results

The Activity of Low-Dose PCA on the Proliferation of A549 and NCI-H1975 Cell Lines in Vitro

Using MTT assay, we found that PCA significantly inhibited the proliferation of NCI-H1975 (Figure 1A) and A549 (Figure 1B) cells in a concentration of 100 nM, but not in the concentration of 100 μM or a higher dose of 10 mM. These results indicate lower dose of PCA (about 100 nM) might after cell proliferation via a mechanism depending on some EGFR signaling pathway inhibition.

Low-dose PCA for the Treatment of A549 Tumor Model Depending on EGFR Transcription

We examined the anti-tumor efficacy of low-dose PCA *in vivo*. Nude mice were inoculated into the right flank with A549 cells in 0.1 ml PBS. Eight mice per group were administrated

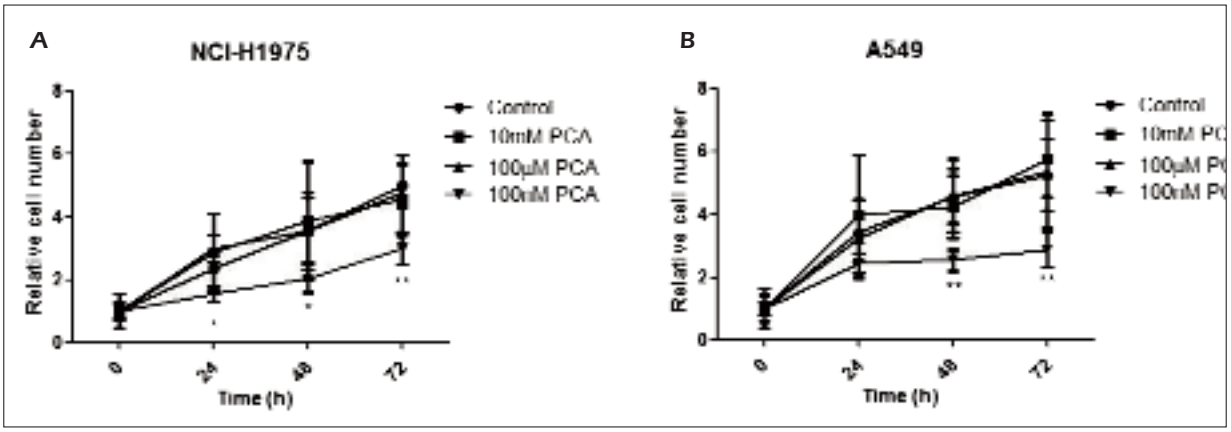

Figure 1. The effect of PCA on cell proliferation in vitro. ***A,*** The cell proliferation of NCI-H1975 cell in the presence (10 mM, 100 μM, 100 nM) or absence (*Control*) of PCA for 24h, 48h, 72h was evaluated by MTT assay. ***B,*** The cell proliferation of A549 cell in the presence (10 mM, 100 μM, 100 nM) or absence (*Control*) of PCA for 24h, 48h and 72h was evaluated by MTT assay. Student's *t*-test $*p < 0.05$, $**p < 0.01$, experiment was performed in triplicate.

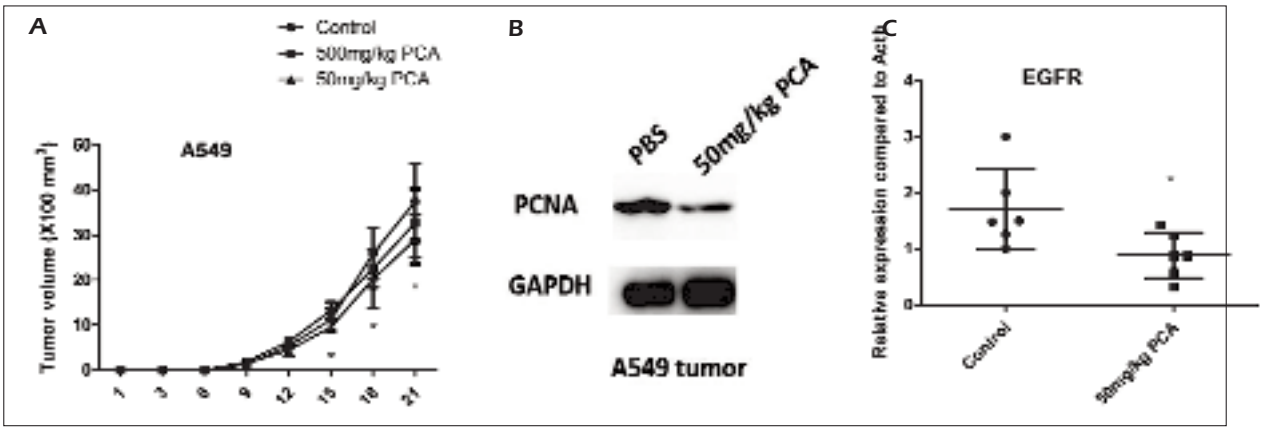

Figure 2. The effect of PCA on A549 tumor model. ***A,*** The tumor volume of PCA treated or PBS treated (control) A549 transplantation lung cancer mice. ***B,*** The protein expression of PCNA between the tumor of PCA treated mice and vehicle control. ***C,*** The mRNA expression of EGFR between the tumor of PCA treated mice and vehicle control. Student's *t*-test $*p < 0.05$, experiment was performed in triplicate.

orally with low-dose PCA (50 mg/kg/day), high dose PCA (500 mg/kg/day) or PBS (vehicle control group) until 20 days. It was found that low-dose PCA with 50 mg/kg/day could markedly reduce tumor growth compared to high dose PCA or vehicle control ($p < 0.05$) (Figure 2A). After sacrifice, tumor samples for each group were isolated and proteins extracted were subjected to Western blotting analysis of cell proliferation marker PCNA (Figure 2B). Furthermore, the mRNA expression of EGFR in tumor samples was downregulated. 50 mg/kg/day PCA significantly reduced the PCNA protein expression meanwhile the EGFR expression was downregulated after the low-dose PCA treatment ($p < 0.01$) (Figure 2C). These data demonstrate that *in vivo* administration of low-dose PCA (50 mg/kg/day) exhibits therapeutic potential on NSCLC via proliferated EGFR pathway.

In vivo Anti-Tumor Efficacy of Low-Dose PCA on NCI-H1975 Xenograft Mouse Lung Cancer Model Independent of EGFR Transcription

Similar to A549 xenograft model, 50 mg/kg/day dose of PCA ameliorated the tumor development in another NSCLC cell line NCI-H1975 xenograft model, PCA treatment also re-

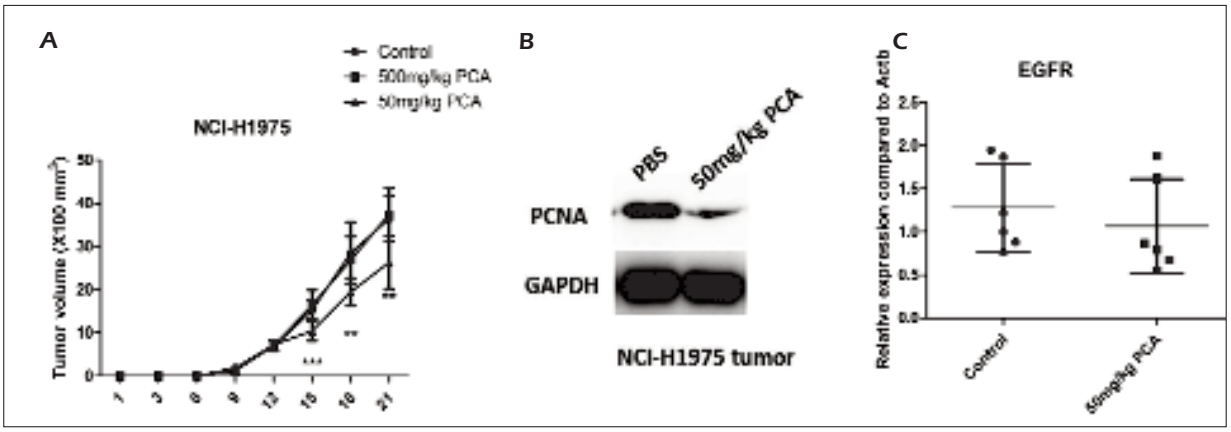

Figure 3. The effect of PCA on NCI-H1975 tumor model. ***A,*** The tumor volume of PCA treated or PBS treated (control) NCI-H1975 transplantation lung cancer mice. ***B,*** The protein expression of PCNA between the tumor of PCA treated mice and vehicle control. ***C,*** The mRNA expression of EGFR between the tumor of PCA treated mice and vehicle control. Student's *t*-test $**p < 0.01$, $***p < 0.001$, experiment was performed in triplicate.

duced the tumor volume in a lower concentration (Figure 3A). Consistently the cell proliferation marker PCNA expression was impaired by PCA treatment compared with vehicle group (Figure 3B). However, PCA could not affect the EGFR mRNA level (Figure 3C), suggesting that PCA involved in a pathway in the NCI-H1975 independent of EGFR transcription.

Procaine or procainamide are usually used for the therapy of cardiac arrhythmias. And the local anesthetic drugs are reported to inhibit DNA methylation by promoting demethylation of series of silenced genes[9-11]. Recently, PCA was also found as demethylation drugs on the breast cancer cell MCF-7 by inhibition of cell proliferation. Procaine can epigenetically demethylate promoter region and thus amplify gene expression of repressed genes. PCA also suppressed the viability of liver cancer cell lines by reactivating the genes transcriptionally suppressed by DNA hypermethylation[12,13]. Although procaine has been reported useful in different types of tumors including liver cancer and breast cancer, the effect of procaine on lung cancer remains uncertain. *In vitro*, procaine and procainamide can inhibit canonical Wnt signaling through promoter demethylation of WIF-1 in lung cancer cells H460 and A549 cell. Here we mainly tested the *in vivo* anti-tumor efficacy of procaine in the murine model of NSCLC[10]. It was showed that in nude mice inoculated with A549 or NCI-H1975 cells, treatment with PCA at 50 mg/kg per day markedly attenuated tumor growth, while PCA at higher dosage doesn't show therapeutic benefit, which was consistent with the *in vitro* data that low-dose of PCA (100 nM) inhibit the cell proliferation of the two types of lung cancer cells. Furthermore, it was found that the PCA could downregulate the tumor PCNA expression that is a marker of cell proliferation, suggesting it also mediate the cell proliferation *in vivo*. Meanwhile, PCA reduced the gene expression of EGFR in the A549 xenograft model. The tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib as typical EGFR targeting drugs[14], benefit about 10-30% of patients especially that never smoke. However, treatment with traditional TKIs would eventually not succeed resulting from the acquired drug resistance by tumor cells. Although there were novel targeting drugs in the research and development, the high price and low efficacy have not been approved by the patients. Here we also found that PCA with lower dose could selectively reduce EGFR in A549 transplantation mouse model but not in NCI-H1975. The mechanism needs further study. The DNA methylation and demethylation may attribute to the gene expression in the EGFR of A549.

Conclusions

Taken together, in both cell lines and the animal model of lung cancer, these results imply that procaine may have a potential therapy for the development of lung cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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