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Short Communication

Areca nut extract may be a potentially driving force for tumors to become autophagy addicted

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Abstract We have previously described the mechanisms of action and composition of the autophagy-inducing ingredients found in areca nut (AN). These ingredients are 30–100 kDa molecules present in AN extract (ANE), referred to as ANE 30–100K. In other studies, we demonstrated that chronic stimulation with sub-lethal doses of ANE or ANE 30–100K resulted in increased tolerance to environmental challenges, including serum deprivation, hypoxic conditions, anti-cancer drugs, and accelerated tumor growth in nude mice, through upregulated autophagy activity. Such tumor cells were highly sensitive to the chemical inhibition of autophagy both *in vitro* and *in vivo*. Here, we further demonstrated similar inhibitory effects on the growth of stimulated CE81T/VGH cells in mice using *atg5* shRNA. In contrast, the growth of non-stimulated control cells in mice was shown to be resistant to 3-methyladenine (3-MA). These findings suggest that autophagy blockade might be particularly effective for treating autophagy-dependent tumors in patients with AN-chewing habits.

Introduction

Autophagy intervention has been explored as a strategy for cancer treatment for nearly two decades; however, the results remain controversial and unpredictable. Over 100 clinical trials have been initiated (some completed, others not), using various autophagy modulators as adjuvant therapy, but the outcomes have been inconsistent.[1] Therefore,

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further careful investigation is needed to determine when and how to activate or inhibit autophagy to enhance therapeutic efficacy in cancer patients.

We have been studying the effects of areca nut (AN, the fruit of *Areca catechu*) ingredients on tumor cells and identified that the partially purified 30–100 kDa fraction from AN extract (ANE), designated as ANE 30–100K, could induce autophagic cell death in various cell types.[2,3] In contrast, tumors chronically and sub-lethally stimulated with ANE 30–100K and ANE exhibited higher autophagic activity and increased resistance to anticancer drugs and environmental stresses compared to unstimulated controls. Chemical inhibition of autophagy significantly reduced the enhanced resistance of these stimulated tumors both *in vitro* and *in vivo*.[4–6] Here, we further provide evidences that *atg5* shRNA effectively inhibited the growth of ANE 30-100K-stimulated esophageal carcinoma CE81T/VGH cells in mice, while the growth of non-stimulated control cells was unaffected by 3-methyladenine (3-MA). These findings, along with our previous studies,[4–6] suggest that autophagy inhibition may be particularly suitable as an adjuvant therapy for cancer patients with an AN-chewing habit.

Materials and methods

Animal experiments were the continual works of our previous studies and conducted after the permission of the Institutional Animal Care and Use Committee (IACUC) of Chi Mei Medical Center, Approval No. 109112002.[6] In brief, CE81T/VGH cells (2.5×10^6) were stimulated with ANE 30–100K (1.75 μg/ml) for three weeks and inoculated into the right flanks of mice subcutaneously. After one week, the lentivirus containing *atg5* shRNA (CCGGCCTTTCATTCAGAAGCTGTTTCTC, NM_004849.2, 942–962 bp) or scramble shRNA was intratumorally injected in 100 μl of phosphate-buffered saline (multiplicity of infection = 2) into 6-week-old male Balb/c nude mice from BioLASCO Taiwan Co., Ltd (n = 5). Both shRNAs were administered every 6 days, for a total of three treatments. After completion of the treatment (18 days), tumor volumes were calculated by caliper measurements [volume = $(W^2 \times L)/2$] (Fig. 1A). Mice were then sacrificed, and their tumors were collected for homogenate preparation. Homogenate proteins (20 μg) were subjected to immunoblot with the antibodies of Atg5 (2630, Cell Signaling Technology, Danvers, MA, USA), LC3 (L7543, Sigma–Aldrich, St Louis, MO, USA) and β-actin (A5441, Sigma–Aldrich) as described in our previous studies.[5,6] Signals of proteins were developed and digitalized with UnSCAN-IT software Automated Digitizing System, version 5.1 (Fig. 1B). Responses of the viability of ANE 30–100K stimulated and non-stimulated CE81T/VGH cells to 3-MA was performed by following our previous works (6) (Fig. 2). All data were analyzed by one-way analysis of variance (ANOVA) and Tukey multiple comparison test using IBM SPSS Statistics 20.0.0. The *P* value less than 0.05 was regarded as statistically significant.

Results

We have previously demonstrated that shRNAs targeting *atg5* and *beclin 1* effectively knocked down their protein expression in different cell types including CE81TVGH.[5] Here, we first selected the same *atg5* shRNA for our animal experiment. Intratumoral injection of *atg5* shRNA significantly reduced the size of ANE 30-100K-stimulated CE81T/VGH-derived tumors in mice compared to those injected with scramble shRNA (Fig. 1A, n = 5). In these tumors, the levels of LC3-II and Atg5 proteins were more effectively knocked down by *atg5* shRNA than in those treated with scramble shRNA (Fig. 1B). Furthermore, a single treatment with 3-MA significantly inhibited the growth of ANE 30-100K-stimulated CE81T/VGH-derived tumors, while it had no effect on the growth of non-stimulated control cells *in vitro* (Fig. 2).

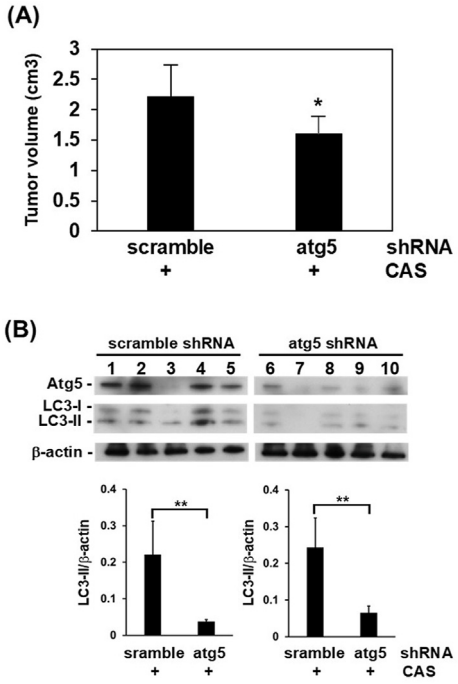

Figure 1 Treatment of nude mice bearing ANE 30–100K-stimulated CE81T/VGH-derived tumors with *atg5* shRNA and scramble shRNA. (A) ANE 30-100K-stimulated CE81T/VGH cells (2.5×10^6) were subcutaneously inoculated into the right flanks of mice. Intratumoral injections of *atg5* shRNA (n = 5) or scramble shRNA (n = 5) were administered as described in the Materials and Methods section. Tumor sizes were measured, and their volumes were plotted. *$P < 0.05$ (B) After tumor volume measurements, homogenates were prepared from the tumors and subjected to immunoblot analysis as described in the Materials and methods section. Signals for Atg5, microtubule-associated protein light chain 3 (LC3), and β-actin proteins were quantified and plotted. **$P < 0.01$.

Discussion

Although numerous clinical trials have used autophagy inhibitors as adjuvant therapy for various cancer patients, the therapeutic responses remain unpredictable.[1,7] However, our studies demonstrated that a single treatment with chloroquine (CQ) or 3-MA effectively blocked the growth of ANE 30-100K-stimulated CE81T/VGH-derived tumors under

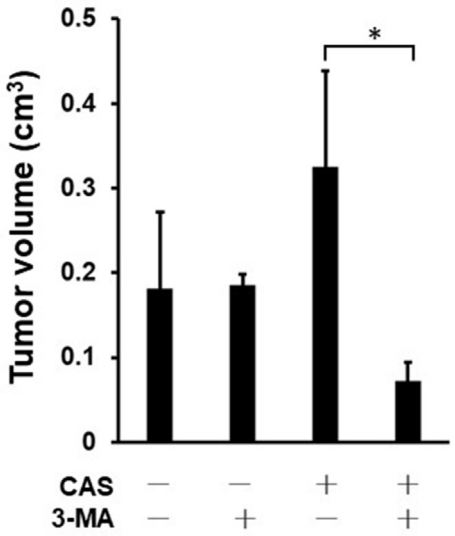

Figure 2 Differential response of ANE 30—100K-stimulated and non-stimulated CE81T/VGH cells to 3-MA therapy. ANE 30-100K-stimulated or non-stimulated CE81T/VGH cells were cultured overnight in serum-free medium. They were then treated with 3-methyladenine (3-MA) (1 mM) for an additional day, and their viabilities were measured as described in the Materials and methods section.

serum-free and low-oxygen conditions *in vitro*. Meanwhile, both agents also showed a synergistic antitumor effect when combined with cisplatin in mice.[6] Here, we further demonstrated that genetic knockdown of Atg5 provided a similar inhibition of tumor growth in mice as seen with CQ and 3-MA. These results suggest that although chronic ANE 30—100K stimulation may enhance tumor growth *in vivo*, it also makes these tumor cells highly sensitive to autophagy inhibition. The 3-MA resistance of non-stimulated CE81T/VGH cells in mice might explain why some clinical trials have failed to achieve the expected responses in patients treated with autophagy inhibitors.[1] Finally, our previous and current studies suggest that chronic stimulation by ANE 30—100K may cause tumor cells to become autophagy-addicted, and that autophagy inhibition may be particularly suitable for cancer patients with areca nut-chewing habits.

Declaration of competing interest

All authors have no conflicts of interest related to this article.

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