Radiotherapy-induced Gadd45a impairs lacrimal gland epithelial cell migration and proliferation

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Abstract. Radiotherapy-induced lacrimal gland injury is a serious clinical problem currently lacking satisfactory therapeutic strategies. Exploring the mechanisms underlying secondary injuries caused by radiation may aid in the development of novel targeted medicine. In the current study, growth arrest and DNA damage-inducible 45 α (Gadd45a), a gene which is upregulated in irradiated skin, was observed as being overexpressed in the irradiated lacrimal gland. Moreover, overexpressed Gadd45a may impair lacrimal gland repair by inhibiting lacrimal gland epithelial cell migration and proliferation. Further signalling pathway analyses indicated that Gadd45a overexpression suppresses Akt (protein kinase B, PKB), P38 and JNK phosphorylation. Thus, the results of the current study suggested that Gadd45a may be a therapeutic target in radiation-induced lacrimal gland injury.

Introduction

Lacrimal gland injury secondary to the radiotherapy of a malignant orbital tumour is a significant clinical problem (1-3). Radiation therapy damages the lacrimal gland cells, resulting in cell degeneration, necrosis and apoptosis (4) and thus, impairs tear secretion and induces xerophthalmia (1,3,5-7). Satisfactory treatment strategies for this clinical problem are lacking due to a poor understanding of the complex process. Exploring the underlying mechanisms may aid in the identification of novel therapeutic targets.

Lacrimal gland epithelial (LGE) cells are primary lacrimal gland cells (8) and are involved in lacrimal gland physiological homeostasis (9) and pathology (10), including radiotherapy-induced lacrimal gland injury. LGE cells are necessary for tear secretion and their proliferation and migration are crucial for lacrimal gland repair (11,12).

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Previous studies focusing on the skin epithelium have found that expression of specific genes are associated with radiation (13-15) and that these genes may be involved in the pathogenesis of radiation-induced skin injury (14). Growth arrest and DNA damage-inducible 45 α (Gadd45a) is one of these genes (14). Previous studies established that radiation upregulates Gadd45a expression in the skin (14,16,17). Gadd45a is also involved in inflammation (18) and numerous fundamental cellular behaviors associated with injury repair, including cell migration and proliferation (16,19-21). However, the roles of the Gadd45a gene in lacrimal gland radiation injury have not yet been studied.

To investigate whether Gadd45a has functional roles in radiation-induced lacrimal gland injury repair, the expression level of Gadd45a in radiation-treated mouse lacrimal glands was observed by quantitative PCR and immunohistochemical analysis. Gadd45a was overexpressed in isolated mouse LGE cells and the cell migration and proliferation ability was determined, as well as the associated signalling pathways.

Materials and methods

Animal preparation and irradiation. Ten healthy female C57BL/6 mice at 8 weeks of age were used for the study. Experimental procedures were approved by the Fudan University Animal Care and Use Committee and all animals were housed under standard conditions according to institution-approved guidelines. Mice underwent initial lacrimal gland scintigraphy. After 1 week, the first group (n=5) was irradiated with a total dose of 15 Gy under general anaesthesia using a combination of 3 mg/kg (S)-ketamin-hydrochloride (Ketanest-S®; Hoofddorp, The Netherlands) and 0.1 mg/kg xylazin-hydrochloride (Rompun®; Bayer, Leverkusen, Germany). Scintigraphy was performed 3 days following irradiation, for a second time, with a subsequent excision of the left side inferior lacrimal gland for histological examination. The same procedure was performed 7 days later with the removal of the contralateral lacrimal gland. The second group (n=5) was sham-treated and were unirradiated for use as the control glandular tissue.

Lacrimal gland scintigraphy. Following intravenous administration of 3.7 MBq (1 mCi = 37 MBq and 100 μCi = 3.7 MBq) $Na^{99m}TcO_4$ as a tracer, the mice underwent sequential scintigraphy in a prone position and frontal projection of the head

using a four-head camera (Picker CX 250 compact, LEHR collimator and field-of-view 25 cm Nano SPECT/CT Plus, Bioscan Inc., Washington DC, USA). Time-activity curves were additionally registered and analyzed. The lacrimal ejection was also observed.

Surgical harvesting of the inferior lacrimal gland. The inferior lacrimal gland was surgically exposed and excised. The harvested glands were divided into two parts. One part was fixed immediately with neutral phosphate-buffered 4% formalin and the other was fixed for transmission electron microscopy.

Transmission electron microscopy. Following embedding in Araldite, semi-thin sections were stained with methylene blue to visualize the region of epithelial cells of interest. Ultrathin sections were sliced and stained with lead citrate and examined using a transmission electron microscope (Philips, CM 120, Amsterdam, The Netherlands).

Quantitative PCR. Total RNA was isolated from mouse lacrimal glands using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was conducted using SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. The obtained cDNA was diluted 1:40 for quantitative PCR. The primers used for the amplification were: mouse Gadd45a: forward, 5'-ggaggaagtgctcagcaaag-3' and reverse, 5'-gcaggatccttccattgaga-3' and mouse GAPDH, forward, 5'-tgtt gccatcaatgacccctt-3' and reverse, 5'-ctccacgacgtactcagcg-3'. The reactions were performed using an ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR-Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions were 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The relative expression levels of Gadd45a were determined by normalizing to the expression of the internal control gene GAPDH.

Immunohistochemistry. Paraformaldehyde-fixed, paraffin-embedded mouse lacrimal gland sections (4 μm) were incubated with a primary antibody against mouse Gadd45a (Millipore, Billerica, MA, USA) overnight at 4˚C and incubated with the appropriate secondary antibodies. The sections were developed with diaminobenzidine and counterstained with haematoxylin and eosin (H&E). Quantification of the staining intensity was conducted by two independent investigators.

Cell isolation and culture. Primary mouse LGE cell isolation and culture were performed as previously described (22). LGE cells at passages 2-6 were used for the subsequent experiments.

Cell transfection. Mouse Gadd45a cDNA was cloned into the CD510B-1 lentivector. The CD510B-1, REV, GAG and VSVG plasmids were then transfected into HEK 293 cells using Lipofectamine 2000™ transfection reagent (Invitrogen Life Technologies). Lentiviral infection was performed in the presence of polybrene (6 μg/ml). Then, following 48 h lentiviral infection, primary LGE cells were treated with puromycin (2 μg/ml) for 10 days. The empty CD510B-1 vector was used as a control. Transfection efficiency was evaluated by western blot analysis.

Scratch-wound healing assay. LGE cells overexpressing Gadd45a or a vector control were seeded in 24-well plates ($2.5x10^5$ cells/well) and cultured to confluency. Next, the monolayer was gently scratched across the centre with a 10 μl pipette tip. The gaps were imaged at 0, 12 and 24 h post-scratch using a live-cell imaging system (Olympus Corporation, Tokyo, Japan).

Cell proliferation assay. Proliferation was determined using a standard CCK-8 cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. LGE cells overexpressing Gadd45a or a vector control ($4.0x10^4$ cells/ml) were seeded in 96-well plates (100 μl/well). The optical density values were measured at days 0, 2, 4 and 6 by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. Cells were lysed with RIPA lysis buffer (Beyotime Biotech, Jiangsu, China) supplemented with 1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA). The primary antibodies used were against the following proteins: Gadd45a (Millipore), Akt, p-Akt, P38, p-P38, JNK, p-JNK, Erk 1/2 and p-Erk 1/2. All antibodies, with the exception of the antibody against Gadd45a, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Signals were detected by an Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA) following incubation with IRDye 800 anti-rabbit (LI-COR) secondary antibodies. Quantification was conducted using ImageJ software.

Statistical analysis. Statistical analyses were performed with a two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Radiotherapy impairs lacrimal secretion and induces lacrimal gland injury in vivo. To evaluate the adverse effect of radiation therapy on the lacrimal gland, a lacrimal gland scintigraphy assay was performed and the histological and structural changes were observed by H&E staining and transmission electron microscopy prior to and following irradiation. The tracer uptake of the lacrimal glands prior to irradiation was mounted before the administration of carbachol. Lacrimal ejection was then induced and the amount of tracer decreased with the passage of time. Thus, the time-activity curve had a parabolic shape. Three days following irradiation, primary tracer uptake was reduced and lacrimal ejection was significantly lower. The time-activity curve showed an ascending tendency. This reduction in lacrimal ejection remained 7 days later and primary uptake remained reduced (Fig. 1A).

In normal lacrimal gland tissue, typical histology shows the tubulo-acinar structure of the inferior lacrimal gland, with a cubic, regular shape for the acinar cells and basally located nuclei (Fig. 1B). However, secretory retention was observed in the majority of acinar and tubular cells in the irradiated inferior lacrimal gland 3 days following irradiation. Scattered vacuolopathy and an increase in the number of aberrant nuclei

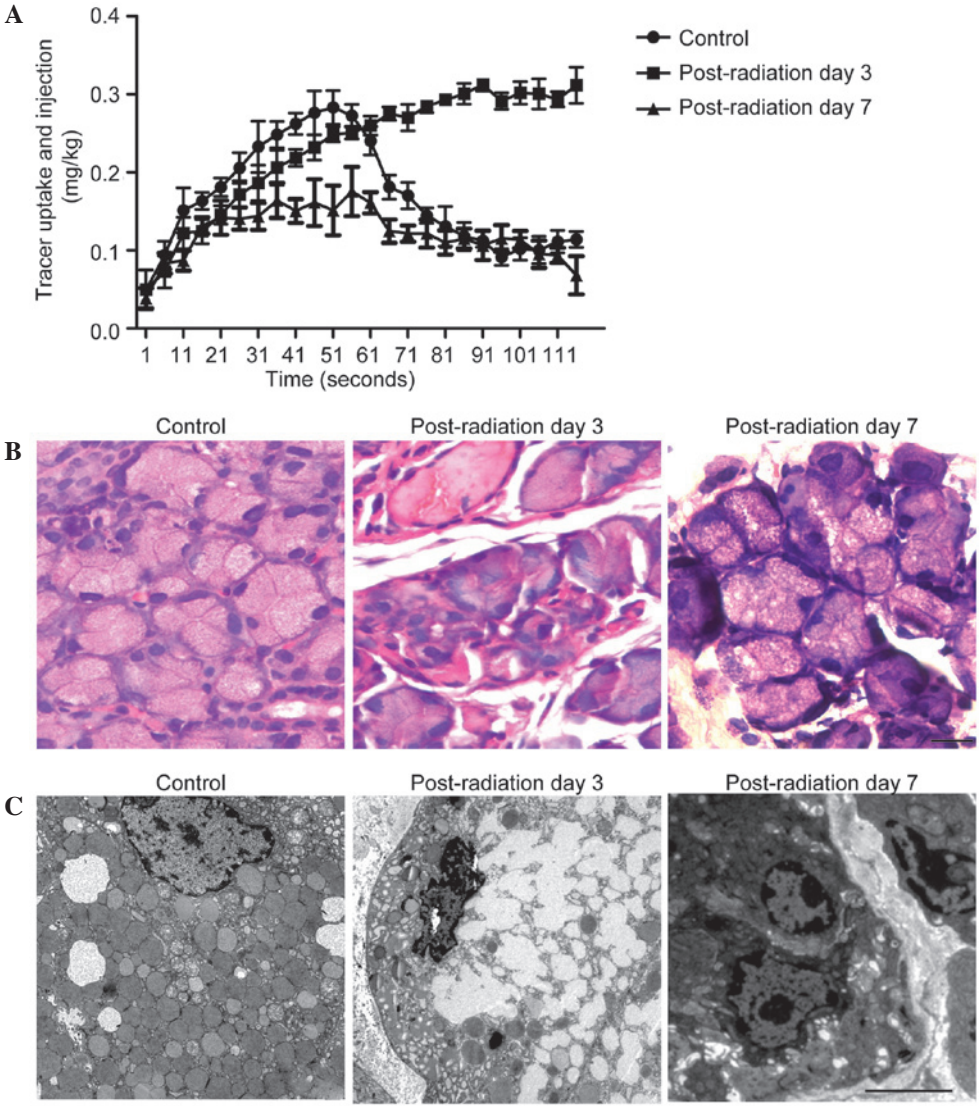

Figure 1. Radiotherapy impaired lacrimal secretion and induced lacrimal gland injury *in vivo*. (A) Scintigraphic data from the lacrimal glands, n=5. The values are means ± SD. (B) H&E staining of irradiated lacrimal glands. Scale bar, 25 μm. (C) Electron microscopy images. Scale bar, 5 μm.

in apoptotic acinar cells were observed, along with extracellular oedema and increased congestion of the interlobular blood vessels (Fig. 1B). The majority of acute changes in the appearance of the acinar cells remained evident seven days later.

Transmission electron microscopy revealed the intracellular retention of secretory granules, with subsequent displacement of the acinar nuclei in the lacrimal gland 3 days following irradiation (Fig. 1C). In addition, 7 days following irradiation, apoptotic acinar nuclei were observed and partial remission was noted, including a reduction in secretory retention (Fig. 1C).

These results confirmed that radiation substantially damages lacrimal gland function and structure.

Radiation upregulates Gadd45a expression in mouse lacrimal glands. To determine the roles of Gadd45a in radiation-induced lacrimal gland injury, Gadd45a gene expression was first observed in a radiation-induced lacrimal gland injury mouse model. At post-irradiation days 3 and 7, lacrimal glands were collected and the mRNA and protein expression levels of Gadd45a were detected by quantitative PCR and western blot analysis. The results showed that Gadd45a was expressed at a low level in normal lacrimal glands, however, following local radiotherapy, mRNA and protein expression levels of Gadd45a were significantly upregulated *in vivo* (Fig. 2A-C). This observation indicated that Gadd45a expression may be induced by radiation, which is consistent with previous reports (14,16,17,23).

Gadd45a overexpression suppresses LGE cell migration and proliferation. LGE cells are the primary cell type in the lacrimal gland (8) and normal motility and proliferation of LGE cells is pivotal for lacrimal gland repair following local radiotherapy. Studies have demonstrated that Gadd45a has negative roles in cell proliferation and migration (20,21,24). To explore the functional roles of Gadd45a in radiation-induced lacrimal gland injury, the effects of the overexpression of Gadd45a on the migration and proliferation of isolated primary LGE cells were observed. The results revealed that following the overexpression of Gadd45a (Fig. 3A), LGE cell migration (Fig. 3B and C) and proliferation (Fig. 3D) were

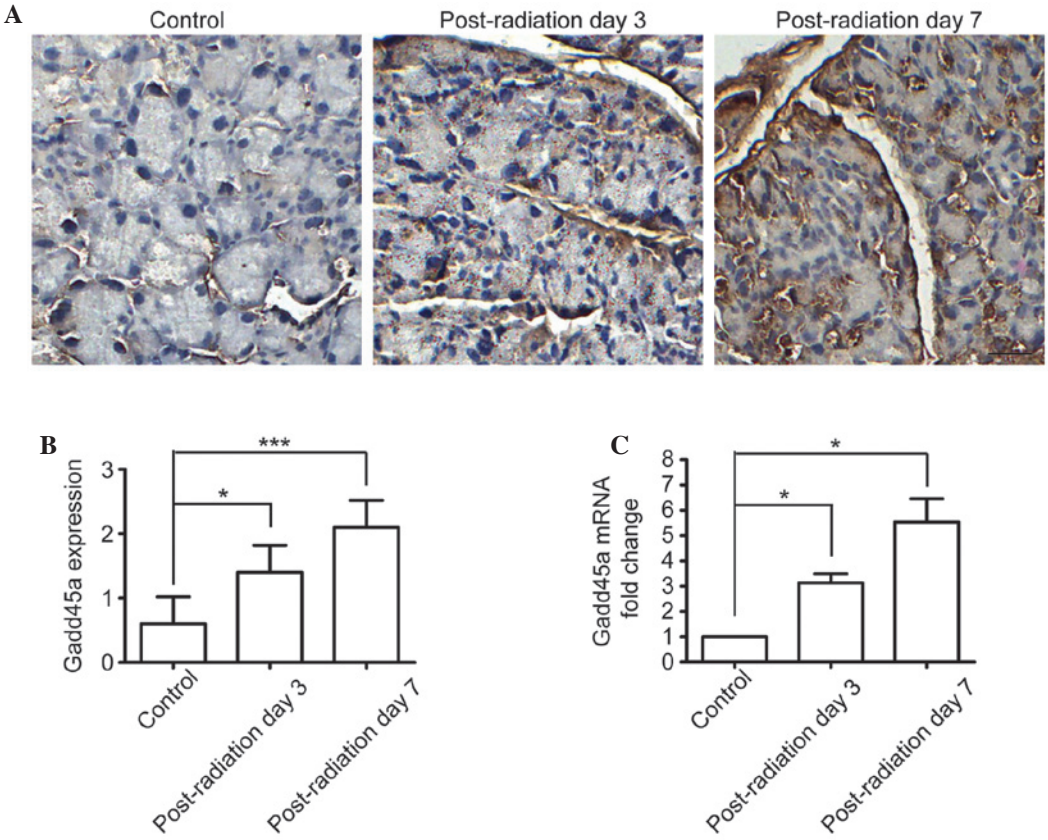

Figure 2. Radiotherapy upregulated growth arrest and Gadd45a expression *in vivo*. (A) Immunohistochemical staining for Gadd45a in a radiation-treated mouse lacrimal gland. Scale bar, 25 μm. (B) Quantification of immunohistochemical staining for Gadd45a, n=5. Values are means ± SD. *P<0.05 vs. control; ***P<0.001 vs. control. (C) Gadd45a mRNA relative expression levels in radiation-treated mouse lacrimal glands. n=5. Values are means ± SD. *P<0.05 vs. control; Gadd5a, DNA damage-inducible 45 α.

significantly inhibited *in vitro*. This observation indicated that the radiation-induced upregulation of Gadd45a may damage the recovery potentially induced by LGE cell migration and proliferation.

Gadd45a inhibits JNK, P38 and Akt phosphorylation in LGE cells. Gadd45a has been reported to be associated with Akt signalling (18) and MAPK stress signalling (17,24), including P38 and JNK. To determine whether these kinases are affected by the overexpression of Gadd45a in LGE cells, western blot analysis of these signalling kinases was performed. Akt, P38 and JNK phosphorylation were observed to be significantly promoted by Gadd45a overexpression (Fig. 4A and B), but Erk1/2 was not markedly affected (Fig. 4A and B).

Discussion

Findings of the current study revealed that Gadd45a is upregulated in lacrimal gland tissue by radiotherapy. Overexpressed Gadd45a impaired two main events in lacrimal gland repair, LGE cell migration and proliferation. This observation indicated that Gadd45a prohibits lacrimal gland repair. Thus, this gene may be used as a therapeutic target in lacrimal gland injury secondary to orbital radiotherapy.

The present study indicated that beyond directly damaging the lacrimal gland, radiotherapy also impairs the reparative ability of the gland by upregulating Gadd45a expression. Microarray analysis of irradiated skin cells revealed a number of up- and downregulated genes (13-15,25-27), however, the roles of the majority of these genes are not well characterized, particularly in the lacrimal gland, another superficial organ that is also affected by radiation. Studies focused on these genes may aid in the understanding of radiation-induced secondary damage.

The roles and mechanisms of Gadd45a have been observed in various physiological and pathological situations. Previous findings have demonstrated that Gadd45a is upregulated following irradiation (14,16,23) and in UV radiation-treated skin, Gadd45a may induce damaged keratinocyte apoptosis via p38 and JNK activation, thus inhibiting tumorigenesis (17). Gadd45a may be induced by the stress of DNA damage and regulated by the tumour suppressor gene p53, through which Gadd45a modulates tumour angiogenesis (28). The effects of Gadd45a on tumour cell migration and invasion have also been shown (20). The roles of Gadd45a in cell proliferation and cell cycle arrest have been comprehensively studied (23,24). The current study on radiation-induced lacrimal gland injury found that Gadd45a may also significantly affect LGE cell migration and proliferation and suppress JNK, P38 and Akt phosphorylation, which is consistent with the observations of previous studies on other cell types (17,18,24).

Radiotherapy-induced lacrimal gland injury is difficult to treat but has a relatively low incidence rate (6). However, with the increasing use of computers, radiation-induced

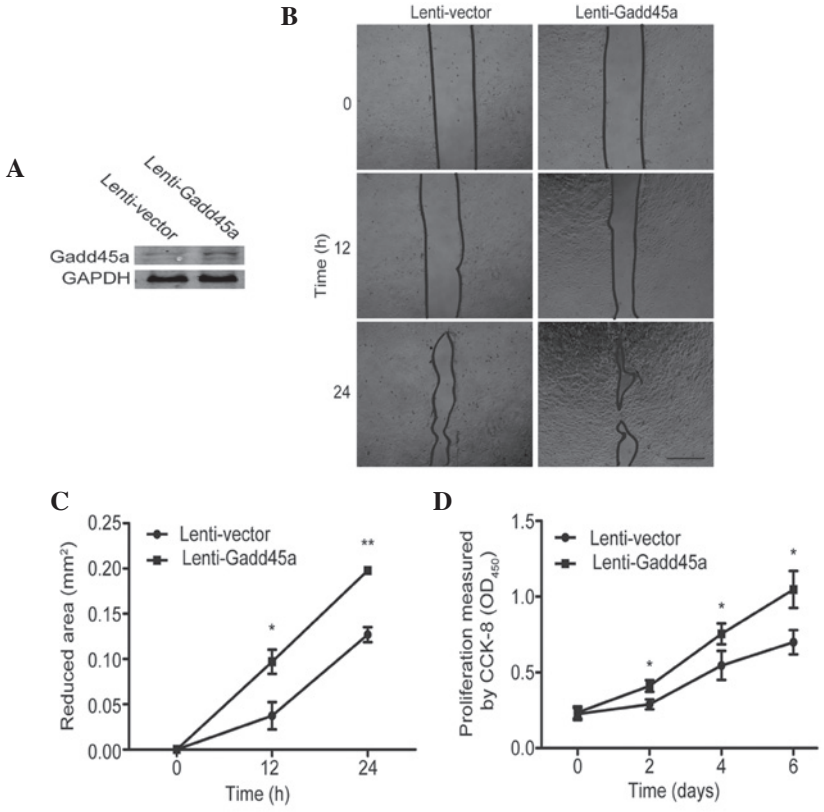

Figure 3. Overexpression of growth arrest and Gadd45a inhibited LGE cell migration and proliferation *in vitro*. (A) Establishment of Gadd45a overexpression efficiency. (B) Images of LGE cell scratch-wound healing assays. Scale bar, 250 μm. (C) Quantification of reduced area. n=3. Values are means ± SEM. *P<0.05, **P<0.01. (D) *In vitro* proliferation assay for LGE cells overexpressing Gadd45a or a vector control. n=3. Values are means ± SEM. *P<0.05. P<0.05 was considered to indicate statistically significant differences. Gadd45a, DNA damage-inducible 45 α; LGE, lacrimal gland epithelial.

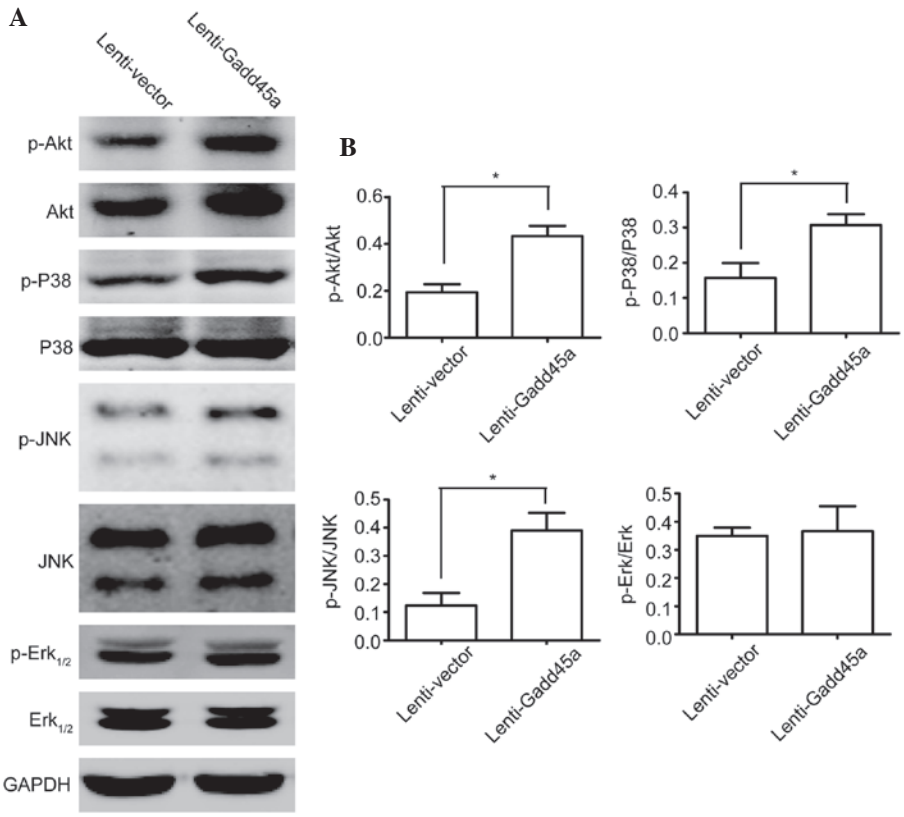

Figure 4. Overexpression of growth arrest and Gadd45a suppressed Akt, P38 and JNK phosphorylation in LGE cells. (A) Images of SDS-PAGE bands for Akt/p-Akt, P38/p-P38, JNK/p-JNK and Erk1/2/p-Erk1/2. (B) Quantification of SDS-PAGE bands, n=3. values are means ± SEM. *P<0.05 was considered to indicate statistically significant differences. Gadd45a, DNA damage-inducible 45 α; LGE, lacrimal gland epithelial.

xerophthalmia is becoming increasingly common clinically (29,30). Gadd45a is involved in solar UVB-induced cell impairment (19), thus, further studies on Gadd45a are required to explore the role of Gadd45a on lacrimal gland dysfunction induced by computer-related radiation.

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