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EGCG inhibits growth of tumoral lesions on lip and tongue of K-Ras transgenic mice through the Notch pathway

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Abstract

Epigallocatechin-3-gallate (EGCG), the main active ingredient of green tea, exhibits low toxic side effect and versatile bioactivities, and its anti-cancer effect has been extensively studied. Most of the studies used cancer cell lines and xenograft models. However, whether EGCG can prevent tumor onset after cancer-associated mutations occur is still controversial. In the present study, Krt14-cre/ERT-Kras transgenic mice were developed and the expression of K-RasG12D was induced by tamoxifen. Two weeks after induction, the K-Ras mutant mice developed exophytic tumoral lesions on the lips and tongues, with significant activation of Notch signaling pathway. Administration of EGCG effectively delayed the time of appearance, decreased the size and weight of tumoral lesions, relieved heterotypic hyperplasia of tumoral lesions, and prolonged the life of the mice. The Notch signaling pathway was significantly inhibited by EGCG in the tumoral lesions. Furthermore, EGCG significantly induced cell apoptosis and inhibited the proliferation of tongue cancer cells by blocking the activation of Notch signaling pathway. Taken together, these results indicate EGCG as an effective chemotherapeutic agent for tongue cancer by targeting Notch pathway.

Keywords: Apoptosis; EGCG; KRas mutation; Notch signaling pathway; Tongue squamous cell carcinoma.

1. Introduction

Green tea is one of the most popular beverages and many epidemiological studies show benefits associations with tea consumption for those affected with cardiovascular diseases [1], diabetes [2], cognitive disorders [3], and various cancers [4]. In a recent umbrella review [5], the most prominent association identified was between tea consumption and diverse cancers, including gastric cancer, oral cancer, lung cancer, biliary tract cancer, thyroid cancer, ovarian cancer, leukemia, glioma, esophageal cancer, and endometrial cancer. It is generally agreed upon that many of the cancer chemopreventive effects of green tea are mediated by epigallocatechin-3-gallate (EGCG), the major catechins in green tea [4,6]. Since the first report of its chemopreventive effect in 1983 [7], EGCG has been extensively studied all over the world, and its anti-cancer effect has been studied on a variety of cancer cells *in vitro*, in animal-based experiments, and some clinical trials [4-6,8,9]. For the *in vivo* tumor formation assay, as summarized in a recent review [6], most studies employed xenograft tumor models by subcutaneous injection of cancer cells into nude mice. All cancers are caused by alterations and mutations in DNA [10,11], and gene–environment interactions have been traditionally understood to promote the acquisition of mutations that drive multistage carcinogenesis [12]. Whether EGCG can prevent or delay tumor onset after cancer-associated mutations occur is still controversial and needs more experimental data.

Ras proteins are small GTPases that contains three RAS proto-oncogenes named HRAS, KRAS, and NRAS in *Homo sapiens*. They play a crucial role as a central component of several signaling pathways that regulate cell proliferation, survival, differentiation, adhesion, and motility [13]. Ras genes are among the earliest, if not the first, genes mutated in a variety of cancers [14], and KRAS is found to be the most commonly mutated oncogene, which accounts for 23% mutations in human cancers [13]. KRasG12D-mutated mice mimic cancer development in humans and have been widely used in cancer research and drug discovery.

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Several studies have reported deregulation of the Notch signaling pathway in KRas-induced tumoral tissues, but the results were still controversial [15-17]. Chung et al. found that the Notch signaling pathway was downregulated in Kras-induced gastric metaplasia [15], but upregulated in gallbladder cancer [16]. Kong et al. showed that inhibition of Notch signaling pathway via DNMAML expression or Pofut1 deletion significantly blocks the oncogenic Kras-induced leukemogenesis [17]. In the present study, the KRasG12D mutation was introduced to mice epidermis by tamoxifen induction. We found the mutated mice can develop exophytic tumoral lesions on the lips and tongues shortly after induction and Notch signaling pathway was activated in these tumoral tissues. We explored the effect of EGCG on tumor initiation and progress and the underlying mechanism in these mice as well as tongue cancer cells.

2. Materials and methods

2.1. Generation of Krt14-cre/ERT-Kras mice

Animal procedures were approved by the Biomedical Ethics Research Committee of Fujian Medical University School and Hospital of Stomatology (Approval Number: FMUSS-2018-35) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health, United States. The LSL-KrasG12D strain mice (Jackson Lab, Stock No. 019104) carry a point mutation (G12D) in the Kras gene and its expression is blocked by the presence of a loxP-flanked stop codon. These mice were crossed with K14-CreERT mice (Jackson Lab, Stock No. 005107) to generate tamoxifen-induced keratinocyte-specific Cre-mediated KRas G12D-mutated mice. All of these genetically engineered mice were bred and genotyped for the presence of Kras and K14-Cre using the protocol recommended by Jackson lab. Tamoxifen induction was initiated at the age of 4 weeks.

2.2. Administration of EGCG

The day after tamoxifen induction, EGCG treatment was initiated. Krt14-cre/ERT-Kras double mutant transgenic mice were randomly assigned to receive either mock treatment (n=10) or EGCG (n=10). EGCG (Selleck, #S2250, 99.68% purity) was dissolved at the concentration of 5 mg/mL in saline solution, and injected every 2 d by intraperitoneal injection at a dosage of 25 mg/kg body weight for 2 months, and the mice in the control group were injected with same volume of saline solution. After exophytic tumoral lesions were visible on the lip and tongue, and interfered the intake of feed, all the mice were fed with 10% milk in the drink water in addition to the regular feed from week 6. The mice were euthanized when they could not feed autonomously or 30% of body weight was lost, which was approved by the Biomedical Ethics Research Committee of Fujian Medical University School and Hospital of Stomatology (FMUSS-2018-35). To measure the mice serum concentrations of EGCG, blood samples were collected by the "retro-orbital bleed" and the serum was separated, by allowing the blood to clot and centrifuging it for 20 min at 4°C. For each sample, 100 μL serum was extracted with 400 μL methanol, and the serum levels of EGCG was analyzed by HPLC–MS/MS.

2.3. Histology and immunohistochemistry

Formalin-fixed paraffin-embedded murine tongue tissue was processed by standard methods and stained with hematoxylin and eosin. The expression of Notch1, Notch2, and Hes1 in animal model samples was measured by immunohistochemical staining as described previously [18].

2.4. Cell lines and cell cultures

The CAL-27, SCC9 cell lines were bought from American Type Culture Collection and maintained in the suggested media and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All cell lines were STR-authenticated annually by Shanghai Biowing Applied Biotechnology Co. LTD, Shanghai, China.

2.5. siRNA transfection

The siRNA was designed and synthesized by GenePharma, Shanghai, China). The specific sequences of siRNA are shown in previous report [19]. We transfected the CAL-27 cells and SCC-9 cells according to the instruction manual of Lipofectamine RNAiMAX (Invitrogen, Catalog # 13778150).

2.6. Cell viability assay

Cell proliferation was measured by counting viable cells with a Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan). At the same time each day for 5 consecutive days, the original culture medium was removed, and 10 μL CCK8 and 90 μL fresh media were added into each well. The cells were incubated at 37°C for 1 h. The absorbance of each well was measured with a microplate reader (Pharmacia Biotech, USA) at 450 nm.

2.7. Colony formation assay

Twenty-four hours after transfection, the cells were plated into 6-cm plates (2,000 cells per plate), treated with EGCG, and cultured for 2 weeks. Colonies were fixed with cold methanol for 10 min and stained with 1% crystal violet for 30 min.

2.8. Apoptosis assay

Cells were seeded in six-well plates (5×10^5 cells/well) and exposed to EGCG for 48 h. Apoptotic cells were detected by FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, Catalog # 556547) followed by flow cytometer analysis.

2.9. Cell cycle test

After treatment with EGCG for 48 h, cells were digested, collected, and fixed with 70% ethanol at 4°C overnight then washed with PBS and stained with 400 μL PI/RNase Staining Buffer (BD Pharmingen, Catalog # 554655). The percentage of each phase of cells was quantified using a BD FACS Verse flow cytometer.

2.10. Quantitative real-time PCR

The expression of Notch relevant mRNA was tested by quantitative reverse-transcription PCR assay. The sequences of primers are listed in Supplementary data Table S1. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [20].

2.11. Western blotting

Total proteins were separated by 4–15% SDS-PAGE and transferred onto PVDF membranes (Amersham, USA). Membranes were blocked with 10% non-fat milk then incubated with primary antibodies (listed in Supplementary data Table S2) in 5% bovine serum albumin overnight. The membranes were then washed three times with Tris-buffered saline containing 0.1% Tween-20 and incubated with the secondary antibody (1:2,000 dilution, Boster, China). The immunoreactive protein bands were visualized using CDP STAR reagent (Roche, IN, USA), and signals were scanned with a densitometer for the semiquantification of signal intensity.

2.12. Subcellular localization analysis

The cells were cultured on glass coverslips in six-well plates and transfected pcDNA3.1-NICD1 or empty vector with Lipofectamine 2000 (Invitrogen, Catalog # 11668019) according to the manufacturer instruction. Forty-eight hours later, the cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed four times with PBS, and then permeabilized with PBS containing 5% BSA and 0.1% Triton X-100 for 10 min on ice. After this step, the cells were washed with PBS and incubated with rabbit anti-Notch1 (1:100, Sigma, N4788) diluted in PBS containing 5% BSA and 0.1% Tween-20 for 1 h at room temperature. After washing, the cells were incubated with goat anti-rabbit labeled with Alexa Fluor 488 (1:500, Abcam, ab150077). The cells were washed and mounted in glycerol mounting medium with DAPI (Abcam, ab188804). The slides were imaged with a confocal microscope (Leica TCS SP8) and the images were analyzed using ImageJ (NIH, Maryland, USA) by determining the colocalization coefficient of Notch1 and DAPI.

2.13. Statistical analysis

The statistical analyses for real-time PCR, Western blot, the colony formation assays, apoptosis, and cell cycle experiments were determined by one-way ANOVA followed by Tukey's multiple comparison test, and the growth curves measured by CCK8 reagent were determined by two-way ANOVA followed by Bonferroni post-test. The survival of the mice was analyzed by Mantel–Cox test. $P<.05$ was considered statistically significant and significance is indicated in the figures as n.s. (not significant) when $P>.05$, * when $P<.05$, ** when $P<.01$, and *** when $P<.001$.

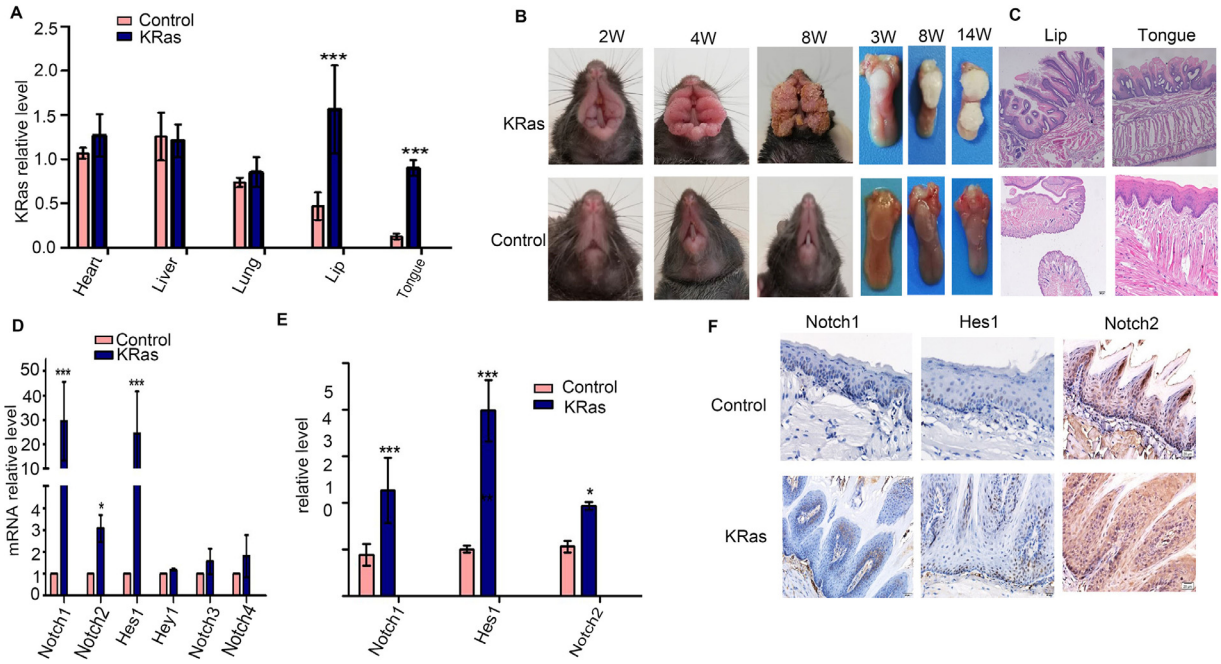

Fig. 1. Development of oral tumoral lesions in K14-CreERT/KRasG12D mice. (A) The expression of KRasG12D in different tissues was detected by Western blot. (B) The appearance of exophytic tumoral lesions on lips and tongues of KRasG12D mice. (C) The exophytic tumoral lesions on the lips and tongues were verified by HE staining. (D–F) The expression of Notch signaling pathway genes in exophytic tumoral lesions was measured by real-time PCR (D), Western blot (E), and IHC (F). $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ when compared with control group.

3. Results

3.1. Development of oral tumoral lesions in K14-CreERT/KRasG12D mice

To mimic tumorigenesis of oral squamous cell carcinoma, LSL-KRasG12D mice were crossed with K14-CreERT mice, which carried a tamoxifen inducible Cre-mediated recombination system driven by the human keratin 14 (K14) promoter. This promoter is supposed to be active in dividing cells of epidermis and some other stratified squamous epithelia. As validated by Western blot, KRasG12D was significantly upregulated in mice lips and tongues, but not in the livers, lungs, or hearts (Fig. 1A and supplementary Fig. S1A) of K14-CreERT/KRasG12D (KRas) mice after induction with tamoxifen. As early as 2 and 3 weeks after the tamoxifen induction, all KRas group mice developed exophytic tumoral lesions on the lip and tongue, which were visible by the naked eye (Fig. 1B). Grossly, the lesions form exophytic individual plaques in the lip at 2 weeks. The individual lesions fused producing nodular neoplasms, which account for two-thirds of the mice lips at 4 weeks. Furthermore, the obviously papillary projections were seen in the whole lip at 8 weeks. However, in the control groups, the lips of mice do not have obviously lesion even at 8 weeks. Similarly, the tongue lesions have the same tendency. At 3 weeks, white speckled lesions were seen in the back of the tongue. However, the lesions were enlarged and characterized as homogeneously white verrucous areas involving the whole tongue at 8 weeks. As the same results of controls, no obvious lesion was seen (Fig. 1B). Microscopically, in comparison to the controls, projections and invaginations of well-differentiated squamous epithelium with low–moderate dysplasia were both seen in lip and tongue lesions (Fig. 1C). These results confirmed that the expression of KRasG12D proteins in the lips and tongue lead to tumoral lesions including hyperkeratotic squamous cell papilloma, papillary structures lined by cuboidal or columnar epithelial cells with low–moderate atypia.

Several studies have reported deregulation of the Notch signaling pathway in KRas-induced tumoral tissues, but the results were still controversial [15-17]. Our previous studies have demonstrated that the aberrant expression of Notch signaling pathway is associated with tumorigenesis of tongue cancer [19, 21]. To explore the role of the Notch signaling pathway in our KRas-induced oral tumoral lesions, the expression levels of Notch receptors and the two key downstream genes, Hey1 and Hes1, were measured in these tissues by real-time PCR and the results showed that Notch1, Notch2, and HES1 were significantly upregulated in KRas-mutated tissues (Fig. 1D). The protein levels of Notch1, Notch2, and Hes1 were also upregulated in KRas-mutated tissues, as validated by Western blot (Fig. 1E and supplementary Fig. S1B) and immunohistochemistry (Fig. 1F). These results suggest that activation of the Notch signaling pathway may contribute to the KRas-induced tumoral proliferation.

3.2. EGCG inhibited the growth of oral tumoral lesions and prolonged the survival of KRas-mutated mice

To investigate the effect of EGCG on the onset and growth of oral tumoral lesions, we treated the KRas-mutated mice with EGCG after tamoxifen induction via intraperitoneal injection every other day at a dosage of 25 mg/kg body weight for 2 months [22,23]. With this dosage, the circulating concentrations of EGCG in the mice serum were 0.703±0.057 μg/mL 1 h after injection, and 0.239±0.057 μg/mL for 2 h as measured by HPLC–MS/MS. Because of its poor bioavailability, it needs a dosage at 800 mg of EGCG once per day to reach the similar circulating concentration in humans by oral administration [24]. Grossly EGCG delayed the appearance of tumoral lesions to 4 weeks on lips and 8 weeks on tongues after tamoxifen induction compared with the vehicle group (Fig. 2A), in which the tumoral lesions appeared at 2 weeks on the lips and 3 weeks on the tongues, and reduced the volume of tumoral lesions (Fig. 2B). Moreover, EGCG prolonged the overall survival of KRas-mutated mice, in which the median survival of the

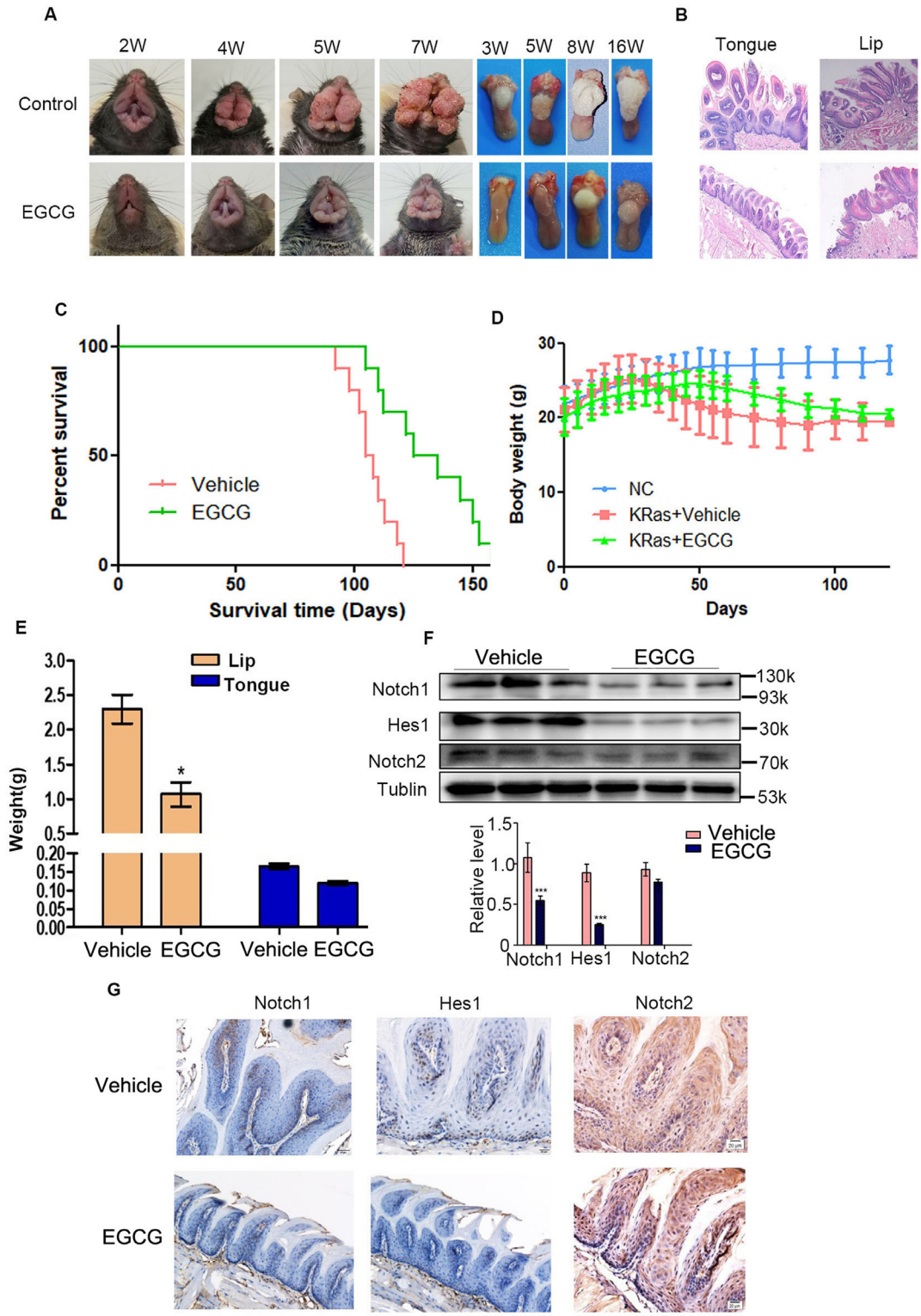

Fig. 2. EGCG inhibited the growth of oral tumoral lesions and prolonged the survival of KRas-mutated mice. (A–B) EGCG delayed the appearance of tumoral lesions (A) and relieved heterotypic hyperplasia of tumoral lesions, as measured by HE staining (B). (C) EGCG administration prolonged the life of mice. (D) The changes of body weights of the mice. (E) EGCG decrease the size and weight of tumoral lesions. (F–G) EGCG inhibited the activation of Notch pathway, as detected by Western blot (F) and IHC (G). NC, negative control, littermates of KRas G12D/K14-Cre^{-} mice with tamoxifen induction. $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ when compared with control group.

EGCG group was 21 weeks as compared to 14 weeks in the control group (Fig. 2C, $P<.001$) and slowed down slightly the body weight loss caused by the oral tumoral lesions (Fig. 2D). After the mice were euthanized, the tongue and the tumoral lesions on the lips of each mouse were dissected and weighed. As shown in Figure 2E, the weight of the tongues and the tumoral lip lesions of mice significantly decreased after EGCG treatment compared with the vehicle group. Microscopically, EGCG significantly inhibited the heterotypic hyperplasia caused by mutated KRas, which was indicated by the thickness of the tumoral lesions (Fig. 2B).

Several studies have shown that EGCG can inhibit the proliferation of cancer cells through suppression of the Notch signaling pathway [25-27]. After proving the activation of Notch pathway in KRas-driven tumoral lesions, we sought to evaluate the expression of Notch in murine tongues after EGCG treatment. The results showed that EGCG significantly decreased the protein levels of Notch1 and Hes1 in the tumoral lesions compared to the vehicle group, but slightly decreased the level of Notch2, as measured by Western blot (Fig. 2F) and immunohistochemistry (Fig. 2G).

3.3. EGCG inhibited cell proliferation and induced apoptosis in tongue cancer cells through suppression of Notch signaling pathway

Our *in vivo* experiments have shown that EGCG inhibits growth of tumoral lesions on lips and tongues of KRas-mutated mice through, at least partially, suppression of the Notch signaling pathway. Next, we want to further explore the effect of EGCG on cell proliferation, apoptosis and the role of Notch pathway in tongue cancer cell lines. As shown in Figure 3A–B, the treatment of EGCG inhibited the proliferation of CAL-27 and SCC9 tongue cancer cells in a dose-dependent manner in a CCK8 assay (Fig. 3A), and colony formation assay (Fig. 3B and supplementary Fig. S2A). The IC50 of EGCG on these two cell lines was around 90 μM (48 h) or 75 μM (72 h), which was similar to the published reports with head and neck cancer cells [28,29] or other cancer cells [30, 31]. To further clarify whether the growth inhibitory effect of EGCG was associated with apoptosis, cells were stained with annexin V-PI and apoptosis was measured by flow cytometry. The results showed that the percentage of apoptotic cells in EGCG treated Cal-27 cells and SCC9 cells were significantly higher than in the control group (Fig. 3C and supplementary Fig. S2B, $P<.05$). Moreover, some of the apoptotic markers, such as cleaved caspase-3, cleaved PARP, and Bax were increased and the anti-apoptosis protein Bcl-2 was decreased after EGCG treatment (Fig. 3D and supplementary Fig. S2C). These results indicated that EGCG could induce apoptosis of tongue cancer cells in a dose-dependent manner.

To explore the role of the Notch signaling pathway in the proliferation inhibition and apoptosis induction by EGCG, the expression of Notch1, Notch2, HES1, the deregulated Notch signaling pathway genes as shown above, and some other reported downstream genes of Notch signaling pathway such as HEY1, Cyclin D1 [32], CDK4 [33] were measured by real-time PCR and Western blot 48 h after EGCG treatment. As shown in the results from the mice tissues above, the expression of Notch1, Notch2, and Hes1 were downregulated in the EGCG group in a dose-dependent manner, both at the mRNA level (Fig. 4A) and protein level (Fig. 4B). Moreover, the expression of Cyclin D1 and CDK4 was decreased upon the treatment of EGCG (Fig. 4A–B), which may be consistent with the growth inhibition of EGCG (Fig. 3A–B). These results indicate that EGCG inhibits cell proliferation and induces apoptosis in tongue cancer cells via suppression of the Notch signaling pathway.

3.4. Notch1 changed the sensitivity of tongue cancer cells toward EGCG

To further validate the role of the Notch signaling pathway in growth inhibition and apoptosis induction by EGCG in tongue cancer cells, the expression of Notch1 in tongue cancer cells was modulated by siRNA knockdown or overexpression which was then followed by EGCG treatment. EGCG can further decrease the Notch1 protein level in Cal-27 cells after Notch1 knockdown (Fig. 5A). As reported previously [19], knockdown of Notch1 by 2 siRNAs (Si1 and Si2) inhibited cell proliferation as shown by the CCK-8 assay (Fig. 5B) and colony formation assay (Fig. 5C), and increased the sensitivity of Cal-27 cells toward EGCG. When co-treated with siRNA and EGCG, the effect of growth inhibition was more significant than that treated with Notch1 siRNAs or EGCG alone (Fig. 5B–C). On the other hand, overexpression of Notch1 intracellular domain (NICD) in Cal-27 cells (Fig. 6A) promoted cell proliferation when compared with empty vector in the CCK8 assay (Fig. 6B) and colony formation assay (Fig. 6C, $P<.05$), and attenuated the proliferation inhibition by EGCG (Fig. 6B–C). To test whether EGCG can inhibit the nuclear translocation of NICD, the immunofluorescence staining of Notch1 was performed after NICD plasmids were transfected. The results indicated that the treatment of EGCG suppressed nuclear translocation of NICD (Fig. 6D) both in the cells with endogenous Notch1 and the cells with overexpressing NICD. These results confirm that EGCG inhibits cell proliferation through suppression of the Notch signaling pathway in tongue cancer cells.

4. Discussion

In the present study, we used tamoxifen inducible KRas G12D-mutated mice which developed exophytic tumoral lesions on the lips and tongue after tamoxifen induction accompanied by a significantly upregulated Notch signaling pathway. EGCG treatment effectively delayed the time of appearance, decreased the size and weight of tumoral lesions, relieved heterotypic hyperplasia of tumoral lesions, prolonged the life of mice and inhibited the activation of the Notch signaling pathway.

Head and neck cancer, arising in the epithelial tissue of the lip, oral cavity, nasal cavity, paranasal sinus, pharynx, and larynx, ranks the sixth most common malignancy worldwide and represents about 4% of all types of cancer [34, 35]. Histologically, 90% of head and neck cancers are squamous cell carcinoma, and the most common subtype of this disease is oral cancer. Although significant progress has been achieved in early detection, diagnosis, surgery, chemotherapy, and radiotherapy, the 5-year survival rates for patients with head and neck cancer have not improved in the past 30 years and remain about 50% [36]. Therefore, a better understanding of the molecular mechanisms involved in the tumorigenesis and progression of oral cancer will provide novel strategies for prevention, diagnosis, and therapy. Genetic alterations commonly found in oral cancer are thought to have a causal role in the disease, and genetically modified mouse models are widely used to explore the role of gene mutation in oncogenesis and progression of different types of cancer, but are not frequently used in the research of oral squamous cell carcinoma [37, 38]. Ras mutations are found in 5–20% of patients with oral cancer from Western countries, but occur in more than 50% of the patients in south Asian populations, where oral cancer accounts for ~50% of all diagnosed cancer cases and is associated with the habit of chewing tobacco [36, 39]. In the present study, we used tamoxifen inducible KRas G12D-mutated mice which develop exophytic tumoral lesions on the lips and tongue after tamoxifen induction, which is consist with previous studies [36, 40, 41]. The results indicate a causal role for oncogenic K-ras in oral tumor development,

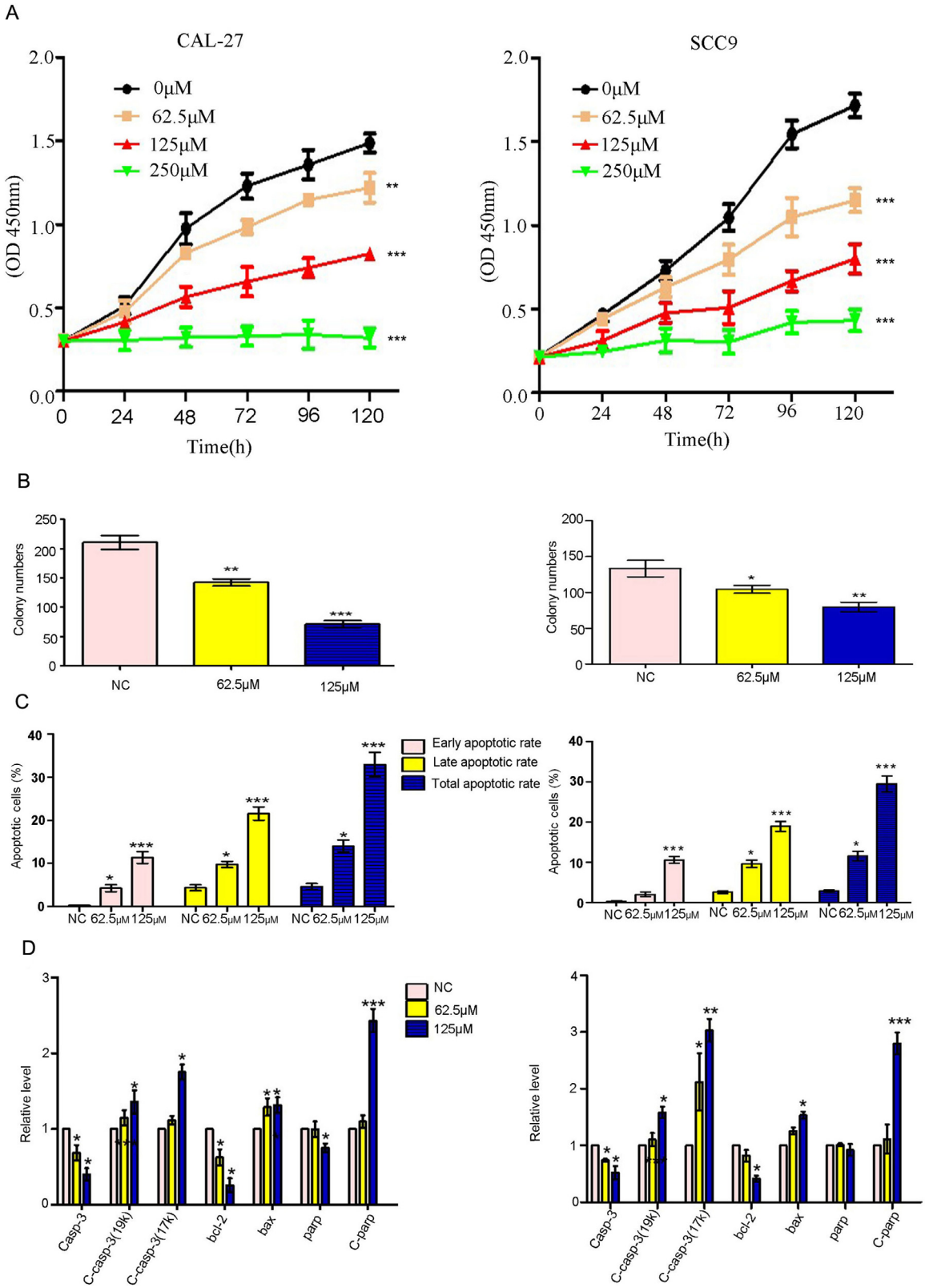

Fig. 3. EGCG inhibited proliferation and induced apoptosis in tongue cancer cells. (A) Cell growth was measured by CCK-8 assay. $^{**}P<.01$ and $^{***}P<.001$ by two-way ANOVA followed by Bonferroni post-test. (B) Apoptotic cells were detected by flow cytometry after ANNEXIN V/PI staining after Cal-27 cells (left) and SCC9 cells (right) were treated with indicated concentrations of EGCG for 48 h. (C) EGCG inhibited colony formation of Cal-27 cells (left) and SCC9 cells (right). (D) The expression levels of apoptosis-related proteins were changed by EGCG treatment at the indicated concentrations for 72 h in Cal-27 cells (left) and SCC9 cells (right). NC, negative control, which was treated with same volume of vehicle (saline solution). Data represent the mean ± S.D. of three independent experiments. $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ when compared with control group.

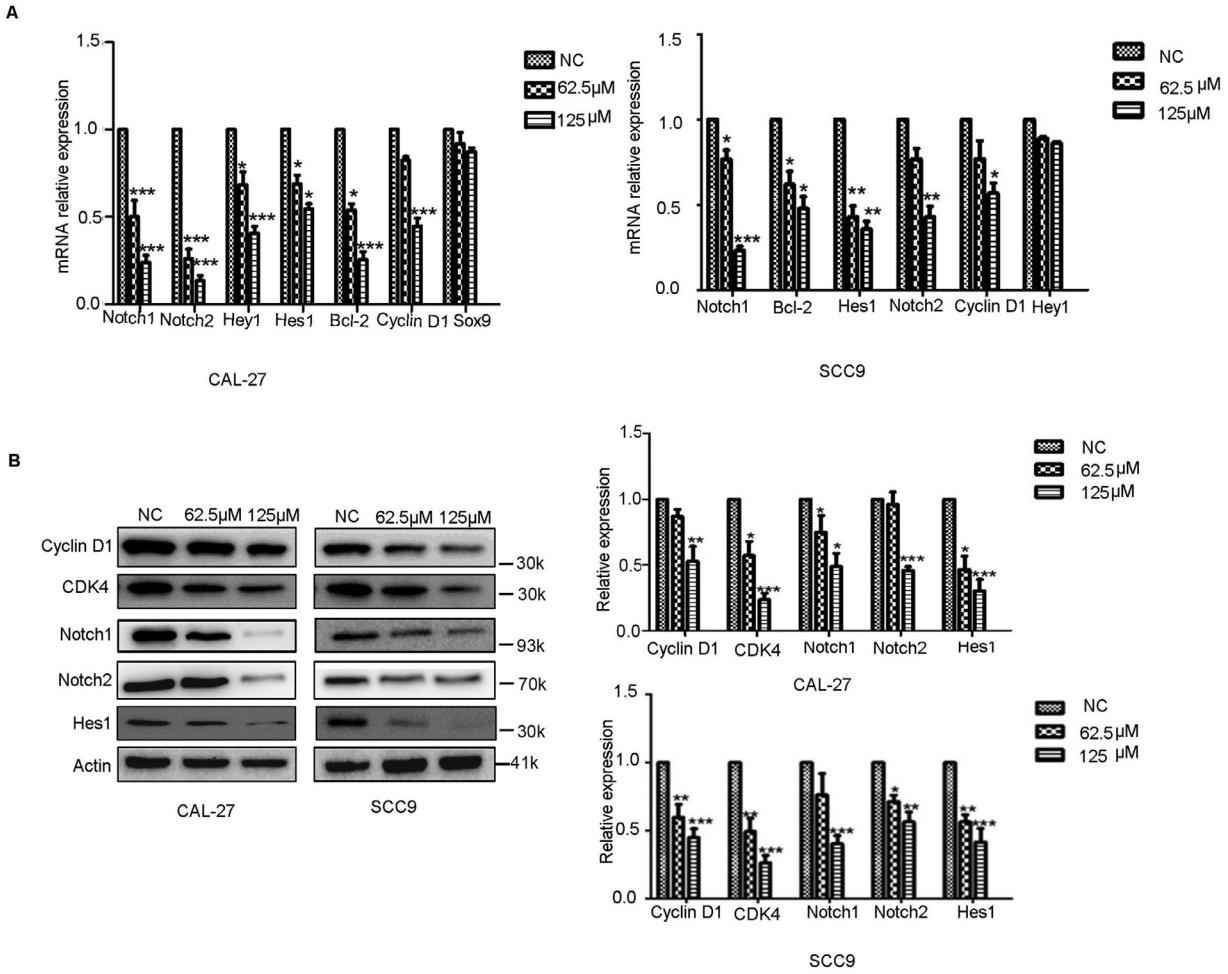

Fig. 4. EGCG inhibited the activation of the Notch signaling pathway in tongue cancer cells. After treated with EGCG at the indicated concentrations for 72 h, the expression of Notch1, Notch2, and some Notch pathway targeting genes in Cal-27 cells and SCC9 cells was measured by real-time PCR (A) and Western blot (B). NC, negative control, which was treated with same volume of vehicle (saline solution). Data represent the mean ± S.D. of three independent experiments. $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ when compared with control group.

and make it an ideal system to study the molecular mechanism of tumorigenesis.

The Notch signaling pathway regulates cell fate decisions, cell differentiation, and proliferation in a variety of tissues during development and homeostasis. Activation of the Notch signaling pathway leads to expansion of stem and progenitor cell population, impaired differentiation, and increased proliferation, ultimately contributing to tumor initiation and progression. Many studies on malignancies, such as T-cell acute lymphoblastic leukemia [42], bladder cancer [43], and prostate cancer [44] have reported that the Notch signaling pathway is oncogenic and could promote cancer progression and metastasis. However, some studies have also stated that the Notch signaling pathway acts as a tumor suppressor, such as in forebrain glioma [45], cutaneous SCC [46], and colorectal cancer [47] (see also recent reviews [48, 49]). In head and neck squamous cell carcinoma (HNSCC), the role of Notch signaling pathway in the carcinogenesis and progress is also inconsistent. Many reports support the tumor suppressive role of the NOTCH pathway in HNSCC [50-53]. A recent study published in Science by Loganathan et al. suggests that NOTCH pathway inactivation is common and a hallmark in HNSCC [54]. The results of our group [19, 21, 55] and other groups [56-60] indicated that Notch signaling pathway was activated in primary HNSCC tumor samples and in some cases correlates with aggressive disease, and its activation can increase cancer stemness and promote EMT and metastasis. The inconsistence indicates that Notch activity regulates tumor biology in a complex, context-dependent manner, the underlying mechanisms are worthy of further investigation. There are several studies [15-17,61, 62] reported that also show that the Notch signaling pathway plays an important role in the tumorigenesis induced by K-Ras mutations but the conclusions are inconsistent. Avila et al found that Notch1 is not required for acinar-to-ductal metaplasia in a mice model of Kras-induced pancreatic ductal adenocarcinoma, in which oncogenic K-ras is sufficient to drive acinar-to-ductal metaplasia both *in vitro* and *in vivo*, and loss of Notch1 has a minimal effect on this process [61]. While in lung cancer, the results from the same lab indicated that Notch1's function is required for tumor initiation via suppression of p53-mediated apoptosis [62]. In a KRAS;TP53-mutant patient-derived lung xenografts, Ambrogio et al. found that the combined inhibition of DDR1 and Notch signaling could be an effective targeted therapy for patients with KRAS-mutant lung adenocarcinoma [63]. In the present study, we found that the Notch signaling pathway was significantly upregulated in KRas-mutated tissues at both the transcriptional level and protein level, which indicates that the Notch signaling pathway plays a key role in mutated Kras-initiated tumorigenesis, and

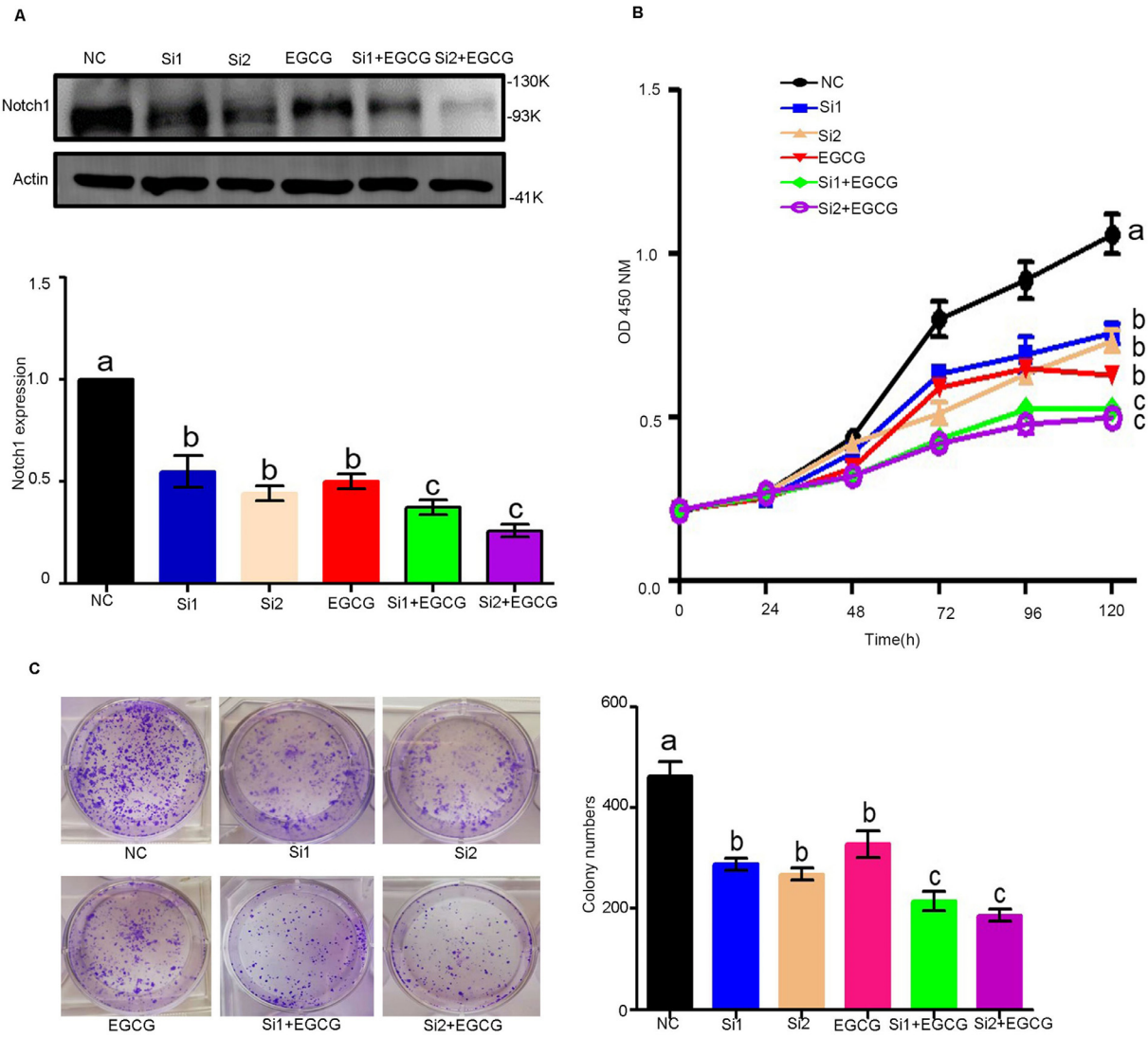

Fig. 5. Knockdown of Notch1 inhibited cell proliferation and increased the sensitivity of CAL-27 cells toward EGCG. (A) The expression of Notch1 in CAL-27 cells was detected by Western blot after EGCG treatment and Notch1 knockdown by two siRNAs (Si1 and Si2). NC, negative control, which was transfected with scrambled small RNA and treated with vehicle. (B) Cell proliferation was measured by CCK-8 assay after transfected with siRNA and treated with or without 62.5 μM EGCG. (C) Colony formation assay for Cal-27 cells after transfected with siRNA and treated with or without 62.5 μM EGCG. Data represent the mean ± S.D. of three independent experiments. Superscripts with different letters (*i.e.*, a–b, b–c, c, d) indicate significant differences between four groups at P=.05.

that inhibition of the Notch signaling pathway may be a potential therapeutic strategy for head and neck squamous carcinoma with KRas mutations.

The cancer-preventive activities of EGCG have been extensively studied. A plethora of biomolecular mechanisms has been demonstrated and proposed to be involved in the usage of EGCG in various types of cancer and the inhibition of ERK1/2, p38 MAPK, JAK/STAT, NF-κB, and VEGF/VEGFR signaling pathways by EGCG have also been reported [64, 65]. It was reported that EGCG can activate the Ras-MAPK pathway [66], but there are also some studies showing that EGCG can inhibit the expression of KRas [67]. In the present study, we found that the treatment of EGCG did not change the activation of Ras pathway in tongue cancer cells (data not shown). Additionally, there are some studies that indicate that EGCG can serve as an inhibitor of the Notch signaling pathway [25–27,65,68]. In HNSCC, there are several publications indicated EGCG can inhibit the proliferation, migration and invasion of cancer cells through suppression of HGF/MMP9 [69], EGFR/ERK [70,71], HER-2/neu [72], Notch [27], Hippo-TAZ [29], JAK/STAT3 [73] pathway, or activation of the proapoptotic proteins such as ATM/p53 [28], BTG2 [74]. EGCG can also sensitizes HNSCC cells to cisplatin [75] and gefitinib [76], or enhance the growth inhibitory effect of 5-fluorouracil [70] and EGFR inhibitor [77,78]. All these studies used cancer cell lines or xenograft models using these cancer cell lines, the various pathways involved in EGCG regulation the growth and motility of cancer cells may be due to the different genetic and epigenetic background of different cell lines. All cancers are caused by mutations in DNA [10,11], whether EGCG can prevent or delay tumor onset after cancer-associated mutations occur is still unclear. In the present study, we confirmed that EGCG suppressed cell proliferation and induced cell apoptosis via inhibiting the Notch signaling pathway in tongue cancer cells and modification of the expression level of Notch1 in tongue cancer cells changed the sensitivity of cells toward EGCG using tongue cancer cell lines. Additionally, using KRasG12D transgenic mice, we found that EGCG significantly decreased the protein levels of Notch1 and Hes1 in the tumoral lesions induced by mutant KRas. Furthermore, the administration of EGCG effectively delayed the time of appear-

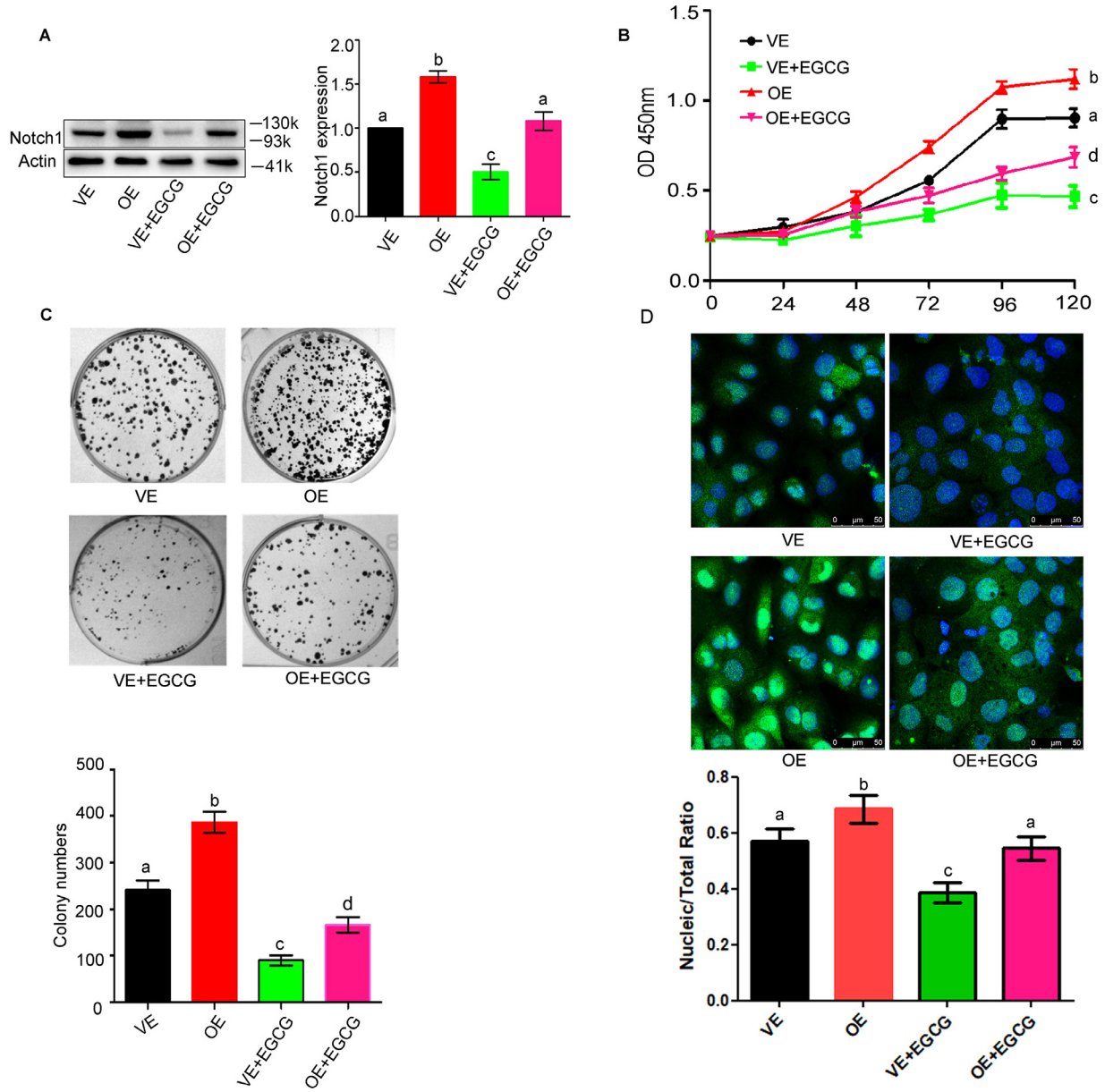

Fig. 6. Overexpression of the Notch1 intracellular domain (NICD) in CAL-27 cells promoted cell proliferation and attenuated the proliferation inhibition by EGCG. (A) The expression of Notch1 in CAL-27 was detected by Western blot after transfected with NICD plasmid and EGCG treatment. VE, vector plasmid; OE, overexpression of NICD. (B–C) Cell proliferation of Cal-27 cells was measured by CCK-8 assay (B) and colony formation assay (C) after NICD or vector plasmid was transfected and treated with 62.5 μM EGCG or same volume of vehicle. (D) Immunofluorescence staining of Notch1 showed the inhibition of nuclear translocation of NICD by EGCG. Data represent the mean ± S.D. of three independent experiments. Superscripts with different letters (*i.e.*, a–b, b–c, c, d) indicate significant differences between four groups at P=.05.

ance, decreased the size and weight of tumoral lesions, and prolonged the life of KRas-mutated mice, which indicates that EGCG can delay the process of tumorigenesis after somatic cancer-related mutation occur and benefit the cancer patients. To our knowledge, our study is the first to address the effect of EGCG on the tumorigenesis after somatic mutation happens.

In summary, in the present study we revealed that Notch signaling pathway was activated in the exophytic tumoral lesions on their lips and tongue of Kras-mutated transgenic mice, and EGCG treatment delayed the time of appearance of lesions, decreased the size and weight of these lesions, relieved heterotypic hyperplasia in the tumor lesions, and importantly, prolonged the life of these transgenic mice via suppression of Notch signaling pathway. Furthermore, EGCG significantly induced cell apoptosis and inhibited the proliferation of tongue cancer cells by blocking the activation of Notch signaling pathway, and modulation the expression level of Notch1 changed the sensitivity of tongue cancer cells toward EGCG. Based on the results of this study, we believe that EGCG should be further explored as a chemotherapeutic agent for tongue cancer patients that present with a significant up-regulation of the Notch signaling pathway.

Ethics approval

This study was approved by the Institutional Review Board of Hospital of Stomatology, Fujian Medical University, and was carried out in accordance with the guidelines for the care and use of human specimens and animals, including in the approved protocol.

Authors' contributions

Concept and design: Y.G.L. and D.L.Z. Experiments and procedures: H.W., Q.G., J.X., R.H.G., and Y.L.Z. Data analysis: H.W., Q.G., J.X., R.H.G., Y.L.Z., Y.G.L., and D.L.Z. Writing and editing the article: H.W., Y.G.L., and D.L.Z. All authors read and approved the final manuscript. All authors have made a sufficient contribution to the work.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declaration of competing interests

The authors declare no potential competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108843.

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