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N-acetylcysteine (NAC) inhibits cell growth by mediating the EGFR/Akt/HMG box-containing protein 1 (HBP1) signaling pathway in invasive oral cancer

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SUMMARY

Objectives: Overexpression of the epidermal growth factor (EGF) receptor (EGFR) gene in the squamous cell carcinomas of the head and neck (SCCHN) is often associated with inauspicious prognosis and poor survival. N-acetylcysteine (NAC), a compound from some vegetables and allium species, appears anti-tumorigenesis, but the underlying mechanism is unclear. The objective of this study is to investigate the role of NAC in EGFR-overexpressing oral cancer.

Materials and methods: Both HSC-3 and SCC-4 human tongue squamous carcinoma cell lines and an HSC-3 xenograft mouse model were used to test the anti-growth efficacy of NAC in vitro and in vivo, respectively.

Results: NAC treatment suppressed cell growth, with concomitantly increased expression of HMG box-containing protein 1 (HBP1), a transcription suppressor, and decreased EGFR/Akt activation, in EGFR-overexpressing HSC-3 oral cancer cells. HBP1 knockdown attenuated the growth arrest and apoptosis induced by NAC. Lastly, NAC and AG1478, an EGFR inhibitor, additively suppressed colony formation in HSC-3 cells.

Conclusion: Taken together, our data indicate that NAC exerts its growth-inhibitory function through modulating EGFR/Akt signaling and HBP1 expression in EGFR-overexpressing oral cancer.

Introduction

N-acetylcysteine (NAC), one of the hydrophilic organosulfur compounds found in allium plants, asparagus, and red pepper,[1,2] provides cysteine for the synthesis of glutathione, a major intracellular antioxidant, which is thereby available as a nutritional supplement. Clinically, NAC has been used as an antidote for acetaminophen hepatotoxicity, a chelating agent for heavy metal poisoning, and a mucolytic drug in the treatment of chronic bronchitis.[3,4] Moreover, NAC has been widely investigated for its anti-cancer efficacy in complementary therapy. The effectiveness of NAC is believed to depend on the nucleophilicity and antioxidant properties.[5,6] Molecular targets of NAC identified thus far, include cyclin D1, IκB, MMP2/9, and angiostatin,[7–10] involved in the regulation of cell cycle progression, differentiation, migration, and angiogenesis. Specifically, NAC has been shown to suppress epidermal growth factor (EGF)-induced EGF receptor (EGFR) phosphorylation,[11] suggesting a potential tumor-suppressive effect on cancers associated with aberrant EGFR activation.

More than 80% of the invasive squamous cell carcinoma of the head and neck (SCCHN) cases overexpress EGFR,[12] where an excess of EGFR is often linked to unfavorable clinical outcome – high recurrence and low survival rates.[13,14] Activated EGFR signaling is often associated with the malignant phenotype, manifested by angiogenesis, inhibition of apoptosis, and increased metastatic potential.[15] Therefore, clinical application of the EGFR-targeting drugs, such as monoclonal antibodies raised against EGFR (e.g. cetuximab) and small molecule tyrosine kinase inhibitors (e.g. erlotinib and gefitinib), has attracted tremendous attention.[16–18] However, developing resistance to the EGFR inhibitors and mutations of the EGFR downstream effectors are often observed.[19] Therefore, to improve therapeutic efficacy, new additions to the existing regimen that target EGFR and/or its closely-related signaling molecules should be considered accordingly.

Abbreviations: ALL, acute lymphoblastic leukemia; DAPI, 4′,6′-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HBP1, HMG box-containing protein 1; ip, intraperitoneal; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription polymerase chain reaction; SCCHN, squamous cell carcinoma of the head and neck.

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HMG box-containing protein 1 (HBP1) is a regulator of cell cycle exit and cell differentiation.[20] HBP1 exerts its repression function through an HMG box DNA-binding domain and an AXH transcriptional repression domain. Genes down-regulated by HBP1 include p47phox, N-myc, c-myc, cyclin D1, and MIF.[21–24] Overexpression of HBP1 leads to an inhibition of the G1-S phase transition.[21,22,25] Loss-of-function HBP1 variants have been isolated in myeloid leukemia and breast cancers.[26,27] Conversely, ectopic overexpression of HBP1 results in growth arrest, apoptosis, or differentiation in various cancer cell lines.[24,28,29] Together, these findings indicate a role of HBP1 in tumor suppression.

This study was to investigate the role of NAC in EGFR-overexpressing oral cancer. We found a novel role of HBP1 in oral cancer such that NAC exerts its anti-growth effect and apoptosis-inducing property by mediating the EGFR/Akt/HBP1 axis. Our data suggest HBP1 as a diagnostic and prognostic marker and NAC as an anti-cancer adjuvant in EGFR-overexpressing oral cancer.

Materials and methods

Reagents and antibodies

All chemicals were purchased from Sigma (St. Louis, MO) and antibodies were from Cell Signaling Technology (Beverly, MA), respectively, unless specified otherwise. PVDF membrane and ECL detection reagents were from Perkin Elmer Life Sciences, Inc. (Waltham, MA). The annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Jose, CA). Dual-light® system was from Applied Biosystems (Foster City, CA). RNase was purchased from Amresco Inc. (Solon, OH); 4′,6′-diamidino-2-phenylindole (DAPI) was from Southern Biotech (Birmingham, AL). Antibodies for HBP1 and cyclin D1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin was from Abcam (Cambridge, MA).

Cell culture and treatment

All cell culture reagents were from Invitrogen (Carlsbad, CA), unless indicated otherwise. HSC-3 human oral squamous carcinoma and HGF human gingival fibroblast cells were kind gifts of Dr. Hsin-Ling Yang at China Medical University (Taichung, Taiwan). SCC-4 human tongue cancer cells were purchased from ATCC (Manassas, Virginia). HSC-3 cells and HGF and SCC-4 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic. All cells were cultured with 5% CO_2 at 37 °C. NAC stock solution of 100 mM in PBS, pH 7.0, was prepared and diluted right before use. Cell viability was measured by trypan blue method.

Establishment of stable HBP1-knockdown cell lines

293T cells were transfected with HBP1 shRNA plasmids (pLKO-HBP1#76, Sigma) and an optimized mixture of three packaging plasmids, PLP1, PLP2, and VSV-G, to produce lentivirus. The pLKO empty vector was used as a control. Briefly, the day following transfection, medium was changed, then collected and filtered through a 0.45-μm filter to condense virus. After addition of 8 g/mL polybrene, the viral supernatant was used for infection. After 5 h of infection, the HSC-3 cells were allowed to recover with fresh medium for 24 h. The infected HSC-3 cells were selected with 0.5 μg/mL puromycin, and the HBP1 expression was assessed by RT-PCR and Western blotting.

Reverse transcription-polymerase chain reaction

Cellular RNA was extracted using RNeasy® Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RT-PCR was performed using SuperScript™ III One-Step RT-PCR System

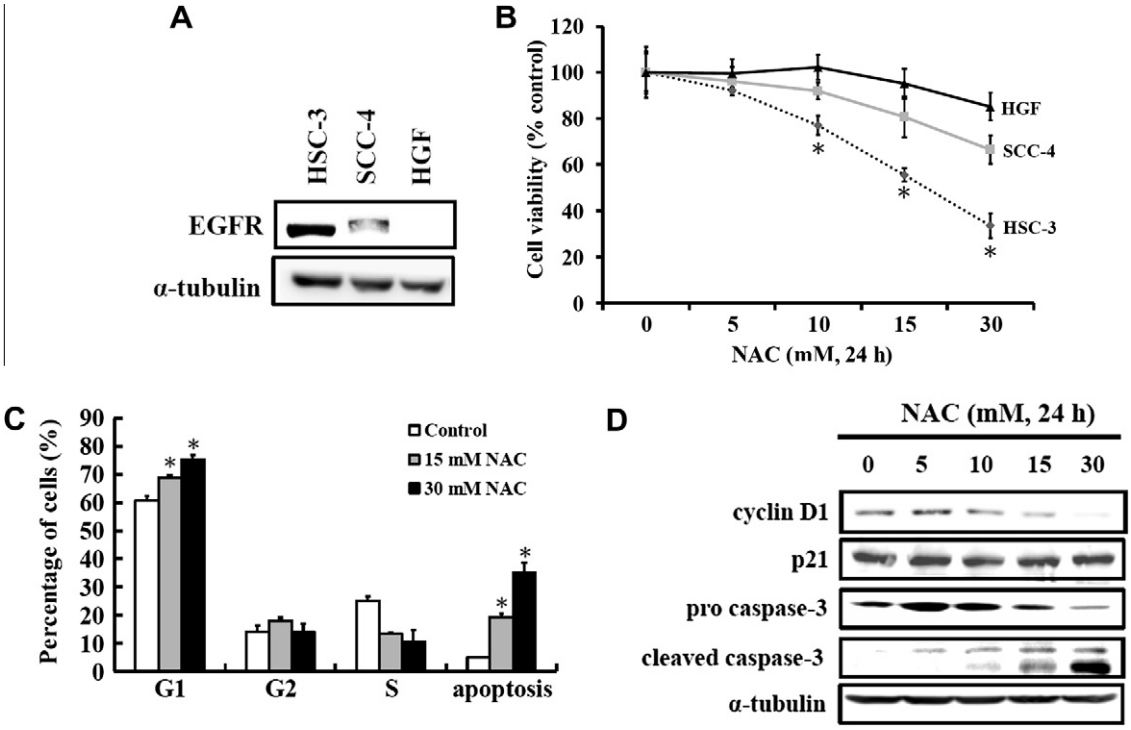

Figure 1 Effect of NAC on cell growth. (A) The endogenous levels of EGFR were detected by Western blotting, where α-tubulin served as a loading control. (B) SCC-4 and HSC-3 cells were treated with NAC for 24 h, where the treatment of HGF cells served as a control (*, $p < 0.05$ as compared with control). (C) HSC-3 cells were treated with NAC for 24 h and then subjected to flow cytometry (*, $p < 0.05$ as compared with control, 0 mM). (D) Expression levels of cyclin D1, p21, and caspase-3 were determined by Western blotting.