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Epigallocatechin-3-gallate attenuates head and neck cancer stem cell traits through suppression of Notch pathway

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Abstract Most solid cancers including head and neck squamous carcinoma (HNSC) are believed to be initiated from and maintained by cancer stem cells (CSCs) that are responsible for treatment resistance, resulting in tumour relapse. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, can potently inhibit cancer growth and induce apoptosis in various cancers, including HNSC. However, its effect on HNSC CSCs is not well elucidated. In this study, we examined the anti-tumour effect of EGCG on HNSC CSCs. We demonstrated that EGCG inhibits the self-renewal capacity of HNSC CSCs by suppressing their sphere forming capacity, and attenuates the expression of stem cell markers, such as Oct4, Sox2, Nanog and CD44. EGCG treatment augmented cisplatin-mediated chemosensitivity by suppressing ABCC2 and ABCG2 transporter genes, which are putative molecules of treatment resistance of CSC. In addition, the combination treatment of EGCG and cisplatin inhibited tumour formation and induced apoptosis in a xenograft model. As one of mechanisms of suppression of HNSC CSC traits, EGCG decreased the transcriptional level of Notch, resulting in the inhibition of Notch signalling. Collectively, our data suggest that EGCG in combination with cisplatin can be used for the management of HNSC CSCs.

1. Introduction

Head and neck squamous cell carcinoma (HNSC) is the sixth most common cancer worldwide, with an annual incidence of more than 500,000 cases.[1] Despite recent advances in the understanding of HNSC progression

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and the development of novel therapeutic targets, HNSC is still a major cause of morbidity and mortality worldwide. Five-year survival rates for HNSC have not improved in more than three decades.[2] Alleviation of HNSC recurrence and mortality requires greater understanding of the biologic behavior and pathologic progression of HNSC.

The cancer stem cell (CSC) hypothesis states that a subpopulation of intratumoural cells is uniquely capable of propagating the tumour, and relies on the hierarchical model to explain tumour heterogeneity and behavior.[3,4] Accumulating evidences have demonstrated that a variety of human malignancies, including HNSC, contain subpopulations of cells that exhibit stem cell-like properties, such as self-renewal and tumour-initiating capabilities.[5,6] It has been suggested that conventional chemotherapies kill differentiated or differentiating cells. CSCs proliferating more slowly appear to be relatively drug resistant and so can be spared, ultimately inducing tumour recurrence after the completion of treatment.[7] Thus, removal of CSCs becomes more and more crucial to chemotherapy and drugs that selectively target CSCs offer a greater promise for cancer treatment.

Notch signalling is activated when Notch ligands including Delta-1, -3, -4 and Jagged-1 and -2 interacts with a Notch transmembrane receptor.[8] This process usually initiates the γ-secretase mediated proteolytic release of the Notch intracellular domain (NICD) and activated NICD migrates to the nucleus.[9] The NICD in the nucleus binds to a transcription factor CSL (CBF1/RBPjk in vertebrates, suppressor of hairless in Drosophila and LAG superfamily in *Caenorhabditis elegans*) and triggers the expression of Notch target genes such as hairy and enhancer of split (HES), which activates stem cell self-renewal.[10,11] In addition, the critical role of Notch signalling in CSCs was demonstrated in oesophageal cancers and breast cancer.[12,13] Furthermore, Notch gene mutations have important roles in the carcinogenesis of HNSC.[14] Thus, targeting the Notch signalling pathway seems to be a novel therapeutic approach of HNSC CSC.

Green tea is one of the most popular beverages in the world, and receives considerable attention because it has many beneficial effects on human health. It contains many catechins such as epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC).[15] EGCG, a major polyphenolic constituent of green tea, potently inhibits cancer growth and induces apoptosis in various cancers by several mechanisms.[16–18] Some recent studies have demonstrated that EGCG induces apoptosis via ATM/p53-dependent NAG-1 expression in HNSC[19] and inhibits HGF-induced tumour growth and invasion in oral and hypopharynx cancer, a subsite of the head and neck area.[20,21] However, the intracellular mechanisms by which EGCG inhibits growth and induces apoptosis in HNSC CSCs have never been examined.

The main objectives of this study were to examine the anti-tumour effects of EGCG on HNSC CSCs and to elucidate its underlying mechanism through the suppression of the Notch signalling pathway.

2. Materials and methods

2.1. Cell lines

HNSC CSCs (K3, K4 and K5) were isolated and characterised from a surgical specimen of a HNSC patient who had provided informed consent, and the CSC phenotype was confirmed by functional assays of self-renewal, stem cell marker expression, chemoresistance and *in vivo* tumour propagation, as previously reported.[6] HNSC CSCs were expanded in serum-free Dulbecco's Modified Eagle Medium (DMEM)/F12 medium supplemented with B27, N2 supplement, 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; 20 ng/ml, R&D systems, Minneapolis, MN, United States of America (USA)), and epidermal growth factor (EGF; 20 ng/ml, R&D systems).

2.2. Sphere forming assay

To assess self-renewal *in vitro*, cells were dissociated into single cells, seeded in a 24-well plate at a density of 200 cells/well and cultured in serum-free media with EGF and bFGF supplementation every other day. Spheres with a diameter exceeding 10 μm were counted after 14 days of incubation.

2.3. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from HNSC CSCs homogenised in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was prepared using a reverse transcriptase kit (Fermentes) according to the manufacturer's instructions. The synthesised cDNA was added to a mixture of 1 U of Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA) and the specific primers, and amplified using the MJ Research Minicycler™ (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were separated by electrophoresis in 1.5% agarose gels and were detected under ultraviolet light (Bio-Rad Laboratories). The sequences of human specific primers used were as follows:

2.4. Transfection of small interfering RNA (siRNA)

Cells were seeded in 6 well plates and cultured overnight in a 5% CO_2 atmosphere at 37 °C. Then the medium was replaced with Opti-MEM containing ABCC2,