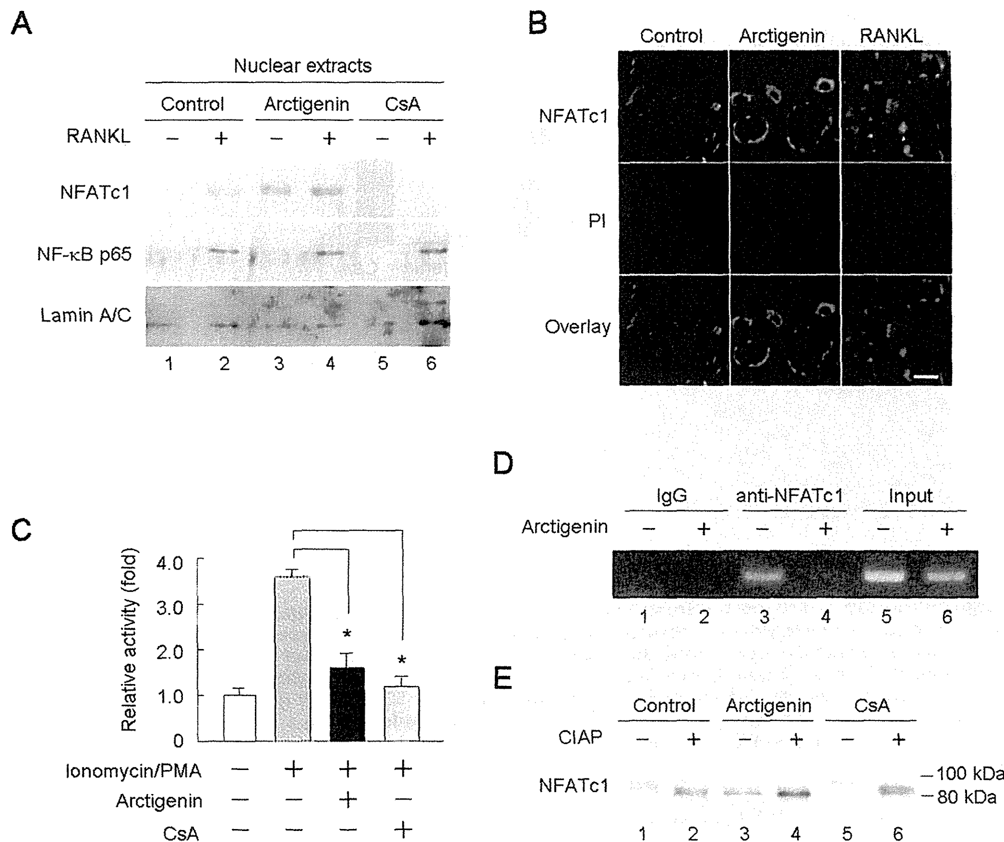

Figure 3. Effects of arctigenin on the nuclear translocation and activation of NFATc1. (A) Effects of arctigenin and CsA on the nuclear translocation of NFATc1 and NF-κB in osteoclast-like cells. Purified osteoclast-like cells were cultured for 16 h in the absence of RANKL. Osteoclast-like cells were then pre-treated for 20 min with or without 1 μM arctigenin or 1 μg/mL CsA, and then further incubated for 20 min with or without RANKL. Nuclear fractions were analyzed by Western blotting with the antibodies indicated. (B) Immunohistochemical detection of NFATc1 in osteoclast-like cells. Purified osteoclast-like cells were cultured on glass plates for 16 h in the absence of RANKL. Cells were then treated for 20 min with or without 1 μM arctigenin or 100 ng/mL RANKL. Cells were fixed and stained for NFATc1 (green). Nuclei were counterstained with propidium iodide (PI, red). Bar = 50 μm. (C) Effects of arctigenin and CsA on NFAT reporter activity. BMMs were transfected with the NFAT-luciferase construct. Forty-eight hours later, cells were incubated for an additional 24 h with or without ionomycin and PMA. Some cultures were treated with 1 μM arctigenin or 1 μg/mL CsA. The fluorescence of the lysates was measured by a luminometer. The results were expressed as means +/− SD (n = 3). *, $p<0.05$. (D) ChIP assay on the *Oscar* promoter. BMMs were cultured for 2 days in the presence of RANKL and M-CSF. Cells were then treated for 1 h with or without 1 μM arctigenin. Chromatin complexes were immunoprecipitated with the anti-NFATc1 antibody or control IgG. Chromatin DNA fragments were subjected to PCR for the *Oscar* promoter. (E) Western blotting of NFATc1 in osteoclast-like cells. Purified osteoclast-like cells were treated for 10 min with or without 1 μM arctigenin or 1 μg/mL CsA. Cell lysates were then collected. Half of the lysates were treated for 30 min with calf intestine alkaline phosphatase (CIAP). Samples were analyzed by Western blotting with the anti-NFATc1 antibody.
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NFATc1 is not involved in the inhibitory effect of arctigenin on osteoclastogenesis.

Effect of arctigenin on osteoclastogenesis induced by the osteoblastic cell-dependent NFATc1 pathway

NFATc1 has also been shown to be activated by the osteoblastic cell-dependent pathway [24,25]. We compared the effect of arctigenin on 1α,25$(OH)_2D_3$-induced osteoclast-like cell formation in co-cultures of osteoblastic cells and bone marrow cells with that of CsA (Fig. 5A). Arctigenin strongly inhibited osteoclast-like cell formation in the co-culture, whereas CsA did not abrogate osteoclastogenesis supported by osteoblastic cells. 1α,25$(OH)_2D_3$ increased *Rankl* expression and decreased *Opg* expression in osteoblastic cells, both of which were not affected by arctigenin (Fig. 5B). The appearance of alkaline phosphatase-positive osteoblastic cells in osteoblastic cell cultures was not affected by arctigenin (Fig. 5C). Arctigenin had no effect on the proliferation of osteoblastic cells at the concentrations examined (Fig. 5D). These results suggest that arctigenin directly acted on precursors of osteoclast-like cells in the co-cultures, and inhibited their differentiation into osteoclast-like cells by suppressing the osteoblastic cell-dependent NFATc1 pathway.

Effect of arctigenin on the pit-forming activity of osteoclast-like cells

There is little conclusive evidence to show that NFATc1 plays an essential role in osteoclast function because an NFATc1 deficiency induces osteopetrosis without osteoclasts. We examined the effect of arctigenin on the pit-forming activity of osteoclast-like cells (Fig. 6). Osteoclast-like cell preparations obtained from co-cultures in collagen gel-coated plates were placed on dentin slices and further cultured for 48 h in the presence of increasing

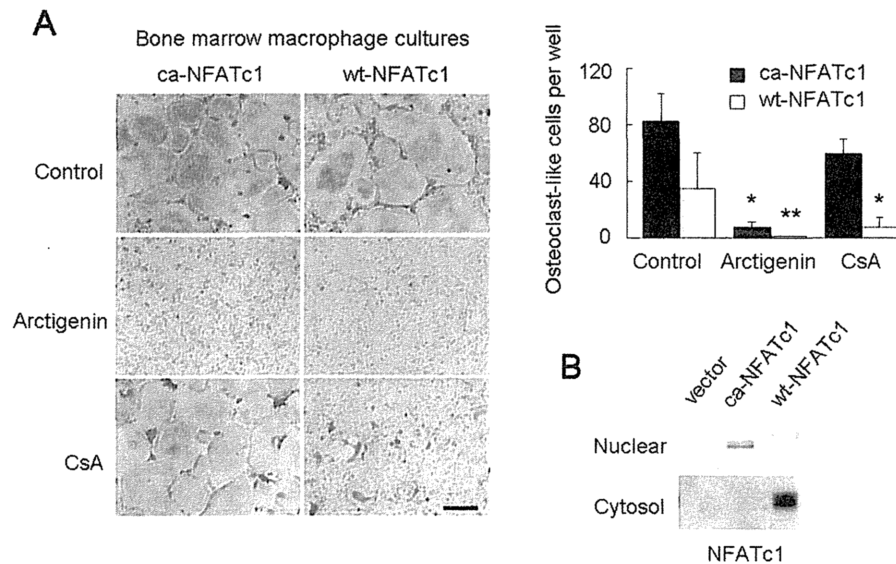

Figure 4. Effect of arctigenin on osteoclast-like cell formation induced by the forced expression of NFATc1. (A) Osteoclast-like cell formation in BMM cultures. BMMs were retrovirally transduced with constitutively active (ca)-NFATc1, wild-type (wt)-NFATc1, or control GFP genes, and cultured for 2 days in the presence of M-CSF. Cells were further cultured in the presence of RANKL and M-CSF together with or without 1 μM arctigenin or 1 μg/mL CsA. After cultivation for 3 days, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclast-like cells. The results were expressed as means +/− SD (n = 4). *, p<0.05. **, p<0.01. Bar = 50 μm. (B) Subcellular distribution of NFATc1. Nuclear and cytosol fractions were prepared from BMMs transfected with or without ca-NFATc1 and wt-NFATc1 and analyzed by Western blotting with the anti-NFATc1 antibody.
doi:10.1371/journal.pone.0085878.g004

concentrations of arctigenin. The number of TRAP-positive cells on dentin slices after cultivation for 48 h was not significantly changed by the treatment with arctigenin (Fig. 6A), which suggested that the survival of osteoclast-like cells supported by osteoblastic cells was not affected by arctigenin. Cells were then removed, and dentin slices were stained with Mayer's hematoxylin (Fig. 6B). Many resorption pits (purple areas) were formed on the slices in control cultures. The pit-forming activity of osteoclast-like cells was inhibited by arctigenin in a dose-dependent manner. Osteoclast-like cells on bone form actin rings (corresponding to sealing zones) to resorb bone. We examined the effect of arctigenin on actin ring formation by osteoclast-like cells. Osteoclast-like cells cultured on dentin slices formed actin rings even in the presence of arctigenin (Fig. 6C). Thus, arctigenin suppressed the bone-resorbing activity of osteoclast-like cells without affecting their survival or cytoskeletal structures.

Discussion

We showed that arctigenin inhibited osteoclast-like cell differentiation and function in a dose-dependent manner. Both the differentiation and function of osteoclast-like cells were almost completely inhibited by 1 μM arctigenin. Arctiin also inhibited osteoclast-like cell differentiation, but a higher concentration was needed to achieve the same effect as that of arctigenin. The chemical structure of arctigenin is similar to that of arctiin, a glucoside of arctigenin, but not to secoisolariciresinol. These results suggest that the target molecule of arctigenin and arctiin is the same in BMMs. Arctigenin has been shown to affect various biological reactions *in vitro*. The minimum effective concentration of arctigenin in most reactions was reported to be much higher than 1 μM [4,39–42]. The differentiation of BMMs into dendritic cells was not affected by arctigenin. These results suggest that the target molecule of arctigenin is very specific for osteoclast lineage cells.

Kim *et al.* previously reported that arctigenin suppressed activation of NF-κB, phosphorylation of ERK, and amplification of NFATc1 in BMMs [5]. However, significant suppression of NF-κB and ERK signals by arctigenin was not observed in our cultures. Such a difference may be due to the different experimental conditions employed. The arctigenin-induced suppression of NFATc1 amplification was similarly observed in both Kim *et al.*'s and our experiments. Several lines of evidence in our experiments have indicated that the target molecule of arctigenin was NFATc1 in BMMs and osteoclast-like cells. First, arctigenin specifically suppressed the RANKL-induced up-regulation of NFATc1 in BMMs. Arctigenin had no inhibitory effect on the RANKL-induced activation of NF-κB and MAP kinases or the expression of *c-Fos*. Second, arctigenin as well as CsA could suppress the osteoclastic differentiation of BMMs even when added at the late stage of the culture. BMMs in the late stage of cultures are committed osteoclast precursors, which already express high levels of NFATc1. Third, similar to CsA, arctigenin suppressed the transcription activity of NFATc1 in a luciferase reporter assay. However, the reappearance of IκBα in BMMs treated with RANKL was not affected by arctigenin (Fig. 2B). This suggests that arctigenin does not inhibit the transcriptional activity of NF-κB. Fourth, arctigenin inhibited the RANKL-induced transcriptional activity of NFATc1 on the *Oscar* promoter. Fifth, arctigenin inhibited osteoclast-like cell formation in co-cultures with osteoblastic cells, in which osteoblastic cell-dependent NFATc1 signaling had been activated. In addition, arctigenin completely suppressed the pit-forming activity of mature osteoclast-like cells. Interestingly, these effects of arctigenin were observed at a concentration as low as 1 μM. Taken together, these results suggest that the target molecule of arctigenin is the same in BMMs and mature osteoclast-like cells, and NFATc1 is the most possible target of arctigenin in osteoclast-lineage cells (Fig. 6D).

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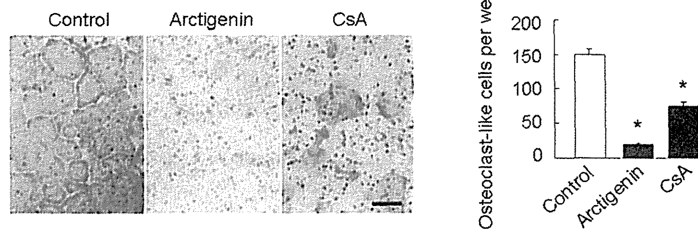

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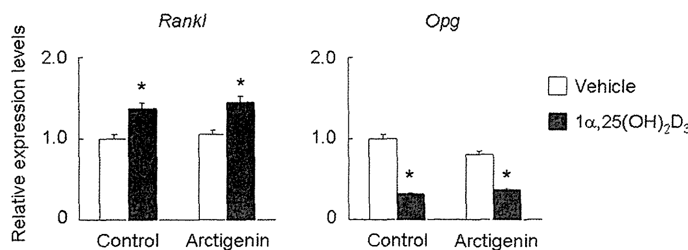

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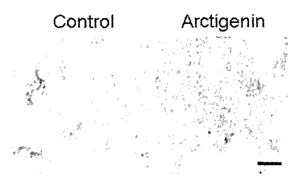

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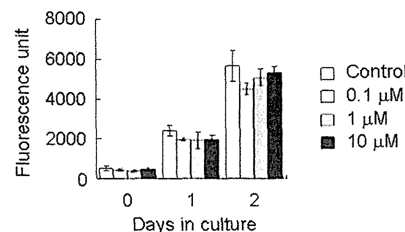

Figure 5. Effect of arctigenin on osteoclast-like cell formation in the co-culture with osteoblastic cells. (A) Effects of arctigenin and CsA on osteoclast-like cell formation in the co-culture. Osteoblastic cells and bone marrow cells were co-cultured in the presence of $1\alpha,25(OH)_2D_3$ together with or without 1 μM arctigenin or 1 μg/mL CsA. After cultivation for 6 days, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclast-like cells. Bar = 50 μm. (B) Effects of arctigenin on RANKL and OPG expression in osteoblastic cells. Osteoblastic cells were cultured for 24 h in the presence or absence of 10 nM $1\alpha,25(OH)_2D_3$ together with or without 1 μM arctigenin. The expression of *Rankl* and *Opg* mRNAs was analyzed by quantitative RT-PCR. The expression level was normalized to *Gapdh* and expressed relative to the vehicle control. (C) Alkaline phosphatase staining of osteoblastic cell cultures. Osteoblastic cells were cultured for 6 days in the presence or absence of 1 μM arctigenin. Cells were then fixed and stained for alkaline phosphatase. Alkaline phosphatase-positive cells appeared as blue cells. Bar = 50 μm. (D) Effects of arctigenin on the proliferation of osteoblastic cells. Osteoblastic cells were cultured with increasing concentrations of arctigenin. Cell proliferation was evaluated on days 0, 1, and 2 using an Alamar Blue assay. The results were expressed as means +/− SD (n = 3). $p<0.05$.
doi:10.1371/journal.pone.0085878.g005

How does arctigenin inhibit the action of NFATc1 in BMMs and mature osteoclasts? Arctigenin induced the nuclear translocation of NFATc1, but not that of NF-κB in osteoclast-like cells in the absence of RANKL. The molecular weight of NFATc1 in osteoclast-like cells was reduced to approximately 85 kDa by the treatment with arctigenin, but not with CsA, which indicated that arctigenin-induced modification of NFATc1. These results suggested that arctigenin-treated NFATc1 acted as the dominant-negative form of NFATc1. In support of this hypothesis, the forced expression of a ca-NFATc1 mutant failed to rescue arctigenin-inhibited osteoclastogenesis. The amount of force-expressed ca-NFATc1 was much higher than that of endogenous NFATc1 (Fig. 4B). This finding may also indicate that arctigenin inactivate even the ca-NFATc1 mutant. In addition, arctigenin, but not CsA strongly inhibited osteoclast-like cell formation in co-cultures with osteoblastic cells. These results suggest that a dominant-negative form of NFATc1 processed by arctigenin can suppress both calcineurin-dependent and osteoblastic cell-dependent NFATc1 signals.

Arctigenin induced the nuclear localization of 85 kDa NFATc1 in osteoclast-like cells even in the absence of RANKL. In order to determine the mechanism of the production and nuclear localization of 85 kDa NFATc1, we performed two additional experiments. In one experiment, hemagglutinin (HA)-tagged ca-NFATc1 (HA-ca-NFATc1) was expressed in BMMs, which were treated with or without arctigenin. HA-ca-NFATc1 protein was similarly detected as an 85 KDa protein in BMMs even in the presence of arctigenin (Fig. S4). These results suggest that arctigenin treatment does not cause the fragmentation of NFATc1. NFATc1 also shifted to approximately 85 kDa after treatment

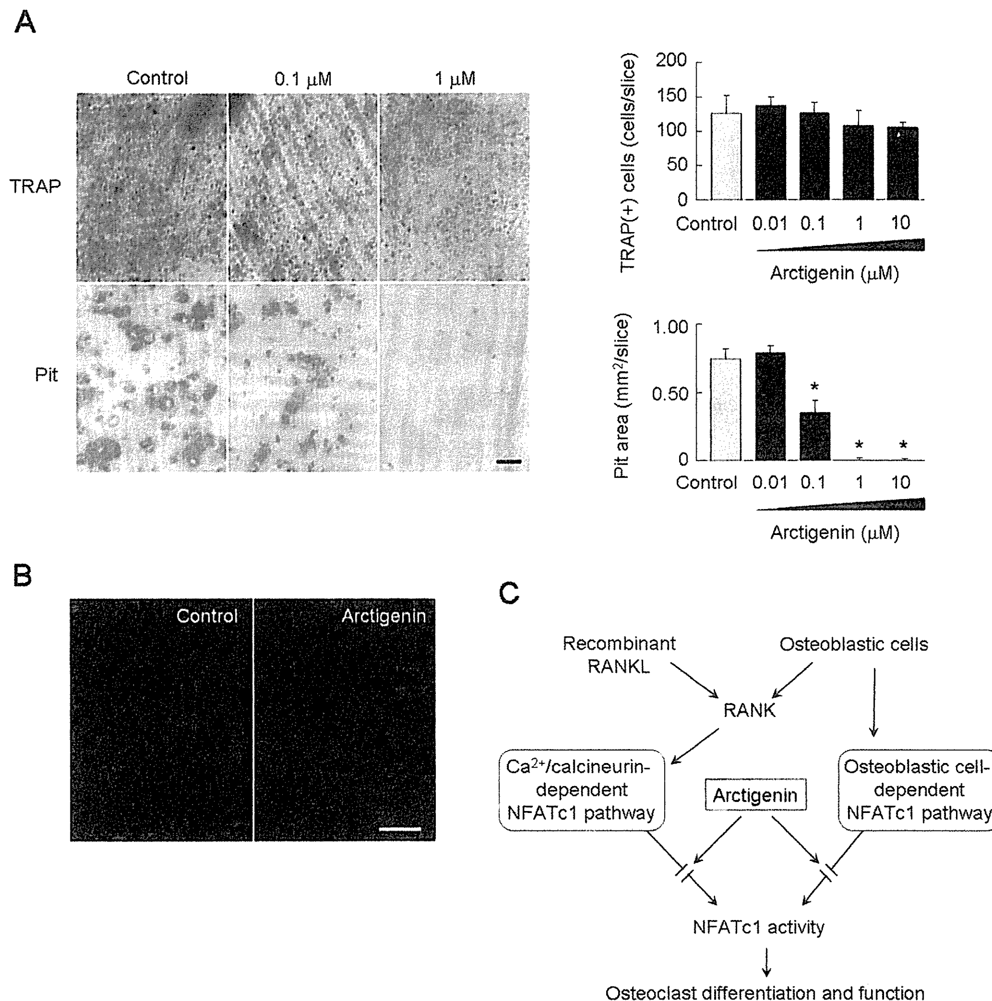

Figure 6. Effect of arctigenin on osteoclast-like cell function. (A) Effects of arctigenin on the pit-forming activity of osteoclast-like cells cultured on dentin slices. Osteoclast-like cell preparations were cultured on dentin slices in the presence or absence of 1 μM arctigenin. After cultivation for 48 h, cells on the slices were fixed and stained for TRAP. TRAP-positive cells were counted as osteoclast-like cells. Cells were then removed from dentin slices, and slices were stained with Mayer's hematoxylin to visualize resorption pits. Resorption pit areas were measured using ImageJ software. The results were expressed as means +/− SD (n = 6). *, $p<0.01$. Bar = 50 μm. (B) Effect of arctigenin on actin ring formation by osteoclast-like cells. Osteoclast-like cell preparations were cultured on dentin slices in the presence or absence of 1 μM arctigenin. After cultivation for 24 h, cells on the slices were fixed and stained with Rhodamine-phalloidin. Bar = 50 μm. (C) A hypothetical model for the action of arctigenin on the differentiation and function of osteoclasts. Arctigenin inhibited RANKL-induced osteoclast-like cell formation in BMM cultures, in which the Ca^{2+}/calcineurin-dependent NFATc1 pathway was activated. Arctigenin also inhibited osteoclast-like cell formation in co-cultures with osteoblastic cells, in which the osteoblastic cell-dependent NFATc1 pathway was activated. The pit-forming activity of osteoclast-like cells was also inhibited by arctigenin. Arctigenin induced a lower molecular weight species of NFATc1, which may act as the dominant negative inhibitor of NFATc1.
doi:10.1371/journal.pone.0085878.g006

with CIAP (Fig. 3E). Therefore, it is possible that arctigenin enhances dephosphorylation of NFATc1. We then examined effects of phosphatase inhibitors such as 3,4-dephostatin [protein tyrosine phosphatase (PTP) inhibitor], NSC87877 [SH2 domain-containing inositol phosphatase (SHIP) 1/2 and PTP1B inhibitor], okadaic acid (protein phosphatase 2A inhibitor), and sodium stibogluconate (SHIP1 inhibitor) on the arctigenin-induced conversion of NFATc1. However, those phosphatase inhibitors failed to suppress the arctigenin-induced production of 85 kDa NFATc1 (Fig. S5). Thus, the mechanism of the arctigenin-induced production and nuclear localization of 85 kDa NFATc1 and the structure-function relationship of NFATc1 are still unknown. The mass spectrometry analysis on the NFATc1 protein to determine the phosphorylation status will elucidate the mechanism of arctigenin-induced modifications of NFATc1.

Interleukin 2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were reported to be the target genes of NFATc1 in T cells [35,36,43]. Our preliminary experiments showed that neither the expression of IL-2 nor that of GM-CSF in splenic T cells was inhibited by arctigenin, while CsA suppressed the expression of both genes (Fig. S6). Unlike osteoclasts, T cells have been shown to express NFATc2 and NFATc3 as well as NFATc1 [35,44]. Therefore, other NFAT family members may compensate for the loss of NFATc1 activity in T cells treated with arctigenin. The differentiation of BMMs into osteoclasts is shown to be tightly regulated by NFATc1. These results suggest that the

inhibitory action of arctigenin on NFATc1 is specific for osteoclast lineage cells. The calcinurin-dependent activation of NFATc1 was shown to be necessary for the survival of thymocytes treated with IL-7 [38]. Treatment of thymocytes with IL-7 induced the phosphorylation of Tyr-371 in NFATc1, and the expression of Y371F-NFATc1 enhanced the survival of thymocytes. However, the expression of Y371F-NFATc1 in BMMs failed to rescue arctigenin-induced inhibition of osteoclastogenesis (Fig. S3). These results suggest that the action of arctigenin on NFATc1 signals is regulated differently in osteoclast lineage cells and thymocytes.

Although NFATc1 signaling was previously proposed to be involved in the bone-resorbing activity of osteoclasts, conclusive evidence has not yet confirmed this. The genetic deletion of NFATc1 in the osteoclast lineage cells resulted in a deficiency in osteoclasts [15]. Therefore, difficulties are associated with investigating the precise role of NFATc1 in the bone-resorbing activity of osteoclasts. Komarova et al. demonstrated that local acidification enhanced the bone-resorbing activity of osteoclasts through the Ca^{2+}/calcineurin-dependent NFATc1 pathway [45]. Treating osteoclast-like cells with RANKL induced the nuclear translocation of NFATc1 and their pit-forming activity. These results suggest that NFATc1 is also involved in the activation of osteoclast-like cells. We showed that arctigenin inhibited the pit-forming activity of osteoclast-like cells cultured on dentin slices. This inhibitory effect of arctigenin was observed at a concentration as low as 1 μM. Interestingly, arctigenin showed no inhibitory effects on actin ring formation or the survival of osteoclast-like cells. Calcitonin and bisphosphonates, well-known inhibitors of osteoclast function, were shown to disrupt actin rings in osteoclast-like cells [7]. These results suggest that NFATc1 signals are involved in the bone-resorbing activity of osteoclasts without affecting the survival or cytoskeletal structure of osteoclasts (Fig. 6D).

In conclusion, arctigenin inhibited osteoclast-like cell formation in BMM cultures and co-cultures with osteoblastic cells by suppressing NFATc1 signals. Arctigenin appeared to induce the dominant negative species of NFATc1. Thus, arctigenin promises to be a useful agent for investigating the role of NFATc1 in osteoclastic bone resorption. Further experiments will elucidate the molecular mechanism for the inhibitory action of arctigenin on the differentiation and function of osteoclasts.

Supporting Information

Figure S1 Effect of secoisolariciresinol on osteoclast-like cell formation. The chemical structure of secoisolariciresinol was shown. BMMs were cultured in 96-well culture plates in the presence of RANKL and M-CSF together with increasing concentrations of secoisolariciresinol. After cultivation for 3 days, cells were stained for TRAP. TRAP-positive cells appeared as dark red cells. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclast-like cells.
(TIF)

Figure S2 Effect of arctigenin on the differentiation of dendritic cells. BMMs (1.5×10^5 cells) were cultured for 1 week in 60-mm dishes in the presence of 20 ng/mL GM-CSF with or without 1 μM arctigenin. Cells were analyzed for the expression of CD11c and CD86 by fluorescence-activated cell scanning. The numbers in the top right corners indicate the percentages of CD11c/CD86 double positive cells as differentiated dendritic cells. Experiments were performed four times, and representative data are shown.
(TIF)

Figure S3 Effect of arctigenin on osteoclast-like cell formation induced by the forced expression of an Y371F-NFATc1 mutant. BMMs (3×10^4 cells) were retrovirally transduced with Y371F-NFATc1 or control GFP, and cultured for 2 days in the presence of 5000 U/mL M-CSF in 96-well culture plates. Cells were further cultured in the presence of 100 ng/mL RANKL and/or 5000 U/mL M-CSF together with or without 1 μM arctigenin. After cultivation for 3 days, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclast-like cells. The results were expressed as means +/− SD (n = 4).
(TIF)

Figure S4 Effect of arctigenin on processing NFATc1. BMMs (3×10^4 cells) were retrovirally transduced with a hemagglutinin (HA)-tagged ca-NFATc1 cDNA, and cultured for 2 days in the presence of 5000 U/mL M-CSF in 96-well culture plates. Cells were further cultured in the presence of 100 ng/mL RANKL and 5000 U/mL M-CSF together with or without 1 μM arctigenin. After cultivation for 2 days, total cell lysates were analyzed by Western blotting analysis using an anti-HA antibody or an anti-β-actin antibody.
(TIF)

Figure S5 Effect of phosphatase inhibitors on the conversion of lower molecular species of NFATc1 induced by arctigenin. Purified osteoclast-like cells (2000 cells) were cultured in 24-well culture plates in the presence or absence of 10 μM 3,4-dephostatin [protein tyrosine phosphatase (PTP) inhibitor], 10 μM NSC87877 [SH2 domain-containing inositol phosphatase (SHIP)1/2 and PTP1B inhibitor], 0.5 μM okadaic acid (protein phosphatase 2A inhibitor), or 100 μM sodium stibogluconate (SHIP1 inhibitor) together with or without 1 μM arctigenin. After cultivation for 10 min, whole cell lysates were harvested and analyzed by Western blotting using an anti-NFATc1 antibody.
(TIF)

Figure S6 Effect of arctigenin on the expression of T cell-related genes. Mouse splenocytes (2×10^6 cells) were activated for 4 h in the presence of 1 μM ionomycin and 20 nM PMA in 24-well culture plates. Cells were further cultured with or without 1 μM arctigenin and 1 μg/mL CsA. After cultivation for 2 h, *IL-2* and *GM-CSF* mRNA levels were analyzed by quantitative RT-PCR. Expression levels were normalized to *Gapdh* and the values were relative to unstimulated controls. The results were expressed as means +/− SD (n = 3). *, $p<0.05$; NS, not significant.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TY NU YK NT. Performed the experiments: TY SU FL YK. Analyzed the data: TY NU YK NT. Contributed reagents/materials/analysis tools: SK HE. Wrote the paper: TY YK NU NT.

References

1. Awale S, Lu J, Kalauni SK, Kurashima Y, Tezuka Y, et al. (2006) Identification of arctigenin as an antitumor agent having the ability to eliminate the tolerance of cancer cells to nutrient starvation. Cancer Res 66: 1751–1757.
2. Kudou N, Taniguchi A, Sugimoto K, Matsuya Y, Kawasaki M, et al. (2012) Synthesis and antitumor evaluation of arctigenin derivatives based on antiausterity strategy. Eur J Med Chem 60C: 76–88.
3. Cho MK, Jang YP, Kim YC, Kim SG (2004) Arctigenin, a phenylpropanoid dibenzylbutyrolactone lignan, inhibits MAP kinases and AP-1 activation via potent MKK inhibition: the role in TNF-alpha inhibition. Int Immunopharmacol 4: 1419–1429.
4. Kou X, Qi S, Dai W, Luo L, Yin Z (2011) Arctigenin inhibits lipopolysaccharide-induced iNOS expression in RAW264.7 cells through suppressing JAK-STAT signal pathway. Int Immunopharmacol 11: 1095–1102.
5. Kim AR, Kim HS, Lee JM, Choi JH, Kim SN, et al. (2012) Arctigenin suppresses receptor activator of nuclear factor kappaB ligand (RANKL)-mediated osteoclast differentiation in bone marrow-derived macrophages. Eur J Pharmacol 682: 29–36.
6. Boyle WJ, Simonet WS, Lacey DL (2003) Osteoclast differentiation and activation. Nature 423: 337–342.
7. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, et al. (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20: 345–357.
8. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, et al. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 95: 3597–3602.
9. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, et al. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93: 165–176.
10. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, et al. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397: 315–323.
11. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, et al. (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci U S A 96: 3540–3545.
12. Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, et al. (1993) Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. J Clin Invest 91: 257–263.
13. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, et al. (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89: 309–319.
14. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, et al. (1998) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 139: 1329–1337.
15. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, et al. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3: 889–901.
16. Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, et al. (2004) Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem 279: 45969–45979.
17. Kim Y, Sato K, Asagiri M, Morita I, Soma K, et al. (2005) Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. J Biol Chem 280: 32905–32913.
18. Ishida N, Hayashi K, Hoshijima M, Ogawa T, Koga S, et al. (2002) Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. J Biol Chem 277: 41147–41156.
19. Cella M, Buonsanti C, Strader C, Kondo T, Salmaggi A, et al. (2003) Impaired differentiation of osteoclasts in TREM-2-deficient individuals. J Exp Med 198: 645–651.
20. Paloneva J, Mandelin J, Kiialainen A, Bohling T, Prudlo J, et al. (2003) DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. J Exp Med 198: 669–675.
21. Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, et al. (2003) The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA 101: 6158–6163.
22. Koga T, Inui M, Inoue K, Kim S, Suematsu A, et al. (2004) Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 428: 758–763.
23. Barrow AD, Raynal N, Andersen TL, Slatter DA, Bihan D, et al. (2011) OSCAR is a collagen receptor that costimulates osteoclastogenesis in DAP12-deficient humans and mice. J Clin Invest 121: 3505–3516.
24. Kuroda Y, Hisatsune C, Nakamura T, Matsuo K, Mikoshiba K (2008) Osteoblasts induce Ca2+ oscillation-independent NFATc1 activation during osteoclastogenesis. Proc Natl Acad Sci U S A 105: 8643–8648.
25. Kuroda Y, Hisatsune C, Mizutani A, Ogawa N, Matsuo K, et al. (2012) Cot kinase promotes Ca2+ oscillation/calcineurin-independent osteoclastogenesis by stabilizing NFATc1 protein. Mol Cell Biol 32: 2954–2963.
26. Mizoguchi T, Muto A, Udagawa N, Arai A, Yamashita T, et al. (2009) Identification of cell cycle-arrested quiescent osteoclast precursors in vivo. J Cell Biol 184: 541–554.
27. Takahashi N, Udagawa N, Kobayashi Y, Suda T (2007) Generation of osteoclasts in vitro, and assay of osteoclast activity. Methods Mol Med 135: 285–301.
28. Koide M, Kinugawa S, Ninomiya T, Mizoguchi T, Yamashita T, et al. (2009) Diphenylhydantoin inhibits osteoclast differentiation and function through suppression of NFATc1 signaling. J Bone Miner Res 24: 1469–1480.
29. Ishida N, Hayashi K, Hattori A, Yogo K, Kimura T, et al. (2006) CCR1 acts downstream of NFAT2 in osteoclastogenesis and enhances cell migration. J Bone Miner Res 21: 48–57.
30. Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7: 1063–1066.
31. Asagiri M, Sato K, Usami T, Ochi S, Nishina H, et al. (2005) Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 202: 1261–1269.
32. Jimi E, Akiyama S, Tsurukai T, Okahashi N, Kobayashi K, et al. (1999) Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. J Immunol 163: 434–442.
33. Matsumoto M, Sudo T, Saito T, Osada H, Tsujimoto M (2000) Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). J Biol Chem 275: 31155–31161.
34. Li X, Udagawa N, Itoh K, Suda K, Murase Y, et al. (2002) p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. Endocrinology 143: 3105–3113.
35. Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15: 707–747.
36. Hogan PG, Chen L, Nardone J, Rao A (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17: 2205–2232.
37. Beals CR, Clipstone NA, Ho SN, Crabtree GR (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev 11: 824–834.
38. Patra AK, Avots A, Zahedi RP, Schuler T, Sickmann A, et al. (2013) An alternative NFAT-activation pathway mediated by IL-7 is critical for early thymocyte development. Nat Immunol 14: 127–135.
39. Jeong JB, Hong SC, Jeong HJ, Koo JS (2011) Arctigenin induces cell cycle arrest by blocking the phosphorylation of Rb via the modulation of cell cycle regulatory proteins in human gastric cancer cells. Int Immunopharmacol 11: 1573–1577.
40. Huang SL, Yu RT, Gong J, Feng Y, Dai YL, et al. (2012) Arctigenin, a natural compound, activates AMP-activated protein kinase via inhibition of mitochondria complex I and ameliorates metabolic disorders in ob/ob mice. Diabetologia 55: 1469–1481.
41. Kim Y, Hollenbaugh JA, Kim DH, Kim B (2011) Novel PI3K/Akt inhibitors screened by the cytoprotective function of human immunodeficiency virus type 1 Tat. PLoS One 6: e21781.
42. Tang X, Zhuang J, Chen J, Yu L, Hu L, et al. (2011) Arctigenin efficiently enhanced sedentary mice treadmill endurance. PLoS One 6: e24224.
43. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5: 472–484.
44. Macian F, Garcia-Rodriguez C, Rao A (2000) Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. EMBO J 19: 4783–4795.
45. Komarova SV, Pereverzev A, Shum JW, Sims SM, Dixon SJ (2005) Convergent signaling by acidosis and receptor activator of NF-kappaB ligand (RANKL) on the calcium/calcineurin/NFAT pathway in osteoclasts. Proc Natl Acad Sci U S A 102: 2643–2648.

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Mucosal Defect Repair with a Polyglycolic Acid Sheet

Takeshi Shinozaki*, Ryuichi Hayashi, Mitsuru Ebihara, Masakazu Miyazaki and Toshifumi Tomioka

Department of Head and Neck Surgery, National Cancer Center Hospital East, Kashiwa City, Japan

*For reprints and all correspondence: Takeshi Shinozaki, Division of Head and Neck Surgery National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. E-mail: tashinoz@east.ncc.go.jp

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Objective: Early-stage oral or oropharyngeal carcinomas are often treated with surgical resection. Resulting wounds that are too large for primary closure can be covered with skin grafts or patches made from various biomaterials. Recently, polyglycolic acid sheets have been used for this purpose.
Methods: We treated six patients with large wounds resulting from the resection of oral or oropharyngeal squamous cell carcinoma by grafting polyglycolic acid sheet patches. All patients were initially treated at the National Cancer Center East Hospital from March 2010 through July 2012. After mucosal resection, the wounds were covered with polyglycolic acid sheet patches attached with fibrin glue. Oral intake was started 4 days after surgery.
Results: Mucosal resection was the initial treatment in seven patients (five with oral squamous cell carcinoma and two with oropharyngeal squamous cell carcinoma). The polyglycolic acid sheet patches became detached in two patients (on the day of surgery and on postoperative day 6), who then required large doses of analgesics. A patient who underwent tooth extraction also required large doses of analgesics. The other four patients required only small doses of analgesics. One patient had bleeding at the surgical site. No adverse effects were caused by the polyglycolic acid sheet patch or by fibrin glue.
Conclusions: Our study has shown that grafting of a polyglycolic acid sheet patch is effective and provides good pain control for patients with large, open wounds after mucosal resection of oral or oropharyngeal squamous cell carcinoma. We plan to evaluate tissue contraction and oral intake after polyglycolic acid patch grafting.

Key words: polyglycolic acid sheet – fibrin glue – good pain control

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OBJECTIVE

Early-stage oral or oropharyngeal carcinomas are often treated with surgical resection. Because the resulting wounds are often too large for primary closure, they can be covered with skin grafts and patches made from various biomaterials. Skin graft leave donor-site wound and often detach within a few days. Some biomaterials often detach in the early postoperative period. Early detachment causes pain and tissue contraction. Recently, polyglycolic acid (PGA) sheets have been used to cover wounds and to prevent bleeding and leakage in liver and lung surgery (1–4). We evaluated the use of PGA sheets to reduce postoperative complications after the resection of oral or oropharyngeal carcinomas.

PATIENTS AND METHODS

We reviewed seven patients with large wounds resulting from the resection of an oral or oropharyngeal squamous cell carcinoma (SCC) which were repaired with PGA sheet grafts. All patients were initially treated at the National Cancer Center East Hospital from March 2010 through July 2012 (Table 1).

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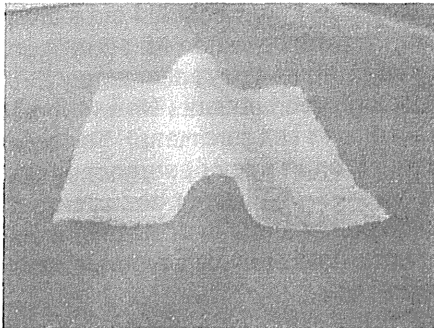
Table 1. Patients' courses and outcomes

Case	Sex	Age	Tumor site	Stage	Resection	Mucosal defect (mm)	Oral feeding start	Analgesic (Loxoprofen) use	Complications	Discharge
1	M	86	Retromolar	T2N0	Mucosa	22 × 42	POD 10	None	None	POD 11
2	M	62	Oral floor	T1N0	Mucosa	34 × 31	POD 7	None	None	POD 13
3	F	64	Buccal mucosa	T2N0	Mucosa	10 × 22	POD 10	None	None	POD 13
4	M	78	Hard palate	T1N0	Mucosa, hard palate	34 × 34	POD 6	POD 2,9	None	POD 12
5	M	62	Soft palate	T1N0	Mucosa	35 × 32	POD 4	Every meal	PGA detached, day of surgery	POD 10
6	F	60	Soft palate	T2N0	Mucosa, hard palate	42 × 26	POD 8	Many times	Bleeding, POD7; PGA detached, POD 10	POD 12
7	M	52	Lower gingiva	T2N0	Mucosa, tooth extraction	40 × 35	POD 4	Many times	None	POD 9

Patients 1–4, without early sheet detachment, required no analgesics or only small doses of analgesics. In contrast, patients 5 and 6, with early sheet detachment, required large doses of analgesics. A patient who underwent tooth extraction also required large doses of analgesics.
POD, postoperative day.



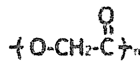


Figure 1. Polyglycolic acid (PGA) sheet. Soft nonwoven fabric with elasticity.

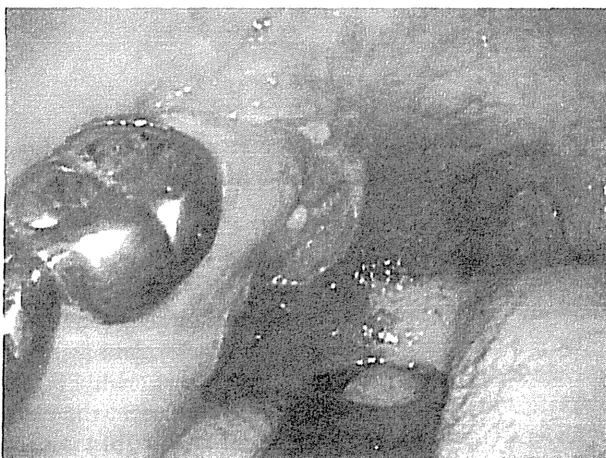


Figure 2. Case 7, before surgery. Lower gingiva T2N0 squamous cell carcinoma.

We indicate the patient with the defects resulting from SCC resection could not be closed primarily and when transfer of a bulky free cutaneous flap would interfere with oral function.

After mucosal resection, the wounds were covered with PGA sheet patches attached with fibrin glue. First, a solution of fibrinogen was applied to the wound. Secondly, the wound was covered with a PGA sheet that cut off one or many pieces slightly smaller than the area of resection. Finally, the wound was sprayed with a solution of fibrinogen and thrombin. Oral intake was started 4 days after surgery, and analgesics (nonsteroidal anti-inflammatory agent loxoprofen) were administered as required. At the surgical site, the edges of separated PGA sheets were cut off.

Equipment

Polyglycolic acid felt sheet is a soft nonwoven fabric that becomes elastic through a special process. (Neoveil®, Gunze Co., Ltd., Tokyo, Japan) (Fig. 1). The PGA sheet was attached with fibrin glue derived from human blood (Bolheal®, Chemo-sero-Therapeutic Research Institute, Kumamoto, Japan).

RESULTS

Mucosal resection was the initial treatment in seven patients (five with oral SCCs and two with oropharyngeal SCCs; Figs 2 and 3). The PGA sheet patches became detached in two patients (on the day of surgery and on postoperative day 6), who then required large doses of analgesics. A patient who underwent tooth extraction also required large doses of analgesics. The other four patients required no analgesics or only small doses of analgesics (Table 1). The PGA sheets in

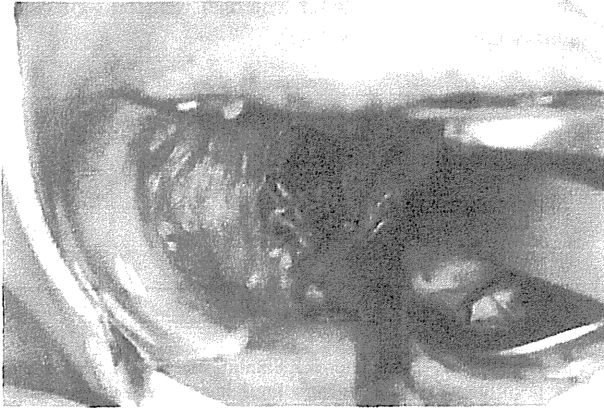


Figure 3. Case 7. Mucosal resection with tooth extraction.

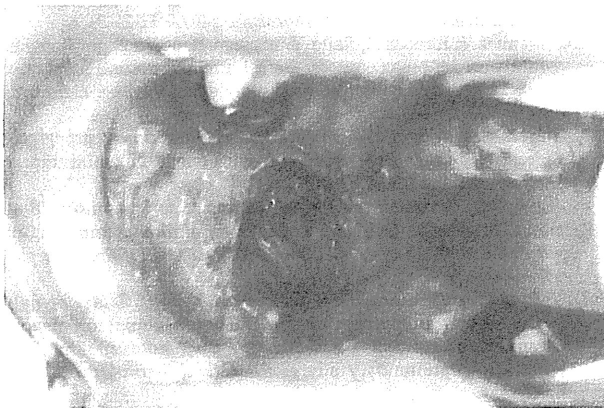


Figure 4. Case 7. The mucosal defect was covered with a PGA sheet attached with fibrin glue.

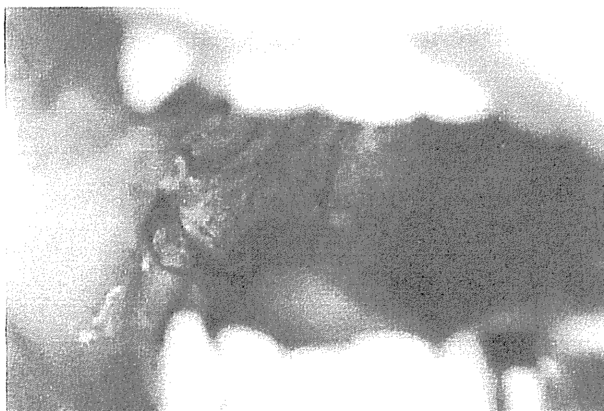


Figure 5. Case 7. Six days after surgery. The PGA sheet and fibrin are observed.

these four patients detached on postoperative days 26 to 38. One patient had bleeding at the surgical site. No adverse events were caused by the PGA sheet patch or by fibrin glue. In patients without early PGA patch detachment, healing was

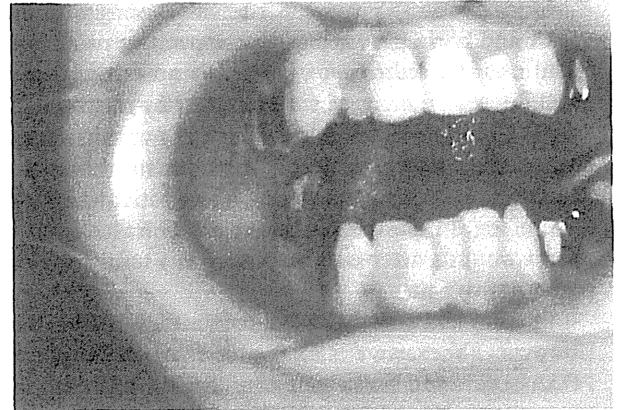


Figure 6. Case 7. Nine days after surgery. The PGA sheet and fibrin are observed. The edge of the PGA sheet is covered with granulation tissue.

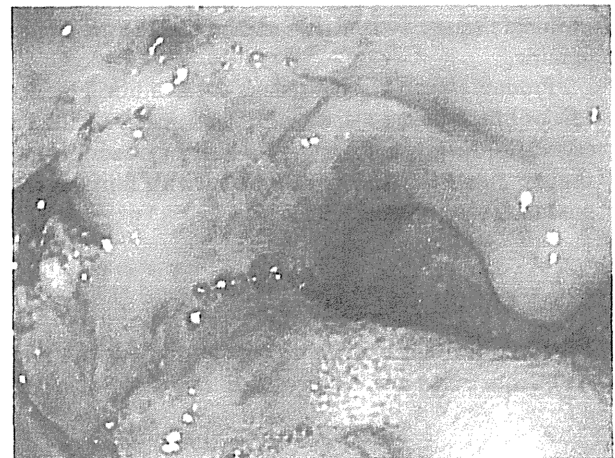


Figure 7. Twenty-two days after surgery. Most of the defect is covered with mucosa and granulation tissue.

excellent. Wound contraction was not observed in any patient, with or without early detachment.

DISCUSSION

After the resection of oral or pharyngeal cancers, mucosal defects are repaired primarily with sutures or covered with skin grafts or various biomaterials. Skin grafts or various biomaterials attached with sutures easily become detached and cause pain. Because adhesion is weak between the materials and the mucosal defects, gaps can open, through which saliva or ingested food can pass. Several authors have reported the usefulness of PGA sheets attached with fibrin glue for covering wounds after oral and pharyngeal surgery (5,6). Covering wounds with PGA sheets and fibrin glue is simple and less time-consuming than the tie-over method or skin grafting or the use of other artificial materials and may avoid the need for microvascular graft reconstruction.

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Murata et al. (5) have reported that covering wounds with PGA and fibrin glue reduces postoperative pain. Our report confirms the reduction of pain, as no analgesics or only small doses of analgesics were required by patients in whom the PGA sheets did not detach early. In contrast, large doses of analgesics were required by patients in whom the PGA sheets had detached early, and one patient had bleeding. Therefore, the use of PGA sheets and fibrin glue for covering open wounds after oral resection was useful for avoiding postoperative pain.

In the present study, tissue contraction was not observed. However, Yonezawa et al. (7) have reported that the use of PGA sheets and fibrin glue causes early epithelialization in experiments in rabbits. Furthermore, in the present study, PGA sheets and fibrin glue seemed to cause early epithelialization and to reduce pain.

We plan to study additional patients in whom wounds have been covered with PGA sheets and fibrin glue and to evaluate tissue contraction and oral intake after PGA patch grafting.

Products from animal tissue, such as fibrin glue, carry a risk of blood-borne disease. However, our patients showed excellent pain control and good healing wound. Our findings suggest that PGA sheets attached with fibrin glue can be used to cover mucosal defects.

CONCLUSION

The use of PGA sheet patch grafts attached with fibrin glue is effective and provides good pain control for patients with large, open wounds after mucosal resection of oral or oropharyngeal SCC (Figs 4–7).

Conflict of interest statement

None declared.

References

1. Hayashibe A, Sakamoto K, Shinbo M, et al. New method for prevention of bile leakage after hepatic resection. *J Surg Oncol* 2006;94:57–60.
2. Kobayashi S, Nagano H, Marubashi S, et al. Experience with the use of fibrin sealant plus polyglycolic acid felt at the cut surface of the liver in laparoscopic hepatectomy. *Surg Endosc* 2011;25:3590–6.
3. Gika M, Kawamura M, Izumi Y, et al. The short-term efficacy of fibrin glue combined with absorptive sheet material in visceral pleural defect repair. *Interact Cardiovasc Thorac Surg* 2007;6:12–5.
4. Maniwa T, Kaneda H, Saito Y. Management of a complicated pulmonary fistula caused by lung cancer using a fibrin glue-soaked polyglycolic acid sheet covered with an intercostal muscle flap. *Interact Cardiovasc Thorac Surg* 2009;8:697–8.
5. Murata M, Umeda M, Takeuchi J, et al. Application of polyglycolic acid sheet and fibrin glue spray for open wounds in oral surgery. *J Jpn Stomatol Soc* 2011;60:232–9.
6. Takeuchi J, Umeda M, Murata M, et al. Application of polyglycolic acid sheet and fibrin glue spray for partial glossectomy: Comparison with artificial dermis. *Jpn J Oral Maxillofac Surg* 2011;57: 394–400.
7. Yonezawa H, Yamada SI, Yanamoto S, et al. Effect of polyglycolic acid sheets with fibrin glue (MCFP technique) on the healing of wounds after partial resection of the border of the tongue in rabbits: a preliminary study. *Br J Oral Maxillofac Surg* 2012;50: 459–63.

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Treatment Strategy for Superficial Pharyngeal Squamous Cell Carcinoma Synchronously Combined with Esophageal Cancer

Kazuhiro Kaneko[a] Tomonori Yano[a] Keiko Minashi[a] Takashi Kojima[a] Miki Ito[a] Hironaga Satake[a] Yoko Yajima[a] Yusuke Yoda[a] Hiroaki Ikematsu[a] Yasuhiro Oono[a] Ryuichi Hayashi[b] Masakatsu Onozawa[c] Atsushi Ohtsu[a]

Departments of [a]Gastroenterology and Gastrointestinal Oncology, [b]Head and Neck Surgery and [c]Radiation Oncology, National Cancer Center Hospital East, Kashiwa, Japan

Key Words
Pharyngeal cancer · Chemotherapy · Esophageal squamous cell carcinoma · Narrow band imaging · Early detection

Abstract
Background: Esophageal squamous cell carcinoma (ESCC) is often synchronously accompanied by pharyngeal squamous cell carcinoma (PSCC). However, treatment strategies for these synchronous cancers have not been established. ***Aim:*** To evaluate retrospectively the effects of both chemoradiotherapy (CRT) targeted for invasive ESCC on synchronous superficial PSCC and additional endoscopic resection (ER) for PSCC. ***Patients and Methods:*** Screening endoscopy in the pharynx was performed in newly diagnosed ESCC patients. CRT combined with 5-fluorouracil (5-FU) and cisplatin (CDDP) was administered to all patients. The effect on superficial PSCC was only evaluated for 5-FU-CDDP chemotherapy that excluded the pharynx from the radiation field. When PSCC was remnant or recurrent in patients evaluated at complete response (CR) of ESCC, ER was performed on the PSCC. ***Results:*** Fourteen cases of superficial PSCC (4.0%) were detected in 348 ESCC patients. Three PSCC reached CR in 8 ESCC-CR patients, while all 3 lesions recurred. No treatment response was found in the remaining 11 PSCC. As a second treatment, ER for 8 PSCC was completed in the 8 ESCC-CR patients, with one complication due to pneumonia. ***Conclusions:*** Standard 5-FU-CDDP CRT targeted for invasive ESCC did not demonstrate a sufficient efficacy for superficial PSCC, while ER even for PSCC after chemotherapy was curative.
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Introduction

Esophageal squamous cell carcinoma (ESCC) is often accompanied by pharyngeal squamous cell carcinoma (PSCC) either simultaneously with the primary lesion (synchronously) or after a period of time (metachronously). These findings have been explained by the 'field cancerization' theory that describes how repeated local exposure to carcinogens contributes to the occurrence of multiple cancers in the esophageal and head and neck regions [1]. For more than 5 decades many epidemiological studies have attributed the increased cancer risks associated with alcohol drinking and smoking to this phenomenon

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Kazuhiro Kaneko, MD, PhD

[2–6]. In 2009, the Working Group of WHO-IARC concluded that acetaldehyde associated with alcoholic beverages was carcinogenic to humans and confirmed the group 1 classification of alcohol consumption [7]. In addition, heterozygous traits found in 40% of Asians, who have an inactive alcohol metabolizing enzyme of aldehyde dehydrogenases 2, accumulate acetaldehyde, with higher relative risks of these cancers [7, 8]. Furthermore, the prevalence of multiple Lugol-unstained lesions (LULs) [9, 10], which are caused by repeated exposure to acetaldehyde, was strongly related to the occurrence of synchronous or metachronous cancers in the esophagus and head and neck regions [11].

In contrast, most patients with PSCC are detected at an advanced stage with a poor prognosis. Even in an operable PSCC case, the extensive surgical resection required may cause a loss of function with respect to swallowing and/or speaking and can lead to cosmetic deformities. Thus it is difficult to determine a final treatment from the viewpoints of both curability and retaining organ function. In cancers combining ESCC and PSCC, the selection of treatment is even more critical. Because of this, the ability to detect pharyngeal lesions at an earlier stage, e.g. as carcinoma in situ, would be of clear benefit to patients. Recently, superficial PSCC has been detected by NBI endoscopy [12].

Systemic 5-fluorouracil-cisplatin (5-FU-CDDP) chemotherapy combined with radiotherapy is the standard treatment for ESCC, and the same treatment is also effective for PSCC patients [13, 14]. The radiation field used in radiotherapy for ESCC does not generally reach the region of the larynx and pharynx, while chemotherapy acts systemically. There have been no reports regarding the efficacy of systemic chemotherapy for patients with superficial PSCC. In this study, we examined the effect on superficial PSCC of chemoradiotherapy (CRT) targeted for invasive ESCC.

Patients and Methods

Patients

Between January 2003 and December 2006, concurrent CRT was performed in 348 patients with invasive ESCC who met the following criteria of this study: (1) newly diagnosed thoracic ESCC; (2) aged between 20 and 75 years; (3) clinical stage I to IVA according to the UICC-TNM classification; (4) absence of previous chemotherapy for malignancy; (5) absence of radiation or surgical treatment for head and neck, and esophageal cancers, and (6) absence of active malignancy except ESCC and PSCC. All patients with invasive ESCC visited our hospital to receive treatment after histological diagnosis of ESCC by endoscopy at another hospital.

Endoscopic Observation of the Oral Cavity and Pharynx

Since January 2003, endoscopic screening of the oral region has been performed in all ESCC patients in order to detect synchronously superficial PSCC. In the initial endoscopic observation in our hospital, narrow band imaging (NBI) or conventional endoscopy was used because both evaluation of ESCC and gastroduodenal screening including oral cavity and pharynx are performed in all patients. When a mucosal abnormality in the oral cavity or pharynx, or multiple LULs in the esophagus, were found in initially conventional endosopcy, the oral cavity and pharynx were observed again by magnifying NBI endoscopy within 2 weeks. Figure 1 shows the NBI findings of an oral cavity and pharynx using a video endoscope system (EVIS LUCERA CV-260, Olympus Optical Co. Ltd., Tokyo, Japan). When a brownish area and an enhancement of the intraepithelial papillary capillary loop were found in the pharynx (fig. 2), an endoscopic biopsy was performed to histologically confirm the carcinoma.

Lugol chromoendoscopy was performed in all patients for both diagnosis of the correct cancer region and evaluation of LULs in the background esophageal epithelium. After ordinary endoscopic observation, 5–10 ml of 2.0% glycerin-free Lugol iodine solution, which is a brown liquid consisting of 2.0 g potassium iodine and 4.0 g iodine in 100 ml distilled water, was sprayed from the upper thoracic esophagus to the gastroesophageal junction using a plastic spray catheter passed through the biopsy channel of the endoscope. Multiple LULs were defined as described in our previous study [15].

Definition of Superficial Pharyngeal Cancer

According to the Japan Society for Head and Neck Cancer [16], a superficial pharyngeal lesion is defined as one in which the invasion depth is comparatively limited and visual changes do not indicate an advanced cancer. The pharynx has no muscularis mucosa, so this somewhat vague definition suggests that the depth of invasion is limited to the epithelium or just beneath the epithelium, but does not extend to the muscle layer.

Treatment Schedule of CRT for ESCC

Chemotherapy consisted of a protracted infusion of 5-FU at a dose of 1,000 mg/m^2 per day on days 1–5 and 22–26, combined with a 2-hour infusion of CDDP at 75 mg/m^2 on days 1 and 22. A 10-MV radiation treatment was administered for 6 weeks (5 days/week) at 1.8 Gy/day with a total radiation dose of 50.4 Gy, concomitantly with chemotherapy.

Patients who were evaluated for an objective response to this treatment received additional chemotherapy consisting of a continuous infusion of 5-FU at a dose of 1,000 mg/m^2 on days 1–5 and CDDP at a dose of 75 mg/m^2 on day 1. This treatment schedule was administered for 1 week followed by a 3-week break. All patients receiving CRT were monitored by neck, chest and abdominal computed tomography, and by endoscopy to evaluate the efficacy of the treatment on both ESCC and PSCC.

As for response for ESCC, objective responses of measurable metastatic lesions were evaluated according to the response evaluation criteria in solid tumors (RECIST v 1.0) guideline. Response of the primary tumor was evaluated by the criteria of the Japan Esophageal Society [17, 18].

Evaluation of Response for PSCC

All follow-up evaluations after 5-FU-CDDP chemotherapy for PSCC were performed every 2 months for the first year and every 6 months thereafter by magnifying NBI endoscopy, with the same periods of evaluation as for ESCC. For PSCC, complete response (CR) was defined as the disappearance of all visible tumors (brownish areas), including ulceration, for at least 4 weeks, confirmed by normal endoscopic biopsy specimens. The recurrence was defined as the reappearance of a brownish area accompanied by an enhancement of intraepithelial papillary capillary loop by NBI endoscopy, and was confirmed in histological findings by endoscopic biopsy. Non-CR for PSCC was defined as the remnant of brownish areas and was classified into a partial response, stable disease or progressive disease.

In the case of non-CR for PSCC, the second treatment was selected according to the efficacy of CRT for ESCC. When ESCC reached CR with remnant or recurrence of PSCC, endoscopic resection (ER) was performed for PSCC. When the ESCC was evaluated for non-CR, thereafter treatment for ESCC, such as second-line chemotherapy, salvage surgery or palliation was performed.

ER for PSCC after CRT

The ER involved endoscopic mucosal resection using the cup method or an endoscopic subepithelial dissection method with the patient under general anesthesia. An important consideration was that ER for PSCC should be performed with cooperation from the endoscopists and the head and neck surgeons. Some head and neck surgeons participated in the ER to prepare emergency treatment, such as tracheostomy, with evaluation of the degree of laryngeal edema after the procedure.

Statistics

All statistical analyses were performed using IBM SPSS Statistics 18 software (SPSS Inc., Tokyo, Japan). Overall survival data were calculated from the date of commencement of CRT to the date of death or the most recent follow-up visit. Survival curves were plotted according to the Kaplan-Meier method. The significance of differences was assessed using the log-rank test. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics

Fourteen patients (4.0%) with synchronous superficial PSCC were found among the 348 patients with invasive ESCC (table 1). Of the 14 patients, 13 (93%) were male and the median age was 62 years. The number of patients for ESCC clinical stage I, II, III, and IVA were 5, 2, 6 and 1, respectively. All 14 patients had both daily alcohol consumption and multiple LULs of the esophagus. All PSCC lesions were detected at our institute with no prior detection in other hospitals. Twelve (86%) PSCC lesions were detected using magnifying NBI endoscopy and the other 2 (14%) by conventional endoscopy. The latter 2 lesions were reevaluated with magnifying NBI endoscopy before

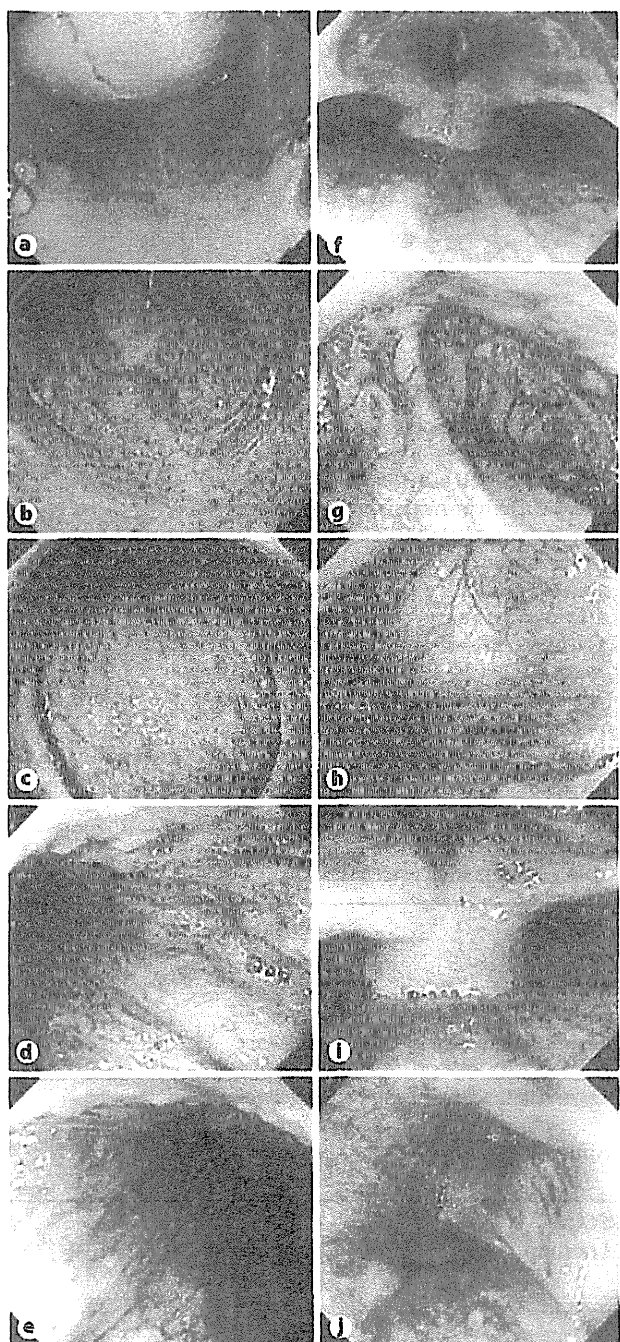

Fig. 1. Narrow Band Imaging observations in individual regions from the oral cavity to the pharynx. **a** The view seen from the entrance of the oral cavity: dorsal side of tongue, hard palate and soft palate. **b** Uvula, palatoglossal arch and lateral walls of oropharynx. **c** The posterior wall of oropharynx. **d** The right side of base of tongue and lateral wall of oropharynx. **e** The left side of base of tongue and lateral wall of oropharynx. **f** Posterior wall of hypopharynx and larynx. **g** Vallecula of epiglottis, median glossoepiglottic fold. **h** The lateral wall and apex of right piriform sinus. **i** Arytenoids. **j** The lateral wall and apex of left piriform sinus.

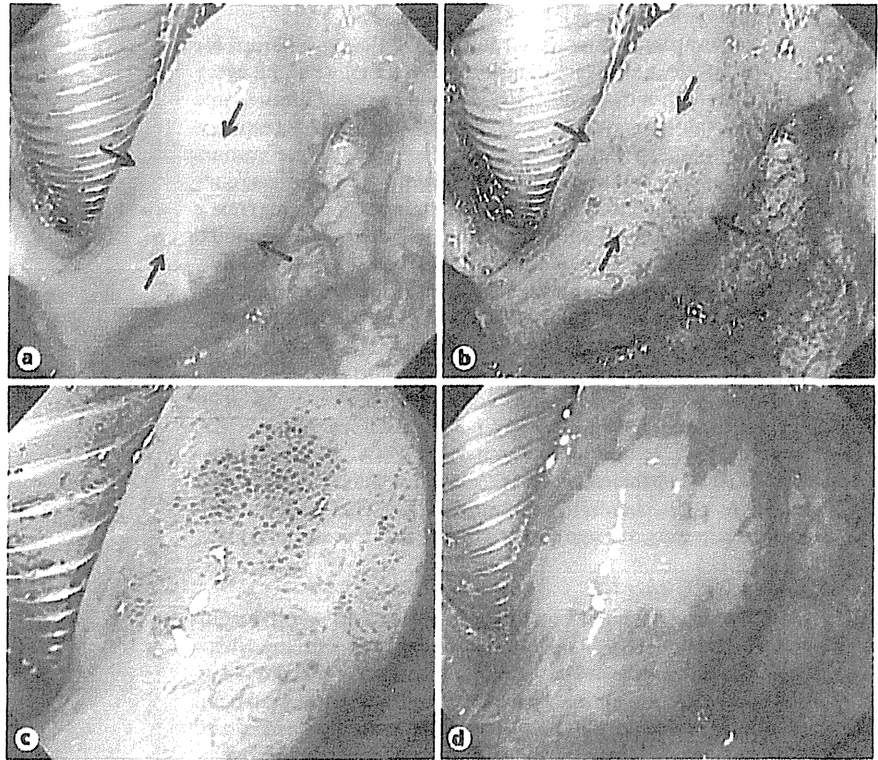

Fig. 2. Superficial cancer of the right arytenoid. a Conventional endoscopic observation. The margin of the cancer is unclear (black arrows). b NBI observation. Cancer is shown as a brownish area (black arrows) and the margin is clear. c Magnifying NBI observation. The enhanced intraepithelial papillary capillary loop is seen in the cancer area. d The view of Lugol staining. Lugol-unstained lesion coincided with the cancer area. Lugol staining method was used to improve lesion visualization during endoscopic treatment. Color refers to the online version only.

Table 1. Patient characteristics

Age, years	Median	62
	Range	47–71
Gender	Male	13
	Female	1
Alcohol consumption	Presence	14
	Absence	0
Cigarette smoking	Presence	12
	Absence	2
Multiple LULs	Presence	14
	Absence	0
PSCC		
Location	Hypopharynx	10
	Oropharynx	4
Size, mm	Median	20
	Range	5–50
Macroscopic findings	Elevated type	5
	Flat type	4
	Depressed type	5
ESCC		
Clinical stage	I	5
	II	2
	III	6
	IVA	1

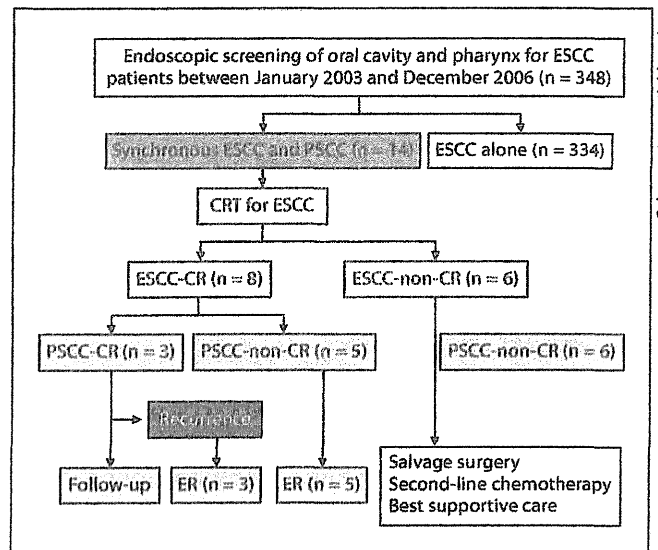

Fig. 3. Flow chart of this study.

CRT. The majority of PSCCs were located in the hypopharynx. In macroscopic findings, there were various lesion types. The median lesion diameter was 20 mm, ranging from 5 to 50 mm. All PSCC lesions were superficial cancers with no advanced cancers.

Efficacy of 5-FU-CDDP Chemotherapy for PSCC

The treatment for PSCC was determined according to response to CRT for primary ESCC (fig. 3). CRT for ESCC resulted in CR in 8 of the 14 patients. In contrast, only 3 of 14 PSCC lesions were evaluated as CR. The 3 PSCC-CR lesions (38%) were found in the ESCC-CR patients (fig. 3). However, the 3 PSCC-CR lesions were only transiently disappeared, and local recurrence was found in the same region. In the 6 ESCC-non-CR patients, there were no PSCC-CR lesions. Of the 6 patients, 2 who were finally evaluated as partial response for ESCC had transformation of their superficial PSCC to invasive lesions. Therefore, active salvage surgery with laryngopharyngeal and esophageal resection was undertaken in these 2 patients. Of the remaining 4 patients, 2 lesions had no change in size and shape while the other 2 were evaluated as partial response because of decreased tumor size.

ER for PSCC and Complications

ER for PSCC was performed in the 8 patients with ESCC-CR. Histologic findings showed the depth of infiltration was invasive PSCC in 2 patients and cancer in situ in 6 patients. However, no lymphovascular involvement was found in any of the 8 cases with PSCC.

Major complications associated with ER included 1 case of aspiration pneumonia. There were no severe complications such as subcutaneous emphysema, post-ER stricture or delayed bleeding. Of the 8 PSCC lesions, 1 was recurrent 4 months after ER. Because the recurrent lesion was superficial and small, an additional ER was performed with complete resection. The median duration of follow-up after ER was 28 months ranging from 12 to 39 months, and no more recurrences of the PSCC were found.

Survival

The 8 ESCC-CR patients received ER for PSCC and the remaining 6 ESCC-non-CR patients did not. The pretreatment clinical stages of ESCC in 8 ER and 6 non-ER patients were 4 and 1 patient in stage I, 1 and 1 in stage II, 2 and 4 in stage III and 1 and 0 in stage IVA, respectively. There were no differences in clinical staging variety in the ESCC pretreatment evaluation between ER and non-ER patients. Median survivals of ER and non-ER patients were 51 and 14 months, respectively (p = 0.002; log-rank test; fig. 4). The 3-year survival rates of ER and non-ER patients were 63 and 0%, respectively. In contrast, 4 of the 8 ER patients died during follow-up periods. Preclinical stages of the 4 patients were 2 patients in stage I, 1 in stage II and 1 in stage IVA, respectively. The 2 patients in stage I died of radiation-induced pneumonia and cerebral infarction. The patient in stage II died of ESCC progression with lymph node metastases and the remaining patient in stage IVA died of multiple lung metastases.

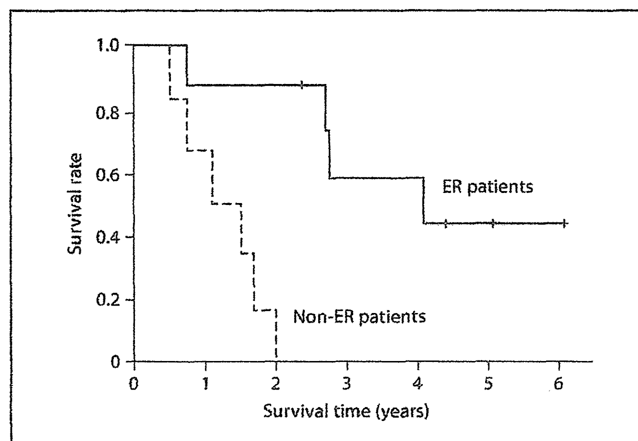

Fig. 4. Overall survival. Median survivals of ER and non-ER patients were 51 and 14 months, respectively (p = 0.002; log-rank test).

After CR confirmation in ESCC, ER was performed in PSCC. The median duration from commencement of CRT to ER in the 8 patients receiving ER was 5.4 months, ranging from 3.8 to 18.9 months. ER was performed in 5 of the 8 patients immediately after CRT since PSCC lesions of the 5 patients were not evaluated as CR. However, the time periods to perform ER after CRT were extended in the remaining 3 PSCC-CR patients from 10 to 18.9 months due to following-up for PSCC-CR. There were no cases in which superficial PSCC transformed to an advanced stage during the follow-up periods. Thus, no functional disorder caused by progression of PSCC, such as difficulty swallowing or speaking, were found in ER patients during all follow-up periods.

Discussion

Of 348 patients with invasive ESCC, 14 (4%) had superficial PSCC detected through endoscopic screening of the oral cavity and pharynx. Standard 5-FU-CDDP CRT targeted for invasive ESCC was administered to the 14

patients with synchronous superficial PSCC and invasive ESCC. After CRT, 8 (57%) were evaluated as CR for invasive ESCC, while only 3 patients with superficial PSCC (21%) achieved transient CR despite receiving 5-FU-CDDP chemotherapy. Therefore, systemic 5-FU-CDDP chemotherapy had no CR potential for superficial PSCC. In contrast, ER for superficial PSCC is quite effective even in a situation after chemotherapy because of minimally invasive treatment with no functional disorder in the pharyngeal region. We propose using novel treatment strategies for synchronous superficial PSCC and invasive ESCC.

Acetaldehyde associated with alcoholic beverage and aldehyde dehydrogenases 2 heterozygous traits can cause pharyngeal and esophageal cancers [7]. According to recent reports regarding multiple cancers, the prevalence of multiple LULs is a biomarker of synchronous or metachronous cancers in the esophagus and head and neck regions [19–21]. In our present study, all 14 patients with synchronous ESCC and PSCC had both daily alcohol consumption and multiple LULs in their esophageal background epithelium. Lugol chromoendoscopy is useful not only to detect superficial ESCC but also to understand the risk of multiple cancers. However, the Lugol solution cannot be routinely sprayed in the region of the pharynx and larynx of patients under conscious sedation because of the stimulation caused by the application of the solution. Thus, we suggest that detecting superficial PSCC by NBI is useful in ESCC patients, especially those with both an alcohol drinking habit and multiple LULs in their esophagus.

5-FU-CDDP treatment has been performed in PSCC patients since the 1980s. The CR rate of this therapy without radiotherapy was 17–20% of locally advanced or metastatic PSCC cases in phase I–II studies [13, 14], and was 5–7% of metastatic or recurrent cases in phase III studies [22, 23]. 5-FU-CDDP treatment alone is likely to be more effective in locally advanced PSCC than in metastatic PSCC. In contrast, there has been no study of 5-FU-CDDP alone in PSCC of early clinical stage, especially stage 0–I. Therefore, the 5-FU-CDDP treatment efficacy in superficial PSCC is uncertain. If the therapy had a high efficacy for superficial PSCC, overlooked superficial PSCC would be cured by the systemic 5-FU-CDDP therapy given to treat ESCC. This is quite a benefit for the patients with these synchronous cancers. As a result, PSCC-CR was found, while no efficacy in continuing CR for superficial PSCC was found in 5-FU-CDDP treatment. In contrast, no progression of PSCC was found in patients having excellent efficacy with CRT for ESCC, although the time periods until CR confirmation for ESCC were required to be at least several months. A good correlation in treatment efficacy between PSCC and ESCC was indicated. It seems that 5-FU-CDDP chemotherapy has a potential in restraining the progression of PSCC. In some recent reports, platinum-based chemotherapy or CRT plus cetuximab were more effective in esophageal and the head and neck cancers [24–26]. CRT combined with cetuximab, a molecular targeted drug, may contribute to a novel treatment strategy for patients with synchronous PSCC and ESCC.

The outcomes of ER for superficial PSCC have been reported [27]. Complications, such as laryngeal edema requiring overnight intubation, aspiration pneumonia and sustained dermatitis around the mouth caused by backflow of Lugol solution from the pharynx, were found in 13% of patients after ER [27]. Complications are transient and tolerable in most of cases, and feasibility is confirmed with no functional disorder. In our study, there were no severe complications, with high treatment efficacy for ER during long follow-up periods, although ER was performed in the condition after 5-FU-CDDP chemotherapy. It is important to maintain function with respect to swallowing and/or speaking, and to perform ER under cooperation with head and neck surgeons.

Regarding the treatment strategy, CRT for ESCC should be the initial therapy in patients with both superficial PSCC and ESCC. As the second step, ER for PSCC should be determined after evaluation of CRT for ESCC. A factor deciding the prognosis of patients with the synchronous cancers depends on the CRT effects for ESCC. In our previous study, the prognosis between CR and non-CR cases of ESCC was quite different [28]. In our present study, the median survival time of ER (ESCC-CR) patients was also significantly longer than that of non-ER (ESCC-non-CR) patients. Furthermore, 5-FU-CDDP chemotherapy showed potential in restraining the progression of PSCC including transient CR. If ER was performed initially, the period before commencement of CRT would be delayed. In addition, when complications occurred in ER, the commencement would be further delayed. Therefore, ER for superficial PSCC should be secondary to CRT for invasive ESCC. We suggest that the ER for PSCC contributed to the beneficial prognosis in patients with synchronous superficial PSCC and invasive ESCC. It is uncertain whether all superficial PSCC lesions progress to an advanced stage in the natural history. However, if superficial PSCC was overlooked and progressed to an advanced stage in ESCC-CR patients, it would be difficult to achieve long survival. Furthermore,

13 Gibson MK, Li Y, Murphy B, Hussain MHA, DeConti RC, Ensley J, Arlene A: Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an Intergroup Trial of the Eastern Cooperative Oncology Group. J Clin Oncol 2005;23:3562–3567.
14 Jacobs C, Lyman G, Velez-Garcia E, Sridhar KS, Knight W, Hochster H, Goodnough LT, Mortimer JE, Einhorn LH, Schacter L, Cherng N, Dalton T, Burroughs J, Rozencweig M: A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. J Clin Oncol 1992;10:257–263.
15 Kaneko K, Katagiri A, Konishi K, Kurahashi T, Ito H, Kumekawa Y, Yamamoto T, Muramoto T, Kubota Y, Nozawa H, Makino R, Kushima M, Imawri M: Study of *p53* gene alteration as a biomarker to evaluate the malignant risk of Lugol-unstained lesion with non-dysplasia in the oesophagus. Br J Cancer 2007;96:492–498.
16 Japan Society for Head and Neck Cancer: General Rules for Clinical Studies on Head and Neck Cancer. Tokyo, Kanehara, 2005.
17 Diseases JSFE: Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus (in Japanese), ed 9. Tokyo, Kanehara Shuppan, 1999, pp 59–79.
18 Takubo K, Makuuchi H, Fujita H: Japanese Classification of Esophageal Cancer, tenth edition: part 1. Esophagus 2009;6:1–25.
19 Hori K, Okada H, Kawahara Y, Takenaka R, Shimizu S, Ohno Y, Onoda T, Shirakawa Y, NaomotoY, Yamamto K: Lugol-voiding lesions are an important risk factor for a second primary squamous cell carcinoma in patients with esophageal cancer or head and neck cancer. Am J Gastroenterol 2011;106:858–866.
20 Muto M, Hitomi Y, Ohtsu Y, Ebihara S, Yoshida S, Esumi H: Association of aldehyde dehydrogenase 2 gene polymorphism with multiple esophageal dysplasia in head and neck cancer patients. Gut 2000;47:256–261.
21 Muto M, Hironaka S, Nakane M, Boku N, Ohtsu A, Yoshida S: Association of multiple Lugol-voiding lesions with synchronous and metachronous esophageal squamous cell carcinoma in patients with head and neck cancer. Gastrointest Endosc 2002;56:517–521.
22 Rowland KM Jr, Taylor SG 4th, Spiers AS, DeConti RC, O'Donnell MR, Showel J, Stott PB, Milner LM, Marsh JC: Cisplatin and 5-FU infusion chemotherapy in advanced, recurrent cancer of the head and neck: an Eastern Cooperative Oncology Group Pilot Study. Cancer Treat Rep 1986;70:461–464.
23 Caponigro F, Comella P, Rivellini F, Avallone A, Budillon A, Di Gennaro E, Mozzillo N, Ionna F, De Rosa V, Manzione L, Comella G: Cisplatin, raltitrexed, levofolinic acid and 5-fluorouracil in locally advanced or metastatic squamous cell carcinoma of the head and neck: a phase I–II trial of the Southern Italy Cooperative Oncology Group (SICOG). Ann Oncol 2000;11:575–580.
24 Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, Raucourt DD, Bokemeyer C, Schueler A, Amellal N, Hitt R: Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008;359:1116–1127.
25 Safran H, Suntharanlingam M, Dipetrillo T, Ng T, Doyle A, Krasna M, Plette A, Evans D, Wanebo H, Akerman P, Spector J, Kennedy N, Kennedy T: Cetuximab with concurrent chemoradioation for esophagogastric cancer: assessment of toxicity. Int J Radiat Oncol Biol Phys 2008;70:391–395.
26 Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky ER, Ang KK: Radiotherapy plus cetuximab for squamous cell carcinoma of the head and neck. N Engl J Med 2006;354:567–578.
27 Suzuki H, Saito Y, Oda I, Nonaka S, Nakanishi Y: Feasibility of endoscopic mucosal resection for superficial pharyngeal cancer: a minimally invasive treatment. Endoscopy 2010;42:1–7.
28 Ohtsu A, Boku N, Muro K, Chin K, Muto M, Yoshida S, Satake M, Ishikura S, Ogino T, Miyata Y, Seki S, Kaneko K, Nakamura A: Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. J Clin Oncol 1999;17:2915–2921.
29 Shimizu Y, Tsukagoshi H, Fujita M, Hosokawa M, Watanabe A, Kawabori S, Kato M, Sugiyama T, Asaka M: Head and neck cancer arising after endoscopic mucosal resection for squamous cell carcinoma of the esophagus. Endoscopy 2003;35:322–326.
30 Katada C, Tanabe S, Koizumi W, Higuchi K, Sasaki T, Azuma M, Katada N, Masaki T, Nakayama M, Okamoto M, Muto M: Narrow band imaging for detecting superficial squamous cell carcinoma of the head and neck in patients with esophageal squamous cell carcinoma. Endoscopy 2010;42:185–190.

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ORIGINAL ARTICLE

Clinical significance of *KRAS* gene mutation and epidermal growth factor receptor expression in Japanese patients with squamous cell carcinoma of the larynx, oropharynx and hypopharynx

Satoshi Fujii · Hideoki Uryu · Ken Akashi · Kensuke Suzuki · Manabu Yamazaki · Makoto Tahara · Ryuichi Hayashi · Atsushi Ochiai

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Abstract
Purpose The significance of epidermal growth factor receptor (EGFR) signaling has been recognized in various cancers and anti-EGFR therapies in Japan are currently under consideration in squamous cell carcinoma of the head and neck (SCCHN) similar to colorectal cancer. However, there was no established survey regarding heterogeneous EGFR protein expression in Japanese SCCHN patients. The purpose of this study is to examine the relationship between EGFR expression or *KRAS* mutation (related to the alteration of EGFR pathway) and the clinicopathological characteristics of SCCHN.
Materials and methods We retrospectively examined the expression of EGFR protein by immunohistochemistry and *KRAS* gene mutation at codons 12 and 13 by using paraffin-embedded and formalin-fixed primary tumor tissues from 205 patients with SCCHN who underwent surgery at National Cancer Center Hospital East.
Results In 200 of the 205 patients (97.6 %), EGFR protein was expressed despite intratumoral heterogeneity. No patients had *KRAS* mutation at codons 12 or 13, and all 183 tumors showed wild-type *KRAS*. Positive rate of EGFR protein expression was significantly associated with better disease free survival (DFS) ($P = 0.0471$) and the intensity of EGFR protein expression showed a tendency for better DFS ($P = 0.1034$). Both higher EGFR positive rate and more intense EGFR expression were significantly associated with well differentiated subtype of squamous cell carcinoma ($P = 0.0003$ and $P = 0.0007$, respectively).
Conclusion Most SCCHN patients may be good candidates for the anti-EGFR therapies.

Keywords Squamous cell carcinoma · KRAS · EGFR · Larynx · Pharynx

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S. Fujii · M. Yamazaki · A. Ochiai (✉)
Pathology Division, Research Center for Innovative Oncology, National Cancer Center at Kashiwa, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
e-mail: aochiai@east.ncc.go.jp

H. Uryu · K. Akashi · K. Suzuki · R. Hayashi
Department of Head and Neck Surgery, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

M. Yamazaki
Division of Oral Pathology, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

M. Tahara
Department of Head and Neck Medicine, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

Introduction

Head and neck carcinoma (HNC) is the seventh most common malignancy and around 600,000 cases are diagnosed annually [1]. Its incidence increases with aging and HNC occurs predominantly from 40 to 60 years of age, affecting at least twice as many men as women. Tobacco smoking and alcohol consumption are risk factors for HCN, including squamous cell carcinoma of the head and neck (SCCHN), as is known for esophageal squamous cell carcinoma [2]. In Europe, 132,000 new cases of HNC were diagnosed and 63,000 patients died of HNC in 2008 [3]. In Japan, 13,026 cases of SCCHN were diagnosed and deaths

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caused by SCCHN have increased recently [4], with 6,255 and 6,768 deaths from SCCHN in 2001 and 2005, respectively [4]. As this increase continues, it is estimated that the annual number of deaths from SCCHN will rise to 10,700 by 2020 [5, 6]. Also, only 40 % of SCCHN patients have early disease (Stages I or II) that can benefit from surgical intervention and/or radiation therapy. The five-year survival rate of those patients ranges from 60 to 90 % [2], but over 50 % of SCCHN patients are in Stages III or IVA/B with locally advanced cancer [7, 8]. Local recurrence affects 60 % of patients with locally advanced cancer, while distant metastases and second cancers occur in about 30 and 5–13 %, respectively [9]. There is an urgent need for novel chemotherapy regimens to treat locally advanced cancer.

Erbitux® (cetuximab) is a chimeric IgG1 mAb that binds specifically and with high affinity to the extracellular domain of human EGFR. Preclinical studies demonstrated synergy of cetuximab with topoisomerase I inhibitors against human colorectal cancer (CRC) cell lines in vivo [10]. Subsequent clinical trials revealed that cetuximab can overcome resistance to topoisomerase I inhibitors and has modest activity as monotherapy, resulting in accelerated provisional FDA approval for the treatment of irinotecan-refractory CRC [10]. Full FDA approval was subsequently granted to cetuximab after a randomized clinical trial (The National Canadian Institute of Cancer Study CO.17) demonstrated improvement of overall survival (OS) in patients receiving cetuximab plus best supportive care compared with best supportive care alone [11]. Cetuximab is the second mAb approved for treating CRC and the first anti-EGFR antibody approved for clinical use in Japan. Clinical utility has been improved by the discovery of negative biomarkers since cetuximab is less effective against tumors harboring *KRAS* mutation [12]. Thus, CRC patients with tumors bearing *KRAS* mutations do not benefit from cetuximab, unlike patients whose tumors have wild-type *KRAS*. In contrast, *KRAS* mutation has no influence on the survival of patients receiving best supportive care alone [12]. Although *KRAS* mutation was reported to be rare in SCCHN overseas, there have been no reports about its influence on survival in SCCHN patients receiving cetuximab.

The EGFR is a 170,000 dalton cell surface tyrosine kinase transmembrane receptor from the ErbB family [13]. It is normally expressed by various epithelial cells, and is overexpressed in the epithelium of the bronchial tree, gastrointestinal tract, skin, and gynecologic tract. After the extracellular component of the receptor binds to a natural ligand, such as TGFα, amphiregulin, or epiregulin, homodimerization or heterodimerization occurs with other members of the ErbB family including ErbB2 or HER2/neu, ErbB3, and ErbB4 [14]. Binding and activation of the receptor lead to phosphorylation of tyrosine kinase and subsequent downstream activation of multiple cellular signaling pathways, such as the Ras-Raf-MAP kinase pathway, PI3 K pathway, and protein-serine/threonine kinase Akt pathway. These signal transduction pathways regulate various cellular activities, including replication, invasion, repair, and protection against exogenous insults [15]. The EGFR is an attractive target for anticancer therapy because it is activated in various cancers, including CRC [16, 17]. In fact, CRC patients with a quantitative increase of EGFR expression demonstrated by immunohistochemistry (IHC) have a worse prognosis [18, 19]. Therefore, interruption of this signaling pathway could potentially abolish the growth advantage of cancer cells and/or promote tumor cell death. EGFR protein is expressed in normal epithelial cells, such as epidermal cells and hair follicle cells, as well as in various cancers (SCCHN, pancreatic, renal, colorectal, and non-small cell lung cancer) [14, 20]. An increase of the *EGFR* gene copy number is frequent in SCCHN and indicates a poor prognosis, suggesting the potential significance of EGFR inhibitor therapy for SCCHN patients [20].

Immunohistochemical staining of tumor tissue specimens with anti-EGFR antibody is usually done before treating CRC with cetuximab. The prevalence of EGFR expression by CRC was reported as about 80 % [21], although a study of 91 SCCHNs found EGFR expression is 100 % [22]. Therefore, immunohistochemical staining for EGFR protein is not required before treating SCCHN with cetuximab overseas and it is necessary to examine the prevalence of EGFR protein expression among Japanese SCCHN patients. *KRAS* gene mutation has been recognized as a negative predictor for responsiveness of CRC to cetuximab. However, *KRAS* gene mutation was estimated to occur in <3 % of SCCHNs, making it of doubtful value to investigate *KRAS* in all candidates for cetuximab treatment, especially from the perspective of cost versus benefit. Therefore, it is important to examine *KRAS* mutation and EGFR protein expression in Japanese patients with SCCHN.

The present study of EGFR protein expression and *KRAS* gene mutation in Japanese SCCHN patients was therefore expected to assist in avoiding unnecessary examinations before starting anti-EGFR therapy such as cetuximab, which could help to reduce health care costs.

Materials and methods

Tissue samples and patients

Two-hundred and five SCCHN tumor samples surgically resected were collected at the National Cancer Center Hospital East between 1994 and 2006. The tumors were located in the larynx ($n = 51$), oropharynx ($n = 71$), and

hypopharynx ($n = 83$) of 135 males and 70 females. Genomic DNA was isolated from formalin-fixed and paraffin-embedded tissue sections with the maximum amount of cancer tissue as microscopically confirmed by pathologists (non-cancerous tissue accounted for <30 %). Genomic DNA was extracted using a DNA extraction kit from QIAGEN (QIAamp FFPE DNA kit, Valencia, CA, USA). This study received institutional review board approval.

KRAS gene mutation analysis

Tumor samples were analyzed for exon 2 mutations of *KRAS* located within codons 12 and 13. DNA was amplified by the polymerase chain reaction (PCR) using the following sense and antisense primers: 5′-TGTGTGACA TGTTCTAATATAGTCACATTT-3′ and 5′-TTAAAACA AGATTTACCTCTATTGTTGGAT-3′ [23]. PCR was carried out in a reaction mixture (25 μL) with 250 ng of genomic DNA. After purification using Illustra GFX DNA and a Gel Band Purification Kit or Agencourt AMPure XPkit, the PCR products were subjected to direct sequencing using a BigDye V1.1 terminator sequencing kit (Applied Biosystems, Foster, CA, USA), ethanol precipitation, and an automated sequencer (ABI Prism 3100: Applied Biosystems). The sensitivity of direct sequencing has been validated since the mutation contamination rate is kept to be lower than 20 %.

Immunohistochemical staining for EGFR protein

EGFR protein expression was evaluated by immunohistochemical staining of 4 μm sections obtained from paraffin-embedded specimens fixed in 20 % (v/v) formalin [24]. Sections were deparaffinized in xylene and then rehydrated in an alcohol series. Endogenous peroxidase activity was blocked by immersion in methanol containing 0.3 % hydrogen peroxide for 5 min. Then the sections were incubated with protease K, followed by immunohistochemical staining using an EGFR pharmDx kit and Autostainer (Dako, Tokyo, Japan), counterstaining with Mayer's hematoxylin, and mounting. A positive control (HT-29 human colon cancer cell line) and a negative control (CAMA-1 human breast cancer cell line) for EGFR protein expression were included in each staining series. Membranous staining of EGFR was assessed according to the EGFR pharmDx kit protocol and EGFR positivity was judged by two pathologists blinded to the clinical data.

Assessment of immunohistochemical staining

Sections were examined at 400× magnification and staining intensity was evaluated by comparison with noncancerous squamous epithelium, which served as a reference for moderate intensity (M). Tumor staining less intense than the basal layer of noncancerous squamous epithelium was categorized as weak intensity (W), more intense staining was categorized as strong intensity (S), and no staining was categorized as negative (N). We also calculated the percentages of cells with different staining intensities and the predominant intensity was recorded for each tumor. Based on the percentage of cells with EGFR protein expression, tumors were classified into three groups: grade 0 (0–10 %), grade 1 (11–50 %), and grade 2 (51–100 %). We then examined the relationship between EGFR protein expression and overall survival (OS) or disease-free survival (DFS).

Statistical analysis

OS and DFS were analyzed by the Kaplan–Meier method. OS was defined as the period from the operation date to the date of death. DFS is the percentage of individuals in the group who are likely to be free of disease after a specified duration of time from the day of surgical operation to the day of termination of observation without the event such as death or the first recurrence. Comparison between survival functions for different strata was assessed with the log-rank test. Multivariate analysis of prognostic factors was performed by Cox's regression model. Statistical significance was accepted at $P < 0.05$. The software used in this study is JMP9, SAS.

Results

Clinicopathological factors

The clinicopathological characteristics of the patients are shown in Table 1. They were aged from 36 to 85 years, with the mean and median age being 62 and 61 years, respectively. The pT and pN factors, and pStage of patients are shown according to the UICC classification (7th edition). This study did not include any patient with a distant metastasis (M1) and any patient who had received preoperative therapy. The number of patients who had received postoperative therapy such as radiation or chemotherapy was 19 (9.2 %). Tobacco and alcohol consumption (major risk factors for laryngeal, hypopharyngeal, and oropharyngeal squamous cell carcinoma) were reported by 159 (77.6 %) and 139 (87.4 %) patients, respectively. Multiple cancers were present in 69 patients (33.7 %).

KRAS gene mutational analysis

KRAS mutational analysis was done in 183 of 205 patients (89.3 %) including 113 males (83.7 %) and 70 females