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Role of Akt isoforms in HGF-induced invasive growth of human salivary gland cancer cells

Shingo Hara [a,b], Koh-ichi Nakashiro [a,*], Hiroyuki Goda [a], Hiroyuki Hamakawa [a]

[a] Department of Oral and Maxillofacial Surgery, Organ Function Regenerative and Reconstructive Medicine, Ehime University Graduate School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan
[b] Japan Society for the Promotion of Science (JSPS) Research Fellow, 8 Ichiban, Chiyoda, Tokyo 102-8472, Japan

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ABSTRACT

The expression of hepatocyte growth factor (HGF) and c-Met is associated with tumor progression in many human malignancies. A recent study demonstrated HGF and c-Met expression in human salivary gland cancer tissues. Here, we investigated the role of the HGF/c-Met system in the invasive growth of two human salivary gland cancer cell lines: green fluorescent protein-adenoid cystic carcinoma 2 (GFP-ACC2) and GFP-ACCM. HGF enhanced the invasive growth of the two cell lines by activating PI3K/Akt signaling. All Akt isoforms (Akt1, Akt2, and Akt3) were detected in both cell types by Western blot analysis. Knockdown of any of the Akt isoforms using isoform-specific synthetic small-interfering RNAs largely abrogated the invasive growth induced by HGF. Our findings suggest that all of the Akt isoforms are required for the HGF-stimulated invasive growth of human salivary gland cancer cells, and that targeting a single Akt isoform could be effective in treating salivary gland cancers.

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Adenoid cystic carcinoma (ACC) is an uncommon malignancy that arises in secretory glands, particularly the major and minor salivary glands. ACC accounts for around 22% of all salivary gland malignancies, and about 1% of all head and neck malignancies [1]. Most such tumors occur in the minor salivary glands, and the oral cavity is the most common site malignancies. Although ACCs generally grow slowly, they have a high potential for local invasion and distant metastasis. They tend to have a prolonged clinical course and a poor outcome, with local recurrences and distant metastases sometimes occurring many years after presentation. The overall long-term survival rate for ACC is particularly poor, with reported figures of over 50% at 5 years and less than 20% at 10–20 years [2]. Furthermore, ACCs are usually resistant to chemotherapy and radiotherapy. At present, the standard primary treatment for salivary gland ACC is surgery, usually followed by post-operative radiotherapy; however, there is uncertainly about the systemic management of recurrent or metastatic ACC. A novel treatment approach is thus required.

Hepatocyte growth factor (HGF) is a pleiotropic polypeptide growth factor, originally identified as a potent mitogen, which stimulates the growth of hepatocytes, and regulates the cell proliferation, migration, survival, angiogenesis, and invasion of many cells (including epithelial, vascular endothelial, and some stromal cells). These diverse biological effects are mediated through its receptor, the proto-oncogene *c-met* [3]. HGF and c-Met expression are associated with tumor progression in many human malignancies. A recent study reported the expression of HGF and c-Met in human ACC of the salivary gland [4].

The current study investigated the role of HGF/c-Met signaling in the invasive growth of human salivary gland cancer cells, and searched for useful target molecules for the treatment of human salivary gland cancer.

Materials and methods

Cells and cell culture. Two human salivary gland cancer cell lines were used in this study: green fluorescent protein (GFP)-ACC2 and GFP-ACCM. These were generated by the transfection of ACC2 and ACCM cells (gifts from W.L. Qui, Shanghai Second Medical University, Shanghai, China), which were derived from human salivary gland cancers, with a gene encoding GFP. The cells were maintained in RPMI1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biosource International, Camarillo, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), referred to here as complete medium, and were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Western blot analysis. The cells were grown in monolayers, harvested at subconfluence, and lysed with CelLytic M cell lysis reagent (Sigma–Aldrich). The samples were centrifuged at 12,000*g* for 15 min at 4 °C, and the supernatants were electrophoresed on sodium dodecyl sulfate (SDS)–polyacrylamide gels (BioRad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat dried milk (Wako, Osaka, Japan) and 1× T-TBS (25 mM Tris–HCl, 125 mM NaCl, and 0.1% Tween 20 [Sigma–Aldrich]) overnight at 4 °C. They were then probed with primary antibodies overnight at 4 °C, followed by with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were visualized with

* Corresponding author. Fax: +81 89 960 5396.
E-mail address: nakako@m.ehime-u.ac.jp (K. Nakashiro).

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Enhanced Chemiluminescence (ECL) Plus or the ECL Advance detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. As an internal control, the blots were stripped with 62.5 mM Tris–HCl (pH 6.8) buffer containing 0.7% 2-mercaptoethanol and 2% SDS at 50 °C for 30 min, and then re-probed with mouse monoclonal anti-β-tubulin antibody (BD Biosciences, San Jose, CA). Primary antibodies were purchased from commercial sources as follows: polyclonal rabbit anti-human Met (C-12) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal rabbit anti-phospho-Met (Tyr1234) antibody, polyclonal rabbit anti-phospho-Met (Tyr1349) antibody, polyclonal rabbit anti-phospho-Akt (Ser473) antibody, polyclonal rabbit anti-Akt antibody, monoclonal mouse anti-Akt1 antibody, polyclonal rabbit anti-Akt2 antibody, polyclonal rabbit anti-phospho-extracellular signal-regulated kinase 1/2 (Erk1/2 [Thr202/Thr204]) antibody, polyclonal rabbit anti-Erk1/2 antibody, polyclonal rabbit anti-phospho-signal transducer and activated of transcription 3 (Stat3 [Tyr705]) antibody, polyclonal rabbit anti-Stat3 antibody, polyclonal rabbit anti-phospho-inhibitory protein kappa B-alpha (IκB-α [Ser 32]) antibody, and polyclonal anti-IκB-α antibody were from Cell Signaling (Beverly, MA); polyclonal rabbit anti-Akt3 antibody was from Chemicon (Temecula, CA); and secondary antibodies against rabbit or mouse immunoglobulin G (IgG) were from Amersham Biosciences.

Three-dimensional collagen-gel assay. Collagen-gels were prepared as reported previously [5]. In brief, eight volumes of rat tail type I collagen suspension (BD Biosciences) were mixed with one volume of 10-fold concentrated RPMI1640 (Sigma–Aldrich) and one volume of reconstruction buffer (2.2 g $NaHCO_3$ and 4.77 g Hepes in 100 ml of 0.05 N NaOH; Sigma–Aldrich). The collagen-gel and carcinoma cells (5×10^4 cells/well) were together poured into a 24-well plate (0.5 ml/well). After incubation for 30 min at 37 °C to permit complete gelation, RPMI1640 complete medium was added; the medium was changed every other day. After incubation for 4 days, the cells contained within the gel were recovered by treatment with 0.1% collagenase and 0.5% trypsin–5.3 mM EDTA (Invitrogen). The cells were counted with a Z1 Coulter® particle counter (Beckman–Coulter, Fullerton, CA). In some of the experiments, human recombinant HGF (40 ng/ml; R&D Systems, Minneapolis, MN) and/or goat anti-human HGF antibodies (2 μg/ml; R&D Systems) were added to the cultures.

*RNA isolation and real-time quantitative PCR (*TaqMan*RT-PCR).* RNA was prepared by lysing the cells with Isogen (NipponGene, Toyama, Japan). The relative quantitation of the messenger RNA (mRNA) levels using the comparative C_T method ($\Delta\Delta C_T$ method) was carried out by real-time quantitative polymerase chain reaction (PCR), using the TaqMan probe and primers. Specific complementary DNAs (cDNAs) and hydroxymethylbilane synthase (HMBS) cDNA (as an internal control) were PCR-amplified separately using an oligonucleotide probe labeled with a 5′-fluorescent reporter dye and a 3′-quencher dye. Each PCR amplification was performed in a 20-μl final reaction mixture consisting of LightCycler RNA amplification kit hybridization probes (1× LightCycler RT-PCR enzyme mix, 1× LightCycler RT-PCR reaction mix hybridization probe, and 6 mM $MgCl_2$ [Roche Diagnostics, Mannheim, Germany]), 450 nM forward primer, 450 nM reverse primer, 250 nM TaqMan probe, and 500 ng total RNA. The thermal-cycling conditions comprised an initial step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, at 60 °C for 10 s, and at 72 °C for 5 s. The primers and TaqMan probes for the interferon response genes, 2′,5′-oligoadenylate synthetase 1 (OAS1), 2′,5′-oligoadenylate synthetase 2 (OAS2), interferon-induced myxovirus resistance protein 1 (MX1), and interferon-stimulated gene factor 3 gamma (ISGF-3γ, were purchased from Assays-on-Demand Products (Applied Biosystems, Foster City, CA). The 5′-fluorescent reporter dye was cleaved from the cDNA by the 5′-nuclease activity of Taq DNA polymerase, and its fluorescence was detected with the LightCycler (Roche Diagnostics).

Design and transfection of synthetic siRNAs specific for Akt isoforms. To clarify the oncogenic function of the Akt isoforms (Akt1, Akt2, and Akt3) in human salivary gland cancer cells, we designed and synthesized five specific synthetic siRNAs for each Akt isoform (siAkt1, siAkt2, and siAkt3, respectively). The target sequences were optimized for maximum target-gene silencing and also to minimize the sequence-specific cross reactivity (off-target effects) using B-Algo and siPRECISE (B-Bridge, Mountain View, CA). B-Algo is an adaptive learning algorithm that formulates rules based on complex empirical experiments, and siPRECISE is a high-performance BLAST-like search that can find sporadic mismatches in a target sequence. The cells were seeded at a density of 8×10^5 in a 60-mm culture dish in complete medium, and were transfected 24 h later with 10 nM siRNA in Lipofectamine 2000 Reagent (Invitrogen).

Statistical analysis. The student's *t*-test was used to determine the significance of the differences between the groups. $P < 0.05$ was considered statistically significant.

Results

HGF-induced phosphorylation of c-Met and invasive growth of human salivary gland cancer cells

We tested the effect of HGF on the phosphorylation of its receptor, c-Met, by Western blot analysis with polyclonal rabbit anti-phospho-Met (Tyr1234) and polyclonal rabbit anti-phospho-Met (Tyr1349) antibodies. After serum starvation for 48 h, the cells were stimulated with HGF (40 ng/ml) for 30 min. Exogenous HGF markedly activated c-Met on tyrosine residues 1234 and 1349 in GFP-ACC2 and GFP-ACCM cells, and anti-HGF neutralizing antibody drastically reduced the HGF-induced phosphorylation of c-Met in both types of cell. The expression of c-Met protein was detected as two bands corresponding to a precursor form (upper band) and a proteolytically processed mature form (lower band; Fig. 1A).

We then examined the effect of exogenous HGF on the invasive growth of the two cell lines using the collagen gel culture system in the presence of 10% FBS. Exogenous HGF enhanced the invasive growth of GFP-ACC2 and GFP-ACCM cells by 1.9-fold and 2.3-fold, respectively; anti-HGF neutralizing antibody largely abrogated these stimulatory effects ($P < 0.05$; Fig. 1B).

Signaling downstream of HGF/c-Met

To understand the pathway of HGF/c-Met signaling in human salivary gland cancer cells, we investigated the activation of two known major downstream elements, phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), using phospho-specific antibodies against serine residue 473 of Akt and threonine residue 202/204 of Erk1. Exogenous HGF markedly induced the phosphorylation of Akt on serine residue 473, whereas Erk1/2 was constitutively activated in both types of cell (Fig. 1C). Subsequently, we tested the phosphorylation of Stat3 and IκB-α, which are reported to be HGF/c-Met targets in other types of cell. Stat3 and IκB-α were also constitutively activated in both cell lines (Fig. 1C).

Effect of blockade of PI3K signaling on HGF-induced invasive growth

We tested the effect of the PI3K inhibitor LY-294002 on the HGF-stimulated invasive growth of the cells using the collagen gel culture system. LY-294002 largely inhibited HGF-stimulated invasive growth and suppressed the phosphorylation of Akt. These results suggest that the activation of PI3K signaling is essential for the HGF-stimulated invasive growth of human salivary gland cancer cells (Fig. 1D).

Expression of Akt isoforms in normal and malignant epithelial cells of human salivary glands

Overexpression and activation of the proto-oncogene Akt has been reported in many human malignancies, and is associated with tumorigenesis and malignant conversion (including mitosis, anti-apoptosis, and invasiveness) [6]. The proteins of the Akt family are serine/threonine kinases. Mammals have three homologous members, known as Akt1, Akt2, and Akt3. We examined the expression of the Akt isoforms in primary cultured epithelial cells, tissues derived from normal human salivary glands, and salivary gland cancer cells using Western blotting. None of the Akt isoforms was strongly expressed in normal human salivary glands, whereas all three were overexpressed in GFP-ACC2 and GFP-ACCM (Fig. 1E). We also examined the expression of Akt isoforms in human normal salivary gland and human salivary gland cancer (SGC) tumor tissues derived from SGC patients using Western blotting. Any Akt isoform almost was not expressed in human normal salivary gland, but all Akt isoforms (Akt1, 2, and 3) were expressed in human SGC tumors (Fig. 1F).

Optimizing the concentration of synthetic siRNA transfected into human salivary gland cancer cells

The selective knockdown of genes of interest using siRNA has become a powerful tool for studying gene function. However, the

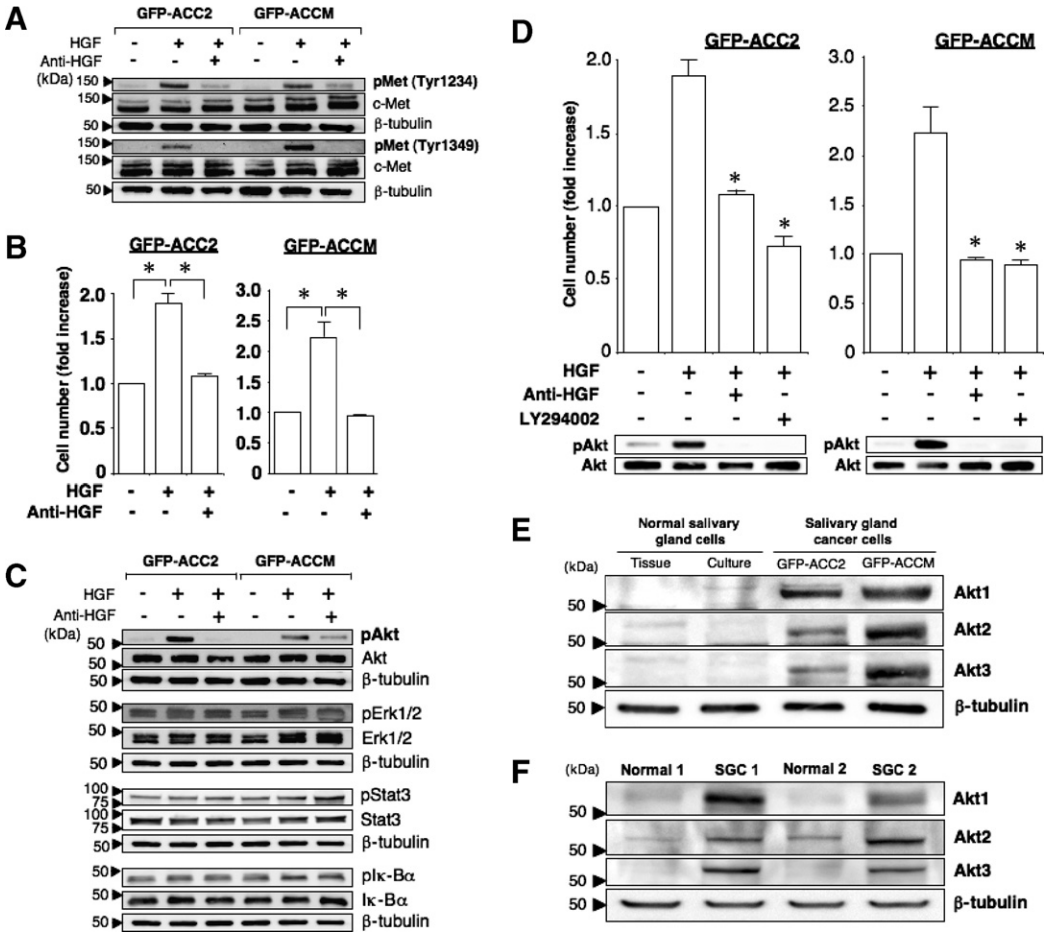

Fig. 1. Role of HGF/c-Met/PI3K/Akt pathway in human salivary gland cancer (SGC) cells. (A) Effect of HGF on the phosphorylation of c-Met in human SGC cells. (B) Effect of HGF on the invasive growth of human SGC cells. (C) Effect of HGF on the phosphorylation of Akt, Erk1/2, Stat3 and IκB-α in human SGC cells. (D) Effect of blockade of the PI3K pathway on the HGF-induced invasive growth of human SGC cells. (E) Expression of Akt1, 2, 3 in human normal salivary glands and SGC cells. (F) Expression of Akt1, 2, 3 in normal salivary gland and human SGC tissues.

transfection of siRNA can also have non-specific effects by activating interferon-stimulated genes involved in the stress response, and can cause the non-specific inhibition of cell growth [7]. These non-specific cellular responses can complicate the interpretation of siRNA-knockdown results. To address this issue, it is necessary to confirm the specificity of responses to the knockdown of genes targeted by experimental siRNA. To test for induction of the interferon response by transfection of synthetic siRNA, synthetic siRNA specific for GFP (siGFP; 0–100 nM) and control siRNA were transfected into GFP-ACCM cells. The induction of the mRNAs of four interferon-response genes, OAS1, OAS2, MX1, and ISGF-3γ, was examined by real-time quantitative RT-PCR. The mRNAs were weakly induced by concentrations of synthetic siRNA up to 20 nM, but were markedly induced by 50 nM siRNA (Supplementary Fig. S1A).

Subsequently, siGFP was also transfected into GFP-ACCM cells, and we examined the effect of the siRNA on the growth of the cancer cells. Synthetic siRNA at concentrations of 50 and 100 nM reduced cell growth by 20% and 50%, respectively (Supplementary Fig. S1B). These results indicated that the concentration of synthetic siRNA for transfection should be less than 20 nM.

Identification of synthetic siRNAs specific for Akt1, Akt2, and Akt3

We designed five specific synthetic siRNAs for each Akt isoform (siAkt1, siAkt2, and siAkt3) using target sequences optimized for maximum gene silencing and minimal sequence-specific cross reactivity (off-target effects) with B-Algo and siPRECISE algorithms (Table 1).

Synthetic siAkt1, siAkt2, and siAkt3 were transfected into GFP-ACCM cells at a concentration of 10 nM, and their effects were examined by Western blot analysis. Each siRNA reduced the expression of the corresponding Akt isoform by 80–90%, and the effects were isoform specific (Supplementary Fig. S1C and D). The effects of RNAi of siAkt1, siAkt2, and siAkt3 on the GFP-ACC2

Table 1
Target sequences of synthetic siAkt1, siAkt2, and siAkt3

siRNA	Target sequence
siAkt1-1	gagcgggaggaguggacaa
siAkt1-2	ccaugaagauccucaagaa
siAkt1-3	ccaaggagaucaugcagca
siAkt1-4	gggguuuacccagugggaca
siAkt1-5	ggacagaggagcaaggguuu
siAkt2-1	ccacaagcguggugaauac
siAkt2-2	gcagaaugccagcugauga
siAkt2-3	cgacugaggagauggaagu
siAkt2-4	gggcuaaagugaccaugaa
siAkt2-5	caagggaaccuuuggcaaa
siAkt3-1	gcaaaaugccaguuaauga
siAkt3-2	agagaaggcaaguggaaaa
siAkt3-3	caccagagguguuagaaga
siAkt3-4	ggcaagauguauaugauaa
siAkt3-5	gcucagacuauuacaauaa

cells were similar to those on the GFP-ACCM cells (data not shown).

Effects of siAkt1, siAkt2, and siAkt3 on the HGF-induced invasive growth of human salivary gland cancer cells

We tested the effects of siAkt1, siAkt2, and siAkt3 on the HGF-stimulated invasive growth of human salivary gland cancer cells using the collagen gel culture system. The knockdown of Akt1 expression largely abrogated the invasive growth induced by exogenous HGF in both GFP-ACC2 and GFP-ACCM cells (Fig. 2A); siAkt2 and siAkt3 also largely suppressed the invasive growth induced by HGF in these cells (Fig. 2B and C).

Discussion

Activation of HGF/c-Met signaling enhances the invasive growth of human salivary gland cancer cells by activating the PI3K/Akt pathway, even in the presence of FBS. To examine the oncogenic function of the Akt isoforms in human salivary gland cancer cells, we constructed synthetic siAkt1, siAkt2, and siAkt3, which had RNAi effects at concentrations less than 20 nM, with little or no off-target effects. siAkt1, siAkt2, and siAkt3 specifically knocked down the expression of the corresponding isoforms. The targeting of any one of the three Akt isoforms largely abrogated the HGF-induced invasive growth of the human salivary gland cancer cells. Therefore, all of the Akt isoforms appear to be required for

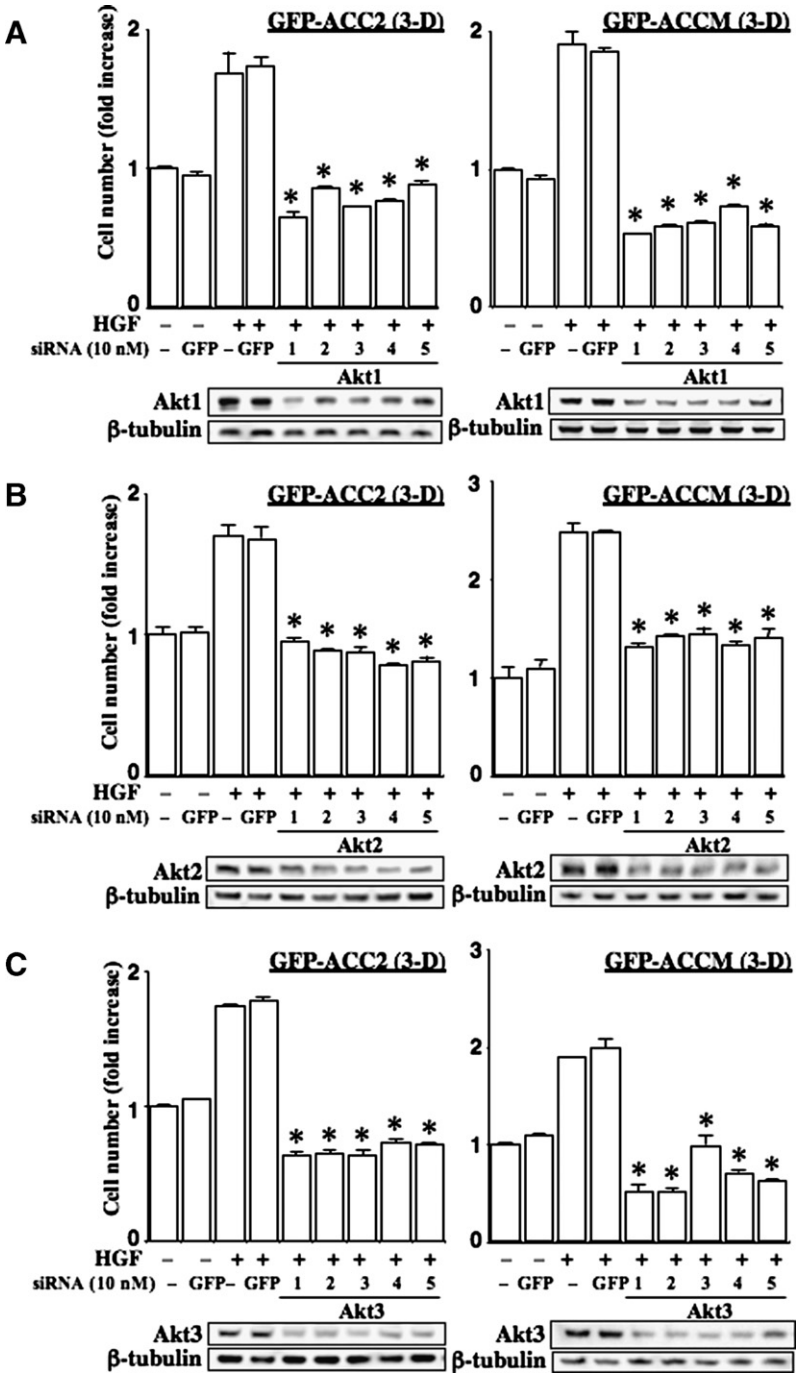

Fig. 2. Effects of siAkt1, siAkt2, and siAkt3 on HGF-induced invasive growth of human salivary gland cancer (SGC) cells. Effects of knockdown of Akt1 (A), Akt2 (B), and Akt3 (C) on HGF-induced invasive growth of human SGC cells in the collagen gel culture system.

the HGF-induced invasive growth of human salivary gland cancer cells. These results suggest that the blockade of HGF/c-Met/PI3K/Akt signaling could be an effective approach for the treatment of salivary gland cancer.

Although the HGF/c-Met system promotes aspects of malignancy (such as invasion, metastasis, and angiogenesis), it also plays major roles in organ formation (tissue organization) during embryogenesis and in adult tissue homeostasis (tissue regeneration) [4–6,8]. Mice lacking HGF or the c-Met/HGF receptor gene fail to complete development and die *in utero* at embryonic day 13–15 due to impaired organogenesis of the liver, kidney, and placenta [8]. In the adult, HGF prevents the onset of acute and chronic renal failure, and acts as a pulmotrophic factor that enhances lung regeneration and suppresses the onset of lung fibrosis [9]. Based on its diverse biological activity, targeting the HGF/c-Met system would be expected to have severe side-effects.

PI3K participates in various signal-transduction pathways that are implicated in many cellular responses, including fibroblast growth, transformation, survival, and chemotaxis [10]. p85α is the most abundantly expressed regulatory isoform of PI3K [11]. Mice with a targeted gene disruption of p85α have impaired B-cell development at the pro-B cell stage, and reduced numbers of mature B cells and B-cell proliferative responses. Although $p85\alpha^{-/-}$ mice were born and grew normally under pathogen-free conditions, 70–80% died within 10 weeks because of bacterial infection. Thus, targeting PI3K could also be dangerous.

Akt lie downstream of PI3K, and is expressed and activated in many human malignancies. Of the three human isoforms, Akt1 is expressed at high levels in most tissues, with the exception of the kidney, liver and spleen. Akt2 expression varies in different tissues, with higher levels in the muscle, intestinal organs, and reproductive tissues [12,13]. Akt3 is most strongly expressed in the brain and testis, with lower expression levels in the intestinal organs and muscle tissues [14]. Increased expression and activation levels have been reported for Akt1 in prostate cancer, breast cancer, and ovarian cancer, for Akt2 in pancreatic cancer, breast cancer, and ovarian cancer, and for Akt3 in prostate cancer and breast cancer [11,15].

Although targeting Akt could be an effective approach for the treatment of human salivary gland cancer, some problems remain to be overcome before its clinical application. Mice lacking Akt1 have increased perinatal mortality and reduced body weight by 20–30% [16]. Akt2-deficient mice are born in the expected Mendelian ratio and display normal growth [17]. Akt3-deficient mice have reduced brain weight resulting from decreased cell size and number, but maintain normal glucose homeostasis and body weight [18]. By contrast, Akt1/Akt2 double-knockout (DKO) mice exhibit severe growth deficiency and die shortly after birth displaying impaired skin development, skeletal muscle atrophy, delayed bone development, and impaired adipogenesis [19]. Akt1/Akt3 DKO causes embryonic lethality at around embryonic days 11–12, with severe developmental defects in the cardiovascular and nervous systems [20]. These data suggested that targeting all of the Akt isoforms simultaneously might cause severe side-effects, even if this approach had good anti-tumor activity. We therefore tested the effect of each isoform separately, using isoform-specific siRNAs, which might provide more useful targets with fewer side effects. We found that the knock down of any one of the Akt isoforms abrogated the invasive growth induced by HGF. Hence, these isoforms could be useful target molecules for the treatment of salivary gland cancer.

In conclusion, our study suggests that the activation of the PI3K/Akt pathway is required for the HGF-stimulated invasive growth of human salivary gland cancer cells. Targeting any single Akt isoform could be an effective approach for the treatment of salivary gland cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.042.

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