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Betel quid extract promotes oral cancer cell migration by activating a muscarinic M4 receptor-mediated signaling cascade involving SFKs and ERK1/2

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ABSTRACT

Betel quid (BQ) is a widely accepted etiological factor for oral squamous cell carcinoma (OSCC) in Southeast Asia, but how BQ chewing leads to oral carcinogenesis remains to be elucidated. We have previously demonstrated that the activation of Src family kinases (SFKs) is critical for BQ-induced oral cancer cell motility. Here we investigate whether this biological effect is mediated by specific membrane receptors in oral cancer cells. We found that BQ-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and cell migration could be inhibited by atropine, suggesting the involvement of the muscarinic receptor family. The enhanced activities of ERK1/2 and cell migration were significantly counteracted by PD102807, the selective antagonist of muscarinic M4 receptor. Moreover, cold BQ extract effectively competed with a known ligand, [^{3}H]-*N*-methyl scopolamine, for binding to muscarinic M4 receptor in vitro, thereby implying that BQ could activate motility-promoting signaling pathways through direct interaction with the receptor. The requirement of muscarinic M4 receptor for BQ-induced oral cancer cell migration was demonstrated by knockdown of the receptor using RNA interference (RNAi). Remarkably, ectopic expression of muscarinic M4 receptor in two oral cancer cell lines, Ca9-22 and SCC-9, further augmented BQ-induced cell migration by 83% and 99%, respectively. Finally, we verified that BQ-induced oral cancer cell migration was mediated through a muscarinic M4 receptor → SFKs → ERK1/2 signaling pathway. Thus, our findings have identified a novel signaling cascade mediating BQ-induced oral cancer cell motility, which could be a therapeutic target for BQ-related oral malignancies.

1. Introduction

Betel quid (BQ) chewing prevails in Southeast Asia, largely accounting for a total of 600 million BQ users worldwide [1]. BQ usually contains areca nut, inflorescence of *Piper betle* and slaked lime. In Taiwan the incidence of oral cancer death has been steadily growing during the past decade. A recent report shows that oral squamous cell carcinoma (OSCC), the most widespread malignant neoplasm of the oral cavity, has become the sixth and the fourth common cancer in total and male population, respectively [2]. There is mounting epidemiological evidence indicating a close causal relationship between BQ chewing and OSCC [3–5]. In fact, approximately 85% of Taiwanese OSCC patients are BQ chewers [6,7]. Despite the fact that smoking and alcohol drinking are also important risk factors for OSCC and may act synergistically with BQ as a carcinogen [8], studies conducted by the International Agency for Research on Cancer (IARC) suggest that BQ without tobacco or areca nut alone is sufficient to cause carcinogenic effects in human. However, the molecular mechanism behind BQ-induced oral carcinogenesis is not fully understood.

G protein-coupled receptors (GPCRs) have been shown to initiate signaling cascades modulating a multitude of physiological responses including cell migration [9,10]. Muscarinic receptors are class I heptahelical GPCRs consisting of five subtypes, namely M1, M2, M3, M4 and M5 receptors [11]. When activated through binding to their canonical agonist, acetylcholine, muscarinic receptors have been shown to regulate many second messengers, such

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as cAMP/calcium-signaling pathway and ion channel activities, by coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins) [12]. Despite the well-characterized functions in neurotransmission, there is emerging evidence indicating that muscarinic receptors play an oncogenic role in many cancer types. For example, previous studies have reported that muscarinic receptors in small cell lung carcinoma are activated by acetylcholine, stimulating cell proliferation in an autocrine manner [13,14]. In addition, activation of M1, M3 or M5 receptors induces transformation of 3T3 cells [15], while activation of M3 and M5 receptors in murine adenocarcinoma cells induce cell growth [16]. The biological roles of muscarinic receptors in malignant tumors are relatively obscure.

We have previously shown that Src family kinases (SFKs) mediate BQ-induced oral cancer cell migration and invasion [17]. In this study, we focus on the identification of upstream membrane receptors that initiate such motility-promoting signaling pathway. Our results suggested that muscarinic M4 receptor mediated BQ-induced oral cancer cell migration through the activation of extracellular signal-regulated kinase 1/2 (ERK1/2). Besides, we found that BQ could physically interact with M4 receptor in vitro. Most importantly, knockdown of M4 receptor by siRNA inhibited BQ-induced cell migration while ectopic expression of M4 receptor further enhanced the effect in two oral cancer cell lines. Furthermore, our data indicated that SFKs acted upstream of ERK1/2 in this BQ-stimulated signaling cascade via M4 receptor. Thus, these results have defined a novel signaling pathway responsible for BQ-induced oral cancer cell motility.

2. Materials and methods

2.1. Antagonists, inhibitors and antibodies

PD102807 were purchased from Tocris Bioscience (Ellisville, MO) while SP600125, PP2, U0126 and PD98059 were from Calbiochem (San Diego, CA). Atropine was obtained from Sigma–Aldrich (St. Louis, MO). Anti-Myc and anti-ERK1/2 antisera were obtained from Cell Signaling Technology (Beverly, MA) whereas anti-M4 antiserum from Chemicon (Temecula, CA). Anti-Src (Src 2) antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2 and anti-phospho-Src family (pY416) antisera were purchased from Cell Signaling Technology.

2.2. BQ extract preparation

Preparation of BQ extract and determination of cytotoxicity were performed as described previously [18].

2.3. Cell culture and transient transfection

Oral cancer cell lines Ca9–22 and SCC-9 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with nutrient mixture F-12 (DMEM/F12; Invitrogen Technologies, Carlsbad, CA) and 10% FBS. For transient transfection, 8×10^5 of Ca9–22 or SCC-9 cells were seeded on 60 mm dishes 24 h before transfection. Later, the cells were transfected with pCMV6-Entry-M4-Myc (OriGene, Rockville, MD) or the control vector using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). 24–48 h after transfection, the cells were harvested for migration assay or Western blot analysis.

2.4. Migration assay

A total of 3×10^4 oral cancer cells in serum-free culture medium containing BQ extract and atropine, PD102807, U0126, or PD98059 were seeded onto the upper chamber of cell migration inserts (BD Biosciences, San Diego, CA). Cell migration assay was done according to our previous studies [19].

2.5. Competitive receptor-binding assay

Membrane homogenates containing human M4 receptor were purchased from PerkinElmer. Competition binding assays were carried out based on the commercialized protocol. Briefly, 500 μl of membranes (24 μg) were incubated with 25 μl of 22 nM [^{3}H]-NMS (Perkin–Elmer) and 25 μl of increasing concentrations of unlabeled BQ extract (final concentrations range from 0.18 to 1.44 mg/ml) in a total of 550 μl reaction for 120 min at 25 °C. The reactions were then filtered over GF/C filters (Perkin–Elmer) and the filters were washed nine times with 0.5 ml ice-cold wash buffer (50 mM Tris–HCl, 0.9% NaCl pH 7.4). The dried filters were assayed by a scintillation counter to determine [^{3}H]-NMS binding to M4 receptor in the presence of cold BQ extract.

2.6. RNA interference and its verification

siGENOME SMARTpool siRNA against M4 receptor was purchased from Dharmacon (Lafayette, CO). This reagent contains four different siRNA duplexes that are targeted to four different sites of M4 mRNA. Ca9–22 cells at 70% confluence were transfected with M4-siRNA (100 nM) using DharmaFECT transfection reagent (Dharmacon) for 48 h. Later, these cells were assayed for BQ-induced cell migration. Knockdown of M4 expression was verified by semi-quantitative RT-PCR using a pair of M4 primers as follows: 5′-CTCGAGATGGCCAACTTCACACCTGTCAAT-3′ (forward) and 5′-TCTAGACTACCTGGCAGTGCCGATGTTCCGA-3′ (reverse). A full-length coding sequence of M4 receptor with 1440 bp was amplified.

2.7. Western blot analysis

Ectopic expression of M4-Myc and total proteins of ERK1/2 and SFKs were detected by anti-Myc, anti-ERK1/2 and anti-Src (Src 2) antisera, respectively. Incubation with the primary antiserum was done at room temperature for 2 h. Detection of phosphorylated ERK1/2 and SFKs were carried out by using anti-phospho-ERK1/2 and anti-phospho-Src family (pY416) antisera, respectively. Primary antibody incubation was done at 4 °C overnight.

2.8. Statistics

Data reported represent the mean ± the standard deviation (SD) of at least three independent experiments that were each performed in triplicate. These results were analyzed by one-way ANOVA followed by a post hoc comparison using Tukey's test. In all cases, a p value of < 0.05 was considered statistically significant.

3. Results

3.1. Muscarinic M4 receptor mediates BQ-induced oral cancer cell migration and activation of ERK1/2

Since the major alkaloids of BQ structurally resemble the classical agonist of muscarinic receptors, acetylcholine, we asked whether these receptors were involved in BQ-induced oral cancer cell migration. Ca9–22 cells were mock treated, treated with BQ extract (0.4 mg/ml) only or together with atropine (200 nM), the general antagonist of the muscarinic receptor family, while such treated cells were allowed to migrate in transwell inserts for 20 h. As shown in Fig. 1A, we found that treatment of atropine resulted in a dramatic reduction of BQ-induced cell migration ($p < 0.001$). Previous reports have shown that GPCRs-triggered sig-

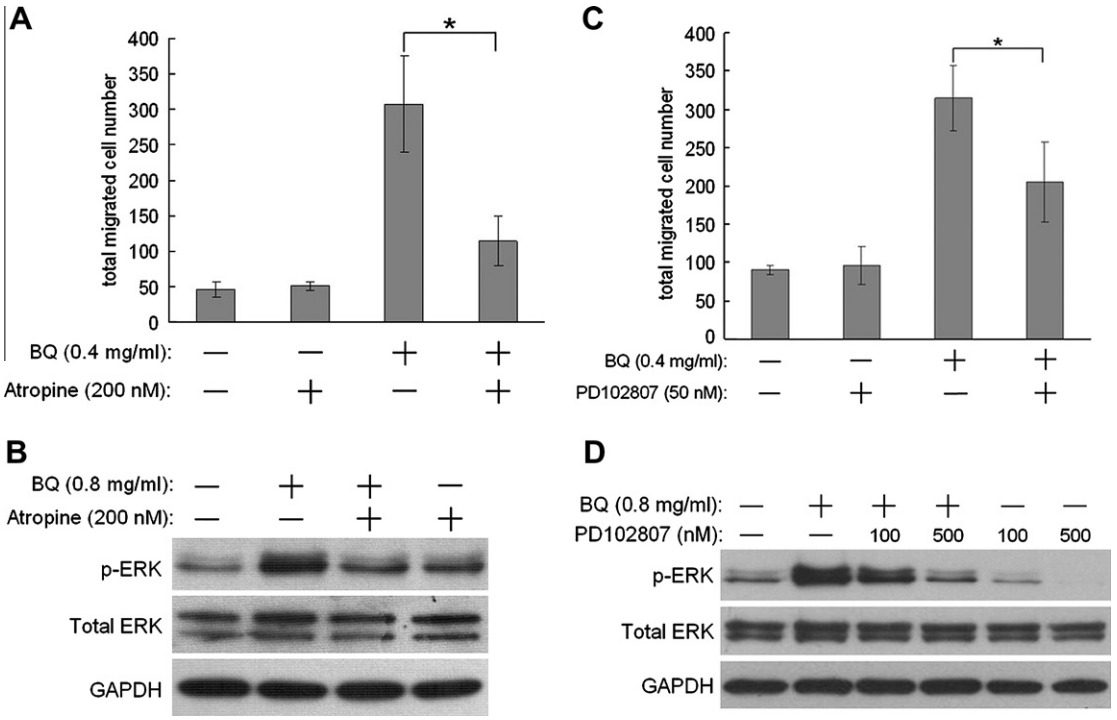

Fig. 1. Muscarinic M4 receptor mediates BQ-induced oral cancer cell migration and ERK1/2 activation. (A) 3×10^4 of Ca9–22 cells treated with BQ extract and 200 nM atropine for 20 h were analyzed for cell migration. $^*p < 0.001$ indicates a significant decrease of BQ-induced cell migration when atropine was present. (B) The effect of atropine on BQ-induced activation of ERK1/2 was examined. Cells were pretreated with atropine for 2 h and 50 min, and then with atropine and BQ for additional 10 min. Phospho-ERK (*p*-ERK) and total ERK protein were analyzed by immunoblotting using anti-phospho-ERK1/2 and anti-ERK1/2, respectively. (C) The inhibitory effect of PD102807 on BQ-induced cell migration ($^*p < 0.05$) and ERK1/2 activation was evaluated similar to (A) and (B). Migration assays were done in triplicate and the results are expressed as means ± SD. Data were analyzed by one-way ANOVA plus Tukey's post hoc test.

naling pathways converge at ERK1/2, suggesting that phosphorylation of ERK1/2 can be an indication of agonist-mediated activation of GPCRs including muscarinic receptors [20,21]. Indeed, our results revealed that BQ extract could dramatically induce phosphorylation of ERK1/2 10 min after the treatment, while this was significantly inhibited by atropine (Fig. 1B).

Muscarinic M4 receptor, a member of the muscarinic receptor family, has been reported to promote keratinocyte migration and wound healing [22]. Thus, we tested if M4 receptor could play a role in BQ-induced oral cancer cell migration. We showed that BQ-induced cell migration was attenuated by a selective antagonist of M4 receptor, PD102807 (50 nM) ($p < 0.05$), and that BQ-induced activation of ERK1/2 was inhibited by PD102807 in a dose-dependent manner (100 and 500 nM) (Fig. 1C and D). These results indicated that the BQ-induced effects could be mediated through M4 receptor.

3.2. Muscarinic M4 receptor is directly involved in BQ-induced oral cancer cell migration

We then investigated if M4 receptor played a direct role in this biological effect by testing physical interaction between BQ and M4 receptor using competitive receptor-binding assay. The data showed that binding between M4 receptor and a tritium-labeled known ligand, [^{3}H]-*N*-methyl scopolamine ([^{3}H]-NMS), was significantly inhibited by unlabeled BQ extract in a dose-dependent manner (0.18–1.44 mg/ml). On the other hand, this ligand-receptor interaction was abolished by atropine (500 nM) but not SP600125 (0.5 μM), a selective inhibitor of Jun *N*-terminal kinase (JNK) served as the irrelevant control (Fig. 2A). These results suggest that BQ extract can specifically interact with M4 receptor.

We then addressed the requirement of M4 receptor in BQ-induced cell migration. Endogenous M4 receptor of Ca9–22 cells was down-regulated by M4-specific siRNA oligos, and such siRNA-treated cells were assayed for cell migration with BQ treatment. We found that knockdown of M4 expression led to reduced BQ-induced cell migration, indicating that M4 receptor was required for the enhanced cell migration induced by BQ (Fig. 2B). Alternatively we used ectopic expression to verify the biological role of M4 receptor. Ca9–22 and SCC-9 cells were transfected with full-length M4 cDNA (pCMV6-Entry-M4-Myc) for 48 h, and BQ-induced cell migration was analyzed. We observed increased cell migration in M4-transfected Ca9–22 ($p < 0.05$) and SCC-9 cells even without BQ treatment. Remarkably, ectopic expression of M4 receptor resulted in an additional 83% increase ($p < 0.05$) for Ca9–22 and 99% increase ($p < 0.01$) for SCC-9 in BQ-induced cell migration (Fig. 2C). Together, these results clearly demonstrate that M4 receptor is directly involved in this biological effect.

3.3. SFKs act downstream of muscarinic M4 receptor but upstream of ERK1/2 in the BQ-activated signaling pathway

We have previously demonstrated the involvement of SFKs in BQ-induced oral cancer cell motility. Thus, we investigated whether SFKs participated in BQ-activated signal transduction emanated from M4 receptor. Ca9–22 cells were treated with BQ extract alone or together with PD102807 (100 nM) for 10 min. Activation of SFKs was evaluated by Western blot analysis using anti-phospho-Src family (pY416). Fig. 3A shows that BQ-induced phosphorylation of SFKs was decreased by PD102807, suggesting that SFKs acted specifically downstream of M4 receptor.

To determine the signaling order between SFKs and ERK1/2, we treated the cells with PP2, the selective inhibitor of SFKs, and asked how this might influence the activation of ERK1/2 induced by BQ. As illustrated in Fig. 3B, we found that BQ-induced phosphorylation of ERK1/2 was significantly inhibited by increasing concentrations of

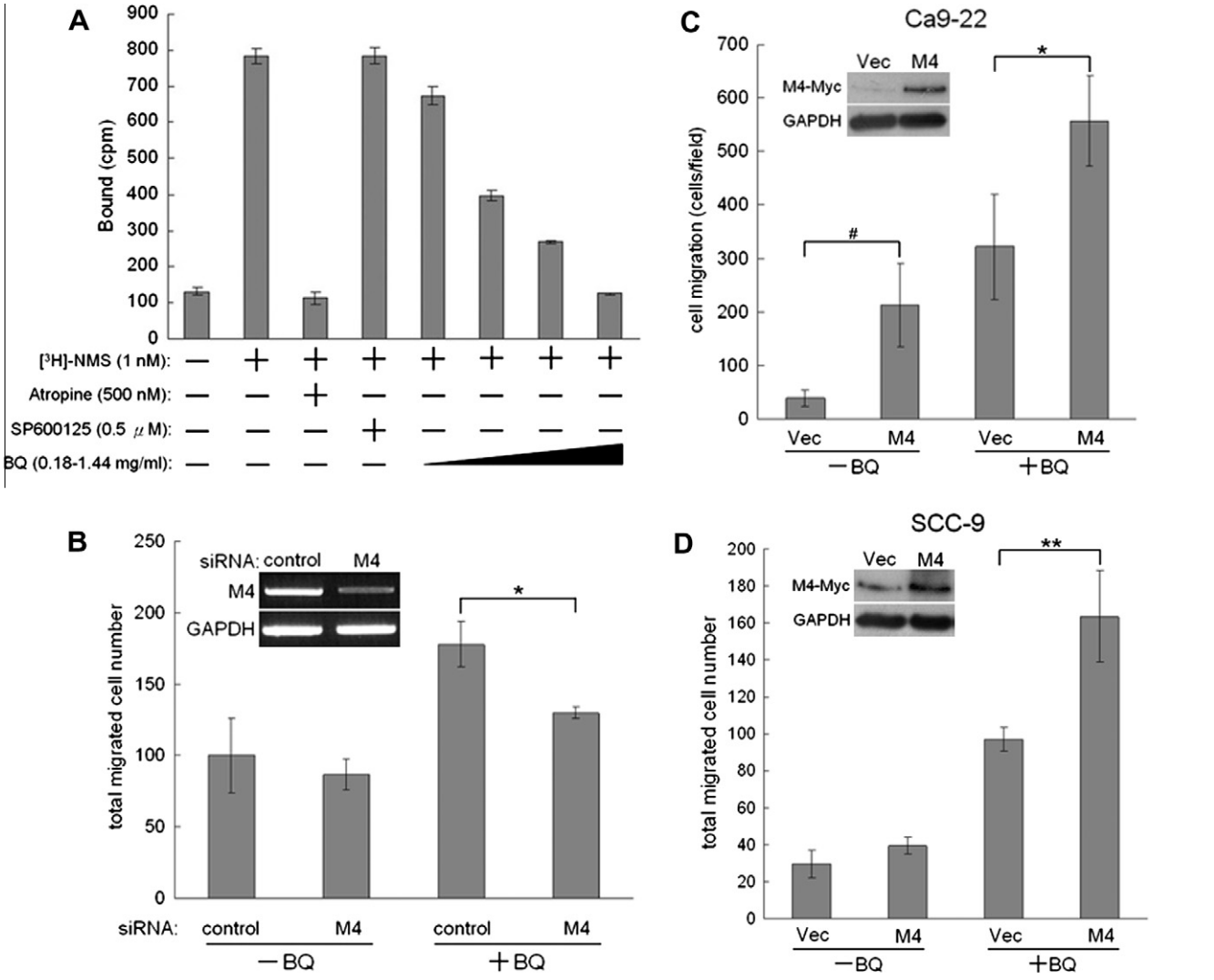

Fig. 2. Direct involvement of muscarinic M4 receptor in BQ-induced oral cancer cell migration. (A) Binding of [^{3}H]-NMS to M4 receptor was examined by in vitro competitive binding assay in the presence of increasing concentrations of cold BQ extract (0.18, 0.36, 0.72 and 1.44 mg/ml). Atropine and SP600125 were the positive and negative control, respectively. (B) Ca9–22 cells were transfected with 100 nM of M4 siRNA oligos or irrelevant siRNA as the control, and then assayed for cell migration with or without BQ treatment. $^*p < 0.05$ indicates the inhibitory effect of M4 siRNA on BQ-induced cell migration compared to the control. Knockdown of M4 expression was confirmed by RT-PCR using specific primers detecting full-length mRNA (upper-left panel). (C) M4 receptor was ectopically expressed in two oral cancer cell lines, Ca9–22 and SCC-9, by transient transfection with pCMV6-Entry-M4-Myc (or M4-Myc) or empty vector (Vec). These cells were assayed for cell migration with or without BQ treatment. $^\#p < 0.05$ shows a significant increase in cell migration in M4-transfectants without BQ addition. $^*p < 0.05$ and $^{**}p < 0.01$ indicate a significant increase in BQ-induced cell migration in M4-transfected Ca9–22 and SCC-9, respectively. Immunoblotting confirmed ectopic expression of M4 receptor by anti-Myc antibody (upper-left panels). Experiments were done in triplicate and the data are presented as means ± SD. Statistical difference was revealed by one-way ANOVA plus Tukey's post hoc test.

PP2 (40 and 60 μM). In contrast, BQ-induced phosphorylation of SFKs was not affected by increasing concentrations of U0126 (0.25 and 0.5 μM), the selective inhibitor of ERK1/2, despite a dramatic inhibition of ERK1/2 phosphorylation by the inhibitor (Fig. 3C). These findings have implicated a signaling order that SFKs act downstream of muscarinic M4 receptor but upstream of ERK1/2 in BQ-activated signal transduction.

3.4. Activation of ERK1/2 is required for BQ-induced oral cancer cell migration

Although ERK1/2 has been suggested to regulate cell motility, whether it is required for BQ-induced oral cancer cell migration needs to be confirmed. Thus, we tested this by using the selective inhibitors of ERK1/2. Ca9–22 cells were treated with BQ extract and increasing concentrations of U0126 (20 and 100 nM) or PD98059 (0.5 and 2 μM), and cell migration was analyzed. Our data revealed that BQ-induced cell migration was significantly inhibited by U0126 in a dose-dependent manner (Fig. 4A). Consistently, treating cells with a higher dose of PD98059 (2 μM) could still result in a significant reduction in BQ-induced cell migration (Fig. 4B). These results indicate that BQ can induce cell migration through the activation of ERK1/2. Together, we have demonstrated that BQ-induced oral cancer cell migration can be mediated through a muscarinic M4 receptor→SFKs→ERK1/2 signaling cascade.

4. Discussion

Cumulative evidence suggests that lymph node metastasis contributes to poor prognosis and reduced 5 year survival rate in OSCC [23,24]. Therefore a better understanding of the molecular mechanism behind tumor metastasis in OSCC is urgently needed. BQ chewing has long been linked to oral carcinogenesis, but the biological role of BQ in promoting malignant progress of OSCC has not been defined. Our previous studies reveal that BQ can promote oral cancer cell motility through the activation of SFKs [17]. How-

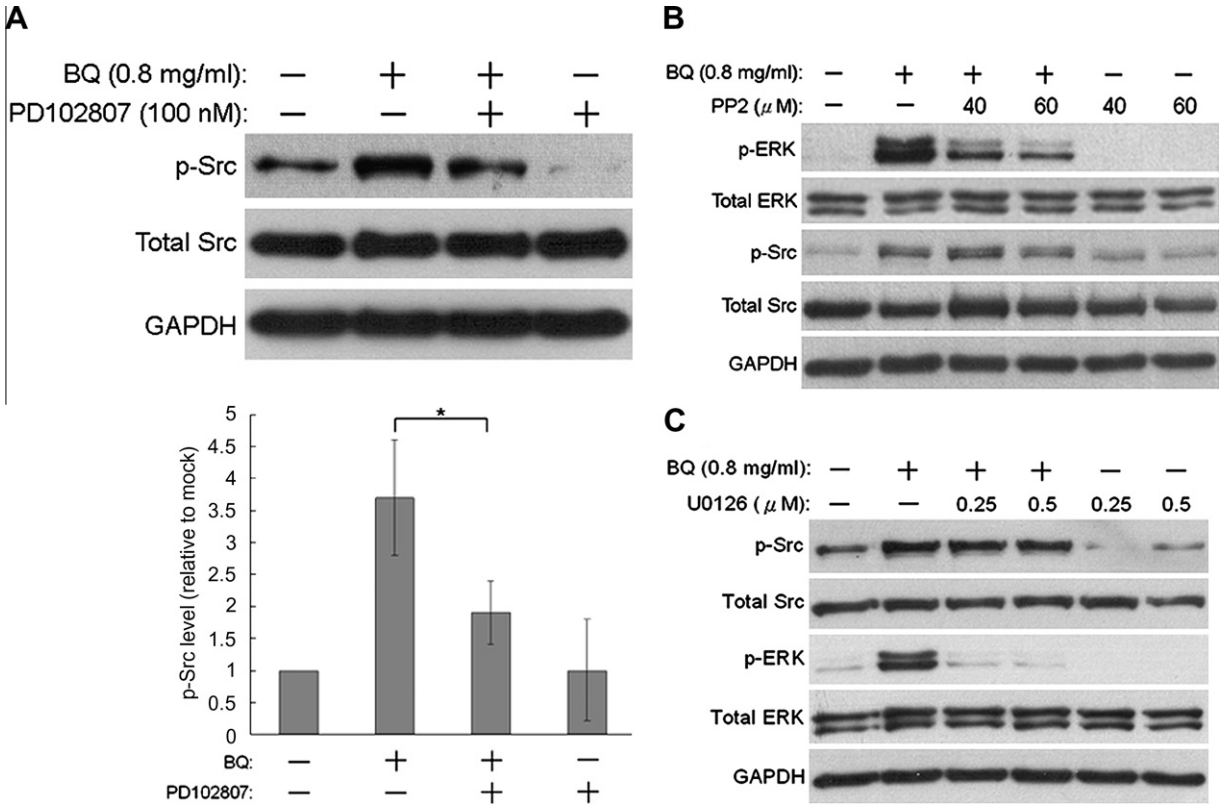

Fig. 3. SFKs are activated by BQ extract through muscarinic M4 receptor and act upstream of ERK1/2. (A) The effect of PD102807 on BQ-induced activation of SFKs was examined as described in Fig. 1. Phosphorylation of SFKs (p-Src) was revealed by Western blot analysis using anti-phospho-Src family (pY416). Experiments were repeated at least three times, and the representative results are shown. Quantification results of p-Src were obtained by densitometry (bottom panel). The intensity values were normalized to those of GAPDH and the data of mock treated were reset to 1.0. $^{*}p \leqslant 0.05$ indicates that PD102807 can significantly inhibit BQ-induced p-Src level. The results were analyzed by one-way ANOVA plus Tukey's post hoc test. The respective effect of increasing concentrations of PP2 (B) and U0126 (C) on BQ-induced activation of ERK1/2 or SFKs was evaluated as mentioned above.

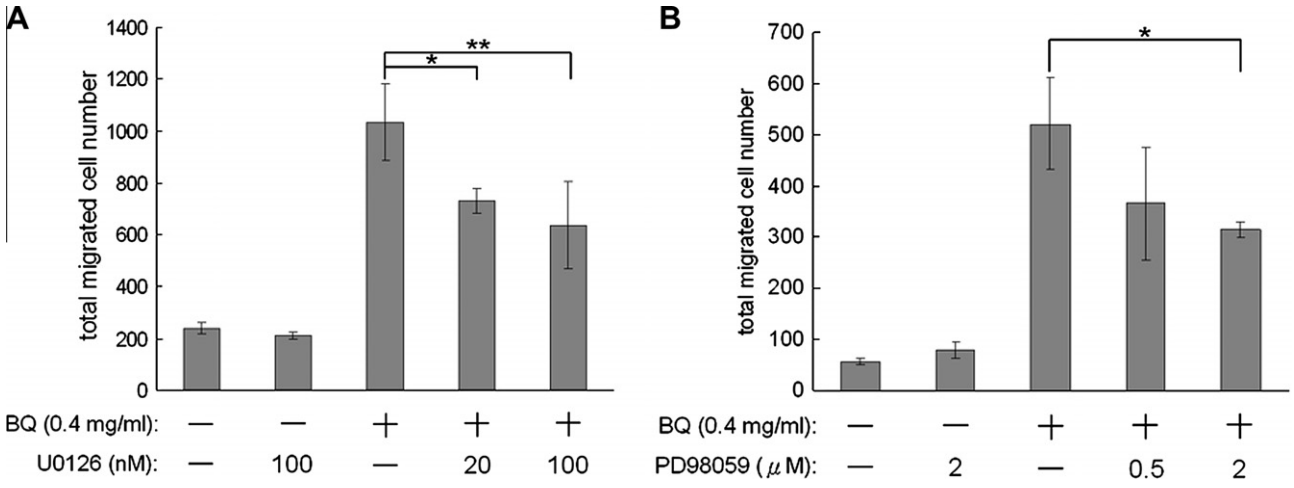

Fig. 4. BQ-induced oral cancer cell migration can be inhibited by the inhibitors of ERK1/2. The effects of increasing concentrations of U0126 (A) or PD98059 (B) on BQ-induced cell migration were examined. Migration assay was done as described. Results were expressed as means ± SD of $n = 3$ independent experiments, each of which was done in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant inhibition in BQ-induced cell migration (one-way ANOVA plus Tukey's post hoc test).

ever, it remains elusive whether BQ may interact with specific membrane receptors and thus activate motility-promoting signaling pathways. Muscarinic receptors are speculated to function in tumor cell motility because of the previous implications in cell movement. In particular, activation of M4 receptor stimulates migration and induces expression of migratory integrins [22]. On the other hand, arecaidine and arecoline, the two major alkaloids of BQ, are structurally similar to acetylcholine and have been shown to regulate signal transduction in brain and nervous system [25,26]. Collectively, we reasonably conjectured that muscarinic receptors were the candidate receptors mediating BQ-induced signal transduction.

We tested this hypothesis by using the selective antagonists of the muscarinic receptor family, and the results suggested that muscarinic M4 receptor participated in BQ-induced cell migration and signal transduction. Whereas the major alkaloids of BQ are

common agonists of muscarinic receptors, little is known about the ligand-binding specificity. Therefore, we examined if BQ could physically bind to M4 receptor in vitro. We found that BQ could significantly compete with a known ligand, [^{3}H]-NMS, for binding to M4 receptor in a dose-dependent fashion. In contrast, the JNK inhibitor SP600125 at 0.5 μM, a concentration 10-fold higher than its IC_{50} (~50 nM), had no effect on the ligand-receptor binding. These results indicate, for the first time, a selective interaction between BQ and M4 receptor. However, the specific ingredients of BQ interacting with the receptor are unknown. Testing of direct binding between the major BQ alkaloids and M4 receptor is under way. Nor is it currently understood if the interaction between BQ and M4 receptor leads to the coupling and activation of G proteins. Our preliminary results indicated that the cellular cAMP level remained unchanged upon BQ treatment, suggesting a lack of involvement of G proteins. Nonetheless subsequent analyses revealed that BQ-induced cell migration could be mediated, at least in part, by M4 receptor presumably through the activation of ERK1/2 signaling pathway. Several evidence indicates that GPCRs relay signals by activating ERK1/2 in a G protein-independent manner [27,28]. It thus appears possible that M4 receptor, upon binding and activation by BQ, signals through G protein-independent pathways to induce cell motility. In addition, we showed that M4 receptor is required for BQ-induced cell migration by siRNA knockdown and ectopic expression, strongly suggesting its direct involvement in this effect. Interestingly, we found that overexpression of M4 receptor seemed to increase cell migration even without BQ treatment, consistent with the previous studies suggesting a promoting role of M4 receptor in basal epithelial cell motility [22].

Muscarinic receptors play an essential role in diverse biological functions coupled to the activation of SFKs [29]. Indeed, by using the selective antagonist of M4 receptor, we demonstrated that BQ could induce activation of SFKs through M4 receptor. Furthermore, PP2 could significantly block the activation of ERK1/2, implicating that SFKs act downstream of M4 receptor but upstream of ERK1/2 in this signaling pathway. This is consistent with the findings by others that recruitment of c-Src at the cell membrane is necessary for subsequent activation of the Ras/ERK1/2 pathway mediated through β_2 adrenergic receptor, a member of the GPCR family [30]. However, it requires further investigations to identify other signaling factors that act between SFKs and ERK1/2 or downstream of ERK1/2 in this BQ-activated pathway via M4 receptor.

In conclusion, we have presented the first evidence that muscarinic M4 receptor can mediate BQ-induced oral cancer cell migration through the downstream motility-promoting signaling effectors including SFKs and ERK1/2. Although we cannot exclude the possibility that other members of the muscarinic receptor family are involved in this biological effect, our data have suggested that blockade of the interaction between BQ components and M4 receptor prevents the ensuing oncogenic signaling events leading to oral carcinogenesis. Antagonists or inhibitors targeting to this receptor or the pathway may thus provide a mechanistic basis for drug development and clinical therapeutics of BQ-related oral cancer.

Acknowledgments

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