Regular Article

Morphine Stimulates Migration and Growth and Alleviates the Effects of Chemo Drugs via AMPK-Dependent Induction of Epithelial–Mesenchymal Transition in Esophageal Carcinoma Cells

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The role of morphine, an opioid analgesic drug, in cancer biology has increasingly garnered attention due to its frequent usage in postoperative period for pain management in cancer patients. In this work, we demonstrated that morphine, at clinically relevant concentrations, stimulated migration and growth, and alleviated chemo drugs’ efficacy in esophageal carcinoma cells. Although morphine did not affect survival, it protected esophageal carcinoma cells from chemo drugs-induced apoptosis. Mechanistical studies showed that morphine increased RhoA but not Rac1 activity. In addition, morphine activated AMP-activated protein kinase (AMPK) pathway, induced epithelial–mesenchymal transition (EMT) via upregulating Snail and Slug levels, and increased oxidative stress in esophageal carcinoma cells. Rescue studies further demonstrated that the stimulatory effects of morphine in esophageal carcinoma cells are through activation of AMPK pathway but not RhoA or opioid receptor. In addition, morphine induced EMT in an AMPK-dependent manner whereas increased RhoA activity in an AMPK-independent manner. Our work demonstrates the protective role of morphine on esophageal carcinoma cells via AMPK activation, which may provide a new guide in clinical use of morphine for patients with esophageal carcinoma.

Key words morphine; epithelial–mesenchymal-transition; esophageal carcinoma; AMP-activated protein kinase

INTRODUCTION

Esophageal carcinoma is one of the leading causes of cancer-related mortality, and the incidence has been increasing in recent years.1 Curative resection is a primary treatment option for esophageal carcinoma with limited and locally advanced stages, but the recurrence is high and less than 25% of patients have long-term survival.2 Substantial evidence have shown that the type of anaesthesia chosen for cancer patients during perioperative and postoperative periods can be crucial and may potentially influence long-term outcomes of the disease.3 Opioids are commonly used medication to manage postoperative pain in patients with esophageal carcinoma. A recent work shows that high-dose (more than 1783.5 mg of total morphine administration over a 10-d period) postoperative opioid use is associated with an increased recurrence risk in patients with esophageal carcinoma.4

Morphine is a main member of the opiate family and acts as an opioid receptor agonist via binding and activating the μ-opioid receptor in the central nervous system.5 Apart from μ-opioid receptors, morphine also binds to N-methyl-D-aspartate (NMDA) receptor in neuronal cells.6 NMDA receptor is a non-classical opioid receptor and essential to the function of nervous system. NMDA receptors are expressed in human ovarian cancer tissues and human ovarian cancer cell lines.7 The effects of morphine on tumor growth is still contradictory, as both growth-promoting and growth-inhibiting effects have been observed.8,9 Apart from suppressing the immune system, accumulating evidence suggests that morphine affects multiple biological activities of tumor cells and tumor microenvironment such as angiogenesis through various signaling pathways.10 Morphin has been reported to promote breast cancer stem cell properties, contributing to cancer chemoresistance.11 Nevertheless, the effects of morphine in cancer biology has remained poorly understood.

In this work, we systematically investigate the effects of morphine alone and its combination with standard of care chemo drugs in esophageal carcinoma cells. We also attempt to dissect the underlying mechanisms of morphine’s action. Our work is the first to demonstrate that morphine stimulates migration and growth, and alleviates the effects of chemo drugs via AMP-activated protein kinase (AMPK)-dependent induction of epithelial–mesenchymal transition (EMT) in esophageal cancer cells.

MATERIALS AND METHODS

Cell Culture and Drugs Human esophageal carcinoma cell lines KYAE-1 and OE33 (ECACC, U.K.) were cultured in RPMI-1640: Hams F12 (1:1, Invitrogen, U.S.A.) and RPMI-1640, respectively, supplemented with 2 mM glutamine and 10% fetal bovine serum (Invitrogen). Both are representative human esophageal carcinoma cell models and are tumorigenic in mice.12 KYAE-1 was derived from a patient with non-Barrett-esophageal adenocarcinoma who was pre-treated with radiation and chemotherapy. KYAE-1 harbours a P53 mutation.13 OE33 was derived from a patient with Barrett-esophageal adenocarcinoma. Cisplatin, 5-FU and paclitaxel,
Compound C (AMPK inhibitor), N-acetyl-L-cysteine (NAC) and naloxone were obtained from Sigma, U.S.A. Morphine-HCL (Sintetica SA, Switzerland) was obtained from the Department of Pharmacy, Xiangyang Central Hospital.

5-Bromo-2'-deoxyuridine (BrdU) Proliferation Assay
Cell proliferation was determined by using BrdU Cell Proliferation Assay Kit (Cell Signaling, U.S.A.) which detects BrdU incorporated into cellular DNA during proliferation using an anti-BrdU antibody. 0.5 to 1 × 10⁴ cells were plated onto 96-well plate in culture medium at 37°C. The next day, drugs at different concentrations or combinations were added to each well and incubated for 3 d. Proliferating cells were then determined as per manufacturer’s protocols and the absorbance at 450 nm was measured on microplate reader.

Apoptosis Assay
Apoptosis was determined by measuring cytosolic oligonucleosome-bound DNA using a Cell Death enzyme-linked immunosorbent assay (ELISA) kit (Roche, U.S.A.) which is based on the quantitative sandwich-enzymeimmunoassay-principle using monoclonal antibodies directed against DNA and histones. 2 × 10⁵ cells were plated onto 6-well plate in culture medium overnight at 37°C. The next day, drugs at different concentrations or combinations were added to each well and incubated for 3d. Proliferating cells were then determined as per manufacturer’s protocols and the absorbance at 450 nm was measured on microplate reader.

Migration Assays
Migration assay was done using Boyden Chambers containing polyethylene membranes (8.0 μm pore size; BD Falcon, U.S.A.). The inserts were placed into BD Falcon cell culture insert companion 24 well plates (Becton Dickinson, U.S.A.). Culturing medium were placed on the lower chamber whereas drugs together with 10⁴ cells were seeded on cell culture insert. After 8 h incubation, cells on the upper surface of the insert were removed with a cotton swab. Migrated cells on the lower surface of inserts were fixed, and stained with Giemsa (Sigma). The migrated cells were counted and quantified using light microscopy.

RhoA and Rac1 Activity Assays
2 × 10⁵ cells were plated onto 6-well plate in culture medium overnight at 37°C. The next day, drugs were added to each well and incubated for 24h. Cells were harvested and homogenized using a standard protocol. RhoA and Rac1 activities were assessed using total cell lysates and were measured using kits from Abcam. Rac1 Activation Assay Kit and RhoA Activity Assay Kit use PAK1 PBD and Rhotekin RBD agarose beads to selectively isolate and pull-down the active form of Rac and RhoA, respectively.

Reactive Oxygen Species (ROS) Measurement
10⁴ cells were plated onto 96-well plate in culture medium overnight at 37°C. The next day, drugs were added to each well and incubated for 24h. Intracellular ROS levels were determined using Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, U.S.A.) which uses a ROS sensor to quantify ROS in live cells. Signals at Ex/Em = 520/605 nm were measured on fluorescence microplate reader.

Denaturing Sodium Dodecyl Sulfate-Polyacrylamide
**Gel Electrophoresis (SDS-PAGE) and Western Blot (WB) Analyses**

10^6 cells were plated onto flask in culturing medium overnight at 37°C. The next day, drugs were added to each well and incubated for 24h. Cells were homogenized in RIPA lysis buffer (Invitrogen) containing 1× protease inhibitor cocktail (Roche). Equal amount of total protein extracts was separated by SDS-PAGE and then processed for Western blot analysis by using antibodies recognizing phospho-AMPKα, -ACC, -myosin phosphatase-targeting subunit 1 (MYPT1) and -MCL, and their corresponding total proteins; E-cadherin, Vimentin, Snail and Slug (Cell Signalling Technology, U.S.A.).

**Statistical Analyses**

All data are expressed as mean ± standard error of the mean (S.E.M.). For comparison between groups of two categorical variables, these were analysed by the Student’s t-test. Across multiple groups, one way ANOVA with post-hoc Tukey was performed. A p-value <0.05 was considered statistically significant.

**RESULTS**

**Clinically Relevant Doses of Morphine Promote Migration and Proliferation of Esophageal Carcinoma Cells while Sparing Survival**

Migration is the common phenotype in epithelial cancers. We firstly investigated the effects of morphine on esophageal carcinoma cell migration using Boyden Chamber migration assay. KYAE-1 and OE33 are representative in vitro esophageal carcinoma cell models with epithelial type but derived from different patients with varying genetic background. We found that morphine at all tested concentrations significantly increased migration in both KYAE-1 and OE33 cells in a dose-dependent manner (Figs. 1A, B). We further found that morphine at 2 and 10 µM but not 0.5 µM significantly stimulated esophageal carcinoma cell growth as measured by BrdU labeling (Fig. 1C). In contrast, morphine up to 10 µM did not affect esophageal carcinoma cell apoptosis (Fig. 1D). Our results demonstrate the stimulatory effects of morphine on esophageal carcinoma cell growth and migration. We further investigated the effects of morphine on the growth and migration of normal human esophageal epithelial cells under the same experimental conditions as esophageal carcinoma cells. We found that morphine also stimulated growth and migration in normal esophageal epithelial cells (Supplementary Fig. 1).

**Morphine Protects Esophageal Carcinoma Cells from Chemo Drugs-Induced Toxicity**

To simulate the clinical settings, we next investigated whether morphine affects standard of care chemo drugs in esophageal carcinoma. We treated KYAE-1 and OE33 cells with morphine or paclitaxel alone or combination of both and measured migration, growth
and apoptosis. We found that paclitaxel significantly inhibited migration and growth, and induced apoptosis in esophageal carcinoma cells (Fig. 2). In addition, morphine at 10 µM remarkably abolished the anti-migratory and anti-proliferative effects of paclitaxel (Figs. 2A, B). Although morphine alone did not affect apoptosis, it significantly reversed the apoptosis induced by paclitaxel (Fig. 2C). These results suggest that morphine protects esophageal carcinoma cells from paclitaxel-induced toxicity. It is worth noting that the protective effects of morphine in esophageal carcinoma is not limited to paclitaxel; similar phenomena were observed to cisplatin and 5-FU (Fig. 2), suggesting a general protective effect by morphine in esophageal carcinoma in response to chemo drugs.

**Morphine Increases RhoA Activity, Activates AMPK, Upregulates EMT and Increases ROS in Esophageal Carcinoma Cells**

Rho guanosine 5′-triphosphatases (GTPases), including RhoA and Rac1, are well known essential molecules regulating all types of cell migration and to contribute to proliferation. We found that morphine significantly increased RhoA but not Rac1 activity in esophageal carcinoma cells in a dose-dependent manner, suggesting the specific stimulatory effect of morphine to RhoA (Figs. 3A, B). In addition, Western blot analysis indicated that morphine dose-dependently increased phosphorylation of AMPK at Thr172 and its downstream substrate ACC at Ser79 (Figs. 3C, D), suggesting the activation of AMPK pathway in morphine-treated esophageal carcinoma cells.

Decreased E-cadherin and increased Vimentin expression were observed in morphine-treated cells (Figs. 3C, D), suggesting that morphine induces EMT in esophageal carcinoma cells. Snail and Slug are zinc-finger transcriptional factors that downregulate E-cadherin expression and upregulate Vimentin expression by binding E-boxes. Consistent with EMT induction, we observed the increased Snail and Slug levels in morphine-treated cells (Figs. 3C, D), suggesting that morphine induces EMT through regulating Snail and Slug. Rho-associated protein kinase (ROCK) is the downstream effector of RhoA pathway. ROCK activation leads to the phosphorylation of MYPT1 on thr853, and subsequently phosphorylates myosin light chain (MLC) at Ser19 to regulate cell migration. We further found that morphine increased phosphorylation of MYPT1 (Thr853) and MLC (Ser19) (Figs. 3C, D), which correlated well with the increased RhoA activity by morphine. Oxidative stress has been reported to be closely associated with EMT induction in tumor. Morphine significantly increased reactive oxygen species (ROS) level in esophageal carcinoma cells (Fig. 3E), indicating that morphine induces oxidative stress. Taken together, morphine increases RhoA activity, activates AMPK, upregulates EMT and increases ROS in esophageal carcinoma cells.

**The Stimulatory Effects of Morphine in Esophageal Carcinoma Cells Are through AMPK-Dependent EMT Induction**

We next attempted to rescue morphine’s effects using NAC (an antioxidant), naloxone (a µ-opioid receptor antagonist) and Compound C (an AMPK inhibitor). The concentrations of NAC, naloxone and Compound C used in our work are referred from the established reported literatures. Compound C has been identified as a pharmacological AMPK inhibitor that efficiently blocks metabolic actions of AMPK and widely used in cell-based, biochemical, and in vivo assays. Experimental evidence also indicated that Compound C at 10 µM remarkably decreased p-AMPK level (Fig.
Compound C but not NAC or Naloxone significantly reversed the stimulatory effects of morphine in migration (A) and proliferation (B) in KYAE-1 cells. Compound C at 10µM, NAC at 10mM, naloxone at 5µM and morphine at 10 nM were used. (C) Representative Western blot images of KYAE-1 cells treated with morphine in the presence of compound C. (D) Quantification of Western blot band densities using Image J software from three independent experiments showing the expression level of p-AMPKα, p-ACC, Vimentin, Snail, p-MYPT1 and p-MLC in KYAE-1 cells exposed to morphine, Compound C alone or both. (E) Compound C does not affect the stimulatory effect of morphine in RhoA activity. *, p < 0.05, compared to morphine.

4C) and NAC at 10mM completed rescued morphine induced-oxidative stress in esophageal carcinoma cells (Supplementary Fig. 2). We found that Compound C but not NAC or naloxone significantly abolished the pro-migratory and pro-proliferative activities of morphine (Figs. 4A, B), indicating that the stimulatory effects of morphine in esophageal carcinoma cells are via activating AMPK but not ROS or opioid receptor. We further observed that Compound C reversed the effects of morphine in increasing p-AMPK, p-ACC, Vimentin and Snail levels but not p-MYPT1 or p-MLC (Figs. 4C, D), suggesting that morphine induces EMT in an AMPK-dependent manner. In contrast, morphine increases RhoA activity in an AMPK-independent manner. This is also consistent with our result that Compound C did not reverse the effects of morphine in increasing RhoA activity in esophageal carcinoma cells (Fig. 4E). Our results demonstrate that the stimulatory effects of morphine in esophageal carcinoma cells are through AMPK-dependent EMT induction.

We further investigated the effect of fasudil, a specific RhoA inhibitor, on the morphine-induced EMT in esophageal carcinoma cells by determining the level of p-MYPT1, vimentin and snail in the cells exposed to morphine or fasudil alone, and both. As expected, fasudil remarkably decreased p-MYPT1 (Supplementary Fig. 3). We found that vimentin and snail levels were significantly lesser in cells exposed to both morphine and fasudil compared to cells exposed to morphine alone, suggesting that fasudil reverses morphine-induced EMT. However, fasudil alone remarkably decreased the levels of vimentin and snail and therefore whether the rescue was due to the direct effect of fasudil or the effect of fasudil on the morphine-induced EMT is inconclusive. We found that compound C partially but significantly abolished the protective effects of morphine in chemo drug-induced inhibition of migration, growth and survival in esophageal carcinoma cells (Fig. 5). These results suggest that AMPK activation is involved in morphine-induced suppression of chemo drug’s toxicity in esophageal carcinoma cells.

DISCUSSION

Opioids such as morphine are frequently given to cancer patients to manage pain associated with comorbid conditions, in particular those with terminal illness. The association of opioids with cancer progression is controversial and importantly, the effect of opioids analgesia on longer-term recurrence, overall survival or disease-free survival requires confirmation in prospective, randomized clinical trials where data may not be available for many years. To explore the role of opioids in tumor development, increasing studies have attempted to evaluate their action and underlying mechanisms using cell culture system and xenograft mouse models. However, the role of morphine in cancer metastasis and recurrence remains poorly understood and appears to differ depending on the cancer cell type. In this work, we clearly demonstrate that morphine has stimulatory effects as a single drug and protective effects as a combination with chemo drugs in esophageal carcinoma cells, via non-classical opioid receptor signaling.

The cell lines, we selected for demonstration of the biological effects of morphine are often used to model esophageal
carcinoma. In our study, morphine at clinically relevant concentrations significantly increased migration and growth while sparing survival in these cells (Fig. 1). In addition, morphine displayed a general protective effect in oesophageal carcinoma in response to all tested chemo drugs (Fig. 2). Notably, although morphine did not affect survival, it significantly protected chemo drugs-induced apoptosis in esophageal carcinoma cells (Figs. 1D, 2C). Our results agree with the previous work on the ability of morphine in promoting cancer cell growth, invasiveness and aggressiveness in breast cancer, renal cell carcinoma and bladder cancer.\(^{13,37,38}\) Our work also supports the notion that morphine acts a potential antagonist of chemo drugs in cancer.\(^{39}\) On the other hands, the anti-migratory, anti-proliferation and pro-apoptotic effects of morphine have been shown in lung and oral cancers.\(^{40,41}\) The differential effects of morphine in cancer might be cancer type-specific or due to the differences in drug dose tested. A recent study on 258 patients show that high-dose postoperative morphine use is a significant factor affecting recurrence and will be associated with an increased recurrence risk in patients with esophageal carcinoma.\(^{42}\) Our work provides preclinical evidence to support this study by demonstrating that the increased recurrence risk is highly likely due to morphine’s stimulatory and protective effects in esophageal carcinoma cells. It is well-known that morphine inhibits immune cell functions leading to immunosuppression and stimulates angiogenesis by activating proangiogenic signalling.\(^{5}\) Our work together with previous findings suggest that morphine plays a positive role in tumor progression.

Our work further revealed that morphine acted on esophageal carcinoma cells via AMPK-dependent induction of EMT. The molecular mechanism of morphine’s action in neuronal cells is via binding and activating the \(\mu\)-opioid receptor.\(^{5}\) Morphine has been shown to suppress lung cancer cell proliferation through the interactions with opioid growth factor receptor.\(^{40}\) We demonstrated that morphine acts on esophageal carcinoma cells in an opioid-independent manner as a \(\mu\)-opioid receptor antagonist naloxone did not abolish the stimulatory effects of morphine (Figs. 4A, B), which agrees with the majority of studies that morphine acts on cancer cells via non-classical opioid receptor signaling.\(^{8,37,42}\) We demonstrated that morphine induced EMT, and furthermore this was dependent on AMPK activation (Figs. 3C, 4C). There are conflicting reports about the role of AMPK signalling in EMT and cancer metastasis, suggesting that AMPK activation might have cell-type- and context-specific effects. Recent studies demonstrated that AMPK activation led to a concomitant induction of EMT including increased migration and invasion in multiple cancer cell types.\(^{43}\) In addition, in cancer cells that have already undergone EMT to varying extents, activation of AMPK further promoted the process. The rescue of morphine’s effects by AMPK inhibitor clearly indicated that AMPK is a molecular target of morphine in esophageal carcinoma cells (Figs. 4A, B). Similarly, morphine can ameliorate...
myocardial contractile dysfunction by mechanism involving activation of AMPK. AMPK has recently emerged as a novel target for the treatment of pain. It would be interesting to investigate whether AMPK activation is involved in morphine’s primary indication as a pain medication. AMPK is a key regulator involved in maintaining energy homeostasis and AMPK is activated by energy deficiency and oxidative stress caused by non-functional mitochondria. Our findings excluded the possibility that morphine activated AMPK via inducing oxidative stress (Figs. 3E, 4A, B). Literature reviews suggest that the engagements of AMPK signalling represent a novel pharmacological method to attenuate morphine tolerance. This also provides a rationale for further exploration of AMPK activators as adjuvants to opioid therapy to reduce development of side effects that limit drug efficacy.

In conclusion, our work is the first to demonstrate the positive role of morphine in esophageal carcinoma by mechanism involving AMPK activation and EMT induction. Our preclinical findings correlate well with the clinical study on the association of morphine usage with cancer recurrence in patients with esophageal carcinoma, and provide a guide in clinical use of morphine, particularly in the treatment of esophageal carcinoma.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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