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T-type Ca^{2+} Channel Expression in Human Esophageal Carcinomas: A Functional Role in Proliferation

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Abstract

In the present study the role of T-type Ca^{2+} channels in cancer cell proliferation was examined. Seventeen human esophageal cancer cell lines were screened for T-type channels using RT-PCR and voltage-clamp recordings. mRNAs for all three T-type channel α_1 -subunits (α_{1G} , α_{1H} , and α_{1I}) were detected in all 17 cell lines: either α_{1H} alone, α_{1H} and α_{1G} , or all three T-type α_1 subunits. Eleven cell lines were further subjected to voltage-clamp recordings: one, *i.e.* the TE8 cell line, was found to exhibit a typical T-type current while others exhibited a minimal or no T-type current. Cell proliferation assays were performed in the presence or absence of T-type channel blocker mibefradil in KYSE150, KYSE180 and TE1 cells expressing mRNA for T-type channel α_1 -subunits but lacking T-type current, and TE8 cells exhibiting T-type current. Only TE8 cell proliferation was reduced by mibefradil. Silencing the α_{1G} -gene that encodes functional T-type Ca^{2+} channels in TE8 cells with type-specific shRNA transduction also significantly decreased TE8 cell proliferation. The reduction of cell proliferation in TE8 cells was found to be associated with an up-regulation of p21^{CIP1}. Moreover, p53 silencing nearly abolished the up-regulation of p21^{CIP1} resulting from mibefradil T-type channel blockade. Together, these findings suggest a functional role of T-type channels in certain esophageal carcinomas, and that pharmacological inhibition of T-type channels reduces cell proliferation via a p53-dependent p21^{CIP1} pathway.

Index Terms

T-type calcium channel; esophageal cancer; proliferation; p21^{CIP1}

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INTRODUCTION

Esophageal cancer, with the two major types being adenocarcinoma and squamous cell carcinoma, is a common malignant solid tumor of the digestive system. It ranks as the fourth cause of cancer death in China and among the ten most frequent cancers in the world [1]. Esophageal cancer is increasing in incidence and is associated with one of the highest cancer mortality rates in the United States [2]. In order to develop potent therapeutic strategies, extensive studies have been performed to identify the crucial gene alterations responsible for initiating and promoting the growth of esophageal cancers. To date, a number of molecular alterations have been reported, which generally comprise genetic lesions or altered gene methylation patterns such as loss-of-function mutation of p53 [3], aberrant expression and mutation of epidermal growth factor receptor [4], alternative cyclin D1 splice variants [5], as well as cyclin D1 mutations [6].

Ca^{2+} signaling directs a wide range of intracellular processes, ranging from short-term control of contraction, synaptic transmission, endo- and exocytosis, modulation of enzyme function, and metabolism, to longer term control of gene expression, cell cycle progression, apoptosis, differentiation, and proliferation [7–9]. For many cells, Ca^{2+} signaling is an important growth signal, as changes in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are critical for linking external stimuli to proliferation and apoptosis. Alterations in Ca^{2+} signaling can be the cause of defects in cell growth and are associated with some cancers. Plasmalemmal Ca^{2+} channels, by conducting Ca^{2+} entry, play an active part in the signaling of diverse cellular mechanisms and are critical in the cell proliferation process. Given that proliferation is a principal characteristic of cancer cells, understanding the molecular identity of the Ca^{2+} channels in cancer cells responsible for proliferation is of potential clinical significance.

A number of prior studies suggest a potential role of voltage-gated T-type Ca^{2+} channels in controlling cell proliferation. The unique biophysical properties of T-type Ca^{2+} channels, *i.e.* low voltage activation ranges, transient kinetics of inactivation, and conducting continuous Ca^{2+} entry in a range of low membrane potentials — so called “window current” range of voltages — confer temporal and spatial control of $[\text{Ca}^{2+}]_i$ that may be important for cell cycle progression. Indeed, many proliferating cells exhibit T-type Ca^{2+} current [10,11]; and a correlation between functional T-type Ca^{2+} channels and the presence of the cell cycle specific markers has been shown in cultured smooth muscle cells [12] and in cardiomyocytes [13]. The pharmacological inhibitors of T-type Ca^{2+} channels, *e.g.* mibefradil and pimozide, have been both demonstrated to be effective in decreasing cell proliferation in smooth muscle cells [14, 15], endothelial cells [16], peripheral blood mononuclear cells [17], liver cells [18], glioma cells [19], as well as breast cancer cells [20].

Expression of T-type Ca^{2+} channels changes during development. Developmental studies have shown that T-type Ca^{2+} channels are preferentially expressed in fetal and newborn neuronal and muscle cells [21–25]; a high level of T-type Ca^{2+} channels expression is observed in early mouse embryos [26] and blastocyst-derived embryonic stem cells [27]. Moreover, T-type Ca^{2+} currents are decreased during the induced differentiation of retinoblastoma cells [28], and are re-expressed in dedifferentiated rat ventricular myocytes [29]. Cancer development can be considered a consequence of abnormal cell differentiation. Alterations in Ca^{2+} -relevant and differentiation-associated genes have been reported in esophageal squamous cell carcinoma [30]. Aberrant up-regulation as well as methylation of the gene encoding T-type Ca^{2+} channel α_{1G} subunit was detected in various human primary tumors, suggesting that T-type Ca^{2+} channels may play a role in cancer development by modulating Ca^{2+} signaling [31]. It has long been hypothesized that there exists a link between T-type Ca^{2+} channels and cancer [32]. Indeed, the T-type Ca^{2+} channel has been implicated in proliferation in several tumors [33]. In the present study, we sought to test the hypothesis that T-type Ca^{2+} channels are expressed

in esophageal cancer cells, and the functionally expressed T-type Ca^{2+} channels play a critical role in cancer cell proliferation.

MATERIALS AND METHODS

Culture of esophageal cancer cell lines

Seventeen human esophageal cancer cell lines were examined in this study: fourteen were derived from squamous cell carcinoma, *i.e.* COLO-680N, KYSE30, KYSE70, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510, TE1, TE3, TE8, TE10, TE11, and TE12; three were from esophageal adenocarcinoma, *i.e.* TE7, SEG1, and SKGT4. These cell lines were previously established from primary human esophageal squamous cell carcinomas and adenocarcinomas [34,35]. Cells were maintained in either RPMI 1640 or combination of RPMI 1640 and Ham's F-12 medium (1:1, v/v) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (all from Gibco/Invitrogen, Carlsbad, CA), and passaged at a ratio of 1:3 after reaching confluency in a 75-cm² culture flask.

Patients and samples

Paired total RNA samples used in this study were isolated from the primary esophageal cancer and normal mucosa adjacent to the cancer from each of the eight patients pathologically diagnosed with esophageal squamous cell carcinoma. These tissue samples were obtained by micro-dissection at the First Affiliated Hospital of Zhengzhou University, with the approval for using the samples for research from all eight patients as well as the institutional review board of the Institute for Nutritional Sciences, Chinese Academy of Sciences. The samples were examined histologically to characterize the cancer type or normal mucosa.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was prepared from each esophageal cancer cell line, primary esophageal cancer foci, or adjacent normal mucosa using Trizol reagent (Invitrogen), and cDNA was generated from 3 µg of total RNA with oligo dT primers and Superscript II reverse transcriptase (Invitrogen), all according to the manufacturer's instructions. The cDNA product was used as a template for subsequent PCR amplifications for α_{1G} -, α_{1H} -, and α_{1I} -subunit (GenBankTM accession numbers: AF126966, AF073931, and AF211189, respectively), using sequence-specific primers designed to amplify cDNA regions that are conserved by all the known splice variants and cross at least one intron boundary to prevent amplification from genomic DNA (Figure 1A).

Transient transfection experiments

TE8 cells were transfected with expression vector containing small interfering RNA (siRNA) specifically targeted to p53 (unpublished work). For optimal transfection, cells were grown to 60–80% confluency and transduced with LipofectamineTM 2000 transfection reagent (Invitrogen) in serum-free medium. 4 h after transfection, the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (all from Gibco); mibefradil was applied 24 h later when required. TE8 cells transfected with empty vector DNA were used as control.

Constructs of lentiviral-based small hairpin RNA (shRNA) targeting on α_{1G} -gene, production of recombinant lentivirus and transduction of tumor cells with shRNA via viral infection

Five lentiviral-based shRNA constructs directed against different regions of human α_{1G} -gene were obtained from Open Biosystems (Huntsville, AL). Individual transduction of each shRNA into cultured tumor cells was achieved by lentiviral infection. The recombinant lentivirus was produced using a 293FT producer cell line and ViraPowerTM Lentiviral Expression System

(Invitrogen) according to the manufacturer's instructions. Viral supernatant was collected four consecutive times every 12 h beginning at 24 h or 36 h after transfection of lentiviral vector. The collected supernatant was finally pooled and filtered through a 0.45 μm filter. For tumor cell transduction, cells were grown to 20–40% confluency and subsequently incubated with the harvested viral supernatant.

Cell proliferation assay

Esophageal cancer cells were plated into triplicate wells of six-well culture plates at 1×10^4 cells per well. Mibefradil (3 $\mu\text{mol/L}$; Sigma, St. Louis, MO) dissolved in distilled water, or distilled water alone (10 μL in 1 mL media) was added to triplicate wells after cells were attached (approximately 2 to 3 h after plating). Every 24 h for seven days after the plating date, cells were washed with phosphate-buffered saline (PBS), harvested with trypsin, and counted with an automated cell counter (Z1 Coulter Counter, Beckman Coulter, Fullerton, CA). Data are presented as mean $\pm$ S.E.M.; group means were compared using Student's *t*-tests, and *P* < 0.05 considered significant.

Cellular electrophysiology, data acquisition and analysis

Patch-clamp recordings were performed in whole-cell configuration as previously described [36]. In brief, transmembrane currents in single (electrically isolated) cells were measured with an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pulse+PulseFit software (Version 8.5, HEKA Elektronik) was used to generate the voltage stimuli and to acquire the current signals, which were filtered at 2.9 kHz. The currents were evoked by either step depolarization (*i.e.* 100-millisecond testing pulses ranging from -70 to $+60$ mV with 10-mV increments), or a two-step voltage protocol (*i.e.* measuring the deactivating tail current elicited at the peak of $+80$ mV pulse evoked current), sampled at 50 kHz and averaged respectively 4–6 and 10 data points for one sweep during current recordings; the holding potential was set to -90 mV in each protocol. Fast capacity transients during depolarization were minimized by the patch-clamp analog compensation. Residual capacity transients were further eliminated using an on-line *P/4* subtraction paradigm. Cells with leak current greater than 15 pA at holding potential were excluded from the analysis. The extracellular solution contained the following (in mmol/L): 2 CaCl_2 , 110 tetraethylammonium chloride, 10 CsCl, 10 HEPES, pH 7.4 adjusted with tetraethylammonium hydroxide. The intracellular solution contained the following (in mmol/L): 130 *N*-methyl-D-glucamine, 10 EGTA, 5 BAPTA, 10 HEPES, 6 MgCl_2 , 4 CaCl_2 , 2 Mg-ATP, pH 7.2 adjusted with methane sulfonic acid. All solutions were adjusted to 290 to 300 mOsm with sucrose. Recording pipettes were made of hemo capillaries (Warner Instrument, Hamden, CT), pulled by a two-stage puller (PC-10; Narishige, Tokyo, Japan) and heat-polished with a microforge (MF-200; World Precision Instruments, Sarasota, FL). Pipette resistance was in the range of 3–5 $\text{M}\Omega$ when filled with our intracellular solution. All of the experiments were performed at room temperature (22–25°C). Data are expressed as mean $\pm$ S.E.M for the number of cells (*n*) in which whole-cell patch-clamp recordings were obtained.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting analysis

Cells were lysed using Tween 20 buffer containing (in mmol/L) 50 HEPES (pH 7.5), 150 NaCl, 10 MgCl_2 , 1 EDTA, 0.1% Tween 20, 1 phenylmethylsulfonyl fluoride, 20 units/ml of aprotinin, 5 $\mu\text{g/ml}$ leupeptin, 0.4 NaVO_4 , and 0.4 NaF. Western blotting was performed as described previously [5]. In brief, proteins obtained from lysed cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-p21^{CIP1} antibody (Calbiochem, Laeufelfingen, Switzerland) followed by horseradish peroxidase (HRP)-conjugated secondary antibody. The specific bands were detected by an enhanced chemiluminescence system. Equal loading of the gel was confirmed by either ponceau S (Novagen, Madison, WI) staining or antibody specific for β -tubulin (Sigma).

RESULTS

Expression of T-type Ca^{2+} channel α_1 -subunits in esophageal cancer cell lines

Initially, RT-PCR was performed to identify the expression of mRNA for the three T-type α_1 -subunits, *i.e.* α_{1G} , α_{1H} , and α_{1I} , in each esophageal cancer cell line (Figure 1). Sequence-specific primers were designed based on human α_{1G} , α_{1H} , and α_{1I} sequences (GenBankTM accession numbers: AF126966, AF073931, and AF211189, respectively) to amplify, for each α_1 -subunit, the cDNA region conserved by all the known splice variants and spanning at least one intron boundary to prevent amplification from genomic DNA (Figure 1A). Single PCR products of predicted sizes were revealed from the total RNA isolated from cell lines in individually performed RT-PCR with each set of the primers (Figure 1B), and subsequent cloning experiments confirmed that the products corresponded to human α_{1G} , α_{1H} , and α_{1I} subunits (data not shown). Among 17 esophageal cancer cell lines examined, virtually every cell line expressed mRNA for the T-type α_1 -subunit: either α_{1H} alone (*e.g.* TE1, TE10, TE12, KYSE150, KYSE180, and KYSE450), α_{1G} and α_{1H} (*e.g.* SKGT4, TE3, TE7, and KYSE70), or all three T-type α_1 subunits (*e.g.* COLO-680N, SEG1, TE8, TE11, KYSE30, KYSE410, and KYSE510). The expression level of each specific α_1 -subunit varied significantly between cell lines, where: α_{1G} was markedly expressed in COLO-680N, SEG1, TE7, TE8, KYSE410 and KYSE510 but weakly expressed in SKGT4, TE3, TE11, KYSE30 and KYSE70; α_{1I} was markedly expressed in TE8, KYSE30 and KYSE510 but weakly in COLO-680N, SEG1, TE7, TE11 and KYSE410.

Functional expression of T-type Ca^{2+} channels in esophageal cancer cell lines

To evaluate the functional expression of T-type Ca^{2+} channels in esophageal cancer cells, whole-cell patch-clamp recordings were performed in each of the ten cell lines expressing mRNA for T-type Ca^{2+} channel α_1 -subunits. Utilizing a two-step voltage protocol — where we could detect the maximally evoked T-type tail-current [36] — and solutions designed to isolate Ca^{2+} current, we observed a slowly deactivating T-type tail-current in COLO-680N, KYSE70, KYSE180, KYSE510, TE1, TE3, and TE8 cells (Figure 2A, B). While the maximally evoked tail-current detected in cell types other than TE8 was barely discernible even with 10 mmol/L Ca^{2+} as charge carrier, the T-type tail-current detected in TE8 cells was rather large. The macroscopic current revealed by step depolarizations in TE8 cells was consistent with a T-type Ca^{2+} current, *e.g.*, the current displayed low-threshold voltage activation — activated at -60 mV and above, rapid activation and inactivation kinetics that produce a criss-crossing pattern between successive traces of stepwise current-voltage (*I-V*) protocol (Figure 2C).

Both activation and inactivation kinetics for the T-type current in TE8 cells were far more rapid when compared to the T-type current generated by recombinant human α_{1I} -subunit previously reported by Monteil *et al.* [37]. For instance, at test potential -20 mV, the time constant (τ) for decay of the T-type current in TE8 cells, 28.5 ± 2.5 ms ($n=10$), was about 3–4 fold faster than that of the α_{1I} -mediated current [37]. These biophysical features excluded a functional α_{1I} -subunit in the TE8 cell T-type current. Further, we observed that the T-type current in TE8 cells was insensitive to nickel (Ni^{2+}) even at a concentration of 100 $\mu\text{mol/L}$ (Figure 2C) — characteristic of the α_{1G} - but not α_{1H} -subunit [36,38], suggesting that T-type Ca^{2+} current in TE8 cells is mediated by the α_{1G} -subunit.

Expression of T-type Ca^{2+} channel α_1 -subunits in *in situ* esophageal cancer foci

Although the results from RT-PCR cloning and electrophysiology experiments support expression of the T-type Ca^{2+} channels in cultured esophageal cancer cell lines, such expression could be an artifact of *in vitro* systems. Evidence for *in situ* expression of T-type Ca^{2+} channels in human esophageal cancers has been limited to this point by a paucity of antibodies that are suitable for immunohistochemistry analyses. Nonetheless, RT-PCR of

paired total RNA samples isolated from primary esophageal cancer foci and adjacent normal mucosa — both obtained via micro-dissection — showed *in situ* expression of mRNA for T-type α_1 -subunits in esophageal cancer foci in each of the eight patients examined, *i.e.* one possessed all three α_1 -subunits, one α_{1G} and α_{1H} , one α_{1G} and α_{1L} , two α_{1H} and α_{1L} , and three α_{1H} only. No expression of mRNA for T-type α_1 -subunits was observed in normal esophageal mucosa dissected from these patients (Figure 3). These data suggest, as in *in vitro* experiments, that esophageal cancer cells express T-type Ca^{2+} channels.

T-type Ca^{2+} channel inhibition reduces esophageal cancer cell proliferation

To determine whether functional expression of T-type Ca^{2+} channels is involved in esophageal cancer cell proliferation, we performed cell proliferation assays in KYSE150, KYSE180, TE1, and TE8 cells with and without pharmacological T-type Ca^{2+} channel blockade using mibefradil, one of the most potent T-type Ca^{2+} channel blockers. Application of mibefradil at an approximately IC_{75} value ($3 \mu\text{mol/L}$) for all three T-type α_1 -subunits [39] to TE8 cells resulted in a significant decrease in the number of proliferating cells (23% reduction, $P < 0.05$) relative to controls (Figure 4A). Importantly, mibefradil did not induce apoptosis in TE8 cells as no positive staining was found using annexin V (data not shown). The observed anti-proliferative effect of mibefradil was specific for T-type Ca^{2+} channel blockade and not due to toxicity, since mibefradil had no inhibitory effect on the proliferation of cell type lacking expression of the mRNA for T-type α_1 -subunits, *i.e.* KYSE150 cells (Figure 4B) and cell types exhibiting minimal functional T-type Ca^{2+} channels, *i.e.* KYSE180 and TE1 cells (Figure 4C, D). Together, these results suggest that the functional T-type Ca^{2+} channels contribute to proliferation of certain esophageal cancer cells.

Silencing T-type Ca^{2+} channel with RNA interference (RNAi) reduces esophageal cancer cell proliferation

To confirm that the proliferation inhibition induced by mibefradil was indeed due to the blockade of T-type Ca^{2+} channels, we utilized an RNAi approach to silence the α_{1G} -gene, which encodes functional T-type Ca^{2+} channels in TE8 cells, and examined the effect of α_{1G} silencing on cell proliferation. Five different shRNA constructs directed against human α_{1G} were individually introduced into cultured TE8 cells through lentiviral infection. The silencing effect of each individual shRNA was determined in lentivirally infected TE8 cells by examining the deactivating tail current elicited at the peak of +80 mV pulse — where the maximally evoked T-type current could be measured. Figure 5A demonstrates the effect of each individual shRNA on T-type current in TE8 cells. The two most efficient shRNA constructs, *i.e.* shRNA#1 and #3, were chosen for dual transduction to strengthen the α_{1G} silencing effect. Dual transduction of shRNA#1 and #3 in TE8 cells induced ~67% decrease in T-type current amplitude when compared to non-shRNA virus infected TE8 cells (shRNA#1+#3-transduced group *vs.* non-shRNA control group, $P < 0.0001$, Mann-Whitney test). The T-type current elicited in control cells (only infected with lentivirus) retained a similar maximal current amplitude when compared to the data in Figure 2B as well as *I-V* relationship (data not shown). Silencing the functional T-type Ca^{2+} channel via introducing shRNA resulted in a significant reduction in cell proliferation in TE8 cells as demonstrated by the decrease in the number of proliferating cells in dual shRNA-transduced TE8 cells (Figure 5B), *e.g.* ~40% reduction on day 7 (shRNA#1+#3-transduced *vs.* non-shRNA control, $P < 0.01$). Notably, dual shRNA transduction did not alter cell proliferation in KYSE180 cells, which only exhibit minimal functional T-type Ca^{2+} channels (Figure 5C). These results are consistent with data in Figure 4A where pharmacological T-type Ca^{2+} channel blockade with mibefradil reduced TE8 cell proliferation.

Pharmacological T-type Ca^{2+} channel blockade induced inhibition of esophageal cancer cell proliferation is via up-regulation of p21^{CIP1}

To resolve the molecular events that are regulated by functional T-type Ca^{2+} channels and that contribute to cell proliferation in esophageal cancer cells, we examined the protein expression profile of cyclin D1 and E1, cyclin-dependent kinases (CDK) 2 and 4, and p21^{CIP1} using Western blotting analysis in KYSE510, TE8, and TE10 cells that had been subjected to mibefradil treatment (3 $\mu\text{mol/L}$, 48 h). We repeatedly observed that p21^{CIP1} was up-regulated in TE8 cells expressing functional T-type Ca^{2+} channels but not in KYSE510 and TE10 cells exhibiting minimal or no functional T-type Ca^{2+} channels (Figure 5A), whereas the expression levels of cyclin D1, cyclin E1, CDK2, and CDK4 were not significantly different in any of these cell types (data not shown). Further examination using mibefradil over a range of concentrations (0 to 4.5 $\mu\text{mol/L}$) revealed a dose-dependent increase in p21^{CIP1} expression in TE8 cells (Figure 5B), suggesting that mibefradil-induced up-regulation of p21^{CIP1} is due to specific T-type Ca^{2+} channel blockade.

The up-regulation of p21^{CIP1} induced by the blockade of T-type Ca^{2+} channels is p53 dependent

The tumor suppressor p53 is a major transcription factor involved in the regulation of p21^{CIP1} expression, and p53 is expressed in TE8 cells [40]. Consequently, we examined the contribution of p53 to the observed up-regulation of p21^{CIP1} by the blockade of T-type Ca^{2+} channels in TE8 cells. The expression of p53 was effectively silenced in TE8 cells using siRNA specifically directed at p53. p53 silencing reduced the up-regulation of p21^{CIP1} from the T-type Ca^{2+} channel blockade by over 70% ($n=3$, $P<0.05$) (Figure 6). These results suggest that the increased expression of p21^{CIP1} and the inhibition of cell proliferation are induced in a p53-dependent manner by the blockade of T-type Ca^{2+} channels in TE8 cells.

DISCUSSION

Although Ca^{2+} entry has been implicated in cancer cell proliferation, the molecular identity of the ion channels that mediate this process remain poorly understood. In the present study, RT-PCR were performed to examine the presence of the gene(s) encoding T-type Ca^{2+} channels in cultured human esophageal cancer cell lines. These experiments resolved that virtually all the examined human esophageal cancer cell lines express mRNA for the T-type α_1 -subunit genes: either α_{1H} alone in some cell lines, or α_{1H} together with α_{1G} and α_{1I} in other cell lines. Further, whole-cell voltage-clamp recordings were performed in several cell lines confirming the presence of functional T-type Ca^{2+} channels in some but not all cell lines. In most cases the voltage-dependent currents were hardly detectable with the typical stepwise depolarization protocol, and therefore the two-step voltage protocol had to be employed to reveal the maximally evoked T-type tail-current (Figure 2A, B). Nonetheless, there was a rapidly inactivating, low voltage-activated current, characteristic of the T-type Ca^{2+} current, repeatedly detected in one cell type, *i.e.* TE8 (Figure 2C). Consistently, mRNA for α_{1G} , α_{1H} , and α_{1I} was detected in esophageal cancer foci but not immediately adjacent mucosa tissues from patients diagnosed with squamous cell carcinoma. These results have identified the functional expression of T-type Ca^{2+} channels in esophageal cancers which arise from generally considered “electrically non-excitable” epithelial cells. While the T-type current in TE8 cells was identified as an α_{1G} -mediated current based upon its Ni^{2+} insensitivity and kinetics of current inactivation, at this point we are as yet uncertain whether the observed T-type current is carried by single or multiple T-type α_1 -subunits. These data are consistent with previous work by Toyota *et al* [31], who performed RT-PCR using cDNA from various normal human tissues and a panel of 27 tumor cell lines and determined that the expression of α_{1G} was relatively low in normal tissues, but relatively high in most tumor cell lines, suggesting an aberrant up-regulation of α_{1G} expression in tumor cells.

Inconsistent observations have been made over the past decade in studies on the role of T-type Ca^{2+} channels in regulating cell proliferation. Supporting the notion that T-type Ca^{2+} channels regulate cell proliferation: cell cycle-dependent expression of T-type current was observed in rat aortic smooth muscle cell — the current was electrophysiologically non-detectable in the G_0 -phase, appeared in the G_1 -phase, and increased in the S-phase [12]. Using RNA interference (RNAi) directed to α_{1G} -subunit and pharmacological T-type Ca^{2+} channel blockade, Rodman *et al* demonstrated that T-type Ca^{2+} channels are required for cell cycle progression and proliferation in human pulmonary artery smooth muscle cells [15]. Additionally, Li *et al* demonstrated a pathophysiologic role of T-type Ca^{2+} channels in high glucose-induced myocardial hypertrophy, *i.e.* ventricular cell proliferation, in neonatal rat heart [41]. However, conflicting observations were made in studies overexpressing α_{1G} - or α_{1H} -subunit in a human embryonic kidney cell line (HEK 293). Wang *et al* observed that stable overexpression of α_{1G} and α_{1H} conferred cells with a significant growth advantage, *i.e.* shortened G_0/G_1 phase cycling [42,43], whereas Chemin *et al* reported that overexpression of α_{1G} and α_{1H} was insufficient to modulate cell proliferation, although it indeed resulted in an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [44]. Recent work by Panner *et al* demonstrated that upon overexpression of the α_{1H} -subunit in U87MG glioma cells, there was a marked increase in the number of proliferating cells [19]. These discrepancies could be due to variations in signaling pathways or Ca^{2+} compartmentalization in expression systems.

Our results in the present study clearly show that pharmacological blockade of endogenous T-type Ca^{2+} channels leads to inhibition of cell proliferation in esophageal cancer cells. This was demonstrated by a significant decrease in the number of proliferating cells in TE8 cell line exhibiting functional T-type current when T-type Ca^{2+} channel blocker mibefradil was applied. On the other hand, mibefradil had no effect on the number of proliferating cells in KYSE150, KYSE180, and TE1 cells expressing mRNA for T-type α_1 -subunits but exhibiting only minimal functional T-type current. In addition, silencing the α_{1G} -gene that encodes functional T-type Ca^{2+} channels in TE8 cells with type-specific shRNA transduction significantly decreased the number of proliferating TE8 cells when compared to regular TE8, and virus infected (non-shRNA control) TE8 cells. The effect of α_{1G} silencing is specific for cells exhibiting apparent functional α_{1G} channels as shRNA transduction had no impact upon cell proliferation in KYSE180 cells that only exhibit minimal T-type current. These results support our pharmacological analysis of T-type channel function and further indicate that T-type channel inhibition leads to an inhibition of cell proliferation. Collectively, our data suggest that functional expression of T-type Ca^{2+} channels contributes to the proliferation of certain esophageal cancer cells.

The present study demonstrates that the mibefradil-reduced cell proliferation in the cell line exhibiting functional T-type current is specifically associated with an up-regulation of p21^{CIP1}. The response of cell proliferation in TE8 cells to pharmacological T-type Ca^{2+} channel blockade using mibefradil was paralleled with an increase in p21^{CIP1} expression. Further, the resultant up-regulation of p21^{CIP1} from mibefradil application in TE8 cells was in a dose-dependent manner. Mibefradil application had no effect on p21^{CIP1} expression in KYSE510 and TE10 cells exhibiting minimal or no functional T-type Ca^{2+} channels. These observations also support that the inhibition of cell proliferation induced by mibefradil in TE8 cells is indeed due to inhibition of T-type Ca^{2+} channels. Although numerous studies reveal an anti-proliferative effect of mibefradil in various cell types [14–19,41], only a few succeed in demonstrating that the effect was in fact due to the T-type Ca^{2+} channel blockade [15,41]. Nonspecific anti-proliferative effects of mibefradil may be due to accumulation inside cells and hydrolysis to metabolites which exert effects other than blocking T-type Ca^{2+} channels [45], as exemplified by our recent observation in U87MG glioma cells [46]. Nonetheless, the results presented here provide solid evidence that mibefradil prevents cell proliferation via T-type Ca^{2+} channel blockade and consequent up-regulation of p21^{CIP1}.

Silencing p53 in TE8 cells by an RNAi approach revealed that p21^{CIP1} up-regulation induced by T-type Ca²⁺ channel blockade is p53-dependent. As it is well established that p21^{CIP1} is a downstream effector in the tumor suppressor p53 signaling pathway, this result further supports that T-type Ca²⁺ channels play an active part in processes regulating the proliferative phenotype of certain esophageal cancer cells. Note that the TE8 is a wild-type p53-expressing cell line [47], whereas TE1, KYSE150, and KYSE180 are esophageal cancer cell lines which express mutant p53 [48–50].

In conclusion, our present study has established for the first time the existence of functional T-type Ca²⁺ channels in a number of human esophageal cancers *in vitro* as well as *in vivo*. Studies using both pharmacological and molecular strategies present direct evidence that T-type Ca²⁺ channels play an active part in the processes regulating the proliferative phenotype of certain esophageal cancers, and T-type Ca²⁺ channel blockade diminishes cell proliferation in esophageal cancer cells through a p53-dependent p21^{CIP1} up-regulation pathway. Future studies will be required to examine the signaling pathways downstream of Ca²⁺ entry through T-type Ca²⁺ channels in regulating the cell cycle and the process of cell proliferation. Understanding such mechanisms will provide new therapeutic strategies for treating esophageal carcinomas and a variety of other cancers. It can be expected that T-type channel blockers may be utilized as future therapies for neoplasm expressing T-type channels [51].

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Figure 1. Presence of T-type Ca^{2+} channel α_1 -subunits in esophageal cancer cell lines
A, Sequences of oligonucleotides used as primers. **B**, RT-PCR amplification was performed using total RNA isolated from each of the 17 esophageal cancer cell lines and primer sets indicated in **A**.

A

B

C

Figure 2. Functional expression of T-type Ca^{2+} channels in esophageal cancer cells

A, Representative deactivating tail current traces in KYSE180, TE1, and TE8 cells, elicited by repolarization at -120 mV following a short test pulse at $+80$ mV (3.25 ms). No such currents were detected in KYSE150 cells using the same protocol. The time constant (τ) for the decay of the deactivating tail current in TE8 cells is 1.27 ± 0.07 ms at -120 mV repolarization potential ($n = 15$). **B**, Individual data points of maximally elicited T-type tail currents in COLO-680N, KYSE70, KYSE180, KYSE510, TE1, TE3, and TE8 cells. **C**, A set of representative macroscopic current traces in TE8 cells (*Left panel*), and averaged *I-V* relationship (*Right panel*) of peak currents at each depolarizing pulse obtained from normal TE8 cells (open squares, $n = 15$) and TE8 cells in the presence of Ni^{2+} ($100 \mu\text{mol/L}$) (close squares, $n = 6$). The activation and inactivation time constants of the current evoked at test pulse -20 mV were 6.34 ± 0.27 ms and 28.5 ± 2.5 ms, respectively ($n=10$ each), when the current was fit with two exponentials. Ni^{2+} did not block the current ($P = \text{ns}$, Mann-Whitney test). Currents were recorded by increasing depolarization from -60 to $+30$ mV with 10 -mV increments from a holding potential of -90 mV. Ca^{2+} (10 mmol/L) was used as a charge carrier. Temperature was 22 – 25°C . Data represent the mean $\pm$ S.E.M of 4 independent experiments.

Figure 3. Expression analysis of T-type Ca^{2+} channel α_1 -subunits in *in situ* esophageal cancer foci by RT-PCR

RT-PCR was performed to detect mRNA for α_{1G} -, α_{1H} -, and α_{1I} -subunits in paired total RNA samples. Each sample pair was isolated from the primary focus of esophageal cancer and normal mucosa adjacent to the focus from each of the 8 patients pathologically diagnosed with esophageal squamous cell carcinoma. The mRNA for α_{1G} , α_{1H} , and α_{1I} was detected in different combinations in esophageal cancer foci but not normal mucosa.

Figure 4. Effect of pharmacological T-type Ca^{2+} channel blocker mibefradil on cell proliferation in esophageal cancer cells

Cell growth was observed over a 7-day period with and without mibefradil (3 $\mu\text{mol/L}$) application in TE8 (**A**), KYSE150 (**B**), KYSE180 (**C**), and TE1 (**D**) cells. Mibefradil reduced proliferation in TE8 cells by 23% ($n = 6$, $*P < 0.05$ relative to mibefradil non-treated group at the same time points), but had no effect in KYSE150, KYSE180, and TE1 cells ($n = 3$ for each, $P = \text{ns}$ between mibefradil-treated and non-treated groups).

Figure 5. Effect of shRNA-induced α_{1G} -gene silencing on esophageal cancer cell proliferation

A, Individual data points of the deactivating tail current elicited at the peak of +80 mV pulse in TE8 cells transduced with each single shRNA (shRNA#1 - shRNA#5) or dual shRNA (shRNA#1+#3), and TE8 cells only infected with non-shRNA viruses. Lines in each data group are at median with inter-quartile range, *i.e.* for dual shRNA-transduced group (in pA/pF): median -1.6, 25th percentile -2.3, 75th percentile -1.3; for non-shRNA infection control group: median -4.9, 25th percentile -7.4, 75th percentile -3.7 ($P < 0.0001$, Mann-Whitney test). Note the current density distribution in non-shRNA control group is in the same range as those in normal TE8 cells (Figure 2B). **B**, Cell growth was observed over a 7-day period in normal, dual shRNA-transduced, and non-shRNA infection control TE8 cells ($n = 3$ each). Dual shRNA transduction reduced proliferation in TE8 cells by ~40% at day 7. Asterisk (*) indicates $P < 0.01$ relative to non-shRNA control group at the same time points.

Figure 6. Pharmacological T-type Ca^{2+} channel blockade induces up-regulation of p21^{CIP1}
 Whole cell lysates from TE10, TE8, and KYSE510 cells with or without mibefradil (3 μmol/L) treatment for 48 h (**A**), and TE8 cells treated for 48 h with a series of mibefradil concentrations (0, 1.5, 3, and 4.5 μmol/L) (**B**) were separated by 10% SDS-PAGE and immunoblotted with anti-p21^{CIP1} antibody. Equal loading of the gel was confirmed by ponceau S staining in **A** (data not shown) or β-tubulin in **B**. Quantification was performed by densitometry ($n=3$ each; * $P < 0.05$). **A**, Shows that p21^{CIP1} expression was increased in TE8 cells but not in TE10 and KYSE510 cells. **B**, Shows that the increase in p21^{CIP1} expression in TE8 cells following mibefradil treatment was in a dose-dependent manner.

Figure 7. The up-regulation of p21^{CIP1} induced by the blockade of T-type Ca²⁺ channels is p53 dependent

TE8 cells were transfected with or without siRNA specifically directed at p53, then treated for 24 h with or without mibefradil (3 μmol/L). Whole cell lysates from equal number of cells were separated by 10% SDS-PAGE and immunoblotted with anti-p21^{CIP1} antibody. Equal loading of the gel was confirmed by ponceau S staining. Quantification was performed by densitometry ($n=3$ each; * $P < 0.05$). Data illustrate that p21^{CIP1} expression was increased in non-transfected TE8 cells following mibefradil treatment, a response that was nearly abolished in siRNA-transfected TE8 cells.