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Effects of salivary Mg on head and neck carcinoma via TRPM7

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Abstract:	Magnesium (Mg) has been known to play vital roles in regulating growth and various metabolic processes. In recent years, the association between Mg and tumorigenesis has raised more and more attention. However, the effects of Mg on the progression of head and neck carcinoma (HNC) as well as the mechanism behind it remain undefined. In this study, the roles of Mg in tumorigenic activities were tested in CAL27 and FaDu cells, as well as in a xenograft tumor model in nude mice. We demonstrated that moderate increase in extracellular Mg contributed to the proliferation, migration, and invasion of two HNC cell lines, while the addition of Mg in drinking water promoted the growth of xenograft tumor in mice without altering their serum Mg level. Moreover, TRPM7, a major Mg transporter, was shown

	essential for the tumorigenic activities of HNC and the Mg induced promotive effects on HNC cells, which was further shown to be associated with the activation of AKT/mTOR signaling. As a preliminary clinical study, we determined the Mg ion concentrations in the stimulated saliva from 72 nasopharynx carcinoma (NPC) patients and 12 healthy individuals. Our data revealed that the salivary Mg level in NPC subjects was significantly higher than the healthy controls. This is correlated with our finding showing TRPM7 to be overexpressed in tumor tissues harvested from nine HNC patients. Therefore, it can be concluded that salivary Mg level, within a certain range, could act as a risk factor for the progression of HNC, which involves the activation of AKT/mTOR signaling pathways through TRPM7 channel. The control of salivary Mg level and the intervention of TRPM7 should not be ignored during the study of HNC.

Effects of salivary Mg on head and neck carcinoma via TRPM7

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Abstract

Magnesium (Mg) has been known to play vital roles in regulating growth and various metabolic processes. In recent years, the association between Mg and tumorigenesis has raised more and more attention. However, the effects of Mg on the progression of head and neck carcinoma (HNC) as well as the mechanism behind it remain undefined. In this study, the roles of Mg in tumorigenic activities were tested in CAL27 and FaDu cells, as well as in a xenograft tumor model in nude mice. We demonstrated that moderate increase in extracellular Mg contributed to the proliferation, migration, and invasion of two HNC cell lines, while the addition of Mg in drinking water promoted the growth of xenograft tumor in mice without altering their serum Mg level. Moreover, TRPM7, a major Mg transporter, was shown essential for the tumorigenic activities of HNC and the Mg induced promotive effects on HNC cells, which was further shown to be associated with the activation of AKT/mTOR signaling. As a preliminary clinical study, we determined the Mg ion concentrations in the stimulated saliva from 72 nasopharynx carcinoma (NPC) patients and 12 healthy individuals. Our data revealed that the salivary Mg level in NPC subjects was significantly higher than the healthy controls. This is correlated with our finding showing TRPM7 to be overexpressed in tumor tissues harvested from nine HNC patients. Therefore, it can be concluded that salivary Mg level, within a certain range, could act as a risk factor for the progression of HNC, which involves the activation of AKT/mTOR signaling pathways through TRPM7 channel. The control of salivary Mg level and the intervention of TRPM7 should not be ignored during the study of HNC.

Keywords: magnesium (Mg), head and neck carcinoma (HNC), Transient receptor potential melastatin-subfamily member 7 (TRPM7), mammalian target of rapamycin (mTOR).

Introduction

Head and neck carcinoma (HNC) is the 6th most common malignancy with a five-year survival rate of only around 50% (Jemal et al. 2011). The development and progression of HNC are known to be related to the changes in the intraoral environment. On the other hand, HNC has been reported to alter the environment of the oral cavity, which allows early detection of HNC by using potential markers in saliva (Cheng et al. 2014). It was reported that the salivary Mg concentration of patients with parotid malignant tumors at stage II-III was significantly increased when compared to that of healthy individuals (Gradinaru et al. 2007), while in head and neck squamous cell carcinoma (HNSCC) patients, the salivary Mg concentration was found to be around 28% higher than the healthy controls (Shpitzer et al. 2007). However, the correlation between salivary Mg level and the progression of HNC remains unclear.

Mg is known to play vital roles in almost all major cellular metabolic processes, redox reactions, and the activation of various enzymes. Neoplastic cells have been reported to have a higher intracellular Mg concentration, which confers a metabolic advantage to cell proliferation, benefits the alteration of genome and promotes the acquisition of an immortal phenotype (Castiglioni and Maier 2011). Transient receptor potential melastatin-subfamily member 7 (TRPM7) is a ubiquitously expressed cationic ion channel responsible for cellular Mg flux in respect of development and tissue-specific regulation of gene activity (Nadler et al. 2001; Schmitz et al. 2003). In recent years, TRPM7 has been extensively reported to be involved in various cellular processes in cancer, including survival, cell cycle, proliferation, migration, and invasion (Middelbeek et al. 2012; Trapani et al. 2013; Yee et al. 2014). Some clinical studies have also revealed the positive correlation between the overexpression of TRPM7 and pathological parameters, including the increased Ki67 proliferative index, tumor size and patient

survival rate (Dhennin-Duthille et al. 2011; Guilbert et al. 2009; Rybarczyk et al. 2012). Moreover, the blockage of TRPM7 dependent currents or the suppression of TRPM7 expression in HNSCC cells were reported to significantly inhibit the cell proliferation by inducing G0/G1 cell cycle arrest and inhibiting Rb activation (Dou et al. 2013; Jiang et al. 2007). However, since TRPM7 is also permeable to calcium (Ca), a well-known second messenger in numerous fundamental cellular activities, most of the studies have been focusing on the effects of Ca on TRPM7, the vital roles of Mg have been largely overlooked.

Therefore, the aim of this study is to investigate the effects of extracellular Mg concentration on tumorigenic activities of HNC cells and to elucidate the potential mechanisms underlying these effects. We found the tumorigenic activities of human HNC cell lines could be significantly affected by extracellular Mg *in vitro* and *in vivo*. We also confirmed the involvement of TRPM7, as well as the activation of AKT-mTOR signaling in Mg-induced tumorigenesis. These, together with our clinical data showing the elevation of salivary Mg level in NPC patients compared with healthy control and the overexpression of TRPM7 in human HNC surgical specimens, suggesting that the exposure of Mg in oral cavity might act as a risk factor in the progression of HNC.

Materials and methods

Cell culture and in vitro assays

The human tongue squamous cell carcinoma cell line CAL27 and the pharynx squamous cell carcinoma cell line FaDu were obtained from the American Type Culture Collection (USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. Proliferation assay was done by measuring

the cell viability of CAL27 and FaDu cells cultured in DMEM supplemented with different concentrations of $MgCl_2$ (*i.e.*, 0 mM, 0.8 mM, 2.0 mM and 5.0 mM) using a cell counting kit-8 (CCK-8). Cell cycle analysis was done using propidium iodide (ThermoFisher) on a flow cytometry (BD FACSVerse), and analyzed with FlowJo (Tree Star Inc.). Cell migration was measured using a wound healing assay. Images of the wound were captured at baseline and 48 h after the treatment. Cell invasion was evaluated by transwell assay using 8-μm polycarbonate membrane chambers (Corning) coated with Matrigel (Corning). After 24 h, the invaded cells on the lower membrane surface were stained with 0.1% crystal violet. Immunofluorescent staining was done 24 h after the treatment, the cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with rabbit anti-TRPM7 (Abcam) or rabbit anti-mTOR (Abcam) antibody. Then, the cells were incubated with Alexa-Fluor 488 conjugated secondary antibody (ThermoFisher), and counterstained with Hoechst 33342 (ThermoFisher).

Western Blots

At the designated time points, the cells were lysed with RIPA Lysis and Extraction Buffer (ThermoFisher). A total of 30 μg of protein from each sample were subjected to SDS-PAGE electrophoresis and transferred to PVDF membrane. The primary antibodies used included rabbit anti-TRPM7 and rabbit anti-mTOR (Abcam), as well as rabbit anti-p-AKT, rabbit anti-AKT, rabbit anti-p-p38 MARK, rabbit anti-p38 MARK, rabbit anti-p-c-Raf, rabbit anti-p-GSK-3β, and mouse anti-β-actin (CST). The protein bands were visualized by ECL substrate (ThermoFisher) and exposed under ChemiDoc XRS System (BioRad).

TRPM7 silencing and blocking

For TRPM7 silencing, CAL27 and FaDu cells were transfected with 10 nM siRNA targeting human TRPM7 (OriGene) using siTran1.0 (OriGene) as the transfection agent. Cells transfected with nonspecific control siRNA (OriGene) were used as the control. siRNA transfection efficiency was verified 72 h after the transfection by Western blotting before further *in vitro* assays. For the inhibition of TRPM7 activity, cells were pre-treated with 3 μM FTY720 (Sigma-Aldrich) for 2 h before *in vitro* assays.

Xenograft tumor assay

Female BALB/c nude mice, 4-6 weeks of age, were purchased from Charles River Lab (USA) and maintained in the specific pathogen-free facility (Lab animal unit, HKU). Animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR, HKU). In brief, all of the mice were anesthetized via intraperitoneal (i.p.) injection of ketamine hydrochloride and xylazine hydrochloride (Alfasan International B.V.). A total of 8×10^5 FaDu cells suspended in Mg-free and Ca-free Hanks' balanced salt solution (Gibco) were injected into the anterior tongue tissue. For siRNA group, the same number of TRPM7-siRNA transfected cells were injected accordingly. For FTY720 group, the mice were given 10 mg/kg FTY720 daily via i.p. injection from the second week of tumor inoculation till the day of execution. Immediately after the inoculation of FaDu cells, the mice were randomly divided into control or Mg group, and either fed with deionized (DI) water or DI water supplemented with 10 mM $MgCl_2$. Mice were euthanized by cervical dislocation after anesthesia at 21 days after inoculation.

Histological assays

Tongue tissues harvested at the end-point were fixed and embedded in paraffin for the preparation of 5 μm-thick sections. Haematoxylin and eosin (H&E) staining and Immunohistochemistry (IHC) was performed on selected slides from each sample. For IHC, the dewaxed slide was treated with Proteinase K (Sigma-Aldrich) for proteolytic digestion and 3% H_2O_2 for the elimination of endogenous peroxidase activity. The slides were incubated with rabbit anti-Ki67 (Abcam) or rabbit anti-TRPM7 (Abcam) antibodies, and visualized using Alexa-Fluor 555 conjugated secondary antibody (ThermoFisher) and Hoechst 33342. Histological images were captured using a fluorescent microscope (Nikon).

Saliva collection and analysis

Saliva samples were collected from 12 healthy control individuals and 72 newly diagnosed nasopharyngeal carcinoma (NPC) patients, who had no history of chemotherapy or radiotherapy in the head and neck region, from Queen Mary Hospital and Prince of Wales Hospital, Hong Kong. Detailed patient information is shown in Supplementary table 1. The study was approved by the Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster and the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee. For each subject, written informed consent was signed prior to their participation. The salivary electrolyte analysis was performed using an inductively coupled plasma-optical emission spectrophotometry (ICP-OES, Spectro Arcos).

Histological studies of human HNC tissues

Nine pairs of human HNC tissues and corresponding non-tumor normal tissues were collected from HNC patients. Detailed information of the patients is shown in Supplementary

table 2. Informed consent was obtained from the patient, and all procedures were approved by IRB (HKU). The histological methodology was the same as described above, and the expression of TRPM7 was detected using rabbit anti-TRPM7 (Abcam) antibodies and Diaminobenzidine (DAB) staining kit (Santa Cruz).

Statistical analysis

Data were analyzed with SPSS software (IBM SPSS) using either Student *t* test or one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. All data were presented as mean ± standard deviation, and variables with a P value < 0.05 was defined as the level of significant difference.

Results

Effects of Mg on HNC cells

As demonstrated in Fig.1a, the growth of HNC cells in the Mg-free medium was significantly inhibited compared with the control group (*i.e.*, 0.8 mM), while a moderate increase in extracellular Mg to 2.0 mM led to a significant ($p < 0.05$) increase in cell proliferation (day 3 and day 5). However, excessive addition of Mg to 5.0 mM didn't show significant difference in cell proliferation compared to the control group. Cell cycle analysis (Fig. 1b) revealed that the addition of Mg resulted in a marked increase in G2 populations in both HNC cell lines, supportive of the increase in cell growth rate. Moreover, as shown in Fig.1c, the lack of Mg in culture medium dramatically affected the invasion of HNC cells. The number of invaded cells across the transwell membrane in the Mg-free medium was less than 40% of that in the medium containing 2.0 mM Mg. Wound closure assay (Fig.1d) showed that, the depletion of Mg in the medium resulted in much slower closure of wound compared with the control group (i.e., 0.8

mM Mg). The rate of migration was increased in 2.0 mM Mg group, but not in 5.0 mM Mg group.

The involvement of TRPM7

Immunofluorescent images demonstrated that TRPM7 was primarily expressed in the cytoplasm membrane (Fig. 1e). The addition of extracellular Mg was demonstrated to intriguingly increase the expression of TRPM7 (Fig. 1f). After the transfection of TRPM7-siRNA, the expression of TRPM7 in CAL27 and FaDu was successfully inhibited, as shown in Fig. 2a. The cell viability data indicated that both the inhibition of TRPM7 by siRNA and the blockage of TRPM7 by FTY720 led to compromised cell proliferation (Fig. 2b), as well as retarded migration (Fig. 2c) and invasion (Fig. 2e) compared with the control groups. Meanwhile, the effect of additional extracellular Mg on cell proliferation vanished (Fig. 2d).

The regulation of AKT/mTOR signaling

Western blotting data (Fig. 3a, b) suggested that the addition of Mg significantly upregulated the expression of mTOR, while FTY720 downregulated it. Moreover, increased extracellular Mg also upregulated the phosphorylation of AKT and its upstream PDK, as well as its downstream c-Raf and GSK-3β, which were all inhibited by FTY720. Fluorescent images demonstrated mTOR was primarily expressed in vesicles and cytosol in HNC cells (Fig.3c). It can also be noted that the cellular movement is related to the concentration of Mg in the culture medium, manifested by better spreading of cells and extension of lamellipodia.

Xenograft HNC in a murine model

As shown in Fig. 4a and 4b, the size of xenograft tumors grown in nude mice fed with DI water (n=6) were significantly ($p < 0.05$) smaller when compared with that in Mg drinking water

group (n=6), even though there was no significant difference in serum Mg concentration between both groups (Fig. 4c). Meanwhile, both *i.p.* injection of FTY720 and transfection of TRPM7-siRNA in inoculated HNC cells resulted in smaller xenograft tumor at the endpoint, regardless of the addition of Mg in drinking water. The growth of tumor also contributed to decreased body weight, which was more prominent in Mg water fed mice, but less significant in FTY720 and siRNA groups (Fig. 4d). Higher magnification revealed the formation of abundant blood vessels (Fig .4e, yellow arrows) and identical keratinization (Fig. 4e, green arrows) in the xenograft tumor tissues. Fluorescent IHC images demonstrated that Ki67 was highly expressed in the inoculated FaDu cells, especially in Mg water treated group (Fig. 4f). Moreover, TRPM7 was upregulated in the neoplastic cells located at the margin of the xenograft tumors (Fig. 4g).

Changes in salivary Mg level and TRPM7 expression in HNC patients

Salivary Mg level of NPC patients was significantly higher ($P<0.05$) than that of healthy controls (Fig. 5a). Nevertheless, there was no significant difference in salivary Ca level between NPC patients and the healthy individuals. IHC study of surgical specimens from nine HNC patients showed that TRPM7 expression was higher in tumor tissues than in normal tissues (Fig. 5b, Supplementary Fig. 1)

Discussion

Although the effects of Mg on the cell growth, proliferation, differentiation, and migration have been known for decades, the correlation between Mg and the progression of cancer remains controversial due to inconsistent experimental and epidemiological results concerning various cancers. Some studies have indicated Mg as a protective agent against the development and

progression of cancer, while hypomagnesemia participates in both early and late phases of tumorigenesis through promotion oxidative stress and inflammation (Castiglioni and Maier 2011; Tukiendorf and Rybak 2004; Yang et al. 2002). Conversely, there are also studies suggesting the insufficiency of Mg in diet contributed to approximate 60% reduction in the growth of xenografted lung carcinoma, mammary adenocarcinoma, and colon carcinoma (Nasulewicz et al. 2004). A systematic epidemiological study revealed that higher Mg intake led to increased risk of lung cancer, especially in men and current smokers (Mahabir et al. 2010). In this study, we demonstrated that Mg supply is vital for the proliferation, migration and invasion of HNC cells, as well as the growth of xenograft HNC tumors in mice. Thus, excessive Mg exposure in the oral cavity, especially at the site of tumor, should be prevented so as not to trigger the progression of primary tumor and promotion of further genetic instability.

In recent years, with the discovery of Mg as a second messenger in regulating cell functions (Li et al. 2011), TRPM7, the major transporter for Mg homeostasis, has been recognized to participate in tumor biology. Since ion channels play vital roles in a large variety of cell functions, the development and progression of tumor are often associated with the abnormality of ion channels (Visser et al. 2014). Our data indicated that the loss of TRPM7 greatly impaired a series of cellular functions that are pivotal to cancer progression. Moreover, we noticed that when TRPM7 was inhibited in HNC cells, the promotive effects of extracellular Mg on cell proliferation vanished. Given this, TRPM7 appears to be a promising therapeutic target for the intervention of HNC. FTY720 (Fingolimod), a structural analog of sphingosine, is a novel immunosuppressive drug approved by the Food and Drug Administration (USA), which was recently found to have a potential role in cancer therapy (White et al. 2016). However, it was not until most recently did people realize the efficient inhibitory effects of FTY720 on TRPM7.

The addition of FTY720, even at a low dosage (*i.e.*, 1 μM), was shown sufficient to block TRPM7 dependent current, leading to suppressed cell motility (Qin et al. 2013), pro-inflammatory polarization of macrophages (Schilling et al. 2014), and volumetric changes of submandibular gland acinar cells (Kim et al. 2017). In this study, we found that the treatment of FTY720 resulted in significant suppression on the tumorigenic activities of two HNC cells, as well as the growth of xenograft tumor. In addition, subsequent addition of extracellular Mg failed to rescue the cells from growth inhibition, at least within the duration of observation. Therefore, specific TRPM7 inhibitors including FTY720 (Qin et al. 2013), Carvacrol (Chen et al. 2015), Waixenicin (Zierler et al. 2011), etc. may be proposed in HNC therapy in the future.

Mammalian target of rapamycin (mTOR) kinase is emerging as one of the most important regulators of a cascade of signal and effector molecules in the oncogenesis of HNSCC. The activation of AKT/mTOR pathway through multiple components leads to sequential phosphorylation of 3-phosphoinositide-dependent kinase 1 (PDK1) and AKT, as well as the accumulate of mTOR. Meanwhile, AKT can also regulate cell survival through phosphorylation of several targets such as c-Raf and glycogen synthase kinase 3β (GSK3β) (Herzog et al. 2013; Molinolo et al. 2007). The activation of AKT/ mTOR pathway and downstream mediators has been reported in more than 90% of human HNSCC, while the inhibition of AKT or mTOR individually have been shown to contribute to the suppression of tumor growth in human and murine HNSCC models (Herzog et al. 2013; Molinolo et al. 2007). Recently, mTOR was proposed as the target of increased free intracellular Mg for the regulation of protein synthesis and the control of cell proliferation in a membrane, magnesium, mitosis (MMM) model (Rubin 2007). Magnesium-transporting proteins, especially TRPM7, was suggested indispensable for sustaining the activation of AKT/mTOR (Feeney et al. 2016; Sahni and Scharenberg 2008).

Moreover, as a cofactor for ATP, Mg fluxes were proposed able to affect circadian translational regulation via the highly MgATP-sensitive mTOR pathway (Feeney et al. 2016). Here, we demonstrated the addition of Mg contributed to the activation of AKT/mTOR signaling and the phosphorylation of its targets including c-Raf and GSK-3β in both CAL27 and FaDu cells. These, together with the inhibitory effects of FTY720 on the AKT/mTOR signaling pathways, these results revealed the activation of AKT/mTOR signaling by TRPM7 in Mg mediated progression of HNC.

In accordance with previous reports on other types of cancer (Dhennin-Duthille et al. 2011; Guilbert et al. 2009; Rybarczyk et al. 2012), the current study also detected overexpression of TRPM7 in murine xenografted HNC and human HNC tissues. This not only validates the involvement of TRPM7 in the development and progression of HNC, but also offers a strong evidence indicating the vital role of Mg in tumorigenesis. We then demonstrated the salivary Mg level of NPC patients was significantly higher than that of healthy controls. This suggests that higher salivary Mg, but not Ca, could be implicated in the progression of HNC. However, it remains to be clarified whether the progression of HNC is able to influence the Mg level in saliva via altering Mg homeostasis in mucosal epithelial cells or even targeting saliva glands.

Conclusions

Within the limitations of this study, it could be concluded that a moderate increase in the extracellular Mg contributes to the proliferation, migration, and invasion of human HNC cells, possibly involving the activation of AKT/mTOR signaling. TRPM7, the expression of which is upregulated in HNC tissues, is demonstrated essential in the development and progression of HNC, and plays vital roles in the Mg induced promotive effects on the cancer cells. To summarize, our findings reveal the positive correlation between salivary Mg and the progression

of HNC, as well as the potential mechanism behind it. The Mg channel, TRPM7 may be proposed as a potential therapeutic target for the treatment of HNC, which warrants further investigation.

Conflict of interest statement

The authors declare no competing financial interests.

Author Contributions

W. Qiao contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; X. Lan, H. Ma , and Z. Hu contributed to design, data acquisition, and analysis, drafted the manuscript; J. Y.K. Chan contributed to design, data acquisition and interpretation; V. W.Y. Lui and K.W.K. Yeung contributed to design, interpretation, and critically revised the manuscript; D. L.W. Kwong and J.K.H. Tsoi contributed to conception, design, and interpretation; J.P. Matinlinna and Y. Su contributed to conception, design, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Fig. 1. Effects of Mg on cellular activities of two HNC cell lines: **(a)** The addition of $MgCl_2$ in the culture medium significantly increased the proliferation of CAL27 and FaDu cells, especially at the level of 2.0 mM, a concentration slightly higher than physiological Mg level (i.e., 0.8 mM) in body fluid. (n=5, Significant difference was indicated as * $p < 0.05$). **(b)** Representative cell cycle analysis showing that, when compared with control groups, 24 h treatment of Mg contributed to increased ratio of HNC cells at their G2 phase. **(c)** The depletion of Mg dramatically inhibited the invasion of CAL27 and FaDu cells in transwell experiment (n=4, Significant difference was indicated as * $p < 0.05$). **(d)** Representative images of wound healing experiments of two HNC cell lines demonstrated that the addition of Mg also resulted in accelerated cell migration, indicated by earlier closure of wound in 2.0 mM Mg group. **(e)** Representative immunofluorescent images showing positive staining of TRPM7 in green, and counter-stained nuclei in blue. TRPM7 were primarily expressed in the cytoplasmic membrane. **(f)** Representative blots showing the expression of TRPM7 was up-regulated by extracellular Mg level in both cell lines.

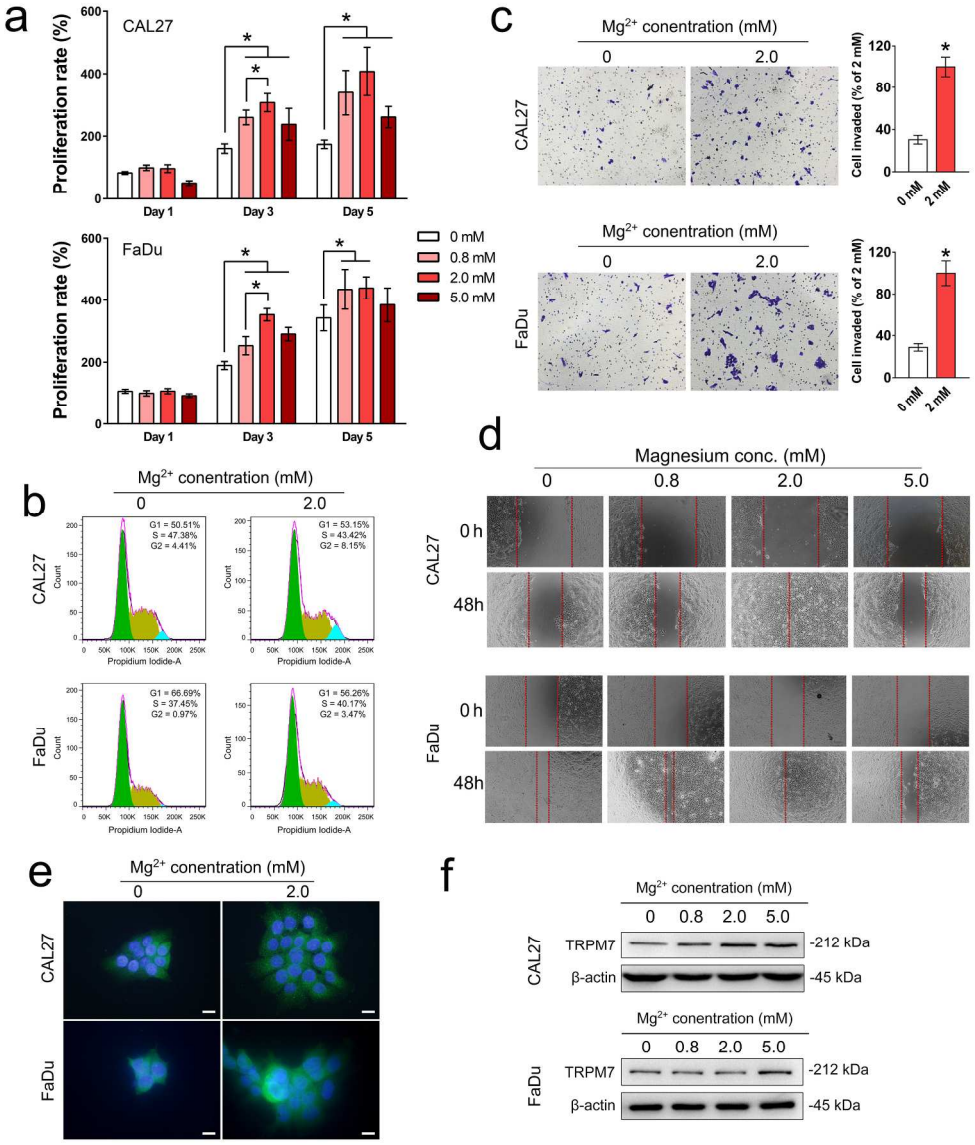
Fig. 2. The effects of TRPM7 blockage and knockdown on the cellular activities of two HNC cell lines. **(a)** Representative blots showing the expression of TRPM7 in TRPM7-siRNA treated CAL27 and FaDu cells were successfully inhibited when compared with non-treated (NT) or control-siRNA (Ctrl) transfected groups. **(b)** Both the blockage of TRPM7 by FTY720 (3 μM for 2 h) and inhibition of TRPM7 by siRNA led to compromised proliferation of CAL27 cells and FaDu cells (n=4, Significant difference was indicated as * $p < 0.05$). **(c)** Both Blockage of TRPM7 and transfection of TRPM7-siRNA resulted in decreased invasion capability of CAL27 and FaDu in transwell assay (n=4, Significant difference was indicated as * $p < 0.05$). **(d)** When TRPM7 was either blocked or inhibited, the proliferation of CAL27 cells and FaDu cells were no longer affected by extracellular Mg level. (n=4, No significant difference was indicated as n.s.) **(e)** Pre-treatment of FTY720 also contributed to retarded migration of CAL27 and FaDu cells.

Fig. 3. The involvement of AKT-mTOR signalling upon stimulation of Mg. **(a)** Representative blots showing the upregulation of mTOR, increased phosphorylation of c-Raf, AKT and PDK, as well as the inhibitory phosphorylation of GSK-3β resulting from the increase in Mg concentration. Meanwhile, the addition of FTY720 demolished the effect of Mg. **(b)** Relative protein level quantified by densitometry showing the fold change in protein level compared with the control group. **(c)** Representative immunofluorescent images showing the expression and localization of mTOR in CAL27 and FaDu cells. mTOR was stained with Alexa-Fluor 488 (green), whereas nuclei were counter-stained with Hoechst 33324 (blue). In both cell lines, mTOR were primarily expressed in the vesicles and cytosol. **(d)** Schematic showing the mechanism in which Mg promotes the proliferation, invasion and migration of HNC cells. In brief, extracellular Mg could trigger the AKT signaling pathway via the transmembrane cation channel TRPM7. The activation AKT signaling then contributes to the activation of c-Raf-MEK-ERK signaling, the upregulation of mTOR, and the inhibitory phosphorylation of GSK-3β. These, collectively, lead to various tumorigenic activities for the growth and progress of HNC.

Fig. 4. The effects of Mg in drinking water on growth of xenograft tumor in nude mice. **(a)** Representative histological images (H&E staining, scale bar = 200 μm) with inserted photos showing the addition of Mg in drinking water contributed to larger xenograft tumor in the tongue tissues of nude mice, meanwhile, both *i.p.* injection of FTY720 (10 mg/kg) and transfection of TRPM7-siRNA in inoculated FaDu cells resulted in decreased size of tumor. **(b)** Statistical analysis confirmed the size of xenograft tumor in mice was affected by the presence of Mg in drinking water and the function of TRPM7 (n=6, Significant difference was indicated as * $p < 0.05$). **(c)** There was no significant difference in the serum Mg concentration regardless of the addition of Mg in the drinking water. **(d)** The growth of xenograft tumor in tongue also led to decrease in body weight of the mice. **(e)** Representative histological images revealing the formation of blood vessels (yellow arrows) and keratinization (green arrows) can be noted in

xenograft tumor in Mg-water fed mice (H&E staining, scale bar = 100 μm). **(f)**. Immunofluorencent images (scale bar = 40 μm) showing higher expression of Ki67 in the xenograft tumor tissues (T) in the tongues of mice fed with Mg containing water, higher magnification was shown as the insertions (scale bar = 5 μm). **(g)** Representative immunofluorencent images (scale bar = 40 μm) showing the upregulation of TRPM7 in the neoplastic cells at the margin of the xenograft tumor tissues (T) in the tongue, lower images (scale bar = 10 μm) are high-resolution versions of the boxed regions.

Fig. 5. (a) The salivary Mg level of NPC patients (n=72) were significantly higher ($P<0.05$) than that of healthy controls (n=12). While there was no significant difference in salivary Ca level between NPC patients and the control individuals. **(b)** Representative IHC images (scale bar = 5 μm) demonstrated that TRPM7 expression was higher in tumor tissues than in normal tissues.



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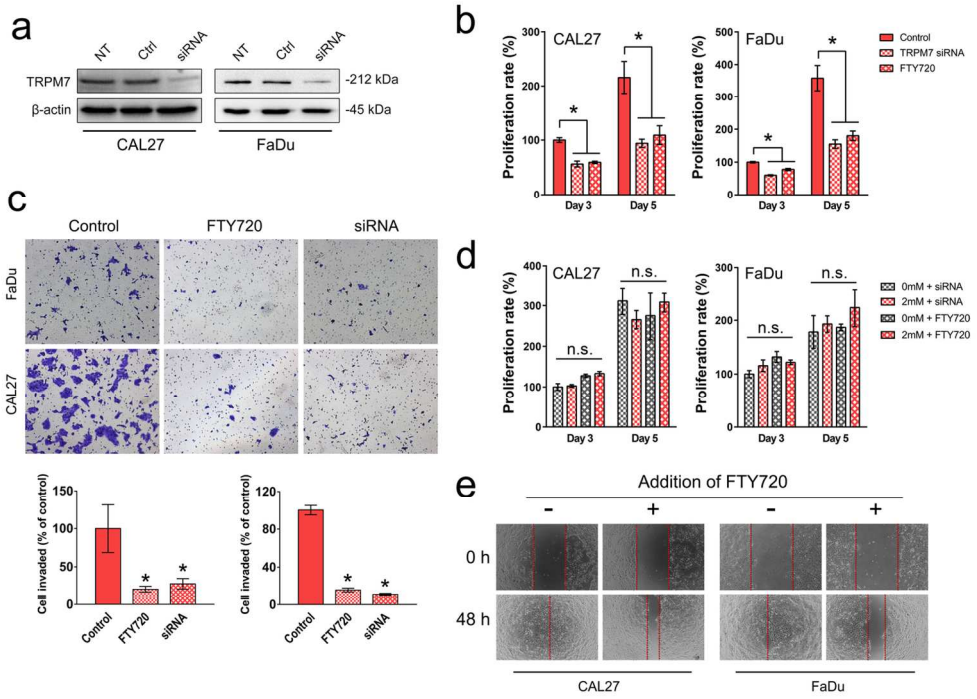

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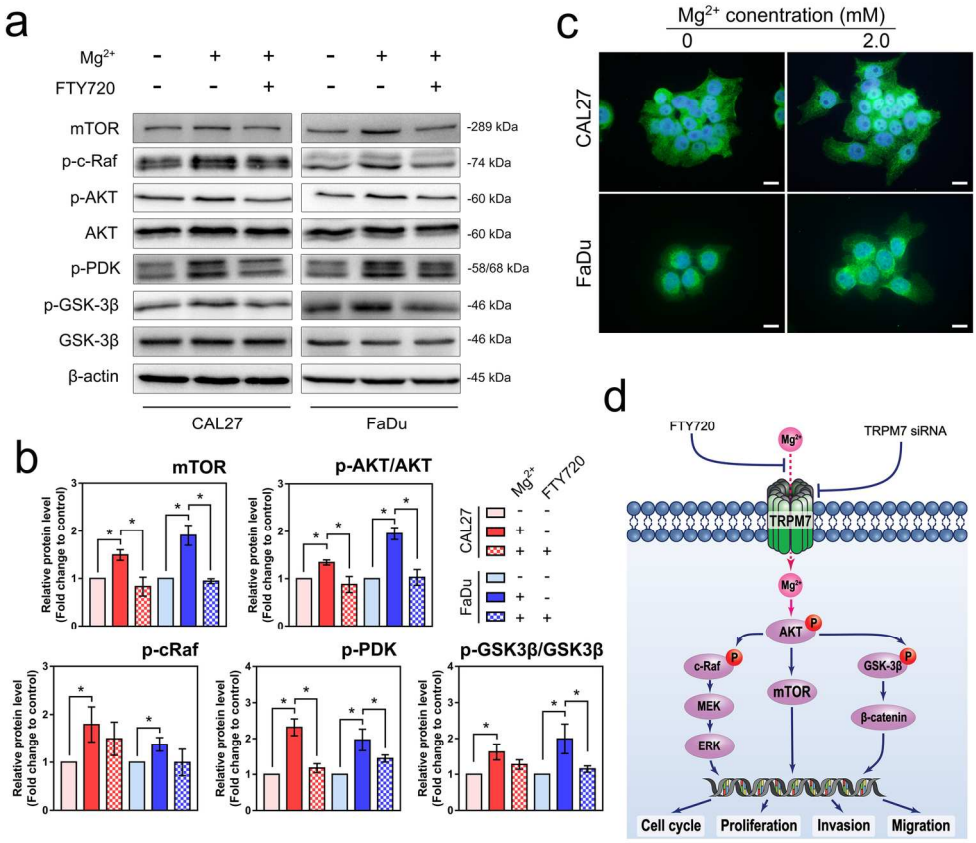

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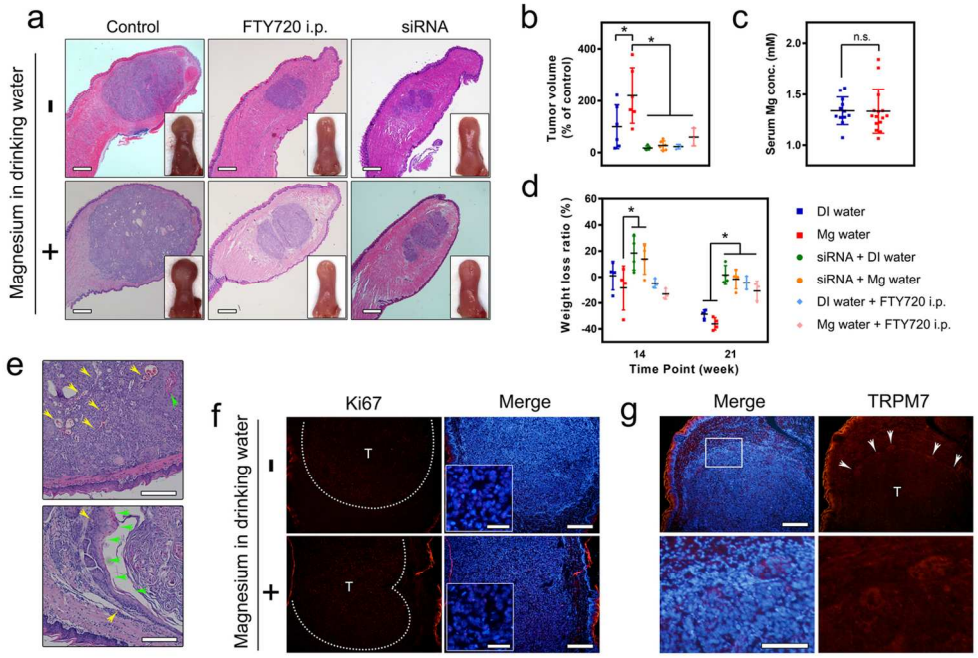

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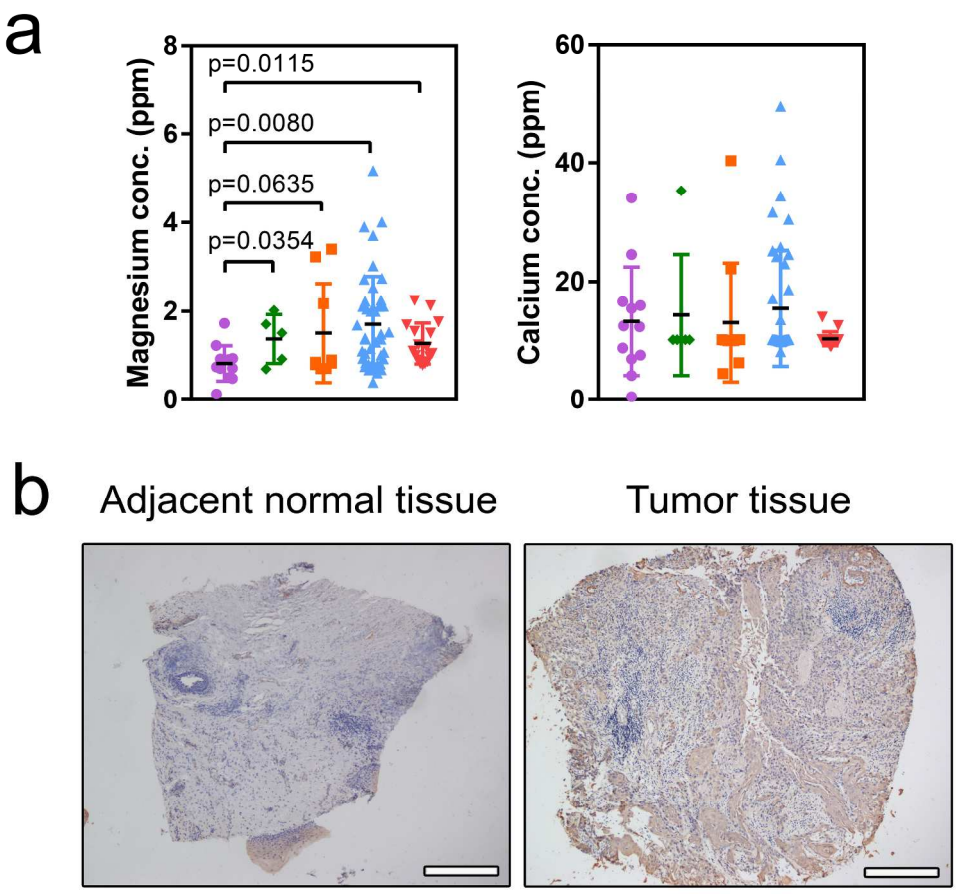

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Supplementary materials

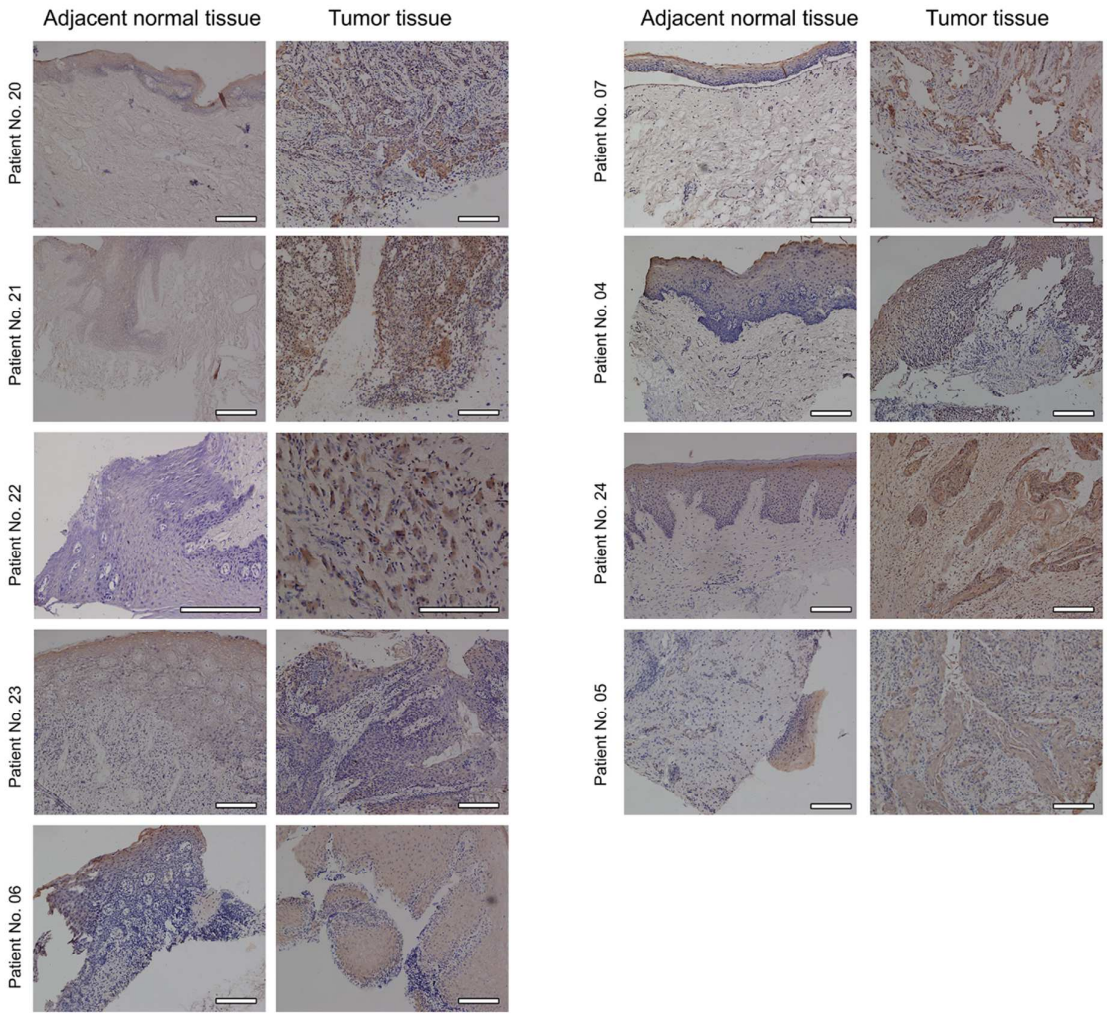
Supplementary table 1. Characteristics of NPC patients.

Characteristics (n=72)	No. of patients (%)
Age (years)	
Mean (SD)	52.32 (11.06)
Gender	
Male	47
Female	25
AJCC* tumor stage	
I	5
II	9
III	41
IV	17
T classification	
1	24
2	8
3	29
4	11
N classification	
0	11
1	14
2	37
3	10
M classification	
0	71
1	1
2	0

*AJCC: American Joint Committee on Cancer

Supplementary table 2. Characteristics of SCC patients.

Patient ID	Origin of tumor tissues	Age	Gender	AJCC tumor stage
04	Mandible	72	Male	T4aN0M0
05	Mandible	80	Male	T4aN2bM0
06	Mandible	65	Male	T2N0M0
07	Maxilla	87	Male	T4aN0M0
20	Maxilla	58	Female	T3N2aM0
21	Tongue	29	Male	T1N2bM0
22	Mandible	65	Female	T1N0M0
23	Buccal	55	Female	T1N0M0
24	Mandible	70	Male	T3N0M0



Supplementary figure 1. Representative IHC images (scale bar = 5 μm) demonstrated that in all of the HNC patients, TRPM7 expression was higher in tumor tissues than in adjacent normal tissues