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Tumor inhibitory effects of intravesical *Ganoderma lucidum* instillation in the syngeneic orthotopic MB49/C57 bladder cancer mice model

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Abstract

Ethnopharmacological relevance

Ganoderma lucidum (GL) has been traditionally used in oriental medicine as superior health tonic, and there are numerous scientific evidences of its antitumorigenic activities.

Aim of the study

To evaluate the intravesical chemopreventive effects of ethanol extract of GL (GLe) on bladder cancer.

Materials and ~~Methods~~methods

Intravesical therapy is defined as the direct instillation of a liquid drug into bladder through a catheter. *Bacille Calmette-Guerin* **(**BCG) solution is applied intravesically as a conventional immunotherapy for preventing recurrence of bladder cancer. By adopting the MB49/C57 bladder cancer mice model, an overall 60 MB49-implanted mice were randomized into 3 groups and treated according to 3 treatment arms, including GLe, BCG and PBS. Additionally, wild-type mice without MB49 cell inoculation and treated with PBS were used as the negative control group. Testing agents were instilled intravesically for 2 h and repeated after one week for evaluating the effects on preventing the tumor formation and growth. The treated-mice were closely monitored for major adverse effects.

Results

GLe demonstrated more potent cytotoxic effects than BCG on MB49 cells, although both in dose-dependent manner. In the MB49-implanted mice, ~~80~~80 μg/ml GLe was shown to delay the tumor formation by one week, whereas the averaged tumor volume measured at endpoint was 3.6-fold and 4.6-fold smaller than that of the BCG or PBS, respectively. However, no significant effects were observed on body weight and hematuria.

Conclusion

Current findings in mice suggested intravesical GLe therapy as an effective and safe chemopreventive strategy for inhibiting bladder tumor formation.

Abbreviations: BCG, Bacille Calmette-Guerin; CIS, Carcinoma in situ; CO_2, Carbon dioxide; DNA, Deoxyribonucleic acid; GL, Ganoderma lucidum; GLe, Ethanol extract of Ganoderma lucidum; HUC-PC, Pre-cancerous human

uroepithelial cells; LDH, Lactate dehydrogenase; PBS, Phosphate-buffered saline

Keywords: Ganoderma lucidum; Bladder cancer; ~~urothelial~~Urothelial carcinoma; MB49; ~~intravesical~~Intravesical therapy

1 Introduction

Ganoderma lucidum (Curtis: Fr.) P. Karst has been proven to possess anti-tumorigenic properties in cancers of various origins from *in vitro* to animal and human *in vivo* (Yuen and Gohel, 2005). Meta-analysis of eligible randomized controlled trials (RCTs) identified in a recent systematic review (Jin et al., 2016) has suggested the adjuvant administration of GL to conventional treatments by enhancing tumor response and stimulating host immunity, yet there was insufficient evidence to justify its usage as a first-line treatment for cancer. The two major bioactive fractions of *G. lucidum* (GL), namely polysaccharides and triterpenes were believed to be responsible for such anticancer activities through immune enhancement of cancer patients as well as inducing cytotoxicity on cancer cells, respectively (Kao et al., 2013). Traditional uses of GL products for anticancer activity were mainly evidenced by its adjuvant roles in increasing immune cell levels (Gao et al., 2003, 2005) and improvements in physical well-being, fatigue and anxiety (Zhao et al., 2012). This medicinal fungus may thus induce anti-cancer effects indirectly through its immunomodulating properties, with particularly the downregulation of cancer T-helper 2 (Th2) cytokines as well as activation of cancer-related immune cells including dendritic cells and natural killer cells (Lin et al., 2005; Chang et al., 2014; Guggenheim et al., 2014). However, its effectiveness in tumor shrinkage in cancer patient were earlier evidenced in two Chinese studies, which have incorporated GL products as part of the mainstream treatment regimen (Yan et al., 1998; Zhang et al., 2000). In addition to the above discussed, a few ongoing clinical trials were registered (clinical trails.org: NCT00575926 and NCT02844114) where results are pending. Particular for bladder cancer, ethanol extracts of the fruiting body and spores of GL were defined as potent cytotoxic agents that exhibited G2/M cell cycle arrest in the low-grade cancerous MTC-11 cells and the chemically transformable pre-cancerous Human Urothelial cells (HUC-PC) (Lu et al., 2004). The cytotoxicity of such extracts seemed to be dependent on the malignancy of the cells, such that the potency was higher in MTC-11 when compared with HUC-PC (Lu et al., 2004). Later on, our research team has adopted the successive ethanol extraction procedure of Lu et al. (2004) to re-extract a commercially available proprietary extract of GL to separate into water-soluble and water-insoluble re-extracts which were named as GLw and GLe, respectively (Yuen and Gohel, 2008; Yuen et al., 2008, 2011, 2012, 2013). Further tested with GLe, more significant cytotoxic effects were obtained when the HUC-PC cells were challenged with the corresponding bladder carcinogen (Yuen et al., 2008). As evaluated in non-cytotoxic dosages, the GLe-treated HUC-PC cells displayed irreversible progressive apoptosis accompanied with a reduction of telomerase activity (Yuen et al., 2008). Free radicals and oxidative deoxyribonucleic acid (DNA) damage were elevated in dose-dependent manner in media harvested from the cultures of GLe-treated apoptotic HUC-PC cell (Yuen and Gohel, 2008; Yuen et al., 2008). Opposite to the genocytotoxic characteristics of GLe, its water-extractable counterpart GLw was demonstrated to be genoprotective while it did not exhibit any growth inhibition (Yuen and Gohel, 2008). Although oxidative stress was postulated to be the mechanism underlying the apoptotic events of GLe in bladder cancer cells, such causal relationship between genotoxicity and growth inhibition has never been proven.

Despite the vast majority of bladder cancer that belongs to the superficial type of urothelial carcinoma that are usually resectable, there are still up to 80% of patients suffering from recurrence (Williams et al., 2010; Chamie et al., 2013; Malats and Real, 2015). Those recurrent cases were dependent on the tumor grading, whereas multiple recurrences and malignant progressions were common (Chamie et al., 2013). Over the past four decades, high-risk patients could opt to receive intravesical therapy with the tuberculosis vaccine *Bacille Calmette-Guerin* (BCG), which was used as the first-line prophylactic agent with an overall effectiveness in reducing about one-half of the recurrences (Brandau and Suttmann, 2007). However, a better chemopreventive agent is still needed, because 30–40% of recurrences remains even with BCG and with its toll of high side effects (Brandau and Suttmann, 2007; Udovicich et al., 2017). Intravesical BCG therapy is referred as a form of “immunotherapy”, since live BCG vaccine was known for triggering non-specific immunity to attract immune cells to come to the bladder wall for eradicating any residual tumor cells (Redelman-Sidi et al., 2014). Our previous studies have reported that GLe was synergistic with BCG to inhibit the growth of HUC-PC cells, whereas GLe was also shown to be immunologically active for inducing neutrophilic migration that may also be favorable for apoptotic clearance (Yuen et al., 2011). Owing to the fact that scientific evidences available so far supporting the chemopreventive effects of GLe on bladder cancer as well as the potential synergism with BCG, were limited to be *in vitro*, GLe and BCG should be first evaluated independently for their effectiveness and safeness by using a relevant animal model. There are many tumor-bearing animal models available for bladder cancer, but very few of these models were both orthotopic and syngeneic to allow implantation of tumor cells in immunocompetent mice to evaluate the tumor behavior at the organ-specific microenvironment (John and Said, 2017). This kind of model is essential for evaluating the chemopreventive properties of BCG, which is conventionally akin to applying to bladder intravesically with an underlying immunological mechanism that requires immunocompetence (John and Said, 2017). The ~~MB49~~MB49/C57 mouse has shown to be the excellent model to fulfill these criteria, with evidences of high up-take rate of the tumor cells as well as the high survival rate (Gunther et al., 1999). Therefore, in this study, the chemopreventive effects of GLe were investigated and compared with BCG using the ~~MB49~~MB49/C57 model.

2 Materials and methods

2.1 Study design

This study adopted the orthotopic ~~MB49~~MB49/C57 bladder cancer mice model (Gunther et al., 1999; Dobek and Godbey, 2011) to evaluate the chemopreventive effects of GLe in terms of tumor formation and growth as well as any major adverse effects. The conventional BCG therapy was also evaluated for comparison. Intravesical instillation was used as the route of administration for both testing regimens. Before evaluating in the animal model, the cytotoxic

effects and effective dosages of GLe and BCG were first determined by *in vitro* experiments with the ~~MB49~~MB49 cell line.

2.2 Preparation of G. lucidum extract and BCG

Three batches of GLe were freshly prepared from the proprietary extract of fruiting bodies and cracked spores (ReishiMax ~~GLp~~GLp™, ~~TM~~ Pharmanex Inc., Hong Kong) according to the successive ethanol extraction procedure published elsewhere (Yuen et al., 2008; Yuen and Gohel, 2008). The industrial product with batch number: DL25351 was used in this study. The filtered water-insoluble brown powder GLe was preserved in a desiccator. Immediately before testing, the GLe was dissolved in appropriate amount of absolute ethanol and then further diluted into working concentrations with incomplete media, whereas the final concentration of absolute ethanol (vol/vol) was below 0.01% to ensure any cytotoxic effects were not due to the alcohol. The water-soluble extract (GLw) was discarded and not used in the present study. The Tokyo 172 strain freeze-dried intravesical use BCG (Immunobladder®, Japan BCG Lab, Japan) was used in this study. This drug is indicative for treating TaT1 papillary tumors and carcinoma in situ (CIS) of the urinary bladder (Ikeda et al., 2005), and it is currently used in Hong Kong as the sole immunotherapeutic agent for bladder cancer. According to the manufacturer's manual, each vial of immunobladder® BCG contains ~~80~~80 mg live BCG and ~~140~~140 mg inactive ingredient as stabilizer, which reconstitutes with ~~2~~2 ml isotonic sodium chloride solution as diluent to make up the ~~1~~1 mg/ml as the clinical dosage.

2.3 Cell culture and assays

The murine urothelial carcinoma ~~MB49~~MB49 cell line was used because of its known compatibility and efficiency for tumor inoculation in C57BL/6 J mice. This cell line was cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (GIBCO BRL Island, New York, NY) and 1% penicillin G/streptomycin (10,000 µg penicillin, 10 mg/ml streptomycin). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Logarithmically growing cells were subcultured when reaching 90% confluence, and culture media were replaced every 48 h. The cytotoxicities of GLe and BCG on MB49 cells were assayed by using the LDH Cytotoxicity Detection Kit (Clontech Laboratories Inc., Mountain View, CA). The LDH assays were performed according to the manufacturer's instruction on the 96-well microplate format. Neither the test substances nor their solvents were demonstrated to have interference to the kit reagents. Trypan blue exclusion assay was performed to determine the cell viability. The percentage distribution of cell cycle phases (G0/G1, S and G2/M) of viable cells were further determined by using the Cell Cycle Assay Kit (Flurometric – Green) (Abcam ab112116, Cambridge, MA) with the BD FACSAria™ ~~TM~~ III Flow Cytometry (BD Biosciences, SAN Jose, CA). All *in vitro* assays were run in triplicate in three independent experiments.

2.4 Animals and ~~MB49~~MB49 tumor cells implantation

All animal experiments conducted in this study were approved by the Animal Subjects Ethics Sub-Committee (ASESC No. 13/22) of the Hong Kong Polytechnic University (HKPolyU). Female ~~C57BL/6~~C57BL/6J mice of 4–6 weeks old with weights in the range of ~~15–20~~15–20 g were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. On arrival, mice were acclimatized for at least 2 weeks prior to any experiments by housing in the Centralized Animal Facilities of the HKPolyU, where they were housed four mice per cage with a 12-h light/dark cycle, and regular diet and water ad libitum. In this study, wild-type non-tumor bearing C57 mice were used as the “Negative Control” of the experiment, where these mice still received the electrocautery procedure and instilled with phosphate-buffered saline (PBS) instead of ~~MB49~~MB49 cell suspension and followed by the same intravesical therapy cycles as other groups but again instilled with PBS. The ~~remining~~remaining animals were ~~MB49~~MB49 tumor-bearing and randomly assigned into one of the three groups: 1) Positive Control, 2) GLe, and 3) BCG. Hence, the “Positive Control” group was defined as ~~MB49~~MB49 tumor-bearing mice receiving intravesical treatments with PBS. All mice were subjected to the electrocautery procedure for inducing an open wound at the urothelium of the bladder wall (Dobek and Godbey, 2011). The procedure was illustrated in Fig. 1. The mice were anesthetized with isoflurane inhalation and stabilized each with its back placing on the ground plate of the cautery unit (Aaron 950 high frequency desiccator, Bovie Medical Co., Clearwater, FL). A 24-gauge SURFLO® I.V. catheter (Terumo Co., Philippines) was inserted into the bladder through the urethra with plenty of K-Y Jelly lubricant, and the bladder was emptied by draining out the urine. Urine specimens were collected once a week to test for hematuria using the Multistix® 10 SG reagent strip (Siemens, Germany). Then, a platinum wire electrode with a diameter of ~~0.1~~0.1 mm was inserted through the catheter lumen, followed by applying a monopolar coagulation at ~~2~~2 W for ~~1~~1 s and the wire was removed. ~~MB49~~MB49 cell suspension ~~(0.1~~(0.1 ml at density ~~1×10~~1×10^6 cells per ml with 95% viability) was instilled into the bladder for ~~2~~2 h through a syringe attached to the catheter in situ. The bladder was rinsed once with PBS before removing the catheter, the mouse was allowed to recover from anesthesia in a small recovery chamber, then returned to the housing facility. Intravesical therapies were scheduled for all mice on day 1 and day 8 following the electrocautery tumor implantation, according to the assigned treatment groups i.e. optimal GLe dose, optimal BCG dose, and PBS for both positive and negative controls. Particularly for the GLe group, a total of 27 mice were required because the GLe was tested in triplicate in 3 different batches of re-extraction. The instillation volume of all groups was fixed as ~~0.1~~0.1 ml, after considering the net ~~50~~50 µl bladder capacity of mice in addition to another ~~50~~50 µl for the deadspace volume of the catheter. The intravesical therapy procedure was followed exactly the same as the ~~catherization~~catheterization and instillation procedures for tumor cells inoculation. All experimental procedures were carried out in the light phase of the dark/light cycle. The tumor growth was monitored by transabdominal micro-ultrasound imaging technique, as the details described below. All animals were sacrificed by carbon dioxide (CO_2) inhalation no later than day 21 as the final follow-up time point for harvesting the bladder for histological examination.

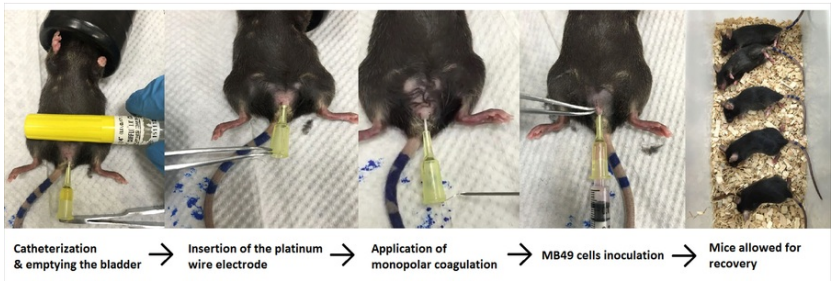

Fig. 1 A photo series of the MB49 inoculation procedure.

alt-text: Fig. 1

2.5 Transabdominal micro-ultrasound imaging technique

The Vevo2100 high-frequency ultrasound system (Visual Sonics, Toronto, Canada) equipped with a real-time micro visualization Transducer MS550D at a frequency of ~~40~~40 MHz was used in this study. An anesthetized mouse was prepared by shaving the abdominal hair with the application of depilatory cream. Then the mouse was secured onto the heated ultrasound platform, where the high viscosity ultrasound gel was applied onto the surface of abdomen skin. Isoflurane inhalation was applied throughout the entire imaging procedure and vital signs including heart and respiratory rates were closely monitored, whereas the procedure was immediately terminated if any abnormalities. The mouse bladder was filled with ~~100~~100 µl PBS through the catheter in place. The pelvic region was scanned in the transverse and sagittal planes, in order to examine the location and size of any visualized tumors in two dimensions. The tumor volumes were determined by measuring the largest longitudinal and the transverse diameters in the anterior-posterior plane, and calculated according to the formula ~~π~~/6π/6 × length × ~~length~~width^2 (Patel et al., 2010). Following the imaging procedure, the mouse was placed in the recovery chamber before returning to the housing facility.

2.6 Statistical analysis

Data collected in the study was managed and analyzed using SPSS Statistics 23.0 (IBM, Armonk, NY) and GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA). All assays were performed in triplicate for reproducibility. Descriptive statistics (such as mean, standard deviation (SD), frequency and percentage) were used to summarize the results. Chi squared (χ^2) test and one-way ANOVA were used to compare the differences between multiple groups for categorical variables (frequency and percentage) and continuous variables (mean and SD), respectively. Statistical significance was sought at two-tailed *p*-value of 0.05.

3 Results

Ethanol extract of *G. lucidum* (GLe) was shown to be more cytotoxic to the ~~MB49~~MB49 cells than the conventional immunotherapeutic BCG *in vitro* (Fig. 2). The cytotoxicity of GLe reached a plateau of 85–88% at concentration 80 µg/ml or above (Fig. 2A). All assayed GLe solutions had ethanol concentrations below 0.01% vol/vol, whereas the cultures treated with solvent control (PBS with 0.01% vol/vol ethanol; negative control) confirmed that such cytotoxicity of GLe was not due to the effects of ethanol. Whilst the clinical dosage of BCG as recommended by the manufacturer (i.e. 1 mg/ml) has demonstrated to eradicate around 21.71 ± 5.78% of the ~~MB49~~MB49 cells, and doubling of the dosage has increased the cytotoxicity 33.31 ± 6.34% (Fig. 2B). Such cytotoxic effects inhibited the growth of ~~MB49~~MB49 cells in the following 24 h post-treatment, which demonstrated in a clear dose-dependent manner (Fig. 2C). However, analysis of the viable treated cells remaining in cultures at 48 h revealed similar cell cycle distribution profile with the propagating control cells and corresponding solvents, typically with 50–52% at G0/G1, 22–25% at S, and 20–23% at G2/M phases (Fig. 2D). The *in vitro* findings herein suggested the intravesical concentration to be tested in the animals at 80 µg/ml for GLe and 1 mg/ml (i.e. recommended clinical dosage) for BCG.

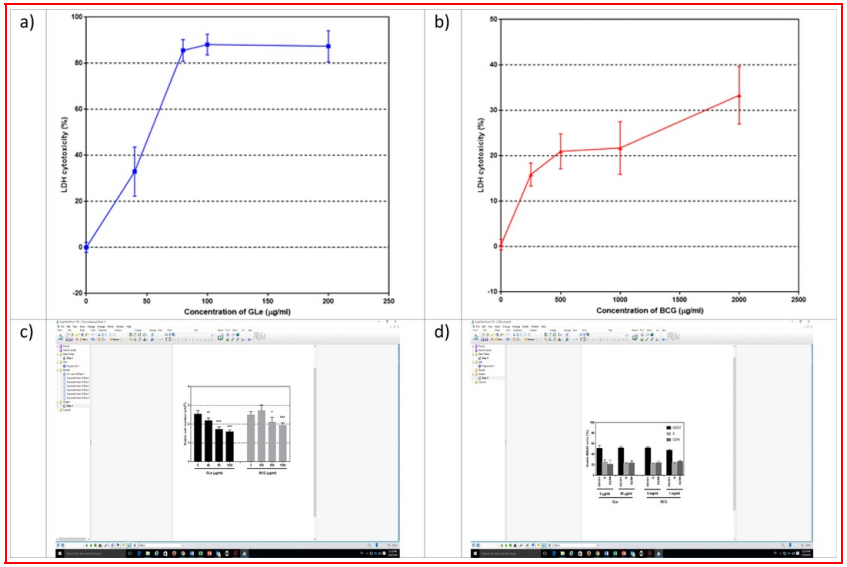

Fig. 2 *In vitro* Cytotoxic effects of **(a)** GLe and **(b)** BCG were evaluated by measuring the amounts of LDH released from the MB49 cells, which were correspondent to **(c)** the reduction of viable cell numbers growing in next 24 h while **(d)** no changes were observed in the cell cycle phase distribution.

alt-text: Fig. 2

Current laboratory setting recorded a 100% successful rate for the ~~MB49/C57~~MB49/C57 model establishment. The earliest full-blown tumors were detected in mice on day 5 following the electrocautery inoculation of ~~MB49~~MB49 cells. Transabdominal micro-ultrasound imaging captured the progressive and rapid growth of the tumors up to day 15, in order to estimate the tumor volumes (Fig. 3). Stereomicroscopic and histologic examinations verified the tumor existence in the bladder harvested not later than day 21 (Fig. 4), and these bladder sections were kept for other molecular analyses. When compared with a normal bladder, the freshly dissected tumor-bearing bladders displayed more erythematous with thickened wall and decreased elastic with edematous tissue (Fig. 4A and B), which suggested the signs of inflammation. The tumor inside the bladder wall could be easily observed as clear shadow under the stereomicroscopy (Fig. 4C). The bladder mucosa and lumens were clearly defined in a normal bladder (Fig. 4D) in contrast to the tumor-bearing bladder lumens that were significantly occupied where the structures of mucosa and associated tissue became more difficult to identify (Fig. 4E and F). The ~~MB49~~MB49 tumors were outgrowths from the bladder wall and clearly distinguished from the normal tissue with strong hematoxylin staining in purple color (Fig. 4E and F).

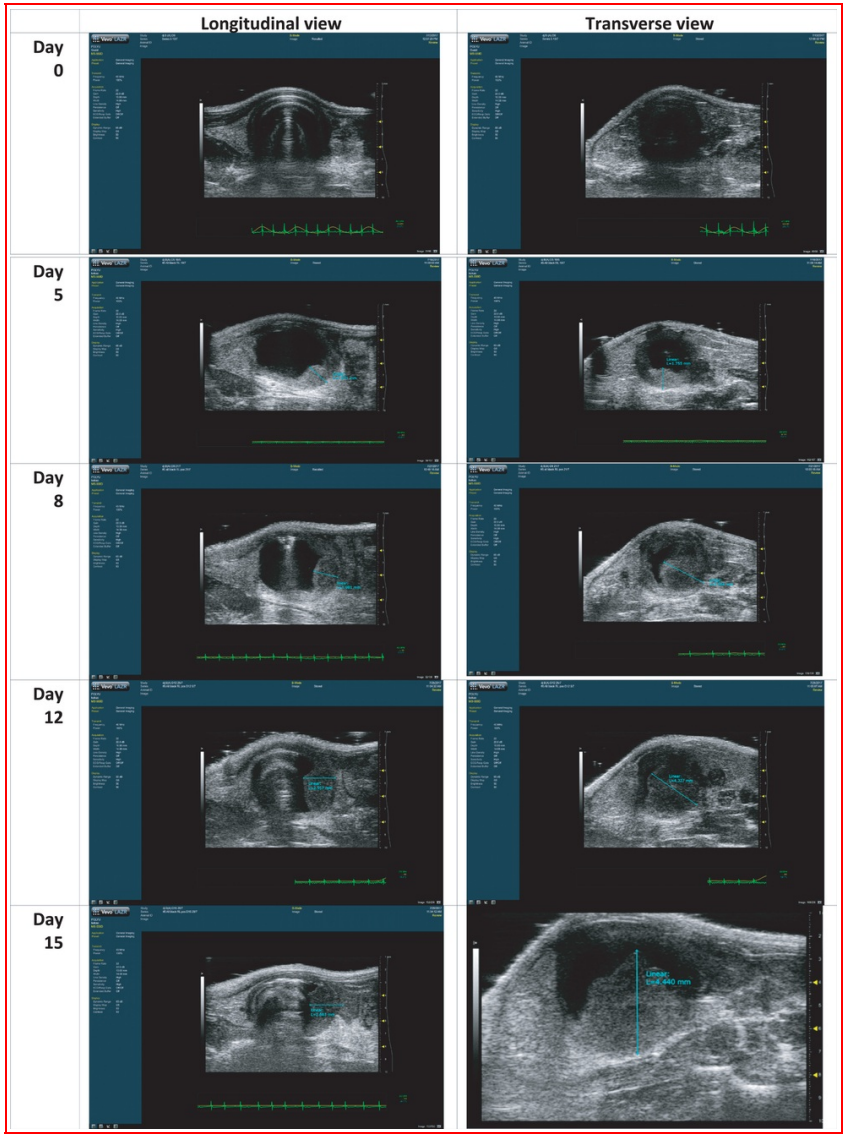

Fig. 3 The two dimensional (longitudinal and transverse) measurement of the tumor volume at different time points, as illustrated by a mouse of the positive control group.

alt-text: Fig. 3

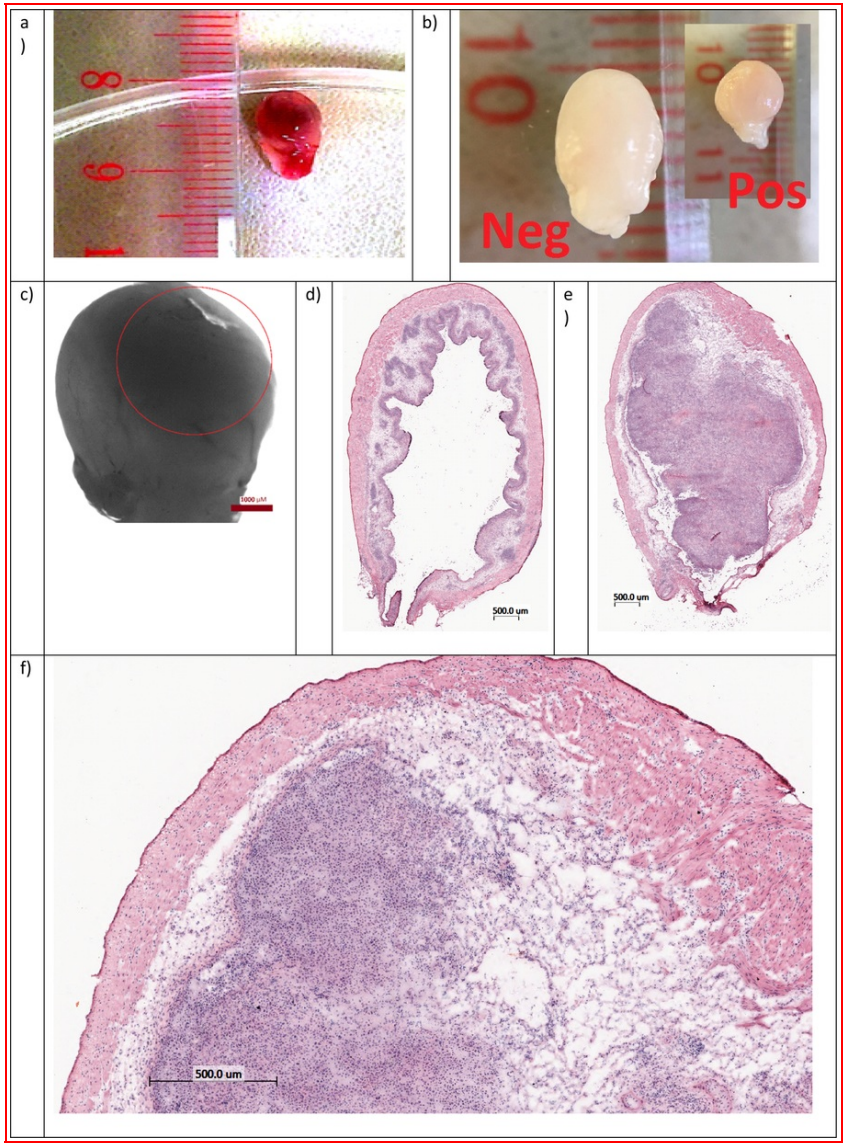

Fig. 4 The examination of urinary bladder harvested at the endpoint, showing the gross appearance of **(a)** a tumor-bearing bladder and **(b)** comparison of the external appearance of the bladder samples of positive and negative controls. The tumor-bearing bladder was examined under **(c)** the stereomicroscopic for the tumor location. Histological examination was performed on tumor sections, showing in **(d)** a negative control section with clear bladder lumen (1.3×) and in **(e)** a large tumor grew occupied the bladder lumen of a positive control section (1.3×), where **(f)** illustrate a closer look of the tumor tissue at higher magnification (8.3×).

alt-text: Fig. 4

A total of 60 ~~MB49-implanted~~MB49-implanted mice were randomized into 3 groups and treated according to 3 treatment arms, in addition to an extra group of 8 negative control mice which neither indicate any tumor formation in the urinary bladder nor any active treatments. The ~~MB49~~MB49 tumors of all groups were observed to follow linear growths (Fig. 5). In the positive control group, ~~MB49~~MB49 tumors were first detected on day 5 post-inoculation in about half of the mice with an average volume of ~~1.13±0.47~~1.13 ± 0.47 mm^3~~mm^3~~, which by day 12, all mice had an average volume of ~~11.25±0.90~~11.25 ± 0.90 mm^3 (enlarged 15.5 times) of the initial tumor size to ~~17.56±0.58~~17.56 ± 0.58 mm^3

on day 15 (Table 1). In Fig. 5, ~~MB49~~MB49 Tumors treated with BCG were shown to be growing in smaller sizes than those of the positive control group at each corresponding time points. BCG did not affect the tumor incidence at various time points while statistically significant ~~(p<0.05)~~(p < 0.05) growth inhibitory effects were only observed at the initial week of tumor inoculation, as measured on day 5 and day 8 (Table 1). Apart from reducing the tumor sizes, GLe was also demonstrated to be effective in delaying the formation time of tumors by one week, from day 5 to day 12 (Fig. 5). As referred to Table 1, although tumors were probably present between day 8 and 12 in mice treated with GLe, the first tumor was observed on day 12, whereas the incidence rate of tumor formation was dramatically increased to 85% on day 12 and all mice became tumor-bearing on day 15. Furthermore, the growth inhibitory effects were shown to be more significant than that exhibited by BCG and the tumor sizes measured on day 12 and day 15 were about 4.6–5.0 folds and 3.4–3.6 folds smaller than those of the positive control group and BCG treatment group, respectively (Table 1). Body weights of mice were found to be correlated with the tumor sizes, particularly on day 12 and day 15, the body weights of all 3 treatment groups were significantly different from the negative control group (Table 1). Furthermore, by using urine strip test, moderate hematuria (at grade 3–4) was observed in up to half of the negative control mice in the first week, which was believed to be caused by the injuries induced by the electrocautery procedure in the bladder wall (Table 1). Whilst hematuria was more commonly occurred in the tumor-bearing mice at higher grades in general, no significant differences were observed between the GLe and positive control groups. However, the BCG-treated mice were shown to have significantly ~~(p<0.001)~~(p < 0.001) lower grade of hematuria than both the GLe and positive control groups on day 15. The voiding volume of urine ranged ~~40–80~~40–80 µl and no significant differences were observed amongst the four groups, except a clear decreasing trend was observed in the positive control mice with the most significant tumor sizes and significantly ~~(p<0.001)~~(p < 0.001) lower volume on day 15 than the negative controls as well as the other two treatment arms (Table 1).

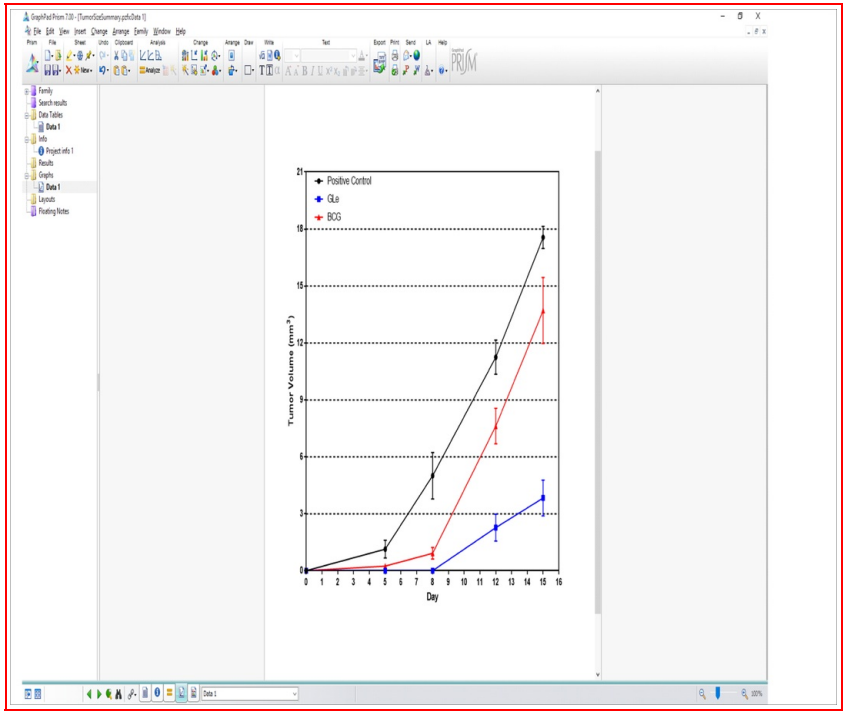

Fig. 5 A plot of tumor volume against the time points amongst the three groups, in order to illustrate the comparison of the time for tumor formation and tumor size.

alt-text: Fig. 5

Table 1 Comparison of major parameters measured in the testing and control groups.

alt-text: Table (Footnote 3 of Table 1 has repeated twice 'df1 =')1 (*p<0.05; **p<0.01; ***p<0.001)

Variables	Day	Negative Control	Positive Control	GLe	BCG	Statistical analysis
		(n = 8)	(n = 18)	(n = 27)	(n = 15)	

Procedure received		Electrocautery w/o ~~MB49~~MB49 cells + PBS	~~MB49~~MB49 inoculation + PBS	~~MB49~~MB49 inoculation + 80 µg/ml GLe	~~MB49~~MB49 inoculation + 1 mg/ml BCG	
		Number (Percentage)				***χ2 p*-value ~~a~~**[1]
Incidence of tumor occurrence	1	0 (0)	0 (0)	0 (0)	0 (0)	–
	5	0 (0)	10 (55.6)	0 (0)	8 (53.3)	~~**<0.0001**~~**< 0.0001**
	8	0 (0)	15 (83.3)	0 (0)	14 (93.3)	~~**<0.0001**~~**< 0.0001**
	12	0 (0)	18 (100)	23 (85.2)	15 (100)	**0.073**
	15	0 (0)	18 (100)	27 (100)	15 (100)	–
Incidence of hematuria	1	3 (37.5)	13 (72.2)	20 (74.1)	9 (60.0)	0.616
	8	4 (50.0)	13 (72.2)	20 (74.1)	11 (73.3)	0.991
	15	0 (0)	15 (83.3)	14 (51.9)	8 (53.3)	0.077
						One-way ANOVA
		~~**Mean±SD**~~**Mean ± SD**				**F(df1,df2); *p*-value**
Tumor volume (mm^3)	1	–	–	–	–	–
	5~~#~~[2]	–	1.13 ± 0.47	–	~~**0.25±0.11***~~**0.25 ± 0.11***	**7.956(2,20);** ~~**p=0.0029**~~**p = 0.0029**
	8~~#~~[2]	–	5.01 ± 1.23	–	~~**0.92±0.31***~~**0.92 ± 0.31***	**7.613(2,20);** ~~**p=0.0035**~~**p = 0.0035**
	12~~#~~[2]	–	11.25 ± 0.90	~~**2.27±0.71****~~**2.27 ± 0.71****	7.62 ± 0.93	**7.052(2,20);** ~~**p=0.0048**~~**p = 0.0048**
	15~~#~~[2]	–	17.56 ± 0.58	~~**3.83±0.95*****~~**3.83 ± 0.95*****	13.71 ± 1.74	**17.87(2,20);** ~~**p<0.0001**~~**p < 0.0001**
Body weight (g)	1~~#~~[3]	19.63 ± 0.98	19.24 ± 0.84	19.69 ± 0.42	19.73 ± 0.74	0.629(3,21); p = 0.6045
	2~~#~~[3]	20.10 ± 1.41	20.53 ± 0.21	19.47 ± 0.65	20.94 ± 0.49	1.477(3,17); p = 0.2563
	8~~#~~[3]	21.10 ± 1.27	20.13 ± 1.27	19.99 ± 0.67	20.18 ± 0.62	2.096(3,14); p = 0.1467
	12~~#~~[3]	21.70 ± 0.85	21.10 ± 0.70	~~**20.42±0.69***~~**20.42 ± 0.69***	~~**20.33±0.73***~~**20.33 ± 0.73***	**3.606(3,13);** ~~**p=0.0430**~~**p = 0.0430**
	15~~#~~[3]	22.05 ± 0.78	~~**19.00±0.52*****~~**19.00 ± 0.52*****	~~**19.24±0.71*****~~**19.24 ± 0.71*****	~~**19.45±0.65*****~~**19.45 ± 0.65*****	**22.73(3,20);** ~~**p<0.0001**~~**p < 0.0001**
Grading of hematuria (Range 0–6)	1~~#~~[3]	3.0 ± 1.41	3.17 ± 2.14	3.90 ± 1.41	3.36 ± 1.12	0.707(3,41); p = 0.5534
	8~~#~~[3]	3.75 ± 0.50	~~**5.33±0.82***~~**5.33 ± 0.82***	4.10 ± 0.44	3.87 ± 2.07	**4.693(3,44);** ~~**p=0.0063**~~**p = 0.0063**
	15~~#~~[2]	–	5.50 ± 0.84	5.21 ± 1.12	~~**2.69±2.61*****~~**2.69 ± 2.61*****	**10.43(2,34);** ~~**p=0.0003**~~**p = 0.0003** ~~**aOnly comparing tumor-bearing.bAs compared with the positive control.cAs compared with the negative control; df1= between group degree of freedom; df2 = within group degree of freedom.**~~

[1] Only comparing tumor-bearing.

[2] As compared with the positive control.

[3] As compared with the negative control; df1 = df1 = between group degree of freedom; df2 = within group degree of freedom.

4 Discussion

This study reports, for the first time, using intravesical instillation as a route of administration for the GL extract GLe in the ~~MB49~~MB49 implanted mice, where the novel *in vivo* tumor inhibitory activities on bladder cancer were demonstrated. During the two-week experimental period, in addition to reducing the tumor size more significantly, GLe was shown to be superior to the conventional BCG therapy by delaying the occurrence of tumor formation. Such chemopreventive properties were accountable to the cytotoxic effects of GLe on the murine urothelial carcinoma ~~MB49~~MB49 cells. However, intravescial GLe therapy neither improved the conditions of hematuria in the treated mice nor indicated any major adverse effects.

As a medicinal mushroom, GL and its products are typically taken by cancer patients orally for supplementary purposes (Yuen and Gohel, 2005; Jin et al., 201~~2~~6). The antitumor effects of GL and underlying mechanisms have been extensively studied in animal models of various cancer origins except bladder cancer (Yuen and Gohel, 2005). Unlike cancers of other origins normally evaluated by oral or intravenous mode of administration, intravesical therapies were indicative for bladder chemoprevention that allow direct contact of drug contents to reach the tumor cells within the bladder wall (Shen et al., 2008; Williams et al., 2010). The same therapeutic approach should also be applied when evaluating the bladder chemopreventive effects of GL, especially for its complexity of containing over 300 chemical constituents, many of which would be eliminated by the first bypass metabolism (Yuen and Gohel, 2005; Boh et al., 2007; Kao et al., 2013). This would be a distinct advantage. GL has not been reported for being administered by intravesical instillation, whereas to our best knowledge this is the first evidence showing herein that the treatment schedule of ~~2~~2 h intravesical instillation of GLe repeated once a week in two consecutive week was feasible and safe in mice. Apart from hematuria that also occurred in other treatment and control groups, mice recovered from intravesical GLe therapy have returned to the normal daily life of living without significant increase of mortality rate and/or any major side effects being observed.

Regarding the antitumor properties on the murine ~~MB49~~MB49 cells, the dose-dependent cytotoxicity demonstrated by GLe was consistent with the previous findings on the human HUC-PC and MTC-11 cells (Lu et al., 2004; Yuen and Gohel, 2008), but no cell cycle arrest was observed supporting apoptosis. Bioactive ingredients of GL have demonstrated to be regulatory to the progression and development of different cancers through different signaling pathways, such as TRAIL-induced signaling, SHH signaling, TGF/SMAD signaling, and mTOR signaling in different cancers (Aras et al., 2018; Gill et al., 2017; Suarez-Arroyo et al., 2013). For instance, such signaling pathways could be studied in ~~MB49~~MB49 cells when treated with GLe and other related molecules, by using western blot and mRNA expression techniques. Furthermore, GLe failed to exhibit a 100% cytotoxicity on ~~MB49~~MB49 cells in cultures at any safety dosages (i.e. < 0.01% v/v ethanol). And it was also unlike the progressive apoptosis and apoptotic clearance that occurred in the viable HUC-PC cells that were remaining after GLe treatment at non-cytotoxic concentrations (Yuen et al., 2008). Owing to the fact that besides the differences between the cell lines of rodent and human origins, the relatively more malignant and aggressive nature of ~~MB49~~MB49 cells suggested mechanisms underlying the different cytotoxic effects may be dependent on the tumor cell behaviors. There was no doubt that the current study has extended the *in vitro* knowledge of GL to animal study, which demonstrates the significant inhibitory activities on tumor formation and growth using the relevant ~~MB49~~MB49 mice model. The *in vitro* and *in vivo* findings together supported the notion that following the GLe instillation, a small amount of ~~MB49~~MB49 cells remained within the bladder wall due to the incomplete cytotoxic activity, and those leftover cells failed to enter apoptosis and allowed cellular multiplication to develop into a full-blown tumor. It is postulated that suboptimal condition of incomplete tumor cells eradication maybe solved by increasing the frequency of GLe instillation (for example, repeated instillation twice a week instead of once per week), and thus to improve the outcome for preventing completely the ~~MB49~~MB49 tumor formation. Nonetheless, in this study, the inhibitory effects of GLe is clearly superior to BCG, in terms of delaying the tumor formation by one week which was absent in the BCG mice group. Otherwise, GLe could also be evaluated with BCG for any synergistic relationships in future studies, since combination GLe+BCG treatment or pre-treatment with GLe followed by BCG has potentiated the inhibitory effects of BCG on HUC-PC cells to reduce the treatment dosage (Yuen et al., 2012). Such synergism was supported by several possible mechanisms including the modulatory activity of GLe on the cytokines being stimulated by BCG (Yuen et al., 2013). In the pre-malignant HUC-PC model, GLe was shown to be immunological active to promote migration of neutrophils in addition to cytokines section (Yuen et al., 2011). BCG is referred as an immunotherapeutic agent indicative for prophylaxis of bladder cancer recurrence following transurethral resection by stimulating non-specific immune response in the bladder wall to eradicate any residual tumor cells (Redelman-Sidi et al., 2014). It is known that the effectiveness of intravescial BCG therapy was largely determined by the type of cytokines being secreted within 48 h in the urine of patients (Liu et al., 2014; Kamat et al., 2016). Urine specimens collected from the mice of this study were saved for determining the levels of key cytokines as well as other parameters. Further experiments with ~~MB49~~MB49 cells were underway for profiling the cytokine secretion as well as gene expression induced by GLe, in order to investigate and compare with BCG for any subsequent antitumor activities. Therefore, the current results were highly agreeable with the previous findings reported on the pre-malignant HUC-PC model, and mutually explainable with each other to frame very well the future research direction towards the goal for incorporating GLe into a cancer treatment regimen for enhancing the effectiveness and safety.

In conclusion, the current results have advanced the scientific evidences to *in vivo* extent in animals, which demonstrated for the first-time intravesical instillation of GLe was effective in inhibiting the ~~MB49~~MB49 tumor formation and its growth. Such chemopreventive activities of GLe were shown to be more superior to that exhibited by the conventional BCG therapy. Further studies are required to investigate the molecular mechanisms underlying such chemopreventive effects.

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Conflict of interest

The authors declare that there are no conflicts of interest. This ~~manuscript/ data,~~ manuscript/data, or parts thereof, has not been submitted for possible publication to another journal or that the work has previously been published elsewhere.

Contributions of authors

The research team designed the study and share equal contributions. J.W.M. Yuen is the project leader who has supervised the project and finalized the manuscript. D.S.Y. Mak carried out all the experiments reported in this manuscript and contributed part of the writing. C.F. Ng and E.S. Chan are urologist specialist who has provided support on the clinical aspects of the study. Particularly, C.F. Ng provided guidance on the intravesical BCG therapy while E.S. Chan also guided the transabdominal micro-ultrasound imaging. M.D.I. Gohel in the expertise of clinical biochemistry supported the biomarkers measurement and results interpretation in this study. All authors have read the manuscript and agreed with the contents.

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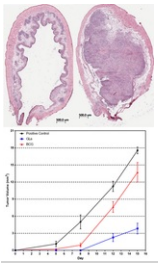

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