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The Hong Kong Polytechnic University
Department of Health Technology and Informatics

Chemopreventive Effects of *Ganoderma Lucidum* on Human Uroepithelial Cell Carcinoma

by

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A thesis submitted to the Hong Kong Polytechnic University for the degree of Doctor of philosophy

October 2007

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DECLARATION

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Signed

____

John Wai Man, Yuen

Dedicated
to my Family
and
my fiancée
Jolene.

Abstract of thesis entitled
"Chemoprentive effects of Ganoderma Lucidum on human uroepithelial cell carcinoma"
submitted by
John Wai Man, Yuen
For the degree of
Doctor of Philosophy
At the Hong Kong Polytechnic University
In 2007

Background: Superficial transitional cell carcinoma (TCC) is the most common form of bladder cancer that can be managed by the transurethral resection (TUR) technique. TCC faces a challenge of exceptionally high recurrence rate, whereby Bacillus Calmette-Guérin (BCG) is used an adjuvant for prophylaxis. As the most effective immunotherapeutic agent, actions of BCG are based on its internalization by urothelium in order to trigger host immune response. However, the efficacy is unsatisfactory and side effects appear in over 90% of the patients, therefore a more powerful but also a safe chemopreventive agent is demanded. **Methodology:** An *in vitro* tumorigenic transferable human uroepithelial cell (HUC-PC) model with carcinogen 4-aminobiphenyl (ABP) was used to evaluate and elucidate the chemopreventive activities of two Ganoderma lucidum extracts – ethanol extract (GLe) and water extract (GLw) for the immunological, oxidative and antioxidant, apoptotic, molecular and cell signaling mechanisms. **Results and Discussion:** Potent cytotoxic and growth inhibitory effects of GLe were demonstrated as compared to GLw. Surprisingly, GLe induced oxidative stress and oxidative DNA damage in HUC-PC cell line, even though both G. lucidum extracts were found to possess rich antioxidant capacities. GLe was also able to induce interleukin-6 (IL-6) cytokine production, which is also an

hallmark of BCG internalization. By using normal HUC-1 cells as control, cytotoxicity of GLe was selective to HUC-PC cells, in particular under ABP challenge. These findings have drawn our attention to demonstrate that the growth inhibition activity of GLe is mainly through (1) telomerase-related apoptosis and (2) up-regulation of intracellular calcium (Ca^{2+}) together with nuclear factor-Kappa B (NF-κB) and protein kinase C (PKC). Furthermore, G. lucidum was capable of modulating the free fibronectin (FN) content as well as the membrane-bound glycosaminoglycans (GAGs) of HUC-PC cells, which may be synergistic to BCG binding efficiency. **Conclusion:** Conclusive findings support G. lucidum as a novel chemopreventive agent for TCC, whereby it may supplement with BCG for better outcome or potentially substitute BCG when more in vivo evidences become available.

SCHOLARSHIP

(1) The Hong Kong Polytechnic University Tuition Scholarship for Research Postgraduate Studies (2003/07)

(2) The Hong Kong Polytechnic University Research Studentship (2003/07)

(3) Sir Edward Youde Memorial Fellowship (2005/06)

(4) The Hong Kong Polytechnic University Research Student Attachment Programme (2006/07)
Funded for an attachment (titled Research Associate) in the Laboratory of Rosalyn Adam in the Children's Hospital (Dept. of Urology) of the Harvard Medical School at Boston(1st July 2007 to 30th September 2007).

PUBLICATIONS

DURING THE COURSE OF STUDY

Journal Articles

[1] **Yuen J.W.M.**, Gohel M.D.I., Ng A.C.F. (2007) *Ganoderma lucidum is a novel chemopreventive agent for urothelial cancer*. J.Urol. (In preparation).

[2] **Yuen J.W.M.**, Gohel M.D.I. (2007) *The dual role of Ganoderma antioxidants on urothelial cell DNA under carcinogenic challenge*. J. Altern. Compl. Med. (In preparation)

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[1] **Yuen J.W.M.**, Gohel M.D.I., Au D.W.T. (2007) *Ganoderma lucidum is a novel candidate for Bladder chemoprevention*, The 66th Annual Meeting of Japanese Cancer Association, Japan (Poster)

[2] **Yuen J.W.M.** Gohel M.D.I. (2006) *The apoptotic chemoprevention effects of Lingzhi during the initial stage of tumorigenic transformation of Human uroepithelial Cells*, 13th HKICC & 3rd Annual Meeting Center for Cancer Research, University of Hong Kong, Hong Kong (Poster)

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Acknowledgments

I wish to express my gratitude to all those who have helped in various ways during the course of this work. Especially to

Dr. Danny Gohel (Dept. of HTI, HKPolyU), who provided the opportunity to carry out this innovative study. His encouraging and enthusiastic supervision, which never seemed to be lacking along with his open-mindedness, leads to the smooth completion of this study. This thesis couldn't be achieved if it wasn't for his invaluable advice and support.

Prof. Thomas Wong (Dean of FHSS, HKPolyU), for his support, advices and recommendations for scholarships.

Dr. Doris Au (Dept. Biology & Chemistry, CityU of HK), for her laboratory protocols, leading equipments, and critical reading of manuscript.

Dr. Anthony Ng (Dept. of Surgery, PWH), for his clinical expertise and advices provided in this study.

Dr. Maureen Boost (Dept. of HTI, HKPolyU), for critical reading of manuscript.

Dr. J.Y. Rao (Dept. of Cellular and Molecular Pathology, UCLA), for providing the HUC-PC cell line and advices in cell culture.

Department of Health Technology & Informatics, HKPolyU, clerical, Technical, academic and research staff members and postgraduate students for their support.

School of Nursing, HKPolyU, clerical, academic and research staff members and postgraduate students for their support.

Dept. of Biology and Chemistry, CityUHK, for their technical support in performing the telomerase assay.

Dr. Rosalyn Adam (Associate Director of Urology Research & Assoistant Professor of Surgery, Harvard Medical School), for hosting myself for an 3-month attachment in her Laboratory.

Dr. Y.T. Szeto and **Dr. W.Y. Chung**, who taught me a lot of research and technical skills to facilitate my work.

Mr. Raymond Liu, who helped for administration and completion of this work.

My Teachers, who were responsible in opening up my interest in science and shaping my motivation and encouragement. In particular, Mr. Y.T. Yuen, my secondary teacher and scout leader who supported me in every aspect and I wouldn't have had such an accomplishment if it wasn't for him.

Mr. Malcolm Long (Emeritus Principal of Shung Tak Catholic English College), provided an enriched school life and a supportive environment for personal development.

My parents and Family, who in their own way have supported.

Jolene, my beloved fiancée who spent a lot of time with me throughout my writing. Thanks for the admonition, encouragement and motivation. This Thesis is dedicated to her.

TABLE OF CONTENTS

Page

Declaration

Dedication

Abstract

Scholarships

Publications during the course of study

Table of Contents…..………………………………………………………... i

Abbreviations…………………………………………………………….. ix

List of Tables…………………………………………………………… xiii

List of Figures………………………………………………………….. xiv

Chapter 1 Literature Review: Bladder Cancer is Demanding for Chemoprevention

1.1 Carcinoma of the Urinary Bladder

1.1.1 The urinary bladder…………………………………….. 1

1.1.2 Malignancy in the urinary bladder……………………... 2

1.2 Bladder Cancer is a Preventable Disease

1.2.1 Epidemiology…………………………………………... 7

1.2.2 Etiology………………………………………………… 8

1.3 The Link between 4-Aminobiphenyl and Human Uroepithelial Cells in Transitional Cell Carcinogenesis

1.3.1 4-Aminobiphenyl is an environmental bladder carcinogen……………………………………………… 12

1.3.2 The fate of 4-Aminobiphenyl in vivo………………….. 14

1.3.3 Initiation of carcinogenesis: ABP-DNA adducts………. 14

1.3.4 Oxidative stress in ABP carcinogenesis………………... 15

1.3.5 Who are the high risk people?....................................... 16

1.3.6 Prime target for ABP: Human uroepithelial cells……… 17

1.4 Molecular Pathogenesis of Bladder Cancer and Markers

1.4.1 Chromosomal and genetic alterations in bladder cancer.. 19

1.4.2 Potential markers for TCC surveillance………………... 20

1.4.3 p53………...……………………………………………. 22

1.4.4 Telomerase……………………………………………... 23

1.4.5 Cycooxygenase-2………………………………………. 25

1.4.6 8-hydroxy-2'-deoxyguanosine…………………………. 26

1.5 Management of TCC

1.5.1 Transurethral resection for treating superficial TCC…... 28

1.5.2 Multifocal TCC is an indication for recurrence risk…… 29

1.5.3 Adjuvant Bacillus Calmette-Guérin immunotherapy…... 30

1.5.4 Urothelial internalization of BCG……………………… 31

1.5.5 Mode of actions for BCG………………………………. 33

1.5.6 Efficacy and side effects of BCG………………………. 35

1.6 Chemoprevention

1.6.1 What is still missing for bladder cancer?....................... 39

1.6.2 Chemoprevention for bladder cancer…………………... 40

Chapter 2 Literature Review: G. lucidum - A Medicinal Mushroom with Anticancer Effects

2.1 Ganoderma lucidum is a Valuable Mushroom

2.1.1 Ganoderma tales and history…………………………… 44

2.1.2 Nutriceutical and medicinal Ganoderma lucidum……… 45

2.2 Anticancer Effects of Ganoderma lucidum

2.2.1 Is G. lucidum a food supplement or chemotherapeutic agent?.. 46

2.2.2 Bioactive components: polysaccharides and triterpenes.. 46

2.2.3 Cancer cell cytotoxicity………………………………… 49

2.2.4 Cell cycle and signaling regulations……………………. 53

2.2.5 Anti-angiogenesis and anti-metastasis…………………. 57

2.2.6 In vivo animal studies………………..………………..... 59

2.2.7 Carcinostatic properties: immuno-enhancement………. 60

2.2.8 Antioxidants……………………………………………. 61

2.2.9 Clinical trials…………………………………………... 62

2.3 Anticancer G. lucidum: Scientific evidence…………………... 63

Chapter 3 Hypothesis, Objectives, Relevance and Significance

3.1 Hypothesis of the Study……………………………………... 65

3.2 Evidence to Propose Hypothesis……………………………… 65

3.3 Objectives to Address the Hypothesis……………………..... 67

3.4 Relevance of the Study………………………………………. 68

3.5 Significance & Value of the Study………………………….. 68

Chapter 4 Experimental Design of the Study

4.1 In vitro Tumorigenic Transformation HUC Culture Model

4.1.1 Introduction………………………………………….. 70

4.1.2 In vitro experimental design……………………………. 71

4.1.3 Culture of HUC-PC and HUC-1 cell lines……………. 72

4.1.4 Testing substances and chemicals……………………… 72

4.1.5 Re-extraction from commercial G. lucidum…………… 73

4.1.6 Preparation of assay media for cultures………………... 74

4.1.7 Statistical analysis……………………………………… 74

4.2 Study Plan and Methodology Outline

4.2.1 Objective 1. Evaluate the effects of G. lucidum on IL-6 secretion from pre-malignant HUC cells...……..………. 76

4.2.2 Objective 2. Investigate the effects of G. lucidum on BCG binding mechanisms, using relevant markers of pre-malignant HUC cells………………………….…….. 76

4.2.3 Objective 3. Evaluate the extent of cytotoxicity of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP…..………………… 77

4.2.4 Objective 4. Determine the roles of G. lucidum antioxidant / oxidant activities on cytotoxicity in pre-malignant HUC cells and determine its cytotoxicity…... 77

4.2.5 Objective 5. Determine the effects of ABP and G. lucidum on two selected biomarkers for bladder cancer in the HUC model system………………….……………… 78

4.2.6 Objective 6. Evaluate the apoptotic effects of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP…..……………………….……… 78

4.2.7 Objective 7. Investigate the cell signaling pathways involved in G. lucidum-induced bladder chemoprevention………………………………………… 79

4.2.8 Summary of measuring the parameters………………..... 80

Chapter 5 G. lucidum Induces IL-6 Production and Facilitates BCG Binding to HUC-PC Cells

5.1 Introduction………………………………………………………….. 82

5.2 Materials and Methods

5.2.1 Cell cultures for assays………………………………….... 85

5.2.2 LDH cytotoxicity assay……………………………….….. 85

5.2.3 ELISA for IL-6 cytokine………………………………… 86

5.2.4 EIA for fibronectin quantitation………...……………….. 86

5.2.5 Dimethylmethylene blue method for GAGs quantitation.. 87

5.3 Results

5.3.1 G. lucidum is cytotoxic to HUC-PC cells……………….. 88

5.3.2 G. lucidum and BCG are active to induce IL-6 secretion from HUC-PC cells……………………………………… 91

5.3.3 G. lucidum affects extracellular Fibronectin and cell-surface GAGs of HUC-PC cells…………………………. 95

5.4 Discussion…………………………………………………………….. 100

Chapter 6 G. lucidum cytotoxicity is selective to the pre-malignant HUC cells

6.1 Introduction………………………………………………………….. 108

6.2 Materials and Methods

6.2.1 LDH cytotoxicity assay………………………………….. 109

6.2.2 Trypan blue exclusion assay for cell viability…………… 109

6.3 Results

6.3.1 GLe is cytotoxic to HUC-PC and HUC-1 cells………..… 111

6.3.2 ABP enhances the proliferation of HUC-PC cells………. 114

6.3.3 GLe inhibits the proliferation of HUC-PC and HUC-1 cells……………………………………………………… 117

6.4 Discussion……………………………………………………….. 122

Chapter 7 The Dual Effects of G. lucidum Antioxidants on Cytotoxicity

7.1 Introduction……………………………………………………….. 127

7.2 Materials and Methods

7.2.1 Measuring the antioxidant activities of G. lucidum extracts and assay media………………………………… 129

7.2.2 Treatment of culture media…..………………………….. 129

7.2.3 FRAP assay…………………………………………… 130

7.2.4 DPPH· free radical scavenging activity assay…………… 131

7.2.5 H_2O_2 assay……………………………………………….. 132

7.2.6 8-OHdG ELISA Assay…………………………………... 132

7.3 Results

7.3.1 GLe and GLw are rich in antioxidant capacity………….. 134

7.3.2 Antioxidant status during the 48-hour culture…………… 136

7.3.3 GLe induces oxidative stress in HUC-PC cells………… 143

7.4 Discussion……………………………………………………… 151

Chapter 8 G. lucidum Inhibits the Telomerase Activity in HUC-PC Cells

8.1 Introduction……………………………………………………….. 157

8.2 Materials and Methods

8.2.1 RQT-TRAP for measurement of telomerase activity……. 159

8.2.2 EIA for COX-2 quantitation…………………………….... 160

8.3 Results

8.3.1 G. lucidum inhibits telomerase activity of HUC-PC cells. 162

8.3.2 COX-2 is undetectable in HUC-PC before and after treatments………………………………………………... 167

8.4 Discussion…………………………………………………….. 168

Chapter 9 G. lucidum is Apoptotic to the Pre-malignant Human Uroepithelial Cells

9.1 Introduction…………………………………………………….. 172

9.2 Materials and Methods

9.2.1 Annexin V-FITC/7-AAD apoptosis assay………………. 174

9.2.2 Isolation of polymorophonuclear neutrophils…………… 174

9.2.3 Cell migration assay……………………………………... 175

9.3 Results

9.3.1 Apoptotic effects of GLe on HUC-PC cells……………... 176

9.3.2 Culture media of apoptotic HUC-PC cells are chemotactic to neutrophils………………………………. 184

9.4 Discussion…………………………………………………….. 186

Chapter 10 The Cell Signaling Pathways Involved in G. lucidum-induced Chemoprevention

10.1 Introduction…………………………………………………….. 190

10.2 Materials and Methods

10.2.1 ELISA-EMSA for measuring NF-κB activity………….. 192

10.2.2 ELISA for measuring PKC activity…………………… 193

10.2.3 Fluo-4 biosensor for measuring intracellular calcium….. 194

10.3 Results

10.3.1 GLe enhances NF-κB activity in HUC-PC cells……….. 195

10.3.2 GLe enhances PKC activity in HUC-PC cells……….… 197

10.3.3 GLe increases intracellular Ca^{2+} in HUC-PC cells…….. 199

10.4 Discussion………………………………………………………. 201

Chapter 11 Summary and Further Discussion

11.1 Summary of Findings…..……………………………………… 208

11.1.1 HUC-PC is sensitive to ABP…………………………… 208

11.1.2 G. lucidum is cytotoxic and active in growth inhibition…………………………………………….. 209

11.1.3 Dual roles of G. lucidum's antioxidants………………... 209

11.1.4 Telomerase-associated apoptosis involving PKC and intracellular Ca^{2+} as the main growth inhibitory mechanism……………………………………………… 210

11.1.5 G. lucidum is immunologically active…………………. 210

11.1.6 G. lucidum may facilitate BCG efficacy……………….. 211

11.1 Justification: G. lucidum -- carcinogenic or anti-carcinogenic?.. 213

11.2 The concept of cellular senescence…………………………… 214

Chapter 12 Conclusions and Future Perspectives…..…………………….. 218

Appendixes……………………………………………………………. 221

Reference list…………………………………………………………… 235

Abbreviations

<u>Numeric / characters</u>

4-HPR	Fenretinide
7-AAD	7-amino-actinomycin D
8-OHdG	8-hydroxy-2’-deoxyguanosine
8-oxo-dG	8-Oxo-7,8-dihydro-2'-deoxyguanosine

<u>A/a</u>

ABP	4-Aminobiphenyl
AC	Adenocarcinoma
ACP	Alginate-chitosan-PEG
AJCC	American Joint Committee on Cancer
AP-1	Activating protein-1
AS-ODNs	Antisense oligonucleotide

<u>B/b</u>

BCG	Bacillus Calmette-Guérin
BTA	Bladder tumor antigen
BTAstat	Bladder tumor antigen stat
BTA TRAK	Complement factor H-related protein

<u>C/c</u>

$[Ca^{2+}]_i$	Intracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
CAM	Chick chorioallantoic membrane
CDKN2	Cyclin-dependent kinase inhibitor 2
CFUs	Colony-forming units
CO_2	Carbon dioxide
Con A	Concannavalin A
COX	Cycooxygenase
CYP	Cytochrome P450

<u>D/d</u>

DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH·	2,2-diphenyl-1-picrylhydrazyl

E/e

EGFR	Epithermal growth factor receptor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic Mobility Shift Assay

F/f

FAP	Fibronectin attachment protein
FasL	Fas/CD95 ligand
FBS	Fetal Bovine serum
FDA	The Food and Drug Administration
Fe^{2+}	Ferrous
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FN	Fibronectin
FRAP	Ferric Reducing Antioxidant Protocol

G/g

G	Guanine
GAGs	Glycosaminoglycans
G. lucidum	Ganoderma lucidum
GLe	Ganoderma lucidum ethanol extract
GLw	Ganoderma lucidum water extract
GST	Glutathione-S-transferase
GSTM1	Glutathione-S-transferase M1

H/h

H_2O_2	Hydrogen peroxide
HKCR	Hong Kong Cancer Registry
hTERT	Human telomerase reverse transcriptase
HTIA	[^{3}H] Thymidine incorporation assay
hTR	Human telomerase RNA
HUC	Human uroepithelial cells
HUVEC	Human umbilical cord vascular endothelial cell

I/i

IARC	International Agency for Research on Cancer
IC	Interstitial cystitis
IC_{50}	Growth inhibitory concentration at 50%
IFNα	Interferon alpha
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
ITCs	Isothiocyanates

J/j

JNK	c-Jun N-terminal kinase

L/l

LD_{50}	Lethal Dose at 50%
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide

M/m

MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
mRNA	Messenger RNA
MTT	Tetrazolium

N/n

NA	Not available
NADH	Nicotinamide Adenine Dinucleotide
MAPK	Mitogen-activated protein kinase
NATs	N-acetyltransferases
NCI	National Cancer Institute
NF-IL6	Nuclear factor Interleukin-6
NF-κB	Nuclear factor KappaB
NK	Natural killer
NMP22	Nuclear matrix protein-22
NO	Nitric oxide

O/o

OSHA	Occupational Safety and Health Administration

P/p

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PHA	Phytohemagglutinin
PKB	Protein kinase B
PKC	Protein kinase C
PS	Phosphatidylserine

R/r

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RTQ-PCR	Real-time quantitative polymerase chain reaction

S/s

SAPK	Stress-activated protein kinase
SCC	Squamous cell carcinoma
SOD	Superoxide dismutase
SRB	Sulforhodamin B Method
SV40	Simian virus 40
SWOG	The Southwest Oncology Group

T/t

TB	Trypan blue exclusion assay
TCC	Transitional cell carcinoma
TGF-β1	Transforming growth factor-Beta-1
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TNFα	Tumor necrosis factor-alpha
TNM	Tumor-Node-Metastasis
TRAIL/Apo-2L	TNF-Related Apoptosis-Inducing Ligand
TRAP	Telomeric repeat amplification protocol
TSG	Tumor suppressor gene
TUR	Transurethral resection

U/u

UD	Undifferentiated
U.K.	United Kingdom
uPAR	Urokinase-type plasminogen activator
U.S.A.	United States of America
UTI	Urinary tract infection

V/v

VEGF	Vascular endothelial growth factor

LIST OF TABLES

		Page(s)
(Table 1.1)	TNM system for classification of bladder tumors	4
(Table 1.2)	Bladder cancer staging refers to the TNM system.	5
(Table 1.3)	Risk factors for bladder cancer.	11
(Table 2.1)	A list of G. lucidum products or isolated compounds that has been tested for effectiveness of inhibiting cancer cell proliferation.	51
(Table 2.2)	A list of G. lucidum products or isolated compounds that has been tested for direct lethal effects on cancer cells.	52
(Table 2.3)	Cell signalling pathways and mechanisms of G. lucidum in control of cancer cell growth.	55-56
(Table 7.1)	Six study groups involved in antioxidant and oxidant experiments	130
(Table 7.2)	Antioxidant activities of ReishiMax and its defined re-extracts.	135
(Table 7.3)	Antioxidant / oxidation properties of freshly prepared assay media	135

LIST OF FIGURES

		Page
(Figure 1.1)	The human urinary tract.	1
(Figure 1.2)	Anatomy of human urinary bladder.	3
(Figure 1.3)	Histological types of bladder cancer reported in the United States of American and Hong Kong.	6
(Figure 1.4)	Chemical structure and physical properties of ABP.	13
(Figure 1.5)	Transurethral resection.	29
(Figure 2.1)	A summary illustration to show all the works related to anticancer activity of G. lucidum, as corresponding to the stages of carcinogenesis.	64
(Figure 4.1)	Seven groups of testing parameters used for investigating the chemopreventive effects of G. lucidum on human uroepithelial cell carcinoma.	81
(Figure 5.1)	Cytotoxicities of BCG and G. lucidum on HUC-PC cells measured by LDH cytotoxicity assay.	89
(Figure 5.2)	Dose-dependent cytotoxic effects of GLe on HUC-PC cells measured by LDH cytotoxicity assay.	90
(Figure 5.3)	Dose-dependent IL-6 secretion induced by BCG.	92

(Figure 5.4)	Dose-dependent IL-6 secretion induced by GLe.	93
(Figure 5.5)	No IL-6 secretion was induced by GLw.	94
(Figure 5.6)	Reduction of extracellular FN induced by GLe.	96
(Figure 5.7)	Reduction of extracellular FN induced by GLw.	97
(Figure 5.8)	Dose-dependent increase of GAGs on the HUC-PC surface induced by GLe.	98
(Figure 5.9)	Increase of GAGs on the HUC-PC surface induced by GLw.	99
(Figure 6.1)	Cytotoxic effects of GLe on HUC-PC and HUC-1 cells.	112
(Figure 6.2)	Cytotoxic effects of GLw on HUC-PC and HUC-1 cells.	113
(Figure 6.3)	Cytotoxic effects of ABP on HUC-PC and HUC-1 cells.	115
(Figure 6.4)	The cell proliferation of HUC-PC and HUC-1 cell lines induced by ABP.	116
(Figure 6.5)	Dose-dependent growth inhibition of HUC-PC cells induced by GLe.	118
(Figure 6.6)	Dose-dependent growth inhibition on ABP-pretreated HUC-PC cells induced by GLe.	119
(Figure 6.7)	Dose-dependent growth inhibition of HUC-1 cells induced by GLe.	120

(Figure 6.8)	No growth inhibition was demonstrated by GLw on HUC-PC and HUC-1 cells after the 48-hour culture in the presence or absence of ABP.	121
(Figure 7.1)	Pattern of Changes in FRAP values in HUC-PC cultures induced by G. lucidum over 48 hours.	137
(Figure 7.2)	Pattern of Changes in FRAP values in HUC-PC cultures induced by G. lucidum and ABP over 48 hours.	138
(Figure 7.3)	Enhancement of FRAP values by G. lucidum in HUC-PC cultures at the 48-hour endpoint.	139
(Figure 7.4)	Patten of Changes in DPPH・scavenging activities in HUC-PC cultures induced by G. lucidum over 48 hours.	140
(Figure 7.5)	Pattern of Changes in DPPH・scavenging activities in HUC-PC cultures induced by G. lucidum and ABP over 48 hours.	141
(Figure 7.6)	Enhancement of DPPH・scavenging activities by GLe in HUC-PC cultures at 48-hour endpoint.	142
(Figure 7.7)	Pattern of changes in H_2O_2 levels in HUC-PC cultures induced by G. lucidum over 48 hours incubation.	144
(Figure 7.8)	Pattern of changes in H_2O_2 levels in HUC-PC cultures induced by G. lucidum and ABP over 48 hours.	145
(Figure 7.9)	Enhancement of H_2O_2 levels by GLe in HUC-PC cultures at the 48-hour endpoint	146
(Figure 7.10)	The effects of G. lcuidum on 8-OhdG.	147

(Figure 7.11)	Dose-dependent increase of 8-OHdG in HUC-PC cells induced by GLe in the presence of ABP.	148
(Figure 7.12)	8-OGdG concentration plotted against LDH cytotoxicity induced by GLe in the presence of 100 μM ABP.	149
(Figure 7.13)	Effects of ABP on 8-OHdG levels in culture media of HUC-PC cells.	150
(Figure 8.1)	Inhibition of base- and ABP-induced elomerase activities by GLe.	163
(Figure 8.2)	Dose-dependent inhibition of telomerase activity induced by GLe in ABP-pretreated HUC-PC cells.	164
(Figure 8.3)	Separation of PCR products on polyacrylamide gel electrophoresis.	165
(Figure 8.4)	The inhibition of telomerase activity was strongly correlated with the growth inhibition induced by GLe.	166
(Figure 9.1)	Photographs of HUC-PC cells taken after the 48-hour treatment with (A) complete medium and (B) 80 μg/ml GLe in complete medium.	177
(Figure 9.2)	Photographs of HUC-PC cells taken after 48-hour treatment with (A) 100 μM ABP in complete medium and (B) 80 μg/ml GLe + 100 μM ABP in complete medium.	178
(Figure 9.3)	The time-dependent progression apoptosis in HUC-PC cells induced by GLe.	179

(Figure 9.4)	Biparametric histogram LOG PMT2 (525nm) vs LOG PMT4 (675nm) showing the time-dependent progression of apoptosis of HUC-PC.	180
((Figure 9.5)	Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 3 hours.	181
(Figure 9.6)	Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 12 hours.	182
(Figure 9.7)	Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 48 hours.	183
(Figure 9.8)	Dose-dependent chemotactic effects of HUC-PC culture media to neutrophils induced by GLe.	185
(Figure 10.1)	Dose-dependent increase of NF-κB DNA binding activity in the nuclear extracts of HUC-PC cells induced by GLe.	196
(Figure 10.2)	Dose-dependent increase of PKC activity in the cytosolic extracts of HUC-PC cells induced by GLe.	198
(Figure 10.3)	Increase of $[Ca^{2+}]i$ in HUC-PC cells induced GLe.	200
(Figure 11.1)	A summary of G. lucium's chemopreventive effects tested on the HUC-PC cells.	212
(Figure 11.2)	An illustration for proposing the conceptual cellular senescence as G. lucidum's chemopreventive strategy.	217

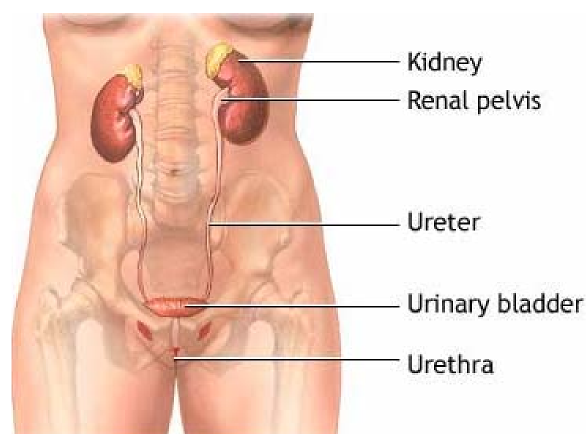
CHAPTER 1

LITERATURE REVIEW: BLADDER CANCER IS DEMANDING FOR CHEMOPREVENTION

1.1 CARCINOMA OF THE URINARY BLADDER

1.1.1 The urinary bladder

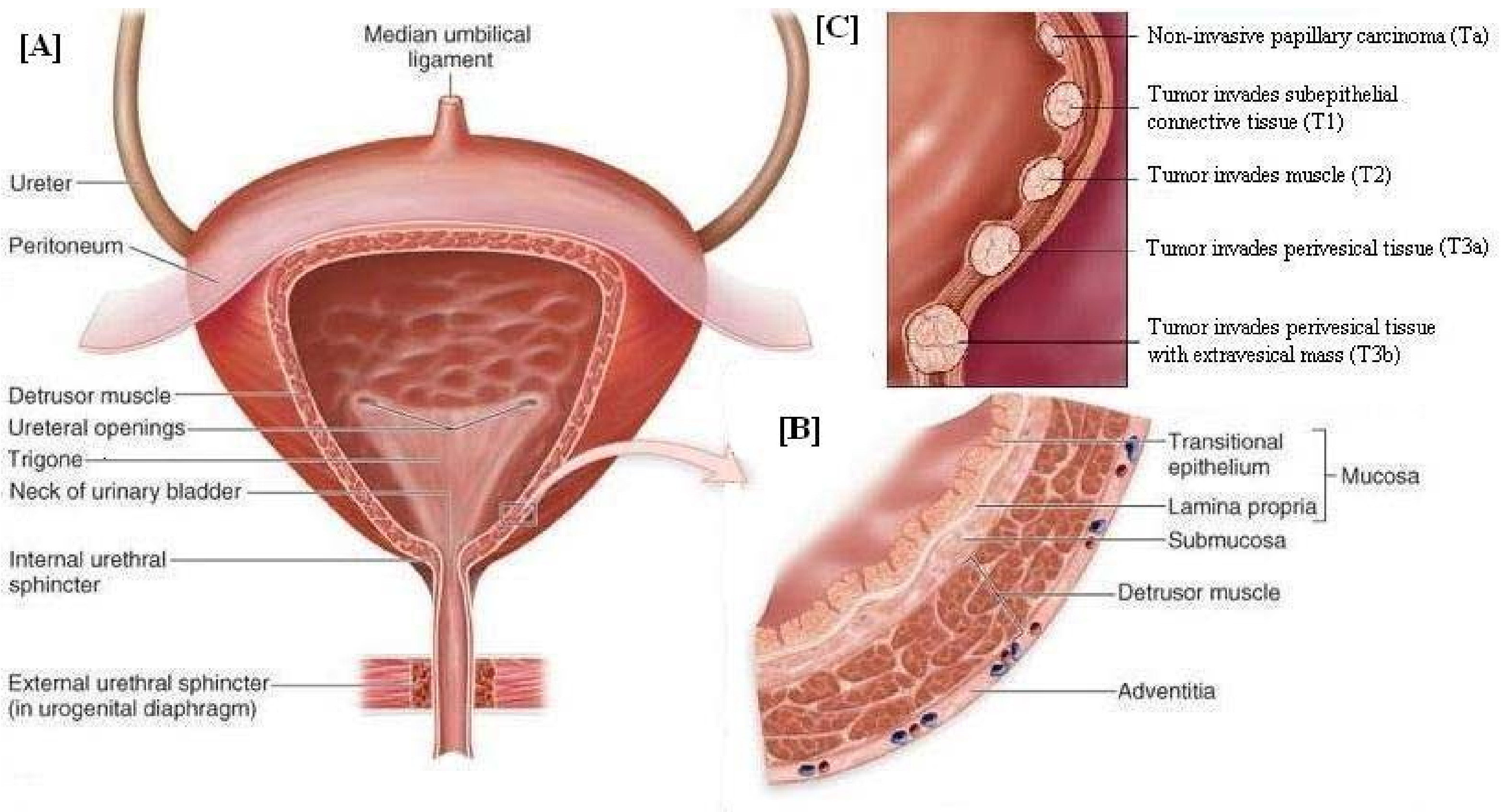
The urinary bladder is a sac-shaped hollow organ that sits on the pelvic floor, posterior to the symphysis and anterior to the vagina in females or the rectum in males. Urine produced by the kidneys is collected into the bladder via the connection of ureters, for storage until eliminating through the urethra (Figure 1.1). As the final urinary reservoir, the distensible bladder consists of a wall of smooth muscle that contains up to 250 cm^3 of fluid under normal conditions, and able to hold up to 450 cm^3 in extreme cases. The urinary bladder is the most common site of malignancy along the human urinary tract [1].



(Figure 1.1) The human urinary tract (*adopted from the Monterey Bay Urology Associates at www.montereybayurology.com*).

1.1.2 Malignancy in the urinary bladder

Bladder cancer is defined as any of the several types of malignant growths of the urinary bladder. The malignancy of urinary bladder gives the cardinal sign of total gross painless hematuria, and sometimes superimposes the symptoms of urinary tract infection (UTI), which are associated with voiding irritabilities such as unexpected frequency, dysuria, urgency and urinary tract obstruction [2-5]. Diagnosis of bladder cancer is based on cytoscopy in combination with urinary cytology findings [5,6]. The Tumor-Node-Metastasis (TNM) system from the American Joint Committee on Cancer (AJCC) is universally adopted for staging the disease. The TNM system is designated by T – the position of the primary tumor, N – the degree of regional lymph nodes (those within the true pelvis) metastasized, and M – whether the tumors is distantly metastasized or not. Their definitions are listed in Table 1.1 and the stage of the bladder cancer is based on the grouping of the TNM (Table 1.2). The inner structure of the bladder and its luminal wall, as shown in Figure 1.2, consists of several layers, which are potential sites for the primary tumor growth.



(Figure 1.2) Anatomy of human urinary bladder. [A] Outer and inner structure of the bladder; [B] The cross-sectional illustration of the luminal layers; [C] Invasiveness of tumors at the luminal wall at different stage (Anonymous).

(Table 1.1) TNM system for classification of bladder tumors.

(T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: “flat tumor”
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
pT2a	- Tumor invades superficial muscle (inner half)
pT2b	- Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
pT3a	- Microscopically
pT3b	- Macroscopically (extravesical mass)
T4	Tumor invades neighbourhood tissues
T4a	Tumor invades prostate, uterus, vagina
T4b	Tumor invades pelvic wall, abdominal wall

(N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Single lymph node (≤2cm) metastasis
N2	Single lymph node (2-5cm) or multiple lymph nodes (≤5cm) metastasis
N3	Single / multiple lymph node(s) (≥5cm) metastasis

(M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

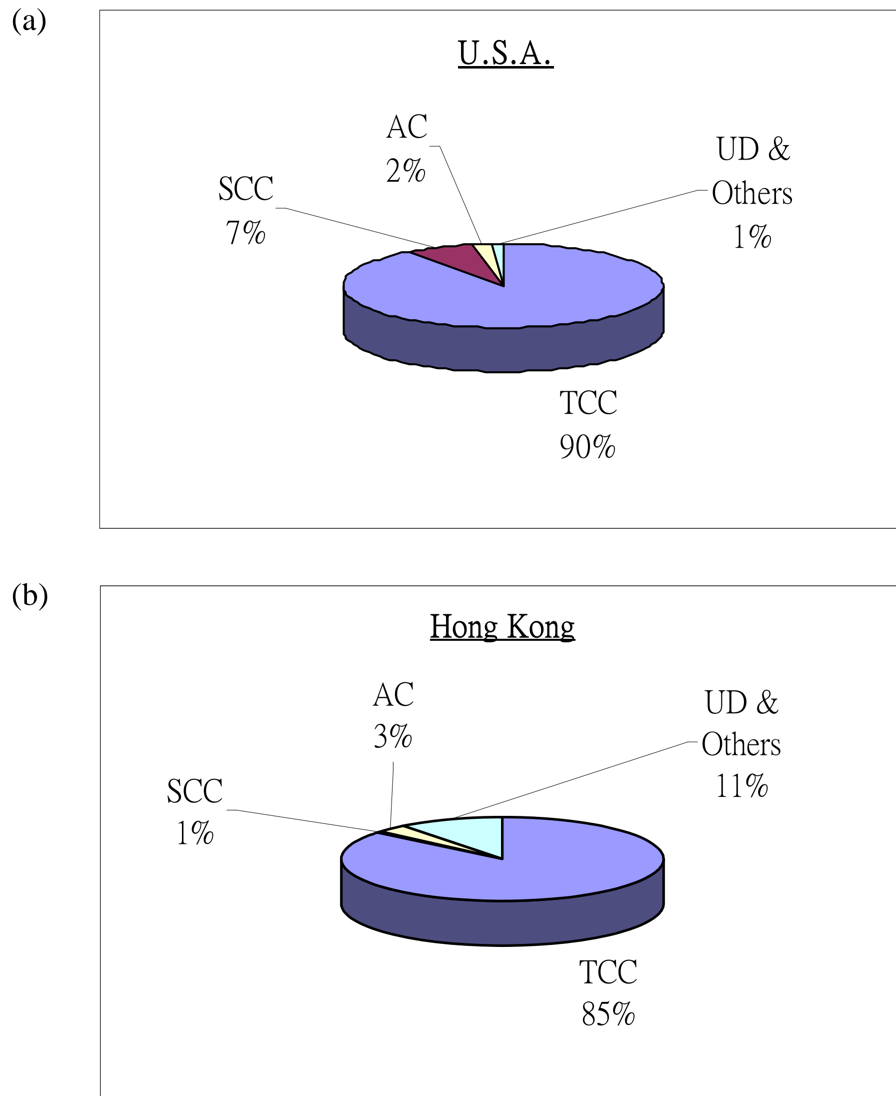
American Joint Committee on Cancer, 2002 [7].

(Table 1.2) Bladder cancer staging refers to the TNM system.

Stage grouping			
Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a	N0	M0
	T2b	N0	M0
Stage III	T3a	N0	M0
	T3b	N0	M0
	T4a	N0	M0
Stage IV	T4b	N0	M0
	Any T	N1	M0
	Any T	N2	M0
	Any T	N3	M0
	Any T	Any N	M1

American Joint Committee on Cancer, 2002 [7].

The urothelium (also called transitional epithelium), formed by multilayered uroepithelial cells, is the most common site for tumor initiation, which accounts for up to 90% of all bladder cancers and are termed transitional cell carcinoma (TCC) particularly in most Western countries [4,8] as well as in Hong Kong [9]. Other types of bladder cancer are squamous cell carcinoma (SCC), adenocarcinoma (AC) and small cell carcinoma. Figure 1.3 exemplifies the common histological types of bladder cancer in two developed areas. TCC can be further subtyped into non-invasive or invasive and papillary or flat. Overall, 70-80% of TCC cases are superficial at presentation and not beneath the lamina propria [6,10,11].



(Figure 1.3) Histological types of bladder cancer reported in the United States of America (U.S.A.) and Hong Kong. Abbreviations are as follows: TCC: Transitional cell carcinoma; SCC: Squamous cell carcinoma (SCC); AC: adenocarcinoma; UD: Undifferentiated.

1.2 BLADDER CANCER IS A PREVENTABLE DISEASE

1.2.1 Epidemiology

Around the world, there are approximately 340,000 new cases of bladder cancer reported every year, accounting for 5% of all diagnosed cancers and associated with overall 40% of the death toll [12-14]. Bladder cancer is the second most frequent malignancy of the genitourinary tract next to prostate cancer [15]. Incidence of bladder cancer in Western countries is high, especially in Europe and the United States, accounting for 5-10% of all malignancies among men [11,16]. This is probably due to socioeconomic reasons as well as the life styles of people in these countries, including cigarette smoking and eating high-calorie diets [17,18].

In the U.S.A., bladder cancer is the fourth most commonly diagnosed malignancy in men and the tenth in women [19]. The National Cancer Institute (NCI) predicted that there will be 67,160 new cases and 13,750 deaths in 2007 (http://www.cancer.gov/cancertopics/types/bladder) in the U.S.A. alone. The government of the United States has spent approximately US$2.9 billion on treatment of bladder cancer. In addition, NCI invested US$30.1 million in bladder cancer research in 2003, which has increased by US$2.9 million since 2001.

Caucasians showed a higher bladder cancer risk than East Asian and Negro people in both sexes [20]. In Hong Kong, bladder cancer is the tenth most common cancer for both sexes, with 584 new cases reported by the Hong

Kong Cancer Registry (HKCR) in 2004. Also for males (with 441 new cases), it ranks as the seventh most common form of cancer, making it 4.1 times more likely to be diagnosed in males than females. This local cancer-sex ratio is comparable with those in Western countries where males have 2.5 to 4 times higher the risk than females [11,18]. Such gender difference of bladder cancer is mainly due to the smoking habits and job nature taken by men, who are more prone to be exposed to carcinogenic agents [11,17,21]. Moreover, bladder cancer is regarded as a late onset disease, morbidity and mortality rates increasing steeply after age 50 in both genders, while it is uncommon in childhood and in young adulthood [18].

1.2.2 Etiology

Asian immigrants in both the U.S.A. and the United Kingdom (U.K.) were shown to have higher risk of bladder cancer than in their original countries, and these risks were directly correlated to their duration of stay [16]. Second-generation immigrants in Sweden had no difference in risk from native Swedes for the incidence of bladder cancer [22]. This suggests the likelihood of environmental etiology rather than race differences for bladder cancer development.

During the period of 1920-40 in Europe, men had the highest mortality rate from bladder cancer, which was seen to be due to the exposure to smoking and industrial carcinogens [16]. The prevalence of bladder cancer was correlated with the contact of mutagenic agents, mainly arylamines and

polyaromatic hydrocarbons sourced from smoking and industries [6,16,23-26]. The major etiological factors for bladder cancer are listed in Table 1.3.

Cigarette smoking contains more than 30 carcinogenic compounds, which increases the risk of developing bladder cancer two- to ten-folds [6,14,23]. In Western countries, tobacco smoking has been regarded as the primary cause, accounting for 40-70% of bladder cancer diagnoses [24,27,28]. In India, the odds of bladder cancer development amongst smokers are significantly higher ($P<0.01$) than that of non-smokers [29]. As demonstrated by studies from the U.S.A., Europe and Japan, the adverse effects of smoking on bladder cancer are similar in both sexes [21,30]. In particular, black tobacco contains more aromatic amines and 2-3 times higher cancer risk than blond tobacco [31] As compared to non-smokers, current and ex-smokers are at a higher risk for bladder cancer [32]. The association between smoking and bladder cancer is related to the number of cigarettes smoked per day, the duration of smoke being retained in the lungs, and the amount of smoke inhaled, along with the length of time a person smokes, the number of years since quitting and age at which the person began to smoke [30,32]. In addition, exposure to environmental tobacco smoke during childhood and adolescence also increases the chance for developing bladder cancer [32].

The etiology of bladder cancer has been extended to certain at-risk occupations, including those in the industries of dye, textile, chemical, painting, leather, aluminum, coal, hair styling and rubber that require long-

term to exposure of chemicals that may be carcinogenic [6,16,23,24,28,33]. The first time a causal relationship between occupation and bladder cancer occurrence was suggested was among German dye industry workers in 1895 [34]. Today in Western Europe the metal, machinists, transportation and mining sectors remain as the major occupational contributors for bladder cancer. [17]. Additionally, professional hairdressers were reported to have five-fold increased risk compared with other people and it is possibly due to the frequent contacts with hair dyes; moreover, hair dye users also have a higher risk than non-users [24,25].

Professional drivers belong to another high risk occupational group due to their long term exposure to diesel exhaust and frequent delay in voiding [24,35]. A meta-analysis concluded that workers exposed to diesel exhaust have a modest increased relative risk of bladder cancer [36]. In addition, chronic arsenic ingestion from contaminated water is considered as a minor environmental factor for bladder cancer [37-42]. However, clinical relevance of carcinogenic contact exposure and urothelial transformation comes from studies that have shown that higher total fluid intake was inversely correlated with the risk of developing bladder cancer through increased frequency of micturition, and thus decreased the contact time between the urothelium and chemical agents contained in the urine [43]. There is no doubt that factors which relate to environmental exposure, habits and job nature are all modifiable, thus making many cases of bladder cancer preventable.

(Table 1.3) Risk factors for bladder cancer.

Risk factors	People at risk	Mechanism of carcinogenesis
(Environmental)		
Tobacco smoking	Active / passive smokers	Exposure to aromatic amine and other carcinogens in smoking
Drinking water	Whoever	Exposure to chlorination by-product and arsenic
Coffee drinking	Coffee drinkers	Exposure to carcinogenic metabolites from coffee
(Occupational)		
Industries	Workers#	Exposure to aromatic amine during work
Dyestuff	Hairstylers / hair dye users / Workers##	Exposure to aromatic amine during work
Delay of voiding	Professional drivers	Aromatic amine and metabolites from diesel combustion staying in the bladder
(Urinary tract diseases)		
Infections	Schistosoma harmatobium Other UTI	Mutations / chronic inflammation
Inflammation	Patients with cystitis	chronic inflammation / altered metabolism
Stone diseases	Kidney / ureter stones	chronic inflammation / altered metabolism
(Genetic)		
Hereditary	Family history	Genetic predisposition
Polymorphisms	Gene polymorphisms	Polymorphism of particular xenobiotic-metabolizing enzymes that responsible the detoxification / metabolism of bladder carcinogens

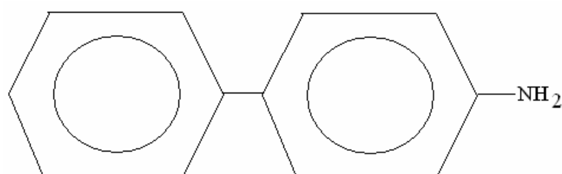
#Rubber, painting, leather, aluminum, coal
##Dye industries
Patton et al., 2002 [24]

1.3 THE LINK BETWEEN 4-AMINOBIPHENYL AND HUMAN UROEPITHELIAL CELLS IN TRANSITIONAL CELL CARCINOGENESIS

1.3.1 4-Aminobiphenyl is an environmental bladder carcinogen

A representative aromatic amine for bladder cancer, 4-Aminobiphenyl (ABP; CAS No. 92-67-1; Figure 1.3), was listed in the First Annual Report on Carcinogens in 1980 [44-46]. The International Agency for Research on Cancer (IARC) defined ABP as carcinogenic to human, as evidenced by experiments on both humans and animals [45]. Neoplasms appeared at various sites after administrating ABP or its metabolites to rabbits, dogs and rodents via different routes. A descriptive study in the mid 1950s showed that 19 out of 171 men developed bladder cancer after exposure to ABP [44]. Moreover, 43 confirmed bladder cancer cases were reported to have been exposed to ABP in a surveillance program launched in 1955 that involved 541 male workers [44].

ABP was used as a rubber antioxidant and a dye intermediate in the past [47]. Although ABP is banned and no longer manufactured or used commercially, it is regarded as an environmental carcinogen that is mainly sourced from tobacco smoking and the combustion of fossil fuels [23]. Mainstream cigarette smoke, per cigarette contains 4.6 ng ABP, while sidestream smoke (the smoke released from a smoldering cigarette, cigar, or other smoking device diluted with ambient air) has 140 ng [48]. However, smokers have higher levels of ABP breakdown products in their blood than non-smokers [47].



Formulae: $C_6H_5C_6H_4NH_2$

Molecular Weight:

169.22 g/mol

Melting point: 52-54^oC

Boiling point: 191^oC (2 kPa)

Storage at room temperature

(Figure. 1.4) Chemical structure and physical properties of ABP. ABP contains two benzoic rings and one amino group, which is a well known human carcinogen considered by Occupational Safety and Health Administration (OSHA). It Occurs as colourless / yellowish-brown crystalline solid having floral odour that turns purple on contact with air and turns dark upon oxidation. The compound is slightly soluble in cold water; soluble in non-polar solvents such as alcohol, ether, chloroform and lipids, and very soluble in hot water. Acute inhalation exposure may cause headaches, lethargy, cyanosis, urinary burning, and hematuria in human. Lethal Dose at 50% (LD_{50}) was defined at 690 mg/kg in rabbits, 500 mg/kg in rats, and 205 mg/kg in mice. [44-46,49,50].

1.3.2 The Fate of 4-aminobiphenyl in vivo

In vivo, ABP is oxidized by the cytochrome P450 (CYP) enzyme - CYP1A2 in the liver to N-hydroxyl metabolites. Then it is esterified to its transport forms with acetate by O-acetyltansferase, glucuronate by glucuronidase and sulfate by sulfotransferase, and is subsequently transported into blood circulation and excreted through urine. By arriving in the urinary bladder, the arylamine conjugates contact directly with the mucosal urothelium lining [51-54]. Bladder carcinogens can also be activated by another CYP enzyme, CYP4B1 in the bladder mucosa [55]. Under the acidic conditions of the urinary environment, these conjugates are hydrolyzed back into their N-hydroxyl forms by respective enzymes and generate reactive arylnitrenium ions and electrophiles to attack the uroepithelial cells [54,56]. In contrast, ABP and its metabolites can be detoxified by N-acetyltransferase (NAT)-mediated competing reactions in the liver by Nicotinamide Adenine Dinucleotide (NADH)-dependent reductase-mediated conversion of N-hydroxyl metabolites into ABP [27,29,42,57].

1.3.3 Initiation of carcinogenesis: ABP-DNA adducts

Chronic exposure of ABP causes the accumulation of primary ABP and its metabolites, which interact with the deoxyribonucleic acid (DNA) bases to form covalent adducts [54,58]. ABP-DNA adducts were first identified in exfoliated urothelial cells [59] and cancer biopsies [23] from smokers. Hemoglobin-ABP and DNA-ABP adducts can be detected in bladder cancer patients with smoking history [23,58,60]. It was proposed that DNA adduct

levels in a specific organ have predictive values for certain cancer risks, including bladder cancer [61].

According to Airoldi et al. [52], the frequency of ABP-adduct occurrence was associated with grading of the tumors, fruit and vegetable consumption and smoking activity. The C-8 position of guanine (G) is the most attributable binding site for carcinogens [62,63]. C8-substituted deoxyguanosine derivatives are the major adducts related to carcinogenic arylamine [62]. Acetyltansferase and horseradish peroxidase play a significant role in the formation of various ABP-DNA adducts [58], whereas xenobiotic polymorphisms have further promotional effects [41].

The occurrence of bladder cancer is related to ABP-DNA adducts, and related concentrations [61], structural conformation [62], metabolic activation phenotypes [51] and the duration of ABP exposure [59]. DNA adducts are essential components for inducing gene mutation [62,64]. Therefore, ABP-DNA is considered an initial event in the multi-step bladder carcinogenesis. The correlation with tumor invasiveness has also been reported [52].

1.3.4 Oxidative stress in ABP carcinogenesis

Plant phenolics, which possess potent antioxidant activities and interferes with the metabolic activation/detoxification of ABP, reduce the levels of ABP-DNA adduct in humans [52]. By exposing ABP metabolites to low- grade bladder cancer cells, the amount of reactive oxygen species (ROS) was

increased, and subsequently induced the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) in dose- and time-dependent manners [58]. Both ABP and metabolites are active in producing reactive oxygen intermediates during bladder carcinogenesis, while N-hydroxyl derivatives have the strongest effects [58]. N-hydroxyl metabolites induce oxidative DNA damage, as assessed by 8-oxo-2'deoxyguanosine (8-OHdG), in cultured cells. This response was inhibited by Copper I-specific chelator, catalase and radical scavenger, and hence hydrogen peroxide (H_2O_2)-mediated autoxidation is involved in the presence of Copper II ions [53]. Again, the C8 position of G is the major site for ROS attack, whereas oxidized forms of G, 8-oxo-dG and 8-OHdG, are the most common oxidative DNA damages that have altered base-pairing and mutagenic properties [63,65,66].

1.3.5 Who are the high risk people?

Bladder carcinogens, such as ABP in vivo are primarily metabolized by the liver and excreted via the urinary tract. The prevalence of bladder cancer is associated with the activities of at least three xenobiotic-metabolizing enzymes N-acetyltransferases (NATs), glutathione-S-transferases (GST) and cytochrome P450 (CYP) enzymes are of particular importance [67-69]. Polymorphisms of the corresponding genes for these enzymes often manifest in bladder cancer upon exposure to certain environmental factors [51,70]. NATs are coded by two distinct genes, designated NAT1 and NAT2, which control the rate of N-acetylation [27,51,69]. NAT1 accelerates the generation of carcinogenic metabolites from pro-carcinogens, while NAT2 reduces the

formation of mutagenic DNA adducts, and therefore polymorphic types of rapid NAT1 and slow NAT2 are at high risk of bladder cancer development [29,51,71,72]. Mutation at the genes controlling expression of CYP enzymes accelerates the activation of bladder pro-carcinogens [51]. The rapid CYP1A2 phenotypes are associated with accumulation of specific carcinogen-DNA adducts in bladder [52,54]. Moreover, the GSTM1 gene encodes the cytosolic enzyme GST-μ, which is highly related to bladder cancer in smokers [42]. Individuals with homozygous deletion of GSTM1 (null GSTM1 phenotype) are less efficient in detoxifying tobacco genotoxins, and thus accumulate polycyclic aromatic hydrocarbons-related DNA adducts [68,73]. Taken together, people bearing such polymorphic enzymes are at high risk for bladder cancer.

1.3.5 Prime target for ABP: Human uroepithelial cells

In the inner surface of the urinary bladder, the outermost transitional layer beneath the mucosal lining composed of multilayer of uroepithelial cells, is the most common site for tumor initiation. In vitro, normal human uroepithelial cells (HUC) were successfully immortalized by the simian virus 40 (SV40), and caused chromosome losses after exposure to ABP and its metabolites [74]. The SV40-immortalized HUC was genomically unstable during continuous passages, positive for SV40 T antigen but non-infectious, and which remained non-tumorigenic when inoculated into athymic nude mice [75]. With this platform, a multistep in vitro/in vivo tumorigenic transformation model system has been developed at the University of

Wisconsin Comprehensive Cancer Center, and used for examining the tumorigenicity of suspected bladder carcinogens [64]. The system consisted of two clonal near-diploid isogenic derivatives of HUC: HUC-PC and HUC-BC which were also SV40 immortalized and, sensitive and refractory respectively, to ABP and its metabolites [76]. A series of mutations were detected when HUC-PC cells were exposed to ABP and its metabolites [60], which induced tumors reproducibly when inoculated into athymic nude mice subcutaneously, while the HUC-BC cells were inert [76]. Carcinogenicity of carcinogens is always correlated with cytoxicity, and ABP is relatively less cytotoxic on HUC when compared with its metabolites [77]. However, ABP metabolites, because they have stronger activities and a shorter half-life than the parent ABP, are more likely to be degraded, or interfered with, by other cellular macromolecules, which prevents them from reaching the target nucleus [23]. Regardless there are differences between the mode of actions for ABP and its metabolites for transformation, HUC cell lines are prime target for ABP tumorigenicity.

1.4 MOLECULAR PATHOGENESIS OF BLADDER CANCER AND MARKERS

1.4.1 Chromosomal and genetic alterations in bladder cancer

Genetic alterations are a hallmark of human cancer; multiple genetic modifications are essential for successful HUC tumorigenesis in vitro [78,79] and are frequently detected in urothelial carcinoma even in the absence of morphological changes [80]. Cytogenetic abnormalities were induced by ABP, including deletions on chromosomes 3p, 4, 6, 8p, 9, 10p, 11p, 13p and 18q as well as gains on chromosomes 3q, 7, 8q and 20q [81]. The most frequent genetic loses in the bladder commonly occur in chromosomes 3, 9, 11, 17 and 18 [82]. In TCC, two cytogenetic pathways have been reported by Höglund et al [83], one initiated by loss of chromosome 9 and one initiated by gain of chromosome 7, both leading to different downstream chromosomal changes for carcinogenesis. Isochromosome 5p, trisomy 7 and loss of 9q are the common chromosomal abnormalities of bladder cancer karyotypes, which hints the biologic diversity of the disease [82,84]. Amongst all, a chromosome 9 aberration regarded as the most common one, accounts for about 70% of bladder cancer cases [41,85]. Whereas, 90% of chromosome arm 9p deletions are associated with 9q deletion in TCC [41]. Loss of heterozygosity (LOH), homozygous deletion and intragenic mutations are major causes for sequential deletion of loci on chromosome 9 [27,81,85]. LOH at D9S113 (9q33-34) can be detected in approximately 30% of normal urothelial samples, and is suggested to be an early event in molecular pathogenesis [80]. A specific pattern of LOH occurs at the region between D9S126 and the interferon alpha

(IFNα) cluster at 9p21, where a tumor suppressor gene (TSG) – cyclin-dependent kinase inhibitor 2 (CDKN2) is located, was commonly identified in primary bladder tumors [27,41]. Genetic alterations in TCC ultimately affect protein expressions that initiate carcinogenesis and deregulate the cell cycle, resulting in cell proliferation and progression of tumors to become metastatic.

1.4.2 Potential markers for TCC surveillance

There are various molecules expressed anomalously in patients with bladder cancer, which can be detected in specimens of serum, bladder wash or urine. An ideal marker for bladder cancer surveillance should have high sensitivity and specificity, be noninvasive and be easy to administer and interpret. Urinary markers for TCC can be broadly categorized into several classes, namely tumor-associated antigens, blood group antigens, proliferation markers, cell cycle and apoptotic regulators, growth factors and their receptors, and other unclassified markers [86]. Out of 18 selected urine markers in a systematic review under strict criteria, van Rhijn and associates [87] suggested microsatellite analysis, ImmunoCyt, nuclear matrix protein-22 (NMP22), CYFRA21-1 and LewisX as the most promising markers with regard to sensitivity and specificity for TCC surveillance. For diagnostic purposes, the Food and Drug Administration (FDA) has approved the following urinary tests: NMP22, bladder tumor antigens stat (BTAstat) and complement factor H-related protein (BTA-TRAK), ImmunoCyt and UroVysion [88,89]. But only a few including hemoglobin stick, NMP22 and modified UroVysion, have been accepted for screening of TCC [89].

Commercial kits for BTA and NMP22 are available for screening new and recurrent cases of TCC [90,91]. NMP22 is one of the most useful biomarkers for TCC surveillance [92], and it has been evaluated in large number of individuals [87], and the cutoff value for monitoring the occurrence of recurrent urothelial tumors has been well defined [93]. However, contradictory results have also been reported regarding poor sensitivity and a high false-positive rate [94]. BTAstat and BTA TRAK are qualitative and quantitative respectively, for detecting a human complement factor H-related protein produced by human bladder cancer cells [95]. These two tests possess basically higher sensitivity but lower specificity than cytology; however, the overall accuracy is decreased by coexistence of non-neoplastic urologic conditions, gross hematuria, stone disease, urinary tract infection (UTI) and other genitourinary malignancies [89]. Administration of intravesical bacillus Calmette-Guérin (BCG) also decreases specificity of BTA-based tests up to 28%, and therefore lowers the reliability of using BTAstat or BTA TRAK for monitoring the disease after intravesical therapy [96]. Furthermore, a number of studies have been conducted for comparing the performance of other urinary biomarkers, but their conclusions were inconsistent. The highest overall sensitivity and specificity has been reported for telomerase and Lewis X by Ramakumar et al [97] and Lee et al. [98], and the microsatellite instability assay by Ross & Cohen [90]. To date, no single marker achieves 100% accuracy and conventional cytology remains as still-the-best option with modest sensitivity but highest specificity ranging from 81% to 100% [89]. Quek et al. [99] suggested that panels utilizing the most promising biomarkers

may be an even better alternative. Microarray technology allows measurements of hundreds or thousands of items, including DNA sequences, RNA transcripts and proteins in a single experiment. This accelerates the discovery of new tumor markers, and perhaps a powerful tool to provide new direction for biomarker identification for TCC. The following molecular markers are reviewed as some of the proposed diagnostic and therapeutic targets for TCC.

1.4.3 p53

Over-expression of mutant p53 protein is a common feature across a wide range of human malignancy [100,101]. Accumulation of nuclear p53 protein is clinically significant in bladder cancer for correlating recurrence and progression, and thus reduces the survival rate [102-104]. Protein p53 is the product of a pivotal TSG, whose inactivation is probably the most frequent single genetic event in human cancer [105]. In bladder carcinoma, a 'two-hit' inactivation of the p53 gene frequently occurs at locus 17p13.1, with one allele being deleted and another one mutated [60,84]. The mutation of p53 caused by adenine to thymine (A→T) transversion occurs in codon 227 and has never been detected in normal bladders but it presents in malignant bladders [41]. Codons 175, 248 and 273 are common hotspots shared by many human cancers; however, codons 280 and 285 lacking CpG sites are unique for bladder cancer pathogenesis [23,106]. In addition, all these hotspots, except codon 273 are preferential sites for ABP adduct formation, which plays a crucial role in mutational spectrum of p53 protein expression [107]. The

function of p53 as a TSG is to prevent excessive cell proliferation through the induction of cell cycle arrest or apoptosis upon activation [108]. Stabilization of p53 protein regulates the cascade of events of downstream WAF1/p21-induced cell cycle arrest, GADD45-mediated DNA repairing and BAX/BCL-2 apoptotic pathway. Therefore, the vital protein p53 has critical roles in protecting cells from mutagenic genotoxic damage in order to maintain genome stability. The absence of p53 TSG brings genetic errors into the subsequent generations, increasing the chance of malignancy.

1.4.4 Telomerase

Programmed cell death is committed when somatic cells reach their replicative cycles, known as the "Hayflick limit", which delineates the shortening of telomeres for achieving its threshold to uncap the telomeric ends [109,110]. Telomerase is a "ribonucleoprotein reverse transcriptase" enzyme that synthesizes DNA onto chromosomal ends, and thus stabilizing the genome by repairing the shortened telomeres after cell division. Elevated telomerase activity is commonly seen in malignancies; whereby continuous telomere repairing leads to uncontrolled cell proliferation, in turn escaping the mitotic clock of senescence [109,111,112].

Numerous studies showed sensitivity of telomerase-based assay in favour of urinary cytology [89,99,113]. The human telomerase enzyme is composed of two essential subunits: human telomerase reverse transcriptase (hTERT) complex and human telomerase RNA (hTR) component. hTR serves as a

template for elongation of telomeric DNA, while hTERT is the catalytic counterpart to promote the telomere synthesis [114,115]. Both subunits can be detected in the urine of patients with bladder cancer by using quantitative real-time polymerase chain reaction (RT-PCR) technique. On the other hand, inhibition of hTERT was induced by transfection of specific antisense oligonucleotide (AS-ODNs) into the human bladder cancer EJ28 cell line [116]. In a comparative study conducted by Weikert and colleagues [113], hTR is more reliable and sensitive than hTERT, and thus hTR is a suggestive target for diagnostic and monitoring analyses and has the potential to replace conventional cytology. Eighty-five percent of patients diagnosed with bladder cancer are positive for telomerase expression [117]. The telomeric repeat amplification protocol (TRAP) is a gold standard, with 90% sensitivity and 88% specificity, for detecting telomerase activity in various specimens of bladder carcinoma [99,118,119].

Up-regulation of telomerase activity can be found in transformed bladder cancer cells [111,120,121], voided urine [118] and bladder-wash specimens [122] of most patients with urothelial carcinoma. Telomerase has been proposed as a urine-based marker for bladder cancer with overall 70-90% sensitivity and 80-95% specificity, depending on the techniques chosen [118,123]. However, telomerase is not grade-sensitive and is relatively a more reliable biomarker for early phase urothelial carcinoma [114]. False-negative results, particularly when TRAP assay is used, results from contaminants such as Taq polymerase inhibitors and ribonucleases that are occasionally present

in high-grade tumors [89,124]. Careful re-evaluation is needed before placing telomerase as a routine marker, since false-negative results with gross hematuria (a cardinal sign for bladder cancer) and false-positive results with bladder inflammation, may be seen [89].

1.4.5 Cycooxygenase-2

Cycooxygenase (COX) is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins during the metabolism of arylamine carcinogen [24,125-127]. There are two common isoforms: COX-1 that expresses constitutively in most tissue as a housekeeping enzyme for mediating physiological responses and COX-2 is rarely expressed under normal physiological conditions [125,126,128]. COX-2 is an immunostaining positive in malignant urothelial carcinoma depending on the grade and stage [24,129-131]. Expression of COX-2 is correlated with the invasiveness of bladder tumors and reduced survival rate [132]. Selected COX-2 inhibitors such as celecoxib, NS398, Rofecoxib, and most over-the-counter nonsteroidal anti-inflammatory drugs possess antiproliferative and antineoplastic properties to reduce the incidence of bladder cancer [24,125,133]. COX-2 inhibitors also induce apoptosis in TCC cell lines, including T24 and TCCSUP through cell cycle phase arrest [125,134]. Some phytochemicals, such as flavonoids and tocopherols, modulate the expression of COX-2 messenger RNA (mRNA) [135-138]. Recently, a combination of inhibitors for COX-1 (catechin) and COX-2 (NS398) was reported to exhibit synergetic antiproliferative effects on bladder cancer cells [127].

Expression of COX-2 is responsible for the synthesis of prostanoids that can be induced by tumor promoters, growth factors, mitogens and inflammatory cytokines [125,126,128,129,139]. The promoter region of the COX-2 gene contains a canonical TATA box and various transcriptional regulatory elements such as nuclear-factor kappa-B (NF-κB) [139]. Polymorphisms of the COX-2 promoter region, especially the nucleotide situated in the NF-κB binding promoter region, cause COX-2 over-expression that is associated with increased risk of bladder cancer [126]. COX-2 expression is regulated by the p53 TSG at transcriptional levels [139,140]. The over-expression of COX-2 increases the synthesis of prostaglandins, which subsequently enhances tumor cell growth, tumor invasiveness, angiogenesis and apoptosis suppression in transformed cancer cells [126,128,129,139].

1.4.6 8-hydroxy-2'-deoxyguanosine

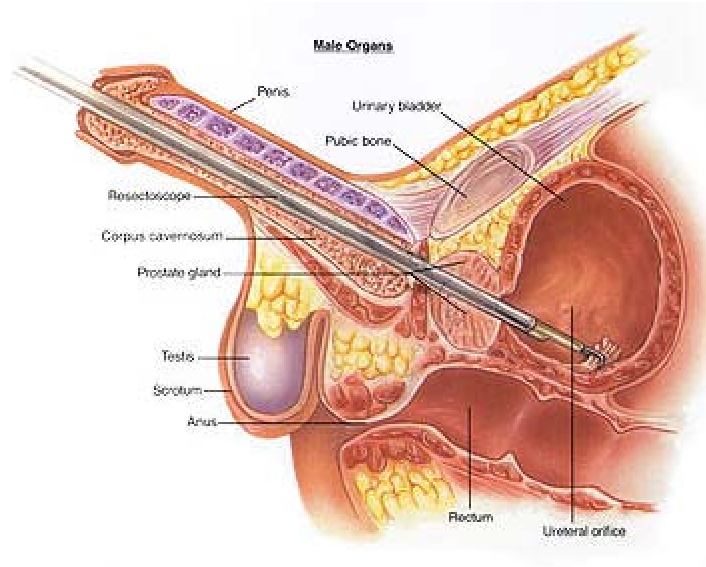
8-hydroxy-2'-deoxyguanosine (8-OHdG) is a biomarker for oxidative DNA damage, which has been related to mutagenesis, and therefore cancer development. The causal relationship between oxidative stress and cancer incidence has been widely supported by epidemiological observations and molecular studies. In the field of oncology, DNA is the most reported macromolecule being attacked by both endogenous and exogenous oxygen radicals [141,142]. However, 8-OHdG is the most well known deoxyribose lesion leading to G:C→A:T transition mutations and being proposed as a candidate biomarker for carcinogenesis [143,144]. High levels of urinary 8-

OHdG is detected in patients with bladder cancer [145]. Moreover, the formation of 8-OHdG can be induced by in vitro exposure to ABP metabolites [53,58]. The proximate metabolites of ABP are active in producing ROS that subsequently attacks the C8 position of guanine [53,58]. 8-OHdG and its analogs have been suggested as biomarkers for oxidative stress to determine the risk of bladder cancer [145].

1.5 MANAGEMENT OF TCC

1.5.1 Transurethral resection for treating superficial TCC

The decision of the course of treatment for bladder cancer is based on how far the tumor has invaded the surrounding tissues and whether there is migration. For superficial TCC, transurethral resection (TUR) is the primary modality effective in tumor removal [6,146]. Figure 1.4 below illustrates how TUR takes place. However, TCC has a high recurrence rate that is probably due to the tumor cell implantation or incomplete resection [147]. Even with complete TUR, up to two-thirds of the patients with TCC will recur in five years and 30% of them develop progressive diseases [6,148]; and the recurrences are in biphasic fashion with an initial peak within three to six months and a second peak between 18 and 30 months [147]. At least 60% to 80% of TCC recurs within the five years following surgical ablation alone [149]. Approximately only 20% of superficial TCC are cured without recurrence or progression by surgical TUR [88]. This has led many urologists to believe that TUR alone is insufficient for treating cases of superficial TCC, and therefore, intravesical therapy has been adapted to adjuvant TUR in the standard protocol of superficial TCC treatment.



(Figure 1.5) Transurethral resection. A rigid 'Resectoscope' introduced down into the urethra to reach the bladder (*sourced from the Perth Urology at www.perthurology.com*).

1.5.2 Multifocal TCC is an indication for recurrence risk

TCC recurrence is probably due to the high propensity to papillary multifocality [6,148,150]. The recurring tumors arise from inadequate resection, areas of dysplastic urothelium or implanted tumor cells, depicting the biological nature of how bladder carcinogenesis occurs after TUR [147]. The presence of chemical carcinogens in the intravesical compartment and the occurrence of metachronous tumors lead to the "Field cancerization hypothesis" which accounts for the multifocal nature of TCC [151]. In addition, molecular studies support that the multifocal tumors are derived from a single lesion originating from a single transformed cell due to clonal

expansion, which is known as “clonal seeding theory” [81,150,152,153]. In addition to the number of tumors, the size of the tumor, the grade of anaplasia and the history of recurrence, multifocal TCC at any grade or stage has an increased risk of recurrence and progression that constitutes an indication for adjuvant intravesical prophylactic therapy [1,146].

1.5.3 Adjuvant Bacillus Calmette-Guérin immunotherapy

Since the late 1950s, chemotherapeutic agents have been practiced through intravesical instillation into the bladder of patients with superficial TCC after TUR, for three designated outcomes: (1) clearance of residual tumor; (2) prevention of recurrence and (3) prevention of progression [154,155]. Theoretically, immediate intravesical chemotherapy after TUR may prevent tumor cell implantation and recent data suggest that a single postoperative instillation can significantly reduce tumor recurrence [6]. Intravesical drug delivery has certain advantages: (1) it avoids initial metabolism of the drug; (2) it localizes the drug to the desirable site to exert therapeutic effects and (3) it minimizes the chance of systemic effects [156]. At present, intravesical immunotherapy with BCG, a live attenuated strain of Mycobacterium bovis, has been seen to be the most effective regimen for the treatment of superficial bladder cancer since the first reported case in 1976 [157]. Action of the most frequently used immunotherapeutic BCG is based on the urothelial internalization to trigger inflammatory response, which ultimately results in tumor necrosis factor-alpha (TNF-α)-induced apoptosis [158].

1.5.4 Urothelial internalization of BCG

Direct contact of live BCG with the urothelium is essential after intravesical instillation to exert the desired outcomes. In vitro, dose-dependent and specific bindings of BCG to urothelial carcinoma cells derived from grade I-III of tumors have been demonstrated [159]. However, electron microscopy failed to reveal adherence of BCG to the surface of either an intact or artificially damaged guinea pig inner bladder wall [160]. This is probably due to the protection of the bladder's luminal wall from toxic compounds and microorganisms by a layer of highly sulphated glycosaminoglycans (GAGs) [161]. Both the GAGs layer and BCG cell wall are highly negatively charged and that generate repellent forces to accumulate BCG bacteria at a close docking distance of 70-100Å without adherence to the bladder wall [162].

A specific receptor-ligand event mediated by fibronectin (FN) in the urothelial mucosa has been proposed by several investigators [1,159,161,163]. FN is an extracellular matrix component equally distributed on normal and malignant urothelium, and its soluble form is present in urine. Two molecules with affinity to FN, namely, the α5β1 integrin present on urothelial cell surface [164] and FN attachment protein (FAP) present on BCG cell wall [165,166], suggests the attachment of FN to urothelial cells via the opsonization process. The cross-linking of the α5β1-integrin triggers various signal transductions to mediate immune responses involved in BCG-induced responses [164,167]. Activation of α5β1-integrin can also induce non-apoptotic cell cycle arrest for controlling cell proliferation [168]. The expressions of α5- and β1- subunits

are correlated with the ability of BCG adherence to the human TCC 253J cell line [169]. However, the α5 and β1 mediated reactions can also be triggered by analogue antibodies, which is not dependent on FN expression [169]. The mandatory role of FN in BCG therapy is contradictory, because some investigators reported the impairment of BCG attachment to urothelial cells and subsequent anti-tumor effects by the presence of anti-FN antibodies and soluble FN (autocrine / exogenous) molecules [169,170], but others could not demonstrate such inhibitory effects [159,171]. Moreover, the interaction between BCG, fibronectin and tumor cells can be altered by antibiotics and fibrin clot inhibitors such as aspirin [172]. There is also evidence that TUR up-regulates the latent expression of transforming growth factor-β1 (TGF-β1) [173]. Consequently, TGF-β1 increases cellular FN production in human TCC cells [174]. Contradictorily, clinical data supported that the failure of BCG therapy may be due to persistent elevation of FN after TUR [175]. Therefore, the effectiveness of BCG therapy is possibly influenced by the increase of TGF-β1 expression after TUR that augments the production of FN in the bladder compartment.

Regardless of the mechanism of how BCG attaches to the urothelial wall, phagocytosis is confirmed to be the ultimate internalization immune process. Electronic microscopic techniques revealed that urothelial carcinoma cells acquired the capacities of “professional” phagocytic cells. Inside the TCC cells, BCG molecules were incorporated in vacuoles to form phagosomes, which subsequently fused with lysosomes to generate phagolysosome where

the mycobacteria finally disintegrated [159,160,176]. From the bladder washes of TCC patients receiving BCG therapy, internalization of BCG was detected in the membrane-bound vesicles in epithelial cells, and degraded mycobacteria were also found [176].

1.5.5 Mode of actions for BCG

Internalized BCG triggers immune responses for the prophylaxis of TCC is well documented. Live BCG and numerous subcomponents are immunologically active [177]. BCG is regarded as a non-specific immuno-stimulant initiating inflammatory response in the bladder lumen. A broad range of cell types: macrophages, T lymphocytes, B lymphocytes, and natural killer (NK) cells infiltrate into the bladder wall after BCG instillation [1]. The antineoplastic effect of BCG is a localized phenomenon such that various arms of the immune system are stimulated [1]. Processed BCG antigens after internalization are associated with major histocompatibility complex (MHC) class II molecules that are recognized by CD4+ T-helper lymphocytes [161]. The mechanism of action of BCG immunotherapy is mainly based on the stimulation of T-helper lymphocytes, followed by the secretion of cytokines, such as interleukin-1 (IL-1), IL-2, IL-6, IL-10, interferon gamma (IFN-γ), and TNF-α [178,179].

During the course of BCG instillation, levels of several cytokines including IL-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α, interferon-gamma (IFN-γ) and macrophage colony-stimulating factor (M-CSF), are elevated

heterogeneously in urine specimens from patients [180,181]. However, only a few, including IL-2, IL-6 and TNF-α, can be detected in urine within the first 24 hours after BCG instillation [182]. Both T helper type 1 (Th1) and type 2 (Th2) cytokines are produced in response to BCG. Many of these secreted cytokines are chemo-attractants (including IL-8 and M-CSF) and inflammatory active (including IL-1, IL-2, IL-6 and TNF-α). Only IL-2 and IFN-γ are increased in serum from TCC patients after BCG therapy [180]. These two cytokines are particularly useful in monitoring the BCG-induced immune reaction [182]. Isolated peripheral blood mononuclear cells (PBMCs) from healthy donors who are positive for mycobacterial antigens are able to enhance IFN-γ in T24 TCC cell line to induce Th1 differentiation and non-MHC-restricted cytotoxicity [177]. IL-2 and IFN-γ are known Th1 cytokines, whereas Th1 is responsible for cell-mediated immune response for developing the protective antitumor response, such as delayed-type hypersensitivity, cytolysis and macrophage activation [1,183]. Evidence suggests that activation of Th1 immune response is essential for BCG efficacy [179]. On the other hand, Th2 is responsible for humoral immune response that produces antibodies upon activation. The absence of urinary IL-2, IL-6 or TNF-α leads to a poor prognosis, which correlates with an increase of urinary tumor markers – UBC, CYFRA21-1 and NMP22 [181]. However, polymorphisms of certain cytokine genes are associated with the failure of BCG immunotherapy [184].

The efficacy of the BCG-provoked immune response relies on the induction of apoptotic cell death to residual tumor cells with minimal cytotoxicity against normal cells or tissues. This can be achieved by stimulating the expressions of TNF-Related Apoptosis-Inducing Ligand (TRAIL/Apo-2L) and Fas/CD95 ligand (FasL) on infiltrated immune cells and Fas expression on tumor cells [158,185]. Positive expression of FasL or TRAIL can be detected on neutrophils, macrophage, T helper, T cytotoxic and natural killer cells [158,185,186]. Cell cycle arrests were observed when BCG was exposed to T24 TCC cell line, but no DNA degradation was shown, and therefore, apoptotic effects could not be confirmed [187]. It was found that voided urine from TCC patients receiving BCG therapy is cytotoxic to RT-4 bladder cancer cells [158]. High levels of TRAIL were expressed, in both surface-bound and functional soluble forms for the isolated neutrophils in the urine specimens [158,186].

1.5.6 Efficacy and side effects of BCG

Intravesical BCG is so far superior for the prophylaxis of superficial TCC [6]. With completion of TUR, at least one to two weeks should elapse before BCG administration in order to prevent BCG sepsis [149]. A retrospective study by Andius & Holmäng [188] indicated that patients with a BCG induction of less than six weeks have a higher risk of progression and local failure. A six-week course of BCG induction is minimal, but most experts agree that it is insufficient for optimal response. Reduction of the BCG dose from six consecutive weekly instillations to two single instillations at six-week

intervals did not diminish the levels of local Th1 cytokines mRNA, but decreased the levels of those for Th2 cytokines, such as IL-10 and IL-4 [183]. A meta-analysis of randomized trials demonstrated that the BCG treatments with maintenance course appeared to produce the best response [189]. The Southwest Oncology Group (SWOG) 8507 protocol, with the three weekly BCG instillations at 3, 6, 12, 18, 24, 30 and 36 months after the initial six-week induction course, significantly lengthens the recurrence-free survival rate [172,190]. However, the maintenance schedule of three years is unrealistic, because only 16% of the patients fulfilled the complete course of the SWOG study [190]. At present, at least one three-week maintenance course is recommended and thereafter at three months for the induction therapy. The usual dwell time for each BCG instillation is two hours [149].

BCG's effectiveness is still controversial, while some studies have shown it to delay or prevent TCC recurrence [189]. The colony-forming unit (CFUs) should be added to measure adequate BCG viability for optimal activity [191]. A systematic review suggests that the combination of intravesical BCG therapy with TUR is more favored to TUR alone in preventing recurrence of superficial TCC [192]. BCG reduced the mortality rate by 18% and the disease progression by 7% [193]. Meta-analysis by Lamm et al. [194] concluded that BCG treatment has an overall 42% prophylaxis to TCC recurrence. Instillation of BCG after TUR reduces 20-40% of recurrence [157,195]. However, approximately 10-30% of recurrent cancers still progress to invasive muscle disease, threatening survival rate [1].

Although dose scheduling and administration have been optimized for years through intensive studies, intravesical BCG instillation is not free of side effects [196]. The process of BCG internalization leaves bacterial cell surface glycoproteins attached to the epithelial cell surface, and this antigen is thought to trigger de novo inflammatory response that consequently leads to dysuria and urinary frequency [1]. Up to 90% of patients receiving BCG instillation experienced cystitis as the major side effect, whereas up to 34% had gross hematuria [157,197]. Other non-specific complications included fever, malaise and appetite loss [198]. Serious and life-threatening adverse reactions of BCG is rare, accounting for 0.5-1% of the patients. Systemic immune response has been revealed to enhance killer cell activity of PBMCs in patients with sufficient BCG instillation, which is associated with some severe side effects, such as sepsis and epidimitis [199-201]. Although the virulence of live BCG organisms is dramatically attenuated, potential regional or systemic infection may occur. Upon instillation, BCG organisms are reported to persist in the urinary tract for at least 16.5 months [1]. BCG is capable of infecting the mucosal layer that may persist for several months after instillation. It is also able to penetrate within the deep layers of the bladder wall and reaching the pelvic lymph nodes [172]. Moreover, intravesical BCG is ‘a double-edged sword’ to be a powerful tool in the treatment of superficial bladder cancer, but it is also capable of triggering autoimmune responses [201]. It is reported that side effects of BCG immunotherapy can be decreased by logarithmic reductions in dose; however, the efficacy may also reduced

[157][202]. No doubt, the effectiveness of BCG immunotherapy largely relies on host-immune response and it is contraindicated in immunosuppressed patients and in those with urethral trauma [199].

1.6 CHEMOPREVENTION

1.6.1 What is still missing for bladder cancer?

Bladder cancer is a preventable disease. Superficial TCC accounts for more than 80% of all bladder carcinoma. Although TUR is effective and simple, the high recurrence rate and potential progression due to the multifocal and clonal seeding nature of TCC calls for a better or more advanced prophylaxis. BCG has become the most effective agent for preventing TCC recurrence and progression, with an optimal dosing schedule; however, the route and mode of action are still not clear. The BCG treatment is not free of side effects because it is a live mycobacterial organism that is potentially infectious. Adverse reactions that come up with BCG are probably due to the inflammatory response based on the internalization of BCG in the urothelial cells. A series of urinary cytokines for both arms of Th1 and Th2 reactions are triggered by the presentation of BCG antigens, for infiltrating immune cells into the bladder wall. The efficacy of BCG relies on persistent mucosal infection as well as the host's immunity. However, the effectiveness of BCG is still unsatisfactory since only less than 40% of recurrence is reduced and still 20-30% of the recurrent tumors progressed to an invasive disease. Therefore, a non-infectious chemopreventive agent containing more powerful activity for residual tumor cell clearance is essential for bladder cancer prophylaxis.

1.6.2 Chemoprevention of bladder cancer

Through advanced surveillance techniques, the mortality of bladder cancer has been largely reduced by chemoprevention strategies in the past decade [10]. Diet could influence bladder carcinogenesis since many compounds in foods and their metabolites are excreted through the urinary tract [26]. Increased intake of fruits, vegetables, vitamins A, B6, C, D and E and a reduction of animal fat are suggested to be useful for prevention [203]. However, it is still insufficient for the high-risk patients of TCC and a safe chemopreventive agent is still demanded for.

Chemoprevention is defined as the use of pharmacological agents for reducing the cancer incidence either through the suppression of established malignant cell clones or through the inhibition of growth and progression of premalignant cells [204]. In the laboratory, chemoprevention is aimed at preventing the formation of carcinogen, avoiding the initiation of carcinogenesis, blocking the interaction with genome and suppressing cancer cell growth [34]. An ideal chemopreventive agent should be easy to administer with minimal side effects and specifically targets the metabolism of internal or external carcinogens, avoiding malignant transformation [10]. In the clinical settings, assessment of chemopreventive efficacy involves three phases, (1) identification of an optimal chemopreventive agent; (2) demonstration of efficacy in human through the modulation of reversal of a tissue, biochemical and molecular surrogates for neoplastic transformation and invasion and (3) demonstration of cancer risk reduction in large cohort

trials [205]. Tertiary chemoprevention aims to prevent the occurrence of second primary tumors after complete resection [206]. A number of natural and synthetic agents are suggested to be chemopreventive for high-risk bladder cancers. The most studied ones include retinoids and isothiocyanates (ITCs).

Retinods are vitamin A derivatives that could be natural or synthetic, which holds promise for bladder cancer chemoprevention [207,208]. Mechanisms are mainly based on alteration of cell cycle distribution and induction of apoptosis [207,209]. Fenretinide (4-HPR), regarded as the most potent apoptosis-inducing retinoid analog to TCC cell lines of varying histological grade, is undergoing clinical trials [209-211]. 4-HPR reduces circulating insulin-like growth factor-1 (IGF-1) in patients with superficial TCC in a time-dependent manner [212]. Recently, a combined use of retinoic acid and interferon-α2a has demonstrated synergistic effects to induce apoptosis in TCC cell lines [213]. This could be a new direction for retinoid-related strategies for bladder chemoprevention.

Dietary isothiocyanates (ITCs) are rich in cruciferous vegetables, in particular *Brassica* vegetables, such as broccoli, cauliflower, cabbage and radish [214]. Chemopreventive actions of ITCs are associated with the modulation of xenobiotic-metabolizing enzymes, including CYPs and GSTs [214,215]. A few ITC compounds have been investigated for chemoprevention, and, amongst all, 4-methylsulphinylbutyl (sulforaphane SFN), the most extensively

studied one, was shown to suppress growth of T24 bladder cancer cells in vitro through induction of apoptosis and G0/G1 cell cycle arrest with up-regulation of cyclin-dependent kinase inhibitor p27 expression in a dose-dependent manner [216]. Other ITCs possess anti-proliferative and apoptotic activities involving a variety of pathways [215]. In addition, ingested ITCs are quickly absorbed and metabolized into N-acetylcysteine conjugates (NAC-ITC), which are efficiently excreted and concentrated in urine, and thus effectively deposited onto bladder urothelium [215].

Several synthetic compounds have provided evidence for bladder chemoprevention. Erlotinb is a specific tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR), which induces actin remodeling and growth inhibitory effects on bladder cancer cell lines [217]. Sulindac and ketoprofen are two non-steroidal anti-inflammatory drugs which effectively reduce the incidence of TCC in the mouse bladder [218]. Moreover, atrovestatin, which is a drug for hyperlipidemia, is also demonstrated to be anti-proliferative and apoptotic in two human TCC cell lines [219].

Besides, other nutritional agents including soy products, garlic and megavitamins are demonstrated to be chemopreventive for urinary tract cancers [220]. Soy bean foods were found to be modulatory to isoflavones, which are present in human urine and possess antitumor activities [221]. From a variety of foods, including meat, poultry, eggs, fish and grains, selenium is an essential trace element that can be metabolized in vivo to restrict cell

proliferation by inhibiting protein kinases and halting cell cycles [222]. Recently, cactus pear and ginger have been demonstrated to be chemopreventive for bladder cancer [223,224]. Taken together, many substances are suggested to prevent the occurrence or recurrence of bladder cancer and most are shown to be cytotoxic. According to the latest systematic review by Busby & Kamat [208], although megadose vitamins, certain vitamin A analogues and pyridoxins have been associated with promising findings, no oral agent has been recommended until now and the best chemopreventive strategy remains to be determined.

CHAPTER 2

LITERATURE REVIEW: G. LUCIDUM - A MEDICINAL MUSHROOM WITH ANTICANCER EFFECTS

2.1 GANODERMA LUCIDUM IS A VALUABLE MUSHROOM

2.1.1 Ganoderma tales and history

The Chinese have centuries' old "anecdotal evidence" that Lingzhi mushrooms possessed supernatural and everlasting properties to bring the dying back to life. In the history of Traditional Chinese medicine, Lingzhi is regarded as a supreme tonic for enhancing the longevity and maintaining the health. Six types of Lingzhi in the colors of red, purple, green, white, yellow and black have been recorded in the Pharmacopoeia Classic of the Divine Husbandman (Shennong Bencaojing) since the 1st century BC [225]. For each type of Lingzhi, detailed explanations have been made according to their names, collective meanings, corrections, handling methods, odors, indications and appended prescriptions, by Li Shizhen in the Classic Compendium of Materia Medica (Bencao Gang Mu) written during the 15th century BC [226]. Nowadays, Lingzhi have also been added to the American Herbal Pharmacopoeia and Therapeutic Compendium [227]. Lingzhi with the genus name Ganoderma is a Basidiomycetes mushroom of woody nature, belonging to the family of Ganodermaceae of Aphyllophorals [227,228]. Moreover, Lingzhi are also called 'Reishi', 'Mannentake' or 'Sachitake' in Japan, and 'Youngzhi' in Korea.

2.1.2 Nutriceutical and medicinal Ganoderma lucidum

Ganoderma lucidum (Curt: Fr.) P. Karst is a representative species of 'Red G. lucidum' which is well known and the one most frequently used for human consumption; its use is supported by a large amount of evidence. Various forms of health products using G. lucidum as the active ingredient have been marketed in recent years. G. lucidum has gained wide popularity as health food supplement in Asia because of its perceived health benefits, which include anti-inflammatory, anticancer, antiviral, antibacterial, antiparasitic, blood glucose regulator, cardiovascular tonic, kidney tonic, nerve tonic, hepatoprotective, chronic bronchitis and immunomodulatory agents [229,230]. Therapeutic values of G. lucidum cannot be underestimated, especially in some areas such as antitumorigenicity which have been extensively studied.

2.2 ANTICANCER EFFECTS OF GANODERMA LUCIDUM

2.2.1 Is G. lucidum a food supplement or chemotherapeutic agent?

The proposed anticancer activity of G. lucidum has prompted its usage by cancer patients, but whether G. lucidum is a food supplement for health maintenance or actually a therapeutic 'drug' for medical purposes is debatable. The use of herbal medicine, such as G. lucidum, in cancer curing and preventive treatment remains questionable and are avenues that have not been scientifically evaluated [231]. Previous studies on anticancer activity of G. lucidum, from experiments in vitro and animals to humans in vivo, merely supported its applicability for cancer treatment and prevention, but the mechanisms of the action have not been fully explored [232-236]. A variety of G. lucidum anticancer activities from prevention to treatment have been extensively and systematically reviewed by Gao et al. [232,237] and Yuen & Gohel [228].

2.2.2 Bioactive components: Polysaccharides and Triterpenes

It is believed that the anticancer properties of G. lucidum are largely contained in its diversified chemical constituents. A large number of chemical compounds could be extracted from the fruiting body, mycelium or spores. The mushroom contains a wide variety of bioactive molecules, including phenols, steroids, amino acids, lignin, mycins, vitamins, nucleosides and nucleotides, whereas polysaccharides and triterpenes are the two major groups of components [205,229,232-241]. Each fraction of polysaccharides and triterpenes has more than 100 molecules that have been isolated; most are

potent immunomodulators and/or antioxidants and are also chemopreventive and tumoricidal [233,240-242].

A polysaccharide extract of G. lucidum mycelia inhibited the formation of ras-induced transformed foci in an R6 embryo fibroblast cell line [243]. The G. lucidum-isolated polysaccharides are macromolecules, having a molecular weight range from 4 x 10^5 to 1 x 10^6 Da in the primary structure, whereas solubility depends on molecular weight and temperature [233]. Most of these polysaccharides are extractable with hot water, salt solutions, alkali solutions and dimethyl sulfoxide (DMSO) solution [229,230,233]. After initial extraction with water, the compounds can be further extracted by ethanol and sodium hydroxide; the purified molecules are less soluble in water and become more soluble in alkali [233]. Among those, neutral polysaccharides *(β-1→3, β-1→6 homo D-glucan)*, acidic glucan and polyglycan are bioactive; but only branched glucan with *(1→3)-, (1→4)- and (1→6)-β-D-linkages* have been characterized with novel antitumor activity against oncogenesis and tumor metastasis [229,238,244]. Such Ganoderma β-D-glucans were reported to have higher antitumor activity and are better absorbed orally than those commercially available in synthetic β-D-glucans [238]. Yet, the antitumor activity of β-D-glucans was exhibited mainly at the *branched (1→3)-chain binding* to complement receptor type 3, triggering a series of molecular pathways such as NF-κB, mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), which in turn, activate the host immune response for immune cell proliferation and cytokine production [233,239]. Therefore, the

polysaccharide compounds have been suggested to be a new type of carcinostatic agent based on their ability to enhance the host's defence system [229]. However, fractionated polysaccharides were not as effective as their equivalent dose in the crude extract of the whole mushroom, suggesting that bioactivity of G. lucidum may be attributed to the synergistic effects of its multiple compounds, such as triterpenes [245].

Triterpenoids are the predominant triterpenes in G. lucidum, possessing multiple bioactivities including immunomodulating, antioxidative and antitumor effects [233,237]. In particular, the highly oxygenated triterpenoids responsible for the bitter taste of G. lucidum are strain specific, suggesting that their quantities are eligible for quality measures [227,229,238,246]. These oxidized species are pharmacologically active, containing a lanostane skeleton (for example, ganoderic acids, lucidone acids, ganodermic acids, ganoderenic acids, lucidone, ganoderal and ganoderols) which can be easily extracted by any organic solvents [233]. It has been demonstrated that *ganoderic acids-R, -T, -U*, *-V*, *-W*, *-X*, *-Y* and -Z, *lucidimol-A*, *-B*, *ganodermanondiol*, *ganoderiol F* and *ganodermanontriol* exert cytotoxic-based carcinostatic effects on cancer cells, and many of them also possess anti-angiogenic activity [239,241]. In the last two decades, the majority of research has been performed on G. lucidum's inhibitory effects on cancer cell growth and angiogenesis and also the underlying mechanisms involved.

2.2.3 Cancer cell cytotoxicity

Of 58 Basidiomycetes species tested, G. lucidum is the most active cytotoxic mushroom with regards to cancer cells [234]. Cytotoxicity can be defined as (1) the direct killing of cells and (2) the inhibition of cell proliferation. With the tetrazolium (MTT) method, inhibition of proliferation has been shown in various cancer cell lines: murine lymphocytic leukaemia L1210 and Lewis Lung carcinoma (LLC) [234]; human hepatoma PLC/PRF/5 and KB [247]; human breast cancer MDA-MB-123 [248]; human prostate cancer PC-3 [249]; human breast cancer MCF-7 [250]; human cervix uteri tumor HeLa [245] and low-grade bladder cancer MTC-11 [251]. The cytotoxic effects of G. lucidum as demonstrated by the studies of Jiang et al. [248,249] and Zhu et al. [251] were dose dependent. Lu et al. [252] have also shown consistent cytotoxic activity with [^{3}H] thymidine incorporation assay. The inhibitory effects of G. lucidum products, extracts or pure compounds isolated on cancer cell proliferation are summarized in Table 2.1.

Additionally, as shown in Table 2.2, G. lucidum has been found to exert direct lethal effects on human hepatoma Hep3B [253] and Huh-7 [254], human cervix tumor HeLa [251], murine sarcoma Meth-A [241,242], murine LLC [241,242], human breast cancer T-47D [242] and mouse sarcoma S-180 [242]. As noted in Tables 2.1 and 2.2, the cytotoxic effects expressed in the concentration that inhibits 50% of cell proliferation (IC_{50}) and the lethal dose to cause 50% of cell death (LD_{50}) varied from 1 to 5000μg/ml by different G. lucidum components. In general, the isolated pure compounds exhibited

cytotoxic activity at very low concentrations, whereas that of the extracts was at relatively high concentrations (Tables 2.1 and 2.2). However, the direct cytotoxicity should not be considered the major mechanism for G. lucidum's anticancer effect because the dosages applied in vitro are seldom achieved in vivo. Besides its tumoricidal activity, G. lucidum was also able to induce apoptosis, for example, in human breast cancer MCF-7 [250] and colonic carcinoma HT-29 [255]. The cellular signalling pathway by which G. lucidum regulates cancer cell death and proliferation has been widely reported [239,248-250,252,254-256].

(Table 2.1) A list of G. lucidum products or isolated compounds that has been tested for effectiveness of inhibiting cancer cell proliferation.

G. lucidum Product / extract / pure compound	Cell line	IC_{50} (μg/ml)	Method	Authors
Ergosta-7,22-diene-2β,3α,9α-triol	PLC/PRF/5	1.2	MTT	[247]
	KB	0.9	MTT	[247]
5α,8α-Epodioxyergosta-6,22-dien-3β-ol	PLC/PRF/5	11.0	MTT	[247]
	KB	9.8	MTT	[247]
Crude methanolic extract	L1210	15.0	MTT	[234]
	LLC	10.0	MTT	[234]
Commercial extract X	MDA-MB-123	NA	MTT	[248,257]
	PC-3	250.0	MTT	[249]
Sporoderm-broken spores alcoholic extract	HeLa	4460.0	MTT	[251]
Fruiting bodies ethanolic extract	MCF-7	NA	MTT	[250]
	MTC-11	129.3	MTT	[252]
	MTC-11	113.0	HTIA	[252]
Fruiting bodies water extract	MTC-11	509.0	MTT	[252]
	MTC-11	990.0	HTIA	[252]
Spores-ethanolic extract	MTC-11	274.7	MTT	[252]
	MTC-11	234.0	HTIA	[252]
Spores-water extract	MTC-11	365.0	MTT	[252]
	MTC-11	465.0	HTIA	[252]

Abbreviations are as follows: NA, not available; MTT: Tetrazolium method; HTIA: [^{3}H] Thymidine incorporation assay; IC_{50}: Growth inhibitory concentration at 50%.

(Table 2.2) A list of G. lucidum products or isolated compounds that has been tested for direct lethal effects on cancer cells.

G. lucidum Product / extract / pure compound	Cell line	LD_{50} (μg/ml)	Method	Authors
Triterpene enriched mycelial ethanolic extract	Huh-7	450	ACP	[254]
Sporoderm-broken spores alcoholic extract	HeLa	4700	TB	[251]
Culture broth of mycelia	Hep3B	NA	TB	[253]
Ganoderic acid G	Meth-A	6.8	SRB	[241]
Ganoderic acid γ	Meth-A	15.6	SRB	[241]
Ganoderic acid ε	Meth-A	12.2	SRB	[241]
Ganoderic acid θ	Meth-A	5.7	SRB	[241]
	LLC	15.2	SRB	[241]
Ganoderic acid C1	LLC	17	SRB	[241]
Ganoderiol F	LLC	6	SRB	[241]
	Meth-A	4.4	SRB	[241]
Ganodermanontriol	Meth-A	5.4	SRB	[241]
	LLC	9.6	SRB	[241]
Ganolucidic acid A	LLC	15.5	SRB	[241]
Lucieric acid α	LLC	17.8	SRB	[241]
Lucidumol A	LLC	2.3	SRB	[241]
	Meth-A	4.2	SRB	[241]
Lucidumol B	Meth-A	8.5	SRB	[241]
	LLC	16.6	SRB	[241]
Ganodermanondiol	LLC	12.5	SRB	[241]
	Meth-A	3.4	SRB	[241]
	Meth-A	9.2	SRB	[242]
	LLC	14	SRB	[242]
	T-47D	4.7	SRB	[242]
	S-180	11	SRB	[242]
Ganodermnonol	S-180	10	SRB	[242]
	T-47D	4.8	SRB	[242]
	Meth-A	2.8	SRB	[242]
Ganodermadiol	Meth-A	10.3	SRB	[242]
Lucialdehyde A	Meth-A	10.4	SRB	[242]
Lucialdehyde C	Meth-A	3.8	SRB	[242]
	LLC	10.7	SRB	[242]
	T-47D	4.7	SRB	[242]
	S-180	7.1	SRB	[242]
Lucialdehyde B	S-180	4	SRB	[242]
	T-47D	15	SRB	[242]
	LLC	14.3	SRB	[242]

Abbreviations are as follows: NA: Not available; ACP: Alginate-chitosan-PEG; LD50: Lethal Dose at 50%; SRB: Sulforhodamin B Method; TB: trypan blue exclusion assay.

2.2.4 Cell cycle and signaling regulations

Proliferation of cells relies on successive cell division cycle, which is tightly regulated to control a balance between cell survival and cell death; otherwise, cells grow out of control and result in "tumors". Several studies correlated the tumoricidal effects of G. lucidum with the regulation of cancer cell cycling and signalling [249-252,254,255,258-264]. Ethanolic G. lucidum extract and purified triterpenes induced apoptosis in several human cancer cell lines by increasing caspase-3 activity that resulted in a series of molecular events such as cytosolic release of cytochrome c and dysfunction of mitochondria [255,258,260,261]. The apoptosis of the human colon cancer cell HT-29 involved an increase of nitric oxide (NO) in a dose-dependent manner [255]. Pro-apoptotic protein Bax was up-regulated in human prostate cancer PC-3, breast cancer MCF-7 cells and lung cancer 95-D by G. lucidum extracts [249,250,258]. In addition, expression of protein p53 was also enhanced by ganodermic acid T in a time-dependent manner [258].

In PC-3 cells, expression of NF-κB related proteins (Bcl-2 and Bcl-xl) and G_2/M transition proteins (cyclin B, Cdc2) was also inhibited by G. lucidum extract, resulting in G_2/M phase cell arrest [249]. When incubated with a triterpene-enriched mycelial extract, hepatoma Huh-7 cells were arrested at G_2 phase due to the deficiency in M-phase promoting factors through the down-regulation of PKC activity and activation of c-Jun N-terminal kainase (JNK) and p38 MAPKs [254]. In several hematologic cell lines related to leukemia, G2/M phase cell arrest was revealed as the principle mechanism for apoptotic

events [259]. Similar G_2/M arrest has also been shown in bladder cancer MTC-11 cells exposed to G. lucidum extract; however, the underlying mechanism involved was not reported [252].

Interestingly, G_1 phase arrest happened in human breast cancer MCF-7 and MDA-MB-231 cell lines exposed to G. lucidum extract [248,250]. The possible mechanism may be mediated through a down-regulation of cyclin D1, which is controlled by the protein kinase B (PKB) and NF-κB pathways [248,251]. Also, sporoderm-broken spores reduced intracellular calcium $[Ca^{2+}]_i$ that also caused G_1 phase arrest in human cervix tumor HeLa cell line [251]. However, the induction of G_1 and G_2/M cell arrests may be specific to cancer types due to the diversity of G. lucidum's diversified chemical constituents. Table 2.3 summarizes the in vitro anticancer studies of G. lucidum in relation to cell signalling controls.

(Table 2.3) Cell signaling pathways and mechanisms of G. lucidum in control of cancer cell growth.

Cancer	Cell line	Sample	Cytotoxic / Apoptotic	Proliferation / Cell growth	Cell cycle arrest at phase G0/G1	S	G2/M	Mechanisms	Reference
Bladder	Low-grade tumour MTC-11	Water and alcohol extract of spores and fruiting bodies	Cytotoxic	Inhibited	↓	↓	↑	F/G-actin ratio was increased that suggested the contribution of actin remodeling in G2/M phase growth inhibition.	[252]
Breast	Human MCF-7	Ethanol extract of fruiting bodies	Both	Inhibited	↑	↓	↓	p21 was up-regulated that inhited cdk4, which led to dephosphorylation of RB and caused the inactivation of E2F, and thus prevent G1 - S transition of cell cycle; In addition, BAX was also up-regualted, which led to mitochondrial dysfunction and released Cyt c and that interacted with Apaf-1 to activate caspase-7, and thus induced apoptosis.	[250]
	Human MDA-MB-231	Boiled water extract of Reishimax G. lucidum	—	Inhibited	↑	—	—	Suggested that Protein kinase B activity was inhited that caused phosphorylation of IκB, which followed by suppression of NF-κB that down-regulated cyclin D1 and cdk4 kinase, and induced G0/G1 phase arrest.	[248]
Cervix uteri	Human HeLa	Broken spores	Cytotoxic	Inhibited	↑	↓	—	The level of intracellular calcium were strongly reduced, and thus it blocked the G1 - S transition of cell cycle and induced the G1 arrest.	[251]
Colon	Human HT-29	ethanolic extract of a commercial powder	Apoptotic	No effects	—	—	—	Apoptosis through increased caspase-3 activity.	[255]

↑: increase; ↓: decrease; -: not studied yet

Cancer	Cell line	Sample	Cytotoxic / Apoptotic	Proliferation / Cell growth	Cell cycle arrest at phase G0/G1	S	G2/M	Mechanisms	Reference
Hepatoma	Human Huh-7	Ethanol soluble fraction of hot water extract	—	Inhibited	—	—	↑	Supressed PKC activity leading to prolonged G2 cell cycle that resulted in delayed cell division caused by a lack of cyclin B, and activation of JNK/SAPK and p38 MAP kinases were also found.	[254]
			Apoptotic	Inhibited	—	↓	—	Activation of ERK and JNK MAPK resulting in decrease of Bcl-xL and activation of caspase-3.	[261]
Leukemia	several hematologic cells	Water extract of fruiting bodies	Both	Inhibited	—	↑	↑	Upregulation of $p21^{WAF1}$ and $p27^{KIP1}$ resutling in decrease of mitochondrial membrane potential and multinucleation.	[259]
Lung	Human 95-D	Purified Ganoderic acid T	Both	Inhibited	↑	—	—	Reduction of mitochondria membrane potential and release of cytochrome c increasing the G1 phase percentage, and expression of p53 and Bax and activity of caspase-3 increased.	[258]
Prostate	Human PC-3	Boiled water extract of Reishimax G. lucidum	Both	Inhibited	—	—	↑	Suggested that inhibition of NF-κB up-regulated the expression of GADD45, then down-regulated the expression of cyclin B and Cdc2, and which induced G2/M phase arrest; In additon, the inhibited NF-κB also slightly down-regualted the proapototic proteins Bcl-2 and Bcl-xl that supprose inhibit apoptosis, however, the another proapoptotic protein Bax was also markedly up-regulated to induce apoptosis with unknown resasons.	[249]

(Table 2.3 continued)

2.2.5 Anti-angiogenesis and anti-metastasis

A growth tumor mass requires continual nutrient supply via vascularization called angiogenesis. Invasive cancer cells then spread out to distant normal tissues through blood and lymphoid vessels – a process known as metastasis. To study the *ex vivo* anti-angiogenic effects of G. lucidum, the chick chorioallantoic membrane (CAM) assay is commonly employed. Polysaccharide-peptides extracted from G. lucidum reduced the formation of microvessels by preventing the proliferation of human umbilical cord vascular endothelial cell on a microfiber filter disc [235,265]. This was also demonstrated by an ethanolic G. lucidum fruiting body extract in a dose-dependent manner, and supported by the inhibition of inducible NO production [266]. The suppression of angiogenesis in PC-3 cells was mediated through the modulation of MAPK and Akt signalling pathways, and resulted in down-regulation of vascular endothelial growth factor (VEGF) and TGF-β1 [267]. Similarly, the inhibition of VEGF secretion by PG lung carcinoma cells was demonstrated by G. lucidum polysaccharides [263]. These polysaccharides also induced apoptosis in human umbilical cord vascular endothelial cell (HUVEC), whereas endothelial growth is essential for angiogenesis [263].

Cell adhesion, invasion and migration are the factors which contribute to the aggressiveness of cancer; hence, control of cell motility should efficiently avoid cancer metastasis. This has been successfully demonstrated in human cancer cells of the breast [268-270], prostate [268,269] and bladder [252]. In

highly invasive breast MDA-MB-231 and prostate PC-3 cells, G. lucidum down-regulated constitutively with active transcription factors active protein-1 AP-1 and nuclear factor NF-κB through the suppression of urokinase-type plasminogen activator and its receptor (uPAR) expressions [268,269]. The behaviour of MDA-MB-231 on cell adhesion, invasion and colony formation was also inhibited upon exposure to G. lucidum extract in vitro [270]. Recently, the F/G-actin ratio and the formation of stress fiber and focal adhesion complex in MTC-11 bladder cancer cells were shown to be increased by G. lucidum extracts, thus actin remodelling and redistribution of focal adhesion complex were associated with the inhibition of carcinogen-induced cell migration [252].

In addition to in vitro and *ex vivo* experiments for anti-angiogenesis and anti-metastasis, studies of G. lucidum have been extended to animals in vivo. A polysaccharide mixture containing isoflavone aglycons produced from cultured G. lucidum mycelia inhibited angiogenesis in vivo was tested using tumor-implanted chambers in mice colons [265]. Angiogenesis induced by Martigel was inhibited by triterpenoid fractions of G. lucidum, and a reduction of primary splenic tumor size, as well as suppression of secondary metastasis in intrasplenic implant mice with LLC cells was seen [271]. G. lucidum also inhibited metastasis of tumors in the lung and prolonged the life span of tumor-transplanted mice [272].

2.2.6 In vivo animal studies

The Yun's model, a medium-term model using benzo(a)pyrene to induce lung tumors in newborn mice, has been shown to reduce lung tumor incidence by G. lucidum [273,274]. Oral administration of sporoderm-broken germinating spores for seven consecutive days was able to inhibit the growth of hepatoma, sarcoma and reticulocyte sarcoma cells inoculated intraperitoneally into mice in a dose-dependent manner [245]. A water-soluble mycelial extract inhibited cell proliferation, aberrant crypts and aberrant crypt foci formations in an inducible colon cancer mouse model [275]. Consistent growth inhibition has also been shown by crude hot water mycelial extract on sarcoma in ICR mice [276] and fibrosarcoma in C3H mice [272]. Female C57BL/6J strain mice given intrasplenic implantation of LLC cells and administration of a triterpenoid fraction for 18 consecutive days had significantly reduced splenic tumor weight and number of tumor cell colonies that metastasized to the liver [271]; histologically, tumor growth and invasion of the white pulp of the spleen was prevented. Lymphocytes were also found to accumulate around the tumor cells in the spleen and metastatic cells of the liver [271]. It was reported that G. lucidum contributes to the reduction of tumor weight and suppression of growth with significant increase of cytokines TNF-α and IFN-γ in sarcoma-180-bearing mice supplemented with Ganopoly (an over-the-counter polysaccharide extract of G. lucidum) [277]. This suggests that the inhibition of tumor growth by G. lucidum in the animal involved the host's immunity.

2.2.7 Carcinostatic properties: Immuno-enhancement

G. lucidum has long been considered as an immunomodulatory agent that may favour the host-mediated anticancer immunity [232,238,278-282]. A commercial G. lucidum extract did not stimulate proliferation of lymphocytes, but it activated the expression of interleukin (IL)-10, IL-1β, IL-6, and TNF-α [283]. A proportion of β-D-glucan moiety isolated from G. lucidum was also rich in polyphenolic substances, which stimulated murine macrophage to produce IL-6 and NO synthesis significantly in vitro [284]. These polysaccharides were also found to be highly suppressive to tumor cell proliferation while enhancing the host's immune response [229,282]. Therefore, G. lucidum has been suggested to have an anticancer effect by exerting a series of immuno-enhancement properties, such as cytokine production and splenic natural killer (NK) cell cytotoxicity [236,272,285].

Furthermore, serum incorporated treatment with a polysaccharide peptide fraction from G. lucidum markedly inhibited the proliferation of human lung carcinoma (PG) cells, but the pure extracted fraction alone did not induce similar effects [235]. G. lucidum extracts were able to stimulate blood mononuclear cells to secrete cytokines against cancer cell proliferation and enhancing phagocytosis by ten-folds [272,286]. Furthermore, incubating human leukaemia HL-60 and U973 cells with G. lucidum-treated conditioned media, which contained pure monocytes-macrophages and lymphocytes, induced with a range of cytokines, such as IL-1β, IL-6, TNF-α, IFN-γ, caused

apoptosis in the cancer cells [278]. Nevertheless, evaluation of any anticancer agents requires trials on human beings in the final stage.

2.2.8 Antioxidants

A number of investigators found that various components of G. lucidum, in particular polysaccharides and triterpenoids, possess excellent antioxidant activities that prevent oxidative damage [240,282,287-294]. Extracts of G. lucidum polysaccharides possess scavenging activities on hydroxyl and superoxide anion radicals, which prevent lipid peroxidation efficiently [287,293,295]. Ethanol extract of G. lucidum also possesses free radical scavenging properties that inhibit the ferrous (Fe^{2+})- ascorbate-induced lipid peroxidation in mouse skin [291]. Ooi & Liu [282] reported that protein-bound polysaccharide and polysaccharide peptide were able to mimic superoxide dismutase (SOD) in cancer-bearing animal in vivo to remove hyperoxide radicals. These polysaccharides protected the immune cells from oxidative damage [282]. The protective effects of G. lucidum on DNA strand scission induced by metal-catalyzed Fenton reactions, ultraviolet irradiation and hydroxyl radical were shown in agarose gel electrophoresis in vitro [289,293]. In DNA in vitro, polysaccharides also protected against hydroxyl radical-mediated strand breakage [289,293] and albumin-induced oxidative DNA damage [296]. Hot water extracts of G. lucidum significantly protected Raji cell from H_2O_2-induced DNA damage [290]; boiling water extracts protected human lymphocyte DNA only at low (< 0.001% w/v) concentration but caused H_2O_2-mediated damage at relatively high concentration (> 0.001%

w/v) [294]. These contradictory results may be explained by the extractability of different components from G. lucidum, where boiling water was able to isolate relatively more organic compounds such as triterpenoids than hot water. Triterpenes isolated from G. lucidum protects erythrocyte membrane against pyrogallol-induced oxidation and liver mitochondria from lipid peroixdation [240]. The potential anticancerous ganoderic acids extracted from the mushroom have powerful free-radical scavenging antioxidants [288].

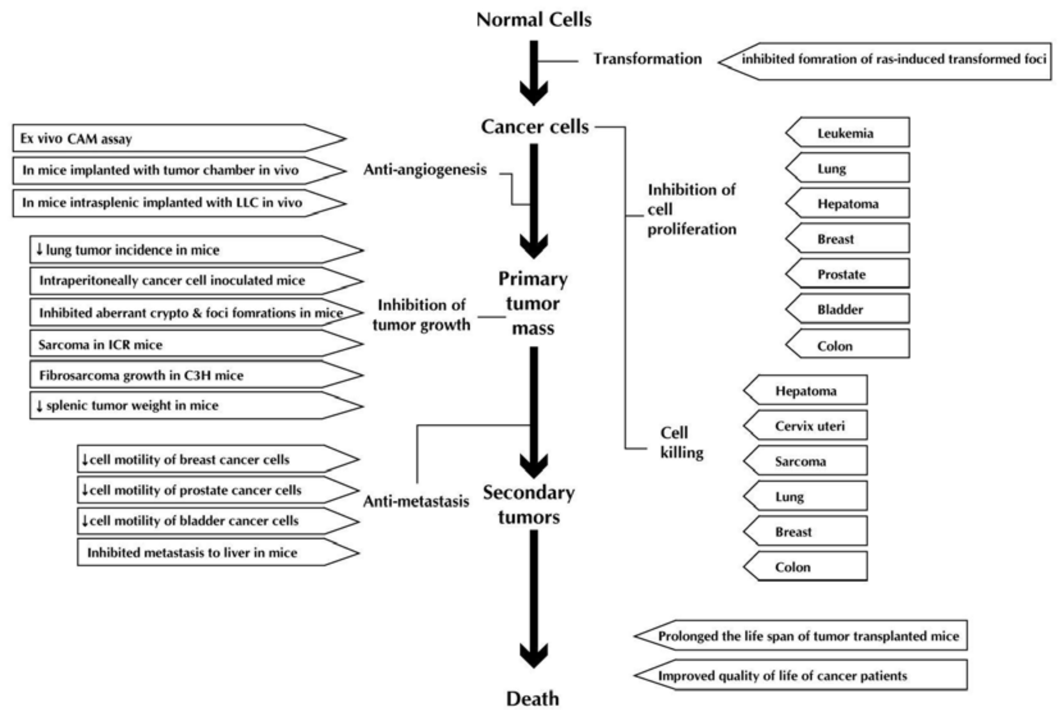
2.2.9 Clinical trials

There were two randomized control trials using over-the-counter Ganopoly as reported by Gao et al. [297,298]. A total of 34 advanced patients of different cancer origins were administered a 12-week course of G. lucidum capsule supplementations at a dosage of 1,800 mg were given; 80% of these patients were found to have a series of cellular immunological enhancements, including IL-2, IL-6 and IFN-γ secretions in plasma and NK cell activity [233,297]. In another study using the same G. lucidum capsule in 68 lung cancer patients, the quality of life, in terms of Karnofsky scores, was improved in about 65% of the patients [298]. Significant increase of total T cells and NK cells and a slight increase of CD4/CD8 ratios were found in the treatment group as compared with the placebo group [298]. Moreover, the mitogenic reactivity to phytohemagglutinin (PHA) and concannavalin A (Con A) was found to be enhanced in previous studies [233,297,298]. Similar enhancements of mitogenic reactivity, as well as NK cells in advanced cancer

patients, have also been demonstrated by Gao et al. [299] in a nonrandomized clinical study using Ganopoly as the single agent.

2.3 ANTICANCER G. LUCIDUM: SCIENTIFIC EVIDENCE

The anticancer potency of G. lucidum, irrespective of whether the fruiting body, mycelium or spore was used, largely relies on the diversity of its chemical constituents. The cytotoxic activity of G. lucidum, which contains a wide range of important bioactive polysaccharides and triterpenes, has been repeatedly demonstrated in most of the human and murine cancer cell lines tested. Lung, liver, breast, prostate and sarcoma were commonly studied; other studies include colon, bladder, cervix and leukaemia. Research on breast and prostate and G. lucidum has focused on the underlying mechanisms involved in signalling and cell cycle regulations. Interestingly, the cytotoxic effects of G. lucidum on prostate cancer brought about G_2/M phase cell arrest; whereas in breast cancer G_1 phase arrest was observed, thus they were controlled by different signalling pathways. The studies on hepatic cancer and sarcoma and G. lucidum have been extended to in vivo animal models that inhibited tumor growth and metastasis. The direct cytotoxic and anti-angiogenesis mechanisms of G. lucidum have been undoubtedly established by in vitro and animal studies. Immunological enhancements have been shown in human cancer patients, particularly in lung cancer patients whose quality of life was seen to be improved. In summary, along the spectrum of carcinogenesis, G. lucidum has exerted a variety of anticancer activities, as illustrated in Figure 2.1.



(Figure 2.1) A summary illustration to show all the works related to anticancer activity of G. lucidum, as corresponding to the stages of carcinogenesis.

CHAPTER 3

HYPOTHESIS, OBJECTIVE, RELEVANCE AND SIGNIFICANCE

3.1 HYPOTHESIS OF THE STUDY

G. lucidum contains chemopreventive activities when tested in pre-malignant human uroepithelial cells undergoing transformation under carcinogenic attack.

3.2 EVIDENCE TO PROPOSE HYPOTHESIS

Urothelium of the urinary bladder is regarded as the prime target for carcinogenic attack. Human uroepthelial cells (HUC) provides a relevant model for studying chemopreventive strategies. A special derivative of HUC, called HUC-PC, is non-tumorigenic but chemically transformable to become tumorigenic. We regard the HUC-PC cell line as pre-malignant cells which represent the residual cells existing in the bladder urothelium after surgical ablations [74]. Exposure to ABP (a relevant bladder carcinogen) to HUC-PC is akin to the presence of carcinogen in the intravesical compartment for occurrence of metachronous tumors. BCG is currently the most effective agent for TCC prophylaxis, but it is not free from side-effects and only reduces less than 40% of recurrence. Anticancer effects of G. lucidum have been demonstrated in tumors arising from different organs. Recently, growth inhibition of HUC-PC and inhibition of ABP-induced migration were

demonstrated by G. lucidum extracts [252]; however, the underlying mechanisms are still unclear. Questions have arisen as to what the extent of G. lucidum cytotoxicity on HUC-PC is when ABP is present? Could G. lucidum be a better option than BCG? Would G. lucidum be also cytotoxic to normal HUC-PC cells? Therefore, we hypothesized that G. lucidum contains chemopreventive activities when tested in the HUC-PC cells undergoing transformation under carcinogenic attack.

3.3 OBJECTIVES TO ADDRESS HYPOTHESIS

In order to address the hypothesis proposed in this study, the following objectives are set:

(1) To evaluate the effects of G. lucidum for cytokine IL-6 secretion from pre-malignant HUC cells.

(2) To investigate the effects of G. lucidum on BCG binding mechanism using relevant markers of pre-malignant HUC cells.

(3) To evaluate the extent of cytotoxicity of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP.

(4) To determine the roles of G. lucidum antioxidant / oxidant activities in pre-malignant HUC cells and determine its cytotoxicity.

(5) To determine the effects of ABP and G. lucidum on two selected biomarkers for bladder cancer in the HUC model system.

(6) To evaluate the apoptotic effects of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP.

(7) To investigate the cell signaling pathways involved in G. lucidum-induced bladder chemoprevention.

3.4 RELEVANCE OF THE STUDY

The novelty of this project lies in evaluating and elucidating the chemopreventive effects of G. lucidum, regarding its immunological, oxidative/antioxidant, apoptotic and molecular mechanisms in ABP-induced bladder carcinogenesis, using a pre-malignant human uroepithelial cell line, which has not yet been reported. Furthermore, the cell growth inhibition elicited by G. lucidum will be investigated whether apoptosis is involved OR due to other mechanisms such as necrosis and cell cycle arrest. Biomarkers relevant to bladder cancer, such as telomerase and cycooxygenase 2, are being used for the first time to study the chemopreventive effects of G. lucidum. Also, to the best of our knowledge, the antioxidant properties of G. lucidum would be used for the first time as a parameter to characterize its direct chemopreventive capabilities. Furthermore, for the first time, a comparison of cytokine secreting activity with BCG will be demonstrated with G. lucidum. The BCG-binding mechanism was also studied in response to G. lucidum. To our best knowledge, there has been no clinical trial or epidemiological studies were regarded to beneficial effects of G. lucidum on bladder cancer. These investigations will support planning of future clinical trials with G. lucidum as a supplement or as a stand-alone novel chemopreventive agent.

3.5 SIGNIFICANCE & VALUE OF THE STUDY

The significance of this project is that should our hypothesis hold true, G. lucidum would be a natural chemopreventive candidate for human bladder

cancer. The resultant outcome would lead to the rational planning of clinical trials in large cohorts of high risk individuals.

CHAPTER 4

EXPERIMENTAL DESIGN OF THE STUDY

4.1 IN VITRO TUMORIGENIC TRANSFORMATION HUC CULTURE MODEL

4.1.1 Introduction

To achieve the proposed objectives, pre-malignant HUC-PC cell line was chosen to establish the cell-culture model system. HUC-1 cell line was regarded as the normal counterpart of HUC-PC. In the 1980s, the first successful immortalization of HUC was achieved after infecting the cell line with a wild type Simian virus 40 (SV40) [300]. The clonal SV40-immortalized HUC cell line with balanced chromosomal translocation was named HUC-1 [75]. Subsequently, the HUC-1 cells was processed by cryopreservation at passage 10, thawed, expanded and cryopreserved at passage 21, for producing the HUC-PC clone [76]. Therefore, HUC-1 and HUC-PC are considered isogenic with each other. The HUC-PC cell line is sensitive to ABP, and has been characterized by using a multistep in vitro/in vivo transformation system [74,76]. Tumorigenic transformed HUC-PC cells induced by ABP are able to reproducibly induce tumors in nude mice [76]. Such tumorigenic transferable characteristics make the cell line most relevant to represent its pre-malignancy status for identifying chemopreventive strategies for bladder cancer. In this study, the HUC-PC cell line was regarded

as pre-malignant, and used for investigating the G. lucidum chemopreventive activities and mechanisms.

4.1.2 In vitro experimental design

The present study used an in vitro experimental design. According to the Taber's Cyclopedic Medical Dictionary, In vitro means 'in the glass' in Latin referring to the technique of performing a given experiment in a test tube, usually involving isolated tissue, organ or cell preparation. More precisely, in vitro includes any experiments being performed in a controlled environment outside the living organism. However, constants estimated in vitro might be very different from those inside the body (in vivo). Results of in vitro studies are normally not corresponding to those carried out inside the body [301].

In the field of pharmacology, in vitro experiments are essential for deducing the mechanisms of action before animal and human clinical trials can be conducted [302-304]. Under well-controlled systems, particularly in cells, cellular components and biomolecules can be focused to provide perceptually amplified reactions to subtle causes, which generally makes results more productive and discernible [304,305]. The desired animal model and human trials for TCC prophylaxis are currently unavailable. Therefore, setup of pre-malignant HUC models, with relevant ABP carcinogens, provides an invaluable system for filling the gaps of understanding the putative chemopreventive mechanisms of G. lucidum before proposing it as a prophylactic agent for testing on human beings.

4.1.3 Culture of HUC-PC and HUC-1 cell lines

The HUC-1 cell line was purchased from the American Type Culture Collection (ATCC, U.S.A.). The HUC-PC cell line (derived from HUC-1) originated from the Department of Human Oncology, University of Wisconsin Medical School, gifted by Dr. Rao from the University of California, Los Angeles. Both cell lines were cultured in F12 Ham enriched Dulbecco's Modified Eagle's Medium (F12/DMEM purchased from Sigma, St. Louis, MO) with 1% penicillin (10,000 μg/ml) and streptomycin (10,000 mg/ml) and 10% Fetal Bovine Serum (antibiotics and FBS purchased from GIBCO BRL Island, New York, U.S.A.), and this was refered to as the complete medium (Appendix I-A). All cell cultures were maintained at 37°C in a water-saturated atmosphere containing 5% carbon dioxide (CO_2).

4.1.4 Testing substances and chemicals

A proprietary extract composed of G. lucidum fruiting bodies and cracked spores in capsules, branded ReishiMaxGLP^{TM}, was purchased from Pharmanex Inc. (Hong Kong, China). ABP and Dimethylsulphoxide (DMSO) were purchased from Sigma Chemical, Co. (St. Louis, MO). BCG (IMMUCYST®, Aventis, Toronto, Ontario, Canada) was provided by Dr. Anthony Ng from the Prince of Wales Hospital, Hong Kong. HPLC grade absolute ethanol was purchased from RDH (Seelze, Germany). MilliQ ultrapure water (18.2 MΩ) was prepared using MilliQ Ultrapure Water System (Millipore Co. Bedford, MA).

4.1.5 Re-extraction from commercial G. lucidum

The active ingredients of the ReishiMaxGLP^{TM} G. lucidum was standardized at 13.5% polysaccharides (β-1,3-glucans) and 6% triterpenes (ganoderic acids and other), which is the highest level of extractable activities, whereas the remaining 80% was composed of nucleosides, fatty acids and amino acids, according to the manufacturer's technical bulletin. Many G. lucidum products contain complex mixtures of triterpenes and polysaccharides as their key active ingredients. We accentuated the importance of studying re-extracted fractions (water and ethanol) of rich active ingredients than selected pure compounds for chemopreventive activities. The powdered extract was sourced from capsules, and was re-extracted as published with minor modification [252], with 95% ethanol by sonication for 30 minutes at room temperature to generate two separate layers, which were centrifuged at 2000 rpm for five minutes at 4°C. The supernatant and the suspension were separately followed by successive sonication for 30 minutes at room temperature, with absolute ethanol and MilliQ water, respectively. The ethanol extract was then filtered through 0.45 µm polypropylene filters (Sartorius, Goettingen, Germany). The filtrate was dried under reduced pressure using DNA110 Speed Vac® with refrigerated Vapor TRAP RVT100 (Savant, N.Y., U.S.A.) to yield a dark brown oily residue and finally a water-insoluble brown powder (final yield of 2.4%). The water extract was also dried under reduced pressure using Savant speed Vac (U.S.A.) to yield a water-soluble black powder (final yield at 16%). We named the ethanol extract and water extract as "GLe' and 'GLw',

respectively. The final yield was calculated by the percentage of total dry powder of re-extracted from the total capsule extract powder used. The protein, carbohydrate and lipids contents in the GLe and GLw fractions have not been characterized yet.

4.1.6 Preparation of assay media for cultures

Test substances (*detailed in 4.1.4 & 4.1.5*) were dissolved in appropriate solvents: ABP in DMSO, GLe in absolute ethanol, GLw in MilliQ water, and BCG in diluent (according to package instructions). Assay media at appropriate concentrations were made immediately before use with complete media unless otherwise stated. The final concentration of solvents in assay media must be less than 0.01% for ethanol, 0.1% MilliQ, and 0.1% DMSO.

4.1.7 Statistical analysis

All experiments were performed in triplicate for reproducibility. Mean and standard error (Mean±SEM) were used to summarize the results of descriptive statistics. GraphPad Prism (GraphPad software, version 3.0 for windows, U.S.A.) was used to perform Student's t-test for statistical comparisons. Pearson's correlation test was carried out to measure the relationship between

measured parameters. Statistical significance was sought at two-tailed P-value ≤ 0.05.

4.2 STUDY PLAN AND METHODOLOGY OUTLINE

4.2.1 Objective 1. Evaluate the effects of G. lucidum on IL-6 secretion from pre-malignant HUC cells.

IL-6 was selected for measurement with the justification detailed in the introduction of Chapter 5. IL-6 levels secreted in the culture media of HUC-PC cells treated with BCG or G. lucidum was quantified by using an Enzyme-linked ImmunoSorbent Assay (ELISA) kit. IL-6 has also been suggested to be responsible for the cytotoxic effects of BCG on certain TCC cell lines. Cytotoxicities of G. lucidum and BCG on HUC-PC cells were also assessed by measuring the lactate dehydrogenase (LDH) secreted from the cells. Detailed methodologies and results are presented and discussed in Chapter 5.

4.2.2 Objective 2. Investigate the effects of G. lucidum on BCG binding mechanisms, using relevant markers of pre-malignant HUC cells.

Urothelium of the bladder is protected by a layer of negatively charged GAGs from direct contact of BCG. Fibronectin forms the bridge between HUC and BCG for internalization. After treatment of G. lucidum, culture media was collected for measuring the free-fibronectin by using Enzyme immunoassay (EIA) kit. Cell surface-bound GAGs were extracted for measurement by using the Dimethylmethylene blue (DMMB) method [306]. Detailed methodologies and results are presented and discussed in Chapter 5.

4.2.3 Objective 3. Evaluate the extent of cytotoxicity of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP.

Cytotoxicity of GLe/GLw with and without ABP was compared using pre-malignant and normal HUC cell lines. The concentrations of test substances were determined and used for subsequent experiments. Cell viability and proliferation after incubation with ABP and/or G. lucidum was further assessed by the trypan blue method. Detailed methodologies and results are presented and discussed in Chapters 5 and 6.

4.2.4 Objective 4. Determine the roles of G. lucidum antioxidant / oxidant activities on cytotoxicity in pre-malignant HUC cells and determine its cytotoxicity.

Oxidative stress and DNA damage are risk factors for bladder carcinogenesis, whereas antioxidants are suggested to be useful to reduce the chance of cancer occurrence/recurrence. Ferric reducing/antioxidant power (FRAP) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) free radical scavenging assay are selected biomarkers for measuring antioxidant capacities [307,308]. H_2O_2 concentration is regarded as an oxidative marker. GLe and GLw were compared with the original capsule extract for antioxidant and oxidative activities. These three biomarkers were also used to monitor the changes of antioxidant and oxidant profile during the 48-hour treatment of HUC-PC cells with G. lucidum in the presence or absence of ABP. Additionally, oxidative DNA damage was also assessed at 48 hours, using the commercial 8-hydroxy-

2'-deoxyguanosine (8-OHdG) ELISA kit. Detailed methodologies and results are presented and discussed in Chapter 7.

4.2.5 Objective 5. Determine the effects of ABP and G. lucidum on two selected biomarkers for bladder cancer in the HUC model system.

Telomerase and COX-2 are proposed as relevant biomarkers for TCC carcinogenesis as reviewed in Chapter 1, Section 4. Telomerase activity of HUC-PC after treatment with ABP and/or G. lucidum was assayed with a Real-Time Quantitative – Telomeric Repeat Amplification Protocol (RTQ-TRAP) [309]. The cells were also extracted to measure the concentration of COX-2 using an enzyme immunoassay (EIA) kit method. Detailed methodologies and results are presented and discussed in Chapter 8.

4.2.6 Objective 6. Evaluate the apoptotic effects of G. lucidum on pre-malignant and normal HUC cells when challenged with carcinogenic ABP.

Apoptosis is proposed as the main cause of HUC cell growth inhibition induced by G.lucidum. Morphological changes of HUC cells were examined using an Olympus CK2 inverted microscope (Microscope-U.K., U.K.). The Annexin V-FITC/7-AAD apoptotic assay kit (Immunotech, France) was assessed by flow cytometry. During the 48-hour treatment with G. lucidum and/or ABP, the extent and progression of apoptosis was assessed in the HUC-PC and HUC-1 cell lines by measuring the exteriorization of phosphatidylserine (PS) and binding of DNA-specific viability 7-

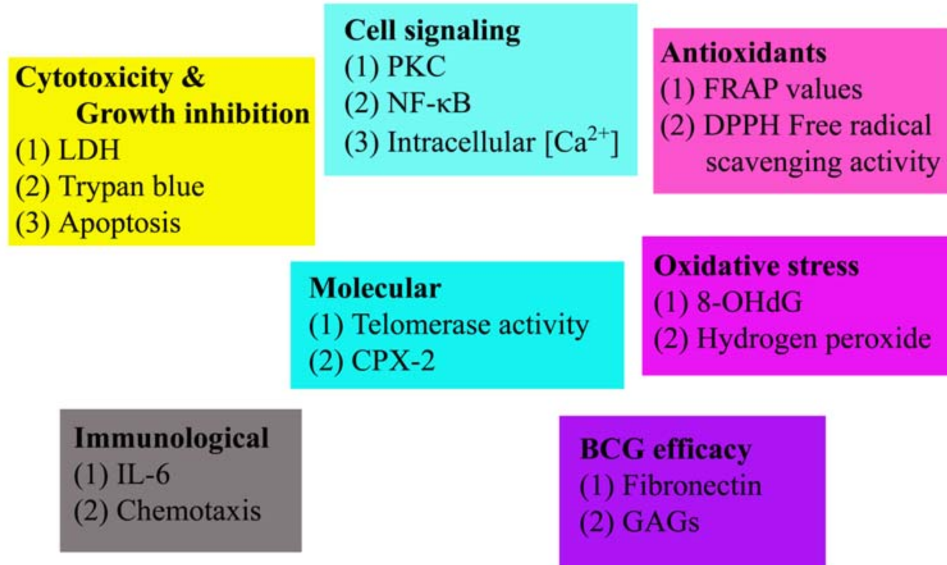
aminoactinomycin (7-AAD) dye. Peripheral polymorphonuclear neutrophils were used in the chemotaxis cell migration assay for assessing the chemotactic activities of culture media after incubation. Detailed methodologies and results are presented and discussed in Chapter 9.

4.2.7 Objective 7. Investigate the cell signaling pathways involved in G. lucidum-induced bladder chemoprevention.

Calcium (Ca^{2+}) is a universal second messenger and the changes of its intracellular concentration play an important role in cellular metabolism. Furthermore, activities of NF-κB in nucleus and PKC in cytoplasm have crucial roles in regulating cell proliferation and apoptosis and other cellular responses. Therefore, these parameters were chosen for studying the signaling pathways involved in the chemopreventive activities of G. lucidum. Nuclear proteins of the cells were extracted for determining NF-κB transcriptional factor activities by using Electrophoretic Mobility Shift Assay (EMSA)-ELISA method [310]. Cytosolic PKC was extracted and assayed using ELISA method [311]. The intracellular Ca^{2+} level of the cells was assessed using specific Fluo-4 dye [312]. Detailed methodologies and results are presented and discussed in Chapter 10.

4.2.8 Summary of measuring the parameters

Cells in the pre-malignant stage are particularly important for studying chemoprevention. Nothing is better than eliminating the adversed cells before they turn malignant, especially true for bladder TCC where the rate of recurrences remain exceptionally high, even after complete transurethral resection. The non-tumorigenic but ABP-transformable characteristic of the HUC-PC cell line provides an excellent model for studying the chemoprevention of bladder cancer since the majority of bladder cancer is diagnosed as TCC at presentation, and residual cells after resection are regarded as the key stimulus factor for recurrence. The superficial mucosal urothelial lining is targeted for attack by carcinogens, including ABP. Biomarkers are essential for testing the proposed hypothesis in the present study. Selected parameters, cover varying aspects, including mode of cell death and cytotoxicity, immunological, antioxidant, oxidative stress, cell signaling and bladder cancer markers (Figure 4.1).



(Figure 4.1) Seven groups of testing parameters used for investigating the chemopreventive effects of G. lucidum on human uroepithelial cell carcinoma.

CHAPTER 5

G. LUCIDUM INDUCES IL-6 PRODUCTION AND FACILITATES BCG BINDING TO HUC-PC CELLS

5.1 INTRODUCTION

The recurrence rate of TCC remains exceptionally high even with the effective transurethral resection (TUR) technique [146]. Referring to the “Field cancerization hypothesis” and “seeding theory”, residued cells at treated and adjacent sites are highly susceptible for mutagenic attacks and can potentially develop into tumors, and hence powerful chemopreventive agents are demanded for prophylaxis [10].

BCG, is the most effective prophylactic agent available and, when introduced intravesically, it triggers a local inflammatory response inside the bladder [156,157]. In response to BCG, host leukocytes infiltrate the urothelial wall and are responsible for most of the cytokine secretion in urine [180,181]. Evidence has also indicated that in situ lymphocytes are able to eradicate BCG-internalizing tumor cells through specific cell lysis against mycobacterial antigens [313]. However, only certain cytokines, including IL-2, IL-6 and TNF-α, are detectable in a patient’s urine within the first 24 hours upon BCG instillation [182]. IL-6 and TNF-α are pro-inflammatory cytokines that can be secreted from human bladder cancer cell lines [314-316]. For example, T24 carcinoma cells produced IL-6 and TNF-α but not IL-1β and IL-2, as a response to BCG [314]. Interestingly, well-differentiated bladder tumor

cells that are incapable of internalizing BCG were also unable to up-regulate IL-6 expression [317]. In contrast, normal urothelial cells and poorly differentiated TCC cells were able to internalize BCG and produce IL-6 [313,317]. Therefore, IL-6 cytokine was considered as an indicative marker for BCG internalization [161].

Given that binding of BCG to the urothelial surface is a pre-requisite for successful internalization [161], the urothelium and mycobacterium are linked through fibronectin (FN) opsonization [163,318,319]. Formation of FN bridges might facilitate the process of BCG internalization [161,163,318]. Such linkage induces the expression of IL-6 gene through NF-κB and AP-1 signal transducers in bladder tumor cells [320]. However, excess free FN was reported to be competitive with each other for the limited binding sites on urothelium and BCG surface, thus impairing the internalization of BCG and its subsequent responses [169]. On the other hand, the luminal wall of the bladder is protected by the mucosal lining that is covered with negatively charged GAGs [161]. Bacteria and toxins are kept away from the anionic barrier of the urothelial mucosa. During the process of BCG internalization, direct adhesion is not required, and a close-docking distance (70-100Å) is set by the repellent force between BCG and urothelium [162,321]. Thus, free FN concentration and cell surface-bound GAGs are relevant biomarkers for BCG binding efficiency.

In this chapter, objectives 1 and 2 of the study are addressed. With the use of HUC-PC cell line, IL-6 cytokine was chosen to compare effectiveness of G. lucidum with that of BCG for immunologic activity. IL-6 has been suggested to be responsible for the cytotoxicity of BCG on several TCC cell lines [168, 323]. Whether BCG and G. lucidum would also be cytotoxic to HUC-PC if they both are capable of inducing IL-6 secretion was determined using the LDH cytotoxicity assay. In addition, the effects of G. lucidum on extracellular FN and cell surface GAGs were investigated. These findings will aid in elucidating whether G. lucidum is a possible candidate to supplement or replace BCG therapy.

5.2 MATERIALS AND METHODS

5.2.1 Cell cultures for assays

Logarithmically growing HUC-PC cells were harvested and seeded in 96-well flat-bottle tissue culture plate (Greiner bio-one, Germany) at a concentration of 5 x 10^4 cells per microtitre well for cytotoxicity, FN and GAGs measurement. Harvested cells were also seeded in 100-mm tissue culture dishes (Greiner bio-one, Germany) for IL-6 and NF-κB assays. Culture conditions are detailed in *Chapter 4.1.3*.

5.2.1 LDH cytotoxicity assay

Direct cytotoxicity of GLe, GLw and BCG was assayed by measuring LDH released from cells with LDH Cytotoxicity Detection kit (TaKaRa Bio Inc., Shiga, Japan). Optimization of the assay is presented in *Appendix II-A*. The manufacturer's instructions were followed. Briefly, the HUC-PC cells were incubated with assay media containing GLe / GLw or BCG for 24 hours in microtitre plate wells (Thermo Labsystems, Franklin, MA). Solvents including DMSO, absolute ethanol and MilliQ water were each tested on cells at 0.01%, 0.1% and 1% concentrations. No significant cytotoxicity was found except at 1% absolute ethanol, therefore, concentrations of solvents higher than 0.1% were not acceptable for any of the experiments. The release of LDH from cells was measured at 490 nm with reference wavelength at 690 nm, using TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). Untreated cells were used as low controls to measure the spontaneous LDH release, and Triton X-100 treated cells were

used as high controls to measure the maximum releasable LDH activity. No interference was observed from any test substances used in the assay. Cytotoxicity was calculated as a percentage of LDH release with the following formula:

$$\text{Cytotoxicity (\%)} = \frac{(A_{490}[\text{Experimental}] - A_{490}[\text{Low control}])}{(A_{490}[\text{High control}] - A_{490}[\text{Low control}])} \text{ X } 100$$

5.2.1 ELISA for IL-6 cytokine

HUC-PC cells were incubated in various assay media for 24 hours, and cultured supernatants were collected to measure the IL-6 secretion with the Endogen® Human IL-6 ELISA kit (Pierce Biotechnology Inc, Rockford, U.S.A.). The manufacturer's instructions were followed. Briefly, culture medium was used to prepare the standard curve *(Appendix III-A)* by serial dilutions (ranging from 0 pg/ml to 400 pg/ml). Absorbance of the reaction microplate wells was measured at 450 nm on microplate reader (TECAN, Austria). Duplicated samples of each study group were run in triplicate.

5.2.5 EIA for fibronectin quantitation

Cell culture media were collected from cell-seeded microtitre plate after four hours of treatment with GLe/GLw. The TaKaRa Fibronectin EIA kit (TAKARA Bio Inc., Japan) was used for assay. The kit instructions were strictly followed. A 100 µl of sample/standard was added into an ELISA well coated with human anti-fibronectin and incubated for one hour at 37°C. The microtitre wells were washed four times and then 100 µl of substrate solution was added and incubated for 15 minutes at room temperature. 1N Sulphuric

acid (H_2SO_4) was added to stop the reaction. Finally, absorbance was read against diluent blank at 450nm, using TECAN SPECTRAFluor Plus microplate reader (TECAN, Austria). FN concentration of each sample was read from the standard curve (*Appendix III-B*). The samples were run in duplicate with three separate trials. The standard curve is shown in *Appendix IIIB*.

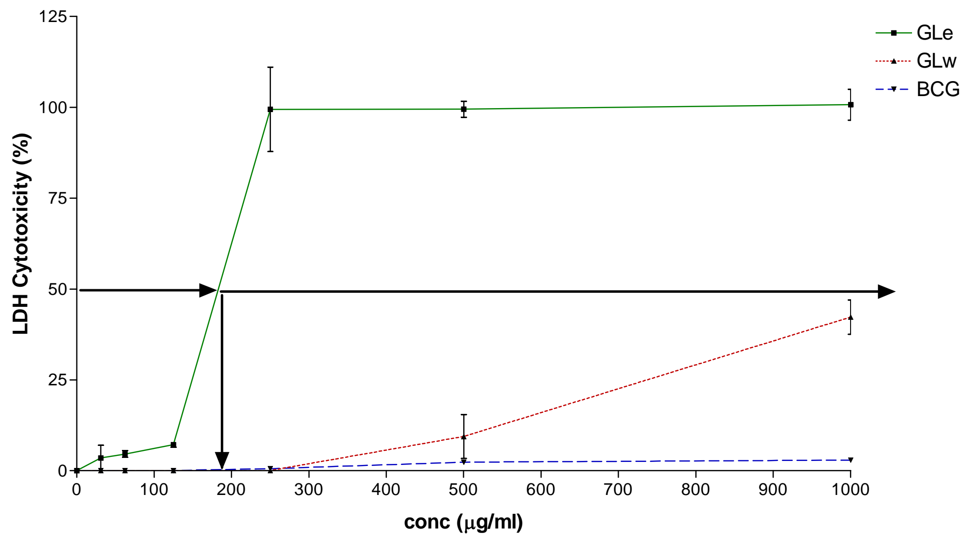
5.2.6 Dimethylmethylene blue method for GAGs quantitation

After four hours of incubation, same as for FN assay, membrane-bound GAGs from HUC-PC cells were extracted by a 0.1M sodium acetate buffer at pH 5.8 in a microtitre plate overnight, in accordance with Farndale et al. [322] with minor modifications. The supernatant was collected and digested overnight with 20μl of papain (Merck, U.K.) at 65°C. The isolated GAGs was assayed by the Dimethylmethylene blue (DMMB) method as published [306]. A 50 μl of sample/standard was added into each well of a new microtitre plate, which was followed by an addition of 200 μl of working DMMB (Aldrich, U.S.A.) reagent. Absorbance of the microtitre wells were read immediately against milliQ blank at 620nm. GAGs solution (mixture of hyaluronate, chondroitin, sulphate, Keratan sulphate and heparan sulphate) at 0, 4, 8, 16, and 32 μg/ml concentrations was used as standards. The standard curve is shown in *Appendix III-C*.

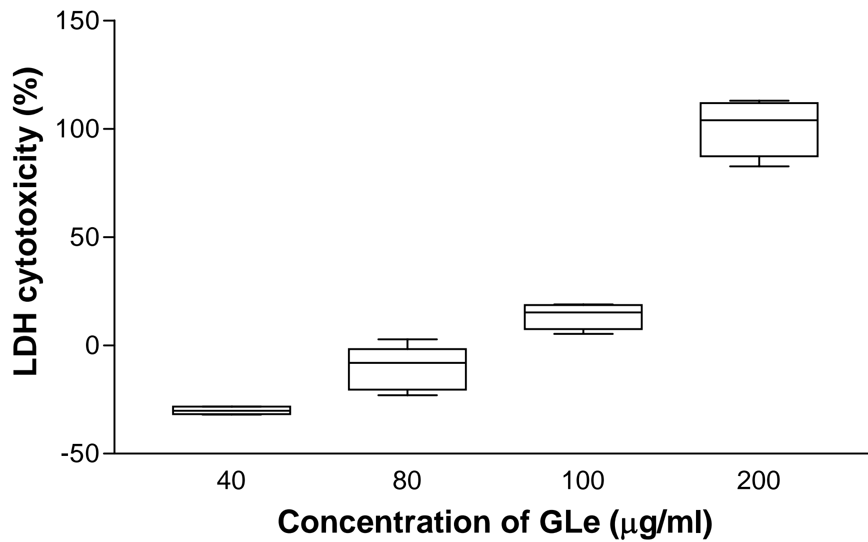
5.3 RESULTS

5.3.1 G. lucidum is cytotoxic to HUC-PC cells

GLe was shown to be cytotoxic to the HUC-PC cell line. By serial dilution, 100%±12% (Mean±SEM) of the cells were killed by 250 µg/ml of GLe, and the cytotoxic effects reached a plateau of 100% at concentrations of 250-1000 µg/ml (Figure 5.1). LD_{50} for GLe is between 180-190 µg/ml for GLe and is higher than 1000 µg/ml for GLw. The LDH cytotoxicity assay was repeated using GLe concentrations at 40, 80, 100 and 200 µg/ml, for confirmation. Results indicated that 100%±5% and 13.8%±2% (Mean±SEM) of cells were killed, by 200 µg/ml and 100 µg/ml of GLe respectively, but no cytotoxicity was found at 80 µg/ml or lower GLe concentration (Figure 5.2). On the other hand, 42%±5% and 9%±6% (Mean±SEM) cells were killed by 1000 µg/ml and 500 µg/ml of GLw, respectively, but no cytotoxicity was found at 250 µg/ml or lower GLw concentration (Figure 5.1). Furthermore, no significant cytotoxicity was shown by BCG up to 1000 µg/ml concentration (Figure 5.1).



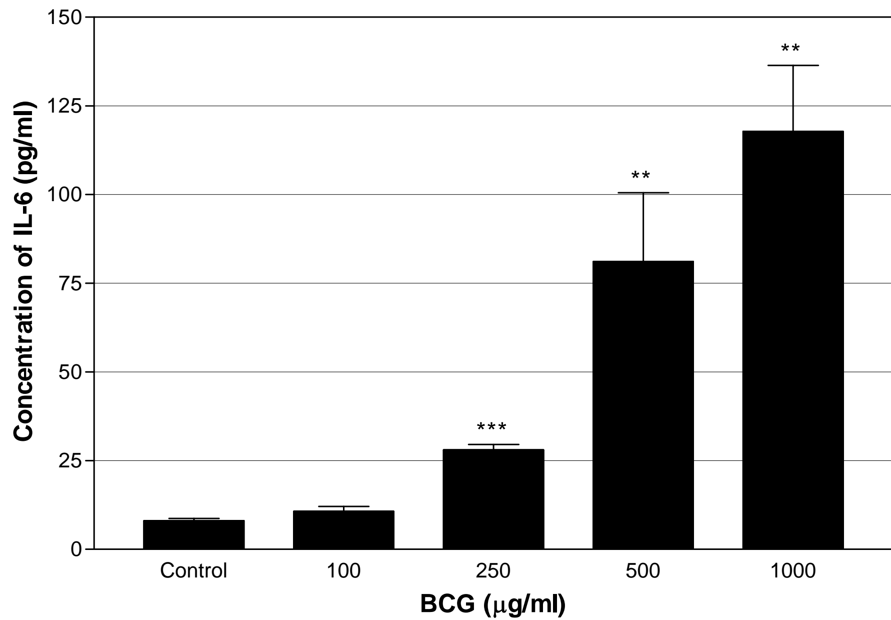
(Figure 5.1) Cytotoxicties of BCG and G. lucidum on HUC-PC cells measured by LDH cytotoxicity assay. Serial dilution of GLe, GLw and BCG (each at starting concentration of 1000 µg/ml) were incubated with the HUC-PC cells for 24 hours. LD_{50} (→) was between 180-190 µg/ml for GLe and >1000 µg/ml for GLw (n=6, error bar: SEM).



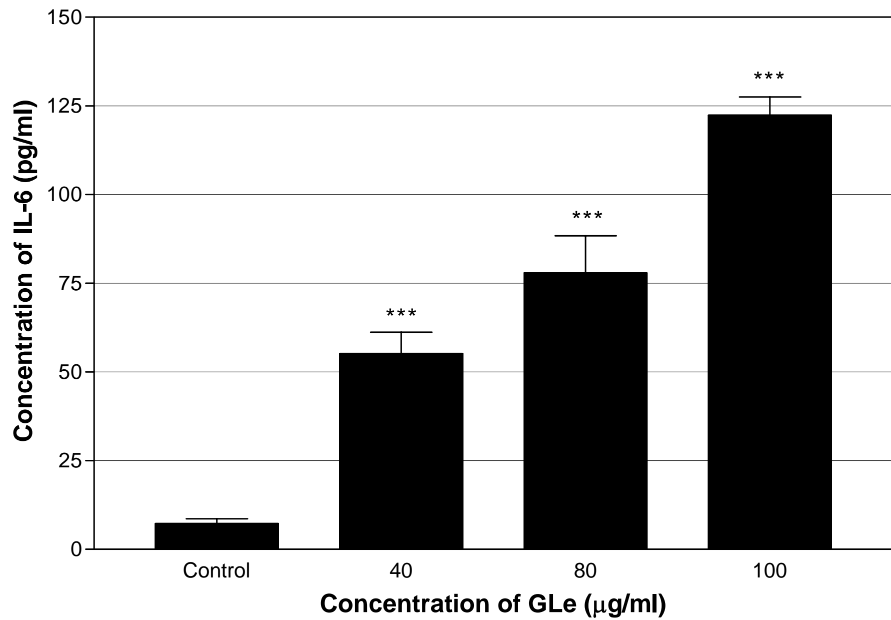
(Figure 5.2) Dose-dependent cytotoxic effects of GLe on HUC-PC cells measured by LDH cytotoxicity assay. GLe at concentrations 40, 80, 100 and 200 μg/ml were incubated with the HUC-PC cells for 24 hours (n=9, error bar: SEM). Negative cytotoxicities were read at 40 and 80 μg/ml of GLe. Smaller absorbances of the sample than that of the control cells may be due to suboptimal condition of the cell culture that released spontaneous LDH.

5.3.2 G. lucidum and BCG are active to induce IL-6 secretion from HUC-PC cells

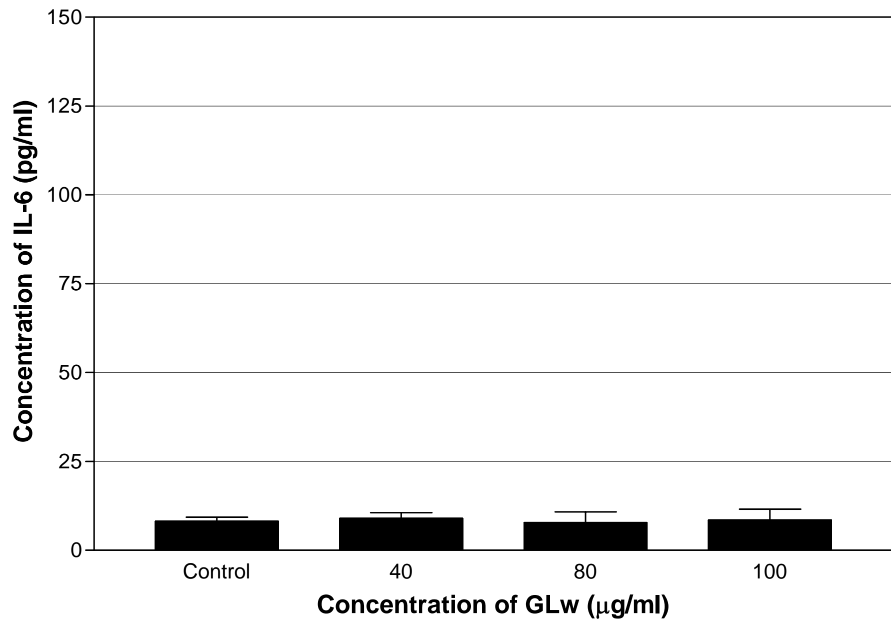
Results indicated that both BCG (Figure 5.3) and GLe (Figure 5.4) were able to induce secretion of IL-6 from HUC-PC cells in a clear dose-dependent manner. But no significant IL-6 production was induced by GLw (Figure 5.5).



(Figure 5.3) Dose-dependent IL-6 secretion induced by BCG. Culture media were harvested and measured at 24 hours after incubating with BCG at concentrations 100, 250, 500 and 1000 μg/ml. Cells growing in complete medium were used as control for comparison (n=3, error bar: SEM, *** P<0.001; ** P<0.01).



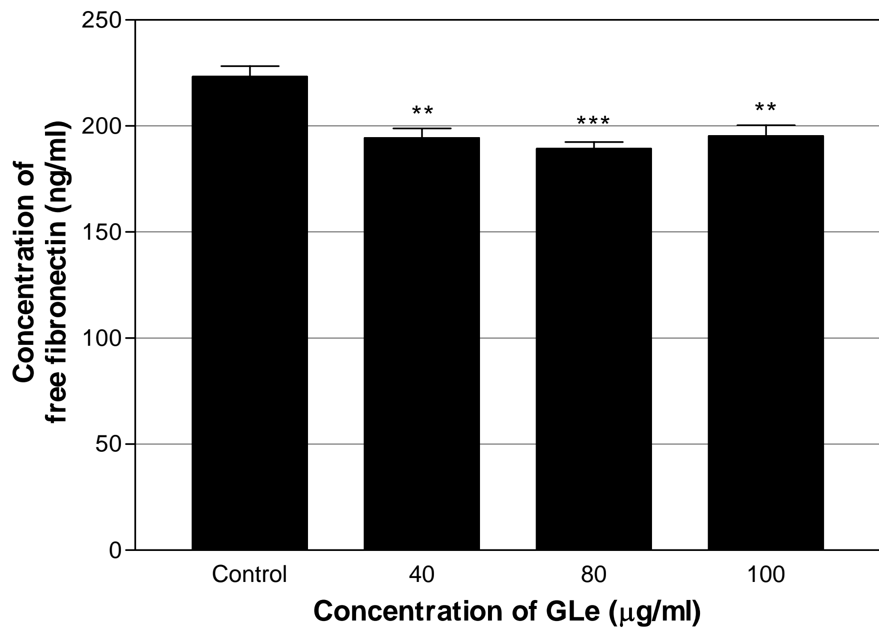
(Figure 5.4) Dose-dependent IL-6 secretion induced by GLe. Culture media were harvested and measured at 24 hours after incubating with GLe at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.01% ethanol were used as control for comparison (n=3, error bar: SEM, *** P<0.001).



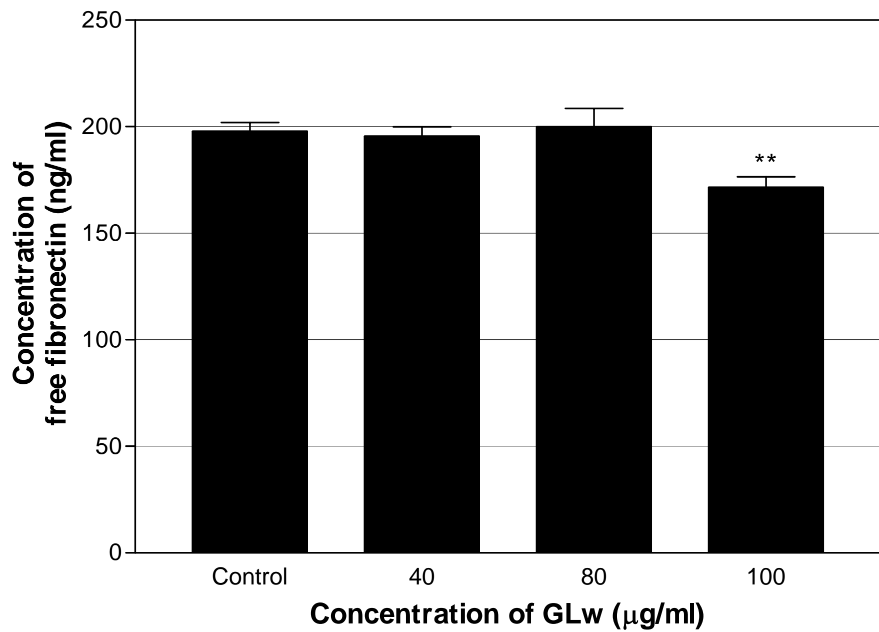
(Figure 5.5) No IL-6 secretion was induced by GLw. Culture media were harvested and measured at 24 hours after incubating with GLw at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.1% MilliQ water were used as control for comparison (n=3, error bar: SEM, statistically insignificant).

5.3.3 G. lucidum affects extracellular Fibronectin and cell-surface GAGs of HUC-PC cells

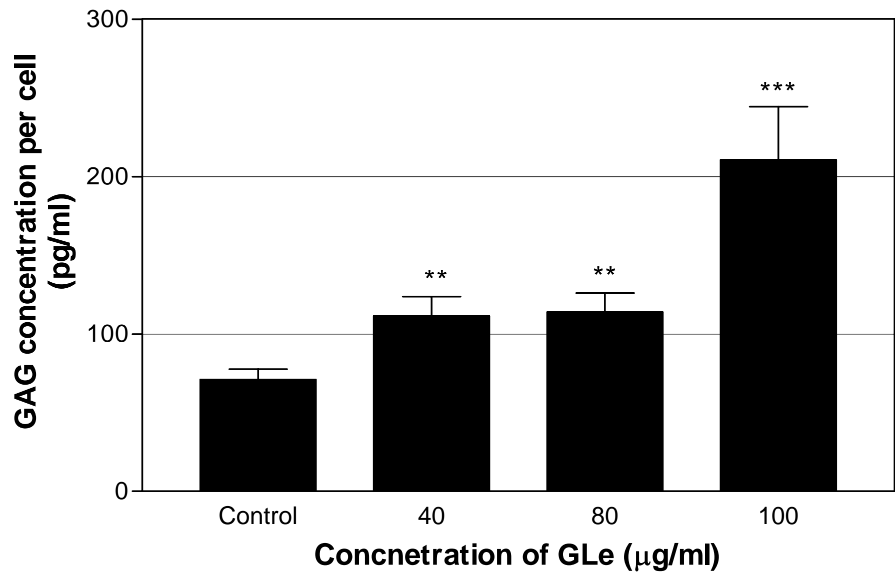
Free FN in the cultured media was significantly reduced by GLe at concentrations of 40-100 μg/ml (Figure 5.6). Significant reduction of the FN was only induced by GLw at 100μg/ml, but no effects were demonstrated at lower concentrations (Figure 5.7). The reduction of FN induced by G. lucidum (Figure 5.6 and 5.7) ranged from 12.5-15.2%, and may not be the maximum effects of G. lucidum, as measured at 4 hours after incubation to avoid any cytotoxic effects. GLe significantly ($P<0.01$) increased the cell-membrane bound GAGs levels on HUC-PC cells. The effects of GLe was statistically significant ($P<0.01$) (Figure 5.8), but the increase in GAGs by GLw was statistically insignificant ($P>0.05$), as shown in Figure 5.9. The GAG values from the controls in experiments (Figure 5.8 and 5.9) were different because of two reasons: Firstly, these two control values come from two separate sets of experiments. Secondly, these two controls contain different vehicle solvent, as 0.01% ethanol for GLe (Figure 5.8) and 0.1% MilliQ water for GLw (Figure 5.9). Thus, the values of the two controls cannot be compared between each other.



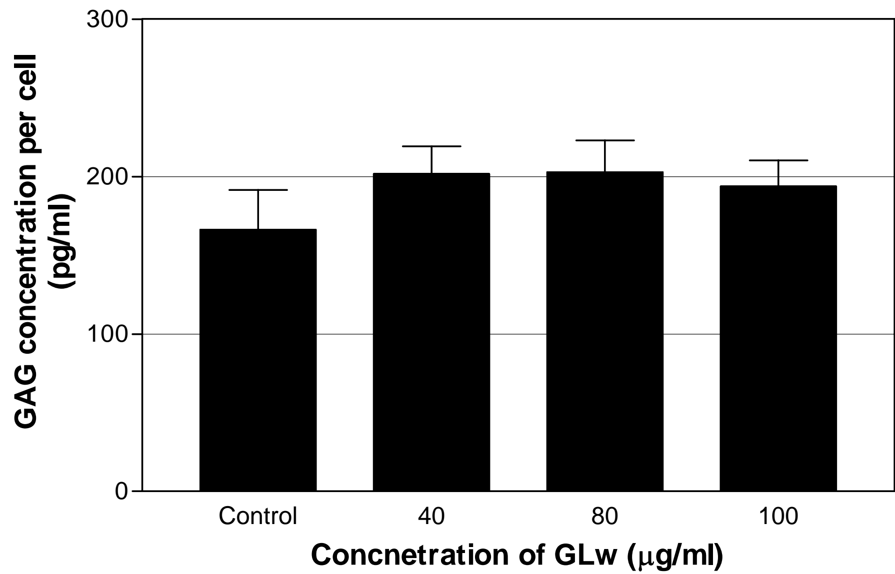
(Figure 5.6) Reduction of extracellular FN induced by GLe. Culture media were harvested and measured at 4 hours after incubating with GLe at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.01% ethanol were used as control for comparison (n=3, error bar: SEM, *** P<0.001; ** P<0.01).



(Figure 5.7) Reduction of extracellular FN induced by GLw. Culture media were harvested and measured at 4 hours after incubating with GLw at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.1% MilliQ water were used as control for comparison (n=3, error bar: SEM, ** P<0.01).



(Figure 5.8) Dose-dependent increase of GAGs on the HUC-PC surface induced by GLe. Cells were harvested and treated for measurement at 4 hours after incubation with GLe at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.01% ethanol were used as control for comparison (n=3, error bar: SEM, *** $P<0.001$; ** $P<0.01$).



(Figure 5.9) Increase of GAGs on the HUC-PC surface induced by GLw. Cells were harvested and treated for measurement at 4 hours after incubation with GLw at concentrations 40, 80 and 100 μg/ml. Cells growing in complete medium containing 0.1% MilliQ water were used as control for comparison (n=3, error bar: SEM, statistically insignificant).

5.4 DISCUSSION

Although some cytotxic effects of BCG have been reported, especially for the high grade tumors [323], BCG has shown to be non-cytotoxic to HUC-PC, in the present study. On the other hand, GLe is at least five-fold more cytotoxic to HUC-PC cells than GLw, which is consistent with the cytotoxic effects demonstrated by Lu et al. [252]. BCG is a well-known immunotherapeutic agent to prevent TCC recurrences. Though its mechanisms are not fully understood, it was reported that BCG had to be active in numerous bladder cancer cell lines to trigger cytokine secretions [161,314,324]. HUC-PC cells are regarded as pre-malignant and have never been used for testing the effects of BCG. As expected, IL-6 secretion from HUC-PC cells was induced by BCG in a dose-dependent manner after 24-hour treatment. This is consistent with other reports based on a HPV-immortalized Hu35E6E7 HUC cell line [320] and other bladder cancer cells [313-317]. On the other hand, two defined G. lucidum extracts – GLe and GLw were also tested. GLe induced IL-6 secretion from HUC-PC cells, but GLw did not. Almost the same levels of IL-6 were produced by GLe and BCG, at 100 μg/ml and 1000 μg/ml respectively, and thus GLe is about ten-fold more potent than BCG in this sense (Figures 5.3 and 5.4). G. lucidum had immunomodulatory effects on stimulation of Th1 and Th2 cytokine mRNA expression [325]. Although induction of IL-6 secretion by G. lucidum from human T cells and mouse splenocytes has been reported [253,326], this is the first demonstration on epithelial cells.

Several investigators have proposed the immunomodulatory roles of Toll-like receptor (TLR) in response to mycobacteria [327]. The expression of IL-6 mRNA in the Hu35E6E7 HUC cells was found to be exclusively triggered by BCG through the TLR signaling [320]. Furthermore, NF-κB and AP-1 are the main signaling pathways responsible for IL-6 expression immediately upon BCG stimulation [328]. Reserachers reported that IL-6 expression in bladder cancer cells induced by BCG is mediated by NF-κB signal transduction [168,329,330]. Furthermore, lipopolysaccharide (LPS) can be easily extracted by ethanol in the GLe fraction. LPS can exhibit many immunomodulatory activities including the activation of NFκB and IL-6 secretion [253. 326]. Therefore, Limulus Amoebocyte Lysate test is suggested to assess any LPS contamination, polymyxin B neutralization and LPS removal gel should be used if LPS is found in GLe. GLe produced similar effects on IL-6 production as with BCG, but whether this activation is also elicited by NF-κB, will be discussed in Chapter 10.

Nonetheless, signaling pathways of NF-κB can be stimulated by the cross-linking of α5β1 integrin on the surface of human TCC cells, which subsequently activates IL-6 promoter constructs to express IL-6 mRNA [164,324]. Interestingly, the α5β1 integrin is a classic cellular receptor present on the malignant urothelium for fibronectin (FN) [164,168]. Expression of α5 and β1 mRNA can be promoted by exogenous and autocrine IL-6, while competitive inhibitors of FN inhibit BCG-induced NF-κB signaling pathways [164,331]. IL-6 possesses multiple-functions including pro- and anti-

inflammatory activities, modulation of bone resorption, promotion of hematopoiesis and induction of plasma cell development [330,332]. Although IL-6 is a Th2-like cytokine, it also plays crucial roles in the initial differentiation of Th1 cells [333]. Prevention of TCC recurrence is mainly relied on Th1 cell mediated immunity (*as detailed in 1.5.5*). In BCG prophylaxis, IL-6 acts as a pro-inflammatory cytokine, further inducing cytokine production, T-cell proliferation, cytotoxic T-cell differentiation and acute phase reaction [334]. Furthermore, autocrine IL-6 enhanced BCG adherence to the 253J TCC cell line through the up-regulation of α5β1 integrin receptor for FN [331].

The idea of "cytokine-activated tumor inhibition" has been recently proposed [335]. The antiproliferative effects of IL-6 were demonstrated in vitro on numerous TCC cell lines [168,187,336,337]. These suggested that IL-6 is the interleukin responsible for the BCG-related cytotoxic effects on tumor cells. Similarly, G. lucidum has been reported to be immunologically active in cytokine production [278,338]. IL-6 was one of the several macrophage cytokines induced by G. lucidum, and possesses inhibitory effects on leukaemia cell growth [45,278]. However, results of the current study are inconsistent with the conceptual "cytotoxic IL-6" theory. Firstly, HUC-PC in response to BCG was active in IL-6 production but no cytotoxicity was shown. Secondly, both G. lucidum extracts was demonstrated to be cytotoxic to HUC-PC cells, but only GLe was active in IL-6 production. Nonetheless, cytotoxicity of BCG was shown to be more potent on high-grade bladder

cancer cells than the low-grade ones [323]. It was also reported that only normal and poorly differentiated bladder cancer cells are able to internalize BCG [317]. Thus, BCG is unable to stimulate IL-6 production in high grade tumors, which are supposed to be killed by BCG. Current and other evidences clearly indicate that it is not necessary for anything that can stimulate IL-6 secretion to be concurrently cytotoxic.

In the present study, the up-regulation of IL-6 secretion in response to BCG suggested HUC-PC cells are capable of internalizing BCG. FN is an essential adhesion glycoprotein for BCG binding to the surface of urothelium, internalization and production IL-6 [161,165,339]. There are two forms of FN: soluble and surface-bound [340]. Loss of cell surface FN on transformed cells is correlated with acquisition of tumorigenicity [341] and metastatic potential [342]. Such FN losses are mainly due to reduced synthesis, reduced binding and increased degradation rate, and increased FN release into the extracellular matrix [340]. These FN molecules facilitate cell-substrate adhesion, and thus enhance the interaction between the urothelium and extracellular matrix, and ultimately affect the cell morphology, cytoskeletal organization, migration and differentiation [343]. Expression of extracellular FN is correlated with tumor progression for invasiveness and aggressiveness [344,345]. 89% of urothelial tumor stroma was positive for FN immunohistochemical expression [344]. Blocking of FN attachment sites on TCC cells inhibits the tumor outgrowth in vivo [346]. The diagnostic roles of soluble FN in urine have been proposed and its elevated levels are associated with tumor stage, degree of

differentiation, tumor size, multifocal nature or macroscopic appearance [343,347]. Clinical data supported that persistent elevation of urinary FN causes BCG failure after complete TUR [175]. Excess soluble FN, whether from exogenous or autocrine origin, also saturates mycobacterial and cell surface FN receptors simultaneously, and precludes the bridging ability of a single FN molecule, impairing α5β1 integrin/integrin mediated NF-κB signal transduction which is critical for BCG prophylaxis [169]. Thus, free FN in the culture media is regarded as a key factor for both carcinogenesis of urothelial cells and BCG binding. In contrast, cell surface expression of FN is comparatively less important regarding its roles in bladder chemoprevention. In the present study, G. lucidum extracts reduced free FN in the culture media of treated HUC-PC cells. This suggests that combinational use of G. lucidum and BCG may enhance the overall efficacy in two ways: (1) facilitating BCG binding to the urothelial surface for subsequent internalization and (2) suppressing the tumor growth and progression by reducing unnecessary cell-substrate interactions.

Furthermore, G. lucidum also increased the expression of GAGs on the HUC-PC cell surface. Formerly called mucopolysaccharides, GAGs are long unbranched polysaccharides, are highly anionic and are often bound to core proteins to become proteogylcans with varying properties of extracellular matrices of tissues [348,349]. GAGs are extremely hydrophilic and trap water at the outer layer of the umbrella urothelium, and this trapped water forms a gel as part of the mucosal barrier that interfaces urine and the bladder wall

[350,351]. This produces a protective barrier that becomes highly impermeable to any solutes, crystals and even bacteria in urine [350,352]. The disruption of this mucosal permeability is pathologically significant such that interstitial cystitis (IC) occurs [350,353]. Elevation of urinary uronate and sulfated GAGs are correlated with the IC severity [353]. GAGs are also able to repair damaged bladder mucusa [350]. Oral or instillated GAGs, as recommended by the European Association of Urology (EAU), are given to patients with chronic cystitis [354]. In addition, anti-adherence properties of GAGs have primary innate defence against bacterial attacks [348,350,352]. Experimental removal of GAGs from the urothelial surface causes a ten-fold higher bacterial adherence [355]. In BCG immunotherapy for TCC, interaction between BCG mycobacteria and urothelial are kept by the repellent force of highly anionic GAGs at a 70-100Å close docking distance [162,321]. In mice liver, polysaccharides isolated from G. lucidum protected hepatocytes from inflammation-induced injury induced by BCG infection [356]. Therefore, the effects of G. lucidum on cell-surface GAGs may strengthen the mucosal barrier of the urothelium, as well as prevent the side effects of BCG therapy, such as cysititis and infections.

In summary, ethanol extract of G. lucidum was a more potent activator for IL-6 production in HUC-PC cells than BCG. This suggested the immunological role of G. lucidum in TCC prophylaxis. IL-6 was the only cytokine selected for measurement because it is the earliest cytokine that can be detected after BCG exposure to urothelial cells and it is also an indicator

for successful BCG internalization by these cells. Other cytokines such as IL-2, IL-8 and TNF-α can be measured, but they show up later. In regards of BCG efficacy, G. lucidum reduced the amount of extracellular free FN concentration, and this might facilitate BCG binding and suppress the process of carcinogensis. Moreover, G. lucidum also increased GAGs expression on the cell surface, and thus strengthened the protection of the pre-malignant cell line from carcinogenic attack and bacterial infection. Altogether these suggest the potential synergy of G. lucidum to BCG therapy and this may reduce the side effects of BCG in clinical practice. G. lucidum would be a good supplement for BCG prophylaxis. This has to be confirmed by further studies. In vitro data reported here is not strong enough to support clinical trials but it is biologically important to make further hypothesis. Combination and ABP-pretreated experiments were not performed because cell culture consisting of monolayer of HUC-PC did not have the 'whole organ structure' for adequate outcome measures. Therefore, an ex-vivo organ culture model involving whole bladder from rats will be planned and designed in the near future for this purpose. Moreover, G. lucidum was demonstrated to be cytotoxic to HUC-PC whereas BCG was not. As reviewed in Section 1.6.2, potential chemopreventive agents are also cytotoxic to remove residual tumor cells after TUR. These residual cells are in pre-malignant or malignant stage that can be triggered by carcinogen to form foci and tumors for recurrence. Therefore, the cytotoxicity of G. lucidum is biologically important as a good sign for effectiveness for

eliminating the adverse cells before it turns tumorigenic. The cytotoxic effects of G. lucidum will be further elaborated and discussed in Chapter 6.

CHAPTER 6

G. LUCIDUM CYTOTOXICITY IS SELECTIVE TO THE PRE-MALIGNANT HUC CELLS

6.1 INTRODUCTION

Many putative prophylactic agents are sought for their cytotoxicity (reviewed *in 1.6.2*), but most of these properties were established on bladder cancer cell lines [10,203,220]. Cytotoxicities of G. lucidum have been demonstrated in a variety of cancer cells through the regulation of different signaling pathways [228]. The inhibitory effects of G. lucidum on HUC-PC growth and ABP-induced migration have been reported [252]. Additional evidence further propose G. lucidum as a novel supplementary chemopreventive agent to BCG, based on its properties of IL-6 induction as well as modulation of FN and GAGs (*discussed in Chapter 5*). G. lucidum is also found to be cytotoxic to HUC-PC cells at concentration 100 μg/ml or above. Would G. lucidum inhibit the proliferation of HUC-PC cells at non-cytotoxic levels? In this chapter, Objective 3 of the study is addressed in hopes of furthering the understanding of the extent of G. lucidum's cytotoxicity on the HUC-PC cells, regarding its effects on cell proliferation especially when challenged with ABP. An ideal chemopreventive candidate should not be cytotoxic to normal tissue. As such, the normal HUC cell line, i.e. HUC-1, was also included for examination for addressing the toxicity issues.

6.2 MATERIALS AND METHODS

6.2.1 LDH cytotoxicity assay

Cell growing conditions and assay procedures were performed as described in the Chapter 5.2.1. Based on the previous LDH findings, which were also presented in Chapter 5, concentrations at 40, 80, 100 and 200 µg/ml were repeated for GLe / GLw when testing the LDH cytotoxicity on HUC-PC and HUC-1 cells. In addition, 100 µM of ABP was the optimal concentration used for inducing the tumorigenic transformation [76]. Concentrations at 40, 80, 100 and 200 µM were used here for ABP for studying the LDH cytotoxicity.

6.2.2 Trypan blue exclusion assay for cell viability

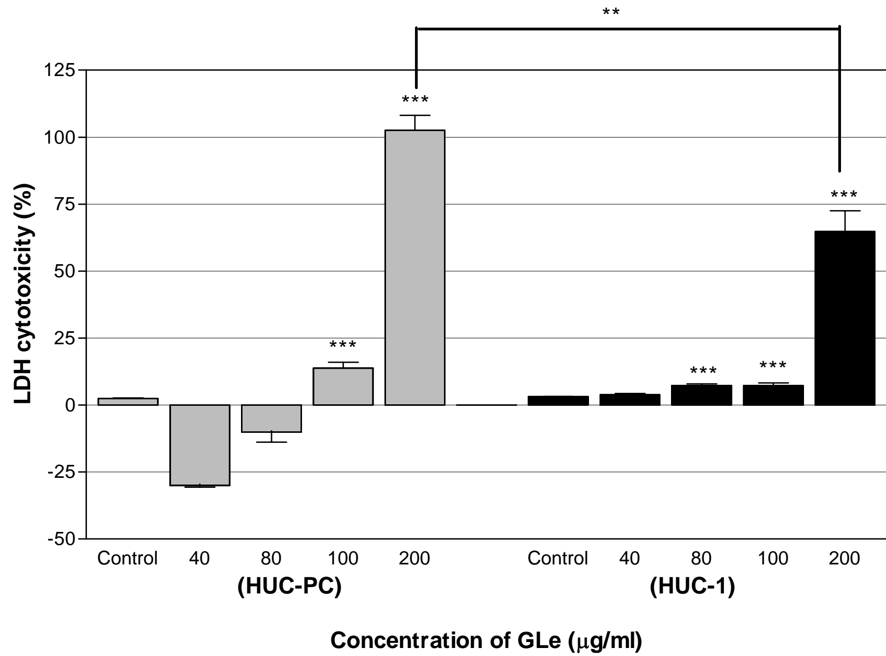
Harvested cells were initially seeded at concentration of 1 x 10^6 cells per 100-mm culture dish [76]. Cells were treated with 100 µM of ABP in two settings: firstly, a 48-hour continuous incubation; secondly, a 24-hour pretreatment with ABP, followed by a change of medium and then a 48-hour incubation without ABP. A set of dishes without any ABP was used as control. Growth inhibitory effects of GLe or GLw (0-100 µg/ml concentrations) were examined by incubating either simultaneously with ABP for 48 hours or post-24hour-ABP-pretreatement for 48 hours. Cell counts were performed using standardized manual trypan blue staining technique and using the automated Beckman Coulter Vi-CELL™ XR Cell Viability Analyzer with its reagent pack (Miami FL). For the manual method, 100 µl of cell suspension in complete medium was mixed with 0.4% (w/v) trypan blue. Stained and unstained cells were counted at the four outer squares of a haemacytometer

under microscope. For the automated method, 500 μl of the cell suspension in complete medium was added into sample cup, then mixed with trypan blue reagent and loaded into the cell counter, 50 pictures were taken for calculating the viable cell number. All cell counting results were verified between the manual and automated methods.

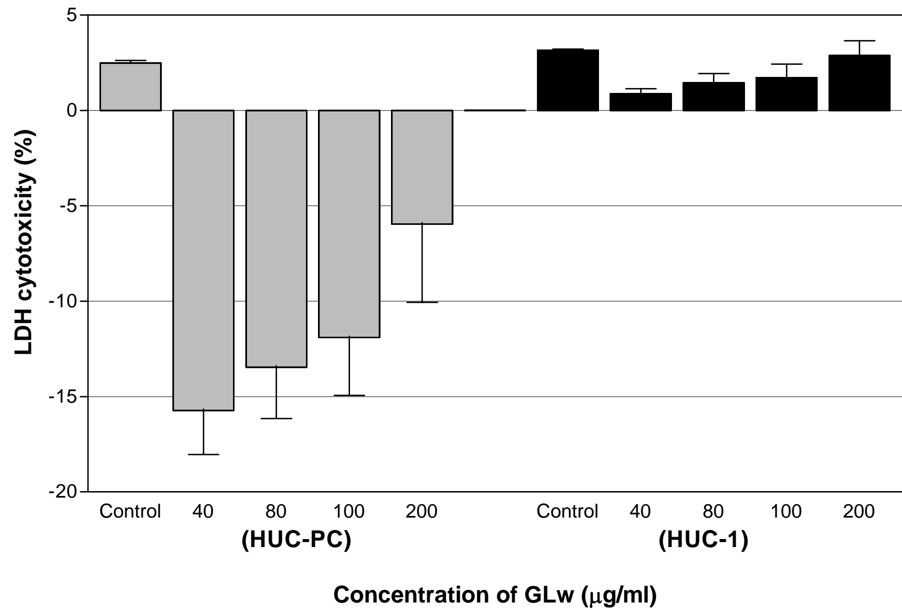
6.3 RESULTS

6.3.1 GLe is cytotoxic to HUC-PC and HUC-1 cells

Although GLe was cytotoxic to both HUC-PC and HUC-1 cells, the potency was shown to be less on HUC-1 (Figure 6.1). Specifically, at 200 μg/ml concentration, all HUC-PC cells were killed (consistent with that reported in Chapter 5) as compared ($P<0.01$) where 65% of the HUC-1 cells were killed. There were 14% of the HUC-PC cells killed by 100 μg/ml GLe, which is statistically significant ($P<0.001$), and no cytotoxicity was seen at 80 μg/ml or below. On the other hand, less than 10% of HUC-1 cells were killed by GLe at 100 μg/ml or below. However, there was no significant cytotoxicity exhibited by GLw on both cell lines at concentrations up to 200μg/ml (Figure 6.2).



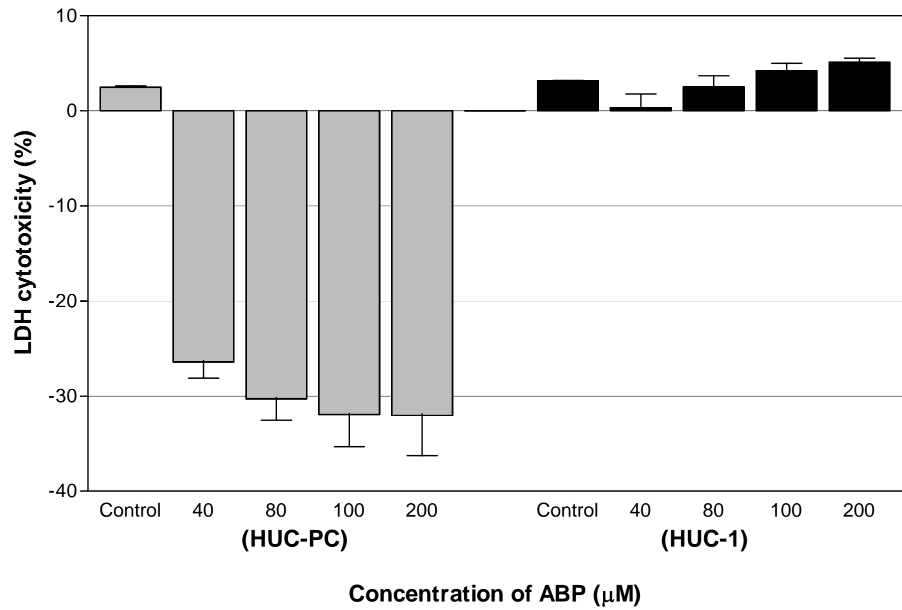
(Figure 6.1) **C**ytotoxic effects of GLe on HUC-PC and HUC-1 cells. Both cell lines were incubated with GLe at concentrations 40, 80, 100 and 200 μg/ml for 24 hours. Cells growing in complete medium containing 0.01% ethanol were used as control (n=3, error bar: SEM, *** P<0.001; ** P<0.01). Negative cytotoxicity explained as same as in 5.2.



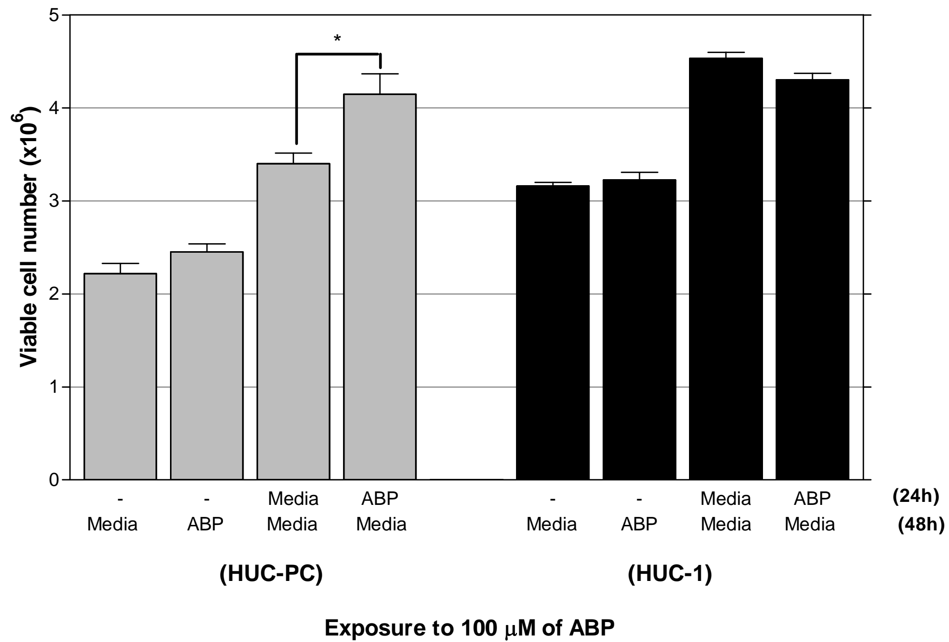
(Figure 6.2) Cytotoxic effects of GLw on HUC-PC and HUC-1 cells. Both cell lines were incubated with GLw at concentrations 40, 80, 100 and 200 µg/ml for 24 hours. Cells growing in complete medium containing 0.1% milliQ water were used as control (n=3, error bar: SEM, statistically insignificant). Negative cytotoxicity explained as same as in 5.2.

6.3.2 ABP enhances the proliferation of HUC-PC cells

ABP was shown to be non-cytotoxic at concentrations up to 200μM to both HUC-PC and HUC-1 cell lines (Figure 6.3). Interestingly, enhancement of HUC-PC growth in the presence of ABP was observed. When compared with the control group, the growth increase (16%) was statistically insignificant after the 48-hour incubation, but becomes significant ($P<0.05$) with 22% increase if cells are pretreated with ABP for 24 hours (Figure 6.4). Such growth enhancement was not seen on HUC-1 cells.



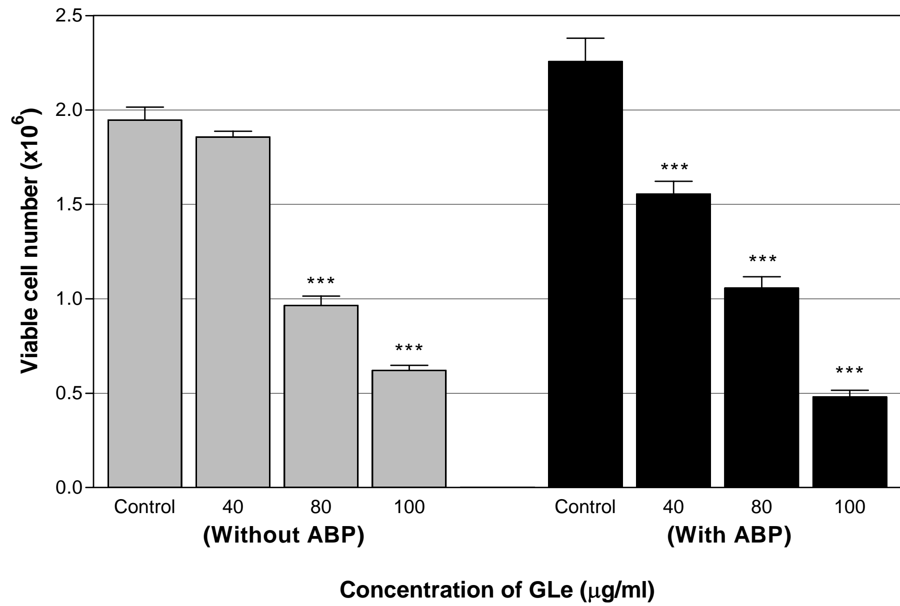
(Figure 6.3) Cytotoxic effects of ABP on HUC-PC and HUC-1 cells. Both cell lines were incubated with ABP at concentrations 40, 80, 100 and 200 μg/ml for 24 hours. Cells growing in complete medium containing 0.1% DMSO were used as control (n=3, error bar: SEM, statistically insignificant). Negative cytotoxicity explained as same as in 5.2.



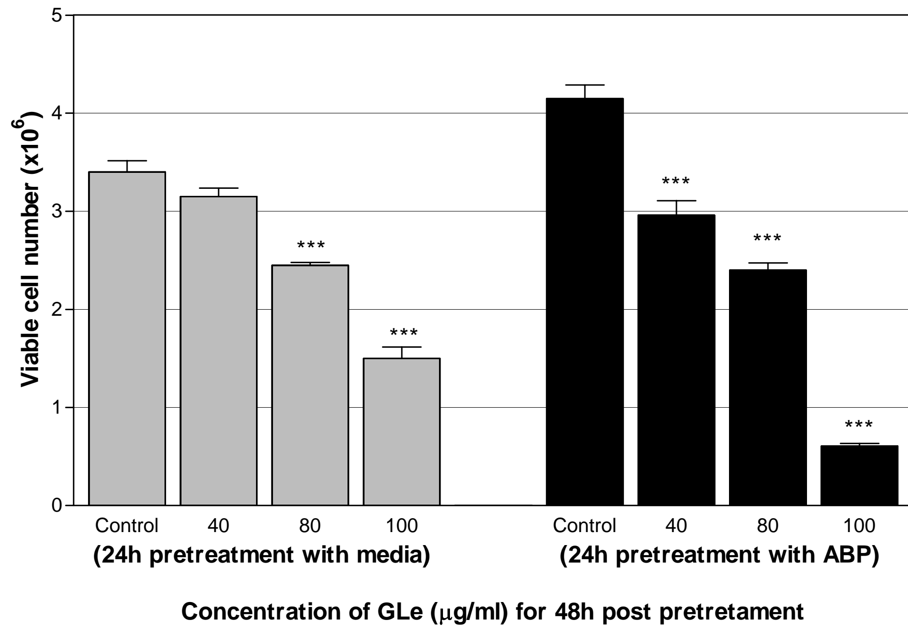
(Figure 6.4) The cell proliferation of HUC-PC and HUC-1 cell lines induced by ABP. Both HUC-PC and HUC-1 cells were exposed to 80 μM of ABP with two experimental settings: (1) 48 hours incubation and (2) 24 hours ABP incubation, followed by 48 hours culture in complete media. Cells growing in medium containing 0.1% DMSO were used as control (n=3, error bar: SEM, * $P<0.05$).

6.3.3 GLe inhibits the proliferation of HUC-PC and HUC-1 cells

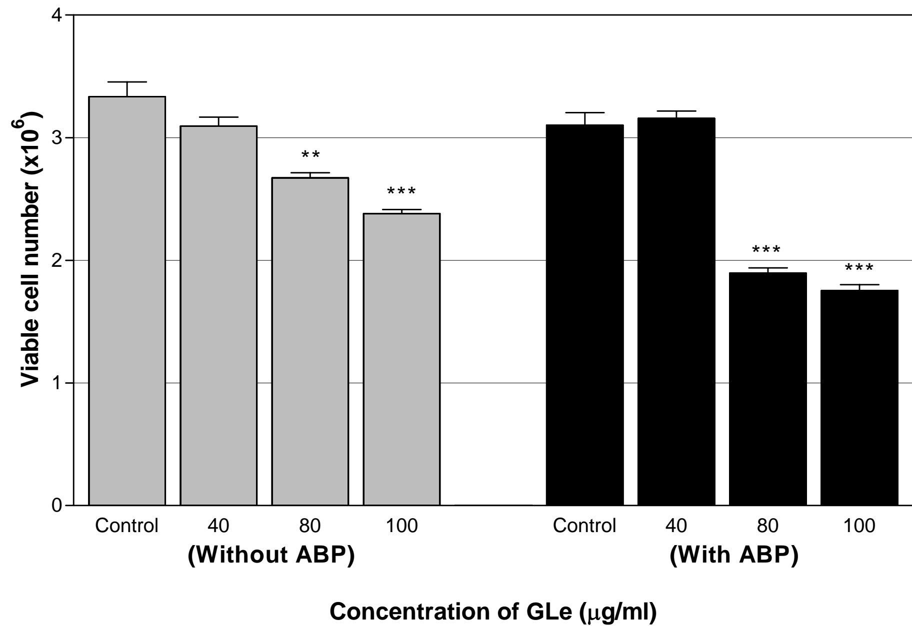
After the 48-hour incubation, HUC-PC growth was significantly ($P<0.001$) inhibited by GLe volume at 80 μg/ml or above when ABP was absent, and 40 μg/ml or above when ABP was present in a dose-dependent manner (Figure 6.5). The same growth inhibition was shown by GLe to the HUC-PC cells pretreated with ABP (Figure 6.6). In particular, at 80 μg/ml concentration, GLe was non-cytotoxic (*presented in 5.3.1 and 6.3.1*); irrespective of the presence of ABP, the cell number of HUC-PC after 48-hour culture remained at the initial seeding, i.e. 1×10^6 cells, and thus cell proliferation was totally suppressed. Growth inhibition by GLe was also demonstrated on HUC-1 cells, but was less potent than that of HUC-PC cells. At 100 μg/ml, the HUC-1 cells were still growing, doubling from the initial seeded cell number, regardless of the presence of ABP (Figure 6.7). However, growth of both HUC-PC and HUC-1 cells were not inhibited by GLw, whether ABP was present or not (Figure 6.8)



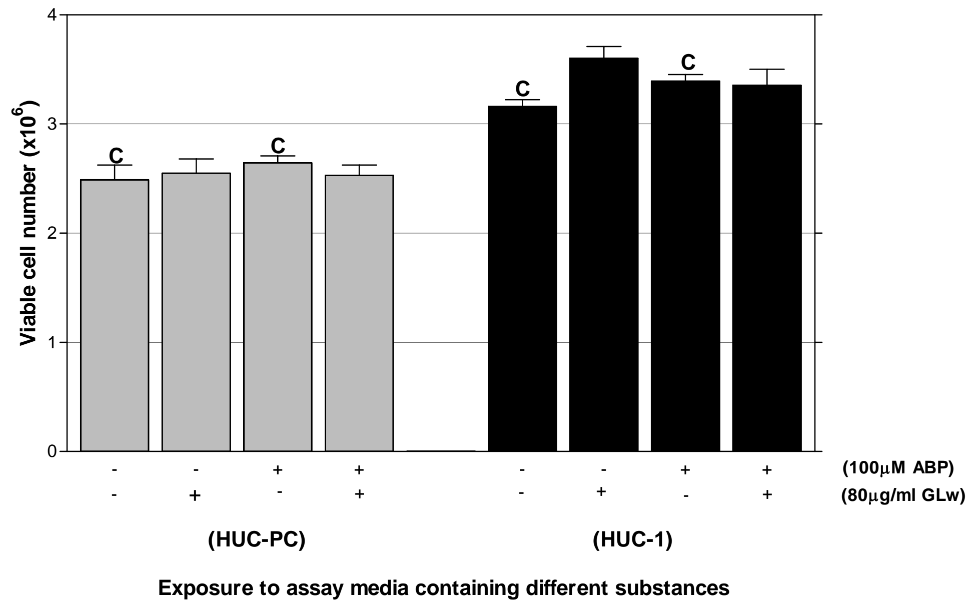
(Figure 6.5) Dose-dependent Growth inhibition of HUC-PC cells induced by GLe. The HUC-PC cells were coincubated with GLe at concentrations 40, 80 and 100 μg/ml with or without 100 μM of ABP for 48 hours. Cells growing in complete medium containing 0.01% ethanol were used as control for the "without ABP" group; Cells growing in complete medium containing 0.01% ethanol + 0.1% DMSO were used as control for "with ABP" group (n=3, error bar: SEM, *** P<0.001).



(Figure 6.6) Dose-dependent growth inhibition on ABP-pretreated HUC-PC cells induced by GLe. The HUC-PC cells were pretreated with either media or ABP for 24 hours. Media- or ABP-pretreated HUC-PC cells were incubated with GLe at concentrations 40, 80 and 100 μg/ml for 48 hours. Cells growing in complete medium containing 0.01% ethanol were used as controls for both the “without ABP” and “with ABP” groups (n=3, error bar: SEM, *** P<0.001).



(Figure 6.7) Dose-dependent Growth inhibition of HUC-1 cells induced by GLe. The HUC-PC cells were coincubated with GLe at concentrations 40, 80 and 100 μg/ml with or without 100 μM of ABP for 48 hours. Cells growing in complete medium containing 0.01% ethanol were used as control for the "without ABP" group; Cells growing in complete medium containing 0.01% ethanol + 0.1% DMSO were used as control for "with ABP" group (n=3, error bar: SEM*** P<0.001).



(Figure 6.8) No growth inhibition were demonstrated by GLw on HUC-PC and HUC-1 cells after the 48-hour culture in the presence or absence of ABP (**C** = Control, error bar: SEM).

6.4 DISCUSSION

Transformed cancer cells are common in vitro tools used for the discovery of new anticancer strategies; however, the purdence of using cells in pre-cancerous stage is particularly important for chemoprevention. Nothing is better than eliminating the adverse cells before they turn malignant, especially when looking at bladder carcinoma where the rate of recurrence remains exceptionally high even after complete TUR. The HUC-PC model simulates the exposure of carcinogens and potential therapeutic agents to the superficial epithelial lining of the urinary bladder. There are basically two types of cytotoxicity assays: (1) quantifying plasma membrane damage and (2) measuring cytoplasmic enzyme release by damaged cells. Both types of assays were used in the current study, including LDH cytotoxicity assay by quantifying LDH enzyme released and trypan blue exclusion assay measuring the uptake of trypan blue dye by damaged cells. Most cytoplasmic enzymes are in low amounts in culture supernatant and require elaborate kinetic assays to quantify, but LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into culture supernatant when the plasma membrane is damaged. Moreover, it involves a simple two-step enzymatic reaction, fast and with high sensitivity to detect low number of dead cells (as low as 2000 cells per microtiter well according to the kit instruction avalible at http://catalog.takara-bio.co.jp).

The HUC-PC cell line has been proven to be sensitive to ABP and able to be transformed into a tumorigenic body and induce tumors in athymic nude mice

[74,76]. At that time, ABP has been shown to be cytotoxic to the HUC-PC cell line [76,77]. In the present study, no cytotoxicity was found; on the contrary, ABP enhanced the HUC-PC proliferation. The enhancement of cell proliferation is consistent with Wang et al. [357], where human hepatoma was grown in serum-supplemented media and the increase of viability after ABP incubation was shown. Despite serum-free techniques having been long established for maintaining human cells, growth and survival of cancer cell lines are still limited when fetal serum is deprived in cultures [358-360]. Serum contains hormones, growth factors, lipids, transport proteins, enzyme cofactors and attachment factors so that it is capable of supplying many in vivo hormonal, nutritional and stromal elements to support the survival and growth of cells in culture [361]. For example, aggressive TCC cell lines, RT4 and J82, were unable to survive when fetal serum content decreased from ten to one percent [358]. Furthermore, serum contributes N-acetyltransferase and oxidase activities, which facilitate the metabolism of ABP in the cultures [362]. Iron, also supplied by FBS in culture, is able to catalyze the production of oxygen radicals and promote biotransformation of carcinogens [363]. High iron stores in vivo increases the overall cancer risk of certain sites [364]. Therefore, the use of complete medium (containing 10% FBS), in the current study, may be the possible reason for enhancing the ABP carcinogenicity in the HUC-PC model, and thus overgrowth seen. Nonetheless, the HUC-1 cell line is known to be insensitive to ABP and no growth enhancement was observed. These new findings suggest that the carcinogenic mechanisms and

characteristics of ABP-induced HUC transformation should be further investigated.

Two G. lucidum extracts were tested at non-cytotoxic levels, growth inhibition was shown by GLe but wasn't by GLw. This is possibly due to the differences of active ingredients being extracted by water and ethanol. Polysaccharides and triterpenes are the two major groups of active components in G. lucidum, and extracts of water and ethanol can be roughly designated as polysaccharides-rich and triterpenes-rich extracts, respectively, because of their solubilities [228]. By exposing the HUC-PC cell line (which is known to be sensitive) to ABP, cell proliferation was enhanced; however, the growth inhibition induced by GLe was not compromised or reduced for ABP-pretreated cells or when ABP was co-existent and the effects were shown to be more significant than in its absence. This means that the action of GLe contributes to HUC-PC growth control, regardless of the presence or absence of ABP, and thus GLe works even on the cells that are under ABP carcinogenic attack. Moreover, massive killing of normal and cancer cells at a site may cause severe side-effects of tissue destruction and serious irritation to the patients [365]. The cytotoxicity and growth inhibitory effects of GLe were less potent on the normal HUC-1 cell line, which suggested the selectivity on pre-malignant instead of normal HUC. This is consistent with the selective cytotoxic effects of G. lucidum on cancer cell lines versus normal cells in other studies [254,264]. The underlying reasons of why there is selectivity iz unknown and requries further studies. The HUC-PC cell line is sensitive to

ABP after 21 passages than the HUC-1 cell line, although both are derived from the latter sharing the same genomic contents [81]. It cannot be excluded of the possible selectivities due to the differentiation of certain cell surface expression of receptors. Findings of the present study suggested that 80 μg/ml should be used as an effective dose that is non-cytotoxic but able to stop the proliferation of HUC-PC cells. Concentrations higher than 100 μg/ml may disturb the tissue integrity of the urothelium because of their cytotoxic effects. Intravesical cytotoxic treatment is commonly used for prophylaxis of superficial TCC because of the efficiency of drug delivery near the site of action and reduction of systemic toxicity [366]. In vitro data herein also supported the intravesical use of G. lucidum for bladder chemoprevention.

In summary, G. lucidum cytotoxicty for the first time tested in relation with a defined bladder carcinogen – ABP, and potent and selective cytotoxic activities confirmed. GLe inhibited the proliferation of HUC-PC cells regardless the presence of ABP. However, the growth inhibitory effects of GLe were not diminished when tested on ABP-pretreated cells. Thus, GLe is able to prevent the enhancement of proliferation induced by ABP. Colony formation experiments could be better designed than the cell proliferation assay, and it is planned for the future study. Possible toxicity to normal tissue cannot be excluded since cytotoxic effects were also shown in the normal cell line. Underlying mechanisms of the cytotoxicity and growth inhibition to HUC-PC are still not clear. Concentrations of GLe at 80 μg/ml were shown to

be non-cytotoxic for exhibiting maximal growth inhibition, which is recognized as an optimal dose to 1 x 10^6 cells population.

CHAPTER 7

THE DUAL EFFECTS OF G. LUCIDUM ANTIOXIDANTS ON CYTOTOXICITY

7.1 INTRODUCTION

Substantial amounts of various reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), nitric oxide (NO) and superoxide (O_2^-) are generated during the process of aerobic metabolism, and redox (oxidation-reduction) reactions are thought to be initiated in cellular signaling [367-369]. The causal relationship between oxidative stress and cancer incidence are supported by epidemiological observations and molecular mechanisms [141,142,370]. Endogenous and exogenous ROS are associated with carcinogenesis by damaging DNA and modifying functional proteins that regulate cell proliferation [371-375]. In the field of oncology, DNA, in relation to mutagenesis, is the most attentive macromolecule being attacked [141,143,376-378].

For bladder cancer, oxidative events are undoubtedly involved. Growth of bladder tumors can be promoted by ROS, such as H_2O_2, through enhancement of the subcellular distribution of heparin-binding EGF-like growth factor (HB-EGF) [379]. Oxidative DNA damage, as assessed by 8-OHdG, was increased in the urine [145] of patients with bladder cancer, as were peripheral blood leukocytes [380]. Bladder cancer is a disease related to environment and human behavior such that the presence of chemical carcinogens triggers

metachronous tumors [381]. One of the most dominant aromatic carcinogens is ABP [23]. Chronic exposure of ABP causes the intravesical accumulation of proximate metabolites, which in turn interacts with the bases in DNA, resulting in covalent adduct formation and thus mutations [53,54,58]. Furthermore, exposure of rat urothelium to this carcinogen triggers inflammatory response and oxidative stress [382]. Growing evidences have emerged to support the involvement of ROS in ABP-induced carcinogenesis (*reviewed in Chapter 1.3.4 & 1.4.6*) [53,58].

Epidemiological studies have shown that diets with high antioxidant content, such as fruits and vegetables, are able to reduce oxidative DNA damage and stimulate DNA repair, which are useful chemoprevention strategies [371,371,375,383-385]. G. lucidum has been documented to contain rich antioxidant capacities [289,290,292,295,386]. Polysaccharides and triterpenes are the two major groups of bioactive components, each fraction consists of more than 100 molecules, and most of these compounds are excellent antioxidants [228,240,292,293,295,387]. At non-cytotoxic concentrations, GLe inhibited the HUC-PC cell growth but GLw does not. In this chapter, Objective 4 of the study is addressed regarding the antioxidant properties of GLe and GLw, as well as their effects on oxidative stress, in particular oxidative DNA damage, will be examined.

7.2 MATERIALS AND METHODS

7.2.1 Measuring the antioxidant activities of G. lucidum extracts and assay media

G. lucidum extracts, including the raw powder extract from a ReishiMax capsule, GLe and GLw, were dissolved in either absolute ethanol or MilliQ water. The assay medium, as detailed in 4.1.6 was freshly prepared. All testing agents were assayed as follows: (1) the automatic Ferric Reducing/Antioxidant Power (FRAP) assay [388-390] for the FRAP values; (2) enzyme-free time-independent chemiluminescence microtitre plate method [308] for DPPH· scavenging activity and (3) modified Ferrous Oxidation-Xylenol orange (FOX) assay [391,392] for H_2O_2 concentration. Methodologies for these assays are detailed below.

7.2.2 Treatment of culture media

There were six study groups in total (Table 7.1), each consisting of three culture dishes in triplicate. Upon exposure, culture media from each study group were collected at 2, 4, 6, 12, 24 and 48 hours. All medium samples were filtered using Centricon YM-10 microconcentrator (Millipore Co., Bedford, MA) and assayed for FRAP, DPPH· scavenging activity and H_2O_2 immediately.

(Table 7.1) Six study groups involved in antioxidant and oxidant experiments.

	Concentration(s) of substances added		
Study groups	ABP (μM)	GLe (μg/ml)	GLw (μg/ml)
Media control	-	-	-
GLe	-	80	-
GLw	-	-	80
ABP	100	-	-
GLe+ABP	100	80	-
GLw+ABP	100	-	80

7.2.3 FRAP assay

FRAP assay [388-390] is a patented and quick, simple redox-linked colorimetric method to measure the ferric reducing (antioxidant) capacity in solutions, which is referred to as the total antioxidant capacity, termed FRAP values. The automated FRAP assay was run and calculated on a Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd., Basel, Switzerland). Ascorbic acid at 100, 250, 500 and 1000 μM concentrations were used for calibration standards. Ferrous suphate·7 hydrate (Fe^{2+}) solution at 1000 μM was used as the assay calibrator. The standard curve is shown in *Appendix III-D*.

For measuring the FRAP values in culture media, the conventional spectrophotometric method was modified into microtitre plate format. 100 μl of media samples were added into 170 μl freshly prepared working reagent in 96-well flat bottomed microtitre plate (Thermo LabSystem, Franklin, MA, USA). Ascorbic acid, at 0, 10, 25 and 50 μM concentrations, was used for calibration standards. Ferrous suphate·7 hydrate (Fe^{2+}) solution at 100 μM was

used as an assay calibrator instead of 1000 μM concentration. The sample-reagent mixture of each culture in the microtitre well was placed in a TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria) for 60 seconds of orbital shaking, followed by additional three minutes' incubation. Absorbance in each well was read at 590 nm against the pure complete media blank using the microplate reader. The standard curve is shown in *Appendix III-E*.

7.2.4 DPPH· free radical scavenging activity assay

DPPH· free radical scavenging activity was measured using an enzyme-free time-independent chemiluminescence microtitre plate method as described previously [308]. A 50 μl of sample was added to 150 μl DPPH· working (400 μM) reagent in a 96-well flat bottomed microtitre plate (Thermo LabSystem, Franklin, MA, USA), mixed and left for 20 minutes at room temperature, and the absorbance at 590 nm was read against a complete medium blank, with the TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). The DPPH· scavenging activity of each sample was read from a standard curve, with 0, 50, 100, 250, 500 μM working standards freshly prepared from a 10 mM butylated hydroxytoluene (BHT, Sigma, Germany) stock solution. Results were expressed in terms of DI% "percentage of DPPH· inhibition". The standard curve is shown in *Appendix III-F*.

7.2.5 H_2O_2 assay

The H_2O_2 assay method was modified from the FOX assay, as previously published [393]. Briefly, 100 µl of sample was added to 170 µl of working reagent in a 96-well flat bottomed microtitre plate (Thermo LabSystem, Franklin, MA, USA), mixed and left for 20 minutes at room temperature, and the absorbance at 590 nm was read against a complete media blank, using the TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). The H_2O_2 concentration of each sample was read from a standard curve (*Appendix III-G*), with 0, 0.5, 1.0, 2.5 and 5.0 µM working standards freshly prepared from a 30% H_2O_2 stock solution (Riedel-deHaën, U.S.A.).

7.2.6 8-OHdG ELISA assay

Oxidative DNA damage was analyzed by measuring free 8-OHdG present in the culture media. A very small quantity of 8-OHdG is expected in the culture media. Filtered medium was assayed immediately using the "Highly Sensitive 8-OHdG Check" kit (Japan Institute for Control of Aging, Shizuoka, Japan). The assay kit is based on competitive ELISA principle with a measurement range of 0.125 ng/ml to 10 ng/ml. The manufacturer's instructions were strictly followed. The culture media were collected after 48 hours' incubation and filtered using the Centricon YM-10 microconcentrator and filtrates were assayed immediately. The absorbance at 450 nm was read against a blank of complete culture medium, on TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). Replicated samples of each study

group were run in triplicate. The outer most wells of the ELISA plate were not used to avoid edge effect, while phosphate buffer was added to the unused wells to maintain the uniform temperature within the wells. The standard curve is shown in *Appendix III-H*.

7.3 RESULTS

7.3.1 GLe and GLw are rich in antioxidant capacity

When dissolved in appropriate solvents, antioxidant properties of both GLe (in ethanol) and GLw (in MilliQ water) were largely enhanced after re-extraction (Table 7.2). In particular, the FRAP value and DPPH· scavenging activity of GLw increased 9.3 and 1.7 times, respectively. And the DPPH· scavenging activity of GLe increased 2.8 times but no increase for FRAP was observed. Of the two re-extracts, GLw contained a higher overall antioxidant capacity (about double for both FRAP and DPPH· scavenging activity) than that of GLe, as shown in Table 7.2. To provide baseline values of the cultures, the antioxidant profiles of working assay media containing different test substances are also presented in Table 7.3. No FRAP value and DPPH· scavenging activity was observed but about 3 μM of H_2O_2 was found in the fresh complete medium. Addition of GLe or GLw separately increased both the FRAP and DPPH· scavenging activity with a slight decrease in H_2O_2 in media. However, ABP increased the FRAP value to a larger extent than G. lucidum but did not increase the DPPH· scavenging activity and decreased 30% of the H_2O_2 in the media. Thus, the majority of ABP antioxidants were of reducing activities rather than of free radical scavenging activity.

(Table 7.2) Antioxidant activities of ReishiMax and its defined re-extracts.

Measurement		DI%	FRAP (µM)
In MilliQ water	ReshiMax powder	45.0±3.61	54.0±32.20
	GLw	76.1±1.27	502.2±5.49
In absolute ethanol	ReshiMax powder	17.7±1.45	260.7±5.49
	GLe	49.2±2.73	244.1±9.84

All results are shown as mean±SEM, n=9 for each measurement.

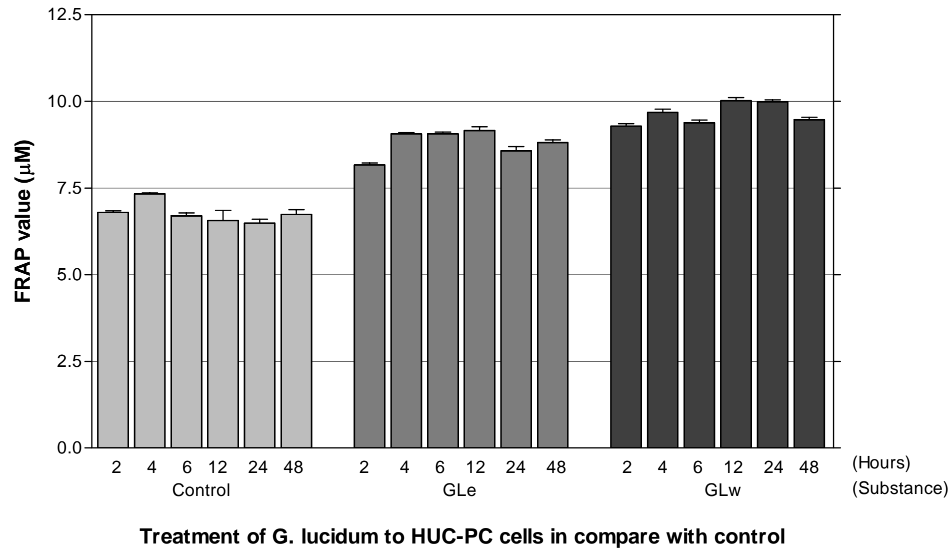
(Table 7.3) Antioxidant / Oxidation properties of freshly prepared assay media.

Assay media (concentration / solvent)	FRAP (µM)	DI%	H2O2 (µM)
ABP (100µM / 0.0002% DMSO)	21.7	0	1.6
	20.9	1	1.9
GLe (80µg/ml / 0.0018% EtOH)	3.4	5	2.7
	4.0	2.5	2.8
GLw (80µg/ml / 0.0018% EtOH)	7.5	3.7	2.3
	8.0	2.9	2.6
ABP + GLe	14.8	5.6	2.8
(0.0002% DMSO / 0.0018% EtOH)	14.8	4.2	2.8
ABP + GLw	23.9	5.6	2.6
(0.0002% DMSO / 0.0018% EtOH)	23.5	4.6	2.7
Pure complete medium (10% FBS)	0	0	3.2
	0	0	3.0

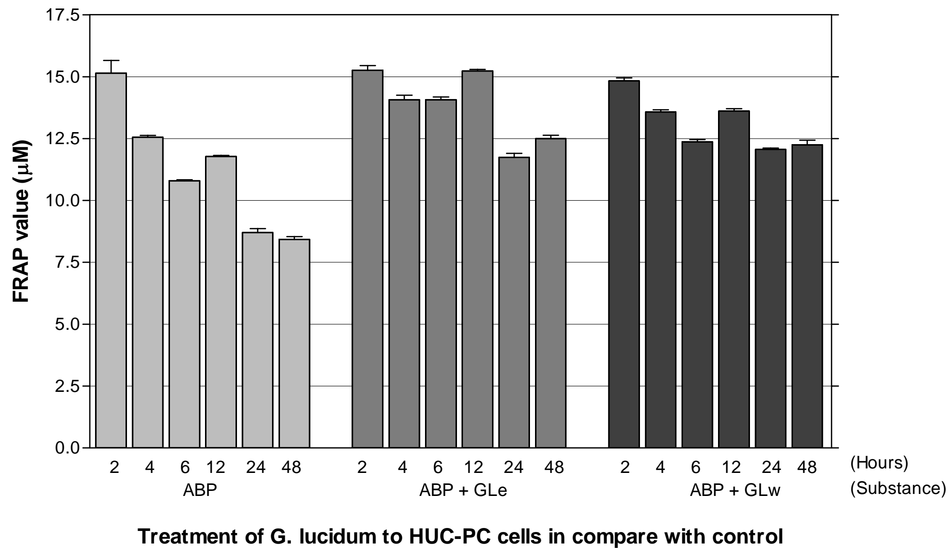
All results are shown as mean of n=2.

7.3.2 Antioxidant status during the 48-hour culture

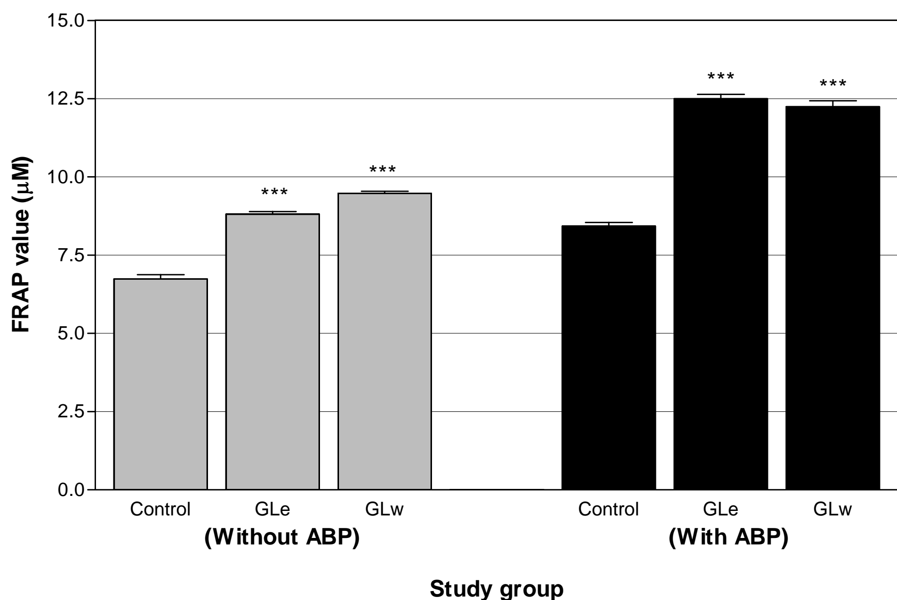
During the course of the 48-hour treatment, FRAP values in the GLe- and GLw- containing media were quite stable and both were maintained at around 9-10 μM levels, as compared to values of < 7.5 μM in the medium control (Figure 7.1). When HUC-PC cells were treated with ABP alone, the FRAP values gradually decreased, whereas GLe and GLw maintained the FRAP values at 12.5-15 μM in the presence of ABP (Figure 7.2). Of all the study groups measured at 48 hours, GLe and GLw enhanced the FRAP values of the cultures, irrespective of the presence or absence of ABP (Figure 7.3). However, the DPPH • scavenging activities during the 48-hour treatments, as shown in Figures 7.4 and 7.5, fluctuated. Since the highest DI% for all study groups was observed at 48 hours (end point of the experiment), only these results were used for comparison. Results indicated that both GLe and GLw enhanced the DPPH • scavenging activities of the cultures, irrespective whether ABP was present or not (Figure 7.6).



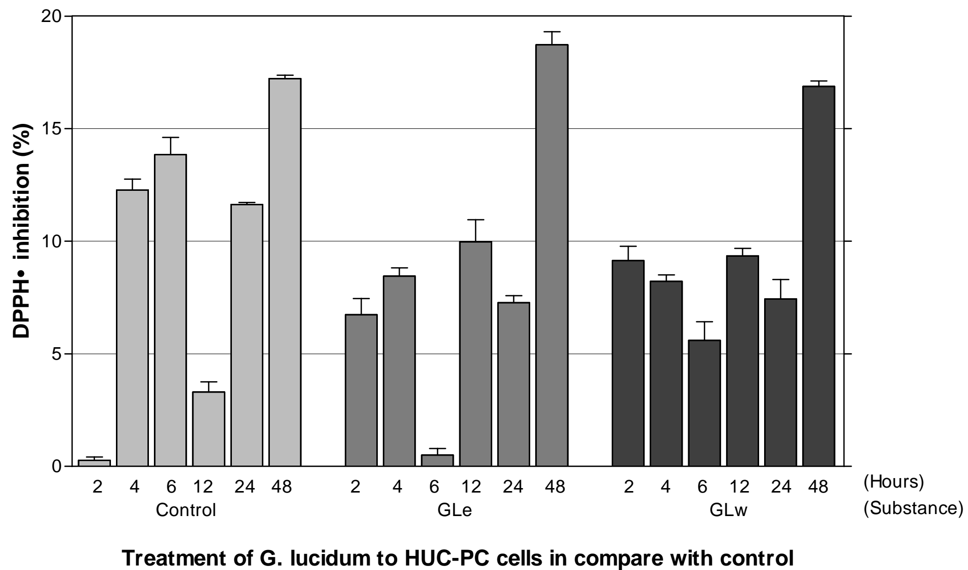
(Figure 7.1) Pattern of changes in FRAP values in HUC-PC cultures induced by G. lucidum over 48 hours. The HUC-PC cells were incubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw for 48 hours. Cuture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in complete medium were used as a control (n=3, error bar: SEM).



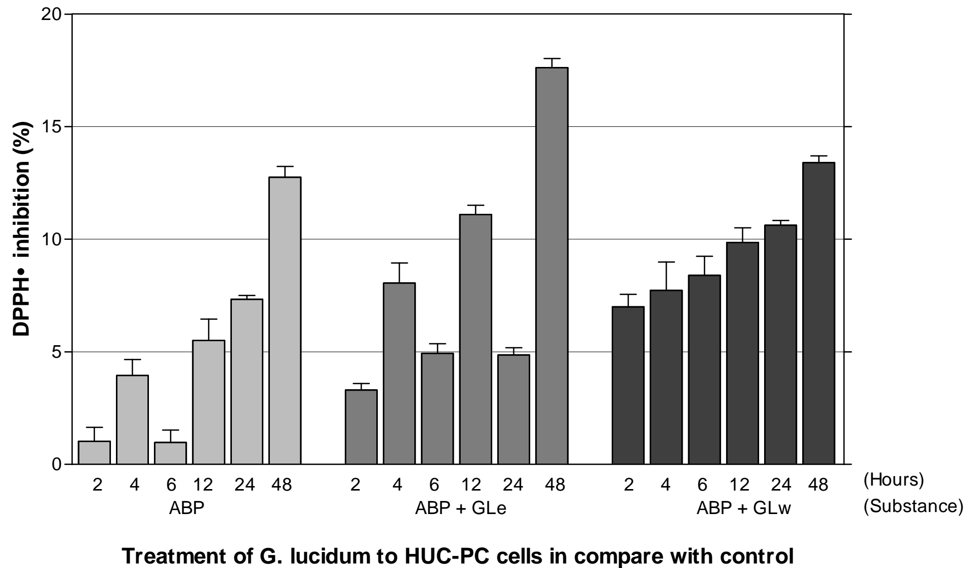
(Figure 7.2) Pattern of Changes in FRAP values in HUC-PC cultures induced by G. lucidum and ABP over 48 hours. The HUC-PC cells were coincubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw with 100 μM of ABP for 48 hours. Culture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in ABP assay media were used as a control (n=3, error bar: SEM).



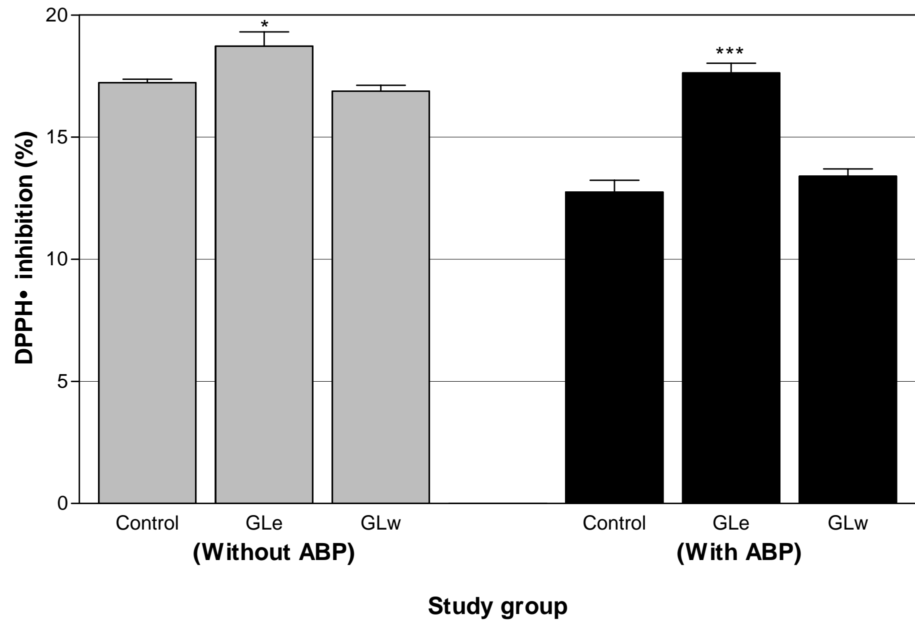
(Figure 7.3) Enhancement of FRAP values by G. lucidum in HUC-PC cultures at the 48-hour endpoint. Cells growing in complete medium was used as a control for the “without ABP” group; Cells growing in assay medium containing 100 μM ABP were used as control for the “with ABP” group (n=3, error bar: SEM, *** P<0.001).



(Figure 7.4) Patten of Changes in DPPH • scavenging activities in HUC-PC cultures induced by G. lucidum over 48 hours. The HUC-PC cells were incubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw for 48 hours. Culture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in complete medium were used as a control (n=3, error bar: SEM).



(Figure 7.5) Pattern of Changes in DPPH • scavenging activities in HUC-PC cultures induced by G. lucidum and ABP over 48 hours. The HUC-PC cells were coincubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw with 100 μM of ABP for 48 hours. Culture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in ABP assay media were used as a control (n=3, error bar: SEM).

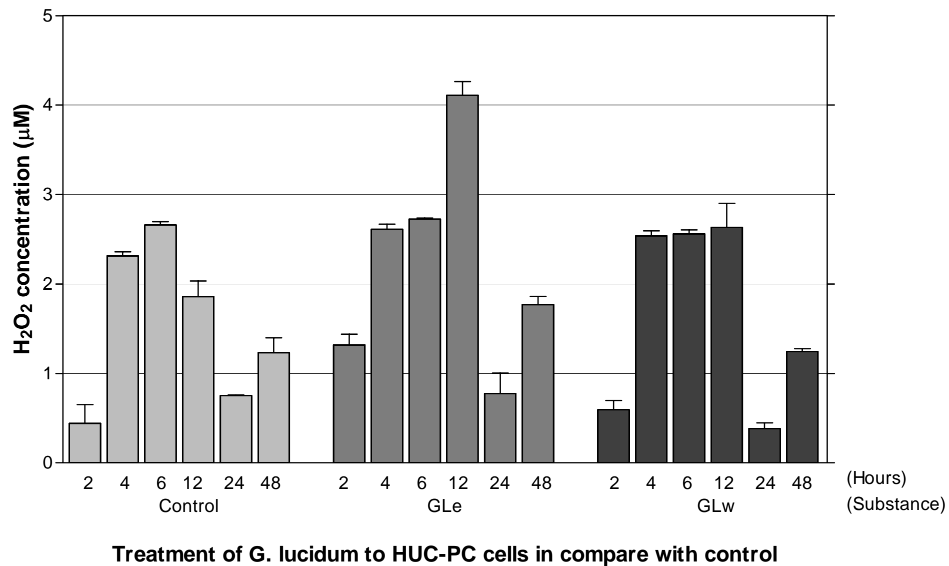


(Figure 7.6) Enhancement of DPPH • scavenging activities by GLe in HUC-PC cultures at the 48-hour endpoint. Cells growing in complete medium were used as a control for the "without ABP" group; cells growing in the assay medium containing 100 μM ABP were used as control for the "with ABP" group (n=3, error bar: SEM, *** P< 0.001; * P<0.05).

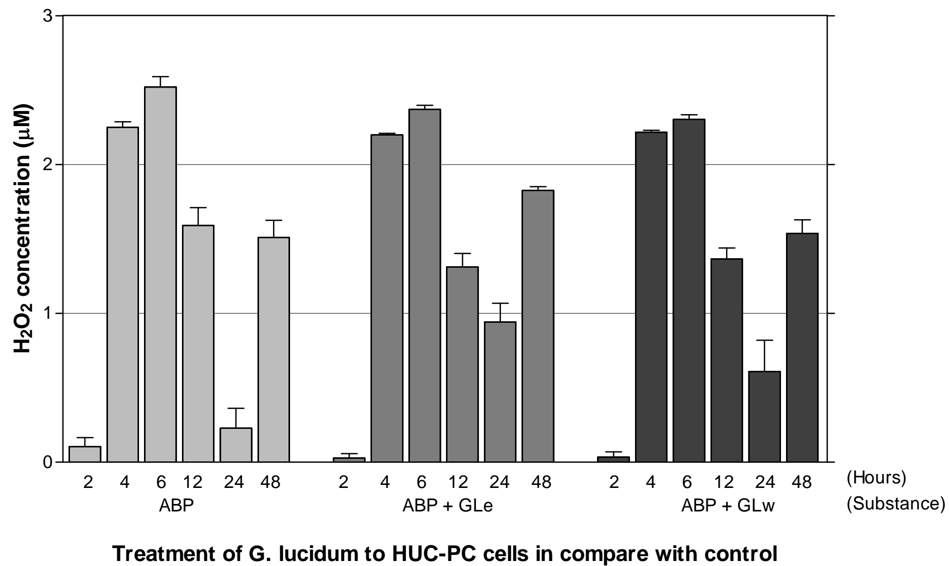
7.3.3 GLe induces oxidative stress in HUC-PC cells

When ABP was absent, H_2O_2 levels were maintained at 2-4 μM during the first 12 hours of culture, and then the H_2O_2 levels dropped below 2 μM until the 48-hour endpoint, but no differences were observed between the control and G. lucidum (Figure 7.7). When ABP was present, higher H_2O_2 levels were shown during the first six hours of culture and dropped slightly until the 48-hour endpoint, but again no differences were observed between control and G. lcuidum (Figure 7.8). After 48 hours' incubation, as shown in Figure 7.9, H_2O_2 levels were significantly increased by GLe but no changes were observed in GLw, irrespective of whether ABP was present or not. Specifically, H_2O_2 was significantly increased by 20% with GLe alone and 43% with GLe+ABP (Figure 7.9).

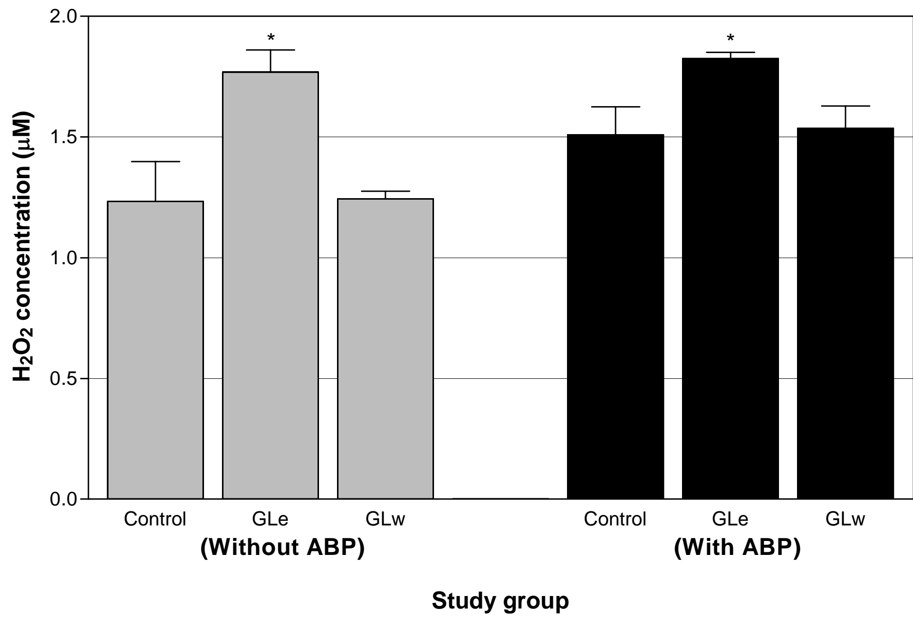
GLe induced 8-OHdG formation when co-incubated with ABP, in contrast to GLw which alone reduced the 8-OHdG formation (Figure 7.10). The 8-OHdG formation induced by GLe was in a clear dose-dependent manner (Figure 7.11). This increase of 8-OHdG level was correlated with the LDH cytotoxicity of GLe with $r = 0.9195$ but statistically insignificant (Figure 7.12). In addition, the effects of ABP on 8-OHdG level were also studied, as shown in Figure 7.13, whereby 8-OHdG concentration was significantly ($P<0.05$) increased by 50 μM of ABP and significantly ($P<0.001$) decreased by 200 μM of ABP.



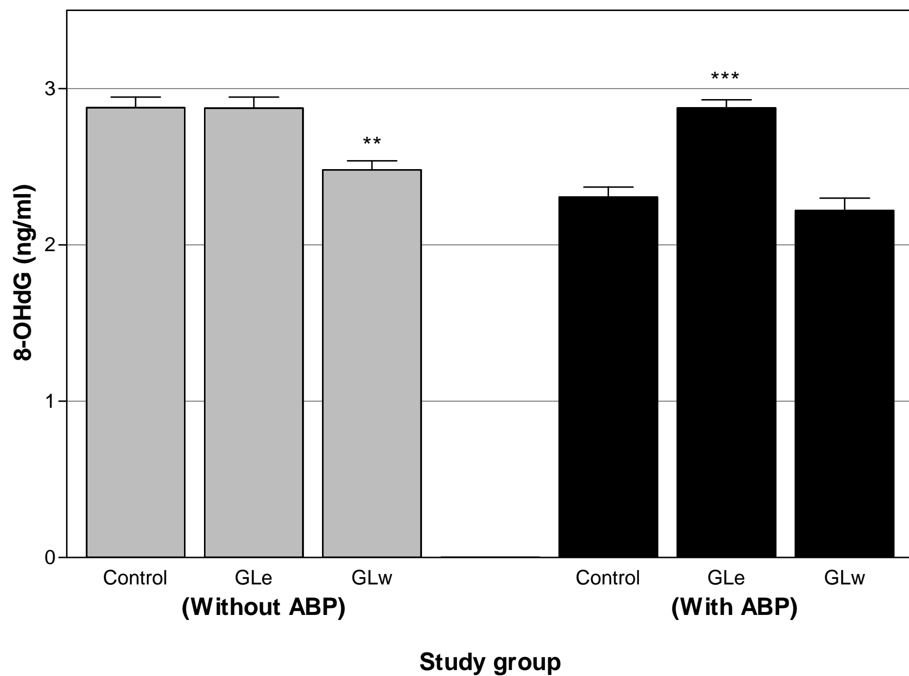
(Figure 7.7) Pattern of changes in H_2O_2 levels in HUC-PC cultures induced by G. lucidum over 48 hours. The HUC-PC cells were incubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw for 48 hours. Culture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in complete medium were used as a control (n=3, error bar: SEM).



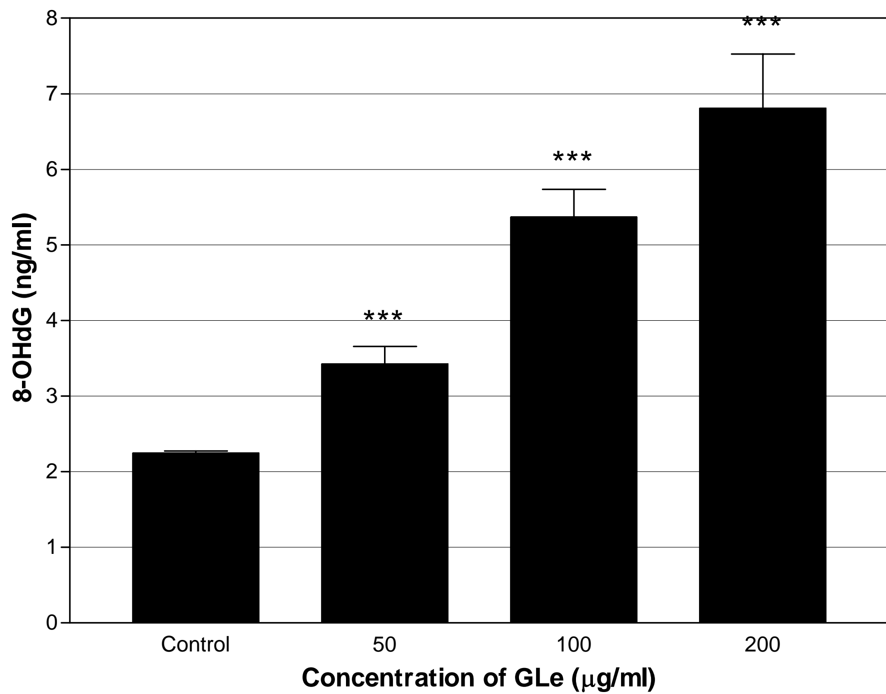
(Figure 7.8) Pattern of changes in H_2O_2 levels in HUC-PC cultures induced by G. lucidum and ABP over 48 hours. The HUC-PC cells were coincubated with complete media, 80 μg/ml of GLe or 80 μg/ml of GLw with 100 μM of ABP for 48 hours. Culture media were harvested at 2, 4, 6, 12, 24 and 48 hours after incubation. Cells growing in ABP assay media were used as a control (n=3, error bar: SEM).



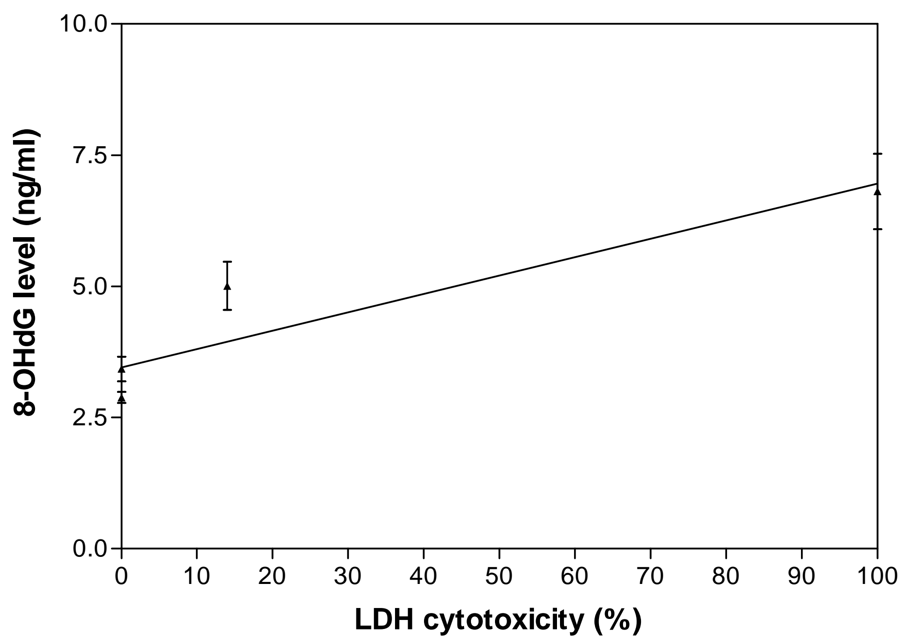
(Figure 7.9) Enhancement of H_2O_2 levels by GLe in HUC-PC cultures at the 48-hour endpoint. The cells growing in complete medium were used as a control for the "without ABP" group; the cells growing in the assay medium containing 100 μM ABP were used as a control for the "with ABP" group (n=3, error bar: SEM, * $P<0.05$).



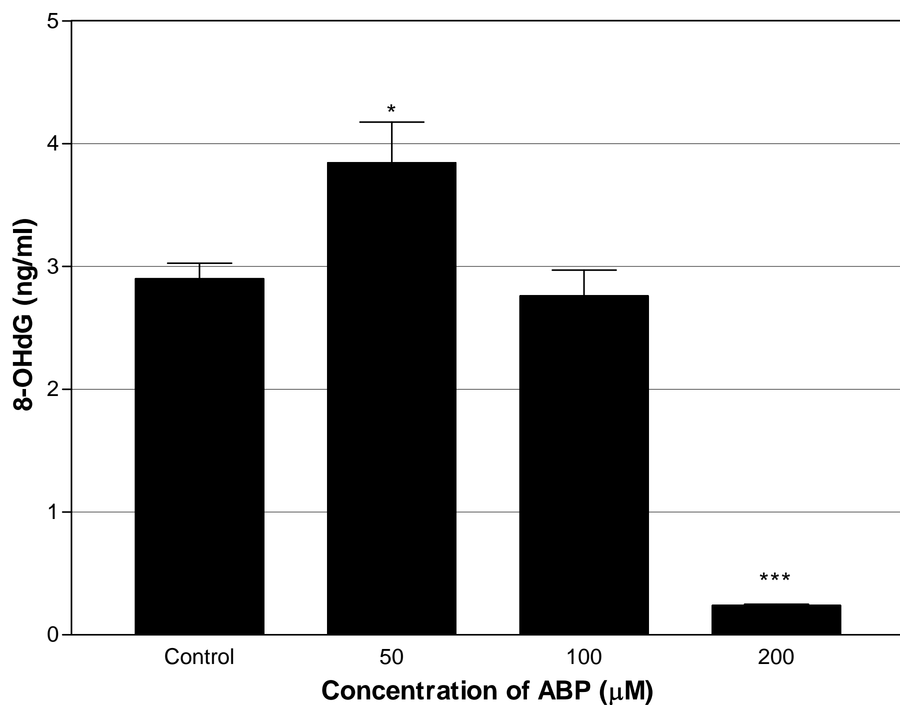
(Figure 7.10) The effects of G. lucidum on 8-OhdG. The HUC-PC cells were incubated with 80 μg/ml of GLe or GLe with or without 100 μM of ABP for 48 hours. The culture media were harvested, treated and measured without delay. The cells growing in the complete medium were used as a control for the "without ABP" group, and cells growing in the assay medium containing 100 μM ABP were used as a control for the "with ABP" group. (n=6, error bar: SEM, *** P<0.001, ** P<0.01).



(Figure 7.11) Dose-dependent increase of 8-OHdG in HUC-PC cells induced by GLe in the presence of ABP. The HUC-PC cells were coincubated with 50, 100 or 200 μg/ml of 100 μM of ABP for 48 hours. The culture media were harvested, treated and measured without delay. Cells growing in the assay medium containing 100 μM ABP were used as a control (n=3, error bar: SEM, *** $P<0.001$).



(Figure 7.12) 8-OHdG concentration plotted against LDH cytotoxicity induced by GLe in the presence of 100 μM ABP (Pearson's correlation r = 0.9195, P = 0.0805).



(Figure 7.13) Effects of ABP on 8-OHdG levels in culture media of HUC-PC cells. The HUC-PC cells were incubated with 50, 100 and 200 μM of ABP for 48 hours. The culture media were harvested, treated and measured without delay. Cells growing in the complete medium containing 0.1% DMSO were used as a control (n=3, error bar: SEM, *** $P<0.001$; * $P<0.05$).

7.4 DISCUSSION

The ReishiMax® capsule of G. lucidum contains reducing and free radical scavenging antioxidants, which is consistent with other publications [287,292,294,295]. These antioxidant activities were enhanced after re-extraction, and the GLw and GLe can be further designated as polysaccharides-rich and triterpenes-rich extracts, as those are the two major groups of G. lucidum's bioactive components [228,252]. According to Halliwell B [394], an antioxidant is defined as "*any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate*". There are several forms of antioxidants, whereby free radical scavengers and reducing antioxidants are the two common types. Scavenging effects of DPPH· radical have been reported for water and ethanol extracts of G. lucidum [292]. The nonenzymatic antioxidants act as reductants that can inactivate oxidants by their reducing capacity, and thus may resist oxidative damage [307,388]. Ferric reducing properties of G. lucidum have been intensively studied in vivo and in vitro [294,386,395]. However, in the present study, GLw has a higher overall antioxidant capacity than GLe, and they exhibited different roles in the HUC-PC culture model.

ROS is believed to be involved during the process of ABP-induced carcinogenesis [58]. N-hydroxyl metabolites of ABP were demonstrated to cause 8-OHdG formation in the human leukemia HL-60 cell line [53]. In the present study, ABP was demonstrated to contain FRAP reducing antioxidant

activities, which reduced the formation of 8-OHdG level in HUC-PC cells. The decrease of 8-OHdG is contradictory to the results of Wang et al. [357], who showed H_2O_2-induced DNA damage after ABP treatment using the comet assay. In vivo, ABP was activated through the process of N-oxidation by a cytochrome P450 isozyme (CYP1A2) in the liver to produce electrophilic intermediates that readily react with critical nucleophiles in cells and initiate carcinogenesis [57,70,396]. Another P450 isozyme CYP4B1 is present in the bladder mucosa and also able to cause mutagenic activation of aromatic amines [55,397]. Therefore, bladder carcinogens are believed to be metabolized in both the liver's and the bladder's mucosal lining. The formation of ABP-specific DNA adducts and subsequent DNA damage is regarded as an essential event for the initiation of ABP carcinogenic effects [52,59]. However, antioxidant properties of ABP and the lack of oxidative DNA damage shown in the present study suggest that ROS may not be essential for the process of ABP mutagenicity.

The formation of base/deoxyribose lesions is one of the most important forms of oxidative DNA modification [372,398]. Guanine (G) is regarded as the most susceptible target for oxidation because it has the lowest oxidative potential among the four nucleic acid bases [399,400]. Specifically, oxidation of G at C8 position forms 8-hydroxydeoxyguanosine (8-OHdG) and 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxo-dG), which are the most well known candidate biomarkers for carcinogenesis [65,143,144,377]. Failure to repair

these oxidative DNA lesions may lead to G:C → T:A transversion mutations, which cause genetic instability and neoplastic development [144,377,401,402].

Previous studies showed that polysaccharides extracted from G. lucidum contain potent antioxidant activities against oxidative process, such as lipid peroxidation and DNA damage [292,293]. G. lucidum's polysaccharides have shown to be genoprotective against oxidative damage [289,290,293]. In the present study, GLw, designated to be polysaccharides-rich, reduced the formation of 8-OHdG in the HUC-PC culture, irrespective of the presence or absence of ABP. On the contrary, GLe did not change the 8-OHdG level when ABP was absence, but increased the formation of 8-OHdG in a dose-dependent manner when ABP was present. As presented in Chapters 5 and 6, GLe induced potent cytotoxicity and growth inhibitory effects on HUC-PC cells, while GLw was shown to be non-cytotoxic. These findings indicate that G. lucidum's antioxidants play dual roles on the growth control of the pre-malignant human urothelial cells.

Recently, a biphasic effect of G. lucidum was demonstrated by comet assay on DNA of human lymphocytes such that, boiling water extract protected the DNA from damage at lower concentration (<0.001% w/v) while it induced oxidative DNA damage at higher concentration (>0.001% w/v), whereas the damage is mediated through production of H_2O_2 [294]. Results of the current study also showed that GLe increased the concentration of H_2O_2 in the HUC-PC after the 48-hour culture, irrespective of the presence or absence of ABP.

Therefore, H_2O_2 is believed as the mechanism responsible for oxidative DNA damage induced by GLe. H_2O_2 and is also a potent cytotoxic agent to tumors in different sites as a novel chemotherapeutic strategy [403,404]. One may argue that the results suggest GLe are mutagenic due to its effect of oxidative DNA damage. However, the fact that the effectivenesses of many cytotoxic-based anticancer drugs are mediated through oxidative events [405-407] should also be considered. Recently, oxidation in green tea and red wines was catalyzed by the culture media to generate H_2O_2, which was shown to be cytotoxic to phenochromocytoma (PC12) cell lines [408]. According to the Fenton's reaction, H_2O_2 is being dissociated to generate hydroxyl radicals that are able to oxidize the C-8 position of G bases to form 8-OHdG [65]. Although oxidative DNA damage, in particular 8-OHdG, is widely accepted to be mutagenic and promote carcinogenesis, it has been proposed as a therapeutic strategy through induction of cell cycle arrest [368,377,409]. These properties facilitate genomic instability in the HUC-PC cells, and consequently arrest the cell cycle process [409-411]. Ramanathan and colleagues [412] also speculated that oxidative DNA damage can initiate programmed cell death. It is well-known that the G_2-M transition of the cell cycle is a critical checkpoint to verify genomic instability, by blocking DNA-damaged cells from entering mitosis [413]. Some potential chemoprevenive agents, including G. lucidum, are genotoxic to the DNA during the synthesis (S) phase of the cell cycle to cause the G_2-M arrest, thus promoting G2-M phase cell arrest in cancer cells [249,254,405,414]. Small amount of H_2O_2 was demonstrated to trigger G2-M arrest in normal uroepithelial cells [415]. In

addition, 6-10% of the HUC-PC cells were induced to undergo G_2-M phase cell cycle arrest were induced by ethanol extract of G. lucidum [252]. Therefore, current and previous findings supported the induction of oxidative stress and oxidative DNA damage as possible mechanisms for G. lucidum's chemoprevention on bladder cancer. Nonetheless, the lacking of 8-OHdG induction in the absence of ABP requires further investigation. Further, the 8-OHdG production induced by GLe is correlated with the LDH cytotoxicity.

In summary, the chemopreventive effects of GLw and GLe from G. lucidum are potentially antagonistic to each other such that the former protected DNA of HUC-PC from oxidative damage, but in contrast the latter caused oxidative DNA damage and inhibited cell growth when HUC-PC cells are under ABP attack. GLe and GLw were expected to protect the HUC-PC cells from oxidative DNA damage because both of them contain momentous antioxidant activities. Further investigations are needed to understand whether the dual effects are suitable for cancer therapies and prevention. What and how G. lucidum could be efficiently used for chemoprevention requires additional investigations. Whether whole extract or specific purified compounds of G. lucidum should be used remains unknown. G. lucidum is a complex mushroom containing diversified chemical constituents and appropriate components should be selected for different purposes, such as triterpenes for anticancer and polysaccharides for genoprotection. More than 300 compounds could be isolated from G. lucidum, whereas the actual compounds responsible for the properties of antioxidant, oxidative damage, cytotoxicity and cell

protection have yet to be characterized. Fractionation and identification techniques are required for this purpose. Underlying mechanisms can be studied accordingly once the corresponding compounds were isolated.

CHAPTER 8

G. LUCIDUM INHIBITS THE TELOMERASE ACTIVITY IN HUC-PC CELLS

8.1 INTRODUCTION

The enzyme telomerase (*detailed in 1.4.5*) plays a vital role in controlling cell proliferation [416,417], and the expression of telomerase is found in over 85% human cancers including 95% of all advanced malignancies [418]. The genomic stabilizing properties of telomerase protect cells from programmed apoptotic cell death, which enables unlimited replicative potential in carcinogenesis [109,120,419,420]. Up-regulation of telomerase activity was commonly seen in different specimens from patients with urothelial cancer [111,118,120-122,421]. For bladder cancer, telomerase is proposed as a urine-based marker with higher sensitivity and specificity than conventional tools for diagnosis and prognosis [123].

Another enzyme - COX-2 (*detailed in 1.4.6*) - is also considered as a key biomarker in bladder cancer because of its ability to induce cell proliferation and transitional cell hyperplasia [422]. COX-2 is rarely expressed under normal physiological conditions [125,126], but its expression in bladder cancers is correlated with the advancing stage and grade of the tumors [130,132]. Furthermore, numerous COX-inhibiting drugs were found to inhibit proliferation and induce apoptosis in vitro, but also delay progression of COX-2-expressing xenografts in mice in vivo [423].

Regarding the implications of cell proliferation and carcinogenesis, both telomerase [115] and COX-2 [423] are preferential targets for anticancer drug development. Since ABP enhanced the proliferation of HUC-PC cells, was enhanced by ABP (*discussed in Chapter 6*), to address Objective 5 of the study, telomerase and COX-2 were used as relevant biomarkers in the HUC-PC model in order to study the effects of G. lucidum in relation to its growth inhibitory properties.

8.2 MATERIALS AND METHODS

8.2.1 RTQ-TRAP for measurement of telomerase activity

Cells (1-5 x 10^6) were lysed in 1X CHAPS buffer (Chemicon, Temecula, U.S.A.) containing RNase inhibitor and incubated on ice for 30 minutes. The lysate was then centrifuged at 12,000g for 40 minutes at 4°C and the supernatant was collected [309]. Total protein concentration of the cell extract was determined using a Bio-Rad Bradford protein assay kit (Hercules, CA). The protein level of each extract was adjusted to 10 ng/μl for telomerase activity analysis. The telomeric repeat amplification protocol (TRAP) is a landmark method for measuring telomerase activity and a real-time polymerase chain reaction (PCR) technique has been incorporated to allow rapid and precise quantitation [309]. TS and ACX primers were purchased from Molecular Information Laboratory (South Korea). TRAP buffer (10X) containing 200 mM Tris, 630 mM KCl, 35 mM $MgCl_2$, 10 mM EGTA, 1 mg/ml BSA and 0.05% Tween 20 was prepared and stored at -20°C until use. The total volume of the reaction mixture was 25 μl, containing SYBR Green (Invitrogen) and 1X Fluorescein (Bio-Rad) master mix, 0.1 μg each of TS (5'-AATCCGTCGAGCAGAGTTAG-3') and ACX (5'-GCGCGG(CTTACC)$_4$-3') primers, 10mM dNTP (Promega), 0.25 μl Hot Star Taq polymerase (Qiagen), 14.75 μl RNase/DNase free distilled water, 2 μl 10X TRAP buffer and 5 μl protein extract. The PCR was performed in a 96-well microtitre plate on a MyiQ Single-Color Real-Time PCR Detection System. The reaction mixture was first incubated at 25°C for 30 minutes to elongate the TS primer. The PCR was started at 95°C for 15 minutes to activate the Hot Star Taq

polymerase, followed by 30-cycle amplification (95°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds). Fluorescence signal generated from SYBR green was collected and analyzed with iCycler iQ Detector software (Ver. 3.0a; Bio-Rad), where measurable threshold intensity (threshold cycle; C_T) was achieved or reached the maximum 30 cycles. Relative telomerase activity, comparing the signals between the samples, was calculated using the derived equation $2^{-\Delta Ct}$ [424].

8.2.2 EIA for COX-2 quantitation

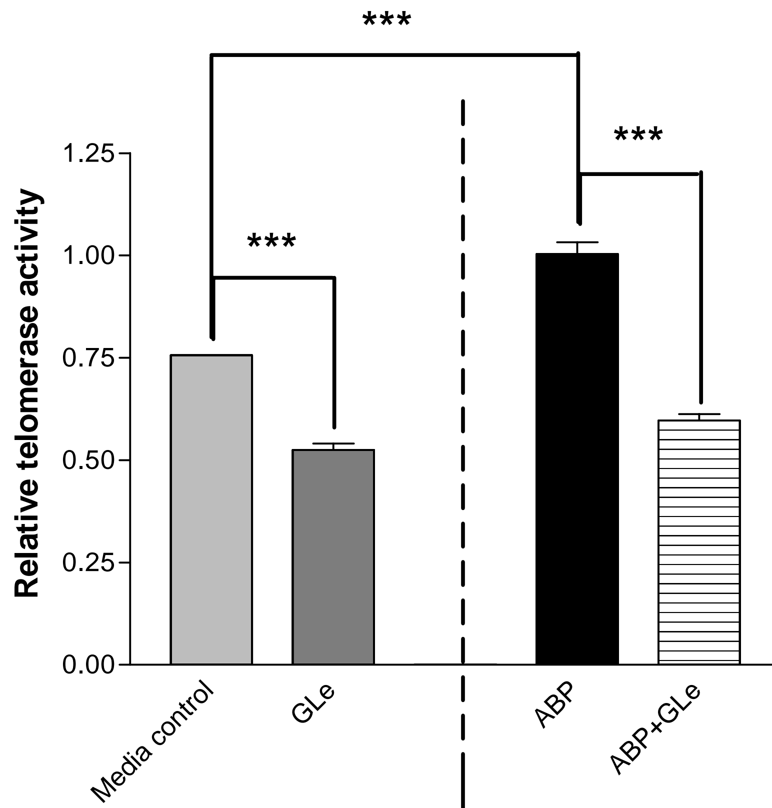
Harvested cells from four 100-mm tissue culture dishes were pooled and washed with PBS. Cell pellets were sonicated with 1ml of TNE (containing 10mM Tris, 0.15M NaCl, 1% NP40 and 1mM EDTA) lysis buffer for five cycles of 30-second bursts at one minute intervals on ice. The cell lysates were then centrifuged at 15,000 rpm for 5 minutes at 4°C and their supernatants were collected for assay without delay. The total protein of the cell lysates was measured using the BCA™ protein assay kit (Pierce, Rockford, U.S.A.). COX-2 concentration in the cell lysates was then quantified by using the TiterZyme® EIA human cyclooxygenase-II EIA kit (Assay Designs, U.S.A.). Kit instructions were precisely followed. At the next step, 100µl of samples or standards were added into appropriate wells of the EIA plate. The plate was tapped gently, sealed and incubated at 37°C for one hour. After washing six times, human COX-II labeled antibody was added into the plate, sealed and incubated at 4°C for an additional 30 minutes. The wells were washed eight times and freshly prepared substrate solution was

added. The plate was incubated at room temperature, in the dark for 30 minutes. The reaction was stopped by 1N sulfuric acid and the plate was read at 450nm against a blank of deionized water. Human COX-2 standard was provided in the assay kit and deionized water was used to make standard concentrations at 0, 2.15, 4.30, 8.59, 17.19, 34.38, 68.75, and 137.5 ng/ml. The standard curve is shown in *Appendix III-I*.

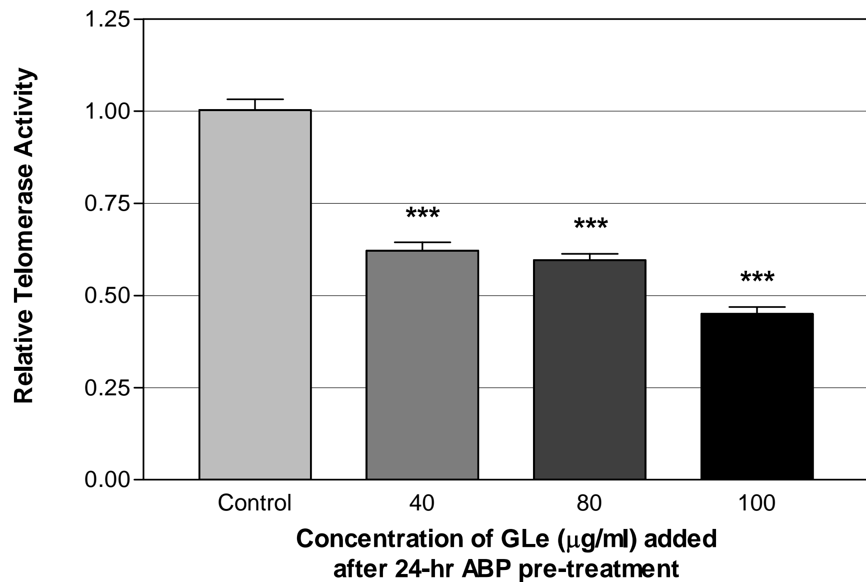
8.3 RESULTS

8.3.1 G. lucidum inhibits telomerase activity of HUC-PC cells

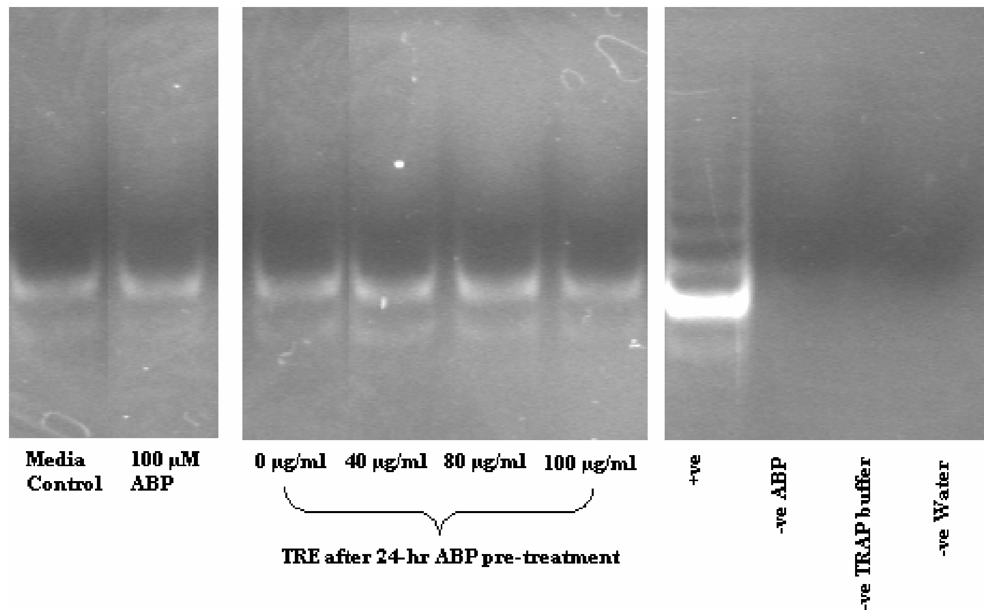
About 25% of telomerase activity in HUC-PC cells was significantly increased by 100 μM of ABP as compared with the media control (Figure 8.1). When HUC-PC cells, alone or ABP-pretreated, are incubated with GLe, there was a 30-40% decrease of telomerase activity respectively ($P<0.001$), as shown in Figure 8.1. The GLe-induced inhibition of telomerase was concentration dependent (Figure 8.2). The purity of the product QRT-PCR products were compared with a positive control using Olf-136 cell extract as shown in Figure 8.3. Moreover, the telomerase activity of the cells was inversely proportional to the growth inhibition exerted by GLe (Figure 8.3), which indicating that the growth inhibition was correlated with the inhibition of telomerase activity.



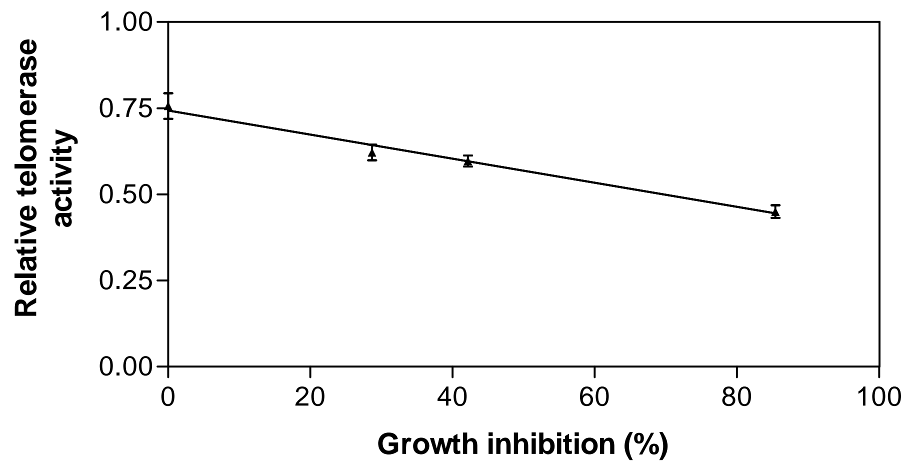
(Figure 8.1) Inhibition of basal- and ABP-induced telomerase activities by GLe. The HUC-PC cells were pretreated with 100 μM ABP for 24 hours and followed by a 48-hour culture with or without 80 μg/ml of GLe alone. GLe-induced inhibition of telomerase activity was compared to cells in complete medium alone and in the presence of ABP formulation (n=3, error bar: SEM, *** $P<0.001$).



(Figure 8.2) Dose-dependent inhibition of telomerase activity induced by GLe in ABP-pretreated HUC-PC cells. The HUC-PC cells were pre-treated with 100 µM of ABP, followed by 48 hours incubation with GLe at concentrations 40, 80 and 100 µg/ml. Cells growing in complete media were used as the control (n=3, *** P<0.001).



(Figure 8.3) Separation of PCR products on polyacrylamide gel electrophoresis; +ve: Olf-136 cell line (Riken Cell bank, Japan); -ve ABP: By boiling the cell lysate of 100 µM ABP at 95°C; -ve TRAP buffer: the 1x TRAP buffer used for cell lysis; -ve Water: the RNase/DNase free distilled water used in the assay.



(Figure 8.4) The inhibition of telomerase activity was strongly correlated with the growth inhibition induced by GLe. Relative telomerase activity plotted against cell growth inhibition induced by GLe (Pearson's correlation r = -0.99, P<0.01).

8.3.2 COX-2 is undetectable in HUC-PC before and after treatments

No COX-2 was detected in any cell lysates. Sensitivity of the ELISA kit used for measurement was at 0.249 ng/ml. Thus, false negative results of COX-2 concentrations below measurable concentration cannot be excluded. Results of total protein measured in each cell lysate are presented in *Appendix IV-A*.

8.4 DISCUSSION

There is an enhancement of HUC-PC growth by ABP as observed and presented in Chapter 6. The HUC-PC cell line is positive for telomerase activity and relative telomerase activity was enhanced significantly ($P<0.001$) by 33%(±4%) for ABP-pretreated cells (Figure 8.1). This is probably due to the immortalization with SV40, where the conjunction of viral small T or large T oncogenes facilitates telomerase expression to transform the human epithelial cells [425-427]. Telomerase activity can be activated in proliferating cultures of normal human uroepithelial cells but not in the uncultured cells, which supports telomerase as a biomarker for cell proliferation instead of malignant transformation [428]. Cytotoxic effects of chemotherapeutic drugs can be enhanced by inhibiting the cellular telomerase activity [429]. However, the HUC-PC cell line has been proven to be sensitive to ABP for tumorigenic transformation [74,76]. It was demonstrated by several investigators that the exposure of ABP and its metabolites to HUC-PC cells caused changes in the proteomic profile [430], alteration of F/G-actin ratio [431], formation of DNA adducts [432] and instability of the genome [433]. Additionally, Hahn and associates [121] have shown that direct tumorigenic conversion of normal human epithelial cells can be achieved by combining ectopic expression of human telomerase catalytic subunit with SV40 large-T oncoprotein. Therefore, the enhancement of telomerase activity and cell proliferation demonstrated here supports our conception that the carcinogenic transformation of HUC-PC cells requires telomerase activation.

In the current study, basal and ABP-induced telomerase activities of HUC-PC were both significantly reduced in a dose-dependent manner. Inhibition of telomerase has been shown to limit human cancer cell growth by disrupting telomere maintenance [434]. It is also demonstrated that telomerase is essential for human tumor cell viability, whereas those cells with shortened telomeres are effectively and rapidly killed after inhibiting the telomerase enzyme [435]. However, a novel structural class of non-peptidic, non-nucleosidic, highly selective inhibitors of human telomerase can only limit cell proliferation after long-term cell culture [436]. There is no direct causal relationship between the telomerase inhibition and growth inhibition because sufficient cell divisions are required, for example, 23-26 doublings for HeLa cells [437], to decrease the proliferation after inactivation of telomerase. The shortening of telomeres is a cumulative process to achieve a threshold to uncap the telomeric ends in order to cause cellular senescence [436,438]. The GLe-induced telomerase inhibition is shown to be directly proportional to the growth inhibition after a 48-hour incubation.

COX-2 is rarely expressed in the normal urothelium of the urinary bladder [125,126,140]. HUC-PC cell line is negative for COX-2 expression, which is expected because of its non-tumorigenic characteristic. Recently, induction of COX-2 expression was successfully demonstrated in an in vitro arsenic-induced bladder carcinogenesis model [439]. This enzyme possesses anti-apoptotic properties that promote cancer development and progression [440]. Over expression of COX-2 is associated with a higher pathological stage of

bladder cancer [140]. In particular, elevated COX-2 expressions are commonly detected in patients with highly invasive tumors [441]. Expression of COX-2 is also related to the poor survival outcome of chemotherapy [442,443], as well as the recurrence in the cases of carcinoma in situ [444,445]. However, immunohistochemical methods revealed that, overall, 60-80% of bladder carcinomas are positive for staining of COX-2 [130,131]. Bladder tumors, in particular non-invasive types, can be negative for COX-2 expression. Transformation of HUC-PC cannot be excluded even when up-regulation of COX-2 was not seen in HUC-PC after exposure to ABP. Results are still important to report that COX-2 is not expressed in the pre-malignant HUC-PC cell line, eventhough after exposure to ABP.

In summary, ABP increases telomerase activity and promotes HUC-PC cell proliferation, as reported for the first time, which supports its tumorigenic transformation. The ability of ABP for tumorigenic transformation of HUC-PC has been well-proven in previous studies [64,75,76]. COX-2 is undetectable in HUC-PC even after exposure to ABP that cannot exclude transformation, because TCC patients could be COX-2 negative and COX-2 expression is correlated with tumor grades. Ethanol extract of G. lucidum (GLe) possesses potent growth inhibitory effects on the HUC-PC cells, in addition to the reduction of the basal and ABP-induced telomerase activity. These findings are novel and have not been reported before, and they support the chemopreventive activities of G. lucidum in TCC in the control of cell

growth and tumor development. However, the mechanisms accounting for the inhibition of telomerase by GLe is unknown and needs further investigations.

CHAPTER 9

G. LUCIDUM IS APOPTOTIC TO THE PRE-MALIGNANT HUMAN UROEPITHELIAL CELLS

9.1 INTRODUCTION

Chromatolysis describes a cell with a 'broken-up' nucleus that ultimately disappears, and was first reported by Flemming in 1885 [446]. Later in 1972, Kerr and colleagues named this phenomenon apoptosis, which means "falling off" in Greek [447]. Apoptosis is an active process of autonomous cellular dismantling, generating apoptotic bodies to be cleared through phagocytosis [448,449]. Specifically, it is programmed cell death involving genetic programs to produce instant cell suicide and is characterized by several morphological features such as cell shrinkage, preserved membrane integrity, budding and DNA condensation [446,449-451].

Apoptosis is fundamental for maintaining cellular homeostasis in normal tissues, while its dys-regulation may lead to pathological conditions, including cancer [452,453]. Evasion from apoptosis forms a hallmark in cancer cells for unlimited replication [454]. Events of apoptosis were first identified in response to cancer therapies in the mid 1970s [455,456]. Nowadays, the concept of apoptosis is widely applied in the design of chemotherapeutic as well as chemopreventive modalities [457].

Emerging evidence supports the theory that the process of apoptosis is not immunologically inert [446][458-460]. Brown et al. [365] suggested that free apoptotic cells in situ may be harmful. However, an interesting feature of apoptosis is its capability of clearing apoptotic bodies by means of phagocytosis, which causes no significant damage to the surrounding tissue architecture [446,457]. The cell debris of apoptotic bodies can be engulfed by both professional and non-professional cells [446].

In this chapter, apoptotic activity of G. lucidum was studied in the pre-malignant HUC-PC cells to address Objective 6 of the study. Normal HUC-1 cell line was also included in order to test the potential toxic effects of G. lucidum on normal tissue. Effects on the cell lines were compared with those challenged with ABP. Furthermore, the chemotactic effect on neutrophils was also studied to demonstrate the potential clearance of apoptotic cells.

9.2 MATERIALS AND METHODS

9.2.1 Annexin V-FITC/7-AAD apoptosis assay

Apoptosis induced by GLe during the 48-hour culture, in the presence or absence of ABP, was assessed using the Beckman Coulter Annexin V-FITC/7-AAD kit (Immunotech, France). Effects were compared between HUC-PC cells and HUC-1 cells. Additionally, dose-response effects were followed up with GLe at 0-100 μg/ml concentrations at 3, 12 and 48 hours of incubation. Cell analysis was performed on a Beckman Coulter COULTER®EPICS®XL™ (Miami, FL) equipped with the Elite software, version 5. A minimum of 10,000 events were collected and measured at FL1 (525nm) and FL4 (675nm). Cells incubated with PBS containing 3% of formaldehyde for 30 minutes on ice were used as positive control. A tube of untreated cells without staining was used as a negative control, and those with staining were used as a background control.

9.2.2 Isolation of polymorophonuclear neutrophils

The procedure is same as Laskin [461] as published. 20 ml of freshly heparinized blood was added into 4 ml of 6% dextran, mixed and allowed to sediment at room temperature for 45 minutes. The buffy coat was collected and washed twice in PBS for eight minutes at 1,000 rpm centrifugation. The cell pellet was resuspended in 20 ml PBS, and then 4 ml of Histopaque was layered on top. Neutrophils were retrieved in the cell pellet by centrifugation at 1,600 rpm for 30 minutes. The remaining red blood cells were lysed by pre-warmed ammonium chloride buffer with continuous shaking at 37°C for 15

minutes. The neutrophils were washed in PBS thrice, and resuspended in assay medium at 1 x 10^6/ml concentration for assay.

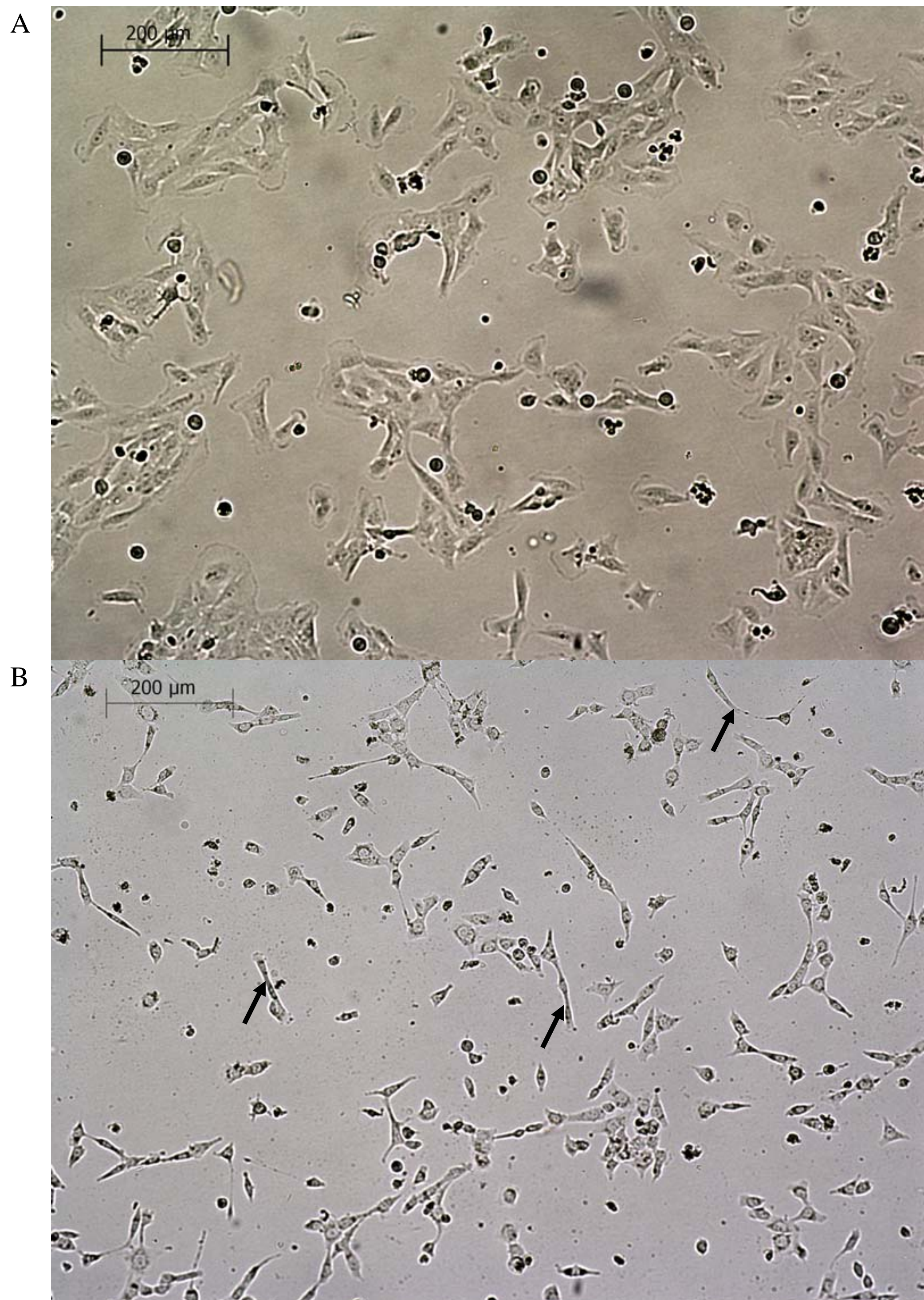
9.2.3 Cell migration assay

The Chemicon®QCMTM Chemotaxis 3µm 96-well Cell Migration Assay kit (Chemicon® International Inc., U.S.A. & Canada) was used to measure the chemotactic activities of the culture media. To start, 150 µl of culture medium, which was collected after a 48-hour incubation was added to the wells of the feeder tray and 100 µl of neutrophils were placed into the migration chamber. The plate was incubated at 37°C with 5% CO_2 for eight hours. The migration chamber plate was then transferred to an unused 96-well feeder tray containing Cell Detachment Solution, followed by incubation at 37°C for 30 minutes. Diluted CyQuant GR dye was added to the detached migratory cells from the migration chamber and the relative fluorescence signal was read on the TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria) with 480/520 nm filter set. Fresh assay media containing 100 µM ABP, 80 µg/ml GLe or GLw was used as a background control.

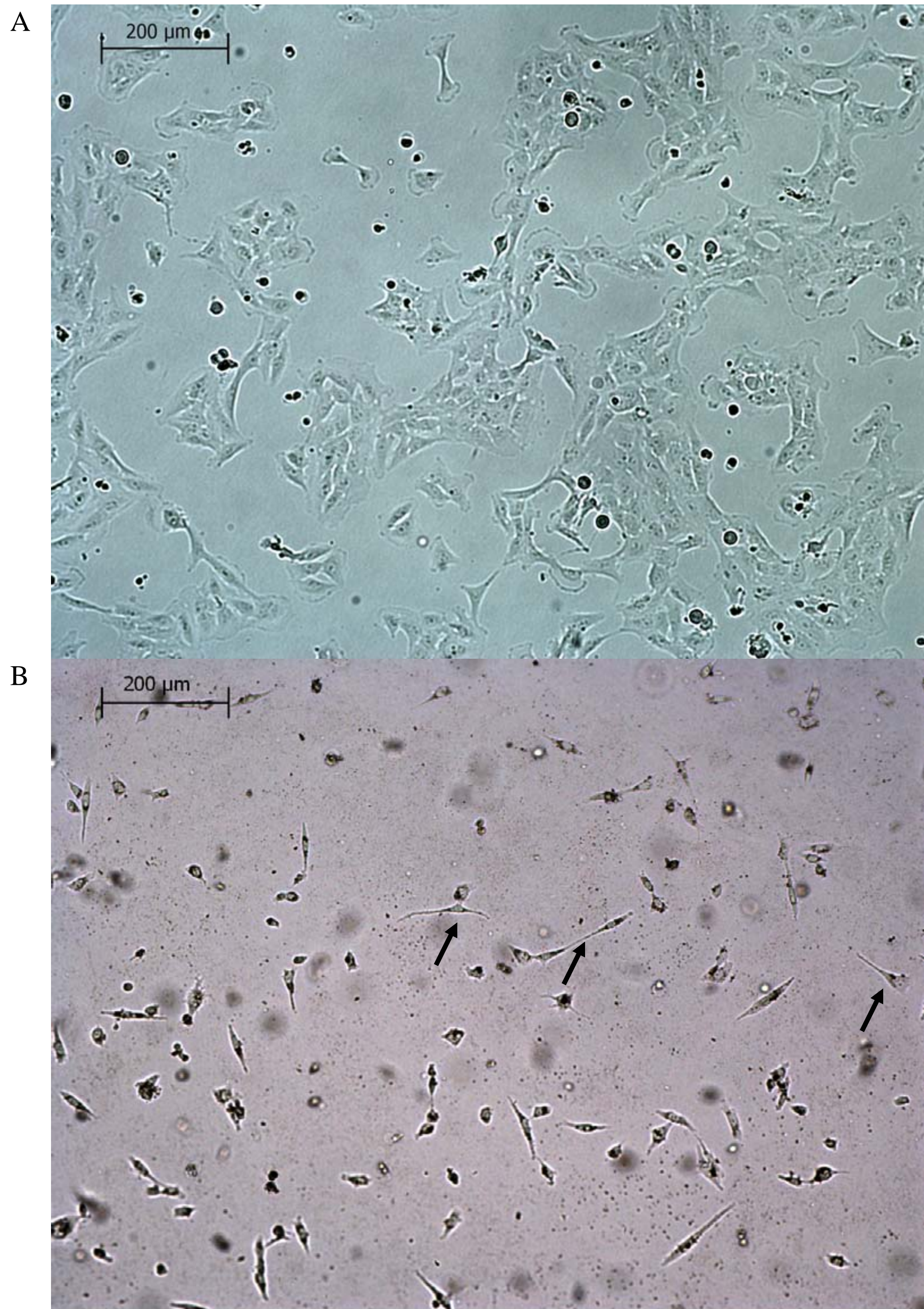
9.3 RESULTS

9.3.1 Apoptotic effects of GLe on HUC-PC cells

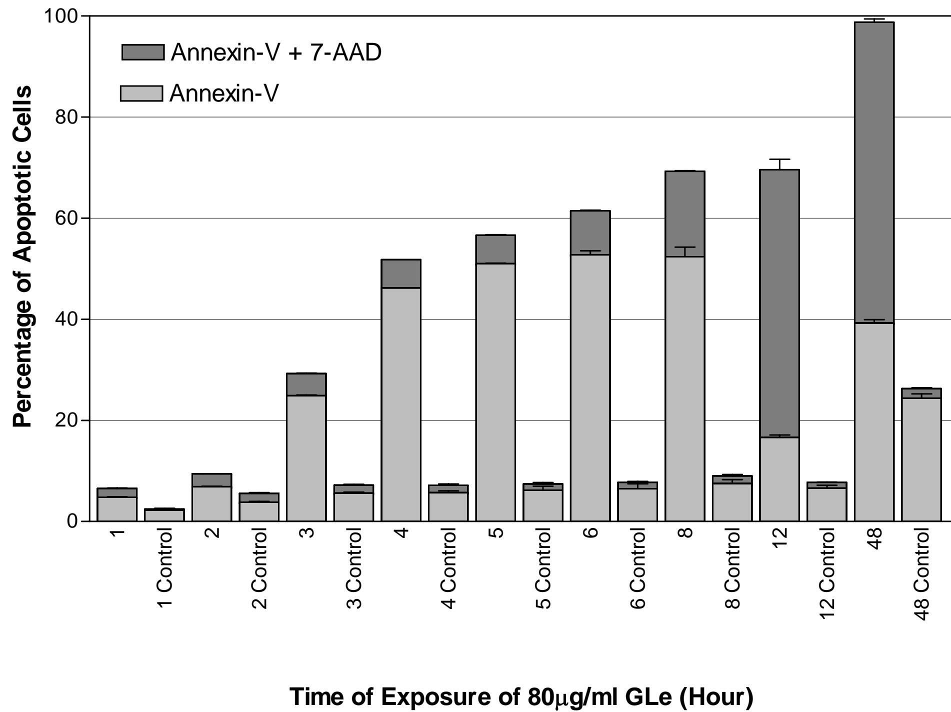
Cell shrinkage, elongation and blebs formation appeared in 48 hours for GLe-treated HUC-PC cell, when ABP was absent (Figure 9.1) and present (Figure 9.2). The effects of GLe on apoptosis in the absence (Figure 9.3) and presence (Figure 9.4) of ABP was demonstrated to be time-dependent and progressive from early phase expressing phosphatidylserine (PS) to late phase uptaking 7-amino-actinomycin (7-AAD) DNA specific dye. Figure 9.4 demonstrated the illustration of flow cytometric results. A gradual increase in apoptosis was observed between three and eight hours. The predominant phenotype of apoptotic HUC-PC cells switched from Annexin-V positive/7-AAD negative at eight hours to Annexin-V positive/7-AAD positive at 12 hours. Since the cell phenotype has been changed, the 24-hour time point was not performed. Subsequently, all experiments were done to the end point of 48 hours. Moreover, cell cultures were incubated for 3, 12 and 48 hours to determine the dose-dependent effect of GLe in the presence or absence of ABP. At both 3-hour and 12-hour test points, effects were in clear, dose-dependent manners (Figures 9.5 and 9.6). However, almost all cells were shown to be apaoptotic at 48 hours (Figure 9.7).



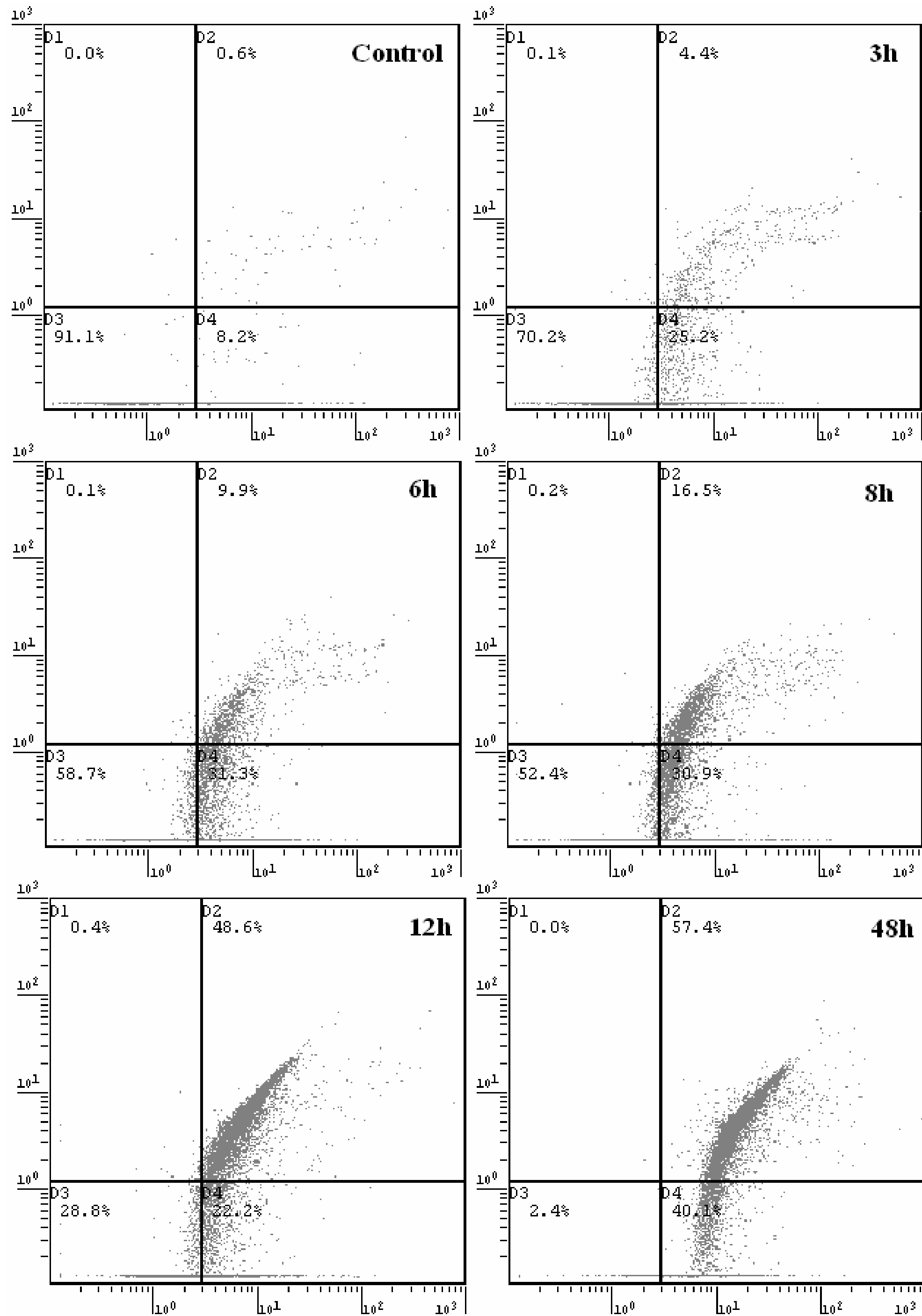
(Figure 9.1) Photographs of HUC-PC cells taken after the 48-hour treatment with (A) complete medium and (B) 80 µg/ml GLe in complete medium (arrows are typical examples of cells in apoptosis with shrinkage and elongation). Magnification: 100x.



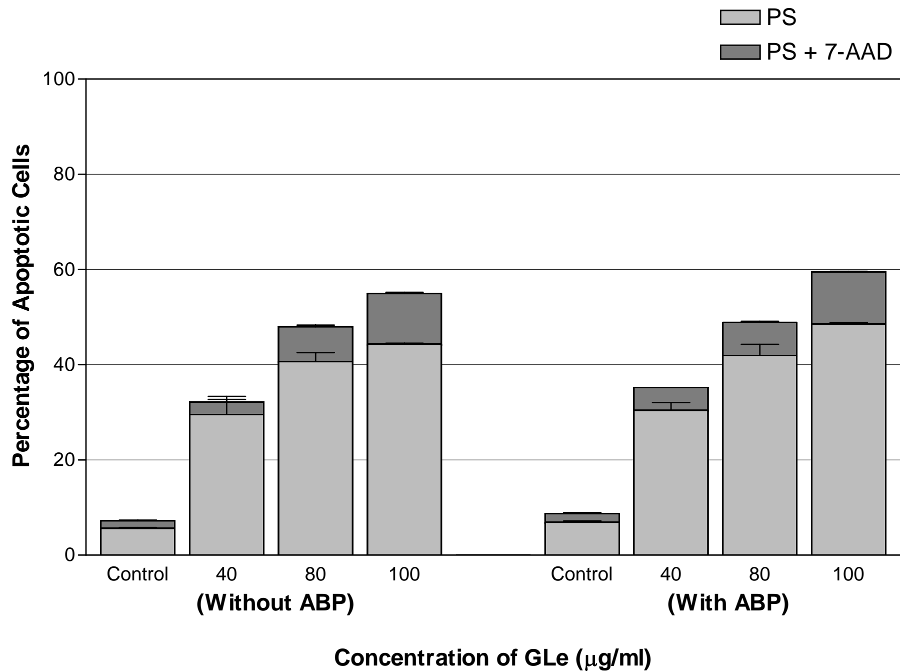
(Figure 9.2) Photographs of HUC-PC cells taken after the 48-hr treatment with (A) 100 µM ABP in complete medium and (B) 80 µg/ml GLe + 100 µM ABP in complete medium (arrows are typical examples of cells in apoptosis with shrinkage and elongation). Magnification: 100x.



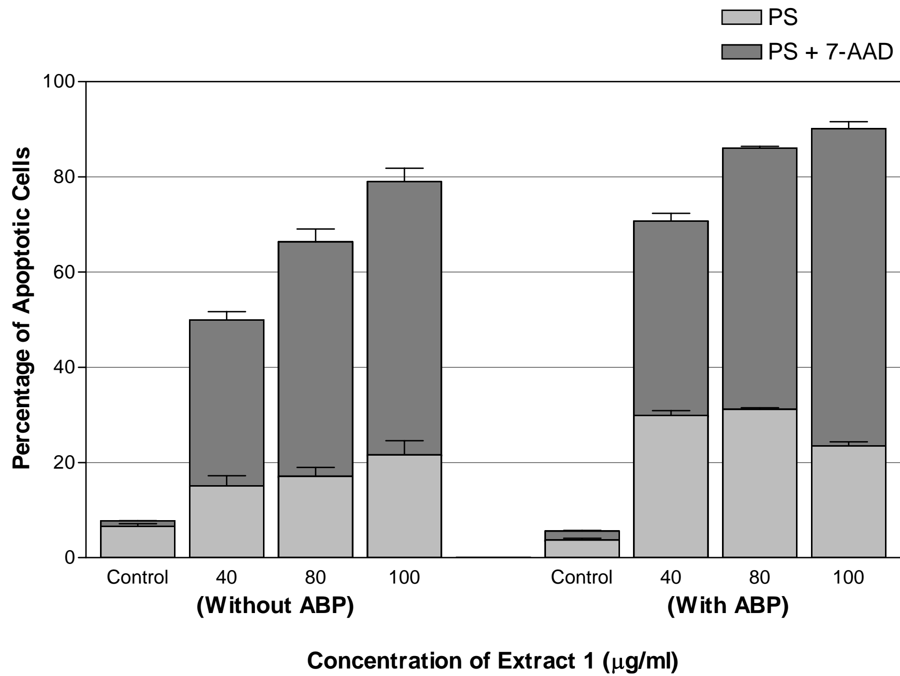
(Figure 9.3) The time-dependent progression apoptosis in HUC-PC cells induced by GLe. The HUC-PC cells were incubated with 80 µg/ml of GLe alone and apoptotic events were measured at 1, 2, 3, 4, 5, 6, 8, 12 and 48 hours. Early apoptosis stains positive with Annexin-V only; late apoptosis stains positive with both Annexin-V and 7-AAD. The cell phenotype was changed from early to late apoptosis inbetween 8 and 12 hours. Cells growing in complete medium containing 0.01% ethanol were used as control for each measured time-point. (Error bar: SEM).



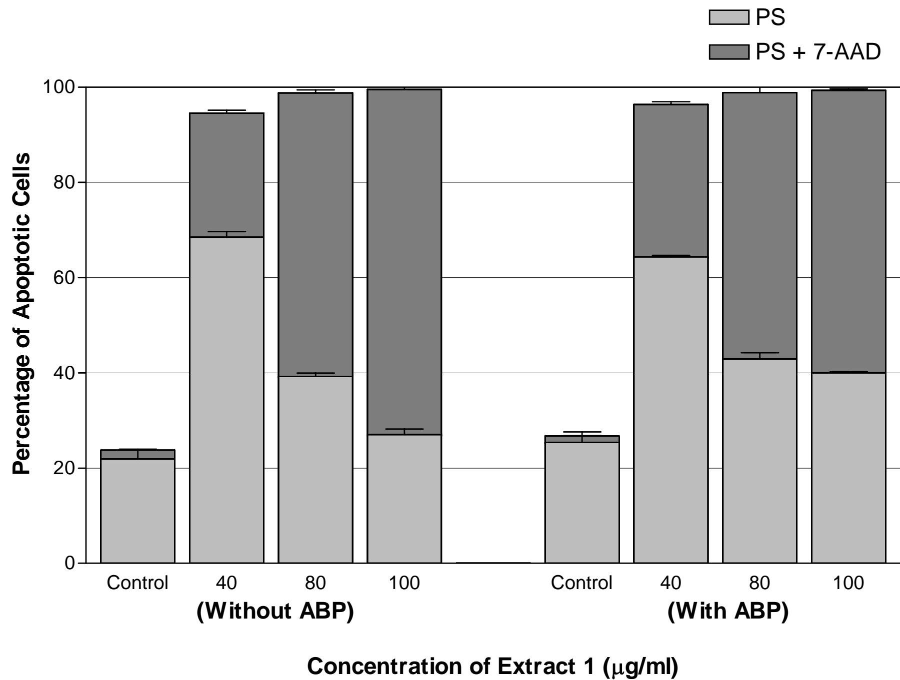
(Figure 9.4) Biparametric histogram LOG PMT2 (525nm) vs LOG PMT4 (675nm) showing the time-dependent progression of apoptosis of HUC-PC. HUC-PC cells were incubated with 80 μg/ml of GLe with 100 μM of ABP and apoptotic events were measured at 3, 6, 8, 12 and 48 hours.



(Figure 9.5) Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 3 hours. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "without ABP" group; cells growing in assay medium containing 100 μM ABP were used as control for the "with ABP" group (n=3, error bar: SEM).



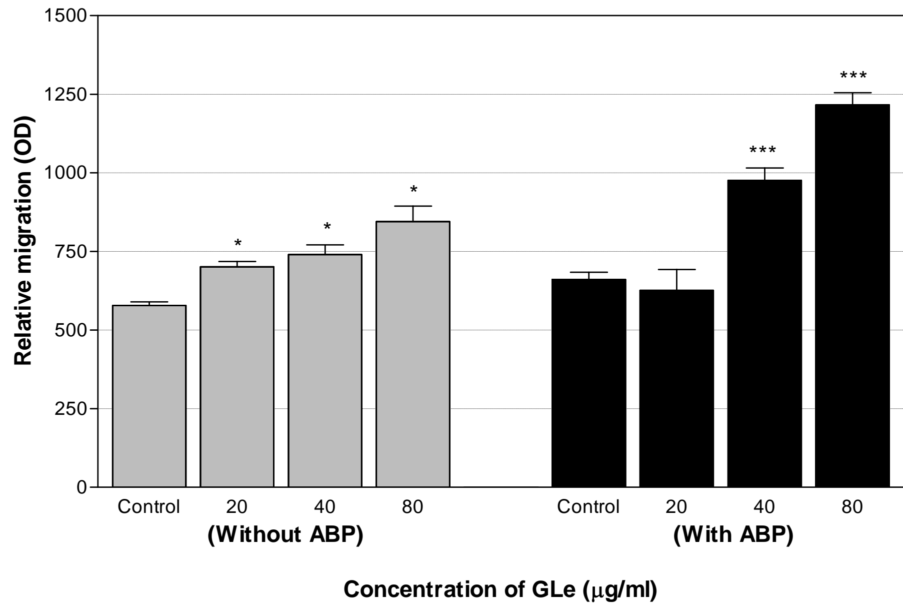
(Figure 9.6) Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 12 hours. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "without ABP" group; cells growing in assay medium containing 100 μM ABP were used as control for the "with ABP" group (n=3, error bar: SEM).



(Figure 9.7) Dose-dependent effects of GLe on apoptosis in the presence and absence of ABP measured at 48 hours. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "without ABP" group; cells growing in assay medium containing 100 μM ABP were used as control for the "with ABP" group (n=3, error bar: SEM).

9.3.2 Culture media of apoptotic HUC-PC cells are chemotactic to neutrophils

After a 48-hour incubation of HUC-PC cells with 0-80 µg/ml GLe, in the presence or absence of ABP, culture medium was collected and shown to be significantly chemotactic to isolated peripheral neutrophils in a dose-dependent manner (Figure 9.8). IL-8, GRO-α/CXCL1 and ENA-78/CXCL5 are common neutrophil chemokines released in the HUC-PC culture media. These chemokines were not measured in the current study, since prelimary data looked at the chemotactic effects of GLe in apoptotic HUC-PC cells instead of its mechanism.



(Figure 9.8) Dose-dependent chemotatic effects of HUC-PC culture media to neutrophils induced by GLe. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP for 48 hours. Culture media were harvested and measured immediately without delay. Cells growing in complete medium containing 0.01% ethanol were used as a control for the “without ABP” group; cells growing in assay medium containing 100 μM ABP were used as a control for the “with ABP” group (n=3, * P<0.05; ***P<0.001).

9.4 DISCUSSION

Apoptosis, as measured by morphological and cytometric changes, is evidenced to be the main cause contributing to HUC-PC growth inhibition induced by GLe. During the early to middle phase of apoptosis, the cells bind positively with annexin V, because of the loss of the asymmetry of cell membrane, which causes the exteriorization of anionic phospholipids-PS-form the inner leaflet [462,463]. Fluorescence-conjugated annexin-V is used for monitoring the externalization of PS [464]. The translocation of PS is revealed as downstream of mitochondria alterations and is capase-dependent [464,465]. At this point, the integrity of the cell membrane and most of the organelles are well preserved [449,450]. But the shape of cells becomes elongated and shrunk because of the dehydration which occurs due to dehydration in the cytoplasm [449]. By triggering a cascade of molecular events, nuclear chromatin condensation and nucleus fragmentation (karyorrhexis) occur [449,466]. Subsequently, there is loss of microvilli resulting in cell blebbing (i.e. contortions of plasma membrane) and release of “apoptotic bodies” [450,467]. At this point, the cellular structural integrity is no longer maintained and the cell membrane becomes permeable to cationic dyes such as trypan blue and 7-AAD [449,468]. As shown in the results, the apoptotic process induced by GLe progressed to late apoptosis, irrespective of the presence or absence of ABP, in a dose- and time-dependent manner in such a way that the cells were continuously losing the cell membrane integrity as demonstrated with the uptake of the 7-AAD DNA-specific viability dye. Ten percent of the HUC-PC cells were shown to be apoptotic at baseline while

25% the cells treated for 48 hours were in an early apoptotic (positive for Annexin-V only) stage (Figure 9.3). This is most likely due to nutritional deficiency in the incessant 48-hour culture.

The current results regarding apoptosis are consistent with other reports, indicating that complex extract or pure Ganoderma triterpenes are able to induce apoptosis in cancer cell lines, including the hematopoietic cell cells [259], the highly metastatic 95-D lung cancer cells [258] and the human hepatoma Huh-7 cells [261]. Disturbed balance of physiological growth control is one of the prerequisite criteria of cancer, indicating cells which are capable of escaping the programmed apoptotic cell suicide [451,454,469]. Despite apoptosis being a major mechanism of chemotherapy-induced cell death, any mistake in the tissue homeostasis is corrected by balancing cell proliferation and cell death [457,470]. For bladder cancer, shifting of adversed cells towards the pro-apoptosis is suggested to be helpful for improving the survival of patients who have a poor prognosis [471]. The apoptotic effects of GLe on normal HUC-1 were not studied, because same apoptotic mechanisms were expected between the HUC-PC and HUC-1 cell lines in response to GLe, since the two cell lines are isogenic but HUC-PC cells are 21 passages older than HUC-1 cells, as the former was derived from the latter.

The progression of apoptosis to the late phase is undesirable since cell rupture causes a release of noxious cellular contents, which results in tissue damage and inflammation [465,468]. Fortunately, several specific "*eat me*" signals,

including PS and annexin I, are expressed on apoptotic cells that facilitate recognition and phagocytic clearance [462,465]. In particular, PS is not just a recognition cue but also serves as a means for the binding of phagocytes [472]. In the connection with the cell recognition, macrophages are the predominant cell type to execute the phagocytosis of apoptotic cells [467]. The engulfment of apoptotic fragments of epithelial cells can be illustrated using immunohistochemical methods [466]. Besides, neighboring cells such as epithelial cells are also capable of engulfing apoptotic bodies [446]. Anti-inflammatory cytokines are actively produced during the process of apoptotic cell clearance [472]. Taken together, in the presence of such clearance mechanisms, inflammation and tissue damage are rarely seen [457,463]. As demonstrated here, the cultured media of HUC-PC cells, during apoptosis, are chemotactic to neutrophils. In vitro and in vivo evidences supported the important role of polymorphonuclear neutrophils in BCG prophylaxis [473]. It was reported that polysaccharides of G. lucidum enhance migration and phagocytic activities of neutrophils [474]. Such chemotactic action was shown to be mediated through the activation of PKC-associated pathways [474]. Apoptotic cells do not attract neutrophils, but rather chemoattractants, i.e. apoptotic bodies, released from dying cells [446]. The natural chemotactic role of G. lucidum could not be excluded. However, the release of PS from apoptotic cells and other chemoattractants may explain the chemotaxis. Phagocytic neutrophils are the major line of defense against bacterial infections and its accumulation is a hallmark of chronic inflammation [475]. Neutrophils contain respiratory burst, which is highly dangerous if the

contents are expelled into the extracellular milieu and cause necrotic damage [476]. A recent study has shown that neutrophils are able to accelerate the complete digestion of apoptotic cells by macrophages [477]. This process is known as "programmed cell clearance" that plays active roles in the resolution of inflammatory drawbacks [467,477]. In vitro evidence herein suggests the potential synergy of neutrophil recruitment and PS expression for programmed cell clearance.

GLe suppresses the HUC-PC cells through promotion of apoptosis. This is the first evidence demonstrating the apoptotic effects of G. lucidum on pre-malignant urothelial cells as well as the recruitment of neutrophils. The outcomes are desirable since the induction of apoptosis and the clearance mechanism resolves the problem of inflammatory response in most immunological-based therapeutic strategies such as BCG.

CHAPTER 10

CELL SIGNALING PATHWAYS INVOLVED IN G. LUCIDUM-INDUCED CHEMOPREVENTION

10.1 INTRODUCTION

Two distinct lines of evidence, presented here, support the chemopreventive effects of G. lucidum on HUC-PC cells: first, the potential synergistic effects with BCG regarding secretion of IL-6 and modulation of FN and GAGs and second, the induction of apoptosis and cell clearance to suppress the cell growth.

The promoter of the IL-6 gene contains binding sites for nuclear factor-kappa B (NF-κB), which is a transcription factor crucial for coordination of the immune response [478-480]. In response to BCG, the initiation of an NF-κB signal transduction requires FN occupancy to cross-link the α5β1 integrin receptor on the urothelium [164]. The critical roles of NF-κB in cancer development and progression have been disclosed in the recent decade [481-483]. On the other hand, Ca^{2+} is a universal, secondary intracellular messenger, whose changes in concentration affects diverse functions in almost all eukaryotic cells [312,484]. Immune response can be regulated by multiple isoforms of protein kinase C (PKC) that are activated by Ca^{2+} [485]. These molecules are all known to be involved in the control of apoptosis. Both NF-κB and PKC have dual properties of anti- and pro-apoptosis, depending on the nature of apoptotic stimulus and cell type [482,486-490]. And these processes can be Ca^{2+} dependent or independent [491].

G. lucidum decreased intracellular calcium ($[Ca^{2+}]_i$) in HeLa and caused G1/S phase arrest [251]. G. lucidum inhibited NF-κB activity, which resulted in suppression of cancer cell proliferation, angiogenesis, invasiveness and migration [249,257,267-269,492]. Triterpene-rich extract of G. lucidum also inhibited hepatoma cell growth through the down-regulation of PKC [254]. In contrast, G. lucidum activated NF-κB in humans to increase the production of TNF-α and IL-6, thus enhancing the innate immunity [493]. G. lucidum polysaccharides enhanced the neutrophil functions in phagocytosis and chemotaxis through the activation of PKC [474]

Depending on the actions to be taken, such signalling pathways can be on or off in response to G. lucidum. Both apoptosis and immunolgical functions are inseparable issues in G. lucidum's anticancer activities. For addressing Objective 7 of the study, NF-κB, PKC and Ca^{2+} were selected for studying and understanding mechanistic insight of the roles in the chemopreventive activities of G. lucidum.

10.2 MATERIALS AND METHODS

10.2.1 ELISA-EMSA for measuring NF-κB activity

HUC-PC cells were grown to 80% confluence, followed by a 48-hour incubation with GLe (0-100 μg/ml) or GLe + ABP (0-100 μg/ml GLe + 100μM ABP) in 100-mm tissue culture dishes. The cells were harvested to generate cell pellet by pooling two dishes for each study group. Nuclear extracts were prepared by a commercially available nuclear extraction kit (Chemicon International Inc., U.S.A. & Canada). Protein concentrations were determined using the BCA™ protein assay kit (Pierce, Rockford, U.S.A.), as presented in *Appendix IV-B*. ELISA-EMSA quantitation was performed using the Chemicon® NF-κB p50/p65 transcriptional factor assay colorimetric kit (Canada & U.S.A.). The manufacturer's instructions were strictly followed. 10 μg of nuclear protein was incubated with 2 pmol of biotinylated double stranded oligonucleotide probe containing a wild-type consensus sequence for NF-κB (5'-GGGACTTTCC-3') in microplate coated with Streptavidin. Then anti-NF-κB p50 primary antibody and anti-NF-κB p65 primary antibody were added, followed by an anti-IgG-HRP conjugated secondary antibody. Colour development was achieved with pre-equilibrated TMB/E substrate and absorbance was read at 450 nm using the TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). Replicated nuclear proteins for each study group were run in triplicate. Relative optical density (OD) values were compared with other test samples to obtain relative activities. TNF-α treated HeLa whole cell extract was used as a positive control. Specific NF-κB competitor oligonucleotide containing the same

consensus sequence as the capture probe was used as competitor control. Biotinylated double stranded oligonucleotide probe without wild-type activity was used as a negative control.

10.2.2 ELISA for measuring PKC activity

ABP-pretreated and non-pretreated HUC-PC cells were incubated with GLe (0-100 μg/ml) for 48 hours. Cytosolic total proteins of cells were extracted in a 100-mm tissue culture dish with 1 ml of lysis buffer [20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 μg/ml leupeptin and aprotinin] on ice for 10 minutes. Cells were scraped and collected into a pre-chilled microcentrifuge tube. Cell lysates were kept on ice and sonicated 3 x 20 second intervals, then centrifuged at 13,000 rpm for 15 minutes at 4°C. Total protein extracts were transferred into another pre-chilled microcentrifuge tube and protein concentrations were determined using Bradford method (Bio-Rad, CA, U.S.A.), as presented in *Appendix IV-C*. Stressgen's StressXpress® non-radioactive PKC kinase activity ELISA kit (BC, Canada) was used for PKC quantitation. Manufacturer's instructions were tightly followed. 10 μg of cytosolic protein was incubated with ATP in the PKC substrate microtitre plate, which followed by Phosphospecific substrate antibody and anti-rabbit IgG: HRP conjugate. Colour development was achieved with pre-equilibrated TMB/E substrate and absorbance was read at 450 nm using the TECAN SPECTRAFluor Plus microplate reader (TECAN

Austria GmbH, Grodig, Austria). Replicated cytosolic proteins for each study group were run in triplicate. Relative optical density (OD) values were compared with other test samples to obtain relative activities. 20ng and 40ng of active PKC were used as two-level controls.

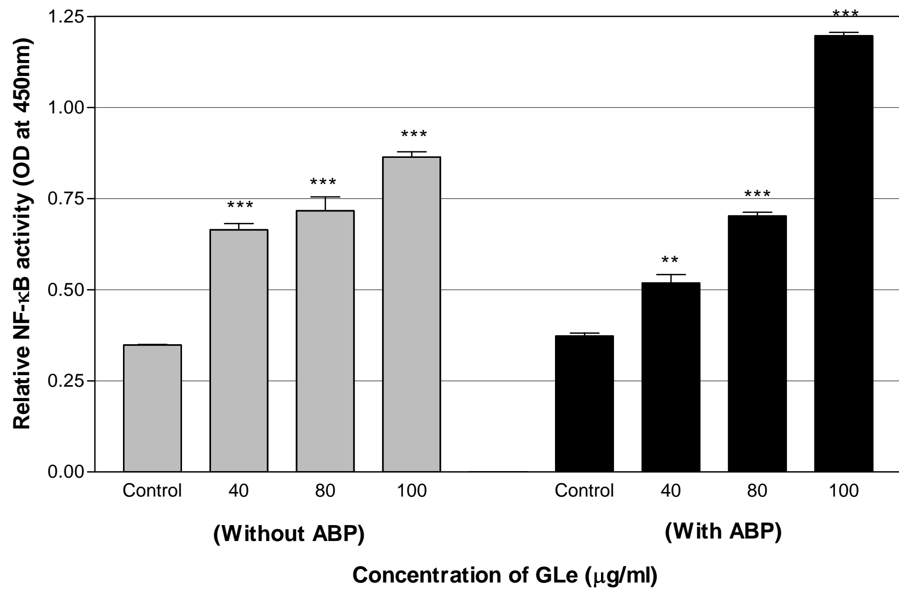
10.2.3 Fluo-4 biosensor for measuring intracellular calcium

HUC-PC cells (2.5 x 10^4 cells / well) were treated with GLe (0-100 µg/ml) in the presence or absence of 100 µM ABP for three hours in a black-colour lumox™ multiwell 96 tissue culture plate (Lumox™, Germany). Cultured cells were washed three times in PBS. Dye loading buffer is 1x HBSS containing 20mM HEPES at pH 7.0. 2.4 µM working Fluo-4 (Sigma) (containing 0.02% (w/v) of Pluronic® F-157 solution (Sigma) in dye loading buffer) was incubated with the cells in dark at 37°C for 45 minutes. The cells were washed three times and 100 µl of dye loading buffer were added for de-esterification at room temperature for 30 minutes. Fluorescence intensity was measured at emission 520 nm with excitation 485 nm using the TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). Replicated nuclear proteins for each study group were run in triplicate. Relative Fluorescence intensity values were compared with other test samples to obtain relative concentrations.

10.3 RESULTS

10.3.1 GLe enhances NF-κB activity in HUC-PC cells

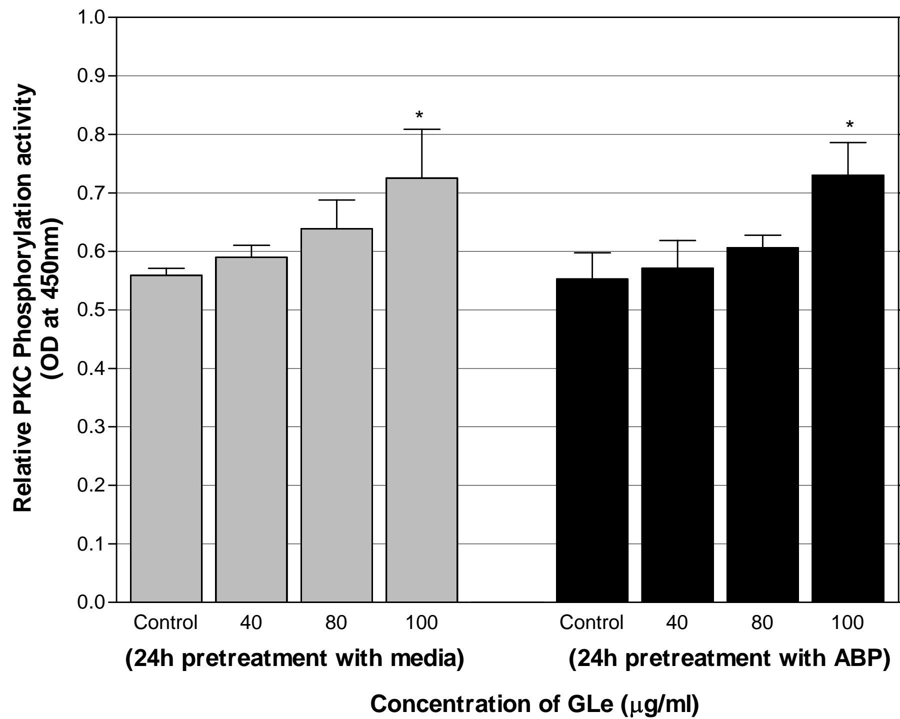
By measuring the nuclear extracts, NF-κB activity in HUC-PC with GLe was relatively and significantly ($P<0.001$) higher than that of control cells, in a dose-dependent manner (Figure 10.1). Similar trends were obtained whether ABP was present or absent. However, at 100μg/ml concentration, increase in the presence of ABP was more significant than in its absence. The ELISA-EMSA technique used for NF-κB involved anti-NF-κB p50 and anti-NF-κB p65 primary antibodies. Thus, the GLe-induced NF-κB binding activity in HUC-PC cells which can activate any p50 or p65 NF-κB dimers.



(Figure 10.1) Dose-dependent increase of DNA binding NF-κB activity in the nuclear extracts of HUC-PC cells induced by GLe. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP for 48 hours. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "without ABP" group; cells growing in assay medium containing 100 μM ABP were used as a control for the "with ABP" group (n=3, error bar: SEM, *** $P<0.001$; ** $P<0.01$).

10.3.2 GLe enhances PKC activity in HUC-PC cells

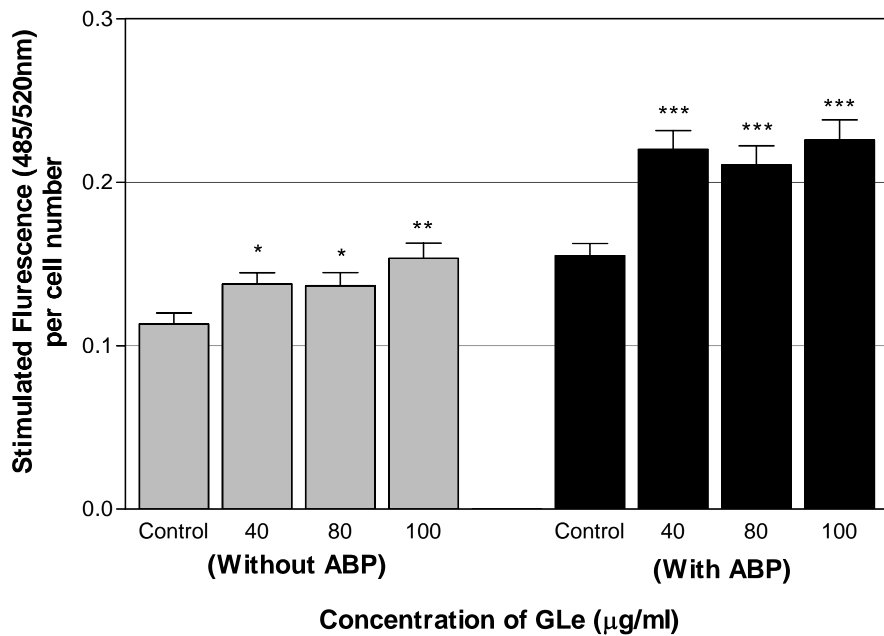
By measuring the cytosolic extracts, PKC activity in HUC-PC with GLe was relatively higher than that in control cells. Similar effects were obtained regardless of whether ABP was present or absent. Significant ($P<0.05$) increase of PKC activity was only observed at 100 μg/ml of GLe concentration. Furthermore, no difference was observed for PKC activity between ABP-treated and untreated cells (Figure 10.2).



(Figure 10.2) Dose-dependent increase of PKC activitiy in the cytosolic extracts of HUC-PC cells induced by GLe. The HUC-PC cells were pretreated with 100 μM of ABP or complete media for 24 hours, and followed by a 48-hour culture with 40, 80 and 100 μg/ml of GLe alone. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "media-pretreated" group; cells growing in assay medium containing 100 μM ABP were used as a control for the "ABP-pretreated" group. (n=3, error bar: SEM, * P<0.05)

10.3.3 GLe increases intracellular Ca^{2+} in HUC-PC cells

By using Fluo-4 biosensor for $[Ca^{2+}]_i$, fluorescence intensity in HUC-PC treated with GLe was relatively higher than that in control cells. The effects were shown to be more significant ($P<0.001$) and stronger when ABP was present. Furthermore, higher fluorescence signal was measured in cells treated with ABP, as compared to cells in pure complete media (Figure 10.3).



(Figure 10.3) Increase of $[Ca^{2+}]i$ in HUC-PC cells induced by GLe. The HUC-PC cells were incubated with 40, 80 and 100 μg/ml of GLe with or without 100 μM of ABP for 3 hours. Cells growing in complete medium containing 0.01% ethanol were used as a control for the "without ABP" group; cells growing in assay medium containing 100 μM ABP were used as a control for the "with ABP" group (n=9, error bar: SEM, ** P<0.01; * P<0.05).

10.4 DISCUSSION

The effects of GLe on nuclear NF-κB activity, cytosolic PKC activity and $[Ca^{2+}]_i$ were tested in the presence and absence of ABP. Results indicated that all these parameters were up-regulated. The up-regulation of NF-κB and $[Ca^{2+}]_i$ was more significant when ABP was present. These results are discussed in the following paragraphs.

NF-κB has a pivotal role in the control of carcinogenic transformation [374]. Bharti & Aggarwal [494,495] listed seven characteristics of NF-κB. NF-κB can be: (1) activated by carcinogens and tumor promotes; (2) involved in cell survival and cell proliferation; (3) involved in tumor cell invasion and angiogenesis; (4) constitutively expressed in tumor cells; (5) suppressed by chemopreventive agents; (6) activated to develop chemoresistance and (7) suppressed to sensitize the tumor cells to chemotherapeutic agents and to ionizing radiation. These properties place NF-κB as a promising target for chemotherapeutic strategies [494-496]. A number of NF-κB-DNA binding inhibitors are in the experimental stage for anticancer therapies [497]. Down-regulation of NF-κB activity has been extensively reported in relation to the anti-tumor effects of G. lucidum [248,249,257,269,492,498,499]. On the contrary, activity of NF-κB was up-regulated in the present study in HUC-PC cells. Under certain circumstances, NF-κB also acted as a promoter of apoptosis, depending on the cell type and nature of stimulus [482,486]. In several reports, activation of NF-κB is a pre-requisite for apoptosis, for example, in neuronal cells induced by glutamate, in human embryonic kidney

cell line triggered by serum withdrawal and in carcinoma cell line stimulated by Sindbis Virus [482]. Nonetheless, the pro-apoptotic role of NF-κB in chemoprevention is of biological importance. And the contradictory findings of G. lucidum on NF-κB activation may be explained by the concurrent induction of apoptosis and immunological activities, as demonstrated in earlier chapters.

NF-κB is an inducible transcription factor under the *Rel* family and is related to the proto-oncogene *c-Rel* [500]. There are five mammalian NF-κB/Rel proteins, namely, Rel(c-Rel), RelA(p65), RelB, NF-κB1(p50 and its precursor p105) and NF-κB2(p52 and its precursor p100) [500,501]. These proteins exist as homo- or hetero-dimer and remain inactive in the cytoplasm by bound inhibitory IκB proteins [310,502]. In the primary culture of human urothelial cells, the inactive NF-κB dimers are readily activated by different stimuli [329].

Upon stimulation, a specific IκB kinase will be activated to phosphorylate IκB, which is then poly-ubiquitinized for degradation by proteosome [500,501]. Subsequently, the NF-κB dimer becomes free and translocates into the nucleus, binds to DNA at target site and initiates gene transcription [500-502]. The promoter of the IL-6 gene contains a binding motif for NF-κB [330,478]. The dose-dependent induction of DNA binding activity of NF-κB in HUC-PC nucleus showed a possible pathway for the dose-dependent IL-6 production induced by GLe, as presented in Chapter 5. This is consistent with the earlier

studies that NF-κB activation by G. lucidum is essential for IL-6 secretion from human whole blood culture [493].

Additionally, the ELISA-EMSA method used here for measuring the DNA binding activity is specific to the putative p50 and p65 subunits of NF-κB [501]. Despite the fact that heterodimers of p50/p65 are the most abundant NF-κB in mammalian cells, several evidences support the binding specifity of the p50/p65 hetero- or homodimer in the IL-6 gene [329,330,478,503]. Both p50 and p65 bound avidly to the κB-like motif of the IL-6 gene in a human monocytic cell line [478]. Blockage of the transcriptional vectors encoding NF-κB p50 or p65 subunits efficiently inhibited the IL-6 expression [504]. Especially in human urothelial cells, translocation of p65 protein to the nucleus was demonstrated upon various immunological stimulations [329]. Therefore, IL-6 production in HUC-PC cells triggered by GLe is believed to be correlated and mediated through specific p65 and p50 NF-κB.

Is the apoptosis in HUC-PC induced by GLe (presented in Chapter 9) NF-κB dependent? The elevation of p50 and p65 NF-κB DNA binding activity is followed by p53 induction, whereby the inhibition of NF-κB in tumors may diminish the wild-type TP53-mediated apoptosis [487]. Thus, the induction of NF-κB-related apoptosis relies on p53 expression. Nonetheless, the p53 gene in HUC-PC contains one allele deleted and another one mutated, which has been proven for lacking wild-type p53 [60,84,505,506]. This is because the immortal cell line was transformed by SV40, whereas the p53 TSG is a

primary binding site for the SV40 large T antigen (TAg) [507]. The p53 TSG is a key regulator of cell cycle arrest in response to DNA damage [508-510]. Activation of NF-κB is known to be Ca^{2+} independent, which in relation to its apoptotic role is not modulated [503]. Up-regulation of $[Ca^{2+}]_i$ was demonstrated by G. lucidum herein. Therefore, the apoptotic effects of GLe on HUC-PC are suggested to be mediated by either p53-independent NF-κB pathways or other pathways. For example, up-regulation of p21 protein is a p53-independent pathway to induce G1/S phase cell arrest [511].

Proteasomal degradation of IκB is not required for PKC-dependent activation of NF-κB [497]. PKC belongs to a superfamily of serine/threonine-specific protein kinases, which play important roles in regulation of apoptosis, and has also been identified as a molecular target for cancer prevention [512]. PKC has capacities of promoting and inhibiting apoptosis depending on which isofrom(s) is triggered [488]. There are eleven PKC isoforms that can be subdivided into three classes: conventional (PKC-α, -βI, -βII, -γ), novel (PKC-δ, -ε, -θ, -η, -μ) and atypical (PKC-ξ, -ι/λ) [489,513]. Both the conventional and novel PKC subtypes are responsive to the second messenger diacylglycerol (DAG) [489,514]. Among all PKC isozymes, only conventional isozymes are Ca^{2+} dependent [489,514]. Referring to the results of Chapter 9, apoptosis has been confirmed as the main role for the growth inhibitory effects of G. lucidum on HUC-PC cells, whereas externalization of PS, a phospholipid, was clearly shown to occur in a dose- and time- dependent manner. Apart from its role on phagocytosis, PS, as an enzyme cofactor, is

also a specific activator of conventional PKC isoforms [463]. Activation of PKC-α has been related to apoptosis in prostate and myeloid cancer cells induced by genotoxic agents [515]. Whether PKC-α is activated by GLe needs further investigation. On the other hand, PKC-δ, having a 40-kd fragment of the novel isoform cleaved by caspase 3 as a key mediator, is the main PKC subtype with pro-apoptotic and growth inhibitory functions, in most cell types, in response to various extracellular stimuli [489,516]. The role of PKC in cell cycle control has been extensively reviewed [517]. It is known that activated PKC-δ decreases mitochondrial membrane potential, resulting in the release of cytochrome c, which ultimately leads to caspase activation and apoptosis [518]. A positive feedback loop of PKC-δ and caspase-3 activation was recently demonstrated by Reyland et al. [515]. Activated PKC-δ inhibits DNA repair and thus enhances DNA fragmentation in apoptosis [519]. Therefore, the activation of PKC by GLe is expected in the process of apoptosis.

Furthermore, the results indicated that $[Ca^{2+}]_i$ was also increased by treating the HUC-PC with GLe. Ca^{2+} is a universal secondary intracellular messenger, playing roles in almost all intracellular signalling mechanisms [312,484]. In resting cells, free Ca^{2+} ion is kept at low concentrations by ATP-driven Ca^{2+} pumps [484]. Ca^{2+} has regulatory activities on protein phosphorylation and dephosphorylation [520]. Such Ca^{2+} mobilization results in an increase of nuclear Ca^{2+}, which is ATP-dependent, and attributed to a selective increase in Ca^{2+} permeability through nuclear pores and/or up-regulation of Ca^{2+} transport from perinuclear pools [488,491]. Moreover, $[Ca^{2+}]_i$ is then targeted to activate cation-dependent endonucleases to cleave nuclear chromatin or

alter the conformation of nuclear chromatin to make it more accessible to endonuclease cleavage [491,521]. In Ca^{2+}-mediated apoptosis, a series of enzymes are activated, including endonuclease, protease, transglutaminase and phospholipase. Further, changes in ionic and water fluxes through plasma membrane causes condensation of cytosol [488]. This targets the core mitochondrial machinery of apoptosis to stimulate effector caspases, thereby activating downstream apoptosis substrates, such as PKC isozymes [453]. Elevation of $[Ca^{2+}]_i$ is one of the key features for apoptosis such that the γ isoform of phospholipase is phosphorylated and tyrosine protein kinase, such as PKC, is activated to hydrolyze phosphoinositide, resulting in D-*myo*-Inositol 1,4,5-trisphosphate ($InsP_3$) production and mobolization of Ca^{2+} from the endoplasmic reticulum and extracellular milieu [521,522]. In addition to $InsP_3$, there are at least two more Ca^{2+}-mobilising intracellular ligands, known as cyclic adenosine diphosphoribose (cADPR) and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), that have been identified to alter Ca^{2+} mobilization, upon stimulation [484].

Apoptosis is the major chemopreventive mechanism of GLe in human uroepithelial cell carcinoma. At 3 hours after incubation with GLe, $[Ca^{2+}]_I$ was increased, which is a typical feature during the early stage of apoptosis. on longer incubation, i.e. 48 hours, PKC and NF-κB were also increased in these apoptotic cells. Since NF-κB-dependent apoptotic event are commonly p53-dependent and Ca^{2+} independent, the HUC-PC is proven to be lacking wild-type p53 gene and $[Ca^{2+}]_i$ was increased, and therefore NF-κB is unlikely to be involved in the GLe-induced apoptosis. On the other hand, the increase

of NF-κB can be correlated with the induction of IL-6 by GLe, because the promoter of IL-6 gene contains a binding site for NF-κB. Furthermore, a Ca^{2+}-dependent PKC pathway is seen to be involved in the GLe-induced apoptosis. Effects of GLe on NF-κB, PKC and $[Ca^{2+}]_i$ were not studied in the present study, because the same mechanisms were expected between HUC-PC and HUC-1 cell lines in response to GLe, since the two cell lines are similar to each other in many molecular aspects. Apart from the tested signalling molecules, mitogen-activated protein kinases and epithelial growth factors are related to the bladder carcinogenesis that should also be investigated in the future. In summary, the present study provides mechanistic insights into the cellular signalling pathways, and further investigation is needed for confirmation.

CHAPTER 11

SUMMARY
AND FURTHER DISCUSSION

11.1 SUMMARY OF FINDINGS

A total of 16 parameters, classified into seven functional categories (*Figure 4.1 on page 79*), were measured for assessing the chemopreventive activities of G. lucidum on human uroepithelial cell carcinoma. All findings were positive except for the expression of COX-2, and these results were grouped, as illustrated in Figure 11.1, and summarized as follows.

11.1.1 HUC-PC is sensitive to ABP

HUC-PC cell line has been proven to be sensitive to ABP in vitro, transformed tumorigenically and induced tumors in nude mice in vivo [74,76]. In response to ABP, the proteomic profile [430] and F/G-actin ratio [431] of HUC-PC were altered, and resulted in the formation of DNA adducts [432] and instability of the genome [433]. The present study provides additional knowledge that telomerase activity of HUC-PC was also enhanced by ABP together with increased proliferation.

11.1.2 G. lucidum is cytotoxic and active in growth inhibition

G. lucidum is cytotoxic to HUC-PC, but GLe is at least five-fold more potent than GLw. At non-cytotoxic levels, GLe exhibited growth inhibitory effects on HUC-PC cells. These results are consistent with previous studies reported by Lu et al. [252]. Additional novel findings, contributed by this research, include the following: firstly, all cytotoxic effects and growth inhibition exerted by GLe were not compromised by the addition of ABP in the cell cultures and secondly, all these effects were shown to be less potent in the parent HUC-1 cells. Intravesical cytotoxic treatment is commonly used for prophylaxis of superficial TCC because of the efficiency of drug delivery near the site of action and the reduction of systemic toxicity [366]. These in vitro properties support the intravesical use of GLe in prevention of bladder cancer recurrence.

11.1.3 Dual roles of G. lucidum's antioxidants

Both GLe and GLw possess significant antioxidant activities, and the antioxidant contents in the culture media were enhanced by addition of these extracts. Therefore, the cells should be protected from oxidative stress and DNA damage [293]. However, in the present study, GLe and GLw exhibited dual roles of inducing and inhibiting oxidative stress, respectively. These findings are novel in that GLe increased the H_2O_2 concentration in the culture media and induced the formation of 8-OHdG in the HUC-PC cells, which were undergoing apoptotic events. In contrast, GLw protected the HUC-PC cells from 8-OHdG formation. The results are difficult to explain using the

current knowledge, and a more intensive study should be undertaken regarding this issue.

11.1.4 Telomerase-associated apoptosis involving PKC and intracellular Ca^{2+} as the main growth inhibitory mechanism

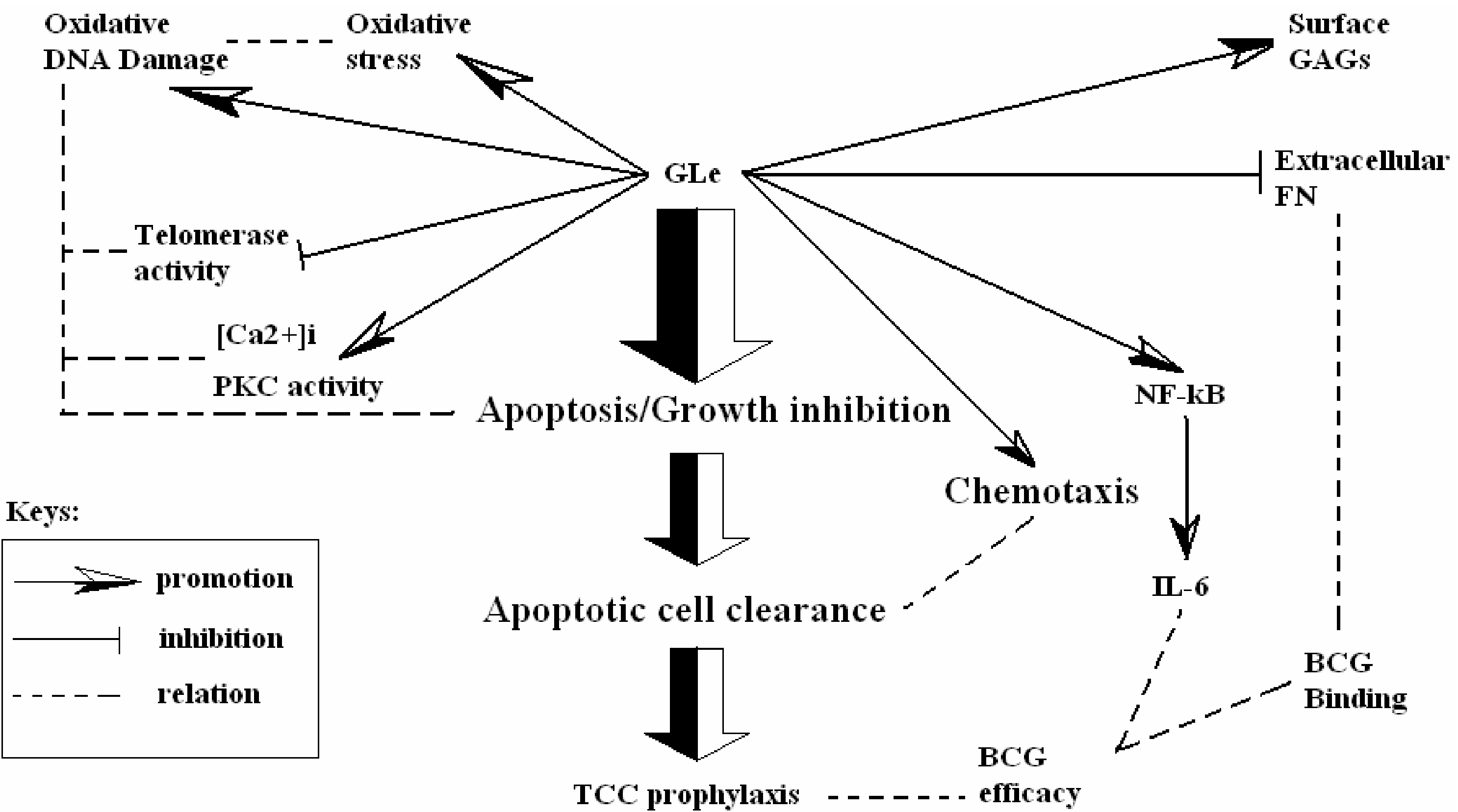
Both basal and ABP-induced telomerase activity in HUC-PC cells were significantly inhibited by GLe. These inhibitory effects were correlated with the markedly apoptotic effects, which is the main mechanism for GLe's growth inhibition on HUC-PC cells. Moreover, the apoptotic events were mediated by up-regulation of PKC activity and intracellular Ca^{2+} ions. The inhibition of telomerase activity is a novel finding for G. lucidum. The apoptotic effect is consistent with earlier reports on cancer cells of various sites [258,259,261]; however, this is the first time it has been demonstrated on urothelial pre-malignant HUC-PC cells.

11.1.5 G. lucidum is immunologically active

This ground-breaking research has shown that the HUC-PC cell line is responsive to BCG for IL-6 production, indicating the capability of BCG internalization by the HUC-PC cells. However, similar effect was shown by GLe at a lower (ten-fold) concentration, which was mediated through the NF-κB signalling pathway. IL-6 contains a diverse range of biological activities, including the activation of IL-2 production for triggering cell-mediated immune response [523].

11.1.6 G. lucidum may facilitate BCG efficacy

Increased extracellular FN concentration is related to the failure of BCG immunotherapy [175]. Both GLe and GLw were shown to reduce the FN levels of culture media of HUC-PC cells. On the other hand, the bladder urothelium is protected by a layer of highly anionic GAGs, whereby subsequent damage is associated with BCG-related side effects, such as cystitis and infections [352,353]. G. lucidum increased the membrane-bound GAG concentration of HUC-PC cells. Taken together, G. lucidum may enhance the efficacy and reduce the side effects of BCG.



(Figure 11.1) A summary of G. lucidum's chemopreventive effects tested on the HUC-PC cells. Apoptosis is the core mechanism, whereas the potential synergistic effects on BCG efficacy are also demonstrated.

11.2 JUSTIFICATION: G. LUCIDUM -- CARCINOGENIC OR ANTI-CARCINOGENIC?

Multiple mechanisms are involved to line up the chemopreventive effects of G. lucidum on HUC carcinoma. However, some of the measured parameters are subject to debate because of their roles in carcinogenesis versus anticancer. The first concern involves extracellular H_2O_2 and 8-OhdG formation in HUC-PC cells that are induced by GLe, as presented in Chapter 7. H_2O_2 is an important ROS that readily attacks DNA to trigger mutagenesis and promote cancer development and progression [66,145,524]. Antioxidants act against oxidative stress and are suggested for cancer prevention [373,375]. Furthermore, activities of PKC and NF-κB were both enhanced by G. lucidum as presented in Chapter 10. One may argue that many anticancer agents target to inhibit PKC and NF-κB for induction of apoptosis [139,488,494,495,525]. Many isoforms of PKC are activated during the process of carcinogenesis [485,489]. ROS [497,526] and DNA damage [527] are known activators for NF-κB, which is a key nuclear factor for activating genes that mediate tumoriegnesis and metastasis [495]. Moreover, ROS are potent stimuli for NF-κB signalling transduction in cell transformation [374]. With these evidences, is G. lucidum carcinogenic?

To answer this question, evidence is provided from other parts of the current research as well as from other studies as follows: (1) Evidence from most recent studies support the induction of apoptosis by oxidative stress as new treatment paradigm for cancer [404]. (2) Both PKC and NF-κB have dual

roles in apoptotic and anti-apoptotic processes, depending on cell type and the nature of stimulus. If G. lucidum is considered as a stimulus, the apoptotic roles of PKC and NF-κB may be exerted in the HUC-PC cell line. (3) However, the activation of NF-κB is correlated with the induction of IL-6, as discussed in Chapters 5 and 10. (4) Activation of PKC is documented to be associated with the expression of COX-2 [139] and telomerase [528] and other malignant-related molecules [488]; however, as discussed in Chapter 9, COX-2 expression was not detected while inhibition of telomerase by G. lucidum was demonstrated in HUC-PC. (5) The most interesting results, presented in Chapter 9, indicated that the induction of apoptosis is the main role in growth inhibition of HUC-PC cells by G. lucidum, which is remarkable and clear. And, (6) further evidence from Chapters 5 and 8 conclusively demonstrate that telomerase inhibition and fibronectin reduction, as well as the increase of GAGs on cell surfaces, protect the HUC-PC from chemical attacks. These pieces of evidence strongly support the multiple chemopreventive activities of G. lucidum to the pre-malignant HUC-PC cell line. G. lucidum is a novel source of chemopreventive agent for uroepithelial cell carcinoma.

11.3 THE CONCEPT OF CELLULAR SENESCENCE

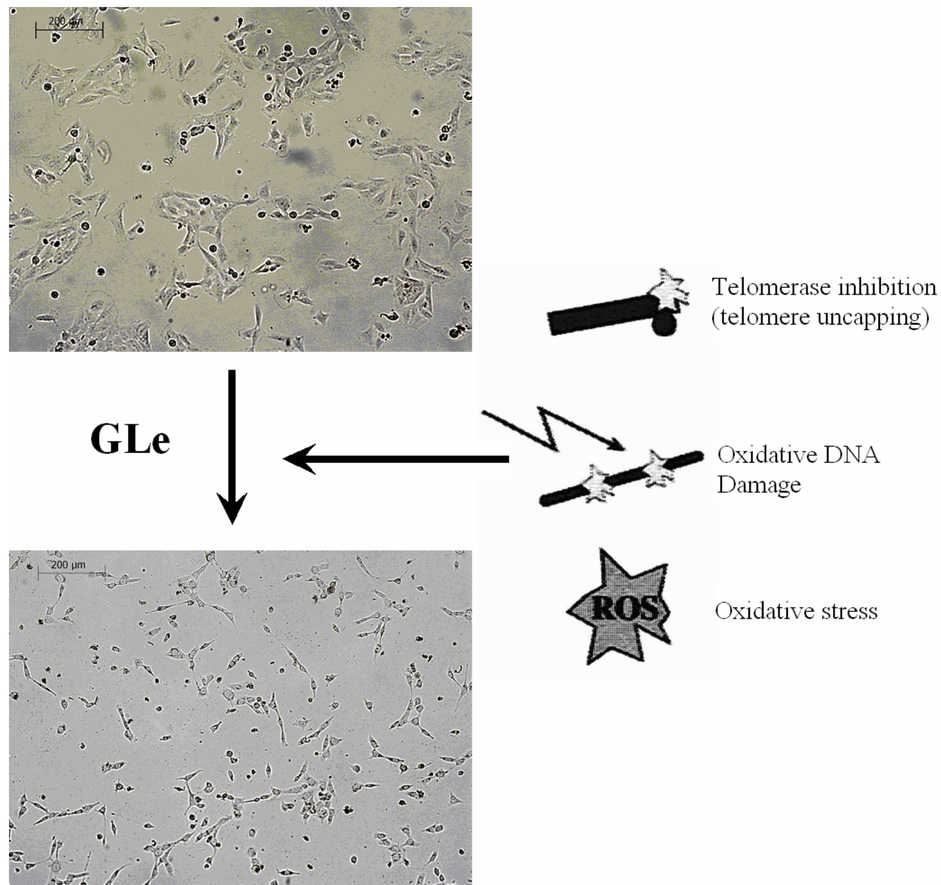
Normal cells have a finite number of cell divisions, an age, if you will, when their replicated capacity is exhausted [109]. Experimental evidences from Hayflick and others [109,529-532] consolidate the concept of replicative senescence in aging and mortality. Mortality is normal, immortality is

abnormal, which is true in the sense that cancer cells must breach the biological clock of senescence to become immortal [454,533]. Science tells us that telomeres are crucial genomic elements, capping the chromosomes, protecting them from degradation, and thereby maintaining the integrity and stability of genomes [534]. Shortening of telomeres during mitosis provides the cell a countable measure for replicative senescence [531,535,536]. On the other hand, the enzyme telomerase repairs the shortened telomere, and is highly implicated in carcinogenesis because of protecting the cells from programme apoptotic cell death [109,418,419]. Expression of telomerase plays a crucial role in about 90% of tumor cells [537]. In the present study, the apoptotic effects and inhibition of telomerase activity was clearly demonstrated by GLe. What is the link or missing link between telomerase activity and apoptosis?

It is now clear that telomere shortening alone is insufficient for triggering senescent arrest [531]. Telomere shortening might act like a semaphore to signal replicative senescence but it is stress-dependent [536,538,539]. There are multiple types of stress that can induce cells to undergo senescence, and one is a DNA-damaged pathway [539]. Many studies have shown that oxidative stress plays a major determinant for rate of telomere shortening [540]. ROS can induce DNA damage and accelerate the rate of loss of telomeric DNA, which involves uncapping the chromosome and results in cellular senescence [110,539,541]. Direct evidence has been shown that ROS accelerates telomere shortening to trigger cell cycle arrest [542]. In the present

study, oxidative DNA damage and oxidative stress were induced by GLe in HUC-PC cells, whereas the telomerase was also inhibited. This could also be explained at the molecular level that, when telomerase protection is deficient, the hexameric repeats of telomeres containing a triple-G-rich (TTAAGGG) structure are highly susceptible and are possible targets for spontaneous attack by ROS that causes DNA damage [110,543-545]. In such situations, telomere shortening may be accelerated and is unlikely to be compensated for when telomerase activity is being suppressed. Despite their roles in carcinogenesis, ROS are natural regulators of multiple cell signaling genes, including NF-κB and PKC [546,547]. PKC is one of many apoptotic substrates that is downstream to the caspases subsequent to the ROS death stimulus [453,489]. Moreover, ROS have been shown to modulate apoptotic cell clearance [548]. Current evidence supports the involvement of PKC in GLe-induced apoptosis of HUC-PC cells. Figure 11.2 illustrates the possible mechanism of cellular senescence in HUC-PC cells induced by GLe.

The combination of telomerase inhibition and DNA damage has been proposed to be a novel anticancer strategy [544]. The actions of GLe support the mechanistic insight of cellular senescence that indicate that oxidative stress could be an extrinsic stimulus to accelerate the telomere shortening in combination of reduced telomerase activity, followed by apoptosis. Last but not the least, the concept of cellular senescence is still a “hypothesis” in its applications in chemoprevention.



(Figure 11.2) An illustration for proposing the conceptual cellular senescence as G. lucidum's chemopreventive strategy. Three types of stress induced by the ethanol extract of G. lucidum can trigger HUC-PC cells to undergo senescence (adpoted and modified from Ben-Porath and Weinberg [539]).

CHAPTER 12

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the hypothesis of the present study has been proven to be true. G. lucidum contains chemopreventive activities towards pre-malignant human uroepithelial cells undergoing carcinogen-induced transformation.

New knowledge has been gained from the current study. Bioactive components for bladder chemoprevention are available in the ethanol fraction of G. lucidum. G. lucidum was shown to be immunologically active in the pre-malignant HUC-PC cells, by inducing interleukin-6 production via NF-κB signalling pathway. G. lucidum also reduces extra-cellular fibronectin concentration and increases GAG expression on the HUC-PC cell surfaces. These findings, supports the potential synergism of G. lucidum with BCG being used for preventing bladder cancer recurrences. The principle chemopreventive mechanism of G. lucidum relies on growth control of pre-malignant HUC-PC cells through the process of apoptosis. In particular, at 80 μg/ml concentration with 1 x 10^6 cells, G. lucidum demonstrated absolute cell growth inhibition (by trypan blue viable cell counting) without release of LDH (biomarker for direct cell killing). G. lucidum also showed selective cytotoxicity to pre-malignant cells (stronger cell killing effect by LDH and apoptosis) than to the normal HUC-1 cells

(weaker cell killing effect by LDH and apoptosis). Additionally, G. lucidum promoted chemotaxis of neutrophils, which possibly facilitates the clearance of apoptotic HUC-PC cells. These evidences supported the desirable chemopreventive activity of G. lucidum, with minimal side effects of general cytotoxic-based chemopreventive agents. Regarding the side effects and the unsatisfactory efficiency of conventional BCG, results collected herein supports the short-term planning of a large cohort clinical trial of using G. lucidum as a supplement to BCG immunotherapy.

Can G. lucidum be a single chemoprevetive agent to substitute conventional BCG? Combined results of telomerase inhibition, oxidative stress and signalling pathways involving Ca^{2+} and PKC, supports the hypothesis of cellular senescence for G. lucidum as new strategy for bladder cancer chemoprevention. Interestingly, significant antioxidant activities were measured in G. lucidum extracts; however, water extract of G. lucidum protected HUC-PC from oxidative DNA damage, and conversely, the ethanol extract induced oxidative stress and oxidative DNA damage. It should be noted that the induction of oxidative DNA damage was only observed when ABP was present. The current knowledge base is insufficient to explain these results adequately, indicating more research is necessary for conclusive information. In particular, the mechanism of the oxidative DNA damage and oxidative stress induced by G. lucidum should be studied in depth.

G. lucidum, alone could be a novel source of chemopreventive substances and as a long-term goal this would be relevant when additional evidences become available. Up-stream and down-stream pathways of PKC and Ca^{2+} mobilization should be focused to fully define the underlying mechanism of G. lucidum's apoptotic and immunological activities. The effects of G. lucidum on BCG binding related to fibronectin and GAGs should be further explored at transcriptional and translational levels. Likewise, the protective mechanisms of GAGs should be followed-up. The effect of telomerase on telomere shortening, as induced by G. lucidum, should also be addressed. Currently, a collaborative study with Harvard Medical School involving ex vivo organ culture technique using mouse bladders is being conducted to address these issues.

Appendi x I

[A] Complete media for HUC cell culture

Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham containing:

2 mM L-Glutamine,

15 mM HEPES,

10% (v/v) FBS,

1% (v/v) penicillin and streptomycin,

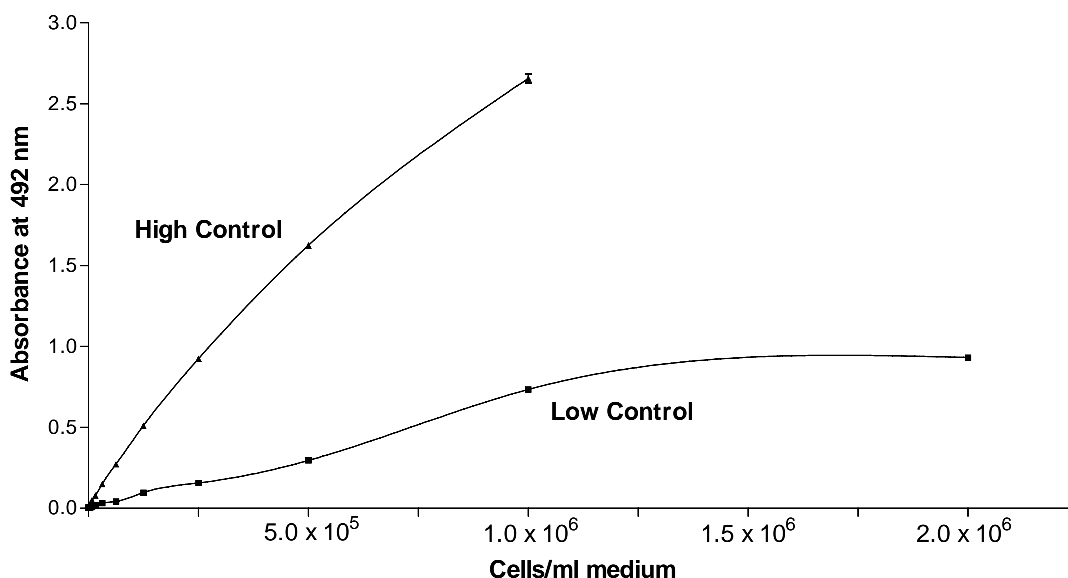
7.5% (w/v) sodium bicarbonate,

prepared with MilliQ water and sterilized by filtration using a membrane with a porosity of 0.22 micons, with final pH 6.9.

Appendix II

[A] Optimization of LDH assay

High control = 100µl cells + 100µl 2% Triton X-100 assay medium
Low control = 100µl cells + 100µl 1% FBS assay medium

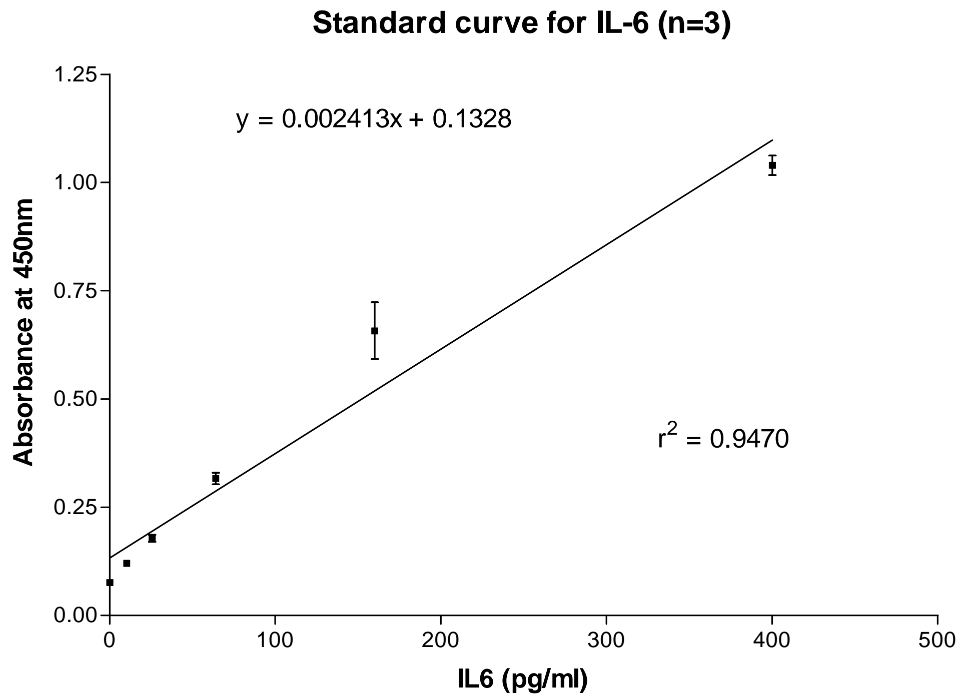


Cells/ml medium	Low control			High control		
	Y1	Y2	Y3	Y1	Y2	Y3
122.1	0.00960	0.00280	0.00190	0.00870	0.00740	0.0060
244.1	0.00270	0.00650	0.00240	0.00870	0.00630	0.0074
488.3	0.00330	0.00250	0.00020	0.00920	0.00630	0.0093
976.6	0.00440	0.00520	0.00430	0.01430	0.01230	0.0139
1953.1	0.00800	0.00410	0.00660	0.01960	0.00750	0.0160
3906.3	0.00720	0.00410	0.00660	0.02830	0.02590	0.0291
7812.3	0.00900	0.00910	0.01320	0.04740	0.04700	0.0536
15625.0	0.01340	0.01900	0.02470	0.07410	0.07410	0.0845
31250.0	0.03060	0.03040	0.03490	0.14780	0.14580	0.1524
62500.0	0.04430	0.04040	0.04220	0.27740	0.27200	0.2675
125000.0	0.10790	0.08870	0.09332	0.50600	0.50250	0.5195
250000.0	0.15770	0.15710	0.15450	0.94630	0.92150	0.9013
500000.0	0.29480	0.29340	0.29760	1.64720	1.60170	1.6226
1000000.0	0.73300	0.73720	0.73180	2.70660	2.60770	2.6554
2000000.0	0.94550	0.91780	0.93127			

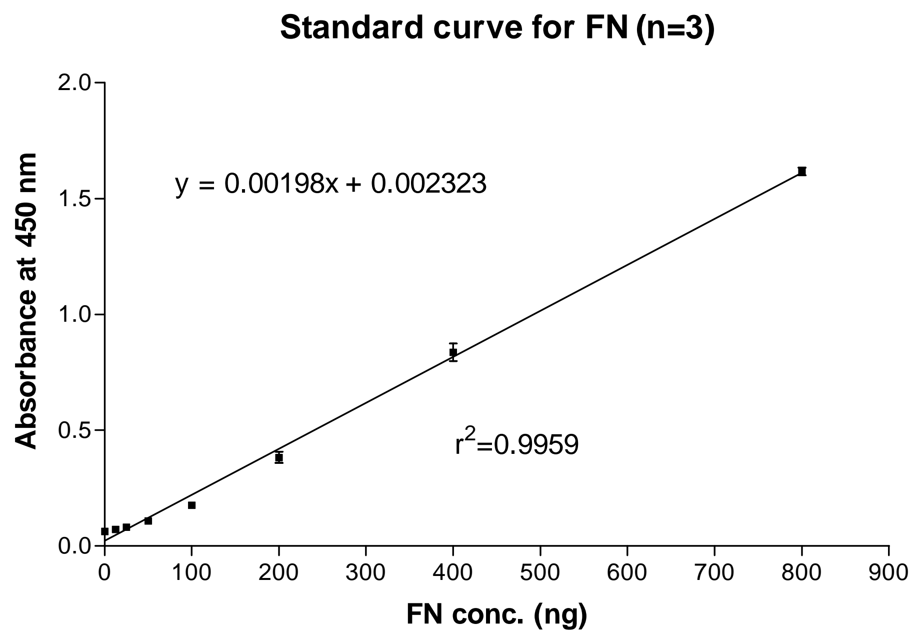
Optimal concentration for HUC-PC cells is **5×10^5 cells/ml assay medium**.

Appendix III

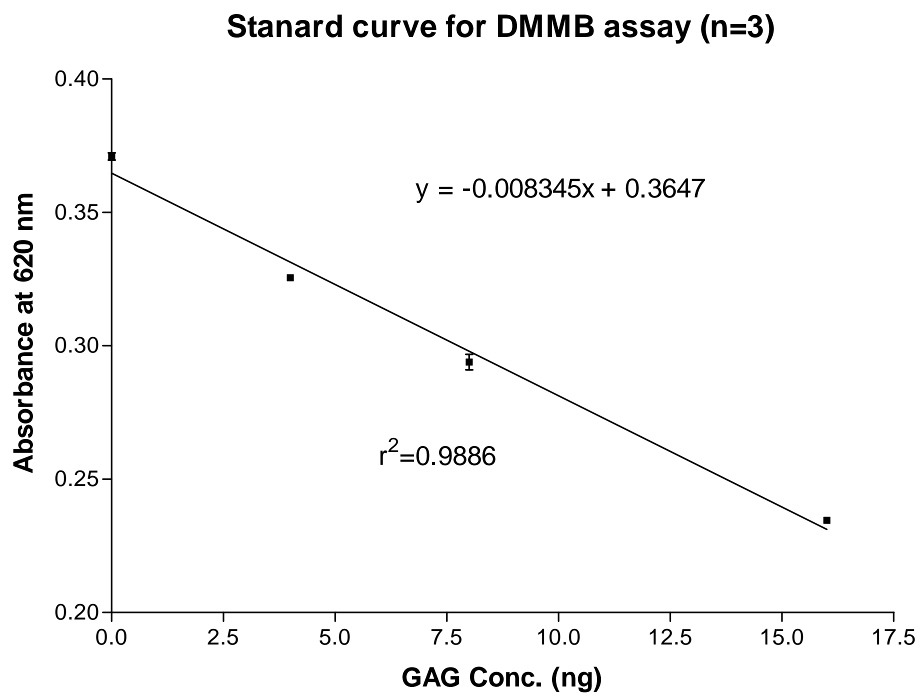
[A] Standard curve for IL-6 assay



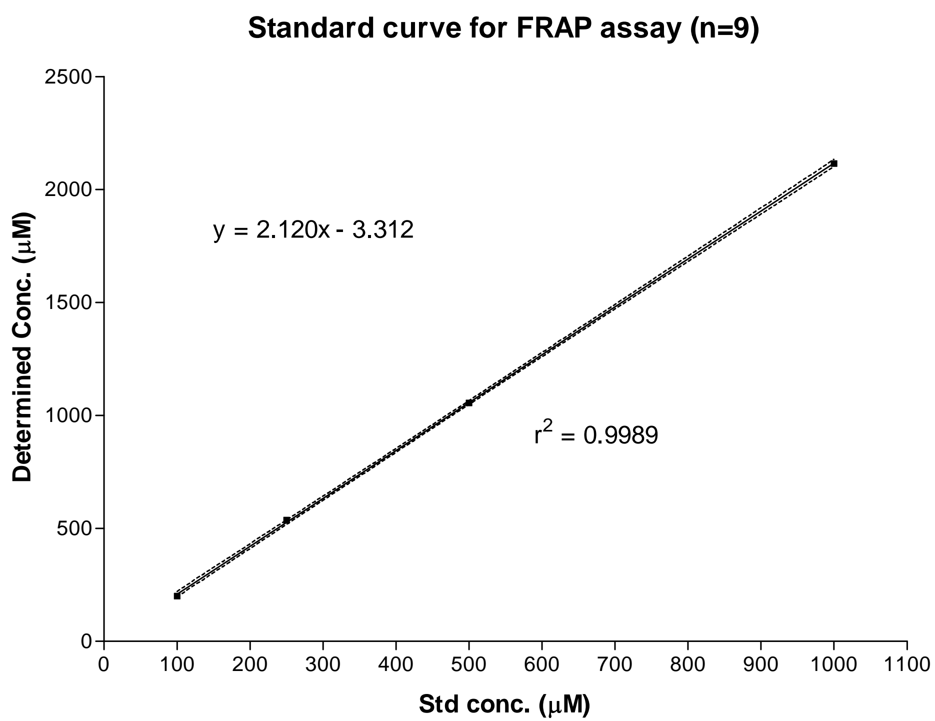
[B] Standard curve for FN assay



[C] Standard curve for GAGs assay

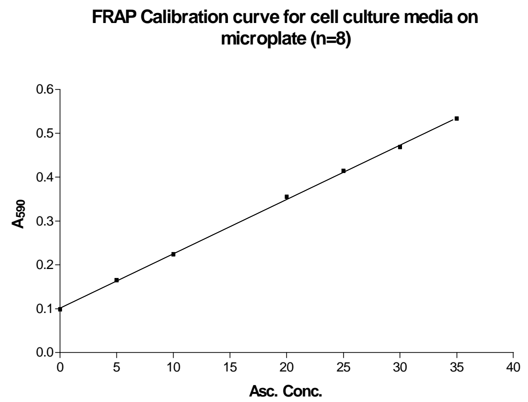


[D] Standard curve for automated FRAP assay



[E] Standard curve for FRAP assay in microplate format

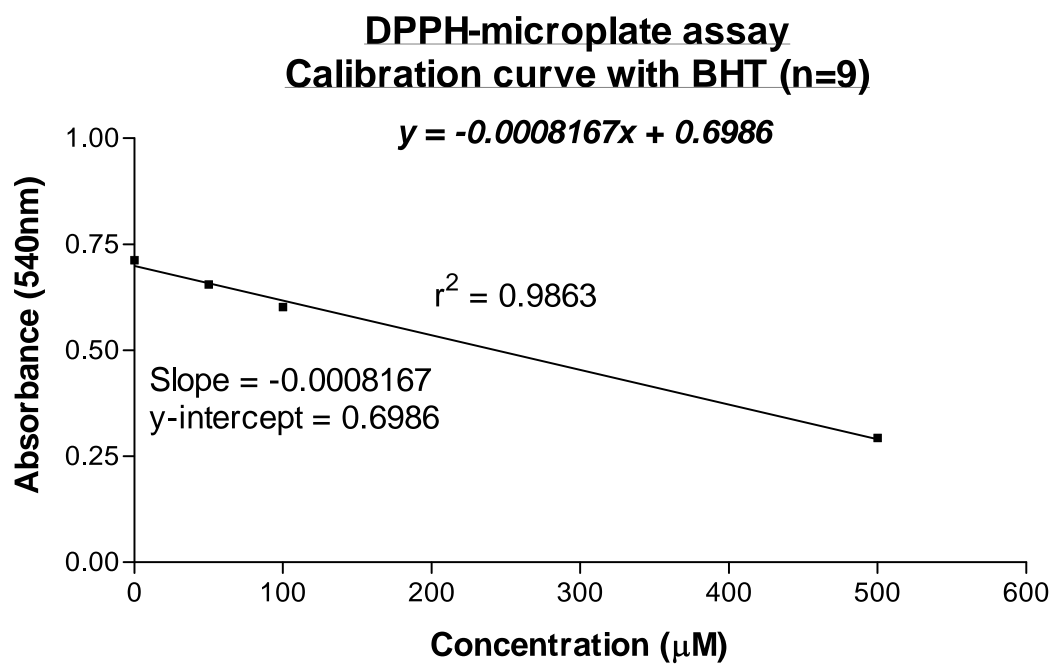
	Set 1
Variables	
Slope	0.01239 ?0.0001092
Y-intercept	0.1016 ?0.002362
X-intercept	-8.201
1/slope	80.71
95% Confidence Intervals	
Slope	0.01217 to 0.01261
Y-intercept	0.09686 to 0.1063
Goodness of Fit	
r2	0.9958
Sy.x	0.009975
Is slope significantly non-zero?	
F	12870
DFn, DFd	1.000, 54.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	7
Maximum number of Y replicates	8
Total number of values	56
Number of missing values	0



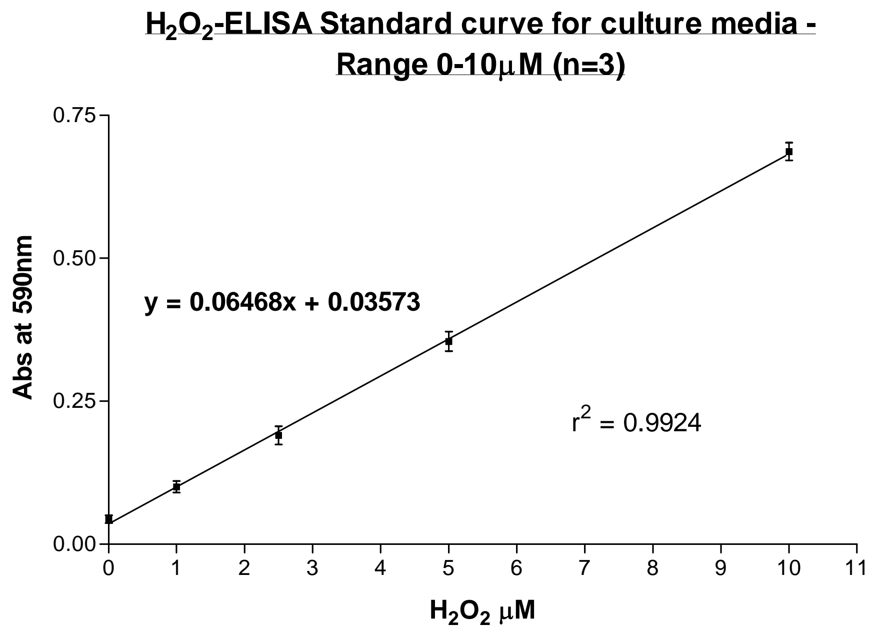
y = 0.01239x + 0.1016

Asc. Conc.	Set 1							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
0.0	0.09540	0.11050	0.09590	0.09940	0.09550	0.10500	0.07390	0.11300
5.0	0.16670	0.16880	0.15510	0.19240	0.15590	0.15820	0.16890	0.15380
10.0	0.22830	0.23460	0.22740	0.21640	0.21910	0.22630	0.22410	0.21750
20.0	0.35540	0.34240	0.36260	0.34660	0.37790	0.36270	0.35380	0.34010
25.0	0.41330	0.42100	0.42310	0.41180	0.40660	0.41190	0.41290	0.41390
30.0	0.48960	0.45580	0.46310	0.46860	0.47220	0.47620	0.46260	0.46400
35.0	0.52500	0.53110	0.52680	0.54100	0.53640	0.53870	0.54030	0.52960

[F] Standard curve for DPPH· assay

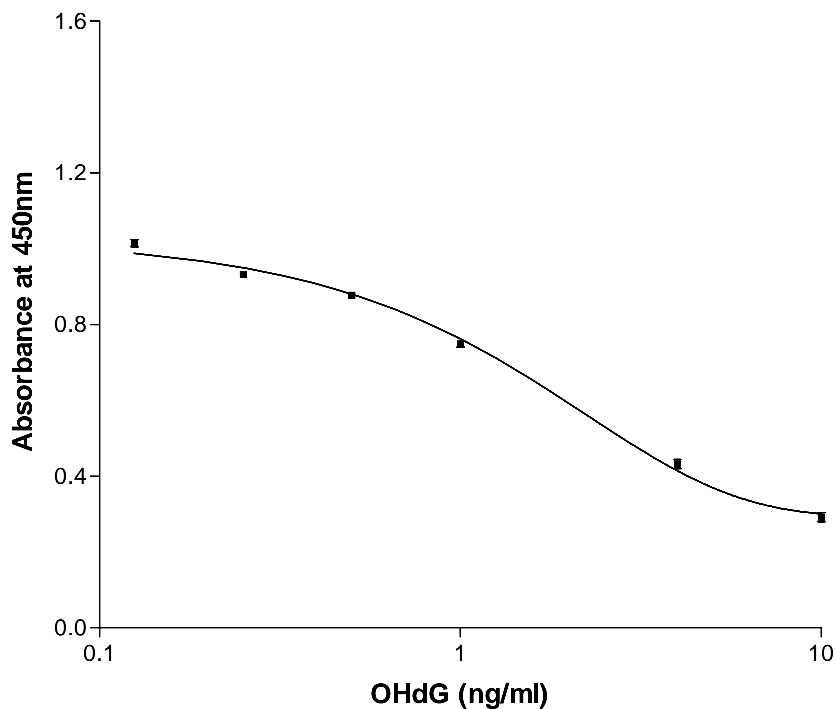


[G] Standard curve for H_2O_2 assay



[H] Standard curve for 8-OHdG assay

OHdG conc	Calibration line		
	Y1	Y2	Y3
0.12500	0.995800	1.024400	1.02400
0.25000	0.933700	0.926100	0.93780
0.50000	0.865900	0.889000	0.87610
1.00000	0.739200	0.741500	0.76550
4.00000	0.411100	0.434000	0.45210
10.00000	0.269700	0.294100	0.31080

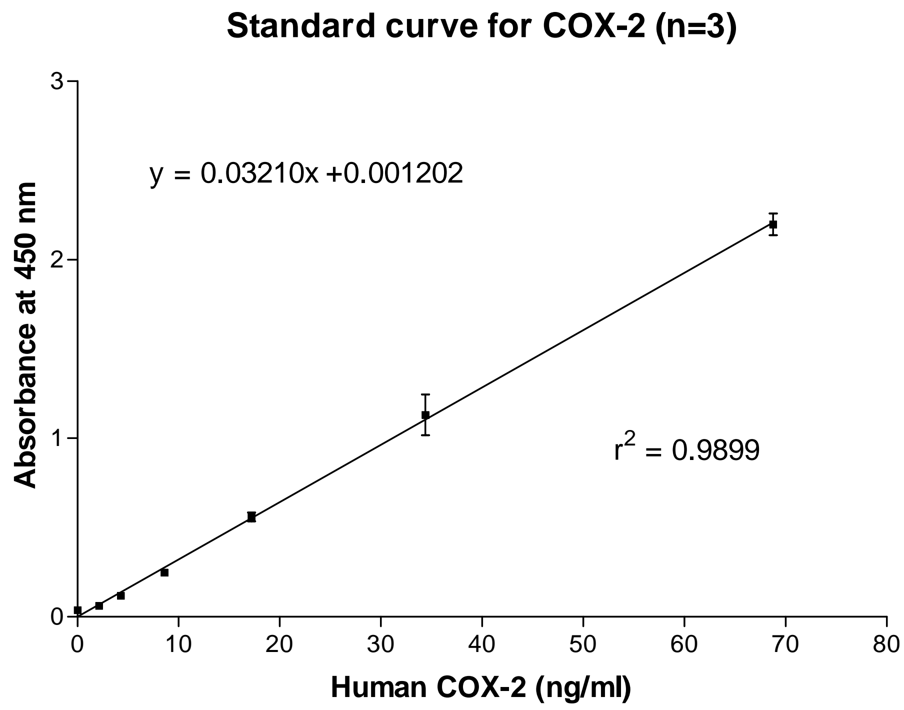


Equation: $y = \text{Bottom} + \dfrac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{logEC50-x})\cdot(\text{Hillslope})}}$

$$y = 40.15 + \frac{39.86}{1 + 10^{1.73+0.20x}}$$

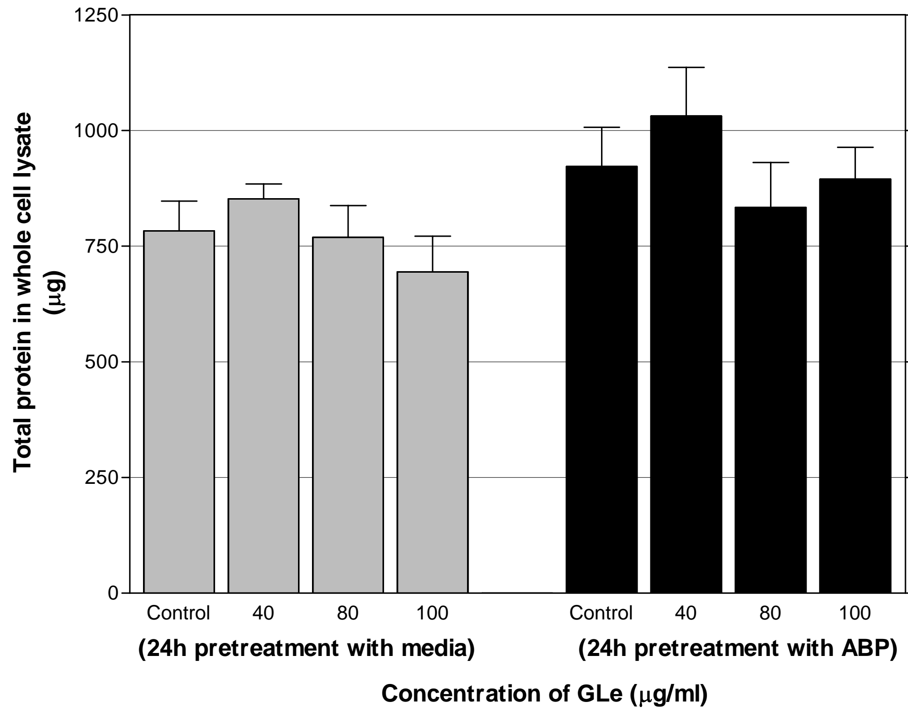
The data fitted on the above equation for Sigmoidal dose-response (variable slope) with P = 0.4194 (P > 0.05).

[I] Standard curve for COX-2 assay

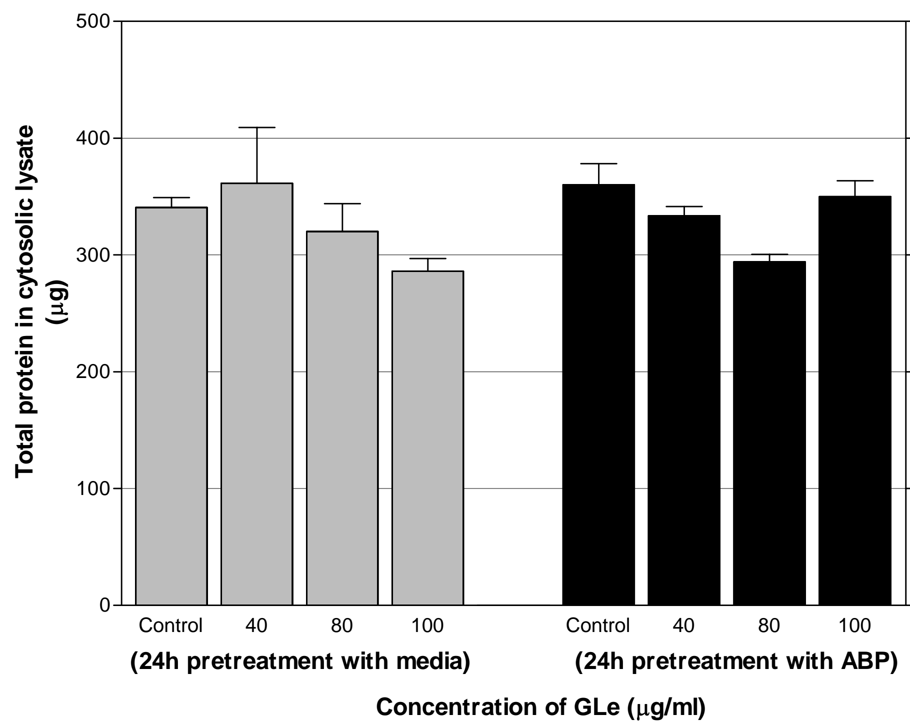


Appendix IV

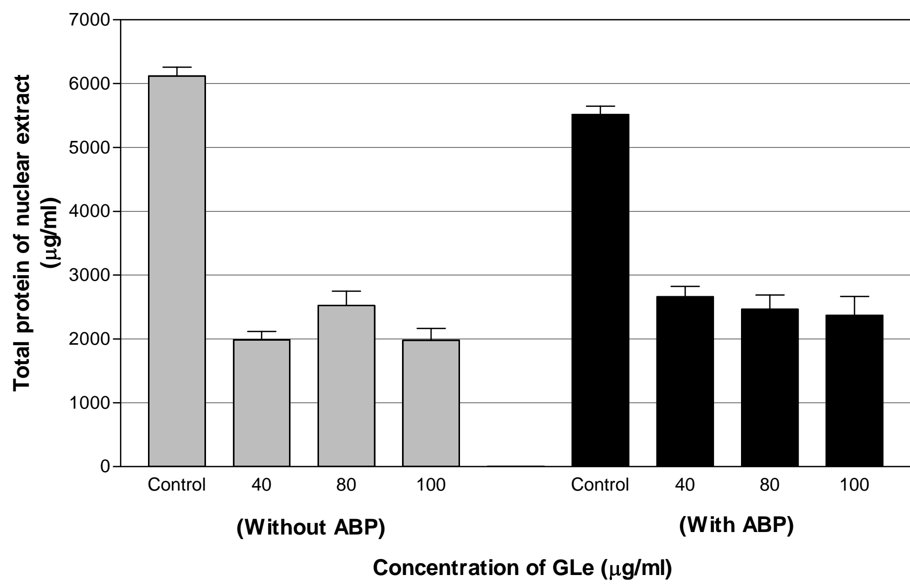
[A] Results of total whole cell protein extracted for COX-2 assay.



[B] Results of total cytosolic protein extracted for PKC assay.



[C] Results of total nuclear protein extracted for NF-κB assay.



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