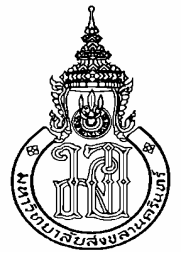


Effects of Cisplatin on Breast Cancer Suppressor Gene 1 (*BRCA1*)

Siriwat Wasiksiri

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biotechnology

Prince of Songkla University

2007

Thesis Title	Effects of Cisplatin on Breast Cancer Suppressor Gene 1 (*BRCA1*)
Author	Mr. Siriwat Wasiksiri
Major Program	Biotechnology

Advisory Committee:

……..……Chairman
(Asst. Prof. Dr. Adisorn Ratanaphan)

……..Committee
(Assoc. Prof. Dr. Poonsuk Prasertsan)

Examining Committee:

…….Chairman
(Assoc. Prof. Dr. Wilaiwan Chotigeat)

…….Committee
(Asst. Prof. Dr. Adisorn Ratanaphan)

…….Committee
(Assoc. Prof. Dr. Poonsuk Prasertsan)

…….Committee
(Asst. Prof. Dr. Aran Hanpongkittikun)

…….Committee
(Asst. Prof. Dr. Varaporn Akkarapatumwong)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy in Biotechnology

……...
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

Thesis Title	Effects of Cisplatin on Breast Cancer Suppressor Gene 1 (*BRCA1*)
Author	Mr. Siriwat Wasiksiri
Major Program	Biotechnology
Academic Year	2007

Abstract

Cisplatin is an anticancer drug for the treatment of various cancers. It essentially exerts its cytotoxicity against cancerous cells via covalent attachment of platinum atom to DNA, generating various platinum-DNA adducts which inhibit biological processes essential for cellular viability. However, cisplatin interacts nonspecifically with DNA, resulting in damaging of normal cell DNA. Potential *in vitro* and cell culture interaction between cisplatin and human breast cancer suppressor gene 1 (*BRCA1*) was herein investigated. The 696-bp fragment of the 3′-region of *BRCA1* gene (nucleotide 4897-5592) was amplified by RT-PCR using mRNA template isolated from human white blood cell. Retardation of the electrophoresis migration on agarose gel of drug-treated *BRCA1*, in the dose-response manner, was observed. An increase in drug concentrations resulted in increased interstrand crosslinks. Restriction analysis with *Pvu*II and *Eco*0109I indicated that the preferential platination site on the DNA fragment was at the dGpG sequence of unique *Eco*0109I-cleaved site. Cisplatin affected the transition temperature of the *BRCA1* gene fragment in a biphasic fashion. DSC thermogram of cisplatin-damaged *BRCA1* was shifted to a lower transition temperature at lower cisplatin concentration of 50 nM. However, at higher cisplatin concentration of 0.5 μM, DSC thermogram peaked at a slightly higher transition temperature with predominantly increased heat specific capacity, compared to the control untreated *BRCA1*. The cellular proficiency in repairing the drug-treated DNA

was evaluated specifically in the 3,426-bp fragment of *BRCA1* exon 11 of the MCF-7 cells using the semi-quantitative PCR assay. The differential reactivation on the drug-treated *Renilla* luciferase encoding plasmid, using the host cell reactivation assay, was a consequence of different platinum levels covalently bonding with the transcribed reporter gene. The GAL4-fused BRCT (BRCA1 C-terminal domain) slightly enhanced the rate of transcription of *Renilla* luciferase gene in the absence of GAL4 binding site. The transcriptional transactivation activity of cisplatin-damaged *BRCA1*, when tested in the GAL4 transcriptional activation system, was inversely proportional to the amount of platinum-*BRCA1* adducts. A significant decrease in transcriptional transactivation of cisplatin-damaged *BRCA1* appeared to be associated with reduced cellular reactivation. Together, these findings suggested that a decrease in transcriptional transactivation of cisplatin-damaged *BRCA1* is associated with DNA repair, DNA conformation and thermal stability of platinated *BRCA1*.

ชื่อวิทยานิพนธ์ ผลของซิสพลาตินที่มีต่อยีนกดมะเร็งเต้านม (*BRCA1*)

ผู้เขียน นาย ศิริวัฒน์ วาสิกศิริ

สาขาวิชา เทคโนโลยีชีวภาพ

ปีการศึกษา 2550

บทคัดย่อ

ซิสพลาตินเป็นยารักษาโรคมะเร็งหลายชนิด ความเป็นพิษของซิสพลาตินต่อเซลล์มะเร็งเกิดจากพันธะโควาเลนท์ระหว่างอะตอมพลาตินัมกับดีเอ็นเอได้เป็น Platinum-DNA adducts ต่างๆ ซึ่งมีผลต่อกระบวนการทางชีววิทยาที่จำเป็นต่อการอยู่รอดของเซลล์ อย่างไรก็ตาม ซิสพลาตินเกิดอันตรกิริยากับดีเอ็นเออย่างไม่จำเพาะเจาะจง ส่งผลให้ดีเอ็นเอของเซลล์ปกติเกิดความเสียหาย งานวิจัยนี้เป็นการศึกษาแนวโน้มของการเกิดอันตรกิริยาระหว่างซิสพลาตินกับยีนกดมะเร็งเต้านม *BRCA1* ในหลอดทดลองและในเซลล์เพาะเลี้ยง เมื่อเพิ่มปริมาณยีน *BRCA1* ขนาด 696 คู่เบสจากบริเวณปลายด้าน 3′ (นิวคลิโอไทด์ 4897-5592) ของ mRNA ที่สกัดจากเม็ดเลือดขาวของมนุษย์โดยใช้เทคนิค RT-PCR พบว่าการเคลื่อนที่ของ *BRCA1* ที่เสียหายด้วยยาซิสพลาตินในอะกาโรสเจลช้าลงเมื่อความเข้มข้นของซิสพลาตินสูงขึ้น ความเข้มข้นของยาที่สูงขึ้นมีผลต่อการเกิด interstrand crosslinks เพิ่มขึ้น จากการศึกษาบริเวณที่เกิดอันตรกิริยาระหว่างอะตอมของพลาตินัมและดีเอ็นเอด้วยการย่อยด้วยเอนไซม์ตัดดีเอ็นเอจำเพาะ *Pvu*II และ *Eco*0109I พบว่าลำดับเบส dGpG ของ *Eco*0109I เป็นบริเวณที่มักจะเกิดพันธะระหว่างอะตอมของพลาตินัมและดีเอ็นเอ ซิสพลาตินมีผลต่อ transition temperature ของ *BRCA1* ในสองลักษณะ จากเทอร์โมแกรมของ *BRCA1* ที่เสียหายด้วยซิสพลาตินพบว่าค่า transition temperature ลดลงเมื่อความเข้มข้นของซิสพลาตินลดลงเหลือ 50 นาโนโมลาร์ อย่างไรก็ตาม ความเข้มข้นของซิสพลาตินที่สูงขึ้นที่ 0.5 ไมโครโมลาร์ transition temperature และความร้อนจำเพาะเพิ่มขึ้น ประสิทธิภาพของการซ่อมแซมดีเอ็นเอของเซลล์ MCF-7 ในส่วนของ *BRCA1* exon 11 ซึ่งมีขนาด 3,426 คู่เบส ด้วยเทคนิค semi-quantitative PCR เมื่อศึกษาการซ่อมแซมที่แตกต่างกันของ reporter *Renilla* luciferase encoding plasmid ที่เสียหายด้วยซิสพลาตินด้วยเทคนิค host cell reactivation assay พบว่าเป็นผลจากปริมาณอะตอมพลาตินัมที่ต่างกันที่เกิดพันธะโควาเลนท์กับ reporter gene สำหรับโปรตีน BRCT (BRCA1 C-terminal domain) ที่เชื่อมกับโปรตีน GAL4 (GAL4-BRCT) พบว่ามีอัตราการถอดรหัสของ reporter gene เพิ่มขึ้นเล็กน้อยเมื่อไม่มี GAL4 binding site การกระตุ้นการถอดรหัสของ *BRCA1* ที่เสียหายด้วยซิสพลาตินเมื่อทดสอบในระบบการกระตุ้นการถอดรหัสที่อาศัย GAL4 เป็นสัดส่วนผกผันกับปริมาณของ platinum-*BRCA1* adducts

การกระตุ้นการถอดรหัสที่ลดลงอย่างมีนัยสำคัญของ *BRCA1* ที่เสียหายด้วยซิสพลาตินดูเหมือนว่าเกี่ยวข้องกับการกระตุ้นการซ่อมแซมดีเอ็นเอของเซลล์ที่ลดลง จากการทดลองโดยรวมชี้แนะว่าการกระตุ้นการถอดรหัสที่ลดลงของ *BRCA1* ที่เสียหายด้วยซิสพลาตินเกี่ยวข้องกับการซ่อมแซมดีเอ็นเอ โครงรูปของดีเอ็นเอ และเสถียรภาพต่อความร้อนของ *BRCA1* เมื่อเกิดอันตรกิริยากับอะตอมพลาตินัมของยาซิสพลาติน

ACKNOWLEDGEMENT

I would like to express my gratitude and sincere appreciation to my advisor, Asst. Prof. Dr. Adisorn Ratanaphan of the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, for his suggestion, comments, correcting and criticizing this manuscript and also his guidance and advice throughout my Ph.D degree study.

I also would like to extend my warm and sincere appreciation to my co-advisor, Assoc. Prof. Dr. Poonsuk Prasertsan of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, for her helpful suggestions of my work.

I am thankful to Assoc. Prof. Dr. Wilaiwan Chotigeat and Asst. Prof. Dr. Aran Hanpongkittikun for their recommendation on my thesis proposal.

My deeply gratitude is also due to all of my family, my friends and staffs at Pharmaceutical Laboratory Service Center (Biotechnology Division), Faculty of Pharmaceutical Sciences who give me their help and enjoyable during a hard time of my study.

I would like to direct my appreciation to Prince of Songkla University for the Graduate Study Scholarship during 2001-2004, and the grant from the National Research Council of Thailand (Grant No. 02011420-0003).

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ADA2	Transcriptional adaptor 2
ADA3	Transcriptional adaptor 3
AP-1	Associated protein 1 (a heterodimer formed by c-jun and c-fos)
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutation
BARD1	BRCA1-associated RING domain 1
BER	Base excision repair
BIC	Breast information core
bp	Base pair
BRCA1	Breast cancer susceptibility gene 1 / breast cancer suppressor gene 1
BRCA2	Breast cancer susceptibility gene 2 / breast cancer suppressor gene 2
BRCT	BRCA1 C-terminal domain
CAK	cAMP-dependent protein kinase
cDNA	Complementary DNA
Cisplatin	*Cis*-diamminedichloroplatinum (II)
$^{\circ}$C	degrees celsius
CBP	CREB binding Protein
CIP1	CDK-interacting protein 1
CMV	Cytomegalo virus
COBRA1	Cofactor of BRCA1
CREB	cAMP response element-binding proteins
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate

dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetate
ERCC1	Excision repair cross-complementing gene1
Fig.	Figure
GADD45	Growth arrest and DNA damage 45
h	Hour
hUBF	Human upstream binding factor
HDAC1	Histone deacetylases 2
HDAC2	Histone deacetylases 2
LOH	Loss of heterozygosity
SAPK	Stress activated protein kinase
MCF-7	Human breast adenocarcinoma cell line
μg	Microgram
μl	Microlitre
μM	Micromolar
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRE11	Meiotic recombinant 11
ng	Nanogram
NELF-B	Negative elongation factor B
NER	Nucleotide excision repair

NHEJ	Non homologous end-joining repair
NLS	Nuclear localization signal
OD_{260}	Optical density at 260 nm
OD_{280}	Optical density at 280 nm
OD_{420}	Optical density at 420 nm
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC4	Positive cofactor 4
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Polα	DNA polymerase alpha
Polβ	DNA polymerase beta
Polδ	DNA polymerase delta
Polη	DNA polymerase eta
Polκ	DNA polymerase kappa
Polζ	DNA polymerase zeta
PTEN	Phosphatase and tensin homologue
QPCR	Quantitative polymerase chain reaction
RAD51	Radiation sensitive protein 51
RB1	Retinoblastoma 1
RNA pol I	RNA polymerase I
RNA pol II	RNA polymerase II
RPB2	Retinol-binding protein 2
RPB10	Retinol-binding protein 10
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SRE	Steroid hormone receptor responsive element

TAE	Tris-acetate-ethylenediaminetetraacetate
TBP	TATA-box binding protein
TE buffer	Tris-ethylenediaminetetraacetate buffer
TEMED	*N, N, N′, N′* -tetramethylethylenediamine
TFIIB	Transcription factor IIB
TFIIF	Transcription factor IIF
TFIIH	Transcription factor IIH
TRAP220	Thyroid hormone receptor-associated protein 220
Tris	Tris (hydroxymethyl) aminomethane
T_m	Melting temperature
UV	Ultraviolet
WBC	White blood cell
XP	Xeroderma pigmentasum
XPB	Xeroderma pigmentasum B
XPD	Xeroderma pigmentasum D

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

The anticancer drug cisplatin [*cis*-diamminedichloroplatinum(II)] is widely used for the treatment of human testicular, ovarian, urinary bladder and head and neck cancers (Frei *et al*., 1985; Loehrer and Einhorn, 1984; Pratt *et al.*, 1994). Cytotoxicity of cisplatin to the cancer cells results from the interaction of the drug to DNA through the covalent bond between a platinum atom and N-7 atom of guanine and guanine or adenine. The intrastrand crosslink is the major form of platinum-DNA adduct. The predominant adducts are Pt-GG (65%), followed by Pt-AG (25%) and Pt-GNG (6%) (Eastman, 1986; Fichtinger-Schepman *et al.*, 1987). The interstand crosslink and monofunctional adduct responses for the minor adduct on DNA strand. The platinum-DNA adducts interfere DNA replication, transcription and translation that initiates apoptosis pathway and cell death.

Cisplatin can be retained in numerous normal tissues during or after treatment. Cisplatin is also carcinogenic both *in vitro* and in laboratory animal. Platinum-DNA adducts cause many mutations in both experimental and clinical trials. The data from the testicular and ovarian cancer patient treated with cisplatin reveal increasing of the frequency of secondary leukemia cancer in many years later. Chemotherapy for testicular cancer has been associated with secondary leukemia risks that range from 20- to 300-folds (Travis *et al*., 2000). Many mutations on tumor suppressor gene of leukemia were found. The p53 mutations are common in leukemia after cisplatin therapy for ovarian cancer (Debra *et al*., 2002). The propencity for G-to-A transitions reflects specific DNA damage in leukemia after treatment with this platinum drug.

Breast cancer susceptibility gene 1 (*BRCA1*) is very important tumor suppressor gene in human. *BRCA1* encodes a protein that controls cell cycle, DNA repair and transcriptional activation of other genes in breast cells. It locates on chromosome 17q21. It comprises 24 exons and 22 introns (Hall *et al.*, 1990; Miki *et al.*, 1994; Smith *et al*., 1992) and codes for a 1863 amino acid polypeptide with molecular weight of 220 kDa. Mutation or malfunction of the gene initiates breast cancer in young germline patient. However, direct effects of cisplatin on *BRCA1* have not been studied previously. Cisplatin may damage *BRCA1*, whose translated product has an important function in DNA repair and transcriptional activation (Ouchi *et al*., 1998; Scully *et al*., 1997a and b; Yarden *et al*., 1999). Damaged *BRCA1* may lead to a serious consequence such as acute toxicity, mutations, degenerative diseases or risk of second malignant neoplasm among long-term survivors of testicular cancer (Travis *et al*., 2000).

In this study, the effects of cisplatin on the specific tumor suppressor gene *BRCA1* are investigated in many aspects both *in vitro* and in cell culture. The cDNA of the 696-bp fragment of the 3′-terminal region of *BRCA1* is derived from messenger RNA of white blood cells and used for biophysical analysis. Cisplatin-*BRCA1* adducts are characterized on native and denaturation gel electrophoresis. The specificity of the adducts is verified by restriction digestion analysis. Thermal stability of cisplatin-induced *BRCA1* is measured by differential scanning calorimetry (DSC). MCF-7 breast cancer cell line is selected as the host cell for transfection. Cellular proficiency in repairing cisplatin-damaged *BRCA1* is specifically evaluated in the 3,426-bp fragment of *BRCA1* exon 11 of the MCF-7 cells and cisplatin-damaged reporter gene encoding plasmid using the quantitative-PCR assay and the host cell reactivation assay, respectively. The transactivation activity of the BRCT domain is performed by “one hybrid assay” of the GAL4 transcription activation system.

CHAPTER 2

LITERATURE REVIEW

2.1 Cisplatin

2.1.1 Introduction

Cisplatin [(*cis*-diamminedichloroplatinum(II)] (Fig. 1), an alkylating agent and anticancer drug, is widely used for the treatment of human testicular cancer, ovarian, bladder cancer and head and neck cancer (Zwelling and Korn, 1982; Loehrer and Einhorn, 1984; Pratt *et al*, 1994). The activity of cisplatin was found in early sixties (Brown *et al*., 1994; Rosenberg *et al*., 1965; Rosenberg *et al*., 1967) and was approved for cancer drug in 1978. Cytotoxicity of cisplatin to the cancer cells results from the interaction of the drug to DNA through the covalent bond between a platinum atom and guanine or adenine.

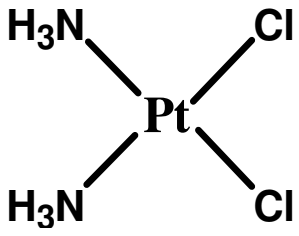


Figure 1. Structure of *cis*-diamminedichloroplatinum (II) (cisplatin)

Cisplatin also crosslinks and interferences the function of DNA, RNA, protein and other biomolecules. The drug is one of a platinum coordination complex (Rosenberg *et al*., 1967). Cis-isomer platinum inhibits cancer growth, whereas trans-compound has no effect. The drug exchanges chloride ions for such nucleophilic groups as RS^-, R-S-CH_3, -N=, and R-NH_2 to form links that can be very stable.

In blood plasma, the high chloride concentration of of apprximately 100 mM would keep cisplatin predominately in the uncharged and relatively unreactive dichloro form. This form may react to some degree with sulfhydryl groups of plasma proteins. The free dichloro form could enter cells by passive diffusion. In the cytoplasm, the relatively low chloride concentration of approximately 1 mM would favor the aquation reaction, which would yield highly reactive species whose ionic charge may retard exit from the cell (Pratt *et al*., 1994).

$$Pt(NH_3)_2Cl_2+H_2O \longrightarrow [Pt(NH_3)_2Cl(H_2O)]^{+}+Cl^{-}$$

$$[Pt(NH_3)_2Cl(H_2O)]^{+}+H_2O \longrightarrow [Pt(NH_3)_2(H_2O)_2]^{++}+Cl^{-}$$

Although, it is possible that a chloride ligand in cisplatin might be displaced directly in a reaction with a macromolecule, it is generally agreed that the more usual part is via an initial aquation reaction in which a chloride is replaced by water molecule. The aquation reaction is driven by the high concentration of water and low concentration of chloride in the tissues. The aquated platinum complex can then react rapidly with a variety of strong binding sites (Pratt *et al*., 1994).

2.1.2 DNA adduct

Cisplatin reacts covalently with DNA and disrupts its secondary structure. It is linked to the N7 atoms of the nucleobases guanine and adenine. The two major adducts are d(pGpG) and d(pApG), both derived from intrastrand cross-links of cisplatin on neighboring nucleobases (Fichtinger-Schepman *et al*., 1985). The minor adduct are d(pGpNpG) and monofunctional bound cisplatin to guanine. The typical proportion is 65, 22, 13 and less than 1 % respectively (Eastman, 1986, Fichtinger-Schepman *et al*., 1987b) (Fig. 2). The cis-GG and cis-AG adducts both unwind DNA by 13 degrees, while the cis-GTG adduct unwides DNA by 23 degrees (Bellon *et al*., 1991). However, the gel electrophoresis technique reveals approximately 40 degree

bend in double helix at the intrastrand crosslink between N7 atoms of GpG (Rice *et al*., 1988). Interstrand cross-links of cisplatin on d(TGCT/AGCA) bends the double helix by approximately 55 degrees toward the major groove (Sip *et al*., 1992). The kinetic of *in vitro* binding of radioactive cisplatin to DNA is studied (Johnson *et al*., 1980a). At 37°C and pH 7 in 0.15 M NaCl, 6-8% of both cis- and trans- isomer bind to DNA in 24 h, suggesting that both compounds should bind to DNA under biological condition. The reaction rate is competitively inhibited by various salts and buffers and is suppressed by raising of the pH (50% inhibition of initial rates at pH 7.3) (Johnson *et al*., 1980a).

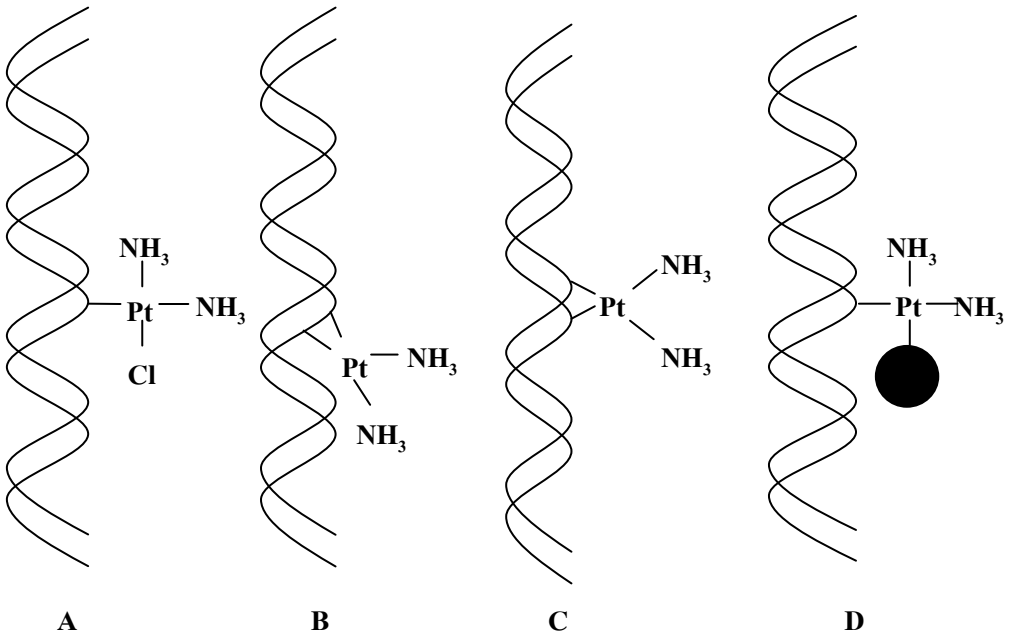


Figure 2. Cisplatin-DNA monocrosslink (A), intrastrand crosslink (B), interstrand cross-link (C) and intermolecular crosslink between cisplatin-DNA and protein (D).

2.1.3 Effects of cisplatin on DNA replication

When the efficiency of cisplatin to inhibit DNA replication is compared to trans-DDP, fourteen times more trans- than cis-DDP in the culture medium is required to inhibit SV40 DNA replication in SV40 infected green monkey CV-1 cell. The two isomers are equally effective at inhibiting replication when equimolar amounts are bound to SV40 DNA *in vivo* (Ciccarelli *et al*., 1985). DNA replication is inhibit at 177 nucleotide 3' the individual adduct and terminated at 25 nucleotode on the 5' side of adducts (Comess *et al*., 1992). The inhibition of DNA replication of DNA polymerase I on M13mp8 viral DNA platinated with cis-diamminedichloroplatinum (II) is occurred on GpG sequence (Pinto and Lippard, 1985b).

Bypass of the cisplatin adducts occurs approximately 10% of the time and that the cis-[$Pt(NH_3)_2$[d(GpG)-N7(1),-N7(2)] intrastrand crosslink is the most inhibitory lesion (Comess *et al*., 1992). The cis-[$Pt(NH_3)_2$[d(GpCpG)-N7(1),-N7(2)] adduct allows a higher frequency of such translesion synthesis (25%) for T7polymerase and *E.coli* DNA polymerase I (Klenow fragment) (Comess *et al*., 1992). The fidelity of replication depends on type of DNA polymerase. The major replicative DNA polymerase has a very limited translesional synthesis capacity. DNA polymerase β (Polβ) has been suggested as a candidate for the role of a DNA polymerase that performs error-prone translesion synthesis of cisplatin-DNA adducts (Hoffmann *et al*., 1995; Hoffmann *et al*., 1996). In addition, polβ is able to elongate the arrested replication products of polymerase α and δ (Hoffmann *et al*., 1995). The full-length DNA products of translesion synthesis by polβ contain a high frequency of mutations in the vincinity of the Pt-DNA adducts (Hoffmann *et al*., 1996). The other family of DNA polymerase that can mediate such bypass replication has been identified in mammalian cell. This family includes Polζ, Polη and Polκ (Friedberg *et al*., 2000). The Polζ plays a role in the replication bypass of cisplatin adducts. It reduces cytotoxicity and enhances mutagenicity of cisplatin in human colon carcinoma cells that have lost

DNA mismatch repair (Lin *et al*., 2006). DNA polymerase η (Polη) is an enzyme with the ability to perform translesion synthesis across pyrimidine dimer (Masutani *et al*., 1999). Mutation of Pol η gene causes xeroderma pigmentasum variant (XP-V) syndrome which patient are highly sensitive to sunlight that prone to skin cancer development (O'Day *et al*., 1992) and increase sensitivity of the XP-V cell to the anticancer drug cisplatin (Chen *et al*., 2006). Polκ is one of polymerase enzyme that has translesional synthesis capacity. However, this enzyme is unable to bypass a cisplatin adduct (Gerlach *et al*., 2001).

2.1.4 Effects of cisplatin on transcription

Cisplatin-DNA adducts form at d(ApG) or d(GpG) sites are not an absolute blocked to formation of a single phosphodiester bond by either of *E.coli* RNA polymerase or wheat germ RNA polymerase II (Corda *et al*., 1992; Corda *et al*., 1993). The binding affinity between *E.coli* RNA polymerase and d(GpG)-containing platinated template is lower 4.5 times than normal template whereas binding between RNA polymerase and d(ApG)-containing template is not affected by modification of cisplatinthe d(ApG) by cis-DDP (Bennett *et al*., 1995). The transcription is impeded more by bifunctional than monofunctional adducts. The GpTpG intrastrand crosslink and GpC/CpG interstrand crosslink are blocked at several nucleotides before the elongation reachs the interstrand crosslink (Corda *et al*., 1993). In the eukaryotic RNA polymerase, the transcription is completed blocked on the strand carrying metal complex, whereas transcription elongation is not blocked on the complementary strand but showed slightly inhibited (Corda *et al*., 1991). The effect of cisplatin damage on RNA pol II elongation is investigated using site-specifically-placed cisplatin adducts. The GTG adduct is an effective block to RNA pol II elongation, inhibiting the polymerase by 80%. In contrast, RNA pol II completely bypassed the cisplatin GG intrastrand adduct. These studies suggest that the inhibition of RNA pol II transcription observed following the treatment of cells with cisplatin is likely to reflect the combined

effects of DNA damage at the level of both transcription initiation and elongation (Cullinane *et al*., 1999). This result differs from the effect of T7 RNA polymerase on site-specifically platinated DNA templates. An elongation complex is strongly blocked by cisplatin 1,2-intrastrand d(GpG) and 1,3-intrastrand d(GpTpG) cross-links located on the template strand (Jung and Lippard, 2003). Moreover, cisplatin treatment triggered ubiquitylation-mediated RNA pol II degradation in HeLa cells. The ubiquitylated polymerase was mostly unbound or only loosely associated with chromatin. This altered polymerase is rapidly destroyed by proteosomes (Jung and Lippard, 2006).

Transcription factor IIH (TFIIH), the transcription initiation complex, is another transcription factor interfered by cisplatin-DNA adduct. TFIIH has a 6-subunit core (XPB, XPD, p44, p34, p52, p62) and a 3-subunit kinase (CAK). TFIIH has roles both in basal transcription initiation and in DNA repair, and several inherited human disorders are associated with mutations in TFIIH subunits (Araújo *et al*., 2000). TFIIH is an intricate component of nucleotide excision repair (NER) (Svejstrup *et al*., 1996). The presence of damage DNA leadS TFIIH to DNA repair function and inhibits of transcription mechanism (Vichi *et al*., 1997).

2.1.5 Effect of cisplatin on protein translation

The effects of cisplatin on *in vitro* translation are studied from guinea pig and rabbit reticulocyte. The peptide synthesis is inhibited on a concentration and time dependent manner. The translation inhibition is nearly maximal after a 30-min exposure. Translation is inhibited by cisplatin at concentrations that have been found after the administration of therapeutic dose. The suppression of peptide synthesis is due to an interaction between cisplatin and mRNA. Large peptide products are inhibited to a greater extent than products with faster electrophoretic mobility (Rosenberg and Sato, 1988). The decrease of protein synthesis started from the inhibition of polyribosome formation in *in vitro* translation. The inhibition is first on initiative step of protein

synthesis. Cisplatin causes an accumulation of 48S particles accompanied by a decreased amount of completed 80S initiation complexes. The cisplatin blocks the initiation of translation by preventing the joining of the 60S ribosomal subunit to the 48S preinitiation subunit (Rosenberg and Sato, 1993). The mean inhibition concentration (IC_{50}) of cisplatin for translation in is 23 μM (Sato *et al*., 1996).

2.1.6 Repair of cisplatin-DNA adduct

Cisplatin is an effective chemotherapeutic agent for the treatment of testicular cancer and is used in combination regimines for a variety of other tumors. Although many patients initially respond to treatment, a common problem is acquired resistance. The intrinsic resistance observed in some patient is multifactorial in nature and includes contribution from differential drug uptake, cellular detoxification systems, and DNA repair mechanism (Reed *et al*., 1996; Zamble and Lippard, 1995). The loss of DNA mismatch repair activity enhanced sensitivity to cisplatin (Fink *et al*., 1996). Nucleotide excision repair is also a major mechanism contributing to cisplatin resistance. The global genome repair and transcription-coupled repair are the major mechanism of nucleotide excision repair. In non-actively transcribed strand of gene, the adduction of cisplatin is firstly recognized by XP (Xeroderma pigmentosum) protein and initiates the process of GGR, which simply means repair anywhere in the genome. However, the repair of adducts on transcribed strand uses TCR that more rapid than GGR (Hartman and Ford, 2003). The cell lines from patient with XP mutation are highly sensitive to cisplatin 4-fold more than normal cell (Dijt *et al*., 1988).

Comprehensive study of yeast mutants reveals that nucleotide excision repair (NER), homologous recombination, and postreplication repair (PRR) play a major role in repairing DNA damage caused by cross-linking agents (Hartwell *et al*., 1997; Perego *et al*., 1998; Simon *et al*., 2000). Cisplatin adducts are eliminated by the NER pathway, which uses Xpc/Hr23B and Xpa to detect the lesions. These proteins

recruit two endonucleases, Ercc1-Xpf and Xpg, leading to incision at the 5' and 3' sides of a DNA lesion and to removal of the lesion. PRR helps restarting replication stalled at lesions that are not repaired before the S phase of cell cycle. This pathway includes two major branches: translesion DNA synthesis (TLS) and homologous recombination (Hochegger *et al*., 2004; Yamashita *et al*., 2002) TLS can fill the strand gap by employing the Rad6/Rad18 complex, an ubiquitin-conjugating enzyme, and specialized TLS polymerases, such as Polη, Polκ, and Polζ (Hochegger *et al*., 2004). Although Rad18 is required for the function of each TLS polymerase in yeast, this may not the case in vertebrate cells, as Rad18-deficient DT40 cells have a less severe phenotype than do Polζ,-deficient DT40 cells (Sonoda *et al*., 2003; Yamashita *et al*., 2002). Homologous recombination can fill the strand gap by using the other intact sister DNA as a template (Sonoda *et al*., 2001; Thompson and Schild, 2001) or could be required to repair replication-induced DSB, and nonhomologous end-joining (NHEJ) might also be employed to religate DNA strands that are broken as a result of replication fork stalling.

In other vertebrate cell, the level of cisplatin adduct repair pathway is different from yeast cell. Chicken-B cell line, DT40 cells, that mutate at various type of DNA-repair gene show that PRR, homologous recombination are mainly responsible for cross-link repair, whereas NHEJ, NER, and BER play only a minor role (Nojima *et al*., 2005). NER is one of the major differences in cross-link repair between yeast and mammal is the extent of contribution by NER. All NER mutants in yeast show a relatively severe sensitivity to cisplatin. However, DT40 deficient in Xpa and Xpg exhibited only mild sensitivity to cisplatin. NER may have a minor role in cross-link repair in higher eukaryotic cells when compared with yeast. BER is another pathway that not role the major pathway on cisplatin-adduct repair in vertebrate. A defect in BER did not result in hypersensitivity to cisplatin. On the other hand, cells deficient in Parp-1, which is involved in a variety of DNA repair pathways, including BER, SSB, stabilization of replication forks, and perhaps DSB repair, showed high sensitivity to

cisplatin as well as IR (Simon *et al*., 2000). The results suggest that the two excision repair pathways play a minor role, if any, in tolerance to cross-linking agents in chicken as well as in mammalian cells. The results are same as NHEJ pathway. NHEJ is preferentially used to repair IR-induced DSBs in mammalian cells, however, mutations in NHEJ have little effect on the sensitivity to cross-linking agents (Biedermann *et al*., 1991). The NHEJ mutants did not show hypersensitivity to killing by cisplatin (Nojima *et al*., 2005). On the other hand, most of the DT40 mutants defective in homologous recombination showed a significant increase in sensitivities to cisplatin in variation in cellular response to cisplatin depend on the type of protein mutation. Rad54 and Nbs1 have a critical role in DSB repair following X-rays. These findings reveal that vertebrate homologous recombination may consist of different subpathways that are employed at different types of damage (Nojima *et al*., 2005).

The increase in DNA repair that accompanies cisplatin resistance could be caused by enhanced expression of other proteins involved in repair such as:

DNA polymerase beta (Polβ) is reported to be the only polymerase that will effiently bypass a cisplatin 1,2-d(GpG) intrastrand crosslink, suggesting that higher expression levels of this protein will increase replicative bypass (Hoffmann *et al*., 1995).

Excision Repair Cross-Complementing gene 1 (ERCC1) is one of the essential components of the mammalian nucleotide excision repair (NER) pathway. When relative expression of the gene encoding for ERCC1 was monitored in tumor tissue from human ovarian patients, the mRNA levels were significantly higher in tissue from patients who were clinically resistant to cisplatin therapy when compared with the tissue of patients who respond favorably to treatment (Selvakumaran *et al*., 2003).

Proliferating Cell Nuclear Antigen (PCNA) is another protein that is required for the NER pathway to function and this protein was also found to be

overexpression in cell lines that showed resistance to cisplatin exposure (Lyakhovich and Shekhar, 2004).

C-fos and C-myc are both proto-oncogenes that have been correlated with cisplatin resistance following drug exposure. Activating the transcription of these genes may lead to a cscade of genes expression that, in turn, stimulates the actyivity of proteins having a direct role in DNA repair (Kashini-Sabet *et al*., 1990).

Finally, p53 is a tumor suppressor gene product that has been linked to the ability of DNA repair to confer cisplatin sensitivity. Disruption of the gene encoding for p53 in human breast cancer cells increased their sensitivity to cisplatin, possibly because of a decrease in DNA repair (Fan *et al*., 1995).

2.1.7 Mutagenicity of cisplatin

In bacteria, the mutation spectrum induced by cisplatin shows that cisplatin[d(ApG)] adducts are approximately 5 times more mutagenic than the major d(GpG) adducts although d(ApG) adducts account for only 25% of the lesions formed (Burnouf *et al*., 1990). In SOS-induced bacteria, mutation frequencies of 1-2% are detected. All these mutations are targeted to the 5′ base of adducts. Single A→T transversions are mainly observed (80%), whereas A→G transitions account for 10% of the total mutation. The high mutation specificity of cisplatin[d(ApG)]-induced mutagenesis is dependent on its structure (Burnouf *et al*., 1990). In human, the effects of cisplatin on hypoxanthine guanine phosphoribosyl transferase gene produce more than one thousand mutants. About 10% of mutation occurs on GGGGGG sequence. G/C→A/T substitutions at the second and third guanines in the 5′-GGGGGG-3′ run made up about 2 and 4% of the induced mutant, respectively. About 4% of the induced mutants contain a G/C→T/A substitution at the sixth guanine. About 1% of the cisplatin-induced mutant has an A/T→T/A transversion in a TAGA sequence. The mutation of guanine and adenine has been identified as the primary sites of cisplatin adduction (Cariello *et al*., 1992). However, the level of mutation depends on the type

of DNA polymerases. The high fidelity *Tli* polymerase shows lower mutation than *Taq* DNA polymerase and T7 DNA polymerase (Cariello *et al*., 1991).

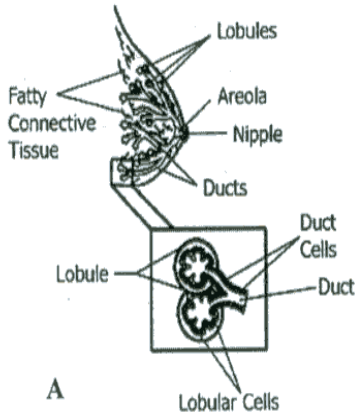
2.2 Breast cancer

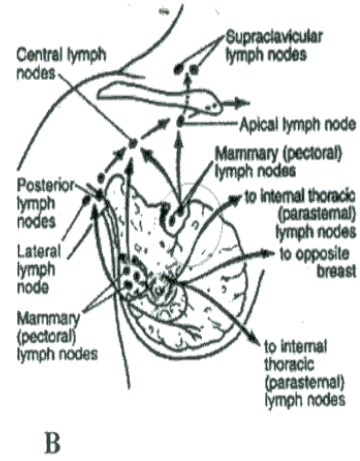
2.2.1 Breast anatomy and physiology

The breast is a mass of glandular, fatty and fibrous tissue. It is positioned over the pectoral muscles of the chest wall and attached to the cheast wall by Cooper's ligaments. A layer of fatty tissue surrounds the breast glands throughout the breast. The breast is composed of: 1) milk gland (lobules) that produce milk, 2) ducts that transport milk from the milk glands to the nipple, areola, 3) fibrous tissue that surrounds the lobules and ducts.

2.2.2 Characteristic of breast cancer

Breast cancer arises in ductal epithelial cells and spread beyond the breast duct or lobule wall. While the breast cancer is increasing in size, it invades the basement membrane, mammary fat, underlying muscle, overlying skin and spreads to the blood vessles and lymph vessels of the dermis (Fig. 3). Dermal lymphatic invasion causes obstruction of lymphartic drainage and therefore, commonly correlates with clinical erythema and induration of the skin of the breast, so called peau d' orange. Cancer of the breast affects the left breast slightly more often than the right. The location of the tumors within the breast and percentage of each location is shown in Fig. 3 and Table 1, respectively.





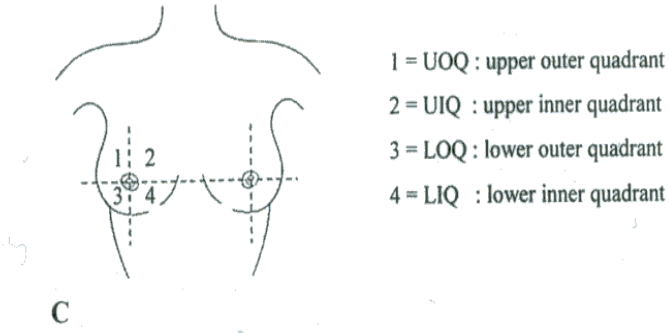


Figure 3. Schematic illustration of breast anatomy (A), lymphatic drainage of the breast (B) and breast position (C)

Table 1. Percentage of breast tumors classified by locations

Locations	Percentage
Upper outer quardrant	50
Central portion	20
Lower outer quardrant	10
Upper inner quardrant	10
Lower inner quardrant	10

2.2.3 Risk factors of breast cancer

A risk factor is anything that increases chance of getting a cancer. But having a risk factor, or even several, does not mean that will get the disease. Most women who have one or more breast cancer risk factors never develop the disease, while many women with breast cancer have no apparent risk factors (other than being a woman and growing older). Even when a woman with breast cancer has a risk factor, there is no way to prove that it actually caused her cancer.

The major risk factors for breast cancer are family history, *BRCA1/BRCA2* mutations, age at menarche, age at menopause, parity, benign breast disease, gender, obesity, high fat diets and radiation (Vogelstein and Kinzler, 2002) (Table 2).

Table 2. Risk factors for breast cancer

Risk Factor	**Risk Category**	**Relative Risk**
Family history	Mother age > 60	1.4
	Two first-degree relatives	4-6
BRCA1/BRCA2 mutations	Carriers	150 (at age 40)
Age at menarche	< 14 y	1.3
Age at menopause	> 55 y	1.5
Parity	No full-term birth	1.9
Benign breast disease	Atypical hyperplasia	4.0
Radiation	Atomic bomb survivor	13
	Mantle radiation for Hodkin's disease	75

2.2.4 Genetic risk factor

Major cause of genetic defect in breast cancer is on the category of tumor suppressor gene mutation. The concept of tumor suppressor genes arose from Knudson's observation that the inactivation of two alleles of the retinoblastoma gene (*Rb*) produces retinoblastoma. All patients with hereditary retinoblastoma carry a germline mutation in one allele of the *Rb* gene, leaving them with only one functional copy. Loss of the remaining functional allele results in the inactivation of both *Rb* alleles: the first one by the inherited germ-line mutation and the second by a somatic event. Thus he proposed a two-hit hypothesis (Knudson, 1971) (Fig. 4). In familial cancers, the first mutation (germ-line mutation) is inherited, and present in all cells of an individual; the second hit is somatic resulting frequently from loss of wild type allele. In the sporadic cancers both hits are somatic. The loss of genetic markers in the

chromosome region of interest within familial cancers is termed loss of heterozygosity (LOH). Consistence LOH for a genetic marker at a given locus in tumors from multiple patients has been considered strong evidence of the presence of a tumor suppressor gene in that region.

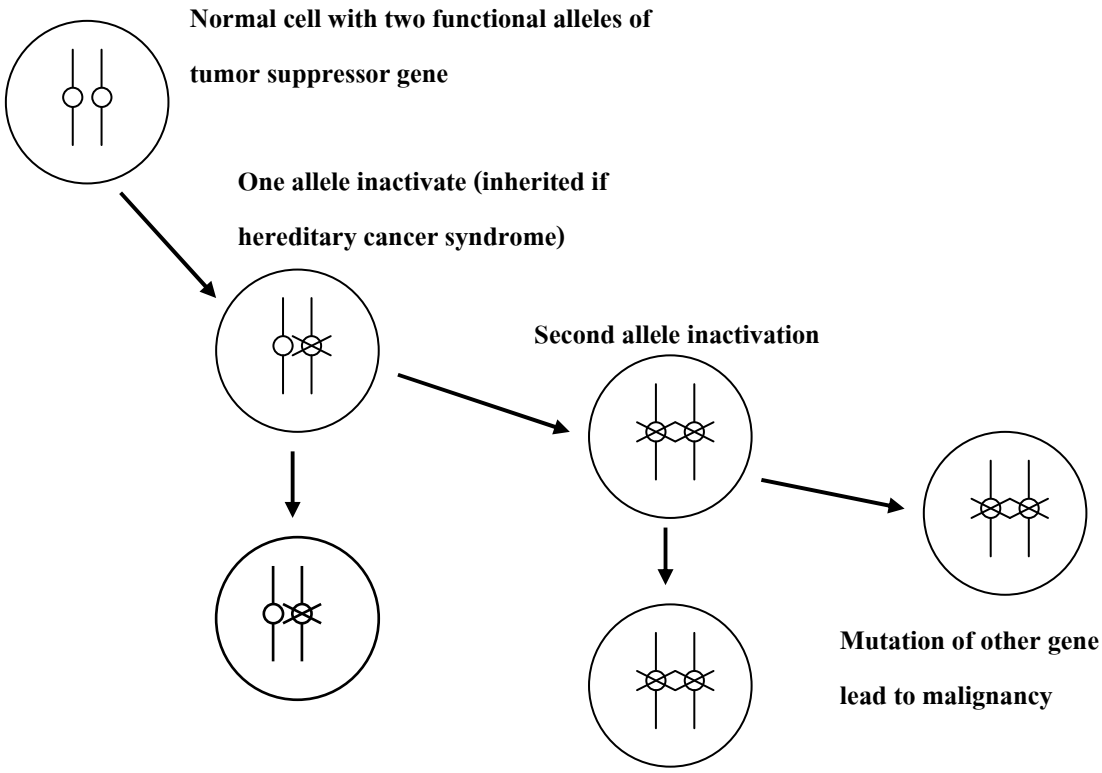


Figure 4. Two-hit hypothesis of tumor suppressor gene.

Recent studies have shown that about 5% to 10% of breast cancer cases are hereditary as a result of gene changes (mutations). The most common mutations are those of the *BRCA1* and *BRCA2* genes. Normally, these genes help to prevent cancer by making proteins that keep cells from growing abnormally. However, if women have inherited either mutated gene from a parent, they are at increased risk for breast cancer.

BRCA1 and *BRCA2* are the two major breast cancer susceptibility genes located on long arms of chromosomes 17 (Hall *et al*., 1990) and 13 (Wooster *et al*.,

1995), respectively. Both genes apparently function as tumor suppressor genes. BRCA1 is a large protein of 1863 amino acids and BRCA2, with 3418 amino acids, is even larger. Both proteins are involved in control of homologous recombination (HR) and double-strand break repair in response to DNA damage (Scully and Livingston, 2000; Scully *et al*., 2000; Wang *et al*., 2000; Welcsh *et al*., 2001; Zhong *et al*., 1999;). Mutations in the *BRCA1* and *BRCA2* genes were first reported in conjunction with their identification in 1994 (Miki *et al*., 1994) and 1995 (Tavtigian *et al*., 1996; Wooster *et al*., 1995). To date more than 1600 distinct mutations, polymorphisms and variants in *BRCA1* and more than 1800 in *BRCA2* have been reported (BIC database, 2007), which are distributed throughout the entire coding regions of both genes. Together, mutations in both genes account for the great majority of families with hereditary susceptibility to breast and ovarian cancer (Ford *et al*., 1998). Epidemiological studies indicate that *BRCA1* mutation carriers have a lifetime risk of breast cancer that is on the order of 60–80% (Antoniou *et al*., 2003; Ford *et al*., 1994; Ford *et al*., 1998). The lifetime breast cancer risk for *BRCA2* mutation carriers approaches that of *BRCA1* carriers: however, disease onset has been documented to be at a later age (Antoniou *et al*., 2003; Ford *et al*., 1994; Ford *et al*., 1998). In other words, women with an altered *BRCA1* or *BRCA2* are 3 to 7 times more likely to develop breast cancer than women without alterations in those genes, with very high relative risks for early disease onset (before age 40) of about 30-fold (Genetic testing for B*RCA1* and *BRCA2*, 2002). Carriers of *BRCA1* and *BRCA2* mutation(s) are also at increased risk for other cancers – in particular, both genes increase the risk of ovarian cancer, while *BRCA2* confers greatly increased risks of male breast cancer. Additional, but more modest risks are found for uterine, cervical, early-onset prostate and pancreatic cancers for *BRCA1* and prostatic, pancreatic, gallbladder, bile duct, stomach cancers and melanoma for *BRCA2* (Thomson *et al*., 2002).

Other genes have been discovered that might also lead to inherited breast cancers such as *AT*, *PTEN* and *p53* (Table 3).

AT (Ataxia telangiectasia) is a rare autosomal recessive disease (heterozygote carrier frequency < ~ 1%) characterized predominantly by severe progressive cerebellar degeneration and increased rates of leukemia and lymphoma (McKinnon, 2004). For nearly 20 years it has been suggested that obligate heterozygote mutation carriers, who are phenotypically normal, are at increased risk of breast cancer (Swift *et al*., 1991). Heterozygote female relatives of *ATM* cases have a 2-7 fold increased risk of breast cancer. High risks of breast cancer also associated with certain *ATM* variants (Thompson *et al*., 2005a). Epidemiologic studies among relatives of *AT* patients have generally confirmed a modest breast cancer risk among obligate heterozygotes (Geoffroy-Perez *et al*., 2001; Olsen *et al*., 2001; Swift *et al*., 1991; Thompson *et al*., 2005b).

PTEN (phosphatase and tensin homologue) encodes a dual-specificity protein phosphatase that negatively regulates phosphatidylinositol 3-kinase/AKT signaling, a pathway with an established role in promoting cell cycle progression and survival (Sansal and Sellers, 2004). Loss of PTEN protein expression in breast carcinomas has been associated with histologic features related to poor prognosis (Bose *et al*., 2002; Tsutsui *et al*., 2005). Rare, germline mutations in *PTEN* have been identified and result in Cowden disease, which is characterized by benign hamartomatous lesions and a 25% to 50% lifetime risk of developing breast cancer. Patients are also at risk for thyroid (and perhaps other) cancers (Pilarski and Eng, 2004).

Another tumor suppressor gene involves in breast cancer is *p53*. The *p53* is the first tumor suppressor gene to be identified. The *p53* functions to eliminate and inhibit the proliferation of abnormal cells, thereby preventing neoplastic development. Abrogation of the negative growth regulatory functions of *p53* occurs in

many, perhaps all, human tumors. Germ-line mutations in *p53* occur in a high proportion of individuals with the Li–Fraumeni cancer susceptibility syndrome, which confers an increased risk of breast cancer (Malkin *et al*., 1990). This implies an important role for *p53* inactivation in mammary carcinogenesis, and the structure and expression of *p53* has been widely studied in breast cancer. In early studies, expression of mutant *p53* was demonstrated in breast cancer cell lines (Bertek *et al*., 1990). Loss of heterozygosity (LOH) in the *p53* gene was shown to be a common event in primary breast carcinomas and colon carcinoma (Arun *et al*., 2003; Davidoff *et al*., 1991; Iacopetta *et al*., 2006; Marchetti *et al*., 2003; Rahman-Roblick *et al*., 2007).

Table 3. Causes of hereditary susceptibility to breast cancer (American Society of Clinical Oncology, 2006)

Gene	**Contribution to hereditary breast cancer**
BRCA1	20%-40%
BRCA2	10%-30%
p53	<1%
PTEN	<1%
Undiscovered genes	30%-70%

2.2.5 Mutation of *BRCA1* gene

Mutations in *BRCA1* account for approximately 2-5% of all breast cancer cases (Easton *et al*., 1993). Mutation of *BRCA1* is predicted to account for 85-90% of hereditary breast and ovarian cancer syndromes (Gangury *et al*., 1997). Among women with breast cancer and a family history of the disease, the percentage with *BRCA1* coding-region mutation is less than the 45% predicted by genetic linkage analysis. The rate is higher among woman from families with history of breast and

ovary cancer (Couch *et al*., 1997; Easton *et al*., 1993). The high probability for detection of *BRCA1* mutation cancer is founded in woman at average age of less than 55 years, the presence of ovarian cancer, the present of breast and ovarian cancer in the same woman (Couch *et al*., 1997). More than one thousand mutations were found worldwild.

Most *BRCA1* mutations lead to frameshifts resulting in missing or nonfunctional protein. In all cancers that have been studied from individuals with a disease-causing mutation, the wild-type allele is deleted, strongly suggesting that BRCA1 is in the class of tumor suppressor genes, i.e., genes whose loss of function can result in neoplastic growth (Smith *et al*., 1992). Additional evidence that *BRCA1* is a tumor suppressor gene is that overexpression of the breast cancer type 1 susceptibility protein leads to growth suppression similar to the paradigmatic tumor suppressors *p53* and the retinoblastoma gene (Holt *et al*., 1996). The pattern of *BRCA1* mutations are shown in Table 4.

2.2.6 Effect of *BRCA1* mutation on breast and ovarian cancer

The study on two subgroups of *BRCA1*-associated breast cancer families that: the first with highly proliferating tumors and, the second composed of cases with a low proliferation. It was found a prevalence of highly proliferating tumors in the mutations on the two terminal conserved domains of BRCA1 protein in the amino and carboxyl termini (Sobol *et al*., 1996). *BRCA1*-associated cancer revealed more genomic instability than sporadic case. The *BRCA1* tumor showed the loss of 5q (86%), 4q (81%), 4p (64%), 2q (40%) and 12q (40%) higher than 7-13% of sporadic tumor (Tirkkonen *et al*., 1997).

Table 4. *BRCA1* mutation database (Breast Cancer Information Core, 2007)

Exon	Total Number of Entries	Distinct Mutations, Polymorphisms and Variants	Alterations reported only once
1	1	1	1
2	2125	52	35
3	167	43	26
5	420	44	21
6	183	29	16
7	157	32	16
8	145	30	18
9	137	18	8
10	42	16	11
11A	1098	210	112
11B	1082	212	117
11C	1385	217	112
11D	1445	202	105
12	108	40	23
13	409	46	27
14	106	27	17
15	214	41	20
16	534	78	45
17	150	48	25
18	218	48	20
19	115	30	18
20	1273	47	26
21	87	28	18
22	158	28	12
23	63	26	14
24	188	50	27
Total	**12010**	**1643**	**890**

The cumulative risk of *BRCA1* mutation was 59% by age 50 years and 82% by age 70 years. The corresponding estimates for the breast-ovary families were 67% and 76%, and those for the families without ovarian cancer were 49% and 90% (Easton *et al.*, 1993). The germline mutation of *BRCA1* confers an increased life time risk for prostate cancer in male. Some mutation was found highly in population such as 185delAG and 5382insC in Ashkenazi Jews (Abeliovich *et al*., 1997; Antoniou *et al*., 2005; Fodor *et al*., 1998; Hodgson *et al*., 1999; Satagopan *et al*., 2002). The probability of death from breast cancer in 5 years was 35.7% in this mutation compare to 4.3 % in non mutation group (Foulkes *et al*., 1997). The 5-year distant disease free survival was 68.2% in *BRCA1* mutation carriers compare to 88.7% in non-carrier group. It means the woman carry this mutation has an adverse prognosis on breast cancer. The lifetime risk of breast cancer and ovarian cancer in women *BRCA1* mutation from is listed in the Table 5 and 6.

2.2.7 Pathology

The histopathology of *BRCA1*-associated breast cancer shows highly proliferating tumor (Eisinger *et al*., 1996). The cancer associated with grade 3 that mean the poorer prognosis than those with sporadic disease (Jarvis *et al*., 1998). Absence of BRCA1 or aberrant subcellular location is observed to a variation extent in histological sections of many breast cancer biopsi. It suggests that BRCA1 abnormalities may be involved in the pathogenesis of many breast cancers, sporadic as well as familial (Chen *et al*., 1995). *BRCA1*-related tumors show an excess of medullary histopathology are more likely to be estrogen receptor-negative and progesterone receptor-negative. At the molecular level there is a higher frequency of p53 mutations, and less HER2/c-erbB-2/neu overexpression (Xing *et al*., 1996).

Table 5. Cumulative risks (standard error) of breast cancer among relative with *BRCA1* mutation (King *et al*., 2003)

Risk by age (year)	**Breast cancer**
	***BRCA1* mutation**
30	0.03 (0.01)
40	0.21 (0.03)
50	0.39 (0.04)
60	0.58 (0.05)
70	0.69 (0.05)
80	0.81 (0.06)

Table 6. Estimated cancer risks in *BRCA1* mutation carriers (White *et al*., 2002)

Cancer type	**Lifetime risk**	
	***BRCA1* mutation carriers**	**General population (non-carrier)**
Breast	56-85%	12.5%
Ovarian	15-60%	1.4%
Contralateral Breast cancer	Up to 65%	15-20%
Ovarian cancer secondary to breast cancer	30-60%	2%-3%

An excess of serous adenocarcinomas of ovarian cancer has been observed in women with *BRCA1* cancer-predisposing mutations compared to controls. Over 90% of tumors in women with *BRCA1* cancer-predisposing mutations are serous,

compared to approximately 50% in women without a *BRCA1* cancer-predisposing mutation (Aida *et al*., 1998; Berchuck *et al*., 1998; Lu *et al* 1999; Rubin *et al.,* 1996). Serous adenocarcinomas are generally of higher grade and are more frequently bilateral.

2.3 BRCA1

2.3.1 Introduction

Human breast cancer is usually caused by genetic alterations of somatic cells of the breast. In occasionally, susceptibility of the disease is inherited. The chromosome appears to be the location of a gene for inherited susceptibility to breast cancer in families with early-onset disease. The linkage between early onset breast cancer and ovarian cancer is found on chromosome 17q21 (Fig. 5).

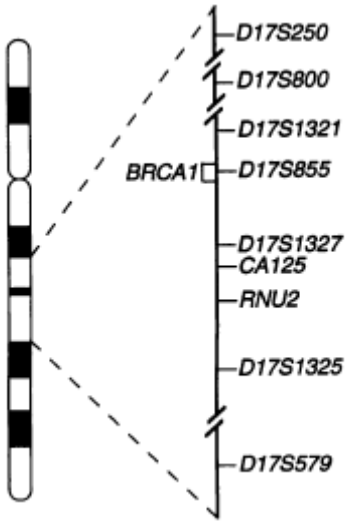


Figure 5. Schematic map of human chromosome 17. D17S855 is located within *BRCA1*. (Miki *et al*., 1994)

The location of a gene *BRCA1* appears to be inherited as an autosomal dominant susceptibility allele (Chamberlain *et al*., 1993). Genetic analysis shows a linkage of breast cancer susceptibility to D17S74 and D17S855 markers on chromosome 17q21 to early-onset families (Feunteun *et al*., 1993; Hall *et al*., 1990; Miki *et al*., 1994; Narod *et al*., 1991). The loss of heterozygosity from familial tumors suggests that *BRCA1* encodes a tumor suppressor protein. The *BRCA1* region is also subjected to allelic loss in sporadic breast and ovarian concern an indication that *BRCA1* mutations may occur somatically in these tumors (Smith *et al*., 1992; Futreal *et al*., 1994).

BRCA1 gene was found in 1994 (Miki *et al*., 1994). The gene comprises 24 exons and 22 introns (Fig. 6) (Hall *et al*., 1990; Miki *et al*., 1994; Shattuck-Eidens *et al*., 1997). *BRCA1* gene codes for 1863 amino acid polypeptide with molecular weight of 220 kDa (Fig. 7). BRCA1 protein contains three regions (1) Zinc finger RING domain at the N-terminal (Brzovic *et al*., 1998; Johnson and Kruk, 2002; Meza *et al*., 1999; Morris and Solomon, 2004) (2) nuclear localization signal (NLS) that leads protein towards to nucleus (Altiok *et al*., 1999; Feng *et al*., 2004; Wilson *et al*., 1997; Thakur *et al*., 1997) and (3) BRCT domain (BRCA1 C-terminal domain) (Anderson *et al*., 1998; Au and Henderson, 2005; Bork *et al*., 1997; Chai *et al*., 1999; Hayes *et al*., 2000; Miyake *et al*., 2000; Welcsh *et al*., 2002; Yaden and Brody, 1999; Yu *et al*., 1998)

The sequence of *BRCA1* is compared in many animal species. The mouse cDNA sequence predicts a protein of 1812 amino acids comparing to 1863 amino acid in human (Bennett *et al*., 1995; Miki *et al*., 1994; Sharan *et al*., 1995). The regions of greatest homology are at the amino and carboxyl terminal. The amino terminal 120 residues of the gene are >80% identical among rat, mouse and human. The C-terminal is also highly conserved, containing an 80 amino acid stretch that is over 80% identical (Szabo *et al*., 1996). The RING finger domain is well conserved.

The expression is highest in testis (Chen *et al*., 1996b). The strong conservation during mammalian evolution reveals the important of this domain (Abel *et al*., 1995). DNA sequence analysis of the entire gene shows the human and mouse coding regions are 75% identical at the nucleotide level while the predicted amino acid identity is only 58% (Bennett *et al*., 1995).

2.3.2 Structure and function of BRCA1

N-terminal of BRCA1 protein contains two RING-finger domains. The zinc-binding sequences of the two RING domains are largely homologous to those of other RING proteins. Thus, the RING sequences of BRCA1 form a central α-helix, a short β sheet of three antiparallel β strands, and two extended loops containing the first and last pairs of metal-binding residues (Fig. 8). This resembles the structurally characterized RING domains of the viral IEEHV polypeptide, the Cbl oncoprotein, and the RAG1 recombination activating polypeptide (Barlow *et al*., 1994; Bellon *et al*., 1997; Zheng *et al.*, 2000). BRCA1 and BRCA1-associated RING domain 1 (BARD1) are multidomain proteins that interact *in vivo* via their N-terminal RING finger motif (Meza *et al*., 1999; Wu *et al*., 1996). The colocalization of BRCA1 and BARD1 proteins are cell cycle dependent. The stedy-stage level of BRCA1 increases in the late G1 and reach maximum during S phase. Moreover, in S phase cells, BRCA1 polypeptides are hyper-phosphorylated and accumulate into discrete subnuclear foci termed “BRCA1 nuclear dots” (Jin *et al*., 1997). BARD1 resides within BRCA1 nuclear dot only during S phase of cell cycle. The progression to S phase by aggregation of nuclear BARD1 and BRCA1 implied the important of both proteins on DNA repair function (Jin *et al*., 1997).

The BRCT domains are at the C-terminal of BRCA1 protein with 217 amino acids long, comprising two repeated motifs of non-identical stretches on amino acids, with the second motif ending eight amino acids short of the carboxy terminus. This motif has been found to occur singly or in multiple copies in other proteins.

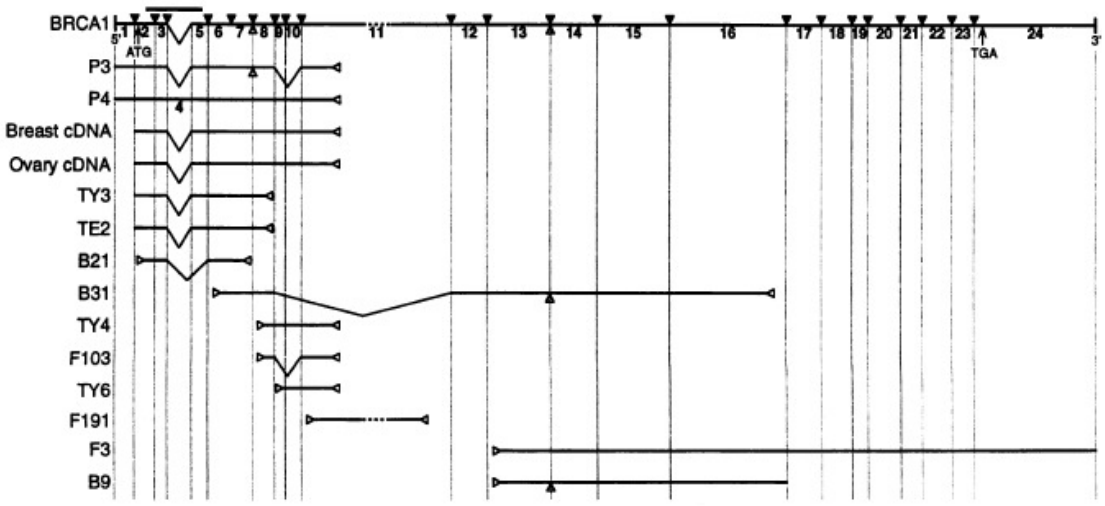


Figure 6. Diagram of BRCA1 mRNA, showing the locations of introns and the variants of BRCA1 mRNA produced by alternative splicing. The top cDNA (BRCA1) is the composite used to generate the protein sequence. Intron locations are shown by filled triangles, and the exons are numbered below the composite cDNA. Alternative mRNAs identified as cDNA clones or in hybrid-selection experiments are shown between the composite. The start codon (ATG) and stop codon (TGA) are indicated. The zinc finger region is denoted note by a bouble line. "V" lines connecting exons indicate the absence of internal exons. All exon are drawn proportionally except exon 11. Upward-pointing unfilled triangles show the position of a single codon (CAG) found at the start site of exons 8 and 14 in some cDNAs. Leftward- and rightward-pointing unfilled triangle represents partial exons in some cDNAs. P3 and P4 are cDNA clones isolated from a placental cDNA libraly; TY3 and TE2 are 5′ RACE clones from thymus and testis, respectively; B21 and B9 are cDNA cloned from a normal breast cDNA library; B31 is a hybrid-selected cDNA clone from breast cDNA; TY4 and TY6 are cDNA cloned isolated from a thymus cDNA library; and F191, F103 and F3 are cDNA clones isolated from a fetal brain library. The *BRCA1* variants labeled breast cDNA and ovary cDNA are the major forms detected in these tissues by PCR (Miki *et al*., 1994)

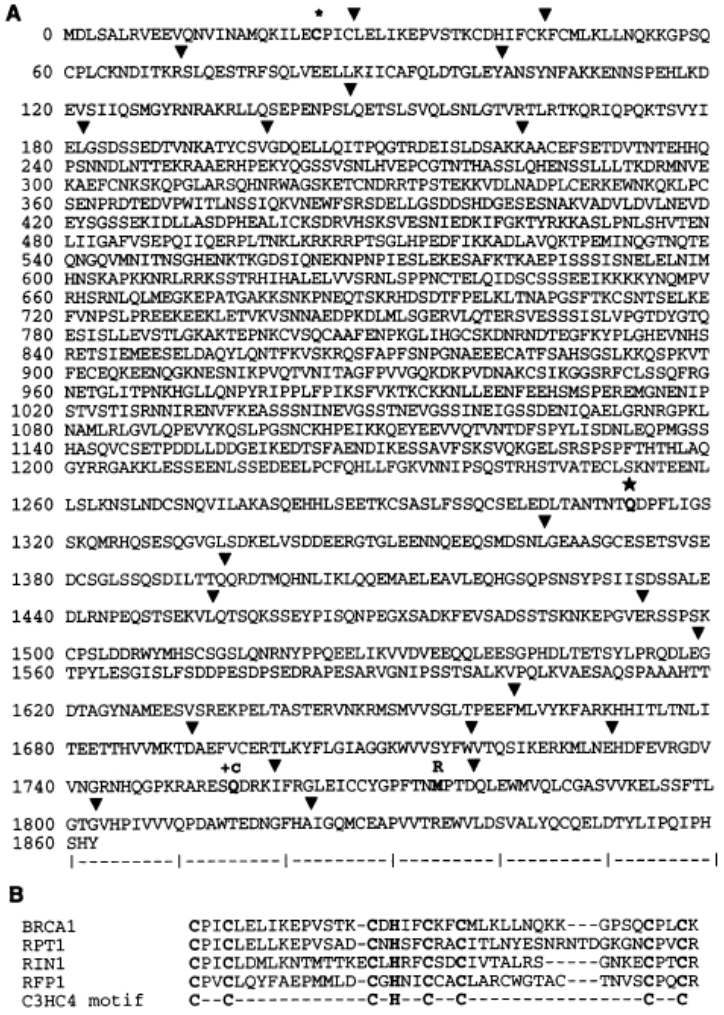


Figure 7. Predicted amino acid sequences for BRCA1. (A) Conceptual translation of the BRCA1 open reading frame, indicating the approximate positions of intron (triangles above sequence) and the locations of some germline mutations (boldface residues). (B) Alignment of the BRCA1 zinc finger domain with three other zinc finger domains that scored highest in a Smith-Waterman alignment. RPT1 is a protein that appears to be a negative regulator of the interleukin-2 receptor in mice. RIN1 is a DNA binding protein that includes a RING finger motif related to the zinc finger. RFP1 is a putative transcription factor comprising the NH_2-terminal domain of the RET oncogene product. The C3HC4 motif shows the positions of the cysteines and the histidine that form the zinc binding pockets (Miki *et al*., 1994).

BRCT domains involves in the function of transcriptional activation, DNA repair and cell cycle regulation (Anderson *et al*., 1998; Chai *et al*., 1999; Chapman and Verma, 1996; Chen *et al*., 1998; Monteiro *et al*, 1996; Neish *et al*., 1998; Ouchi *et al*, 1998; Scully *et al*., 1997c; Somasundaram *et al*, 1997; Yarden and Brody, 1999; Zhang *et al*., 1998). The crystal structure of the two C-terminal BRCT domains of BRCA1 (residues 1646-1859) was presented (Williams *et al.,* 2001). The BRCT motif usually consists of 90-100 amino acids and is found in many eukaryotic proteins, either as an isolated domain or in tandem repeats of two or more BRCT units (Bork *et al*., 1997; Huyton *et al*., 2000). The BRCT domain also presently includes approximately 40 non-orthologous proteins, namely 53BP1, RAD9, RAD4, ECT2, XRCC1, DPB11, deoxynucleotidyltransferases, deoxycytidyl transferase, DNA-ligases III and IV, RAP1, NAD-dependent DNA ligase, eukaryotic replication factor C, poly (ADP-ribose) polymerases, retinoblastoma protein (Anderson *et al*., 2001; Bork *et al*., 1997; Callebaut and Mornon, 1997; Furuya *et al*., 2004; Kim *et al*., 2005; Monaco *et al*., 2005; Soulier and Lowndes, 1999; Yamane *et al*., 2001; Zhang *et al*., 1998). Despite the function diversity of all these protein, participation in DNA damage-responsive checkpoint appears to be a main function (Bork *et al*., 1997). It forms a four stranded parallel β sheet flanked on one side by two α-helices (α1 and α3) and on the other by a third helix (α2). A unique feature of the new structure is that the two tandem motifs of BRCA1 associate with one another in a head-to-tail manner to form a composite domain that is resistant to limited proteolysis (Fig. 8). Significantly, two of the best-characterized missense mutations of *BRCA1* involve residues at the hydrophobic interface between the two BRCT motifs, and it was found that these tumor-associated mutations reduce the proteolytic stability of the composite BRCT domain.

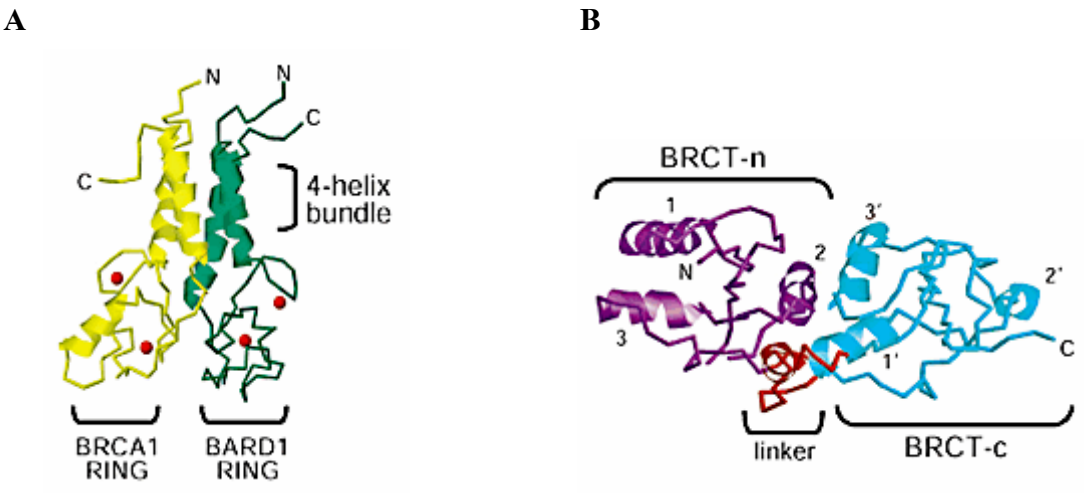


Figure 8. The BRCA1-BARD1 tumor suppressor complex. A. Backbone structure of the dimerized RING domains with helices displayed as ribbons. The core element of each RING motif coordinates two ions of zinc (dot). B. Backbone structure of the two BRCT repeats of BRCA1 with the helices numbered and displayed as ribbons. The N-terminal (BRCT-n) and C-terminal (BRCT-c) repeats are joined through a linker region.: they interact with one another in a head-to-tail fashion by packing the α2-helix of BRCT—n against the α1' and α2'-helices of BRCT-c. The molecule is displayed with the α-helix of the linker perpendicular to the page (Baer, 2001).

2.3.3 BRCA1 and growth control

BRCA1 is expressed in a cell cycle-dependent fashion *in vitro* and *in vivo*. *BRCA1* is expressed concordantly in proliferating cells of embryos, and the mammary gland undergoing morphogenesis in most adult tissue (Blackshear *et al*., 1998). Decrease of *BRCA1* mRNA and protein increase growth rate of both normal and malignant mammary cell. This related to the fact that the mRNA of *BRCA1* decreases during the transition from carcinoma cell *in situ* to invasive cancer. These suggest that *BRCA1* may normally serve as a negative regulator of mammary epithelial cell growth (Thompson *et al*., 1995). In *Saccharomyces cerevesiae*, the expression of BRCA1 or

BRCA1 that fuse with GAL4 activation domain (GAL4-BRCA1) inhibits growth resulting in small colonies easily distinguishable from vector-transformed controls. The growth inhibition effect can be localized to 305 amino acid of BRCT domain (Humphrey *et al*., 1997). Transfected *BRCA1* gene slightly decreases proliferation rate of low BRCA1-expression prostate cell line. Mutation of *BRCA1* has no effect on growth of breast cancer cells. Development of MCF-7 tumors in nude mice is inhibited when MCF-7 cells are transfected with wild-type but not mutant *BRCA1* (Holt *et al*., 1996).

Expression of C-terminal BRCA1 induced alterations in cell cycle control, mainly in G2-M, including a loss of G2-M block by colchicines. It implied that the function of BRCA1 related to growth control by governing checkpoint between DNA replication and mitosis (Larson *et al*., 1997). The arrest of the cell cycle by the tumor-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. BRCA1 does not inhibit the S-phase progression in *p21*-/- cells, unlike *p21*+/+ cells. Tumors associated, transactivation-deficient mutants of BRCA1 are defective in both transactivation of p21 and cell cycle inhibition (Somasundaram *et al*., 1997). However, BRCA1 can activated expression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in both p53-independent and dependent manners and inhibit cell cycle into the S-phase (Chai *et al*., 1999; Somasundaram *et al*., 1997; Zhang *et al*., 1998).

2.3.4 BRCA1 and interacting proteins

BRCA1 interacts with various types of protein in cell cycle check point, transcriptional machinery and DNA repair. Complex of mammalian transcription involving several high molecular weight proteins. BRCA1 has been identified within RNA pol II through RNA helicase, suggesting that it plays a role in the regulation of transcription (Anderson *et al*., 1998: Haile and Parvin, 1999: Krum *et al*., 2003: Scully *et al*., 1997). N-terminal of BRCA1 interacts with BRCA1-associated RING domain

protein (BARD1). The BRCA1:BARD1 heterodimer exhibits ubiquitin ligase activity (Brzovic *et al*., 2001; Hashizume *et al*., 2001; Ruffner *et al*., 2001), suggesting that it may mediate ubiquitination events within the pol II complex. The other cofactor of transcriptional machinery that bind to BRCA1 are ERα (Fan *et al*., 1999), TRAP (Wada *et al.*, 2004), p300 and CBP (Fan *et al*., 2002; Pao *et al*., 2000) RB1 (Aprelikova *et al*., 1999: Fan *et al*., 2001: Yarden and Brody, 1999), RB1 binding protein (HDAC1 and RbAp48) , Histone deacetylases (HDAC1 and HDAC2) (Yarden and Brody, 1999), CiIP (Yu *et al*., 1998), LIM-only protein LMO4 (Sum *et al*., 2002)

BRCA1 also interacts with many proteins in DNA repair patway such MSH2, MSH6 and MLH1 in mismatch repair (Gowen *et al*., 1998). BRCA1 is hyperphosphorylate and relocalized to DNA replication complexes containing proliferating cell nuclear antigen (PCNA), RAD51, BARD1, and BRCA2 (scully *et al.*, 1997A and C). BRCA1 interacts and colocalizes with hRAD51, miotic and mitotic recombination and repair of DNA damage protein, in subnuclear foci in somatic cells and on the axial elements of developing synaptonemal complexes (Chen *et al*., 1998; Scully *et al*., 1997a; Scully *et al*., 1997b). BRCA1 co-localizes with RAD51 in intranuclear structures where DNA replication occurs after treatment with DNA damaging reagents (Scully *et al*., 1997a). BRCA1 deficiency results in decreased RAD51 foci formation in cultured MEFs upon γ-irradiation (Huber *et al*., 2001). Both proteins also co-localize both *in vivo* and to coimmunoprecipitate. BRCA1 residues 758-1064 alone formed RAD51-containing complex *in vitro*. RAD51 is also specifically associated with developing synaptonemal complexes in meiotic cells, and BRCA1 and RAD51 are both detected on asynapsed (axial) elements of human synaptonemal complexes. BRCA1 and RAD51 have function to control recombination and genome integrity (Scully *et al*., 1997a). BRCA1, BARD1 and RAD51 accumulate focally on PCNA replication structure implying an interaction of BRCA1/BARD1/RAD51 containing complexes with damaged replication DNA. The

BRCA1 S-phase foci are dynamic physiological elements that response to DNA damage and participate in a replication checkpoint response (Scully *et al*., 1997c) (Fig. 9 and 10)**.**

Another gene that is important in DSB repair, RAD50, also interacts with BRCA1 both *in vitro* and *in vivo*. Upon ionizing irradiation, wild-type *BRCA1* forms a complex with RAD50, MRE11 and p95/nibrin in discrete nuclear foci. The formation of these irradiation-induced foci was dramatically reduced in BRCA1-deficient HCC1937 breast cancer cells but was restored by transfection of wild-type BRCA1. BRCA1 is important for cellular responses to DNA damage mediated by the RAD50-MRE11-p95 complex (Zhong *et al*., 1999).

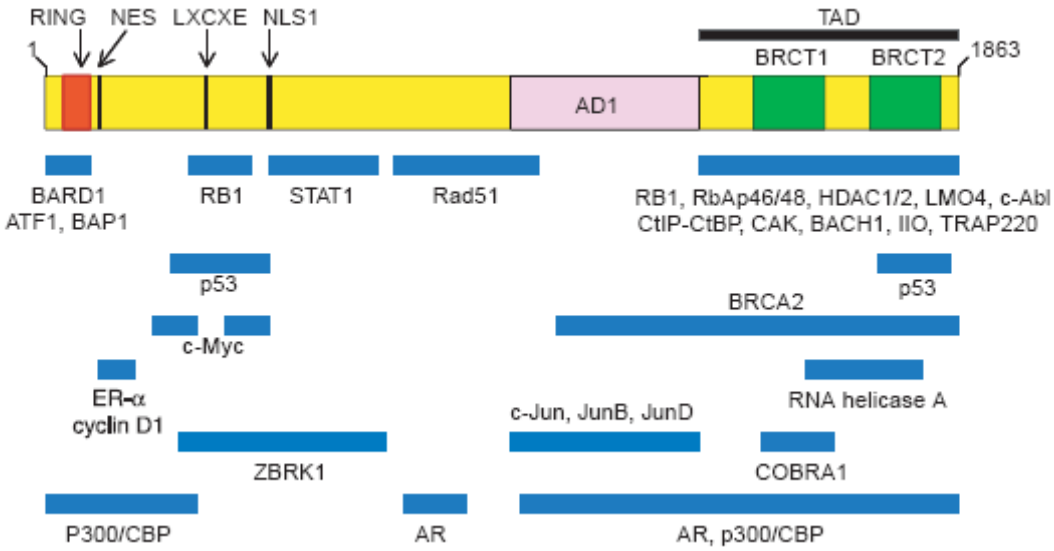


Figure 9. BRCA1 domain structure and sites of interactions. The approximate locations of various protein binding sites are shown with respect to the domain structure of BRCA1 (Rosen *et al*., 2006).

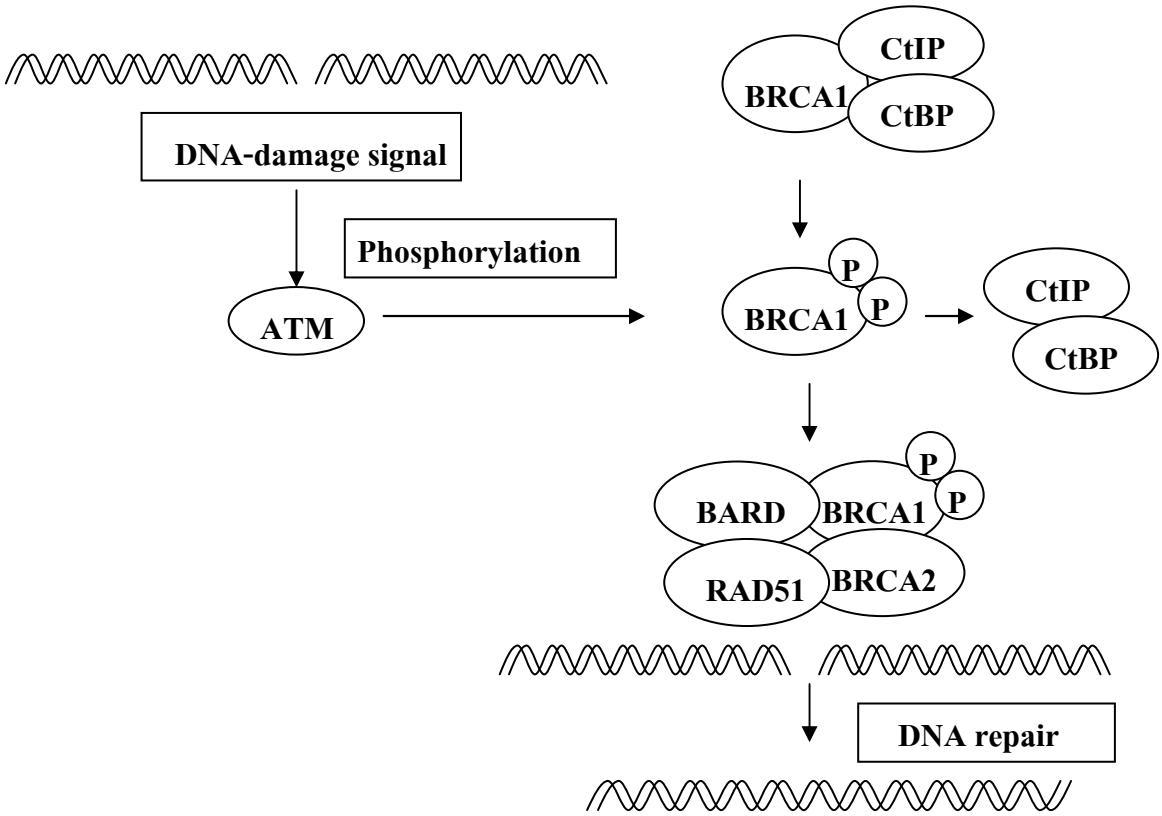


Figure 10. The roles of BRCA1 in DNA repair. This model suggests that a macromolecular complex consisting of BRCA1, BRCA2, BARD1 and RAD51 functions to repair damaged DNA. Complex formation is preceded by phosphorylation of BRCA1 by the kinase ATM. In response to DNA damage, the complex relocates to chromosomal regions undergoing DNA replication marked by proliferating cell nuclear antigen (PCNA). Loss of BRCA1 and/or BRCA2 function leads to inability to repair damaged DNA (Welcsh *et al*., 2000).

2.3.5 BRCA1 and non homologuos end-joining repair (NHEJ)

Mammalian cells can also repair double stranded breaks by non-homologous end joining (NHEJ) (Liang *et al*., 1998). RAD50, Mre11 and p95/nibrin form a complex that functions in homologous recombination and in NHEJ, meiotic recombination, the DNA damage response, and telomere maintenance (Le *et al*., 1999;

Trujillo *et al*., 1998). The association of BRCA1 with these proteins suggests that it may also be involved in repair of DSB by other pathways such as NHEJ (Zhong *et al*., 2002).

To address the role of BRCA1 in NHEJ, the mouse embryonic fibroblasts (MEFs) carrying a p53-null ($p53^{-/-}$) show 50% more accurate of NHEJ repair than *Brca1*-null and p53-null ($Brca1^{-/-}p53^{-/-}$) double mutations (Zhong *et al*., 2002). Using an *in vivo* host cell end-joining assay, a significant decrease in end-joining frequency in $BRCA1^{-/-}$ lymphoblastoid cells was observed (Baldeyron *et al.,* 2002). Moreover, the fidelity of DNA end-joining was strongly reduced in $BRCA1^{+/-}$ cell lines in comparison to control cell lines. They also showed that cell-free $BRCA1^{+/-}$ extracts were unable to promote accurate DNA end-joining in an *in vitro* reaction. Interestingly, the BRCA1 protein was about 20–30% the level of BRCA1 in wild type cells (Baldeyron *et al.,* 2002). This finding provides a basis for the observed defect.

2.3.6 BRCA1 and nucleotide excision repair (NER)

NER is divided into two pathways: global genomic repair (GGR), which removes lesions from the whole genome, and transcription-coupled repair (TCR), which preferentially removes lesions from the transcribed strand of expressed genes (Bernstein *et al*., 2002). Mouse ES cells deficient in BRCA1 are defective in the ability to carry out TCR of oxidative DNA damage, and are hypersensitive to ionizing radiation and hydrogen peroxide (Gowen *et al*., 1998). These results suggest that BRCA1 participates, directly or indirectly, in TCR of oxidative DNA damage. Consistently, expression of BRCA1 restores radiation resistance of a *BRCA1*-deficient human breast cancer cell line, HCC1397, through enhancement of TCR (Abbott *et al*., 1999).

The role of BRCA1 in two human U2OS osteosarcoma cell lines, UBR60 and E621 was study (Hartman and Ford, 2002). Cell line UBR60 carries wild-type p53 and cell line E621 is derived from UBR60, but is deficient for p53 due to

stable expression of the human papillomavirus E6, which targets p53 for degradation. Both cell lines express low levels of endogenous BRCA1 and the exogenously transfected BRCA1 was controlled by tetracycline regulation (about a 4-fold induction of BRCA1 24 h after tetracycline removal). These cells were used as models to study the effects of BRCA1 and/or p53 on NER. The presence of tetracycline (low level of BRCA1), the efficiency of GGR is significantly lower in p53-deficient cells than p53 wild-type cells (3 versus 28% repair at 24 h). This observation is consistent with findings that loss of p53 function results in defective GGR (Amundson *et al*., 2002; Smith *et al*., 2000). Notably, expression of BRCA1 restored GGR in p53-deficient E621 cells with 34% of DNA damages repaired at 24 h versus 3% without BRCA1 induction. Expression of BRCA1 in p53 wild-type UBR cells resulted in greater GGR than the same cells without BRCA1 induction (42 versus 28%) (Hartmann and Ford, 2002). These observations indicate that BRCA1 plays an important role in GGR.

2.3.7 BRCA1 and homologous recombination repair (HRR)

The role of BRCA1 in DNA repair is studied by exposure the cells to DNA damaging agents that hyperphosphorylate BRCA1 and relocalization of BRCA1 with RAD51 to PCNA-containing foci (Scully *et al*., 1997c). RAD51 has been implicated in DNA repair, as demonstrated by the high sensitivity of *S. cerevisiae rad51* mutants to DNA-damaging agents (Shinohara *et al*., 1992). A similar approach has recently shown that BRCA1 can also colocalize in nuclear foci with RAD50 after exposure to ionizing radiation (Zhong *et al*., 1999). Exposure BRCA1 deficient ES cells to DNA damaging agents has revealed that BRCA1 deficient cells display increased sensitivity to DNA damaging agents such as ionizing radiation (Gowen *et al* 1998). These cells have also been shown to be incapable of carrying out TCR after exposure to ionizing radiation and H_2O_2 (Gowen *et al* 1998). This repair system preferentially directs repair of damaged DNA to the transcribed strand of active genes

and is essential for the removal of lesions for which the global repair process is too slow.

Homologous recombination is believed to contribute to the maintenance of genomic integrity through the precise repair of a chromosomal DSB using the sister chromatid as the repair template (Johnson *et al*., 1999). RAD51 has been shown in yeast to promote homology-dependent DNA repair of double strand breaks. A role for BRCA1 in this repair pathway is suggested by its association with this protein, both in somatic cells after exposure to DNA damaging agents, and on the synaptonemal complex during meiosis.

2.3.8 BRCA1 and transcription

BRCA1 is associated with RNA polymerase II holoenzyme such as hSRB7, RNA polymerase II, transcription factor TFIIE, TFIIF, and TFIIH (Scully *et al*., 1997b). BRCA1 activates transcription when linked with a DNA binding domain of RNA polymerase II and is a component of the RNA polymerase II (Pol II) holoenzyme. BRCT domain of BRCA1 protein linked to RNA helicase A (RHA) to the holoenzyme complex. This mean BRCA1 functions as the transcriptional coactivator (Scully *et al*., 1997b). BRCA1 splice variants BRCA1a and BRCA1b interact C-terminal domain of transcription factor CBP (component of the holoenzyme). BRCA1 protein recruits CBP associated HAT/FAT (transcription factor acetyl-transferase) activity for acetylation of either themselves or general transcription factors or both to specific promoters resulting in transcriptional activation (Cui *et al*., 1998).

BRCT domains act as strong transcriptional transactivation when fused to the GAL4-DNA binding domain (Chapman and Verma, 1996; Monteiro *et al*., 1996). The activation of transcription *in vitro* by the BRCT domain in GAL4-BRCA1 protein is tested with several co-activators (Haile and Parvin, 1999). BRCA1 stimulates artificial and genomic promoter construct containing p53-responsive element (Ouchi *et al*., 1998).

The DNA damage-responsive gene *GADD45* in JNK/SAPK pathway can be induced by BRCA1 in response to apoptosis (Harkin *et al*., 1999). The p27 (kip7), the universal cyclin dependent kinase inhibitor factor, is expressed by C-terminal BRCT transactivation and growth inhibitor is induced (Williamson *et al.*, 2002). Full length BRCA1 transactivated the estrogen receptor–alpha and Bcl2 promotor as well as AP-1, SRE and CRE containing promoters (McEachern *et al*., 2003). The transcriptional activation of BRCT domains are suppressed by CtIP and CtBP suppressor proteins both *in vivo* and *in vitro* and mediates transcriptional regulation of p21 in response to DNA damage. The association is abolished in DNA damage manner and correlates with BRCA1 phosphorylation (Li *et al*., 1999).

BRCA1 also transcriptionally regulates DDB2 (a gene defective in Xeroderma Pigmentsum group E cells and encoding the p48 damaged DNA binding protein) in the DNA repair response following UV-irradiation (Hartmann and Ford, 2002; Takimoto *et al*., 2002). The expression of these genes is p53 dependent and is required for efficient GGR, a subpathway of NER (Hwang *et al*., 1999; Adimoolam and Ford, 2002). The overexpression of BRCA1 restored expression of these genes in p53-deficient E621 cells to wild-type levels correlating with a restoration of CGR in these cells (Takimoto *et al*., 2002). Expression of BRCA1 also induced the expression of XPC (Xeroderma pigmentasum group C complimenting protein) in a wild-type p53-dependent manner, as the up-regulation of XPC by BRCA1 is most obvious in p53 wild-type UBR60 cells 48 h post BRCA1 induction, while the up-regulation is moderate in p53-deficient cells. These studies suggest a mechanism, by which BRCA1 functions as a transcriptional regulator of genes involved in NER at p53 dependent and independent ways (Takimoto *et al*., 2002).

2.3.9 BRCA1 is transcriptional activator

The transcription activator is the factor that contact target in the basal transcription machinery directly or indirectly via intermediary protein. Once activator proteins are bound to specific DNA sequences, they interact with the basal transcription machinery, and either recruit it to DNA, stabilize components already recruited to DNA, or cause a productive conformation change or some other kind of modification in one or more components of the basal transcription machinery, and thereby initiate a cascade of events that lead to the increased initiation of transcription. The strong activator protein from virus VP16 activates transcription through many steps of transcription. It is found to be the component of many proteins in transcriptional system such as, RNA polymerase II, TBP, TAF, TFIIB, TFIIH, TFIIF, and many adaptors protein (CBP, ADA2, ADA3, PC4, p300) (Goodbourn, 1996) .

Transactivation activity of BRCA1 gene is predicted by the structure of its protein. The composition of zinc-finger domain, nuclear localization domain and acidic region in the C-terminus correlates with transactivation function of many genes. C-terminus portion of BRCA1 is reported to act as a strong transcriptional transactivation when fuses to the GAL4 DNA-binding domain. The GAL4-BRCA1 (1528-1863) activates transcription of reporter plasmid, containing five GAL4 binding sites and a minimal promoter linked to the chloramphenical acetyltransferase gene in 293T kidney derive cell line. However, a C-terminal portion of BRCA1 amino acids 1760-1863 shows moderate activity on transcriptional activity (Chapman and Verma, 1996). This activation is also found in yeast and other mammalian cell line (Monteiro *et al*., 1996; Nadeau *et al*., 2000). The linkage of exon 16-24 of BRCA1 gene to GAL4 DNA binding domains activate the expression of HIS3 reporter gene under control of the GAL1 upstream activating sequence (UAS), responsive to GAL4 transcriptional activation of *Saccharomyces cerevisiae* strain HF7c (Monteiro *et al*., 1996). The exon 16-24 is also proved in COS-7 mammalian cell line. GAL4 DNA binding domain

activates transcription of luciferase gene as a reporter under control of GAL4 responsive element. In that experiment, the shorter of BRCA1 protein (1760-1863) induces only 60% of growth of yeast compared to the exon 16-24 of *BRCA1*. This reveals that the region of amino acid 1760-1863 is a minimal requirement of the protein to transactivate the other gene (Monteiro *et al*., 1996).

Transactivation of BRCT domain involves many kind of transcriptional factor such as RNA polymerase II (Schlegel *et al*., 2000), RNA helicase A (Scully *et al*., 1997b; Neish *et al*., 1998), CBP/p300 (Pao *et al*., 2000). It has been reported that, when over expressed in mammalian cells, the full-length BRCA1 protein can potentiate transcription several natural promoter in both a p53-depent and independent manner (Ouchi *et al.,* 1998; Somasundaram *et al*., 1997; Yarden and Brody, 1999; Zhang *et al*., 1998). BRCA1 interacts with elements of the RNA pol II holoenzyme linked to post promoter activities of the complex, including interactions with elongation factor NELF-B/COBRA1 and the CstF-50 component of polyadenyl (Kleiman and Manley, 1999). Ectopic expression of full-length human BRCA1 is then shown to increase expression of stress-responsive gene including p21 (Somasundaram *et al*., 1997), *GADD45* (Harkin *et al*., 1999) and decrease expression of other gene, including c-*myc*-regulated gene (Li *et al*., 2002), and certain estrogen regulated genes (Fan *et al*., 1999). The BRCA1 also stimulates transcription without the requirement for a DNA-tethering function both in mammalian and yeast cell (Nadean *et al*., 2000). The BRCA1 can stimulate transcription without the requirement for a DNA-tethering function both in mammalian and yeast cell (Nadeau *et al*., 2000). BRCA1 protein associates specifically with hyperphosphorylated RNA pol II in undamaged cell. The interaction is disrupted by DNA-damaging agent. Preferential interaction processive RNA pol II in undamaged cell placed BRCA1 link to late elongation in transcription with repair processes in eukaryotic cell (Krum *et al*., 2003). Some factors regulate transcription activity of BRCA1 protein. The two subunits of RNA pol II, hRPB2 and hRPB10 block BRCA1

and mediate the regulated stimulation of transcription (Schlegel *et al*., 2000). TRAP220 protein can form complex with BRCA1 and moderate BRCA1 transactivation function (Wada *et al*., 2004).

2.3.10 BRCA1 and hormonal regulation

BRCA1 exhibits a hormone-dependent pattern of expression in oocytes, granulosa cells and theca cells of developing follicles in ovary. In the testis, BRCA1 expressed in mitotic spermatogonia and early meiotic prophase spermatocytes. These observations suggest that it may have distinct roles during meiosis (Blackshear *et al*., 1998). In breast and ovary, the *BRCA1* gene is expressed in hormone dependent manner. The expression is induced during puberty, pregnancy or following treatment of ovariectomized animals with 17 β-estaradiol and progesterone. (Marquis *et al*., 1995). BRCA1 mRNA and protein levels are also regulated by the steroid hormones estrogen and progesterone in human cancer cells. The expression is decreased in estrogen-depleted MCF-7 cell and increased again after stimulation with β-estradiol. The steroid hormone effects BRCA1 expression indirectly by altering the proliferative status of the cells rather than acting directly on DNA sequences in the *BRCA1* motif (Gudas *et al*., 1995). BRCA1 mRNA levels are high in exponentially growing populations of mammalial epithelial cells. The amount of mRNA is decreased upon growth factor withdrawal, and subsequencely increased again in late G1 just prior to S-phase entry. BRCA1 mRNA levels are dramatically reduced in senescent normal human mammary epithelial cells and in normal human mammary epithelial cells treated with transforming growth factor beta 1 (Gudas *et al*., 1996, Vaughn *et al*., 1996).

2.3.11 Phosphorylation of BRCA1 protein

The *BRCA1* gene product is identified as a 220-kD nuclear phosphoprotein in normal cell, including breast ductal epithelial cells (Chen *et al*., 1996a), and cancer cell line (Chen *et al.*, 1995). The phosphorylated BRCA1 is located in nucleus and mitochondria (Coene *et al*., 2005). Phosphorylation of BRCA1 requires

ATM in the DNA damage response to double-strand breaks (Chen, 2000; Cortez *et al*., 1999; Gatei *et al*., 2000). The phosphorylation is cell cycle dependent, with greatest expression and phosphorylation occurring in S and M phase. The BRCA1 is regulated by cyclin-dependent kinases with cyclin D and A (Chen *et al*., 1996a, Ruffner *et al*., 1999). The induction of phosphorylation on BRCA1 occurred during the cell cycle and after DNA treatment (Okada and Ouchi, 2002; Thomas *et al*., 1997; Xu *et al*., 2001, 2002).

2.3.12 BRCA1 on embryonic development

BRCA1 has an important role in early development of embryo. A mouse line carrying a mutation in one mutation allele shows a critical for normal development, as the mice die in utero between 10 and 13 days of gestation. Abnormality in *BRCA1*–deficient embryos is the most evident in the neural tube, with 40% of the embryo presenting with varying degrees of spina bifida and anencephaly. The neuro-epithelium in *BRCA1*-deficient embryo appears disorganize, with signs of both rapid proliferation excessive cell dead (Gowen *et al*., 1996). However, the other investigators show that the heterozygous BRCA1 mutation is normal and fertile and lack tumors by age of eleven months. Homozygous *BRCA1* mutant mice die before day 7.5 of embryogenesis. Mutant embryos are poorly developed with no evidence of mesoderm formation. The extraembryonic region is abnormal. Mutant embryo do not exhibit reduced cell proliferation accompany by decreased expression of cyclin E and mdm-2, a regulator of p53 activity. The expression of cyclin-dependent kinase inhibitor p21 is increased in mutant embryo (Hakem *et al*., 1996). Developmental of embryos are severely retarded in growth as early as embryonic day 4.5 and are complete by day 8.5. They failed to differentiate and form egg cylinder. They cannot form primitive streaks and undergo gastrulation (Liu *et al*., 1996).

2.4 Cisplatin and *BRCA1*

BRCA1 has function on homologous recombinant DNA double strand break repair. BRCA1 and RAD51 protein were found co-localized in cell induced by cisplatin (Zhou *et al*., 2005). C-terminal of BRCA1 is required for assembly of RAD51 and BRCA1 complex. Depletion of BRCA1 foci reduces RAD51 foci formation after cisplatin treatment. C-terminal BRCA1 is also involved in BRCA1 phosphorylation. Cisplatin most likely induced the phosphorylation of tyrosine residue(s) in the BRCA1 protein. It appears that the carboxyl-terminal of BRCA1 is important for this molecule to protect cells from the cytotoxic effect of cisplatin and plays an important role in resistance to cisplatin (Zhou *et al*., 2005).

The overexpression of BRCA1 increases cellular resistant to cisplatin. BRCA1 is overexpressed in cisplatin-resistant MCF-7 cells (Husain *et al*., 1998). The level of BRCA1 protein in cisplatin-resistant breast and ovarian carcinoma cell lines, MCF-7 CDDP/R, and SKOV-3 CDDP/R, is increased. SKOV-3 CDDP/R is significiently more proficient at DNA damage repair. Antisense inhibition of *BRCA1* in this cell line results in an increased sensitivity to cisplatin, a decreased proficiently of DNA repair, and an enhanced rate of apoptosis (Husain *et al*., 1998). The *BRCA1* (-/-) mutants are 5-fold more sensitive to cisplatinum compared with wild-type cells (Bhattacharyya *et al*., 2000). BRCA1 promotes assembly of subnuclear RAD51 foci following x-ray-induced DNA damage and the chemotherapeutic agent cisplatin (Bhattacharyya *et al*., 2000). It is found that although a functional BRCA1 is required for the subnuclear assembly of BRCA1 foci following treatment with either ionizing radiation or cisplatin, a functional BRCA1 is required for RAD51 foci to form following treatment with cisplatin but not with ionizing radiation. Similar results are obtained in SKOV-3 cells when the level of BRCA1 expression is knocked down by stable expression of a retrovirus-mediated small-interfering RNA against BRCA1. The carboxyl-terminal of BRCA1 contains uncharacterized phosphorylation sites that are

responsive to cisplatin. The carboxyl-terminal of BRCA1 is required for the cisplatin-induced recruitment of RAD51 to the DNA-damage site, which may contribute to cisplatin resistance (Zhou *et al*., 2005).

CHAPTER 3

MATERIALS AND METHODS

Materials

Cells

- White blood cell (A normal healthy person)
- MCF-7 cell (The American Type Culture Collection, USA)
- *E. coli* DH5α (New England Biolab, USA)

Chemicals

- Absolute ethanol (Merck, Germany)
- Agarose powder (Analytical grade) (Promega, USA)
- Ammonium acetate (Ajax chemical, Australia)
- Ammonium persulfate (Sigma-Aldrich, USA)
- Ammonium sulfate (Merck, Germany)
- Ampicillin (Sigma-Aldrich, USA)
- Anti-BRCA1 C-20 (Santa Cruz Biotechnology, USA)
- Anti-rabbit IgG (whole molecule) horseradish peroxidase-conjugate (Sigma-Aldrich, USA)
- Bacto™agar (Becton, Dickinson and company, USA)
- Bacto™tryptone (Becton, Dickinson and company, USA)
- Big dye terminator (Applied Biosystem, USA)
- Boric acid (Merck, Germany)
- Bovine serum albumin (Gibco, USA)
- Cisplatin (Sigma-Aldrich, USA)
- Dulbecco's modified eagle medium (Gibco, USA)
- Ethylenediamine tetraacetic acid disodium salt (EDTA) (BDH laboratory supplies, England)

- Calcium chloride (Merck, Germany)
- Difco™LB broth, Lennox (Becton, Dickinson and company, USA)
- D (+) - glucose (Sigma-Aldrich, USA)
- Di - potassium hydrogen phosphate anhydrous (Merck, Germany)
- Di-sodium hydrogen phosphate (Fluka, Switzerland)
- dATP (Promega, USA)
- dCTP (Promega, USA)
- dGTP (Promega, USA)
- dTTP (Promega, USA)
- Ethidium bromide (Gibco, USA)
- Formaldehyde (Merck, Germany)
- Glycine Ultrapure (USB corporation, USA)
- Glycerol (BDH laboratory supplies, England)
- Hydrochloric acid (glacial) 100% (Merck, Germany)
- Isopropanol (J.T. baker, USA)
- Lumino/Enhancer solution (Pierce, USA)
- Magnesium chloride (Merck, Germany)
- Methanol (Labscan Asia, Thailand)
- *N, N′*-methylene-bis-acrylamide (Sigma-Aldrich, USA)
- *N, N, N′, N′*-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, USA)
- Non-fat milk (Mission, Thailand)
- Potassium chloride (Merck, Germany)
- Potassium acetate (BDH laboratory supplies, England)
- Potassium dihydrogen phosphate (Merck, Germany)

- Silver nitrate (Merck, Germany)
- Sodium acetate (APS Finechem, Australia)
- Sodium carbonate anhydrous (Ajax Finechem, Australia)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Promega, USA)
- Sodium hydrogen carbonate (Merck, Germany)
- Sodium hydroxide (Carlo Erba Reagenti, Italy)
- Sodium thiosulfate (Fisher Chemicals, England)
- Tris [hydroxymethyl] aminomethane hydrochloride (Tris-HCl) (Sigma-Aldrich, USA)
- Tris Base (Promega, USA)
- UltraPure™ Acrylamide (Invitrogen, USA)

Enzymes

- *Taq* DNA polymerase and Buffer (QIAGEN, Germany)
- Restriction enzyme *Eco*0109I (New England Biolab, USA)
- Restriction enzyme *Pvu*II (New England Biolab, USA)
- Restriction enzyme *BamH*I (New England Biolab, USA)
- Restriction enzyme *Xba*I (New England Biolab, USA)
- T4 DNA ligase (New England Biolab, USA)

Solutions

- 10x alkaline agarose gel electrophoresis buffer (500 mM NaOH, 10 mM EDTA),
- 6x alkaline gel-loading buffer (300 mM NaOH, 6 mM EDTA, 18% (w/v) glycerol, 0.15% (w/v) bromocresol green, 0.25% (w/v) xylene cyanol)
- Calcium chloride solution (2.5 M $CaCl_2$)
- Calcium chloride solution (50 mM $CaCl_2$)

- Cell lysis buffer (10 mM Tris-HCl, 75 mM NaCl, 25 mM EDTA, pH 8.0).
- Developing solution (6 % sodium carbonate, 0.0004 % sodium thiosulfate, 0.05 % formaldehyde)
- Fix solution (50 % methanol, 12 % acetic acid, 0.05 % formaldehyde)
- 2xHBS buffer (8 g of NaCl, 0.37 g of KCl, 0.201 g of $Na_2HPO_4.H_2O$, 1 g of Glucose, 5 g of HEPES, add water to 500 ml, adjust pH to 7.05 with NaOH and filter sterile)
- Impregnant solution (0.02 % silver nitrate, 0.075 % formaldehyde)
- Neutralizing solution for alkaline agarose gel (1 M Tris-Cl pH 7.6, 1.5 M NaCl)
- Pre-treatment solution (0.02 % sodium thiosulfate)
- Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4)
- Proteinase K solution (1 mg Protease K in 1% sodium dodecyl sulfate and 25 mM EDTA)
- Sample buffer for western blotting (2% SDS, 10% glyceral)
- Sodium acetate solution (3 M NaOAc, pH 5.2)
- Sodium dodecyl sulfate solution (10 % SDS)
- Stop solution (50 % methanol, 12 % acetic acid)
- 5x TBST (35 ml of 2M Tris pH 8.0, 150 ml of 5M NaCl, add distilled water to 1 liter)
- Transfer buffer (3.03 g of Tris base, 14.4 g of glycine, 200 ml of methanol, add distilled water to 1 liter)

- 10x Tris-EDTA, pH 7.6 (100 mM Tris-Cl, pH 7.6, 10 mM EDTA, pH 7.6
- 5x Tris-acetate-EDTA (TAE) (24.2 g of Tris base, 5.71 ml of glacial acetic acid, 10 ml of 0.5 M EDTA, pH 8.0, add distilled water to 1 liter)
- Wash solution I (50 % ethanol)
- Wash solution II (50 % methanol)

Kits

- QIAamp® RNA Blood Mini Kits (QIAGEN, Germany)
- Reverse Transcriptase-Polymerase Chain Reaction QIAGEN OneStep RT-PCR Kit ®
- QIAquick Gel Extraction Kit ® (QIAGEN, Germany)
- Dual-Luciferase® Reporter Assay System (Promega, USA)
- β-galactosidase assay (Promega, USA)

Plasticware

- Acrodisc® syring filter (Pall Corporation, USA)
- Eppendorf tube 1.5 ml (Axygen Scientific Inc, USA)
- PCR tube (Axygen Scientific Inc, USA)
- 6-flat-bottom wells plate (Nunc, Denmark)
- Cell culture flask (Nunc, Denmark)
- CL-X Posure™ Film (Pierce, USA)
- Pipet tip T-300-STK-S (0.2-10 µl) (Axygen Scientific Inc, USA)
- Pipet tip T-200-Y (20-200 µl) (Axygen Scientific Inc, USA)
- Pipet tip T-1000-B (200-1000 µl) (Axygen Scientific Inc, USA)

Instruments

- Agarose gel electrophoresis (EC 370, E-C Apparatus, USA)
- Autoclave (HD-3D, Hirayama Company, Japan)
- CO_2 incubator (ShelLab, Sheldon Manufacturing Inc, USA)
- Deep freezer (-86°C) (Forma Scientific, USA)
- Desk-top centrifuge (VSMC-13 mini-centrifuge, Shelton Scientific, USA)
- Differential Scanning Calorimeter (DSC 7, Perkin-Elmer, USA)
- DNA sequencer (ABI Prism 377, Perkin-Elmer, USA)
- Freezer (-20°C) (Hotpack, Forma Scientific, USA)
- Gel documentation equipment (1000, BIO-RAD, USA)
- Hot air oven (Mammert GmbH Co., Germany)
- Inverted microscope (Ck2, Olympus, USA)
- Laboratory balance (Mettler Toledo, USA)
- Laminar air flow, biosafety carbinet class II (Ultrasafe 48, Faster, Italy)
- Luminometer (TR717, ABI gene system, USA)
- Microplate reader (Power wave X, Biotek, USA)
- pH meter (Mettler Toledo 320, USA)
- Polyacrylamide gel electrophoresis apparatus (AE-6450 Dual mini slab kit, ATTO, Japan)
- Polymerase chain reaction machine (GeneAmp 9600, Perkin-Elmer, USA)
- Power supply (EC 135, E-C Apparatus Company, USA and AE-8150 my power 500, ATTO, Japan)
- Refrigerated microcentrifuge (Kubota 1910, Kubota, Japan)
- Semi-Dry Electroblotter (Owl, Biocompare, USA)
- Shaking bath (SBO 50 BIO, Heto lab equipment, USA)
- UV-spectrophotometer (Genesis 5, Spectronic, USA)
- Vortex (VSM-3 mixer, Shelton Scientific, USA)

- Water bath (Mammert GmbH Co., Germany)
- X-ray cassette (Eastman Kodak, USA)

Methods

3.1 Biophysical characterization of the 696-bp fragment of 3′-terminal region of *BRCA1*

3.1.1 Preparation of the 696-bp fragment of 3′-terminal region of *BRCA1*

RNA isolation

Total RNA was isolated from whole blood by QIAamp® RNA Blood Mini Kits (QIAGEN, Germany). Whole human blood (1.5 ml) was mixed with 7.5 ml of erythrocyte lysis buffer (EL) in erythrocyte lysis tube and incubated for 10-15 min on ice. The mixture was vortexed briefly 2 times during incubation and centrifuged at 400xg for 10 min at 4°C. The supernatant was completely removed. Buffer EL was added again to the cell pellet (3 ml of Buffer EL per 1.5 ml of whole blood). The cells were resuspended by vortexing and centrifuged at 400xg for 10 min at 4°C, and completely removed and discarded supernatant. 600 µl of Leukocyte lysis buffer (RLT) was added to pelleted leukocytes and pipetted to mix. The lysate was pipetted directly into a QlAshredder spin column sitting in a 2-ml collection tube and centrifuge for 2 min at maximum speed to homogenize. QIAshredder spin column was discarded and homogenized lysate was added with one volume of 70% ethanol, mixed by pipetting and carefully pipetted the sample, including any precipitate which may have formed, into a new QIAamp spin column sitting in a 2-ml collection tube without moistening the rim and centrifuged for 15 sec at ≥ 8,000xg. The QIAamp spin column was transferred into a new 2-ml collection tube and 700 µl of washing buffer 1 (RW1) was added, then, centrifuged for 15 sec at ≥ 8000xg to wash RNA sample. The QIAamp spin column was placed in a new 2-ml collection tube and 500 µl of washing

buffer 2 (RPE) was added, then centrifuged for 15 sec at ≥ 8000xg. The QIAamp spin column was added with 500 ul of Buffer RPE and centrifuged again at full speed for 1 min. The QIAamp spin column was transfered into a 1.5 ml microcentrifuge tube and 30-50 µl of RNase-free water was pipetted directly onto the QIAamp membrane and centrifuged for 1 min at ≥ 8000xg to elute. The processes were repeated if whole blood (or > 2 x 10^6 leukocytes) has more than 0.5 ml.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was prepared from total RNA by Reverse Transcriptase-Polymerase Chain Reaction QIAGEN OneStep RT-PCR kit®. The QIAGEN OneStep RT-PCR Enzyme Mix contains a formulated enzyme blend for both reverse transcription and PCR. The combination of Omniscript and Sensiscript Reverse Transcriptases (QIAGEN) transcripts RNA amounts from as little as 1 pg up to 2 µg. After reverse transcription, reactions are heated to 95°C for 15 min to activate HotStar*Taq* DNA Polymerase (QIAGEN) and simultaneously inactivate the reverse transcriptases. The hot start to the PCR eliminates any nonspecific amplification products such as primer-dimers and reduces background smear, ensuring highly sensitive and reproducible RT-PCR. The RT-PCR product is verified by agarose gel electrophoresis.

Template RNA from whole human blood, QIAGEN OneStep RT-PCR enzyme Kit, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, and RNase-free water were thawed and placed on ice. The RT-PCR mixtures were prepared according to Table 7.

Table 7. Reaction components for One-Step RT-PCR

Component	Volume (µl)	Final concentration
RNase-free water	27.0	-
5x QIAGEN OneStep RT-PCR Buffer	10.0	1 x
dNTP Mix (containing 10 mM of each dNTP)	2.0	400 uM of each dNTP
Forward Primer (5′-AGCAGGGAGAAGCCAGAATTG-3′)	3.0	0.6 µM
Reverse Primer (5′-TCAGTAGTGGCTGTGGGGGAT-3′)	3.0	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	2.0	-
RNase inhibitor	1.0	8 units/reaction
Template RNA	2.0	1 pg-2 µg/reaction
Total volume	**50.0**	**-**

All components were mixed thoroughly in PCR tubes. The thermal cycler was programmed according to the program outlined in Table 8. The RT-PCR program was started while PCR tubes were still on ice until the thermal cycler has reached 50°C and then placed the PCR tubes in the thermal cycler. The 696-bp *BRCA1* fragment from the RT-PCR reaction was verified by agarose gel electrophoresis.

Table 8. Thermal cycler conditions for One-Step RT-PCR

Reverse transcription:	50°C, 30 min
Initial PCR activation step:	95°C, 15 min
3-step cycling	
Denaturation:	94°C, 1 min
Annealing:	55, 60, 62, 65 °C min (Variation temperature)
Extension:	72°C, 1 min
Number of cycles:	30 cycles
Final extension:	72°C, 10 min

▼

4862 ATACTGCTGG GTATAATGCA ATGGAAGAAA GTGTG AGCAG GGAGAAGCCA GAATTGACAG
4922 CTTCAACAGA AAGGGTCAAC AAAAGAATGT CCATGGTGGT GTCTGGCCTG ACCCCAGAAG
4982 AATTTATGCT CGTGTACAAG TTTGCCAGAA AACACCACAT CACTTTAACT AATCTAATTA
5042 CTGAAGAGAC TACTCATGTT GTTATGAAAA CAGATGCTGA GTTTGTGTGT GAACGGACAC
5102 TGAAATATTT TCTAGGAATT GCGGGAGGAA AATGGGTAGT TAGCTATTTC TGGGTGACCC
5162 AGTCTATTAA AGAAAGAAAA ATGCTGAATG AGCATGATTT TGAAGTCAGA GGAGATGTGG
5222 TCAATGGAAG AAACCACCAA GGTCCAAAGC GAGCAAGAGA ATCCCAGGAC AGAAAGATCT
5282 TCAGGGGGCT AGAAATCTGT TGCTATGGGC CCTTCACCAA CATGCCCACA GATCAACTGG
5342 AATGGATGGT ACAGCTGTGT GGTGCTTCTG TGGTGAAGGA GCTTTCATCA TTCACCCTTG
5402 GCACAGGTGT CCACCCAATT GTGGTTGTGC AGCCAGATGC CTGGACAGAG GACAATGGCT
5462 TCCATGCAAT TGGGCAGATG TGTGAGGCAC CTGTGGTGAC CCGAGAGTGG GTGTTGGACA
5522 GTGTAGCACT CTACCAGTGC CAGGAGCTGG ACACCTACCT GATACCCCAG ATCCCCCACA
5582 GCCACTACTG A

Figure 11. Alignment of the 696-bp *BRCA1* fragment. It covers exon 16 – 24 (nucleotide 4,897-5,592).

Extraction of the 696-bp *BRCA1* fragment from agarose gel

RT-PCR product was electrophoresed on a 1% agarose gel and extracted by QIAquick gel extraction kit (QIAGEN, Germany). The DNA fragments were excised from the agarose gel with a clean and sharp scalpel. The gel was sliced to a colorless tube. The three volumes of Buffer QG were added to a volume to gel (100 mg ~ 100 μl). The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The mixture was mixed by vortexing the tube over 2-3 min during the incubation to help dissolving gel. A gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in a provided 2-ml collection tube. The mixture sample was applied to the QIAquick column and centrifuge for 1 min. The flow through was discarded and QIAquick column was placed back in the same collection tube. 0.5 ml of buffer QG was added to QIAquick cluumn and centrifuged for 1 min. The flow through was discarded. The QIAquick column was centrifuged for on additional 1 min at ≥ 10,000xg. QIAquick column was placed into a clean 1.5-ml microfuge tube. The 50 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O was added to the center of the QIAquick membrane. The column was centrifuged for 1 min at maximum speed.

Optimization of PCR condition for amplification of the 696-bp *BRCA1* fragment

The One Step RT-PCR product is purified by ethanol precipitation. It is used as the template for *Taq* DNA polymerase. Optimization of PCR condition is nescessery for further DNA amplification. PCR 10x QIAGEN PCR Buffer, dNTP mix, primer solutions and 25 mM $MgCl_2$ were added and placed on ice. A mixture was prepared according to Table 9.

Table 9. Reaction components for amplification of the 696-bp *BRCA1* fragment

Component	Volume (µl)	Final concentration
10x QIAGEN PCR Buffer*	10	1 x
dNTP mix (10 mM each)	2	200 uM of each dNTP
Forward primer (5′AGCAGGGAGAAGCCAGAATT3′)	2	0.1-0.5 µM
Reverse primer (5′ TCAGTAGTGGCTGTGGGGGAT 3′)	2	0.1-0.5 µM
Taq DNA Polymerase	0.5	2.5 units/reaction
Double distilled water	Variable	-
Template DNA	Variable	<1 µg/reaction
Total volume	**100**	

*Contains 15 mM $MgCl_2$

The mixture was mixed thoroughly in PCR tubes. The thermal cycler was programmed according to the manufacturer's instructions (Table 10).

Table 10. Thermal cycler conditions for PCR

Initial denaturation:	94°C 3 min
3-step cycling	
Denaturation:	94°C, 0.5-1 min
Annealing:	50-68°C, 0.5-1 min
Extension:	72°C 1 min
Number of cycles:	30 cycles
Final extension:	72°C, 10 min

The PCR products were verified by agarose gel electrophoresis and extracted by QIAquick Gel Extraction kit and subsequently sequenced.

DNA sequencing of the 696-bp *BRCA1* fragment

The band of PCR product was extracted from the agarose gel. The 200 ng of DNA from gel-extracted band (8 µl) was mixed with 8 µl of Big Dye Terminator and 4 µl of forward primer (0.8 pmol/ µl) in PCR tube. Thermal cycle was set. Initiation of DNA denaturation was performed by rapid temperature up to 96°C for 1 min. Three steps of PCR temperature were set as 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min for 25 cycles.

The unincorperated Big Dye Terminator was removed by adding 80 µl of 75% isopropanol and left the tube at room temperature for 15 min. The tube was spun in a microcentrifuge at 14,000xg for 20 min at room temperature. The supernatant was aspirated and 250 µl of 70% ethanol was added again to the tube and spun at 14000xg for 5 min. The supernatant was aspirated and the sample was dried in a heat block at 90°C for 1 min. The sample was resuspended by 6 µl loading buffer (1 deionized formamide: 5 Blue dextran) and was heated at 90°C for 2 min to denature and the sample was placed on ice. One microliter of sample was loaded into separate lane of gel and run in a sequencing machine. The sequence data was aligned to *BRCA1* cDNA from GenBank (U14680) using SIM-4 program.

3.1.2 Conformational study of cisplatin-*BRCA1* adducts

Conformational study of cisplatin-*BRCA1* adducts on an agarose gel electrophoresis

The 696-bp *BRCA1* fragment was incubated with various cisplatin concentrations (0, 12.5, 25, 50, 100, and 200 µM) at 37°C for 24 h in the dark. Platinated and non-platinated *BRCA1* was electrophoresed on a 1% agarose gel electrophoresis at 80 volt. The gel was stained with ethidium bromide and visualized under UV light (Sambrook *et al*., 2001).

Conformational study of cisplatin-*BRCA1* adducts on a polyacrylamide gel electrophoresis

The 696-bp *BRCA1* fragment was incubated with various cisplatin concentrations (0, 12.5, 25, 50, 100, and 200 μM) 37°C for 24 h in the dark. Platinated and non-platinated *BRCA1* was run on a 4 % SDS-PAGE at 80 volt. The gel was stained by silver nitrate (Sambrook *et al*., 2001).

Interstrand crosslink assay

The 696-bp *BRCA1* fragment was incubated with cisplatin at concentration of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μM at 37°C for 24 h in the dark. DNA was precipitated by absolute ethanol and dissolved in sterile water. Agarose solution was prepared by adding the appropriate amount of powdered agarose to a measure quantity of double distill water and heated until the agarose dissolved. The agarose solution was cooled to 55°C. The 0.1 volume of 10x alkaline agarose gel eletrophoresis buffer was added to the solution and immediately pour the gel in the block. After the gel was completely set, it was mounted in the chamber and the 1x alkaline electropheresis buffer was added until the gel was just covered. DNA sample was run in alkaline agarose gel electrophoresis at 24 volt for 4 h. The gel was neutralized in neutralizing solution for 45 min, stained with ethidium bromide and visualized under UV illumination.

Restriction analysis of cisplatin-treated 696-bp *BRCA1* fragment

The 696-bp *BRCA1* fragment was incubated with various concentrations of cisplatin in a 20 μl of reaction mixture. The reaction mixture consisted of 4.25 μg of DNA and cisplatin at concentration of 0, 12.5, 25, 50 and 100 μM, respectively. The mixture was incubated at 37°C for 24 h in the dark. Cisplatin-treated DNA was precipitated by absolute ethanol and centrifuged at 13,500x g at 4°C for 30 min. The DNA pellet was washed with 70% ethanol, dried in vacuum and

redissolved in distilled deionized water. The platinated DNA was analyzed by restriction analysis using *Pvu*II (recognition sequence: CAG▼CTG) and *Eco*0109I (recognition sequence: PuGG▼NCCPy) (Fig. 12 and 13). The mixtures were incubated at 37°C with *Eco*0109I and *Pvu*II for 3 and 4 h, respectively. DNA adducts were electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized under UV illumination.

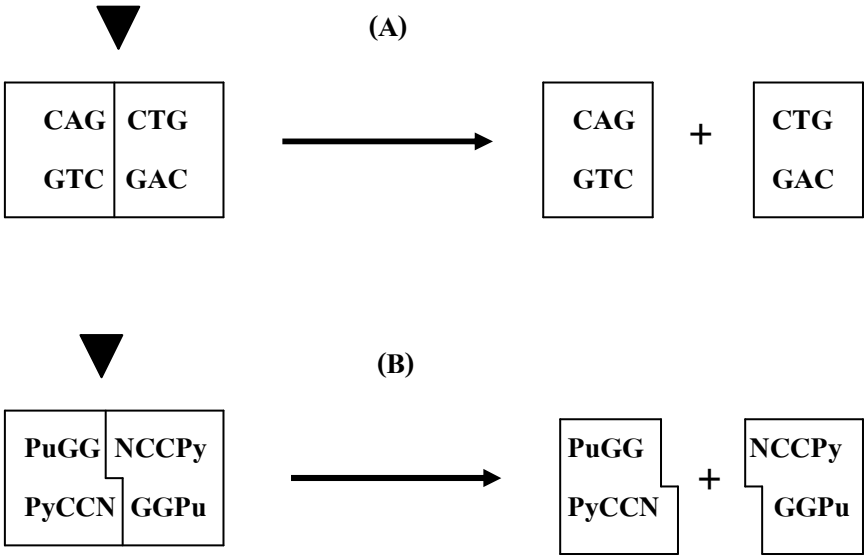


Figure 12. Restriction recognition site of enzyme *Pvu*II (A) and *Eco*0109I (B).

3.1.3 Thermal stability of cisplatin-*BRCA1* adducts

The 696-bp *BRCA1* fragment (425 ng/µl) was incubated with various concentration of magnesium chloride (0.1, 0.5 and 1 mM) and with cisplatin at various concentration (0.005, 0.05 and 0.5 µM), at 37°C for 24 h in the dark. Twenty microliters of all samples were applied to a DSC pan and tightly sealed. The DSC thermogram was obtained by differential scanning calorimeter (DSC-7, Perkin Elmer) with heating from 50°C to 90°C at the scanning rate of 1°C/min.

agcagggagaagccagaattgacagcttcaacagaaagggtcaacaaaagaatgtccatggtggtgtctggcct
gaccccagaagaatttatgctcgtgtacaagtttgccagaaaacaccacatcactttaactaatctaattactgaagagactact
catgttgttatgaaaacagatgctgagtttgtgtgtgaacggacactgaaatattttctaggaattgcgggaggaaaatgggtag
ttagctatttctgggtgacccagtctattaaagaaagaaaaatgctgaatgagcatgattttgaagtcagaggagatgtggtcaa
tggaagaaaccaccaaggtccaaagcgagcaagagaatcccaggacagaaagatcttcaggggctagaatctgttgc

***Eco*0109I** ***Pvu*II**

ta*tgg*▼*gccct*tcaccaacatgcccacagatcaactggaatggatggta*cag*▼*ctg*tgtggtgcttctgtggtgaagga
gctttcatcattcacccttggcacaggtgtccacccaattgtggttgtgcagccagatgcctggacagaggacaatggcttcca
tgcaattgggcagatgtgtgaggcacctgtggtgacccgagagtgggtgttggacagtgtagcactctaccagtgccagga
gctggacacctacctgataccccagatcccccacagccactactga

Figure 13. Deduced nucleotide sequence of the 696-bp *BRCA1* fragment and the recognition site of *Eco*0109I (gggccct) and *Pvu*II (cagctg).

Resulting thermodynamic parameters ΔH_{cal} and ΔS_{cal} were determined from the integrated areas of excess heat capacity versus temperature curves.

The DSC T_m was determined as the temperature at the peak height maximum on ΔC_p^{ex} versus temperature curve. The thermodynamic parameters were obtained from,

$$\Delta G = -RT\ln K = \Delta H - T_m \Delta S \quad (1)$$

The integrated area under the concentration corrected curve provided the reported DSC transition enthalpy,

$$\int \Delta C_p^{ex} \, dT = \Delta H_{cal} \quad (2)$$

The DSC transition entropy values were determined from the integrated area under curves of $\Delta C_p^{ex}/T$ versus temperature,

$$\int \Delta C_p^{ex}/T \, dT = \Delta S_{cal} \quad (3)$$

At mid point temperature that DNA was in equilibrium condition ($\Delta G = 0$), Tm was then determined from,

$$T_m = \Delta H_{cal}/\Delta S_{cal}. \quad (4)$$

Note that for helix-coil transitions of DNA, Cp(coil)-Cp(helix) = 0 (Marky and Breslauer, 1987)

3.2 *BRCA1* damage by cisplatin and its repair

3.2.1 Damage of DNA by cisplatin in MCF-7 cells

Cell culture

MCF-7 was human breast cancer cell line. The cells were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Gibco/BRL, Melbourne, Australia) and incubated at 37^{o}C with 5% CO_2.

DNA preparation

About 1x10^{6} MCF-7 cells were seeded into each well of a 6-flat-bottom wells plate. The cells were treated with cisplatin at the final concentration of 0, 12.5, 25, 50, 100 and 200 μM and incubated at 37^{o}C for 6 and 24 h. Genomic DNA was prepared using a procedure modified from Miller′s method (Miller *et al*., 1988) after drug exposure. The control and drug-treated MCF-7 cells were pelleted by centrifugation at 3,000xg for 5 min and washed twice with 1 ml of phosphate-buffered saline (PBS). The cell pellets were resuspended in a 1.5 microcentrifuge tube with 500 μl of cell lysis buffer. The cell lysates were digested overnight at 37^{o}C with 25 μl of 10% sodium dodecyl sulfate and 2 μl of a proteinase K solution. After digestion was completed, 140 μl of saturated NaCl (6 M) was added and shaken vigorously for 15 sec, followed by centrifugation at 3,000xg for 15 min. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was

transferred to another 1.5 ml microcentrifuge tube. DNA was precipitated by addition of 2 volumes of absolute ethanol and centrifuged at 13,000xg at 4°C for 15 min. The DNA pellet was washed twice with 70% ethanol, dried and resuspended in 100 μl of double distilled water.

Semi-quantitative polymerase chain reaction (Semi-QPCR)

Exon 11 (3426 bp) of *BRCA1* gene is the target for studying of the effect of cisplatin on DNA. The total volume of the PCR mixture was 100 μl. The PCR mixture contained 300 ng of platinated-DNA template 400 pmol of each forward primer (5′-GCCAGTTGGTTGATTTCCACC-3′) and reverse primer (5′-GTAAAATGTGCTCCCCAAAAGC-3′), 400 μM of each dNTP, 2.5 unit of *Taq* DNA polymerase, 3 mM of $MgCl_2$. The 3,426 bp *BRCA1* exon 11 fragment was amplified using GeneAmp 9600 PCR system (Perkin-Elmer) as followed: pre-denature at 94°C for 4 min, 30 cycle of denatureation at 94°C for 2 min, annealing at 60°C for 2 min, extension at 72°C for 3 min, and one cycle of final extension at 72°C for 7 min. PCR products were electrophoresed in a 1% agarore gel at 100 volt and stained with ethidium bromide. The resulting PCR products were visualized and quantitated under UV illumination for the semi-QPCR analysis (Ratanaphan *et al*., 2005).

Product amplification was measured directly from agarose gels using a Bio-Rad Molecular Imager with ImageQuant Software (Molecular Dynamics). The amount of amplification was represented by the units of absorbance of the amplified products. The QPCR assay was further employed to estimate the number of lesions per strand. Based on assumption that the lesions distributed randomly, the Poisson equation (shown below) was used to calculate the lesion frequency per strand

$$S = - \ln Ad/A$$

Symbols in the equation were identified as followed,

S = lesion frequency/strand, A = absorbance units produced from a given amount of non-damaged DNA template and Ad = absorbance unit produced from a given amount of damaged DNA template (damaged by a particular dose of cisplatin), so that Ad/A = fraction of non-damaged template at a given dose.

3.2.2 Repair of cisplatin-treated *BRCA1* exon 11 fragment

MCF-7 cells ($1x10^6$cells/well) were incubated in serum free DMEM medium added with cisplatin concentration of 50, 100 and 200 μM for 6 h, respectively. The cells were washed twice with PBS and medium was replaced by cisplatin-free DMEM. Genomic DNA was harvested at 4, 8, 18 and 24 h and prepared using a procedure modified from Miller′s method (Miller *et al*., 1988). Product amplification was measured as described previously (Ratanaphan *et al*., 2005).

3.3 Construction of the pBIND-BRCT expression vector

The pBIND plasmid is a highly copy number plasmid in which the human cytomegalovirus (CMV) immediate early promoter drives expression of the yeast GAL4 gene that codes for a portion of the protein (amino acid 1-147) containing a DNA-binding domain. The DNA binding domain sequence is flanked by a chimeric intron, a multiple cloning region (MCR), stop codons and an SV40 late polyadenylation region. The fusion gene region is flanked by RNA polymerase T7 and T3 promoters for the purpose of synthesizing sense and antisense RNA products, respectively. The *Renilla* luciferase gene on this vector is preceded by the SV40 early promotor and a growth hormone intron. Introns can increase protein expression through mRNA stability and also increases nuclear to cytoplasmic transport. A synthetic polyadenylation sequence resides the 3′-terminus of the *Renilla* luciferase gene. The plasmid backbone contains an f1 origin of replication for the production of ssDNA and the β-lactamase gene for selection in *E.coli* (Fig. 14).

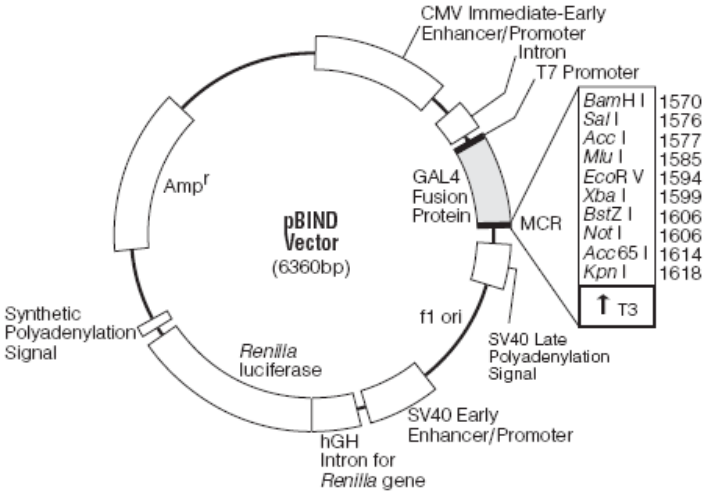


Figure 14. Vector circle map, sequence reference points and multicloning site of pBIND eukaryotic expression vector (Promega, USA)

The pBIND plasmid has been used in "two hybrid assay" system for studying the interaction of any two proteins. The "two hybrid system" comprised pBIND, pACT, and pG5Luc plasmid. The pBIND contains the GAL4-DNA binding domain (GAL4-DBD) that can interact with GAL4 sequence on the pG5Luc plasmid. The interested first protein (geneX) could be cloned to the pBIND plasmid at multi cloning region (MCR) to construct hybrid protein. The pACT plasmid consists of herpes simplex virus VP16 activation domain that has strong transactivation on many promoters. The VP16 could fuse to the second protein (geneY) by cloning to MCR site in the pACT plasmid. The pG5Luc plasmid contains five GAL4 binding domain sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene (*luc*+) (Fig. 15A). The pBIND-geneA and pACT-geneB are transfected along with the pG5Luc plasmid into mammalian cells. Interaction of the two test proteins, expressed as GAL4 and VP16 fusion constructs, results in an increase in firefly luciferase expression as compared to the negative control.

In our modified "one hybrid assay" the BRCT domain fused to GAL4 protein plasmid and is co-transfected with the pG5Luc plasmid. Transcriptional

transactivation activity is observed when both two tandem repeat of the BRCT domain are constructed to the plasmid (Fig. 15B, and 16). The transcriptional transactivation of the BRCT is not as strong as VP16 (Krum *et al*., 2003). This system has been used to study the effects of mutation on BRCT domain, and the interaction of BRCT domain to the other proteins (Monteiro *et al*., 1996; Krum *et al.*, 2003).

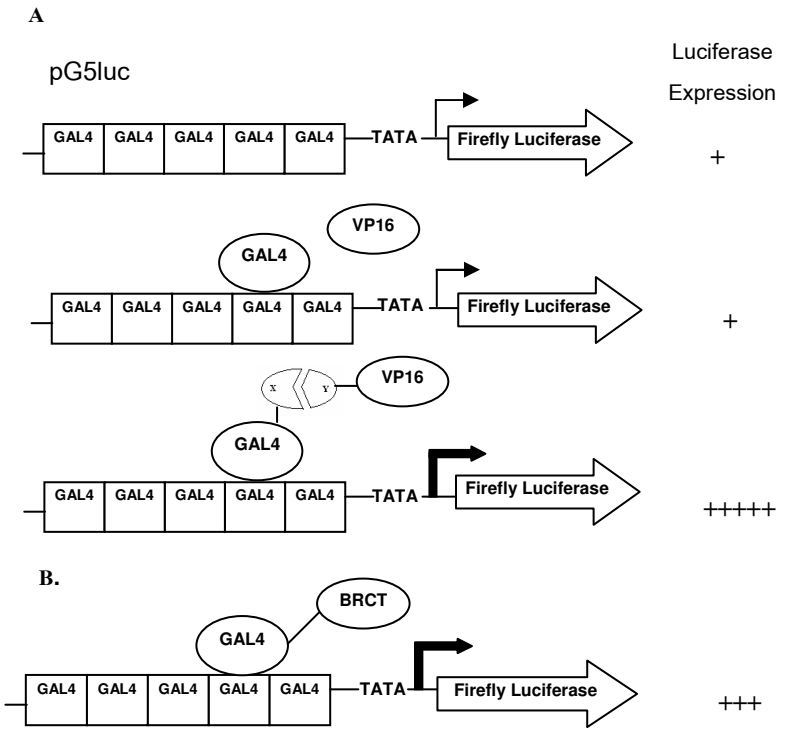


Figure 15. Schematic representation of the CheckMate™ Mammalian Two-Hybrid System (Promega, USA) (A). The pG5Luc vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. In negative control, the background level of luciferase is measured in the presence of GAL4 (from pBIND) and VP16 (from pACT). Interaction between the two test proteins, expressed as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over negative controls. In our modified One-Hybrid System (B), the BRCT domain is fused to GAL4 protein in the pBIND plasmid. This fusion protein also increases the firefly luciferase expression over negative controls.

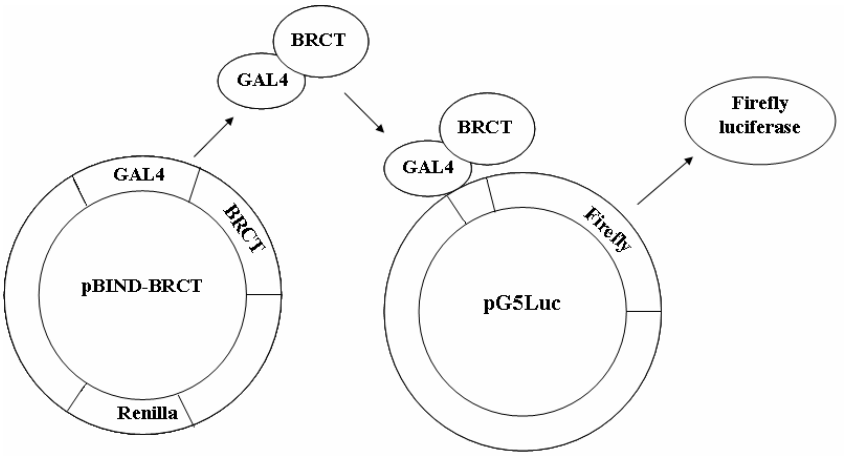


Figure 16. Schematic expression of the GAL4-BRCT from the pBIND-BRCT plasmid. The GAL4-BRCT protein activates transcription of firefly luciferase gene on the pG5Luc plasmid.

3.3.1 Cloning of pBIND-BRCT plasmid

The 696-bp *BRCA1* fragment was amplified by PCR. The 696-bp *BRCA1* fragment, PCR 1xQIAGEN PCR Buffer, dNTP mix, forward primer (5′ ATAAAATCGACAGGGATCCTTAGCAGGGAGAAGCCAGAATTG3′), reverse primer (5′ACTTTGTGTTCATTTTCTAGATCAGTAGTGGCTGTGGGGGAT3′), *Taq* dNA polymerase enzyme were run in 30 cycle of 95°C for denaturation, 58°C for annealing and 72°C for extension. PCR product was purified by absolute ethanol and redissolved by double distill water. PCR product and plasmid pBIND were double digested by restriction enzyme *Bam*HI and *Xba*I (5 µl of 10x restriction enzyme buffer, 0.5 µl of 100x BSA**,** 7.5 µg of pBIND or 696-bp *BRCA1* fragment, 1 µl *Bam*HI (20 unit), 1 µl *XbaI* (20 unit) and sterile water to 50 µl). The reaction mixture was incubated at 37ºC for 4 h. The reaction was stopped by running in 1% agarose gel electrophoresis and both DNAs were further purified using QIAquick DNA Extraction Kit (QIAGEN). The cleaved 696-bp *BRCA1* fragment was then cloned to the *Bam*H1/*Xba*I site of the pBIND plasmid by T4 DNA ligase (Fig. 17) (Sambrook *et al*., 2001).

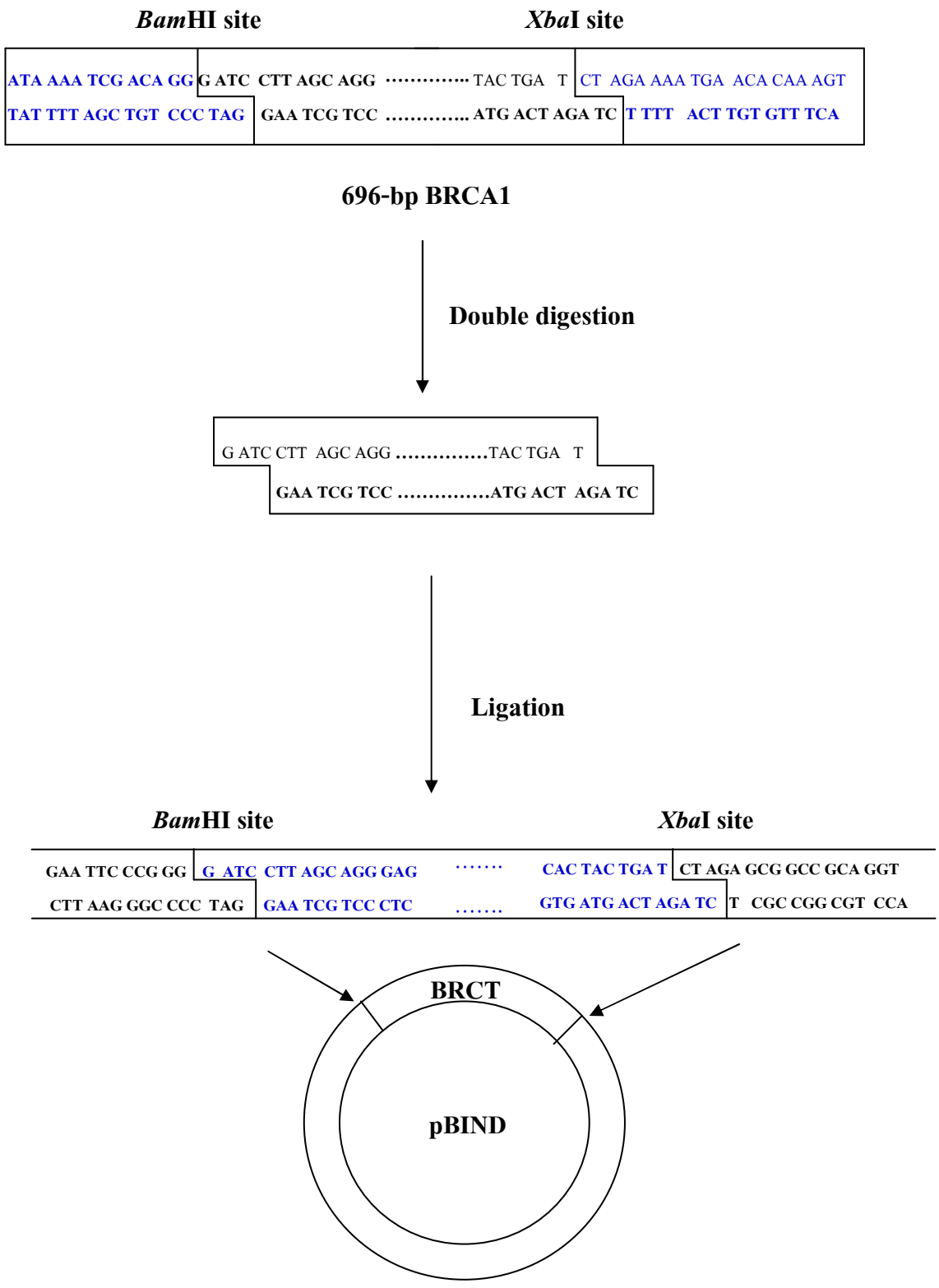


Figure 17. Cloning site of pBIND and the 696 bp-*BRCA1* fragment (*Bam*HI site at 5/ terminal and *Xba*I site at 3/ terminal)

3.3.2 Transformation

The ligation reaction mixture was mixed with 100 µl of competent *E. coli* and incubated on ice for 30 min. Bacterial cells were shocked by incubating at 42°C for 90 sec and replaced on ice suddenly for 5 min. 800 µl of LB broth was added to the mixture and incubated at 37°C for 1 h with shaking water bath. The cells were centrifuged at 5000xg for 1 min. The medium was removed and the cell pellet was resuspended by 100 µl of fresh LB broth. Resuspended cells were spreaded on LB agar with 50 µg/ml ampicillin and incubated at 37°C overnight. Single colony was picked and transfered into LB broth (50 µg/ml of ampicillin) and further incubated at 37°C for 12 h. DNA was isolated from bacterial cells by alkaline lysis method (Sambrook *et al*., 2001).

3.4 Expression of pBIND-BRCT in MCF-7 cell

3.4.1 Transfection

MCF-7 cells were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Gibco/BRL, Melbourne, Australia) and incubated at 37°C with 5% CO_2. About $1x10^5$ MCF-7 cells were seeded into each well of a 6-flat-bottom wells plate and transfected with the pBIND-BRCT plasmid using calcium-phosphate co-precipitation method (Sambrook *et al*., 2001).

For calcium-phosphate co-precipitation method, transfection mixtures were prepared in two tubes. Solution A is the mixture of 4 µg of plasmid DNA and 10 µl of 2.5 M $CaCl_2$ and 90 µl of steriled water. Solution B is the 2xHEPES at the same volume of solution A. The solution A was then added dropwise to the vortexing solution B. The transfection mixture was prepared at room temperature for 20 min and added to 6- cells well plate containing MCF-7 cells and incubated at 37°C with 5% CO_2 for 18-24 h. The calcium phosphate-DNA suspension was transfered into the medium above the cell monolayer. The 0.2 ml of suspension was used for 2 ml of

medium in a well. The plate was rocked gently to mix the medium. After cells have been exposed for 4 h to the calcium phosphate-DNA coprecipitate in growth medium, the medium was removed by aspiration and the cell was washed once with PBS. The 1.5 ml of 15% glycerol in 1xHEPES buffered saline was added to each well, and the cells were incubated for 1 min at 37°C. Glycerol was removed by aspiration, and the monolayers were washed once with PBS. Two ml of warmed complete growth medium was added, and the cells were incubated for 16-36 h at 37°C in 5% CO_2 incubator.

3.4.2 Isolation and detection of BRCT protein

Preparation of cell lysate

MCF-7 cells were collected from the 6-well plate by trypsinization. Cells were lysed with 200 µl/well of lysis buffer (2% SDS, 10% glycerol) for 5 min and spun at 16,000xg in an eppendorf microfuge for 10 min at 4°C. The supernatant was transfered to a new tube. The protein concentration was determined by UV-spectrophotometer (OD_{280}). Cell lysate was mixed with 2x sample buffer, boiled for 5 min and loaded to a 12% SDS-PAGE.

Polyacrylamide gel preparation

Resolving gel was prepared for a 12 % SDS PAGE (10.8 ml Acrylamide / N,N′-methylene bis-acrylamide, 7.5 ml 1 M Tris/HCl (pH 8.8), 1.4 ml distilled water, 200 µl of 10% SDS, 100 µl of 10% Ammonium persulfate, 10 µl TEMED). The ingredients were mixed gently and poured into glass plate assembly. The gel was overlayed with isopropanol to ensure a flat surface and to exclude air. The isopropanol was washed off with water after gel had set. Stacking gels was prepared for a 4.8% SDS-PAGE (Acrylamide / N,N'-methylene bis-acrylamide 2 ml, 1 M Tris/HCl (pH 6.8) 1.25 ml, distilled water 5.6 ml, 10% SDS 100 µl, 10% Ammonium persulfate 50 µl, TEMED 5 µl). The mixture was mixed and then poured onto top of the resolving gel. The comb was inserted, and the gel was allowed to set. When the gel

was set, the comb was removed and the gel was filled with electrophoresis buffer. Protein lysate was applied to each well and run by constant 80 volts for 2-3 h.

Membrane preparation and transfer

A SDS-PAGE gel was applied to a semi-dry electroblotter. A polyvinylidine difluoride (PVDF) membrane was cut to fit the gel exactly. Filter paper, gel and membrane were soaked in a transfer buffer and layered on the semi-dry electroblotter. The separated protein was then transfered to the PVDF membrane using 400 miliampere for 1 h in the semi-dry electroblotter with transfer buffer (3.03 g of Tris base, 14.4 g of glycine, 200 ml of methanol, add distilled water to 1 liter).

BRCA1 antibody and detection

The blot was removed from the transfer apparatus and blocked with a blocking reagent (5% non-fat milk in TBST) overnight at 4°C. The membrane was incubated with a primary antibody, anti-BRCA1 C-20 (Santa Cruz Biotechnology, USA) at dilution of 1:200 in 0.5% non-fat milk in TBST for 1 h. The blot was then washed by 0.5% non-fat milk in TBST for 15 min 3 times, and exposed to an anti-rabbit IgG (whole molecule) horseradish peroxidase conjugated secondary antibody (Pierce, USA) at dilution of 1:20,000 in 0.5% non-fat milk in TBST for 1 h at room temperature with shaking. The blot was washed by 0.5% non-fat milk in TBST for 15 min 3 times before a cheminescence detection. The chemiluminescent substrate was prepared by mixing equal part of the stable peroxide solution and the Lumino/Enhancer solution (SuperSignal™, Pierce, USA) to the final volume of 0.125 ml per cm^2 of the blot. The blot was incubated with working solution for 5 min. The working solution was removed and the blot was placed in a film cassette with X-ray film for 5 min. X-ray film was developed using appropriate developing solution and fixative (Fig. 18).

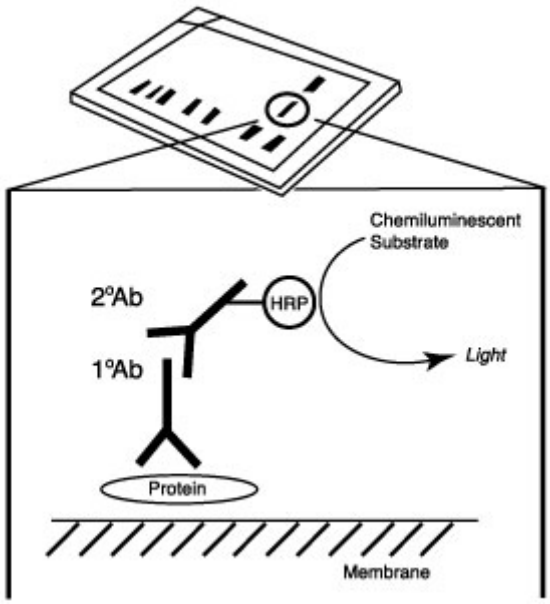


Figure 18. Diagram of western blotting method. After the primary antibody (Anti-BRCA1 C-20) is bound to the target protein, a complex with HRP-linked secondary antibody (anti-rabbit IgG peroxidase conjugate) is formed. The Lumino/Enhancer solution (Pierce) is added and light is emited during enzyme catalyzed decomposition (www.cellsignal.com/products/).

3.5 Host cell reactivation assay

The recombinant plasmid pBIND-BRCT was platinated with cisplatin at concentration of 0, 12.5, 25 and 50 μM for 24 h at room temperature in the dark. The plasmid was precipitated by absolute ethanol and dissolved in double distilled water. Platinated plasmid was transfected with plasmid pSV as a internal control into the MCF-7 cells. *Renilla* luciferase activity was measured by the Dual-Luciferase® Reporter Assay System (Promega, USA).

The amount of activity resulting from transfection with undamaged plasmid, pBIND-BRCT, was defined as 100%. The firefly luciferase activity was

measured as a function of the number of Pt-BRCT adducts in the plasmid. The data were derived from 4 independent experiments ± standard deviation (SD).

3.6 Transcriptional activation of cisplatin-damaged *BRCA1*

3.6.1 The pG5Luc system

The pBIND or pBIND-BRCT was platinated with cisplatin at various concentrations. The plasmid was co-transfected with the pG5Luc into MCF-7 cells. Cell lysates were prepared after transfection for Dual-Luciferase® Reporter Assay System (Promega, USA). The growth medium was removed from the culture cells. Phosphate buffered saline (PBS) was applied to wash the surface of the culture vessel. The vessel was swirled briefly to remove detached cells and residual growth medium. The solution was rinsed completely before applying PLB reagent. One volume of 1x PLB was dispensed into each culture well to completely cover the cell monolayer. The culture plates were placed on a rocking platform for 15 min at room temperature. The lysate was transferred to a tube or vial for further handling.

LARII 100 μl was predispensed into the appropriate number of luminometer plate to complete the desired number of Dual-Luciferase® Reporter Assay System. The luminometer was programmed to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. Cell lysate of 20 μl was transferred into luminometer plate containing LARII and mixed. The plate was placed in the luminometer and initiate reading. The plate was removed from luminometer. The Stop & Glo® reagent 100 μl was added to the plate and mixed. The plate was replaced in the luminometer and initiate reading (Fig. 19.1 and 19.2).

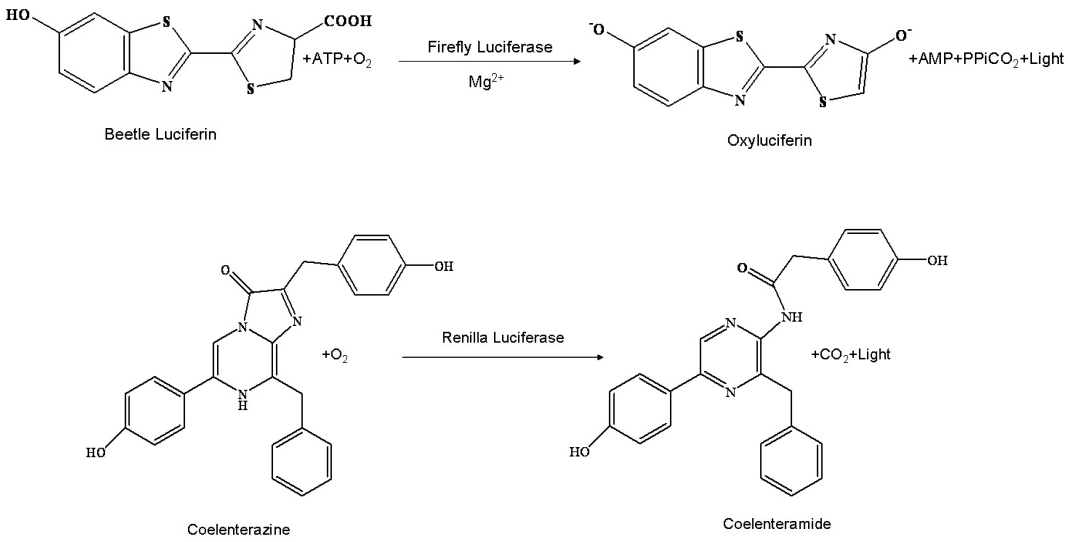


Figure 19.1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferase

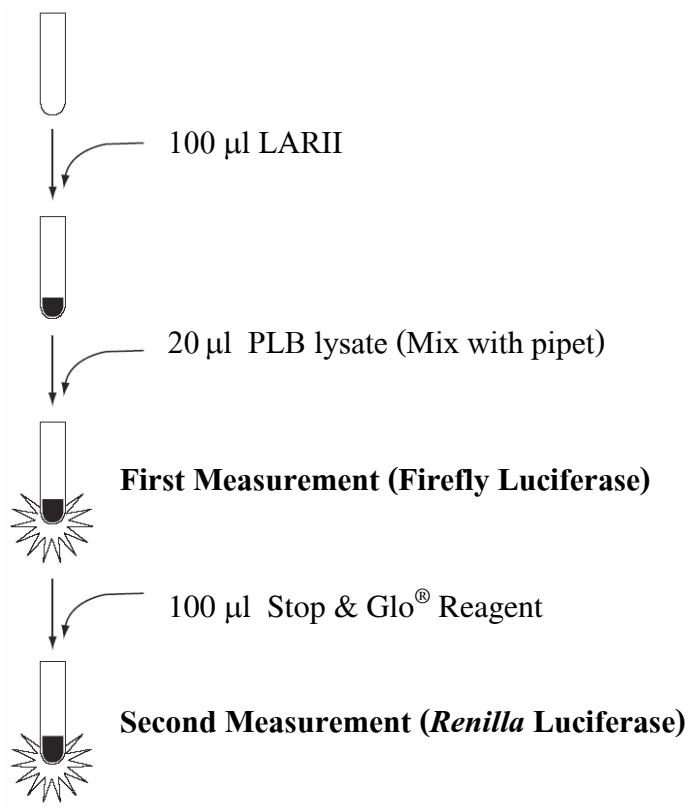


Figure 19.2. Format of the Dual Luciferase Reporter Assay system using a manual luminometer (Promega, USA)

3.6.2 The pSV-β-galactosidase system

The pBIND or pBIND-BRCT was platinated with cisplatin at various concentrations. The plasmid was co-transfected with the pSV-β-galactosidase into MCF-7 cells. Cell lysates were prepared after transfection for β-galactosidase Assay System (Promega, USA). The growth medium was removed from culture plate. The cells were washed twice by 1x PBS. The cell lysis buffer (RLB) was added to the cells at the volumn of 180 μl/well. The plates were incubated at room temperature for 15 min and rocked the plate several times during the incubation. The cells were scraped to the tube, placed on ice, vortexed for 10-15 sec and then centrifuged at 12000xg for 2 min at 4°C. The supernatant was transferred to a new tube for assay.

The assay was performed by adding a dilute sample to an equal volume of Assay 2x Buffer, which contains the substrate ONPG (o-nitrophenyl-beta-D-galactopyranoside). Samples were incubated for 30 min, during which time the β-galactosidase hydrolyzed the colorless substrate to o-nitrophenyl, which was yellow (Fig. 20). The reaction was terminated by addition of sodium carbonate, and the absorbance was read at 420 nm with a spectrophotometer.

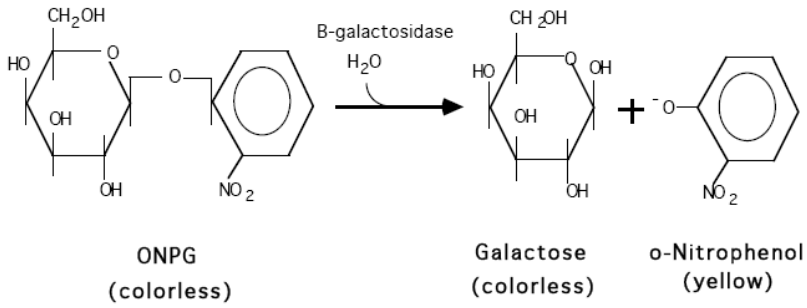


Figure 20. β-galactosidase reaction (Promega, USA)

CHAPTER 4

RESULTS

4.1 Biophysical characterization of the 696-bp fragment of 3′-terminal region of *BRCA1*

4.1.1 Preparation of the 696-bp fragment of the 3′-terminal region of *BRCA1*

RT-PCR product was prepared from RNA of whole human blood as described in chapter 3 (Materials and Methods). PCR product of the 696-bp *BRCA1* fragment was subsequently sequenced by automated DNA sequencer (Perkin-Elmer). The sequence data was completely 100% homology when aligned to *BRCA1* cDNA from the GenBank (accession number U14680) by CLUSTALW program.

4.1.2 Conformational study of *in vitro* platination of *BRCA1* by cisplatin

4.1.2.1 Gel mobility assay

Changes in the mobility of cisplatin-induced *BRCA1* fragment were observed on a 1% agarose gel. The mobility of cisplatin-induced *BRCA1* fragments decreases as the concentration of cisplatin increases, indicating increasing frequency of DNA lesions (Fig. 21A). A decrease in band intensity resulting from the multi-cross link DNAs or aggregates can be observed on an agarose gel as well as a silver-stained polyacrylamide gel (Fig. 21B). This crosslink inhibited the intercalation of ethidium bromide into the DNA molecules at higher levels of the platination.

4.1.2.2 Interstrand crosslink assay

In this study, alkaline gel electrophoresis was used to study the effect of cisplatin on DNA interstrand crosslink. Double stranded DNA was disrupted to a single strand under alkaline denaturation. The rate of migration of both separated single-stranded DNAs was similar on agarose gel because the migration of DNA depended on size, but not the sequence of DNA. At cisplatin concentration of 0.5 μM, initiation of

interstrand crosslinks was occurred. The intensity of interstrand crosslinks increased as cisplatin concentration increased. The complete interstrand crosslinks were formed at cisplatin concentration of 32 μM (Fig. 22).

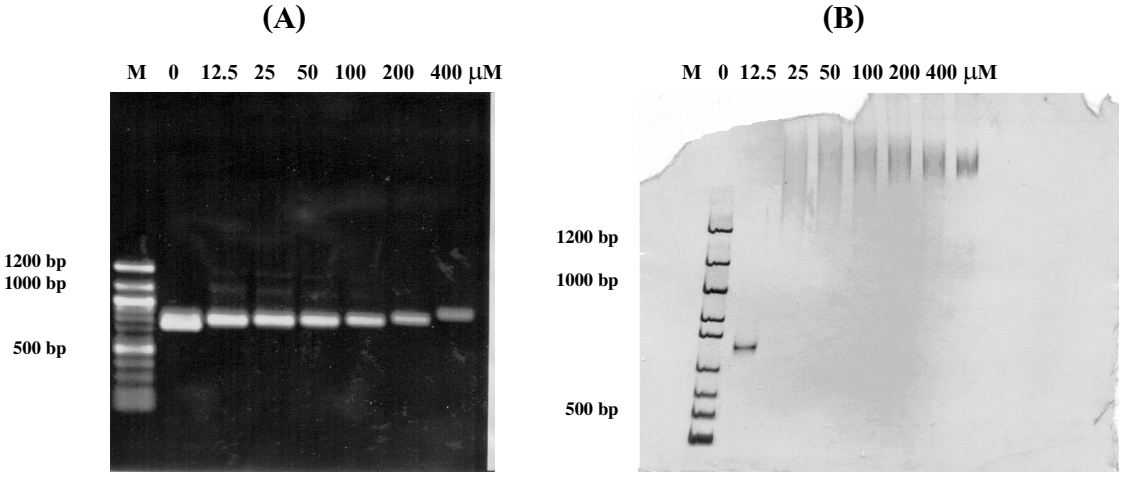


Figure 21. Effects of cisplatin on conformational change of the 696-bp *BRCA1* fragment. The 696-bp *BRCA1* fragment was incubated with various concentration of cisplatin (0-400 μM) at 37°C for 24 h in the dark. (A) Platinated DNA was electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized under UV illumination. (B) Platinated DNA was electrophoresed on a 4 % polyacrylamide gel and stained with silver.

4.1.2.3 Restriction analysis of cisplatin-*BRCA1* adducts

Cisplatin is known to bind covalently to DNA, with the predominant DNA lesion being the (N7, N7)-bidentate cisplatin crosslink between two intrastranded adjacent guanines. These platination sites are known to resist the activities of restriction enzymes which recognize and cleave the native sequence.

C 32 16 8 4 2 1 0.5 (μM)

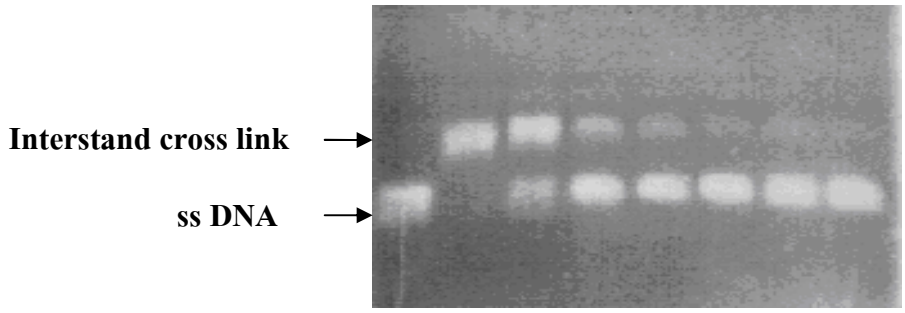


Figure 22. Interstrand crosslink of the 696-bp *BRCA1* fragment. The 696-bp *BRCA1* fragment was incubated with various concentrations of cisplatin (0.5-32 μM) at 37°C for 24 h in the dark. Platinated DNA was electrophoresed on a 1% alkaline gel electrophoresis. The gel was neutralized by neutralizing solution and stained with ethidium bromide and visualized under UV illumination. (C stands for control untreated DNA)

Therefore, preferential platination sites on the 696-bp fragment of the 3′-terminal region of *BRCA1* can be deduced from restriction analysis using only two specific enzymes, *Pvu*II (CAGCTG) and *Eco*0109I (PuGGNCCPy), whose recognition sequences uniquely exist on this DNA fragment. Digestion of the gene fragment by *Pvu*II produces two digestion fragments of 237 bp and 459 bp and that by *Eco*0109I produces two digested fragment of 283 bp and 413 bp. The results revealed that the 696-bp fragment of the 3′-terminal region of *BRCA1* completely resists the activity of *Eco*0109I after treatment with cisplatin at 12.5 μM. Cisplatin-treated *BRCA1* fragment interfered the digestion of *Pvu*II enzyme at higher cisplatin concentration, implying that *Eco*0109I site (dGpG) is favorably platinated more than the *Pvu*II site (dGpC) (Fig. 23).

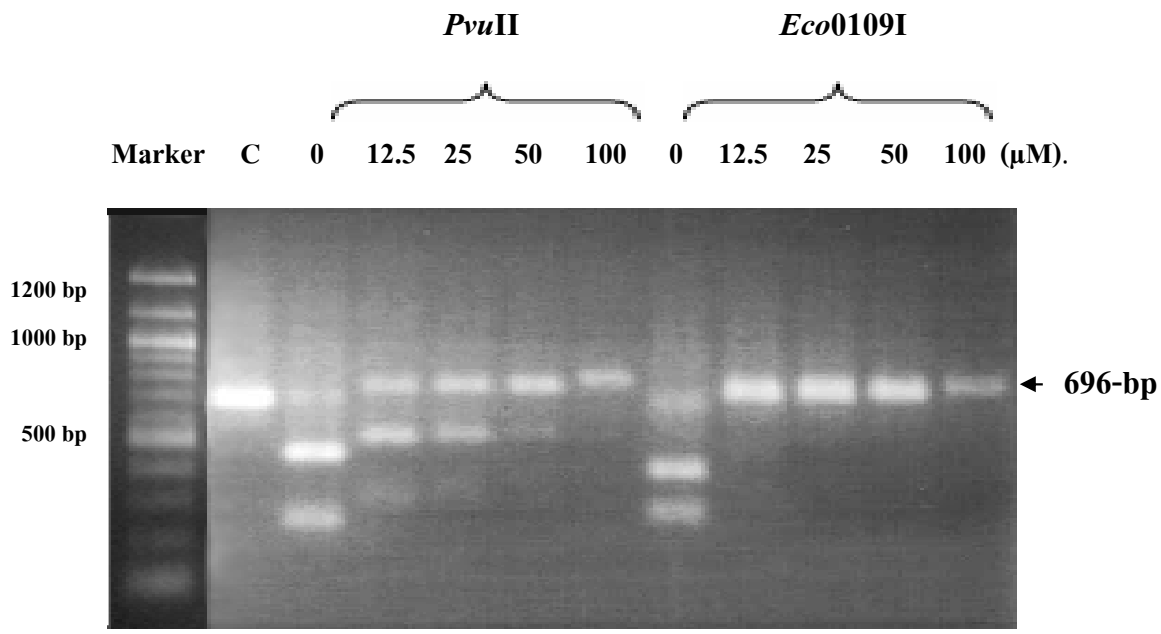


Figure 23. Restriction digestion for platination site of the 696-bp *BRCA1* fragment. The 696-bp *BRCA1* fragment was incubated with various concentrations of cisplatin (0-100 μM) at 37°C for 24 h in the dark (C: untreated DNA). The drug-treated DNA was precipitated, washed and redissolved in double distilled water. The drug-treated DNA was then incubated with either *Pvu*II (CAGCTG) or *Eco*0109I (PuGGNCCPy) at 37°C for 3-4 h, respectively. Restricted products were electrophoresed on 1% agarose gel. The gel was stained with ethidium bromide and visualized under UV illumination.

4.1.2.4 Thermal stability of cisplatin-*BRCA1* adducts

Differential scanning calorimetry (DSC) was used as a tool to study the effect of cisplatin on thermal stability of specified *BRCA1* fragment through the heat absorbed during thermal denaturation (Breslauer *et al*., 1986, Breslauer *et al*., 1992). The effects of alkaline earth magnesium (II) on the *BRCA1* fragment were also compared. Melting temperature of cisplatin-*BRCA1* adducts was decreased from 75.8°C to 75.6°C and 74.9°C when DNA was incubated with cisplatin at concentration of 0.005 and 0.05 μM, respectively (Fig. 24 and Table 11). However, transition

temperature increased to 76.0°C when DNA was incubated with cisplatin at concentration of 0.5 μM.

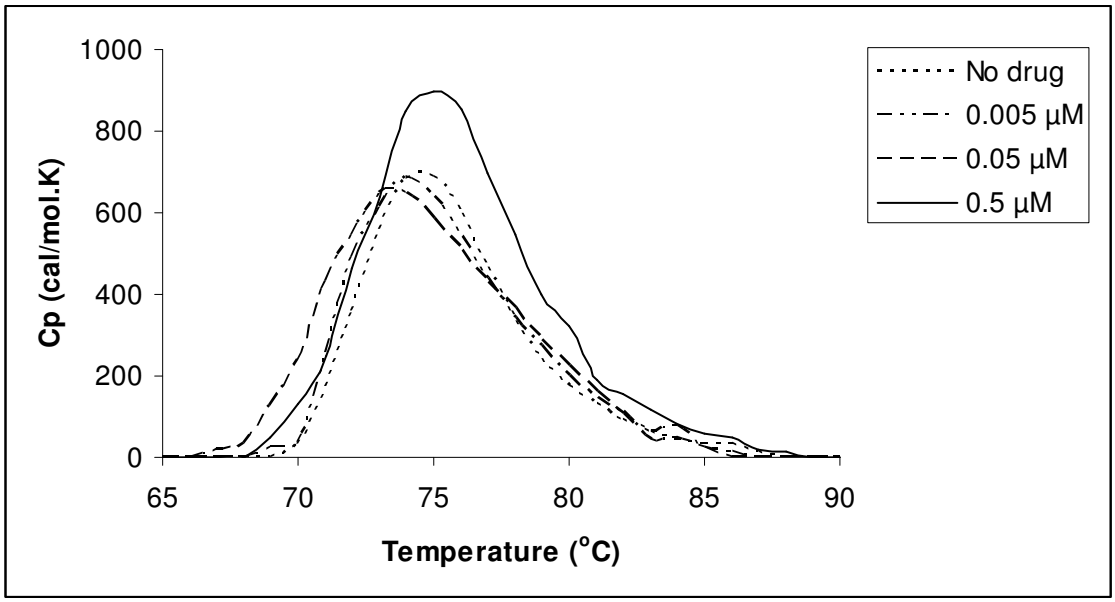


Figure 24. Thermal stability of the 696-bp *BRCA1* fragment at various concentrations of cisplatin

However, DNA melting transition in the presence of magnesium salt solution was different from that observed in the presence of cisplatin. The transition midpoint was raised from 75.8 °C to 76.1 °C and 80.3 °C at magnesium concentration of 0, 0.1 and 0.5 mM, respectively. The higher enthalpy and entropy values indicate the effect of both enthalpy and entropy on stability on melting transition (4.920, 5.120 kcal/mol bp and 13.9, 14.6 cal/moleK). However, at 1.0 mM of $MgCl_2$, the transition temperature of DNA decreased to 74.3°C (Fig. 25 and Table 11).

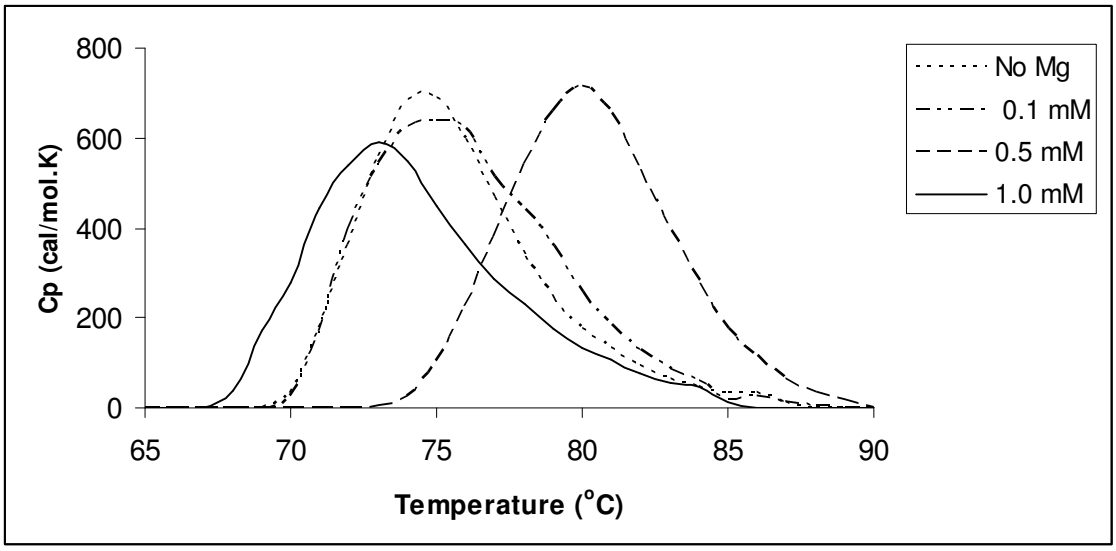


Figure 25. Thermal stability of the 696-bp *BRCA1* fragment at various concentrations of magnesium

Table 11 Thermodynamic data of the 696-bp *BRCA1* fragment incubated with magnesium chloride and cisplatin at various concentrations.

Sample	**T_m [°C]**	**ΔH [kcal/mol bp]**	**ΔS [cal/mole K]**
696-bp *BRCA1*	75.8	4.753	13.6
0.1 mM$MgCl_2$	76.1	5.120	14.6
0.5 mM $MgCl_2$	80.3	4.920	13.9
1.0 mM $MgCl_2$	74.3	4.606	13.2
Cisplatin (0.005 μM)	75.6	4.995	14.3
Cisplatin (0.05 μM)	74.9	5.455	15.6
Cisplatin (0.5 μM)	76.0	6.743	19.3

4.2 *BRCA1* damage by cisplatin and its repair

4.2.1 Cellular *BRCA1* damage

The quantitative polymerase chain reaction (QPCR) method was used to study cellular DNA damage and repair in the specific genes after exposure to DNA damaging agent cisplatin. The drug-treated *BRCA1* fragment with various concentration of cisplatin significiently reduced the amount of amplified DNA (Fig. 26A and B). The level of adduction was time and concentration dependent. The results showed that the total amount of amplified PCR product from a given region was inversely proportional to the amount of DNA adducts within the specified DNA fragment. The amount of DNA amplification was completely diminished at cisplatin concentration of 200 μM for 6 h and 50 μM for 24 h of incubation, respectively (Fig. 26C).

Lesion induction within the 696-bp *BRCA1* fragment can be quantitated by assuming a random (Poisson) distribution of damage. The amounts of platinum atoms in DNA adduct are calculated using Poisson equation as described in Material and Methods. A relation between platinum drug dose and platinum atom in the 696-bp *BRCA1* fragment was plotted. Interestingly, the platination level was almost saturated for 24 h at cisplatin concentration of 50 μM (Fig. 27).

4.2.2 Proficiency in repairing cisplatin-damaged *BRCA1* exon 11

The proficiency of cellular DNA repair after drug treatment of MCF-7 cells was determined by specific amplification of the 3,426-bp *BRCA1* exon 11 fragment using the QPCR. Previous *BRCA1* damage experiments showed that the platinum level increased linearly as the concentration increased (0-200 μM) at 6 h after drug exposure. In this study, MCF-7 cells were treated with cisplatin at the concentration of 50, 100 and 200 μM, respectively. After drug exposure at 37°C for 6 h, the cells were washed twice with PBS and fresh medium was added.

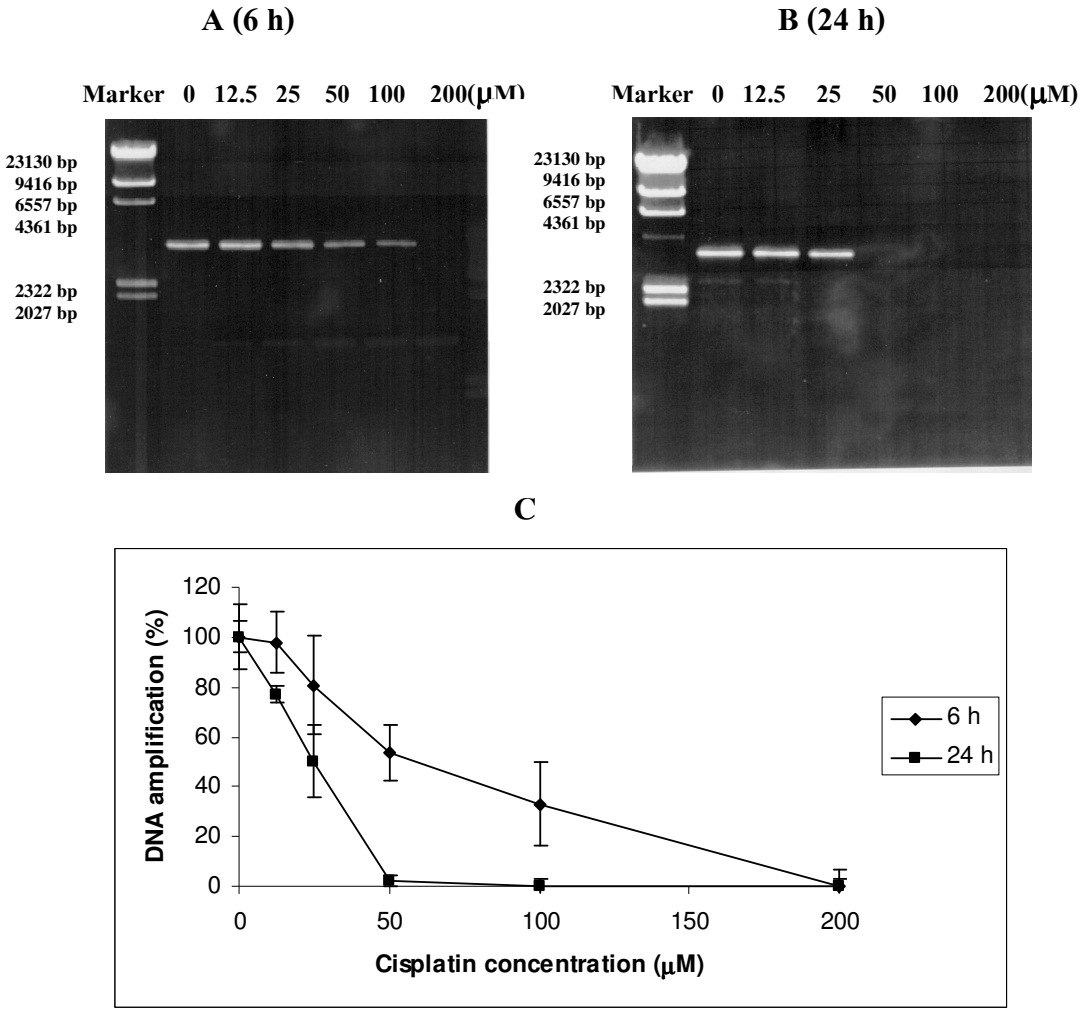


Figure 26. DNA amplification of the 3,426-bp fragment of *BRCA1* exon 11 induced by cisplatin. The MCF-7 cells were incubated with various concentrations of cisplatin (0-200 μM) at 37°C for and 24 h in 5% CO_2 incubator. The 3426-bp *BRCA1* fragment was then amplified by semi QPCR. Semi QPCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized under UV illumination. (A) MCF-7 cells were incubated with cisplatin for 6 h. (B) MCF-7 cells were incubated with cisplatin for 24 h. (C) DNA amplification (%) of the 3426-bp *BRCA1* exon 11 was calculated and plotted against cisplatin concentration. Genomic DNA of cisplatin-treated or untreated cells were isolated after various time intervals of incubation (0-18 h).

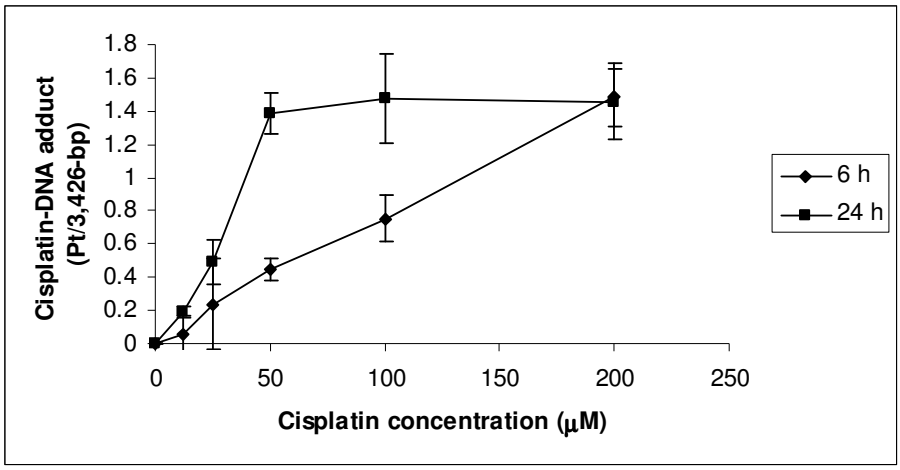


Figure 27. The number of platinum adducts per the 3,426-bp *BRCA1* fragment of exon 11 calculated by Poisson equation.

The 3,426-bp fragment of the *BRCA1* gene was then amplified by PCR using isolated genomic DNA and specific forward and reverse primers. PCR products were electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized under UV illumination (Fig. 28A, B and C). The results indicated a time dependent recovery of cisplatin-modified genomic DNA with an initial low level of lesion removal during the first 4 h (Fig. 28D). A more complete lesion removal was observed with over 90% of 50 μM cisplatin after 18 h of repair time. However, only 30% of the lesion repair was observed at higher cisplatin concentration of 200 μM.

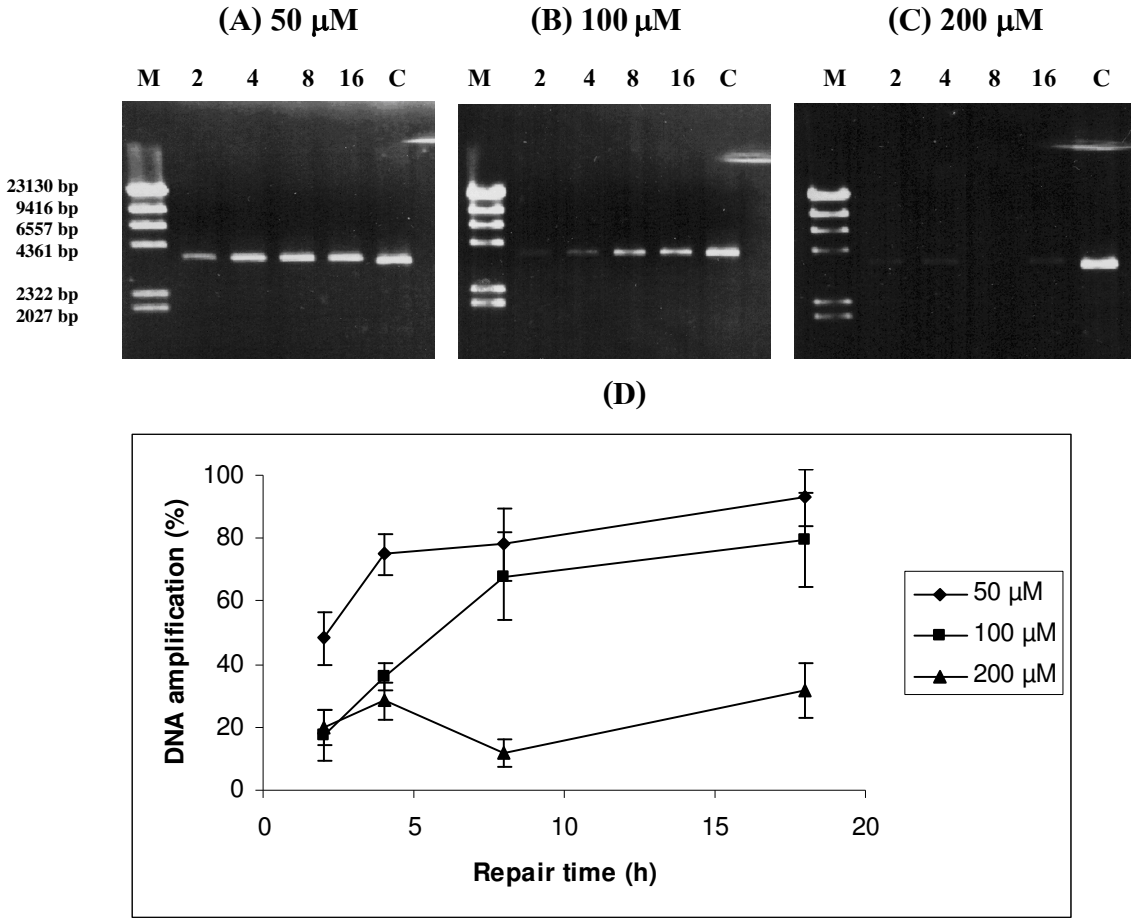


Figure 28. DNA amplification and proficiency in repairing cisplatin-damaged 3,426-bp fragment of *BRCA1* exon 11 of MCF-7 cells. MCF-7 cells were incubated with medium plus cisplatin at various concentrations (50-200 μM) for 6 h. The cells were washed twice with PBS and fresh medium was added. The genomic DNA was extracted at 2, 4, 8, and 18 h and used as the template for the semi QPCR assay (C: the control untreated cells)

4.3 Construction of expression vector pBIND-BRCT

The 696-bp fragment of the 3′-terminal region of *BRCA1* was cloned to the multicloning region of the pBIND at *Bam*HI and *Xba*I site. The plasmid was transformed into *E.coli* strain DH5α for multification and extracted by alkaline lysis

technique. Purified plasmid DNA was then restriction analysed using restriction enzymes such as *Eco*RI, *Eco*0109I, *Pvu*II, and *Bam*HI. Digestion by *Bam*HI produced a 7,027-bp fragment of linearized recombinant pBIND-BRCT plasmid. Digestion by *Eco*RI, *Eco*0109I and *Pvu*II produced 5011 and 2016-bp fragment, 3868 and 3159-bp fragment and 5862 and 1165-bp fragment, respectively. Double digestion by *Bam*HI and *Eco*0109I produced 3455-bp, 3154-bp and 413-bp fragment and that by *Bam*HI and *Pvu*II produced 5403-bp, 1416-bp and 459-bp fragment, respectively. The digested fragments of all enzymes used as expected (Fig. 29).

Purified recombinant plasmid was finally verified using a Big Dye Terminator and sequenced by ABI-prism 377 DNA sequencer (Perkin-Elmer, USA). DNA sequencing confirmed the recombinant pBIND-BRCT. The sequence of GAL4 and 696-bp *BRCA1* fragment was analyzed by Human Genome Sequence Center Program (Fig. 30).

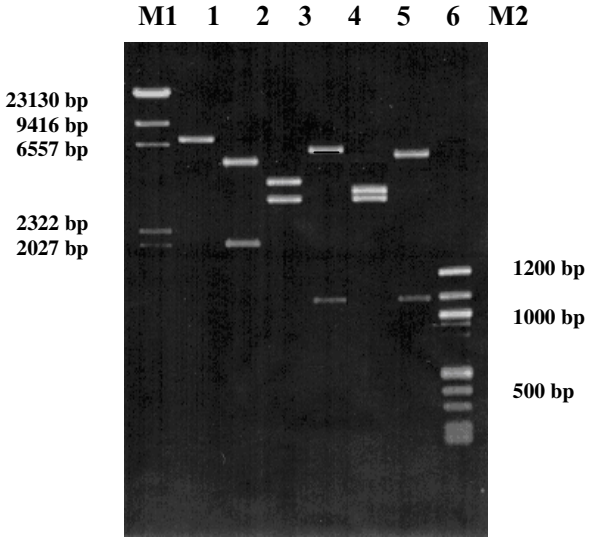


Figure 29. Restriction digestion of the pBIND-BRCT plasmid (7,027 bp). Lane 1: *Bam*HI, Lane 2: *Eco*RI, Lane 3: *Eco*OIO9I, Lane 4: *Pvu*II, Lane 5: *Bam*HI and *Eco*0109I and Lane 6: *Bam*HI and *Pvu*II. M1 is λ-*Hind*III digest marker and M2 is 100 bp ladder marker

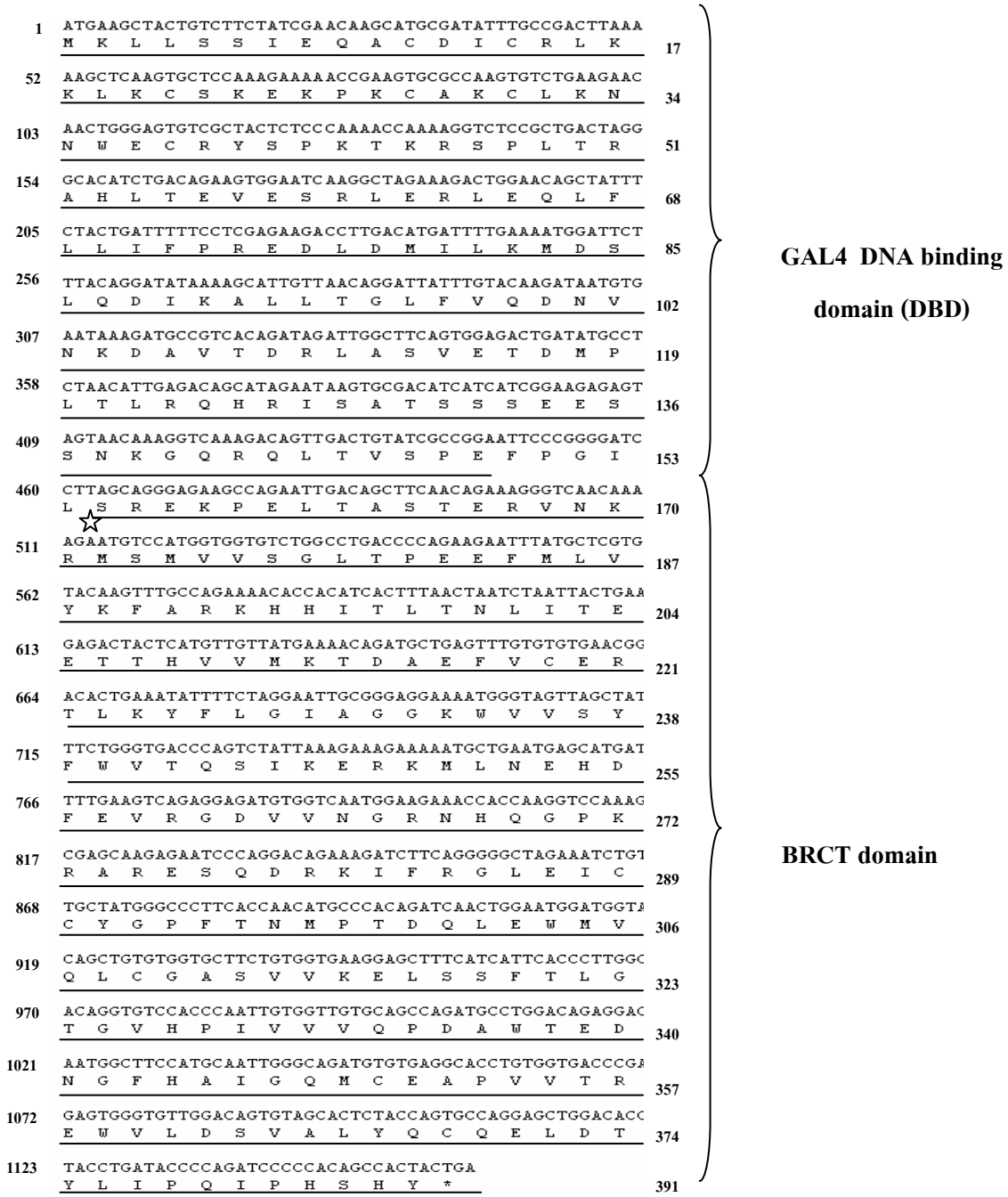


Figure 30. Deduced nucleotides and amino acid sequences of the GAL4 fusion protein containing BRCT domain. (☆ is the first codon of BRCT domain, * is a stop codon).

4.4 Expression of pBIND-BRCT in MCF-7 cells

The expression of GAL4-BRCT protein was performed using western blotting analysis. The band of protein can be observed at 24 h of incubation. The bands of endogenous BRCA1 protein (220 kDa) and GAL4-BRCT protein (43 kDa) were found as expected (Fig. 31).

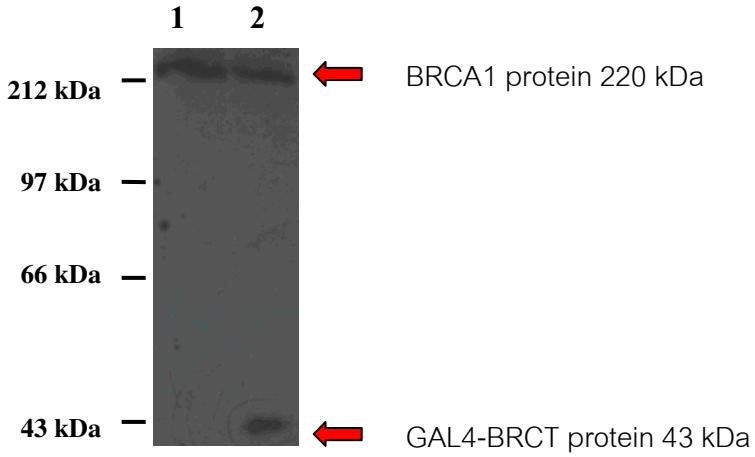


Figure 31. Western blot analysis of the expressed GAL4-BRCT protein. MCF-7 cells were transfected by calcium phosphate co-precipitation with the pBIND-BRCT. Cell lysate was prepared at 24 h after transfection and electrophoresed on a 12 % SDS-PAGE. Cell lysate was fixed on PVDF membrane and blotted by a primary antibody anti-BRCT C-20 and secondary antibody anti-rabbit IgG horseradish peroxidase-conjugate. The emitting light was exposed on X-ray film. Lane 1: a band of endogenous BRCA1 protein (220 kD), Lane 2: endogenous BRCA1 protein (220 kD) and GAL4-BRCT protein (43 kD).

4.5 Cellular reactivation of cisplatin-damaged *BRCA1*

Host cell reactivation assay is the technique used to study the proficiency of the host cells to repair cisplatin-damaged plasmid. In this study, the pBIND-BRCT was *in vitro* platinated at various cisplatin concentrations. The cells were collected at various times after transfection. As shown in Fig. 32, the expression

level of untreated *Renilla* reporter luciferase gen at 10, 16, 24, and 36 h was 70, 89, 100, and 38%, respectively. The *Renilla* luciferase activity of the damaged pBIND-BRCT at cisplatin concentration of 12.5 μM at 10, 16, 24, and 36 h was 10.7, 20.0, 3.5, and 3.6 %, respectively. However, the *Renilla* luciferase activity at 25 μM and 50 μM decreased dramatically. The result indicated that the reduction of cellular reactivation on the the drug-danaged *Renilla* luciferase encoding plasmid was a consequence of an increase in platination levels within the transcribed reporter gene.

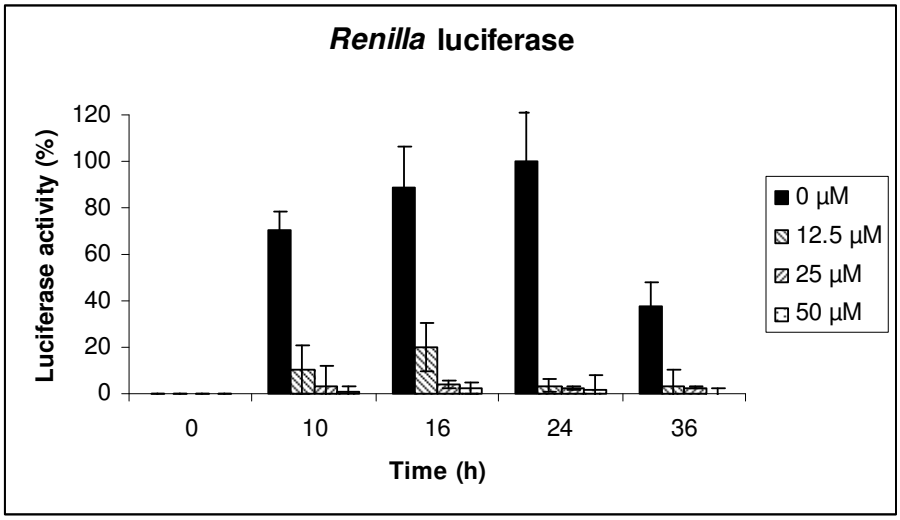


Figure 32. Time-course of *Renilla* luciferase expression of pBIND-BRCT. The plasmid was incubated with cisplatin at the concentration of 0, 12.5, 25 and 50 μM before co-transfecting with the pG5Luc plasmid to $1x10^5$ MCF-7 cells. Cell lysates were collected at 10, 16, 24 and 36 h after transfection. *Renilla* luciferase expression was detected by Stop & Glo® reagent in Dual-Luciferase® Reporter Assay System.

4.6 Transcriptional activation of BRCT on *Renilla* luciferase gene in the absence of GAL4 binding site

The pBIND or pBIND-BRCT was transfected with the pSV-β-galactosidase in MCF-7 cells. Cell lysates were prepared at 10, 16, 24 and 36 h after transfection. *Renilla* luciferase activity was measured by the Dual-Luciferase® Reporter Assay System. The amount of activity resulting from transfection with pBIND or pBIND-BRCT at highest expression was defined as 100%. The data were derived from 4 independent experiments ± standard deviation (SD).

Interestingly, the pBIND-BRCT exhibited more *Renilla* luciferase activity than the parental pBIND at 10 and 16 h post transfection. It is implied that the BRCT domain (amino acids 1631-1863) can enhance the rate of gene transcription at an initial time of transfection (Fig. 33).

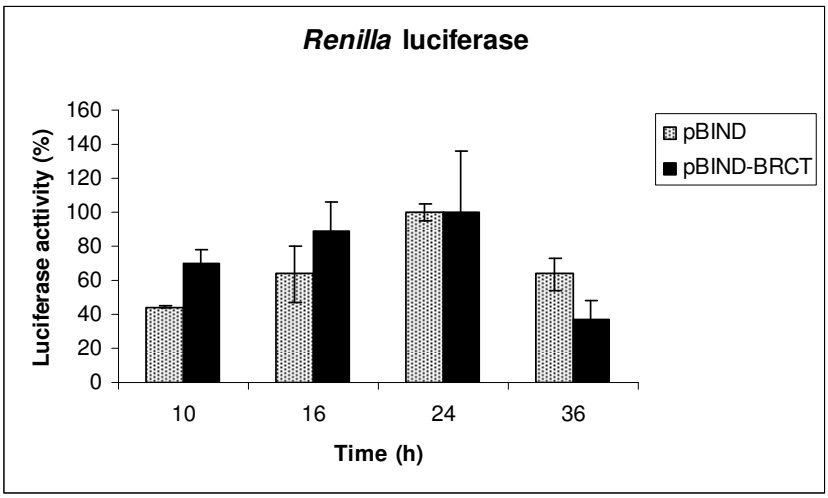


Figure 33. Time-course of *Renilla* luciferase expression of the pBIND and the pBIND-BRCT. The pBIND and pBIND-BRCT were transfected into MCF-7 cells. Cell lysates were prepared at 10, 16, 24 and 36 h after transfection. *Renilla* luciferase expression was detected by Stop&Glo® reagent in Dual-Luciferase® Reporter Assay System. The data were derived from 4 independent experiments ± standard deviation (SD).

4.7 Transcriptional transactivation of the 3′-terminus of *BRCA1*

4.7.1 BRCT transactivated firefly luciferase gene

Previous experiment has demonstrated that the BRCT domain (aa 1528–1863) stimulates gene transcription in transiently transfected human cells (Monteiro *et al*., 1996). We thus hypothesized whether shorter sequence of *BRCA*1 gene covering both N- and C-terminal BRCT domain (aa 1631-1863) could transactivate reporter plasmid. To test this hypothesis, MCF-7 cells with an expression plasmid pBIND-BRCT were used.

The pBIND was co-transfected with the reporter the pG5Luc and the pSV-β-galactosidase in the MCF-7 cells. Cell lysates were prepared at 16, 24 h after transfection. Firefly luciferase activity was measured by the Dual-Luciferase® Reporter Assay System. The data were derived from 4 independent experiments ± standard deviation (SD).

As previously reported (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Anderson *et al.*, 1998), the GAL4–BRCA1 plasmid transactivated a reporter template bearing four GAL4-binding sites upstream of the c-*fos* minimal promoter fused to the luciferase gene. Interestingly, the GAL4-BRCA1 was also able to enhance the level of transcription several-folds in the cells. The level of firefly luciferase expression was measured when the pBIND or the pBIND-BRCA1 and the pG5Luc were co-transfected into the MCF-7 cells. The pBIND plasmid producing only the GAL4 DNA binding protein, can not stimulate expression of firefly luciferase from the pG5Luc plasmid and it was used as negative control. However, when the pBIND-BRCT plasmid was co-tranfected the with pG5Luc plasmid, the expression of firefly luciferase can be observed at 16 h and 24 h after transfection (Fig 34). The pattern of firefly luciferase expression from both co-transfection between the pBIND or the pBIND-BRCT and the pG5Luc plasmid explained the transcriptional activation activity of the BRCT domain as described in Fig. 16.

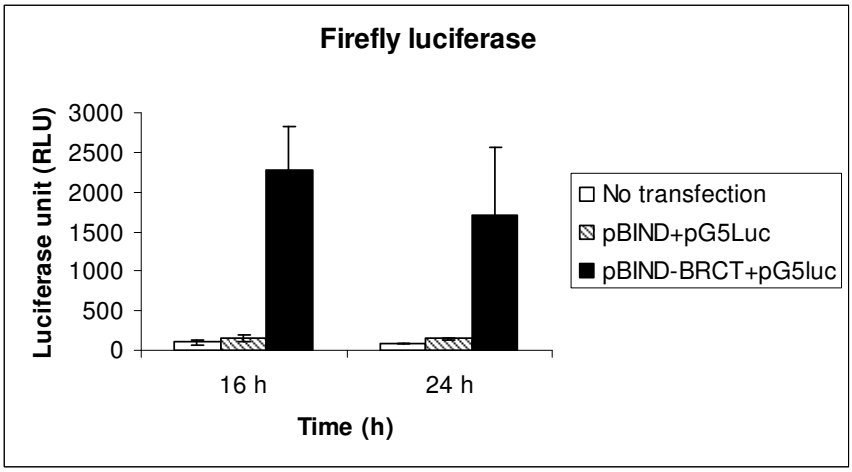


Figure 34. Firefly luciferase expression after transfection. The $1x10^6$ cells of MCF-7 were co-transfected by calcium phosphate precipitation with the pBIND or the pBIND-BRCT along with reporter plasmid pG5Luc and the pSV-β-galactosidase, as transfection control. Cell lysates were prepared at 16 and 24 h after transfection. Firefly luciferase expression was detected using the Dual-Luciferase® Reporter Assay System. The data were derived from 4 independent experiments ± standard deviation (SD).

4.7.2 Effect of cisplatin on transcriptional transactivation of the 3′-terminus of *BRCA1*

To better understanding a role of the C-terminal domain of the protein BRCT as a transcription activator, we hypothesized that such the role could be lose when treating the 3′-terminus of *BRCA1* with cisplation. In order to investigate whether the drug-treated *BRCA1* is able to transactivate the expression of the firefly luciferase gene, DNA repair-proficient MCF-7 cells were transiently transfected with the cisplatin-treated pBIND-BRCT along with the reporter plasmid pG5Luc.

The pBIND-BRCT was platinated with cisplatin at concentration of 0, 12.5, 25 and 50 μM for 24 h at room temperature in the dark. The plasmid was precipitated by absolute ethanol and dissolved in double distilled water. Platinated plasmid was co-transfected with the pG5Luc and the pSV-β-galactosidase in MCF-7

cells. Cell lysates were prepared at 10, 16 and 24 h after transfection. Firefly luciferase activity was measured by the Dual-Luciferase® Reporter Assay System. The amount of activity resulting from transfection with undamaged plasmid, pBIND-BRCT, was defined as 100%. The firefly luciferase activity was measured as a function of the number of Pt-BRCT adducts in the plasmid.

After 10, 16, 24 and 36 h of transfection, the firefly and *Renilla* luciferase activities were measured. The firefly luciferase activity was significantly decreased at cisplatin concentration of 12.5 μM. Maximal luciferase activity can be observed only 10 h after transfection (Fig. 35). A decrease in firefly luciferase activityu can be observed at prolonged transfection.

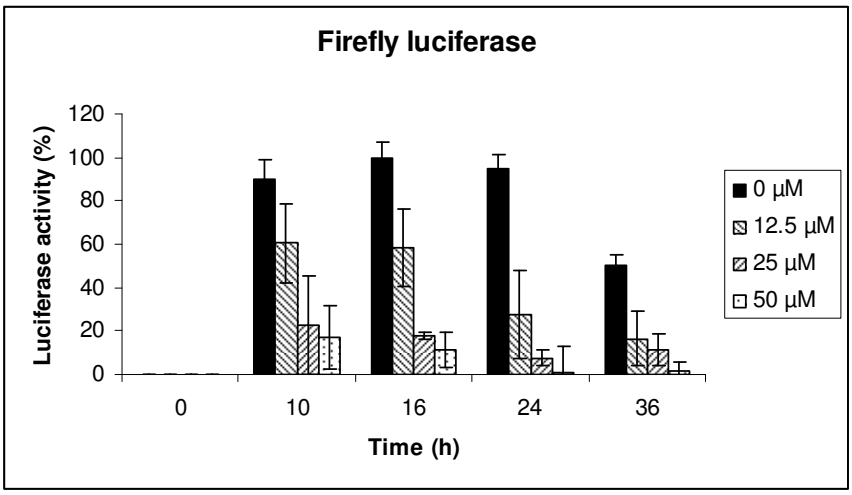


Figure 35. Time-course of Firefly luciferase expression. The pBIND-BRCT was incubated with cisplatin at concentration of 0, 12.5, 25 and 50 μM and then co-transfected with thepG5Luc plasmid into MCF-7 cells. Cell lysate was prepared at 10, 16, 24 and 36 h after transfection. Firefly luciferase expression is detected by the Dual-Luciferase® Reporter Assay System. The data were derived from 4 independent experiments ± standard deviation (SD).

4.7.3 BRCT enhanced transactivation of reporter pSV-β-galactosidase gene

As shown previously that the GAL4-BRCT protein can enhance transcription of *Renilla* luciferase and transactivate the firefly luciferase gene located on the pG5Luc, raises the possibility whether the BRCT domain could transactivate transcription of other types of promoter and/or gene. The pSV plasmid was used on this purpose. The MCF-7 cells were co-transfected with the pBIND or the pBIND-BRCT and the pSV plasmid. The cell lysates were prepared at 16 h after transfection by β-galactosidase assay system (Promega, USA).

The β-galactosidase activity was significantly increased when co-transfected with pBIND. This observation may be resulted from the possibility that the GAL4 protein alone can stimulate the β-galactosidase gene on the pSV. Upon sequence data of pSV, the sequence similarity of GAL4 binding sites was found to be 74 and 77% at nucleotide position of 907-921 inside *LacZ* gene and 6463-6475, respectively (SIM4 DNA alignment program) (Fig. 36). Interestingly, the level of transactivation was significantly higher when co-transfected with the pBIND-BRCT than the parental pBIND plasmid (Fig. 37). This indicated that the GAL4-BRCT domain may stimulate the pSV plasmid. However, when pBIND-BRCT plasmid was platinated by cisplatin at concentration of 12.5 μM, the ability of pBIND-BRCT to enhance expression of pSV was decreased to the same level as that of the pSV alone.

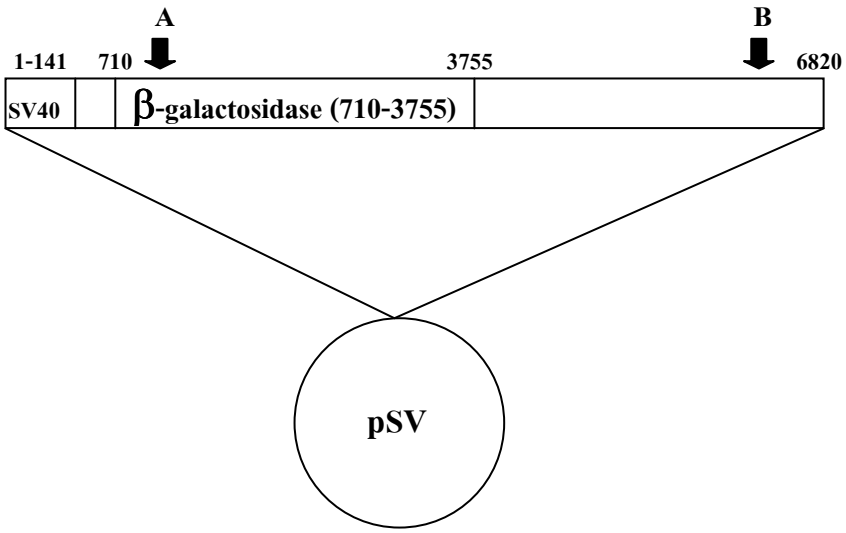


Figure 36. Schematic representation of pSV plasmid, showing the SV40 promoter region followed by the *LacZ* gene. The possibility of GAL4 binding sites is found at nucleotide position of 907-921 inside the *LacZ* gene (arrow A) and nucleotide position of 6463-6475 (arrow B), respectively.

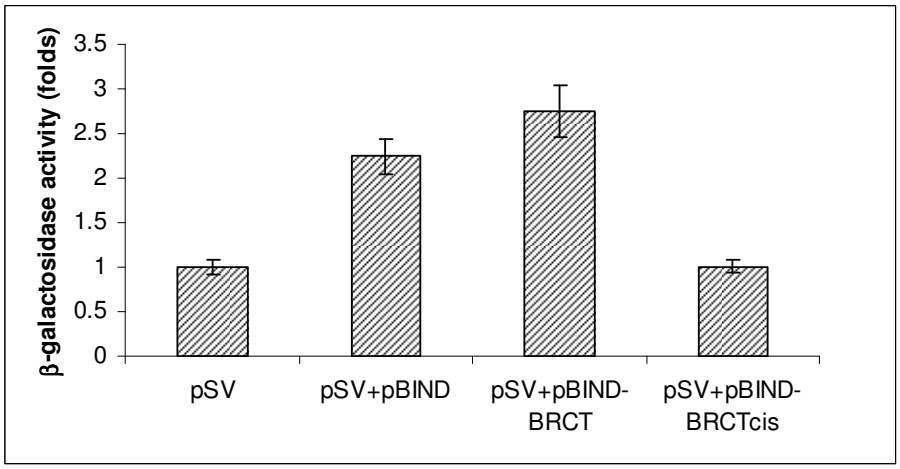


Figure 37. Transcriptional transactivation of BRCT. The pBIND or pBIND-BRCT was co-transfected with the pSV-β-galactosidase. Cell lysates were prepared at 16 h after transfection. β-galactosidase activity was detected using the β-galactosidase assay. The data were derived from 4 independent experiments ± standard deviation (SD).

4.7.4 Transcriptional transactivation activity of cisplatin-damage pBIND-BRCT on platinated pSV-β-galactosidase

To investigate further the role of BRCT as a transactivator, the platinated pSV was co-transfected with pBIND or pBIND-BRCT. The pSV-β-galactosidase was platinated with cisplatin at concentration of 12.5 μM for 24 h at room temperature in the dark. The plasmid was precipitated by absolute ethanol and dissolved in double distilled water. Platinated plasmid was co-transfected with plasmid pBIND or pBIND-BRCT into the MCF-7 cells. Cell lysates were prepared at 16 h after transfection. β-galactosidase activity was measured by β-galactosidase assay system. A decrease in transcriptional transactivation activity of pBIND and pBIND-BRCT was measured. However, no significant difference in transcriptional transactivation can be observed between pBIND and pBIND-BRCT (Fig. 38). Futhermore, when both pSV and pBIND-BRCT were platinated with cisplatin at concentration of 12.5 μM for 24 h at room temperature in the dark and co-transfected into MCF-7 cells, surprisingly, very low β-galactosidase expression was detected.

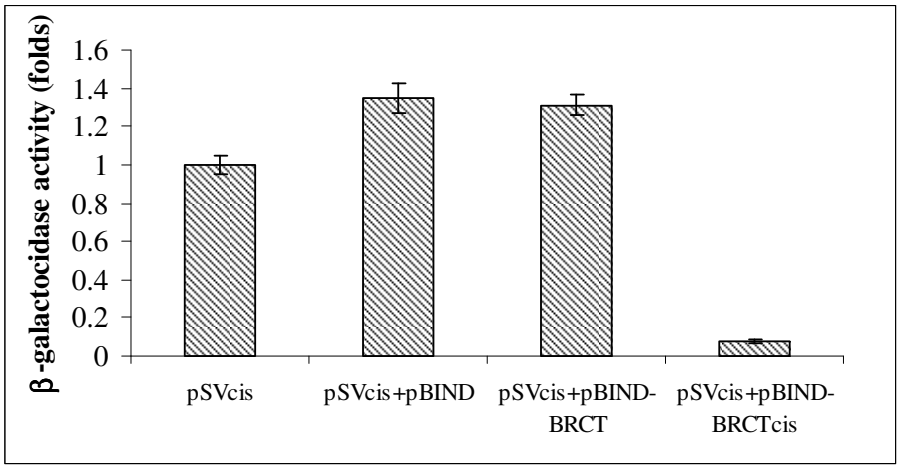


Figure 38. Transcriptional transactivation of platinated pBIND-BRCT plasmid on platinated pSV-β-galactosidase. The platinated pSV (with cisplatin at concentration of 12.5 μM) was co-transfected with non-platinated pBIND and pBIND-BRCT or platinated pBIND-BRCT. Cell lysates were prepared at 16 h after transfection. β-galactosidase expression was detected by the β-galactosidase assay (Promega, USA). The data were derived from 4 independent experiments ± standard deviation (SD).

CHAPTER 5

DISCUSSION

5.1. Conformational change of the 696-bp *BRCA1* fragment

At higher cisplatin concentration, the mobility of platinated DNA is retarded. This may be due to the intrastrand, interstrand and intermolecular cross-links along DNA fragment. Cisplatin forms sequence-specific adducts with DNA (Calvert *et al*., 1982). To confirm the consequence specificity of the intrastrand adduct formation, restriction digestion on platinated DNA is analysed. The platinated sites are known to resist the activities of restriction enzymes which recognize and cleave the native sequence. Therefore, platination site in the gene fragment can be deduced from restriction analysis using specific enzymes whose recognition sequences exist on the tested gene. The *Pvu*II (CAGCTG) and *Eco*0109I (PuGGNCCpy) are the enzyme designed to cleave possibly adducted site of cisplatin on DNA. *Pvu*II digests DNA at the d(GpC) sequence to produce blunt end product whereas *Eco*0109I digests DNA at the d(GpG) sequence to produce sticky end product. From the experiment, digestion of the gene fragment by *Pvu*II produced two digested fragments of 237 bp and 459 bp and that by *Eco*0109I produced two digested fragments of 283 bp and 413 bp. Production of digested fragments from cisplatin-treated DNA in the present of their *Eco*0109I is absolutely inhibited at cisplatin concentration of 12.5 μM. Cisplatin-treated *BRCA1* fragment is less sensitive to *Eco*0109I than to *Pvu*II, implying that the *Eco*0109I site was platinated more easily than the *Pvu*II site. Considering the recognition sequences and restriction sites of these two restriction enzymes, platination of the d(GpG) sequence is preferred to the d(GpC) sequence, which are known target sites of the drug (Calvert *et al*., 1982; Donahue *et al*., 1990; Fichtinger-Schepman *et al*., 1985).

5.2 Interstrand cross-links of cisplatin-*BRCA1* adducts

Although the interaction between a platinum atom to N-7 of guanine of the interstrand cross-links is rare (1%) (Eastman, 1985), it is of interest to determine the extent of the interstrand cross-links of cisplatin-*BRCA1* adducts that may contribute to conformational change of DNA molecule. Formation of interstrand cross-links was analysed by denaturation alkaline gel electrophoresis. Under this condition, double stranded DNA are separated to single stranded DNAs which migrate faster (lower band) in the gel. As shown in Fig. 22, band intensity increased with increased drug concentrations with a concomitant decrease in the intensity of the band corresponding to the non-crosslinked duplex. This observation can be interpreted to mean that the interstrand crosslinks were formed. An increase in drug concentrations resulted in increased interstrand crosslinks. The result showed that the interstrand crosslinks initially formed at cisplatin concentration of 0.5 μM. The interstrand crosslinks completely formed at 32 μM cisplatin. This may be resulted from the multi-crosslinked DNAs or aggregated DNA adducts multi-crosslink is formed and aggregates double-helix.

5.3. Alteration in thermal stability of cisplatin-*BRCA1* adducts

The structure of polynucleotides is dependent on the accompanying counterion. The divalent ions have a significantly influence upon the structure of DNA (Cate *et al*., 1997; Chiu and Dickerson, 2000; Lynch and Schimmel, 1974: Tereshko *et al*., 2001; van de Sande *et al*., 1982). The divalent ions can be grouped into two catagories according to their mode of interaction. Firstly, the transition metal ion that can bind to the base as well as to the phosphate residues along the backbone chain. The interaction of transition metals with the base apparently perturbed hydrogen bonding between base pairs, thus destabilizing the B form of DNA and stabilizing alternative DNA structures (van de Sande *et al*., 1982). The N-7 site of guanine is recognized as an important site of metal interaction. Secondly, alkaline cations present affinity and

greater binding specificity for the phosphates which stabilizing the B form of DNA, apparently through charge neutralization of sugar-phosphate backbone (Duguid *et al*., 1993).

The transition anticancer platinum drug interacts with DNA through its coordination between a platinum atom and guanine or adenine bases to form the intrastrand crosslink, interstrand crosslink or monoadduct (Donahue *et al*., 1990; Fichtinger-Schepman *et al*., 1985). The crosslink alters the structure, thermal stability and thermodynamic stability of the host duplex (Hofr and Brabec, 2001a and b). The energetic consequences of the crosslink are related to the structural perturbations of DNA. These platinum-DNA adducts can interfere DNA replication, transcription, and translation (Poklar *et al*., 1996). In addition, both intra- and interstrand adducts affect nucleotide excision repair of DNA and cause mutation of genes (Zamble *et al*., 1996).

The thermal and thermodynamic properties of DNA duplex containing intra- and interstrand crosslink of cisplatin have been studied in details (Malina *et al*., 2000; Pilch *et al*., 2000; Poklar *et al*., 1996; Zamble *et al*., 1996). The intrastrand crosslink destabilizes DNA structure by changing a B-form to A-form of duplex, lowering the thermal stability by $\sim 9^{\circ}$C and reducing the thermodynamic stability by enthalpy in origin. On the other hand, the interstrand crosslink increases the thermal stability by entropically stabilized the duplex (Hofr and Brabec, 2001).

At low content of cisplatin, the melting temperature of the 696-bp *BRCA1* fragment lowered transition temperature from non-platinated DNA at 75.8°C to 75.6°C and 74.9°C at cisplatin concentration of 0.005, 0.05 μM, respectively. This implies the predominant intrastrand crosslink involved in bending, unwinding and destabilizing the double helix structure of DNA, resulting in decreased DSC endotherms and melting temperature. This result corresponds to the influence of cisplatin intrastrand crosslink on the conformation, thermal stability, and energetics of a 20-mer DNA duplex (Poklar *et al*., 1996). The intrastrand crosslink to DNA through

the covalent bond between cisplatin and the nucleobases such as GG, AG and GNG accounts for more than 90% of lesions on DNA. It alters the structure of duplex and lowers both thermal and thermodynamic stability, most of which is enthalpy in origin (Poklar *et al*., 1996). At cisplatin concentration of 0.5 μM, a shift to a slight increase in transition temperature to 76.0°C can be observed. The result suggests that additional interstrand and intermolecular crosslinks can attribute to the observed higher T_m and increased heat capacity. This data is correlated to our previous data obtained from an alkaline gel that interstrand crosslink is initially form at cisplatin concentration of 0.5 μM and completely formed at 32 μM cisplatin.

Our data suggests that the predominant intrastrand crosslink changes double-helix structure of DNA at lower cisplatin concentrations, resulting in decreased DSC endotherms. Whereas, at higher cisplatin concentrations, interstrand crosslinks between the GG sequences of an opposite DNA strand and intermolecular crosslinks are induced with a slight increase in enthalpy and heat capacity. The energetic consequences of this crosslinks were most likely related to the structure perturbations of the specified *BRCA1* coding sequence (Hofr *et al*., 2001; Poklar *et al*., 1996; Zamble *et al*., 1996). Our model for additional intermolecular crosslinks is proposed as shown in Fig. 39. Low concentration of cisplatin conducts a few platinums adduct on DNA duplex especially the intrastrand crosslink. This adduct may distort, disrupt and change DNA structure and reduces melting temperature of DNA complex. Cisplatin at higher concentration increases the possibility of interstrand formation of the duplex. Although the interaction between a platinum atom to N-7 of guanine of interstrand crosslink is found in few amounts (1%) (Eastman, 1986; Eastman and Schulte, 1988; Fichtinger-Schepman, *et al*., 1987), it can raise the T_m to higher temperature (Hofr *et al*., 2001). The structural alteration of cisplatin-*BRCA1* adducts may interfere biological processes essential for cellular viability including the bindig of adducts with relevant proteins (Jakupec *et al*., 2003).

However, more thermodynamic data and accurate model are required for predicting the drug-gene relationship. The thermal disruption of the hydrogen bonds in solution appears to be a complex multi-step process, very much affected by the ionicity, pH, the composition of the solvent, the length of chain and the particular sequence of the bases in the paired strains (Zamble *et al*., 1996). An approach to understanding the thermal stability of complex DNA structure has been based on the experimental study of both defined length and sequence oligonucleotides (Duguid and Bloomfield, 1996; Mrevlishvili *et al*., 1996; Rouzina and Bloomfield, 1999; Santa-Lucia *et al*., 1996).

DNA melting transition in aqueous magnesium salt solution is dependent on the concentration of salt. The derived heat capacity curve for the 696-bp *BRCA1* fragment in three Mg^{2+} concentrations is measured. The transition midpoint is raised from 75.8°C to 76.1°C and 80.3°C at 0, 0.1 and 0.5 mM, respectively.

The addition of magnesium ion stabilizes DNA against melting at low salt level by reducing the repulsion between phosphate backbone (Clement *et al*., 1973). The ion does not perturb DNA structure. Magnesium stabilizes the DNA structure by neutralizing negative charge of phosphate backbone and consequently resistant to thermal denaturation (Duguid *et al*., 1993, 1995). The data from Raman spectroscopy, UV difference spectra, circular dichroism, sedimentation measurement, laser Raman spectroscopy, optical densitometry, pH potentiometry, and DSC indicated preference of magnesium ion on phosphate backbone that stabilizing the B-DNA. Mg^{2+} is found to interact very weakly with the base, but has higher affinity for the phosphates (Duguid *et al*., 1995; Eichorn, 1962; Langlais *et al.*, 1990; Zimmer *et al*., 1974). These studies indicate that metal ions bind preferentially to phosphates relative to bases in following order: Ba^{2+},Mg^{2+}>Ca^{2+}>Fe^{2+}>Co^{2+}, Ni^{2+}>Mn^{2+}>Zn^{2+}>Cd^{2+}>Pb^{2+}>Cu^{2+}>Hg^{2+} (Duguid *et al*., 1993). However, extremely high magnesium concentrations (1.0 mM) may rupture hydrogen bonds and decreases

thermal stability of DNA. High ionic concentrations may modify the stability of DNA by changing water activity as well as by screening electrostatic interactions, leading to decreased thermal stability of DNA (Duguid and Bloomfield, 1996).

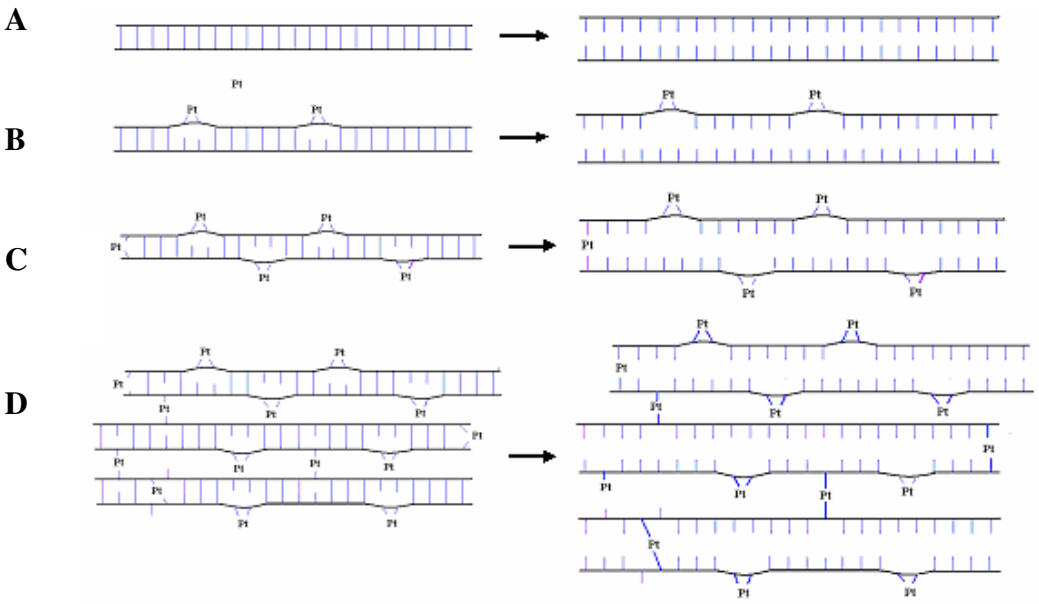


Figure 39. Schematic model of DNA conformational change induced by cisplatin. Double strand DNA is separated into single stranded structure at high temperature (A). An increment of cisplatin concentration increases intrastrand crosslinks (B) that reduce thermodynamic property of DNA. At high cisplatin concentration, interstrand (C) and intermolecular crosslinks or aggregated cisplatin-DNA adducts (D) are formed.

5.4. Cellular *BRCA1* damage and its repair

In order to detecting the degree of DNA damage, the semi-quantitative PCR method is used to monitor the progress of *Taq* DNA polymerase in PCR utilizing DNA adducts as templates. Thus, it is important to establish that the majority of the lesions formed will effectively block the polymerase with minimal bypass. The sensitivity for detection of DNA damage is dependent upon the size of the target sequence. At any level of platination, there is a greater probability that an adduct lies

within longer DNA than shorter sequence. When cisplatin is added to cells culture medium, the drug can be uptaked to cytoplasm and DNA. The level of cisplatin-DNA adduct varies depending on both time and concentration of cisplatin. As shown in Fig. 24, the level of platination linearly increased when MCF-7 cells were incubated with cisplatin for 6 h and cisplatin-DNA adducts completely formed at cisplatin concentration of 200 μM. However, the level of platination for 24 h incubation reached highest at cisplatin concentration of 50 μM. As a result, the supsequent repair experiment was designed after 6 h of drug exposure. The QPCR assay had previously been used to study cellular DNA damage and repair in the specific genes after exposure to DNA damaging agents, including an anticancer drug cisplatin (Kalinowski *et al*., 1992; Grimaldi *et al*., 1994). As shown in Fig. 28D, DNA amplification was reduced dramatically at higher cisplatin concentration of 200 μM. This implies that cisplatin could strongly inhibit cellular DNA machineries required for repair activity in the presence of DNA damage and induce apoptosis. Such cellular proficiency in DNA repair is similarly observed for the 523-bp *N-ras* gene fragment of K562 cells (Dempke *et al*., 1999) and that for the 2.6-kb *DHFR* gene fragment of murine leukemia L1210 cells after being treated with cisplatin (Kalinowski *et al*., 1992).

5.5. Host cell reactivation of cisplatin-damaged pBIND-BRCT

The effects of cisplatin on transcriptional reactivation are reported by host cell reactivation assay (Dabholkar *et al*., 1990). The assay involves host cell reactivation of a cisplatinum-damaged transient expression vector. This technique provides information concerning the cellular responses to damaged DNA without the confounding effects of other damaged cellular molecules. In transcriptional reactivation assay, plasmid DNA is treated *in vitro* with cisplatin and then introducing into the cells of interest. The plasmid DNA originally contains a reporter gene whose expression can easily be quantified. Platination of DNA inhibits expression of reporter gene, but after transfection into repair-proficient cells, removal of the platinum adducts increase

reporter gene expression. Increased reactivation of a cisplatin-treated reporter gene has been observed in cisplatin-resistant cells but not cisplatin-sensitive cells, supporting the involvement of enhanced DNA repair in cisplatin resistance (Calsou *et al*., 1993; Parker *et al*., 1991; Selvakumaran *et al*., 2003). The adduction of cisplatin is randomly on the plasmid. Cisplatin forms Pt-DNA randomly on the pBIND-BRCT plasmid with approximately 10% of cisplatin-BRCTA1adducts (696-bp *BRCA1* fragment in 7027-bp pBIND-BRCT). From our previous experiments, It is suggested that the intrastrand, interstrand and intermolecular adducts were formed along the plasmid molecule at each concentration. The reduction of expression of *Renilla* luciferase reveals the effect of the drug on plasmid transcription. *Renilla* luciferase protein is simultaneous expresses under control of strong SV40 early enhancer/promoter. The reduction of *Renilla* expression results from the inhibition of the drug on any sequence of luciferase gene and enhancer/promoter. The inhibition of function of RNA polymerase and limitation of the involved transcription factors in both transcription and repair machineries required for repair activity in the presence of DNA damage. Cisplatin-DNA adducts block a variety of RNA polymerase including those present in *E.coli*, T7 and mammals (Corda *et al*., 1991; Cullinane *et al*., 1999; Mello *et al*., 1995; Tornaletti *et al*., 2003). This reveals that the transcription derived from a strong promoter is preferentially reduced by cisplatin treatment and this inhibition correlates with the presence of more potential cisplatin modification sites within the promoters.

It has been reported that *in vitro* transcription by RNA pol II in human cell extracts is strongly inhibited by a 1,3-intrastrand but not a 1,2-intrastrand adduct, whereas both types of crosslink effeciently block T7 and SP6 RNA pol (Corda *et al.*, 1991; Cullinane *et al*.,1999). Cisplatin-modified plasmid DNA has been reported to be repaired *in vitro* by nucleotide excision activity in extracts of human cells (Hansson and Wood, 1989). Most cells are capable of some degree of replication bypass as well as transcription bypass of RNA polymerases (Corda *et al*., 1992; Corda *et al*., 1993).

However, in intact cells transcription bypass has been estimated to occur only with much lower frequency (Mello *et al*., 1995)

5.6 Transcriptional transactivation activity of cisplatin-damaged *BRCA1*

The one hybrid assay system is used to study the effect of cisplatin on transcriptional transactivation. DNA damaging agent such as cisplatin interferes transcriptional transactivation of the GAL4-BRCT protein and reduces ability of transcription on reporter plasmid. The data demonstrate that the transcriptional transactivation ability of cisplatin-damaged *BRCA1*, when tested in the GAL4-BRCT transcriptional activation system, reduces transactivation activity of firefly luciferase. The level of reduction is inversely proportional to the amount of platinum-*BRCA1* adducts. The transcriptional transactivation activity of *BRCA1* has previously been reported by fusing the C-terminal domain of *BRCA1* to a heterogenous DNA-binding domain (Monteiro *et al*., 1996). The BRCT domain (amino acids 1380-1863) of human *BRCA1* scores positively in transcriptional activation trap experiments using various forms of so-called "one hybrid assay" (Chapman and Verma, 1996; Hu *et al*., 2000; Miyake *et al*., 2000; Monteiro *et al*., 1996). *BRCA1*-fused DNA-binding domain activates transcription in cell-free system to a similar extent as dose the powerful activator, VP16 (Schlegel *et al*., 2000, Haile and Parvin, 1999). A *GAL4:BRCA1* has also been introduced in yeast- and mammalian-based transcription assays to characterize the deleterious mutations in the 3′-terminal region of the *BRCA1* gene (Vallon-Christersson *et al*., 2001). The transcriptional activity reflects a tumor-suppressing function of the BRCA1 protein.

Expression of β-galactosidase from the pSV plasmid can be transactivated both by the GAL4 domain of the pBIND and pBIND-BRCT. Upon GAL4 DNA sequence similarity as shown in Fig. 36, the GAL4 protein alone can stimulate the expression of reporter gene. However, the degree of transactivation was slightly higher by pBIND-BRCT than pBIND. This may imply that the transcriptional

transactivation activity of BRCT domain on fusion protein increases transcription of β-galactosidase gene-bearing pSV. The transcriptional transactivation activity of the pBIND-BRCT was dramatically reduced when platinated with cisplatin. Although the β-galactosidase activity was measured at 16 h after transfection, no more significantly increased expression was detected.

When platinated pSV is co-transfected with the pBIND or pBIND-BRCT, relatively lower expression of β-galactosidase was observed. The data from the cellular proficiency of DNA repair shows that over 80% of DNA-lesion was repaired after 8 h of cisplatin removal. The platinated DNA must be repaired prior to transcription. As a result, the transcription level of β-galactosidase expression was reduced from 2-2.5 folds to 1.3 folds in both plasmids. During repairing time, RNA polymerase II may be blocked at any lesions on DNA and transcription was terminated (Jung and Lippard, 2003; Jung and Lippard, 2006; Tornaletti *et al*., 2003).

A decrease in transcription level might directly or indirectly result in a deficiency of some components of the basal transcriptional machinery (Laine and Egly, 2006). The TBP, a TATA-box binding factor, inhibits a strong affinity for the "kink" induced by the cisplatin-DNA lesion (Coin *et al*., 1998; Jung *et al*., 2001). It is found sequestered at sites of DNA damage, and is therefore unavailable for transcription by RNA pol II (Vichi *et al*., 1997). Similary, cisplatin adducts inhibit rRNA synthesis by RNA pol I, by hijacking the human upstream binding transcription factor (hUBF) (Zhai *et al*., 1998) and reducing the amount of hUBF available to bind to the promoter. The loss of transcription factors may decrease transcription activity of the GAL4-BRCT protein on the pSV. In addition, loss of β-galactosidase expression was observed when co-transfectd platinated pBIND-BRCT with platinated pSV. It implies that the lack of cellular transcription factors could decrease the transcriptional transactivation activity of the pBIND-BRCT on the pSV.

Together, the data suggest that a repair-mediated transcription transactivation of cisplatin-damaged *BRCA1* appears to be associated with altered thermal stability and DNA conformation. It is likely that a significant decrease in the transcriptional transactivation of the drug-damaged *BRCA1* may be, in part, due to the preferential adduction of platinum atoms at the d(GpG) sequence of unique *Eco*0109I-cleaved site of the 696-bp fragment of the 3′-terminal region of *BRCA1*. Several translesion DNA polymerases have been shown to bypass Pt-GG intrastrand di-adducts (Bassett *et al*., 2003, 2004; Chaney *et al*., 2005). The efficiency and fidelity of bypass by these translesion polymerases is likely to affect the mutagenicity of cisplatin-DNA adducts. It has been reported that the mutation in human *H-ras* proto-oncogene induced by cisplatin is believed to initiate tumorgenesis or secondary cancer (Pillaire *et al*., 1994). Damaged *BRCA1*, if not properly repaired, may lead to serious consequences such as point mutations which have previously been examined in prokaryotic and eukaryotic system with the known mutation spectrum for cisplatin-DNA adducts (Brouwer *et al*., 1981; Burnouf *et al*., 1987), loss of transcriptional activation and repair and neoplastic transformation.

CHAPTER 6

CONCLUSION

The data presented herein revealed the effect of cisplatin on the specific *BRCA1* fragment of coding exons of 16-24. Changes in the mobility of cisplatin-damaged *BRCA1* fragment on agarose gel and polyacrylamide gel implied the structure perturbation of the drug on double helix structure. Predominant DNA lesion d(GpG) intrastrand crosslinks resisted the activities of restriction enzymes *Pvu*II at cisplatin concentration of 12.5 μM. However, interstrand crosslinks were also formed at lower concentration of 0.5 μM. These crosslinks affected the transition temperature of the *BRCA1* fragment in a biphasic fashion. The results suggested that predominant intrastrand crosslinks were formed at lower cisplatin concentrations, resulting in decreased DSC endotherms. Whereas, at higher cisplatin concentrations, interstrand and intermolecular crosslinks were also formed, leading to a slight increase in enthalpy and heat capacity. The energetic consequences of the crosslinks were most likely related to the structural perturbations of the specified *BRCA1* fragment.

The repair-proficiency of cellular DNA-damaged repair was determined in MCF7 cells. The genomic 3,426-bp *BRCA1* exon 11 fragment was used as template for QPCR assay. Cisplatin-modified genomic DNA was repaired with an initial low level of lesion removal during the first 4 h, however, more complete lesions were removed within 18 h. The cells were used for investigating the effect of cisplatin on the 696-bp *BRCA1* fused with eukaryotic expression vector pBIND in one hybrid assay. Co-transfection of pBIND-BRCT with reporter plasmid pG5Luc showed the transcriptional transactivation activity of BRCT domain on firefly luciferase gene. BRCT domain also enhanced transcription of *Renilla* luciferase within pBIND-BRCT.

Host cell reactivation of cisplatin-damaged *BRCA1* was determined on platinated pBIND-BRCT. The expression of platinated pBIND-BRCT decreased at

cisplatin concentration of 12.5 μM. Loss of transcription activation was found at higher concentration of cisplatin. Cisplatin also interfered transcriptional transactivation activity of the GAL4-BRCT on reporter plasmid pG5Luc. Platinated pBIND-BRCT decreased firefly luciferase expression at cisplatin concentration of 12.5 μM.

GAL4-BRCT protein can activate the transcription of other types of promoter and/or genes. β-galactocidase expression of the pSV was significantly increased in the presence of the pBIND or the pBIND-BRCT. The level of transcription is slightly higher when stimulated by pBIND-BRCT than parental pBIND plasmid. However, when pBIND-BRCT plasmid was platinated by cisplatin at concentration of 12.5 μM, the ability of pBIND-BRCT to enhance expression of β-galactosidase gene was significantly lost. pBIND and pBIND-BRCT also transactivated platinated pSV-β-galactosidase plasmid. However, no significant difference in transcriptional transactivation can be observed. β-galactosidase expression was significantly diminished when co-transfecting platinated pSV-β-galactosidase with platinated pBIND-BRCT. It is implied that blockage of transcription machinery reduced transcription ability on both platinated plasmids. A decrease in transcriptional transactivation of platinated *BRCA1* may be, in part, due to the inhibition of function of RNA polymerase and limitation of transcription initiation of involved transcription factors through "transcription factor hijacking" in both transcription and repair machineries required for repair activity in the presence of cisplatin lesions.

REFERENCES

Abbott, D.W., Thompson, M.E., Robinson-Benion, C., Tomlinson, G., Jensen, R.A. and Holt, J.T. 1999. BRCA1 expression restores radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. J. Biol. Chem. 274:18808–18812.

Abel, K.J., Xu, J., Yin, G.Y., Lyons, R.H., Meisler, M.H. and Weber, B.L. 1995. Mouse Brca1: localization sequence analysis and identification of evolutionarily conserved domains. Hum. Mol. Genet. 4: 2265-2273.

Abeliovich, D., Kaduri, L., Lerer, I., Weinberg, N., Amir, G., Sagi, M., Zlotogora, J., Heching, N. and Peretz, T. 1997. The founder mutations 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2 appear in 60% of ovarian cancer and 30% of early-onset breast cancer patients among Ashkenazi women. Am. J. Hum. Genet. 60:505-514.

Adimoolam, S. and Ford, J.M. 2002. p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proc. Natl Acad. Sci. USA. 99:12985–12990.

Aida, H., Takakuwa, K., Nagata, H., Tsuneki, I., Takano, M., Tsuji, S., Takahashi, T., Sonoda, T., Hatae, M., Takahashi, K., Hasegawa, K., Mizunuma, H., Toyoda, N., Kamata, H., Torii, Y., Saito, N., Tanaka, K., Yakushiji, M., Araki, T. and Tanaka, K. 1998. Clinical features of ovarian cancer in Japanese women with germ-line mutations of BRCA1. Clin. Cancer Res. 4:235-240.

Altiok, S., Batt, D., Altiok, N., Papautsky, A., Downward, J., Roberts, T.M. and Avraham, H. 1999. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells. J. Biol. Chem. 274:32274-32278.

Amundson, S.A., Patterson, A., Do, K.T. and Fornace, A.J. Jr. 2002. A nucleotide excision repair master-switch: p53 regulated coordinate induction of global genomic repair genes. Cancer Biol. Ther. 1:145–149.

Anderson, S.F., Schlegel, B.P., Nakajima, T., Wolpin, E.S. and Parvin, J.D. 1998. Brca1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. Nature Genet. 19:254-256.

Anderson, L., Henderson, C. and Adachi, Y. 2001. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol Cell Biol. 21:1719-1729.

Antoniou, A., Pharoah, P.D., Narod, S., Risch, H.A., Eyfjord, J.E., Hopper, J.L., Loman, N., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D.M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjakoski, K., Kallioniemi, O.P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D.G. and Easton, D.F. 2003. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am. J. Hum. Genet. 72:1117–1130.

Antoniou, A.C., Pharoah, P.D., Narod, S., Risch, H.A., Eyfjord, J.E., Hopper, J.L., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D.M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjakoski, K., Kallioniemi, O.P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D.G. and Easton, D.F. 2005. Breast and ovarian cancer risks to carriers of the BRCA1 5382insC and 185delAG and BRCA2 6174delT mutations: a combined analysis of 22 population based studies. J. Med. Genet. 42:602-603.

Aprelikova, O.N., Fang, B.S., Meissner, E.G., Cotter, S., Campbell, M., Kuthiala, A., Bessho, M., Jensen, R.A. and Liu, E.T. 1999. BRCA1-associated growth arrest is RB-dependent. Proc. Natl. Acad. Sci. USA. 96:11866-11871.

Araújo, S.J., Tirode, F., Coin, F., Pospiech, H., Syväoja, J.E., Stucki, M., Hübscher, U., Egly, J.M. and Wood, R.D. 2000. Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. Genes. Dev. 4:349-359.

Arun, B., Kilic, G., Yen, C., Foster, B., Yardley, D., Gaynor, R. and Ashfaq, R. 2003. Correlation of Bcl-2 and p53 expression in primary breast tumors and corresponding metastatic lymph nodes. Cancer. 98:2554-2559.

Au, W.W. and Henderson, B.R. 2005. The BRCA1 RING and BRCT domains cooperate in targeting BRCA1 to ionizing radiation-induced nuclear foci. J. Biol. Chem. 280:6993-7001.

Baer. R. 2001. With the ends in sight: images from the BRCA1 tumor suppressor Nature Structural Biology. 8: 822 – 824.

Baldeyron, C., Jacquemin, E., Smith, J., Jacquemont, C., De Oliveira, I., Gad, S., Feunteun, J., Stoppa-Lyonnet, D. and Papadopoulo, D. 2002. A single mutated BRCA1 allele leads to impaired fidelity of double strand break end-joining. Oncogene. 21:1401–1410.

Barlow, P.N., Luisi, B., Milner, A., Elliott, M. and Everett, R. 1994. Structure of the C3HC4 domain by 1H-nuclear magnetic resonance spectroscopy. A new structural class of zinc-finger. J. Mol. Biol. 237: 201-211.

Bassett, E., King, N.M., Bryant, M.F., Hector, S., Pendyala, L., Chaney, S.G. and Cordeiro-Stone, M. 2004. The role of DNA polymerase eta in translesion synthesis past platinum-DNA adducts in human fibroblasts. Cancer Res. 64:6469-6475.

Bassett, E., Vaisman, A., Havener, J.M., Masutani, C., Hanaoka, F., Chaney, S.G. 2003. Efficiency of extension of mismatched primer termini across from cisplatin and oxaliplatin adducts by human DNA polymerases beta and eta in vitro. Biochemistry. 42:14197-14120.

Bellon, S.F., Coleman, J.H. and Lippard, S.J. 1991. DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug cis-diamminedichloroplatinum(II). Biochemistry. 30: 8026-8035.

Bellon, S.F., Rodgers, K.K., Schatz, D.G., Coleman, J.E. and Steitz, T.A. 1997. Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. Nature Struct. Biol. 4: 586-591.

Bennett, L.M., Haugen-Strano, A., Cochran, C., Brownlee, H.A., Fiedorek, F.T. Jr. and Wiseman, R.W. 1995. Isolation of the mouse homologue of BRCA1 and genetic mapping to mouse chromosome 11. Genomics. 29:576-581.

Bernstein, C., Bernstein, H., Payne, C.M. and Garewal, H. 2002. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat. Res. 511:145-78.

Berchuck, A., Heron, K.A., Carney, M.E., Lancaster, J.M., Fraser, E.G., Vinson, V.L., Deffenbaugh, A.M., Miron, A., Marks, J.R., Futreal, P.A. and Frank, T.S. 1998. Frequency of germline and somatic BRCA1 mutations in ovarian cancer. Clin. Cancer Res. 4:2433-2437.

Bhattacharyya, A., Ear, U.S., Koller, B.H., Weichselbaum, R.R. and Bishop, D.K. 2000. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J. Biol. Chem. 275:23899-23903.

Biedermann, K.A., Sun, J.R., Giaccia, A.J., Tosto, L.M. and Brown, J.M. 1991. scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc. Natl. Acad. Sci. USA. 88:1394–1397.

Blackshear, P.E., Goldsworthy, S.M., Foley, J.F., McAllister, K.A., Bennett, L.M., Collins, N.K., Bunch, D.O., Brown, P., Wiseman, R.W. and Davis, B.J. 1998. Brca1 and Brca2 expression patterns in mitotic and meiotic cells of mice. Oncogene. 16:61-68.

Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F. and Koonin, E.V. 1997. A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. 11:68-76.

Bose, S., Crane, A., Hibshoosh, H., Mansukhani, M., Sandweis, L. and Parsons, R. 2002. Reduced expression of PTEN correlates with breast cancer progression. Hum. Pathol. 33:405–409.

Breast Cancer Information Core, 2007. *http://research.nhgri.nih.gov/bic/*

Breslauer, K.J., Frank, R., Blocker, H. and Markey, L.A. 1986. Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA. 83:3746-3750.

Breslauer, K.J., Freire, E. and Staume, M. 1992. Calorimetry: a tool for DNA and ligand-DNA studies. Methods. Enzymol. 211:533-567.

Brouwer, J., van de Putte, P., Fichtinger-Schepman, A.M. and Reedijk, J. 1981. Base-pair substitution hotspots in GAG and GCG nucleotide sequences in Escherichia coli K-12 induced by cis-diamminedichloroplatinum (II). Proc. Natl. Acad. Sci. USA.78:7010-7014.

Brzovic, P.S., Meza, J., King, M.C. and Klevit, R.E. 1998. The cancer-predisposing mutation C61G disrupts homodimer formation in the NH2-terminal BRCA1 RING finger domain. J. Biol. Chem. 273:7795-7799.

Brzovic, P.S., Meza, J.E., King, M.C. and Klevit, R.E. 2001. BRCA1 RING domain cancer-predisposing mutations. Structural consequences and effects on protein-protein interactions. J. Biol. Chem. 276:41399-41406.

Burnouf, D., Duane, M. and Fuchs, R.P. 1987. Spectrum of cisplatin-induced mutations in Escherichia coli, Proc. Natl. Acad. Sci. USA. 84:3758-3762.

Burnouf, D., Gauthier, C., Chottard, J.C. and Fuchs, R.P. 1990. Single d(ApG)/cis-diamminedichloroplatinum(II) adduct-induced mutagenesis in Escherichia coli. Proc. Natl. Acad. Sci. USA. 87:6087-6091.

Calsou, P., Barret, J.M., Cros, S. and Salles, B. 1993. DNA excision-repair synthesis is enhanced in a murine leukemia L1210 cell line resistant to cisplatin. Eur. J. Biochem. 211:403-409.

Calvert, A.H., Harland, S.J., Newell, D.R., Siddik, Z.H., Jones, A.C., McElwain, T.J., Raju, S., Wiltshaw, E., Smith, I.E., Baker, J.M., Peckham, M.J. and Harrap, K.R. 1982. Early clinical studies with cis-diammine-1,1-cyclobutane dicarboxylate platinum II. Cancer. Chemother. Pharmacol. 9:140-147.

Callebaut, I. and Mornon, J.P. 1997. From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. 400:25-30.

Cariello, N.F., Swenberg, J.A., De Bellis, A. and Skopek, T.R. 1991. Analysis of mutations using PCR and denaturing gradient gel electrophoresis. Environ. Mol. Mutagen. 18:249-254.

Cariello, N.F., Swenberg, J.A. and Skopek, T.R. 1992. In vitro mutational specificity of cisplatin in the human hypoxanthine guanine phosphoribosyltransferase gene, Cancer Res. 52:2866-2873.

Cate, J.H., Hanna, R.L. and Doudna, J.A. 1997. A magnesium ion core at the heart of a ribozyme domain. Nat. Struct. Biol. 4:553-558.

Chai, Y.L., Cui, J., Shao, N., Shyam, E., Reddy, P. and Rao, V.N. 1999. The second BRCT domain of Brca1 proteins interacts with p53 and stimulates transcription from the p21WAF1/CIP1 promotor. Oncogene. 18:263-268.

Chamberlain, J.S., Boehnke, M., Frank, T.S., Kiousis, S., Xu, J., Guo, S.W., Hauser, E.R., Norum, R.A., Helmbold, E.A., Markel, D.S., et al. 1993. BRCA1 maps proximal to D17S579 on chromosome 17q21 by genetic analysis. Am. J. Hum. Genet. 52: 792-798.

Chaney, S.G., Campbell, S.L., Bassett, E., Wu, Y. 2005. Recognition and processing of cisplatin- and oxaliplatin-DNA adducts. Crit. Rev. Oncol. Hematol. 53:3-11.

Chen, Y., Chen, C.F., Riley, D.J., Allred, D.C., Chen, P.L., Von Hoff, D., Osborne, C.K. and Lee, W.H. 1995. Aberrant subcellular localization of BRCA1 in breast cancer. Science. 270: 789-791.

Chen, Y., Farmer, A.A., Chen, C.F., Jones, D.C., Chen, P.L. and Lee, W.H. 1996a. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. Cancer Res. 56: 3168-3172.

Chen, K.S., Shepel, L.A., Haag, J.D., Heil, G.M. and Gould, M.N. 1996b. Cloning, genetic mapping and expression studies of the rat Brca1 gene. Carcinogenesis. 17:1561-1566.

Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M.and Scully R. 1998. Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. Mol. Cell. 2: 317-328.

Chen, J. 2000. Ataxia telangiectasia-related protein is involved in the phosphorylation of BRCA1 following deoxyribonucleic acid damage. Cancer Res. 60:5037-5039.

Chen, Y.W., Cleaver, J.E., Hanaoka, F., Chang, C.F. and Chou, K.M. 2006. A novel role of DNA polymerase eta in modulating cellular sensitivity to chemotherapeutic agents. Mol. Cancer Res. 4:257-265

Chiu, T.K. and Dickerson, R.E. 2000. 1°A crystal structures of B-DNA reveal sequence-specific binding and groove-specific bending of DNA by magnesium and calcium. J. Mol. Biol. 301:915-945.

Chapman, M.S. and Verma, I.M. 1996. Transcriptional activation by BRCA1. Nature. 382:678-679.

Ciccarelli, R.B., Solomon, M.J., Varshavsky, A. and Lippard, S.J. 1985. In vivo effects of cis- and trans-diamminedichloroplatinum(II) on SV40 chromosomes: differential repair, DNA-protein cross-linking, and inhibition of replication. Biochemistry. 24:7533-7540.

Clement, R.M., Sturm, J. and Daune, M.P. 1973. Interaction of metallic cations with DNA. VI. Specific binding of Mg^{++} and Mn^{++}. Biopolymers. 12:405-421.

Coene, E.D., Hollinshead, M.S., Waeytens, A.A., Schelfhout, V.R., Eechaute, W.P., Shaw, M.K., Van Oostveldt, P.M. and Vaux, D.J. 2005. Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. Mol. Biol. Cell 16:997-1010.

Coin, F., Frit, P., Viollet, B., Salles, B., Egly, J.M. 1998. TATA binding protein discriminates between different lesions on DNA, resulting in a transcription decrease. Mol Cell Biol. 18:3907-3914.

Comess, K.M., Burstyn, J.N., Essigmann, J.M. and Lippard, S.J. 1992. Replication inhibition and translesion synthesis on templates containing site-specifically placed cis-diamminedichloroplatinum(II) DNA adducts. Biochemistry. 31:3975-3990.

Corda, Y., Job, C., Anin, M.F., Leng, M. and Job, D. 1991. Transcription by eukaryotic and prokaryotic RNA polymerase of DNA modified at a d(GG) or a d(AG) site

by the antitumor drug cis-diamminedichloroplatinum (II). Biochemistry. 30: 222-230.

Corda, Y., Anin, M.F., Leng, M. and Job, D. 1992. RNA polymerases react differently at d(ApG) and d(GpG) adducts in DNA modified by cis-diamminedichloroplatinum(II). Biochemistry. 31:1904-1908.

Corda, Y., Job, C., Anin, M.F., Leng, M. and Job, D. 1993. Spectrum of DNA-platinum adduct recognition by prokaryotic and eukaryotic DNA dependent RNA polymerase. Biochemistry. 32:8582-8588.

Cortez, D., Wang, Y., Qin, J. and Elledge, S.J. 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks, Science. 286:1162-1166.

Couch, F.J., DeShano, M.L., Blackwood, M.A., Calzone, K., Stopfer, J., Campeau, L., Ganguly, A., Rebbeck, T. and Weber, B.L. BRCA1 mutations in women attending clinics that evaluate the risk of breast cancer. N. Engl. J. Med. 336:1409-1415.

Cui, J.Q., Shao, N., Chai, Y., Wang, H., Reddy, E.S. and Rao, V.N. 1998. BRCA1 splice variants BRCA1a and BRCA1b associate with CBP co-activator. Oncol. Rep. 5:591-595.

Cullinane, C., Mazur, S.J., Essigmann, J.M. Phillips, D.R. and Bohr, V.A. 1999. Inhibition of RNA Polymerase II Transcription in Human Cell Extracts by Cisplatin DNA Damage. Biochemistry. 38: 6204 -6212.

Dabholkar, M., Eastman, A. and Reed, E. 1990. Host-cell reactivation of cisplatin-damaged pRSVcat in a human lymphoid cell line. Carcinogenesis. 11:1761-1764.

Davidoff, A.M., Kerns, B.J., Iglehart, J.D. and Marks, J.R. 1991. Maintenance of p53 alterations throughout breast cancer progression. Cancer Res. 51:2605-2610.

Debra, G., Leonard, B., Lois, B., Travis, Kathakali Addya, Graca M. Dores, Eric J. Holowaty, Kjell Bergfeldt, David Malkin, Betsy A. Kohler, Charles F. Lynch, Tom Wiklund, Marilyn Stovall, Per Hall, Eero Pukkala, Diana J. Slater and Carolyn A. Felix. 2002. *p53* Mutations in leukemia and myelodysplastic syndrome after ovarian cancer clinical. Cancer Res. 8:973-985.

Dempke, W., Voigt, W., Grothey, A. and Schmoll, H.J. 1999. Preferential repair of the N-ras gene in K 562 cells after exposure to cisplatin. Anticancer Drugs. 10: 545-549.

Dijt, F.J., Fichtinger-Schepman, A.M., Berends, F. and Reedijk, J. 1988. Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. Cancer Res. 48:6058-6062.

Donahue, B.A., Augot, M., Bellon, S.F., Treiber, D.K., Toney, J.H., Lippard, S.J. and Essigmann, J.M. 1990. Characterization of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. Biochemistry. 29:5872-5880.

Duguid, J.G., Bloomfield, V.A., Benevides, J.M. and Thomas, Jr. G.J. 1993. Raman spectroscopy of DNA-metal Complexes. I. Interactions and conformational effects of the divalent cations: Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Pd^{2+}, and Cd^{2+}. Biophys. J. 65:1916-1928.

Duguid, J.G., Bloomfield, V.A., Benevides, J. and Thomas, Jr. G.J. 1995. Raman spectroscopy of DNA-metal complexes. II. The thermal denaturation of DNA in the presence of: Sr^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+}. Biophys. J. 65:2623-2641.

Duguid, J.G. and Bloomfield, V.A. 1996. Electrostatic effects on the stability of condensed DNA in the present of divalent cations. Biophys. J. 70: 2838-2846.

Eastman, A. 1986. Reevaluation of interaction of cis-dichloro (ethylenediamine) platinum (II) with DNA. Biochemistry. 25:3912-3915.

Eastman, A. and Schulte, N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). Biochemistry. 27:4730-4734.

Easton, D.F., Bishop, D.T., Ford, D. and Crockford, G.P. 1993. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. Am. J. Hum. Genet. 52:678-701.

Eichhorn, G.L. 1962. Metal ions as stabilizers or destabilizers of the deoxyribonucleic acid structure. Nature. 194:474-475.

Eisinger, F., Stoppa-Lyonnet, D., Longy, M., Kerangueven, F., Noguchi, T., Bailly, C., Vincent-Salomon, A., Jacquemier, J., Birnbaum, D. and Sobol, H. Germ line mutation at BRCA1 affects the histoprognostic grade in hereditary breast cancer. Cancer Res. 56:471-474.

Fan, S., Smith, M.L., Rivet, D.J., Duba, D., Zhan, Q., Kohn, K.W., Fornace, A.J. and O'Connor, P.M. 1995. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res. 55:1649-1654.

Fan, S., Wang, J., Yuan, R., Ma, Y., Meng, Q., Erdos, M.R., Pestell, R.G., Yuan, F., Auborn, K.J., Goldberg, I.D. and Rosen, E.M. 1999. BRCA1 inhibition of estrogen receptor signaling in transfected cells. Science. 284: 1354-1356.

Fan, S., Ma, Y.X., Wang, C., Yuan, R.Q., Meng, Q., Wang, J.A., Erdos, M., Goldberg, I.D., Webb, P., Kushner, P.J., Pestell, R.G., Rosen, E.M. 2001. Role of direct interaction in BRCA1 inhibition of estrogen receptor activity. Oncogene. 20:77-87.

Fan, S., Ma, Y.X., Wang, C., Yuan, R.Q., Meng, Q., Wang, J.A., Erdos, M., Goldberg, I.D., Webb, P., Kushner, P.J., Pestell, R.G. and Rosen, E.M. 2002. p300 Modulates the BRCA1 inhibition of estrogen receptor activity. Cancer Res. 62:141-151.

Feng, Z., Kachnic, L., Zhang, J., Powell, S.N., Xia, F. 2004. DNA damage induces p53-dependent BRCA1 nuclear export. J. Biol. Chem. 279:28574-28584.

Feunteun, J., Narod, S.A., Lynch, H.T., Watson, P., Conway, T., Lynch, J., Parboosingh, J., O'Connell, P., White, R. and Lenoir, G.M. 1993. A bBreast-ovarian cancer susceptibility gene maps to chromosome 17q21. Am. J. Hum. Genet. 52:736-742.

Fichtinger-Schepman, A.M., van der Veer, J.L., den Hartog, J.H., Lohman, P.H. and Reedijk, J. 1985. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. Biochemistry. 24: 707-713.

Fichtinger-Schepman, A.M., van Oosterom, A.T., Lohman, P.H. and Berends, F. 1987a. Interindividual human variation in cisplatinum sensitivity, predictable in an in vitro assay. Mutat. Res. 190:59-62.

Fichtinger-Schepman, A.M., van Oosterom, A.T., Lohman, P.H. and Berends, F. 1987b. cis-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of cis-diamminedichloroplatinum(II). Cancer Res. 47:3000-3004.

Fink, D., Nebel, S., Aebi, S., Nehme, A. and Howell, S.B. 1997. Loss of DNA mismatch repair due to knockout of MSH2 or PMS2 results in resistance to cisplatin and carboplatin. Intl. J. Oncol. 11:539-542.

Fodor, F.H., Weston, A., Bleiweiss, I.J., McCurdy, L.D., Walsh, M.M., Tartter, P.I., Brower, S.T. and Eng, C.M. 1998. Frequency and carrier risk associated with common BRCA1 and BRCA2 mutations in Ashkenazi Jewish breast cancer patients. Am. J. Hum. Genet. 63:45-51.

Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A. and Goldgar, D.E. 1994. Risks of cancer in BRCA1-mutation carriers, Breast Cancer Linkage Consortium. Lancet. 343:692–695.

Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M.D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T.R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B.A., Gayther, S.A. and Zelada-Hedman, M. 1998. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am. J. Hum. Genet. 62:676–689.

Foulkes, W.D., Wong, N., Brunet, J.S., Bégin, L.R., Zhang, J.C., Martinez, J.J., Rozen, F., Tonin, P.N., Narod, S.A., Karp, S.E., Pollak, M.N. 1997. Germ-line BRCA1 mutation is an adverse prognostic factor in Ashkenazi Jewish women with breast cancer. Clin. Cancer Res. 3:2465-2469.

Frei, E. 3rd, Cucchi, C.A., Rosowsky, A., Tantravahi, R., Bernal, S., Ervin, T.J., Ruprecht, R.M. and Haseltine, W.A. 1985. Alkylating agent resistance: in vitro studies with human cell lines. Proc. Natl. Acad. Sci. USA. 82:2158-2162.

Friedberg, E.C., Feaver, W.J. and Gerlach, V.L. 2000. The many faces of DNA polymerases: strategies for mutagenesis and for mutational avoidance. Proc. Natl. Acad. Sci. USA. 97:5681-5683.

Furuya, K., Poitelea, M., Guo, L., Caspari, T. and Carr, A.M. 2004. Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of $Rad4^{TOPBP1}$. Genes Dev. 18: 1154–1164.

Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z., Soderkvist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J., Marks, J., Ballinger, D., Barrett, J.C., Skolnick, M., Kamb, A. and Wiseman, R. 1994. BRCA1 mutations in primary breast and ovarian carcinomas. Science. 266:120-122.

Ganguly, A., Leahy, K., Marshall, A.M., Dhulipala, R., Godmilow, L. and Ganguly, T. Genetic testing for breast cancer susceptibility: frequentcy of BRCA1 and BRCA2 mutations. Genet. Test. 1:85-90.

Gatei, M., Scott, S.P., Filippovitch, I., Soronika, N., Lavin, M.F., Weber, B. and Khanna, K.K. 2000. Role for ATM in DNA damage-induced phosphorylation of BRCA1. Cancer Res. 60:3299-3304.

Genetic Testing for BRCA1 and BRCA2: It's Your Choice Cancer Facts. 2002. *http://www.cancer.gov/cancertopics/factsheet/Risk/BRCA*

Geoffroy-Perez, B., Janin, N., Ossian, K., Lauge, A., Croquette, M.F., Griscelli, C., Debre, M., Bressac-De-Paillerets, B., Aurias, A., Stoppa-Lyonnet, D. and Andrieu, N. 2001. Cancer risk in heterozygotes for ataxia-telangiectasia. Int. J. Cancer. 93:288–293.

Gerlach, V.L., Feaver, W.J., Fischhaber, P.L. and Friedberg, E.C. 2001. Purification and characterization of pol kappa, a DNA polymerase encoded by the human DINB1 gene. J. Biol. Chem. 276:92-98.

Goobourn, S. 1996 Eukaryotic gene transcription. Oxford university press oxford. Newyork. USA.

Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K. and Koller, B.H. 1996. Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. Nat. Genet. 12:191-194.

Gowen, L.C., Avrutskaya, A.V., Latour, A.M., Koller, B.H. and Leadon, S.A. 1998. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science. 281:1009–1012.

Grimaldi, K.A., Bingham, J.P., Souhami, R.L. and Hartley, J.A. 1994. DNA damage by anticancer agents and its repair: mapping in cells at the subgene level with quantitative polymerase chain reaction. Anal. Biochem. 222:236-242.

Gudas, J.M., Nguyen, H., Li, T. and Cowan, K.H. 1995. Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res. 55:4561-4565.

Gudas, J.M., Li, T., Nguyen, H., Jensen, D., Rauscher, F.J. 3rd. and Cowan, K.H. 1996. Cell cycle regulation of BRCA1 messenger RNA in human breast epithelial cells. Cell Growth Differ. 7:717-723.

Haile, D.T. and Parvin, J.D. 1999. Activation of Transcription *in vitro* by the BRCA1 Carboxyl-terminal Domain. J. Biol. Chem. 274:2113 –2117.

Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., Firpo, E., Hui, C.C., Roberts, J., Rossant, J. and Mak, T.W. 1996. The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. Cell. 85:1009-1023.

Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B. and King, M.C. 1990. Linkage of early-onset familial breast cancer to chromosome 17q21. Science. 250:1684-1689.

Harkin, D.P., Bean, J.M., Miklos, D., Song, Y.H., Truong, V.B., Englert, C., Christians, F.C., Ellisen, L.W., Maheswaran, S., Oliner, J.D. and Haber, D.A. 1999. Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell. 97:575-586.

Hartman, A.R., Ford, J.M. 2002. BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. Nat. Genet. 32:180-184.

Hartman, A.R. and Ford, J.M. 2003. BRCA1 and p53: compensation roles in DNA repair. J. Mol. Med. 81:700-707.

Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W. and Friend, S.H. 1997. Integrating genetic approaches into the discovery of anticancer drugs. Science. 278:1064–1068.

Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H. and Ohta, T. 2001. The RING heterodimer BRCA1-BARD1 is a ubiquitin

ligase inactivated by a breast cancer-derived mutation. J. Biol. Chem. 276:14537-14540.

Hansson, J. and Wood, R.D. 1989. Repair synthesis by human cell extracts in DNA damaged by cis- and trans-diamminedichloroplatinum(II). Nucleic Acids Res. 17:8073-8091.

Hayes, F., Cayanan, C., Barilla, D. and Monteiro, A.N. 2000. Functional assay for BRCA1: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation. Cancer Res. 60:2411-2418.

Hochegger, H., Sonoda, E. and Takeda, S. 2004. Post-replication repair in DT40 cells: translesion polymerases versus recombinases. Bio. Essays. 26:151–158.

Hodgson, S.V., Heap, E., Cameron, J., Ellis, D., Mathew, C.G., Eeles, R.A., Solomon, E. and Lewis, C.M. 1999. C.M. Risk factors for detecting germline BRCA1 and BRCA2 founder mutations in Ashkenazi Jewish women with breast or ovarian cancer. J. Med. Genet. 36:369-373.

Hofr, C. and Brabec, V. 2001. Thermal and thermodynamic properties of duplex DNA containing site-specific interstrand cross-link of antitumor cisplatin or its clinically ineffective trans isomer. J. Biol. Chem. 276:9655-9661.

Hofr, C., Farrell, N. and Brabec, V. 2001. Thermodynamic properties of duplex DNA containing a site-specific d(GpG) intrastrand crosslink formed by an antitumor dinuclear platinum complex. Nucleic Acids Res. 29:2034-2040.

Hoffmann, J.S., Pillaire, M.J., Garcia-Estefania, D., Lapalu, S. and Villani, G. 1996. In vitro bypass replication of the cisplatin-d(GpG) lesion by calf thymus DNA polymerase beta and human immunodeficiency virus type I reverse transcriptase is highly mutagenic. J. Biol. Chem. 271:15386-15392.

Hoffmann, J.S., Pillaire, M.J., Maga, G., Podust, V., Hubscher, U. and Villani G. 1995. DNA polymerase beta bypasses in vitro a single d(GpG)-cisplatin adduct

placed on codon 13 of the HRAS gene. Proc. Natl. Acad. Sci. USA. 92:5356-5356.

Holt, J.T., Thompson, M.E., Szabo, C., Robinson-Benion, C., Arteaga, C.L., King, M.C. and Jensen, R.A. 1996. Growth retardation and tumour inhibition by BRCA1. Nat. Genet. 12:298-302.

Hu, Y.F., Miyake, T., Ye, Q. and Li, R. 2000. Characterization of a Novel Trans-Activation Domain of BRCA1 That Functions in Concert with the BRCA1 C-terminal (BRCT) Domain. J. Biol. Chem. 275:40910–40915.

Huber, L.J., Yang, T.W., Sarkisian, C.J., Master, S.R., Deng, C.X. and Chodosh, L.A. 2001. Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci. Mol. Cell Biol. 21:4005–4015.

Humphrey, J.S., Salim, A., Erdos, M.R., Collins, F.S., Brody, L.C. and Klausner, R.D. 1997. Human BRCA1 inhibits growth in yeast: potential use in diagnostic testing. Proc. Natl. Acad. Sci. USA. 94:5820-5825.

Husain, A., He, G., Venkatraman, E.S. and Spriggs, D.R. 1998. BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II). Cancer Res. 58:1120-1123.

Huyton, T., Bates, P.A., Zhang, X., Sternberg, M.J. and Freemont, P.S. 2000. Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. Mutat. Res. 460:319-332.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. 1999. Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc. Natl Acad. Sci. USA. 96:424–428.

Iacopetta, B., Russo, A., Bazan, V., Dardanoni, G., Gebbia, N., Soussi, T., Kerr, D., Elsaleh, H., Soong, R., Kandioler, D., Janschek, E., Kappel, S., Lung, M., Leung, C.S., Ko, J.M., Yuen, S., Ho, J., Leung, S.Y., Crapez, E., Duffour, J.,

Ychou, M., Leahy, D.T., O'Donoghue, D.P., Agnese, V., Cascio, S., Di Fede, G., Chieco-Bianchi, L., Bertorelle, R., Belluco, C., Giaretti, W., Castagnola, P., Ricevuto, E., Ficorella, C., Bosari, S., Arizzi, C.D., Miyaki, M., Onda, M., Kampman, E., Diergaarde, B., Royds, J., Lothe, R.A., Diep, C.B., Meling, G.I., Ostrowski, J., Trzeciak, L., Guzinska-Ustymowicz, K., Zalewski, B., Capella, G.M., Moreno, V., Peinado, M.A., Lonnroth, C., Lundholm, K., Sun, X.F., Jansson, A., Bouzourene, H., Hsieh, L.L., Tang, R., Smith, D.R., Allen-Mersh, T.G., Khan, Z.A., Shorthouse, A.J., Silverman, M.L., Kato, S. and Ishioka, C. 2006. TP53-CRC Collaborative Group. Functional categories of TP53 mutation in colorectal cancer: results of an International Collaborative Study. Ann. Oncol. 17:842-847.

Jakupec, M.A., Galanski, M., Keppler, B.K. 2003. Tumour-inhibiting platinum complexes--state of the art and future perspectives. Rev. Physiol. Biochem. Pharmacol. 146:1-54.

Jin, Y., Xu, X.L., Yang, M.C., Wei, F., Ayi, T.C., Bowcock, A.M. and Baer, R. 1997. Cell cycle-dependent colocalization of BARD1 and BRCA1 proteins in discrete nuclear domains. Proc. Natl. Acad. Sci. USA. 94:12075-12080.

Johnson, N.P., Hoeschele, J.D. and Rahn, R.O. 1980a. Kinetic analysis of the in vitro binding of radioactive cis- and trans-dichloroamminoplatinum (II) to DNA. Chem. Biol. Interact. 30:151-169.

Johnson, N.P., Hoeschele, J.D., Rahn, R.O., O'Neill, J.P. and Hsie, A.W. 1980b. Mutagenicity, cytotoxicity, and DNA binding of platinum (II)-chloroammines in Chinese hamster ovary cells. Cancer Res. 40:1463-1468.

Johnson, R.D., Liu, N. and Jasin, M. 1999. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature. 401:397-399.

Johnson, N.C. and Kruk, P.A. 2002. BRCA1 Zinc RING Finger Domain Disruption Alters Caspase Response in Ovarian Surface Epithelial Cells. Cancer Cell Int. 2:7.

Jordan, P. and Carmo-Fonseca, M. 1998. Cisplatin inhibits synthesis of ribosomal RNA *in vivo*. Nucleic Acids Res. 26:2831-2836.

Jung, Y. and Lippard, S.J. 2003. Multiple states of stalled T7 RNA polymerase at DNA lesions generated by platinum anticancer agents. J. Biol. Chem. 278:52084-52092.

Jung, Y. and Lippard, S.J. 2006. RNA polymerase II blockage by cisplatin-damaged DNA. Stability and polyubiquitylation of stalled polymerase. J. Biol. Chem. 281:1361-1370.

Kalinowski, D.P., Illenye, S. and Van Houten, B. 1992. Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. Nucleic Acids Res. 20:3485-3494.

Kashani-Sabet, M., Lu, Y., Leong, L., Haedicke, K. and Scanlon, K.J. 1990. Differential oncogene amplification in tumor cells from a patient treated with cisplatin and 5-fluorouracil. Eur. J. Cancer. 26:383-390.

Kim, J.E., Billadeau, D.D. and Chen, J. 2005. The tandem BRCT domains of Ect2 are required for both negative and positive regulation of Ect2 in cytokinesis. J. Biol. Chem. 280:5733-5739.

King, M-C., Marks, J.H. and Mandell, J.B. 2003. Breast and ovarian cancer risk due to inherited mutations in *BRCA1* and *BRCA2*. Science. 302:643-646.

Kleiman, F.E. and Manley, J.L. 1999. Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50. Science. 285:1576-1579.

Knudson, A. 1971. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA. 68:820-823.

Krum, S.A., Miranda, G.A., Lin, C. and Lane, T.F. 2003. BRCA1 associates with processive RNA polymerase II. J. Biol. Chem. 278:52012-52020.

Krum, S.A. Womack, J.E, and Lane, T.F. 2003. Bovine BRCA1 shows classic responses to genotoxic stress but low in vitro transcriptional activation activity. Oncogene. 22:6032-6044.

Laine, J.P. and Egly, J.M. 2006. When transcription and repair meet: a complex system. Trends Genet. 22:430-436.

Langlais, M., Tajmir-Riahi, H.A. and Savoie, R. 1990. Raman spectroscopic study of the effects of Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cd^{2+} ions on calf thymus DNA: Binding sites and conformation changes. Biopolymers. 30:743-752.

Larson, J.S., Tonkinson, J.L. and Lai, M.T. 1997. A BRCA1 mutant alters G2-M cell cycle control in human mammary epithelial cells. Cancer Res. 57:3351-3355.

Le, S., Moore, J.K., Haber, J.E. and Greider, C.W. 1999. RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. Genetics. 152:143-152.

Li, S., Chen, P.L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C.K., Sharp, Z.D. and Lee, W.H. 1999. Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. J. Biol. Chem. 274:11334-11338.

Li, H., Lee, T.H. and Avraham, H. 2002. A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer. J. Biol. Chem. 277:20965-20973.

Liang, F., Han, M., Romanienko, P.J. and Jasin, M. 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc. Natl. Acad. Sci. USA. 95:5172-5177.

Lin, X., Trang, J., Okuda, T. and Howell, S.B. 2006. DNA polymerase zeta accounts for the reduced cytotoxicity and enhanced mutagenicity of cisplatin in human

colon carcinoma cells that have lost DNA mismatch repair. Clin. Cancer Res. 12:563-568.

Liu, C.Y., Flesken-Nikitin, A., Li, S., Zeng, Y. and Lee, W.H. 1996. Inactivation of the mouse Brca1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. Genes. Dev. 10:1835-1843.

Loehrer and Einhorn, 1984 P.J. Loehrer and L.H. Einhorn, Drug five years later. Cisplatin, Annals of Internal Medicine 100 (1984), pp. 704–713.

Lu, K.H., Cramer, D.W., Muto, M.G., Li, E.Y., Niloff, J. and Mok, S.C. 1999. A population-based study of BRCA1 and BRCA2 mutations in Jewish women with epithelial ovarian cancer. Obstet. Gynecol. 93:34-37.

Lyakhovich, A. and Shekhar, MP. 2004. RAD6B overexpression confers chemoresistance: RAD6 expression during cell cycle and its redistribution to chromatin during DNA damage-induced response. Oncogene. 15:23:3097-3106.

Lynch, D.C. and Schimmel, P.R. 1974. Cooperative binding of magnesium to transfer ribonucleic acid studied by a fluorescent probe. Biochemistry. 13:1841- 1851.

Malina, J., Hofr, C., Maresca, L., Natile, G. and Brabec, V. 2000. DNA interactions of antitumor cisplatin analogs containing enantiomeric amine ligands. Biophys. J. 78:2008-2021.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F. Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A. and Friend, S.H. 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. 250:1233-1238.

Marchetti, P., Cannita, K., Ricevuto, E., De Galitiis, F., Di Rocco, Z.C., Tessitore, A., Bisegna, R., Porzio, G., De Rubeis, G.P., Ventura, T., Martinotti, S. and Ficorella, C. 2003. Prognostic value of p53 molecular status in high-risk primary breast cancer. Ann. Oncol. 14:704-708.

Marky, L.A. and Breslauer, K.J. 1987. Calculating thermodynamic data for transitions of any molecularity from equilibrium melting curves. Biopolymers. 26:1601-1620.

Marquis, S.T., Rajan, J.V., Wynshaw-Boris, A., Xu, J., Yin, G.Y., Abel, K.J., Weber, B.L. and Chodosh, L.A. 1995. The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. Nat. Genet. 11:17-26.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F. 1999. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature. 399:700-704.

McEachern, K.A., Archey, W.B., Douville, K. and Arrick, B.A. 2003. BRCA1 splice variants exhibit overlapping and distinct transcriptional transactivation activities. J. Cell. Biochem. 89:120-132.

Mello, J.A., Lippard, S.J. and Essigmann, J.M. 1995. DNA adducts of cis-diamminedichloroplatinum(II) and its trans isomer inhibit RNA polymerase II differentially in *vivo*. Biochemistry. 34:14783-14791.

Meza, J.E., Brzovic, P.S., King, M.C. and Klevit, R.E. 1999. Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1. J. Biol. Chem. 274:5659-5665.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P.K., Norris, F.H., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J.C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R.,

Kamb, A. and Skolnick, M.H. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 266:66-71.

Miller, S.A., Dykes, D.D. and Polesky, H.F. 1988. A simple salting out procedure for extracting DNA from human nucleated cell. Nucleic Acids Res. 16:1215.

Miyake, T., Hu, Y.F., Yu, D.S. and Li, R. 2000. A Functional Comparison of BRCA1 C-terminal Domains in Transcription Activation and Chromatin Remodeling. J. Biol. Chem. 275:40169–40173.

Monaco, L., Kolthur-Seetharam, U., Loury, R., Murcia, J.M., de Murcia, G. and Sassone-Corsi, P. 2005. Inhibition of Aurora-B kinase activity by poly(ADP-ribosyl)ation in response to DNA damage. Proc. Natl. Acad. Sci. USA. 102:14244-14248.

Monteiro, A.N., August, A. and Hanafusa, H. 1996. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc. Natl. Acad. Sci. USA. 93:13595-13599.

Morris, J.R. and Solomon, E. 2004. BRCA1 : BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. Hum. Mol. Genet. 13:807-817.

Mrevlishvili, G.M., Razmadze, G.Z., Mdzinarashvili, T.D., Metreveli, N.O. and Kakabadze, G.R. 1996. Calorimetric investigation of DNA in the native and denatured states. Thermochim. Acta. 274:37-43.

Nadeau, G., Boufaied, N., Moisan, A., Lemieux, K.M., Cayanan, C., Monteiro, A.N. and Gaudreau, L. 2000. BRCA1 can stimulate gene transcription by a unique mechanism. EMBO J. 1:260-265.

Narod, S.A., Feunteun, J., Lynch H.T., Watson, P., Conway, T., Lynch, J. and Lenoir, G.M. 1991. Familial breast-ovarian cancer locus on chromosome 17q12-q23. Lancet. 338:82-83.

Neish, A.S., Anderson, S.F., Schlegel, B.P., Wei, W. and Parvin, J.D. 1998. Factors associated with the mammalian RNA polymerase II holoenzyme. Nucleic Acids Res. 26:847-853.

Nojima, K., Hochegger, H., Saberi, A., Fukushima, T., Kikuchi, K., Yoshimura, M., Orelli, B.J., Bishop, D.K., Hirano, S., Ohzeki, M., Ishiai, M., Yamamoto, K., Takata, M., Arakawa, H., Buerstedde, J.M., Yamazoe, M., Kawamoto, T., Araki, K., Takahashi, J.A., Hashimoto, N., Takeda, S., Sonoda, E. 2005. Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. Cancer Res. 65:11704-11711.

O' Day, C.L., Burgers, P.M. and Taylor, J.S. 1992. PCNA-induced DNA synthesis past cis-syn and trans-syn-I thymine dimers by calf thymus DNA polymerase delta *in vitro*. Nucleic Acids. Res. 20:5403-5406.

Okada, S. and Ouchi, T. 2002. Cell cycle differences in DNA damage-induced BRCA1 phosphorylation affect its subcellular localization. J. Biol. Chem. 278:2015-2020.

Olsen, J.H., Hahnemann, J.M., Borresen-Dale, A.L., Brondum-Nielsen, K., Hammarstrom, L., Kleinerman, R., Kaatriainen, H., Lonnqvist, T., Sankila, R., Seersholm, N., Tretli, S., Yuen, J., Boice, J.D., Tucker, M. 2001. Cancer in patients with ataxia-telangiectasia and in their relatives in the Nordic countries. J. Natl. Cancer Inst. 93:121–127.

Ouchi, T., Monteiro, A.N., August, A., Aaronson, S.A. and Hanafusa, H. 1998. BRCA1 regulates p53-dependent gene expression. Proc. Natl. Acad. Sci. USA. 95:2302-2306.

Pao, G.M., Janknecht, R., Ruffner, H., Hunter, T. and Verma, I.M. 2000. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. Proc. Natl. Acad. Sci. USA. 97:1020-1025.

Parker, R.J., Eastman, A., Bostick-Bruton, F. and Reed, E. 1991. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. J. Clin. Invest. 87:772-777.

Perego, P., Zunino, F., Carenini, N., Giuliani, F., Spinelli, S. and Howell, S.B. 1998. Sensitivity to cisplatin and platinum-containing compounds of Schizosaccharomyces pombe rad mutants. Mol. Pharmacol. 54:213–219.

Pilarski, R. and Eng, C. 2004. Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. J. Med. Genet. 41:323–326.

Pilch, D.S., Dunham, S.U., Jamieson, E.R., Lippard, S.J. and Breslauer, K.J. 2000. DNA sequence context modulates the impact of a cisplatin 1,2-d(GpG) intrastrand cross-link on the conformational and thermodynamic properties of duplex DNA. J. Mol. Biol. 296:803-812.

Pillaire, M.J., Margot, A., Villani, G., Sarasin, A., Defais, M. 1994. Gentil, A. Mutagenesis in monkey cells of a vector containing a single d(GPG) cis-diamminedichloroplatinum(II) adduct placed on codon 13 of the human H-ras proto-oncogene. Nucleic Acids Res. 22:2519-2524.

Pinto, A.L. and Lippard, S.J. 1985. Sequence-dependent termination of in vitro DNA synthesis by cis- and trans-diamminedichloroplatinum (II). Proc. Natl. Acad. Sci. USA. 82:4616-4619.

Poklar, N., Pilch, D.S., Lippard, S.J., Redding, E.A., Dunham, S.U. and Breslauer, K.J. 1996. Influence of cisplatin intrastrand crosslinking on the conformation, thermal stability, and energetics of a 20-mer DNA duplex. Proc. Natl. Acad. Sci. USA. 93:7606-7611.

Pratt, W.R., Ruddon, R.W., Ensminger, W.D and Maybaum, J. 1994. The anticancer drugs 2nd , Oxford university press, Newyork, USA.

Rahman-Roblick, R., Roblick, U.J., Hellman, U., Conrotto, P., Liu, T., Becker, S., Hirschberg, D., Jörnvall, H., Auer, G., Wiman, K.G. 2007. p53 targets identified by protein expression profiling. Proc. Natl. Acad. Sci. USA. 104:5401-5406.

Ratanaphan, A., Canyuk, B., Wasiksiri, S. and Mahasawat, P. 2005. In vitro platination of human breast cancer suppressor gene1 (BRCA1) by the anticancer drug carboplatin. Biochimica et Biophysica Acta. 1725:145-151.

Reed, E., Dabholkar, M. and Chabner, B.A. 1996. Platinum analogues. In: Chabner, B.A. and Longo, D.L. (Eds.) Cancer Chemotherapy and Biotherapy, 2nd Edition. Lippincott-raven Publishers, Philadelphia, USA.

Rice, J.A., Crothers, D.M., Pinto, A.L. and Lippard, S.J. 1988. The major adduct of the antitumor drug cis-diamminedichloroplatinum(II) with DNA bends the duplex by approximately equal to 40 degrees toward the major groove. Proc. Natl. Acad. Sci. USA. 85:4158-4161.

Rosen, E.M., Fan, S. and Ma, Y. 2006. BRCA1 regulation of transcription. Cancer Letters. 236:175-185.

Rosenberg, B., VanCamp, L. and Krisgas, T. 1965. Inhibition of cell devision in *Escherichia coli* by electrolysis products from a platinum electrod. Nature. 205:698- 699.

Rosenberg, B., Renshaw, E., VanCamp, L., Hartwick, J. and Drobnik. J. 1967. Platinum induced filamentous growth in *Escherichia coli*. J. Bacteriol. 93:716-721.

Rosenberg, B., VanCamp, L., Trosko, J.E. and Mansour, V.H. 1969. Platinum compounds: a new class of potent antitumor agents. Nature. 222:385-386.

Rosenberg, J. and Sato, P. 1988a. Messenger RNA loses the ability to direct in vitro peptide synthesis following incubation with cisplatin. Mol. Pharmacol. 33:611-616.

Rosenberg, J. and Sato, P. 1988b. Hemolysates from guinea pig reticulocytes also effiently translate added mRNA. Comp. Biochem. Physiol. B. 91:33-37.

Rosenberg, J.M. and Sato, P.H. 1993. Cisplatin inhibits in vitro translation by preventing the formation of complete initiation complex. Mol. Pharmacol. 43:491-497.

Rouzina, I. and Bloomfield, V.A. 1999. Heat capacity effects on the melting of DNA. 2. Analysis of nearest neighbor base pair effects. Biophys. J. 77:3252-3255.

Rubin, S.C., Benjamin, I., Behbakht, K., Takahashi, H., Morgan, M.A., LiVolsi, V.A., Berchuck, A., Muto, M.G., Garber, J.E., Weber, B.L., Lynch, H.T. and Boyd, J. 1996. Clinical and pathological features of ovarian cancer in women with germ-line mutations of BRCA1. N. Engl. J. Med. 335:1413-1416.

Ruffner, H., Jiang, W., Craig, A.G., Hunter, T., Verma, I.M. 1999. BRCA1 is phosphorylated at serine 1497 in vivo at a cyclin-dependent kinase 2 phosphorylation site. Mol. Cell Biol. 19:4843-4854.

Ruffner, H., Joazeiro, C.A., Hemmati, D., Hunter, T. and Verma, I.M. 2001. Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. Proc. Natl. Acad. Sci. USA. 98:5134-5139.

Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd edition, Cold spring Habor Laboratory Press, USA.

Sansal, I. and Sellers, W.R. 2004. The biology and clinical relevance of the PTEN tumor suppressor pathway. J. Clin. Oncol. 22:2954–2963.

Santa-Lucia, J.J., Allawi, H.T. and Seneviratne, P.A. 1996. Improved nearest-neighbor parameters for predicting DNA duplex stability. Biochemistry. 35:3555-3562.

Satagopan, J.M., Boyd, J., Kauff, N.D., Robson, M., Scheuer, L., Narod, S. and Offit, K. 2002. Ovarian cancer risk in Ashkenazi Jewish carriers of BRCA1 and BRCA2 mutations. Clin. Cancer Res. 8:3776-3781.

Sato, P.H., Rosenberg, J. and Sato, R.I. 1996. Differences in the inhibition of translation by cisplatin, transplatin, and certain related compounds. Biochem. Pharmacol. 52:1895-1902.

Schlegel, B.P., Green, V.J., Ladias, J.A.A. and Parvin, J.D. 2000. BRCA1 interaction with RNA polymerase II reveals a role for hRPB2 and hRPB10α in activated transcription. Proc. Natl. Acad. Sci. USA. 97:3148-3153.

Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T. and Livingston, D.M. 1997a. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell. 88:265-275.

Scully, R., Anderson, S.F., Chao, D.M., Wei, W., Ye, L., Young, R.A., Livingston, D.M. and Parvin, J.D. 1997b. BRCA1 is a component of the RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. USA. 94:5605-5610.

Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. 1997c. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell. 90:425-435.

Scully, R. and Livingston, D.M. 2000. In Search of the tomour-supperssor function of BRCA1and BRCA2. Nature. 408:429–432.

Scully, R., Puget, N. and Vlasakova, K. 2000. DNA polymerase stalling, sister chromatid recombination and the BRCA genes. Oncogene. 19:6176–6183.

Selvakumaran, M., Pisarcik, D.A., Bao, R., Yeung, A.T. and Hamilton, T.C. 2003. Enhanced cisplatin cytotoxicity by disturbing the nucleotide excision repair pathway in ovarian cancer cell lines. Cancer Res. 63:1311-1316.

Sharan, S.K., Wims, M. and Bradley, A. 1995. Murine Brca1: sequence and significance for human missense mutations. Hum. Mol. Genet. 4:2275-2278.

Shinohara, A., Ogawa, H. and Ogawa, T. 1992. Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. Cell. 69:457-470.

Simon, J.A., Szankasi, P., Nguyen, D.K., Ludlow C, Dunstan HM, Roberts CJ, Jensen EL, Hartwell LH, Friend SH. 2000. Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of *Saccharomyces cerevisiae*. Cancer Res. 60:328–333.

Sip, M., Schwartz, A., Vovelle, F., Ptak, M. and Leng, M. 1992. Distortions induced in DNA by cis-platinum interstrand adducts. Biochemistry. 31:2508-2513.

Smith, S.A., Easton, D.F., Evans, D.G. and Ponder, B.A. 1992. Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. Nat. Genet. 2:128-131.

Smith, M.L., Ford, J.M., Hollander, M.C., Bortnick, R.A., Amundson, S.A., Seo, Y.R., Deng, C.X., Hanawalt, P.C. and Fornace, A.J. Jr. 2000. p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol. Cell Biol. 20:3705–3714.

Sobol, H., Stoppa-Lyonnet, D., Bressac-de-Paillerets, B., Peyrat, J.P., Kerangueven, F., Janin, N., Noguchi, T., Eisinger, F., Guinebretiere, J.M., Jacquemier, J. and Birnbaum, D. 1996. Truncation at conserved terminal regions of BRCA1 protein is associated with highly proliferating hereditary breast cancers. Cancer Res. 56:3216-3219.

Somasundaram, K., Zhang, H., Zeng, Y.X., Houvras, Y., Peng, Y., Zhang, H., Wu, G.S., Licht, J.D., Weber, B.L. and El-Deiry, W.S.1997. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. Nature. 389:187-190.

Sonoda, E., Takata, M., Yamashita, Y.M., Morrison, C., Takeda, S. 2001. Homologous DNA recombination in vertebrate cells. Proc. Natl. Acad. Sci. USA. 98:8388–8394.

Sonoda, E., Okada, T., Zhao, G.Y., Tateishi S, Araki K, Yamaizumi M, Yagi T, Verkaik NS, van Gent DC, Takata M, Takeda S.et al. 2003. Multiple roles of

Rev3, the catalytic subunit of polζ in maintaining genome stability in vertebrates. EMBO. J. 22:3188–3197.

Soulier, J. and Lowndes, N.F. 1999. The BRCT domain of the S. cerevisiae checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. Curr. Biol. 9:551-554.

Svejstrup, J.Q., Feaver, W.J., Kornberg, R.D. 1996. Subunits of yeast RNA polymerase II transcription factor TFIIH encoded by the CCL1 gene. J. Biol. Chem. 271:643-645.

Swift, M., Morrell, D., Massey, R.B. and Chase, C.L. 1991. Incidence of cancer in 161 families affected by ataxia-telangiectasia. N. Engl. J. Med. 325:1831–1836.

Szabo, C.I., Wagner, L.A., Francisco, L.V., Roach, J.C., Argonza, R., King, M.C. and Ostrander, E.A. 1996. Human, canine and murine BRCA1 genes: sequence comparison among species. Hum. Mol. Genet. 5:1289-1298.

Takimoto, R., MacLachlan, T.K., Dicker, D.T., Niitsu, Y., Mori, T. and el-Deiry, W.S. 2002. BRCA1 transcriptionally regulates damaged DNA binding protein (DDB2) in the DNA repair response following UV-irradiation. Cancer Biol. Ther. 1:177–186.

Tereshko, V., Wilds, C.J., Minasov, G., Prakash, T.P., Maier, M.A., Howard, A., Wawrzak, Z., Manoharan, M. and Egli, M. 2001. Detection of alkali metal ions in DNA crystals using state-of-the-art X-ray diffraction experiments. Nucleic Acids Res. 29:1208-1215.

Thakur, S., Zhang, H.B., Peng, Y., Le, H., Carroll, B., Ward, T., Yao, J., Farid, L.M., Couch, F.J., Wilson, R.B. and Weber, B.L. 1997. Localization of BRCA1 and a splice variant identifies the nuclear localization signal. Mol. Cell. Biol. 17:444-452.

Thomas, J.E., Smith, M., Tonkinson, J.L., Rubinfeld, B. and Polakis, P. 1997. Induction of phosphorylation on BRCA1 during the cell cycle and after DNA damage. Cell. Growth. Differ. 8:801-809.

Thompson, M.E., Jensen, R.A., Obermiller, P.S., Page, D.L. and Holt, J.T. 1995. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat. Genet. 9:444-450.

Thompson, L.H. and Schild, D. 2001. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutat. Res. 477:131–153.

Thompson, D. and Easton, D.F. 2002. Breast Cancer Linkage Consortium. Cancer Incidence in BRCA1 mutation carriers. J. Natl. Cancer Inst. 94:1358–1365.

Thompson, D., Duedal, S., Kirner, J., McGuffog, L., Last, J., Reiman, A., Byrd, P., Taylor, M., Easton, D.F. 2005. Cancer risks and mortality in heterozygous ATM mutation carriers. J. Natl. Cancer Inst. 97:813–822.

Tirkkonen, M., Johannsson, O., Agnarsson, B.A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R.B., Borg, A. and Kallioniemi, O.P. 1997. Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. Cancer Res. 57:1222-1227.

Tornaletti, S., Patrick, S.M., Turchi, J.J. and Hanawalt, P.C. 2003. Behavior of T7 RNA Polymerase and Mammalian RNA Polymerase II at Site-specific Cisplatin Adducts in the Template DNA. J. Biol. Chem. 278:35791–35797.

Travis, L.B., Andersson, M., Gospodarowicz, M., van Leeuwen, F.E., Bergfeldt, K., Lynch, C.F., Curtis, R.E., Kohler, B.A., Wiklund, T., Storm, H., Holowaty, E., Hall, P., Pukkala, E., Sleijfer, D.T., Clarke, E.A., Boice, J.D. Jr., Stovall, M. and Gilbert, E. 2000. Treatment-associated leukemia following testicular cancer. J. Natl. Cancer Inst. 92:1165-1171.

Treiber, D.K., Zhai, X., Jantzen, H.M. and Essigmann, J.M. 1994. Cisplatin-DNA adducts are molecular decoys for the ribosomal RNA transcription factor hUBF (human upstream binding factor). Proc. Natl. Acad. Sci. USA. 91:5672-5676.

Trujillo, K.M., Yuan, S.S., Lee, E.Y., Sung, P. 1998. Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. J. Biol. Chem. 273:21447-21450.

Tsutsui, S., Inoue, H., Yasuda, K., et al. 2005. Reduced expression of PTEN protein and its prognostic implications in invasive ductal carcinoma of the breast. Oncology. 68:398–404.

Vallon-Christersson, J., Cayanan, C., Haraldsson, K., Loman, N., Bergthorsson, J.T., Brondum-Nielsen, K., Gerdes, A.M., Moller, P., Kristoffersson, U., Olsson, H., Borg, A. and Monteiro, A.N. 2001. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. Hum. Mol. Genet. 10:353-360.

van de Sande, J.H., McIntosh, L.P. and Jovin, T.M. 1982. Mn^{2+} and other transition metals at low concentration induce the right-to-left helical transformation of poly[d(G-C)]. EMBO J. 1:777-782.

Vaughn, J.P., Davis, P.L., Jarboe, M.D., Huper, G., Evans, A.C., Wiseman, R.W., Berchuck, A., Iglehart, J.D., Futreal, P.A. and Marks, J.R. 1996. BRCA1 expression is induced before DNA synthesis in both normal and tumor-derived breast cells. Cell Growth Differ. 7:711-715.

Vichi, P., Coin, F., Renaud, J.P., Vermeulen, W., Hoeijmakers, J.H., Moras, D. and Egly, J.M. 1997. Cisplatin- and UV-damaged DNA lure the basal transcription factor TFIID/TBP. EMBO J. 16:7444-7456.

Vogelstein, B. and Kinzler, K.W. 2002. The genetic basic of human cancer 2^{nd}, McGraw-Hill Medical Publishing Division, Newyork, USA.

Wada, O., Oishi, H., Takada, I., Yanagisawa, J., Yano, T. and Kato, S. 2004. BRCA1 function mediates a TRAP/DRIP complex through direct interaction with TRAP220. Oncogene. 23:6000-6005.

Wang, Q., Zhang, H., Fishel, R. and Greene, M.I. 2000. BRCA1 and cell signaling. Oncogene. 19:6152–6158.

Welcsh, P.L, Owens, K.N. and King, M.C. 2000. Insights into the functions of BRCA1 and BRCA2. Trends. Genetics. 16:69-74

Welcsh, P.L. and King, M.C. 2001. BRCA1 and BRCA2 and genetics of breast and ovarian cancer. Human Molecular Genetics. 10:7705–7713.

Welcsh, P.L., Lee, M.K., Gonzalez-Hernandez, R.M., Black, D.J., Mahadevappa, M., Swisher, E.M., Warrington, J.A. and King, M.C. 2002. BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. Proc. Natl. Acad. Sci. USA. 99:7560-7565.

White, M., Bradbury, A. and Olopade, O.I. 2002. Breast and ovarian cancer risk assessment: strategies for the primary care clinician. *Woman's health in primary care*. 5:519-925.

Williams, R.S., Green, R. and Glover, J.N.M. 2001. Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. Nature Struct. Biol. 8:838-842.

Williamson, E.A., Dadmanesh, F. and Koeffler, H.P. 2002. BRCA1 transactivates the cyclin-dependent kinase inhibitor p27 (Kip1). 21:3199-3206.

Wilson, C.A., Payton, M.N., Elliott, G.S., Buaas, F.W., Cajulis, E.E., Grosshans, D., Ramos, L., Reese, D.M., Slamon, D.J. and Calzone, F.J. 1997. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b. Oncogene. 14:1-16.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C. and Micklem, G. 1995. Identification of the breast cancer susceptibility gene BRCA2. Nature. 378:789–792.

Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C., Hwang, L.Y., Bowcock, A.M. and Baer, R. 1996. Identification of a RING protein that can interact in vivo with the BRCA1 gene product. Nat. Genet. 14:430-440.

Xing, W.R., Gilchrist, K.W., Harris, C.P., Samson, W. and Meisner, L.F. 1996. FISH detection of HER-2/neu oncogene amplification in early onset breast cancer. Breast Cancer Res. Treat. 39:203-212.

Xu, B., Kim, S.T. and Kastan, M.B. 2001. Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. Mol. Cell. Biol. 21:3445-3450.

Xu, B., O'Donnell, A.H., Kim, S.T. and Kastan, M.B. 2002. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. Cancer Res. 62:4588-4591.

Yarden, R.I. and Brody, L.C. 1999. BRCA1 interacts with components of the histone deacetylase complex. Proc. Natl. Acad. Sci. USA. 96:4983-4988.

Yamane, K., Katayama, E. and Tsuruo, T. 2001. p53 contains a DNA break-binding motif similar to the functional part of BRCT-related region of Rb. Oncogene. 20:2859-2867.

Yamashita, Y.M., Okada, T., Matsusaka, T, et al. 2002. RAD18 and RAD54 cooperatively contribute to maintenance of genomic stability in vertebrate cells. EMBO. J. 21:5558–5566.

Yarden, R.I. and Brody, L.C. 1999. BRCA1 interacts with components of the histone deacetylase complex. Proc. Natl. Acad. Sci. USA.96:4983-4988.

Yu, X., Wu, L.C., Bowcock, A.M., Aronheim, A., Baer, R. 1998. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in

the CtBP pathway of transcriptional repression. J Biol Chem. 273:25388-25392.

Zamble, D.B. and Lippard, S.J. 1995. Cisplatin and DNA repair in cancer therapy. Trends. Biochem. Sci. 20:435-439.

Zamble, D.B., Mu, D., Reardon, J.T., Sancar, A. and Lippard, S.J. 1996. Repair of cisplatin-DNA adducts by the mammalian excision nuclease. Biochemistry. 35:1004-1013.

Zhang, X., Morera, S., Bates, P.A., Whitehead, P.C., Coffer, A.I., Hainbucher, K., Nash, R.A., Sternberg, M.J., Lindahl, T. and Freemont, P.S. 1998. Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. EMBO J. 17:6404-6411.

Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, BL. and El-Deiry, W.S. 1998. BRCA1 physically associates with p53 and stimulates its transcriptional activity. Oncogene. 16:1713-1721.

Zheng, N., Wang, P., Jeffrey, P.D. and Pavletich, N.P.2000. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell. 102:533-539.

Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P.L., Sharp, Z.D. and Lee, W.H. 1999. Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science. 285:747–750.

Zhong, Q., Chen, C.F., Chen, P.L. and Lee, W.H. 2002. BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. J. Biol. Chem. 277:28641–28647.

Zhou, C., Huang, P. and Liu, J. 2005. The carboxyl-terminal of BRCA1 is required for subnuclear assembly of RAD51 after treatment with cisplatin but not ionizing radiation in human breast and ovarian cancer cells. Biochem. Biophys. Res. Commun. 336:952-960.

Zimmer, C.H., Luck, G. and Triebel, H. 1974. Conformation and reactivity of DNA. IV. Base binding ability of transition metal ions to native DNA and effect on helix conformation with special references to DNA-Zn(II) complex. Biopolymers. 13:425-453.

Zwelling, L.A. and Kohn, K.W. 1982. Platinum complexes. In: Chabner BA, ed Pharmacologic principle of cancer treatment. Philadelphia: WB Saunders: USA.

VITAE

Name Mr. Siriwat Wasiksiri

Student ID 4383006

Education Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Veterinary Medicine (D.V.M)	Kasetsart University	1989

Work-Position and Address

Lecturer, Department of Animal Sciences, Faculty of Natural Resources, Prince of Songkla University, Hat-yai, Songkhla 90112, Thailand. 1995-present

List of Publication and Proceeding

Wasiksiri, S. and Ratanaphan, A. 2002. Breast Cancer Susceptibility Gene 1 (*BRCA1*) (Review article), Songklanakarin J Sci Technol, 24:509-531.

Ratanaphan, A., Canyuk, B., **Wasiksiri, S.** and Mahasawat, P. 2005. In vitro platination of human breast cancer suppressor genel (*BRCA1*) by the anticancer drug carboplatin. Biochimica et Biophysica Acta. 1725:145-151.

Wasiksiri, S., Canyuk, B., Prasertsan, P. and Ratanaphan, A. 2007. Repair-mediated transcriptional transactivation of cisplatin-damaged *BRCA1* is associated with altered DNA conformation and thermal stability (Submitted to Nucleic Acids Research).

Wasiksiri, S., Canyuk, B., and Ratanaphan, A. 2001. Thermal Stability of Human Breast Cancer Suppressor Gene (BRCA1). Poster presentation in The 13th Annual Meeting of the Thai Society for Biotechnology, November12-15 2001, Hotel Sofitel Raja Orchid, Khon Kaen, Thailand.

Wasiksiri, S., Sriroongrueng, W., Canyuk, B., Prasertsan, P. and Ratanaphan, A. 2002. Thermal Characterization of the Breast Cancer Susceptibility Gene 1 (*BRCA1*), The 14th Annual Meeting of the Thai Society for Biotechnology, Biotechnology for Better Living in the New Economy, November12-15 2002, Hotel Sofitel Raja Orchid, Khon Kaen, Thailand.

Ratanaphan, A., Canyuk, B., **Wasiksiri, S.,** Atipairin, A. and Mahasawat, P. 2003. Human Breast Cancer Suppressor Gene 1 (*BRCA1*): Analysis of DNA Damage and Gene Expression, The 3rd Indochina Conference on Pharmaceutical Sciences (Pharma Indochina III), Pharmacy for Better quality of Life, May 21-23 2003, Bangkok, Thailand.

Ratanaphan, A., Canyuk, B., Mahasawat, P. and **Wasiksiri, S.** 2003. Analysis of the breast cancer suppressor gene (*BRCA1*) damaged by cisplatin using gel eletrophoresis, restriction digestion and quantitative polymerase chain reaction, XIX International Congress of Genetics, Genome-The Linkage of Life, July 6-12 2003, Melbourne. Australia.

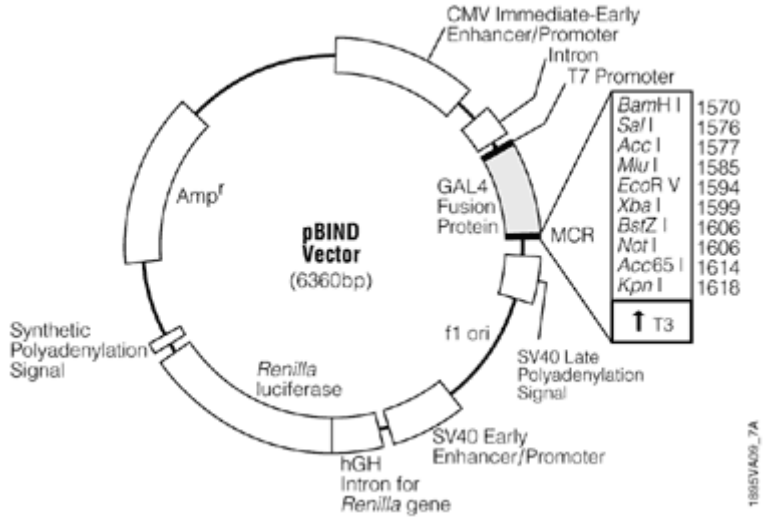
Ratanaphan, A., Canyuk, B., Atipairin, A. and **Wasiksiri, S.** 2003. Molecular cloning and expression of the C-terminal region of human BRCA1 (BRCT) protein induced by the bacterial *pho* A promoter, XIX International Congress of Genetics, Genome-The Linkage of Life, July 6-12, 2003, Melbourne. Australia.

Ratanaphan, A., **Wasiksiri, S.** and Canyuk, B. 2004. Thermal stability of human breast cancer suppressor gene 1 (*BRCA1*), 10th Congress of Federation of Asian & Oceanian Biochemists and Molecular Biologists, December 7-11, 2004, Bangalore, India.

Wasiksiri, S., Canyuk, B. and Ratanaphan, A. 2005. Effects of cisplatin on breast cancer suppressor gene (*BRCA1*), The 4rd Indochina Conference on Pharmaceutical Sciences (Pharma Indochina IV), November 10-16, 2005, Ho Chi Minh, Vietnam.

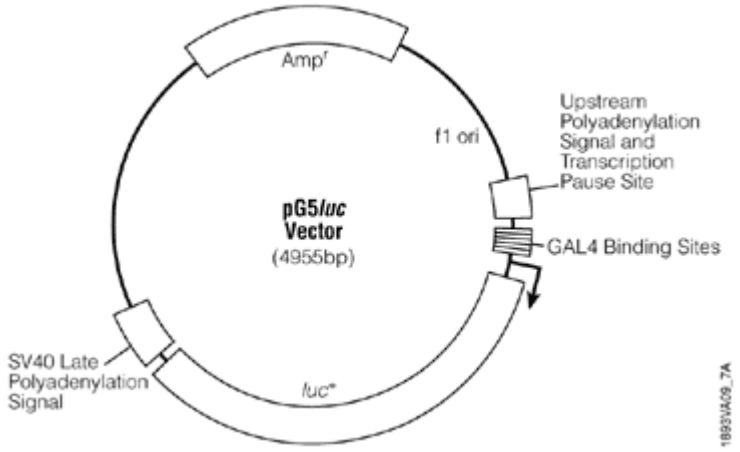
Appendix

Figure 40. pBIND Vector (Promega, USA)



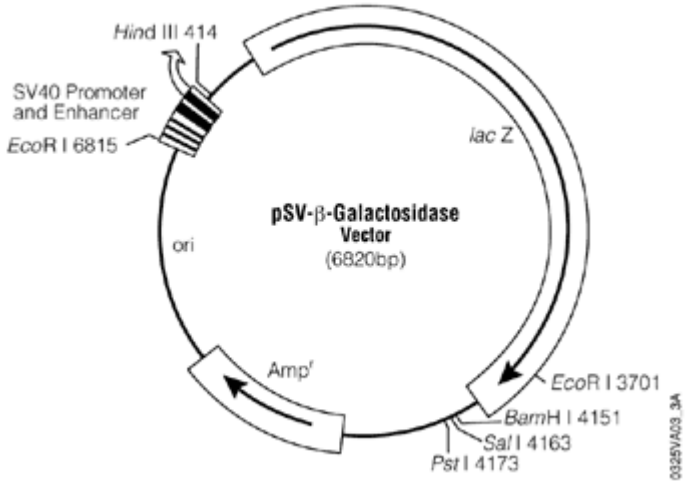
CMV immediate-early enhancer	1-659
CMV immediate-early promoter	669-750
Chimeric intron	890-1022
T7 RNA polymerase promoter (-17 to +2)	1067-1085
GAL4 fusion protein	1116-1556
Multiple cloning regions	1570-1619
T3 RNA polymerase promoter (-16 to +3)	1639-1657
SV40 late polyadenylation signal	1666-1887
Phage f1 origin of replication	1982-2437
SV40 early enhancer/promoter	2502-2872
SV40 minimum origin of replication	2768-2833
hGH intron for Renilla gene	2924-3183
Renilla luciferase gene coding region	3208-4143
Synthetic polyadenylation signal	4201-4249
β-lactamase (AmpR) coding region	4668-5528

Figure 41. pG5luc Vector (Promega, USA)



Five GAL4 binding sites	18-120
Major late promoter of adenovirus	132-172
Predicted transcriptional start site	168
Luciferase (luc+) gene	225-1874
SV40 late polyadenylation signal	1909-2130
Beta-lactamase (Ampr) coding region	4077-3220
Phage f1 origin of replication	4210-4664
Upstream polyadenylation signal and transcriptional pause site	4795-4948

Figure 42. pSV-β-Galactosidase Vector (Promega, USA)



Base pairs	6820
SV40 early promoter and enhancer seqment	1-419
Transcription start sites	354, 360, 365
Possible start codons (ATG)	500, 530, 569
gpt promoter (-10 region)	428-433
lacZ start site	710
lacZ stop site (TAA)	3755
lac operon sequences	709-4020
lacY	3809-4011
SV40 small T antigen	4021-4156
beta-lactamase (AmpR) coding region	4784-5644

Figure 43. Recognition site of *Bam*HI, *Eco*RI, *Eco*0109I and *Pvu*II on recombinant plasmid pBIND-BRCT

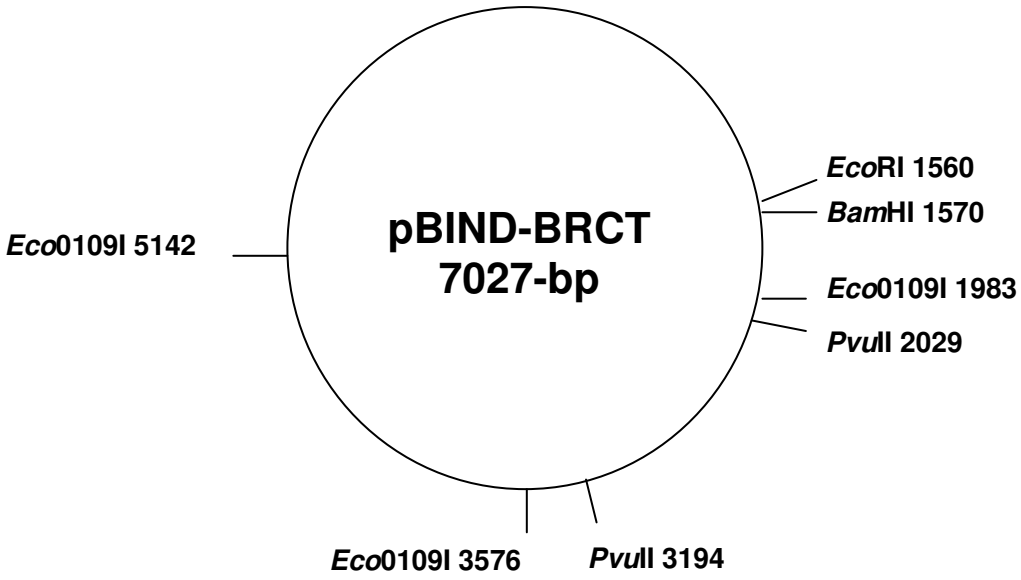


Figure 44. DNA sequence of pBIND-BRCT showing a *Bam*HI cloning site (G GATCC) in the plasmid pBIND (black triangle).