

**DNA REPAIR GENETIC POLYMORPHISMS IN CHILDHOOD
ACUTE LYMPHOBLASTIC LEUKEMIA**



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Thesis

Entitled

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ACUTE LYMPHOBLASTIC LEUKEMIA**



Tanasan Sirirat
Mr. Tanasan Sirirat
Candidate

Suntree Apibal
Assoc.Prof. Suntree Apibal,
M.Sc.
Major-Advisor

Samart Pakakasama
Lect. Samart Pakakasama,
M.D.
Co-Advisor

Rassmidara Hoonsawat
Assoc.Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

Punnee Butthep
Assoc.Prof. Punnee Butthep,
Ph.D
Chair
Master of Science Program
In Clinical Pathology
Faculty of Medicine
Ramathibodi Hospital

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Clinical Pathology)

on

9 September, 2004

The seal of Mahidol University is a large, circular emblem in the background. It features a central golden stupa-like structure with a flame-like base, surrounded by Thai script. The outer ring of the seal contains the university's name in Thai characters.

Tanasan Sirirat.

Mr. Tanasan Sirirat
Candidate

Suntree Apibal.

Assoc.Prof. Suntree Apibal, M.Sc.
Chair

Samart Pakakasama.

Lect. Samart Pakakasama, M.D.
Member

Suradej Hongeng.

Assist.Prof. Suradej Hongeng, M.D.
Member

Rassmidara Hoonsawat.

Assoc.Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Prakit Vathesatogkit.

Prof. Prakrit Vathesatogkit,
M.D., ABIM., FRCP
Dean
Faculty of Medicine
Ramathibodi Hospital,
Mahidol University.

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Tanasan Sirirat

DNA REPAIR GENETIC POLYMORPHISMS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

TANASAN SIRIRAT 4536245 RACP/M

M.Sc.(CLINICAL PATHOLOGY)

THESIS ADVISORS: SUNTAREE APIBAL M.Sc.,

SAMART PAKAKASAMA M.D.

ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. It has been suggested that this malignancy arises by a combination of genetic susceptibility and environmental exposure early in life. The repair of DNA damage has an important role in protecting individuals from cancer-causing agents. Polymorphisms in DNA repair genes XRCC1 and XPD have recently been identified (codon 194, 280, and 399 for XRCC1; codon 312 and 751 for XPD) and may affect DNA repair capacity and modulate cancer susceptibility. The purpose of this study is to determine whether XRCC1 and XPD genetic polymorphisms play a role in childhood leukemogenesis. This study performed genotyping of the polymorphisms by using PCR-RFLP in 108 childhood ALL patients and 317 controls. The presence of XRCC1 codon 194 T allele indicated a protective effect against childhood ALL (OR = 0.673, 95%CI, 0.468-0.967). XRCC1 codon 399 A allele, GA and GA or AA genotypes showed an increased risk of childhood ALL (OR = 1.674, 95%CI, 1.203-2.330; OR = 2.171, 95%CI, 1.365-3.454; OR = 2.180, 95%CI, 1.383-3.422, respectively). This study also found no differences in distribution of the XRCC1 codon 280, XPD codon 312 and XPD codon 751 genotypes between cases and controls. In conclusion, this study demonstrated for the first time that XRCC1 codon 194 and 399 polymorphisms may influence the risk of childhood ALL.

KEY WORDS: ACUTE LYMPHOBLASTIC LEUKEMIA / DNA REPAIR /
GENETIC POLYMORPHISM / XPD / XRCC1

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DNA repair genetic polymorphisms ในมะเร็งเม็ดเลือดขาวเฉียบพลันชนิด Lymphoblastic ในเด็ก (DNA REPAIR GENETIC POLYMORPHISMS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA)

ชื่อนสาร ศิริรัตน์ 4536245 RACP/M

วท.ม. (พยาธิวิทยาคลินิก)

คณะกรรมการควบคุมวิทยานิพนธ์ : สุนทรื อภิบาล, วท.ม., สามารถ ภคกษมา, พ.บ.

บทคัดย่อ

มะเร็งเม็ดเลือดขาวเฉียบพลันชนิด lymphoblastic หรือ ALL เป็นโรคมะเร็งที่พบบ่อยในเด็กซึ่งสาเหตุของการเกิดโรคนี้อยู่ไม่ทราบแน่ชัดแต่พบว่าปัจจัยทางด้านพันธุกรรมและการสัมผัสสิ่งแวดล้อมที่เป็นพิษต่อยีนโดยเฉพาะในช่วงที่ร่างกายเจริญเติบโตอาจเป็นสาเหตุของการเกิดโรคนี การซ่อมแซมดีเอ็นเอที่ได้รับความเสียหายมีหน้าที่สำคัญในการปกป้องการเกิดมะเร็งจากสารก่อมะเร็งในแต่ละบุคคล polymorphisms ของ XRCC1 (ตำแหน่ง codon ที่ 194 280 และ399)และ XPD (ตำแหน่ง codon ที่ 312 และ751) อาจส่งผลต่อความสามารถในการซ่อมแซมดีเอ็นเอและง่ายต่อการเกิดมะเร็ง จุดประสงค์ในการศึกษาครั้งนี้เพื่อทดสอบบทบาทของ XRCC1 และ XPD genetic polymorphisms ต่ออัตราเสี่ยงการเกิดเป็นมะเร็งชนิด ALL ในเด็กโดยวิธี PCR-RFLP ในการทำ genotyping เปรียบเทียบระหว่างกลุ่มผู้ป่วยเด็กจำนวน 108 รายและกลุ่มควบคุมจำนวน 317 ราย ซึ่งผลการศึกษาพบว่า T allele ของ XRCC1 ตำแหน่ง codon ที่ 194 แสดงบทบาทในการปกป้องความเสี่ยงต่อการเกิดมะเร็งเม็ดเลือดขาวชนิด ALL โดยมีค่า OR = 0.673, 95%CI, 0.468-0.967 และพบว่า XRCC1 ตำแหน่ง codon ที่ 399 A allele GA และ GA หรือ AA genotypes มีการแสดงบทบาทในการเพิ่มความเสี่ยงต่อการเกิดมะเร็งเม็ดเลือดขาวชนิด ALL โดยมีค่า OR = 1.674, 95%CI, 1.203-2.330; OR = 2.171, 95%CI, 1.365-3.454 และ OR = 2.180, 95%CI, 1.383-3.422 ตามลำดับ นอกจากนี้ยังพบว่าจำนวนความถี่ของ genotype ของยีน XRCC1 ตำแหน่ง codon ที่ 280 รวมทั้งยีน XPD ตำแหน่ง codon ที่ 312 และ 751 ไม่มีความแตกต่างกันระหว่างกลุ่มผู้ป่วยและกลุ่มควบคุม โดยสรุปการศึกษานี้เป็นการศึกษาครั้งแรกที่แสดงให้เห็นว่า polymorphism ของยีน XRCC1 ตำแหน่ง codon ที่ 194 และ 399 น่าจะมีอิทธิพลต่อความเสี่ยงเป็นมะเร็งเม็ดเลือดชนิด ALL ในเด็ก

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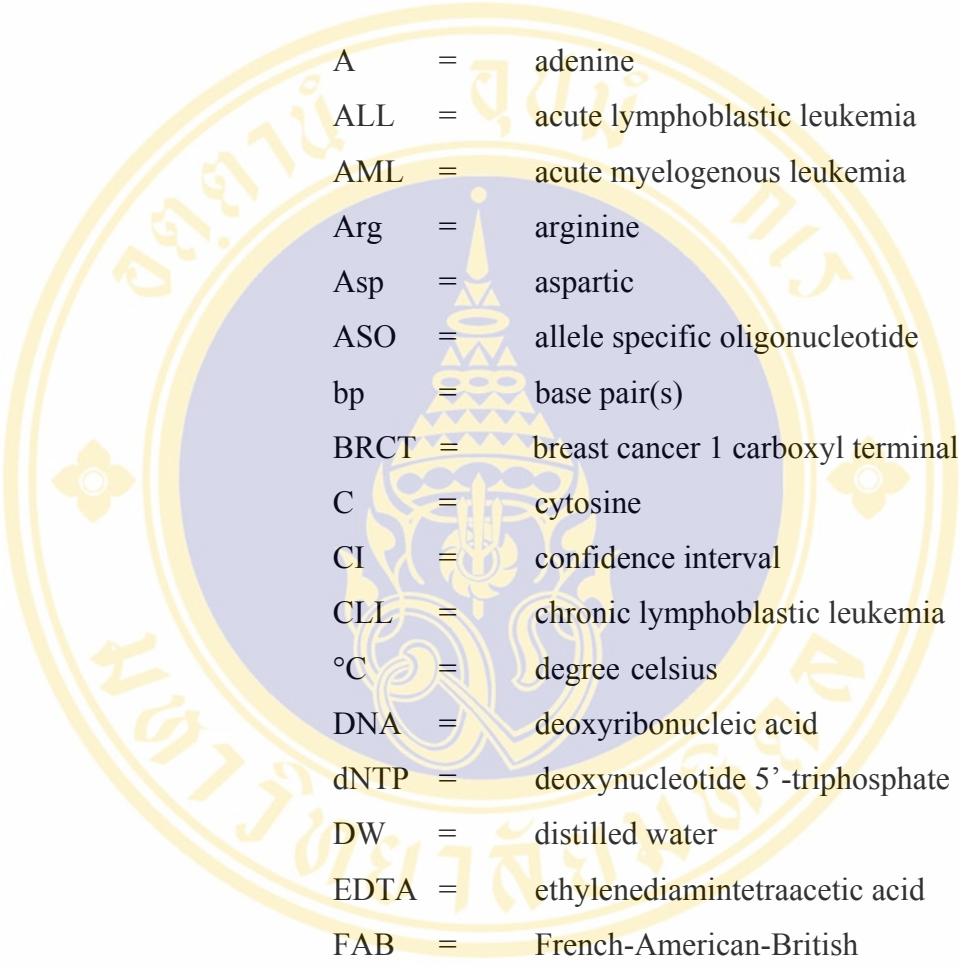
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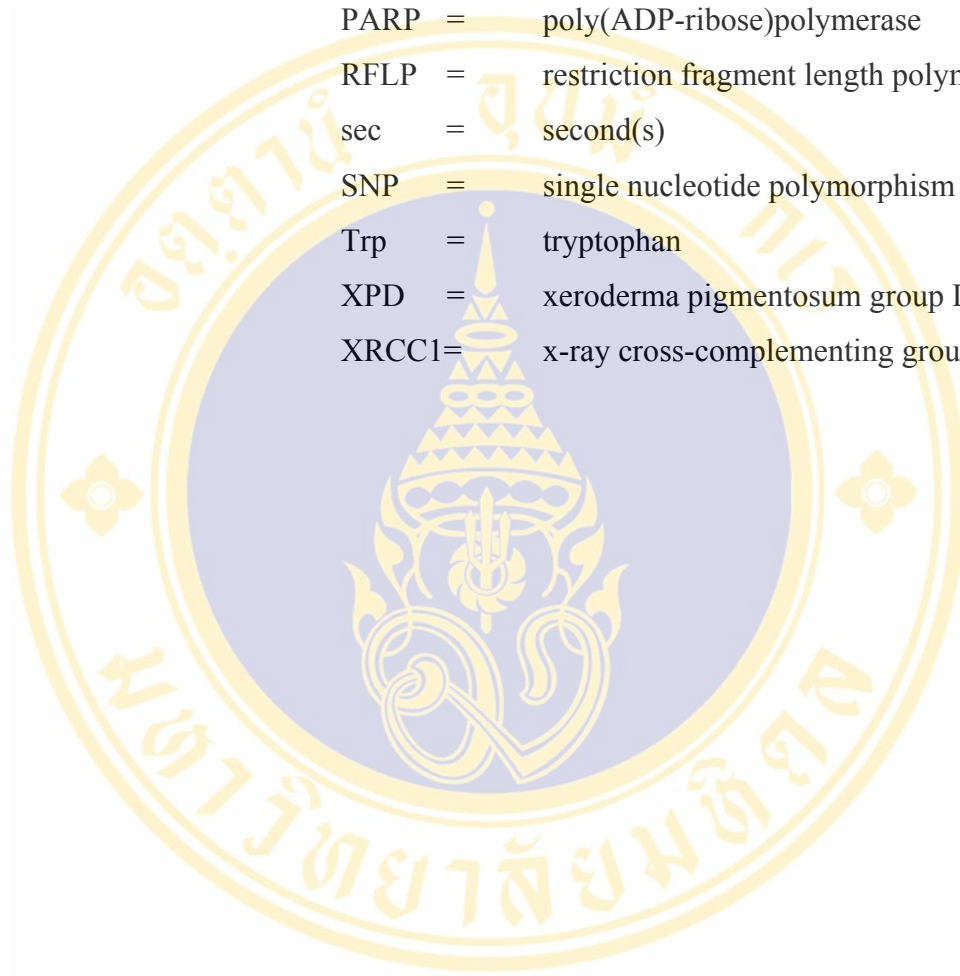
LIST OF ABBREVIATIONS



A	=	adenine
ALL	=	acute lymphoblastic leukemia
AML	=	acute myelogenous leukemia
Arg	=	arginine
Asp	=	aspartic
ASO	=	allele specific oligonucleotide
bp	=	base pair(s)
BRCT	=	breast cancer 1 carboxyl terminal
C	=	cytosine
CI	=	confidence interval
CLL	=	chronic lymphoblastic leukemia
°C	=	degree celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide 5'-triphosphate
DW	=	distilled water
EDTA	=	ethylenediaminetetraacetic acid
FAB	=	French-American-British
g	=	gram
G	=	guanine
Gln	=	glutamine
His	=	histidine
Lys	=	lysine
min	=	minute(s)
μl	=	microlitre
mM	=	millimolar
μM	=	micromolar
OR	=	odds ratio

LIST OF ABBREVIATIONS (Continued)

PCR	=	polymerase chain reaction
PARP	=	poly(ADP-ribose)polymerase
RFLP	=	restriction fragment length polymorphism
sec	=	second(s)
SNP	=	single nucleotide polymorphism
Trp	=	tryptophan
XPD	=	xeroderma pigmentosum group D
XRCC1	=	x-ray cross-complementing group1



CHAPTER 1

INTRODUCTION

1.1 Background and Problems

Acute leukemia is the most common cancer in children and represents 30% of cases of childhood malignancy. Seventy percent of childhood acute leukemia cases are acute lymphoblastic leukemia (ALL). Although no one really knows the cause of ALL in human, many certain risk factors make individuals more prone to developing leukemia. Many factors, such as age and genetics, are probably beyond our control. Other factors, such as environmental or lifestyle-related variables, may be more correctable. It is now known that all cancer, including leukemia, begins as a mutation in the genetic material, the DNA (deoxyribonucleic acid), within certain cells. The external or internal causes of such change probably add up over a lifetime. Leukemia begins when one or more white blood cells experience DNA loss or damage. Those errors are copied and passed on to the subsequent generations of cells. The abnormally leukemic cells remain in an immature blast form that never matures properly. They do not die off like normal cells, but tend to multiply and accumulate within the body.

DNA in most cells is regularly damaged by endogenous and exogenous mutagens. Unrepaired damage can result in apoptosis or may lead to unregulated cell growth and cancer. If DNA damage is recognized by cell machinery, several responses may occur to prevent replication in the presence of genetic errors. At the cellular level, checkpoints can be activated to arrest the cell cycle, transcription can be up-regulated to compensate for the damage, or the cell can apoptose. Alternatively, the damage can be repaired at the DNA level enabling the cell to replicate as planned. Complex pathways involving numerous molecules have evolved to perform such repair. Because of the importance of maintaining genomic integrity in the general and specialized functions of cells as well as in the prevention of carcinogenesis, genes

coding for DNA repair molecules have been proposed as candidate cancer-susceptibility genes (1). These repair systems include nucleotide and base excision repair, mismatch repair and double strand break repair, depend on type of damaged DNA.

Base excision repair (BER) operates on small lesions such as oxidized or reduced bases, fragmented or nonbulky adducts, or those produced by alkylating agents and ionizing radiation. XRCC1 gene is located on q13.2 region of 19th chromosome. The XRCC1 protein plays an important role in BER; after excision of the damaged base, it stimulates endonuclease action and acts as scaffold in the subsequent restoration of the site (1). XRCC1 was identified by its ability to restore DNA repair activity in Chinese hamster ovary cell lines, EM-9 and EM-1, which are hypersensitive to ionizing radiation and alkylating agent (3-5). These cells have increased spontaneous and mutagen-induced sister chromatid exchange and have defects in rejoining single-strand breaks after exposure to X-ray (5, 6). Both EM-9 and EM-11 cells contain a mutated XRCC1 gene and lack XRCC1 protein (7). The absence of XRCC1 activity in the mouse is an embryo-lethal condition (8). The XRCC1 protein complexes with DNA ligase III via a BRCT domain in its COOH terminus and with DNA polymerase β via the NH₂ terminus domain to repair gaps left during BER (9). PARP detects DNA strand breaks induced by ionizing radiation and is believed to participate in BER (10). XRCC1 negatively regulates PARP by binding to it via the XRCC1 central domain (amino acids 301-402 (10)). This central region also includes a BRCT domain and shares homology to the yeast *rad4/cut5* DNA repair gene (11, 12). Functional importance of this region is also suggested by the determination that the DNA repair-deficient EM-11 cell line contains a cysteine to tyrosine mutation at codon 399 (7).

NER is a general repair pathway that is able to remove a variety of bulky DNA lesions, such as the UV-induced lesions, or adducts produced by chemical carcinogens, such as polycyclic aromatic hydrocarbons, aflatoxins, cis-platinum or psoralen crosslinks (13). The XPD gene is located on q13.2-13.3 region of 19th chromosome. The XPD protein is part of the basal transcription factor TFIIH with 5'-3' helicase activity necessary to separate the two DNA strands during both DNA repair and RNA transcription in NER pathway (14-16) and in the p53-dependent

apoptotic pathway(17-19). Identified defects at specific sites within XPD cause defects in transcription and NER, and also cause on of three different major diseases including xeroderma pigmentosum,Cockayne's syndrome and trichothiodystrophy (20).

Hereditary genetic defects in DNA repair lead to a marked increased risk of developing cancer. Although DNA repair deficiency often arises from mutations in genes that result in the loss of the DNA repair protein, DNA polymorphisms may alter the structure of the DNA repair enzymes and modulate cancer susceptibility. Mutations and polymorphisms have been identified in many of the genes coding for DNA repair enzymes such as XRCC1 and XPD (21-23).

Shen et al. (21) identified three coding polymorphisms in the XRCC1 gene at codons 194 (Arg to Trp), 280 (Arg to His) and 399 (Arg to Gln), occurring at conserved sequences. Two of the variants (Arg194Trp and Arg280Gln) reside in the linker regions separating the DNA polymerase β domain from the PARP-interacting domain (24,25). The Arg194Trp change is a nonconservative substitution occurring within a hydrophobic core. The Arg399Gln change resides at the COOH-terminal side of the PARP-interacting domain and within an identified BRCT I domain. The Arg399Gln substitution is within a relatively nonconserved region between conserved residues of the BRCT I domain. The Arg280His variant is another nonconservative substitution. Single amino acid substitutions in both the BRCT I domain and in the DNA polymerase β -interacting regions in the hamster XRCC1 have been shown completely disrupt the functionality of the XRCC1 protein (7). Arg194Trp studies reported a reduced risk of cancer associated with Trp/Trp allele (26-31). Only one small study of SCCHN (98 cases, 161 controls) estimated and increase in risk associated with Trp/Trp allele (32). No associations with Arg194Trp were seen in small studies (≤ 125 cases) of esophageal cancer (33), non-small cell lung cancer (34), or melanoma (35). A second XRCC1 polymorphism (Arg399Gln) has also been well studied: however, the results suggested associations in different directions for different cancers: decreases risk for nonmelanoma skin carcinoma (36), esophageal cancer (33), and bladder cancer (26, 37); increased risk for breast cancer (27), and stomach cell cancer (31). There were inconsistent results for squamous cell carcinoma of head and neck (SCCHN) (30, 32) and lung cancer (28, 29, 34, 38, 39), and no association was seen with melanoma (35). Several polymorphisms in the XPD gene including the most

common Lys751Gln and Asp312Asn have been reported (21). The 312 variant has the acidic moiety of aspartic acid removed and the 751 variant completely changes the electronic configuration of the amino acid. This is a major change, located in the important domain of interaction between XPD protein and its helicase activator, p44 protein inside the TFIIH complex (40). An Asn/Asn at codon 312 increases protection from lung cancer (34). In addition an alteration at amino acid 751 give some protection against basal cell carcinoma (41), but possibility increases risk among smokers and drinkers for squamous cell carcinoma of the head and neck (42). The XPD polymorphic variant at amino acid 312 did not have an altered binding efficiency for p53. However, when present in lymphoblastoid cell lines, this XPD variant produced a 2.5-fold higher apoptotic response to UV- induced damage than that shown by the majority type of XPD (43). This higher apoptotic response may account for the protective effect of this variant against lung cancer.

This thesis hypothesized that patient with polymorphisms in XRCC1 and XPD would have been associated with de novo childhood ALL compared with the control group. Furthermore, we would then know the frequencies of XRCC1 and XPD polymorphisms in Thai population, which would be useful pharmacogenetics study. The polymorphisms analyses are performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

CHATER 2

OBJECTIVES

Genetic approaches to understanding the etiology of the childhood acute lymphoblastic leukemia is beginning to deliver meaningful insights. Polymorphisms in DNA repair genes were a natural starting point to study the relevance of these changes. Biological processes are important in tumorigenesis that exhibit substantial interindividual differences, which may function as susceptibility factors. The major objective of this thesis was the identification of genetic polymorphisms of DNA repair genes, that affect the risk of childhood acute lymphoblastic leukemia.

More specifically, the overall aims were:

1. To studying the effect of the polymorphisms in DNA repair gene XRCC1, XPD on risk of childhood acute lymphoblastic leukemia.
2. To evaluate the relevance of genetic polymorphisms of DNA repair gene including XRCC1 and XPD in the Thai population to be useful in pharmacogenetics studies.

CHAPTER 3

LITERATURE REVIEW

3.1 Childhood acute lymphoblastic leukemia

Among the 12 major types of childhood cancers, leukemias (blood cell cancers) represent one-third of childhood cancers. The most common type of leukemia in children is acute lymphocytic leukemia (ALL), represent of 70% of case of childhood leukemia. ALL is a malignant neoplasm of lymphocytes characterized by clonal accumulation of immature blood cells in the bone marrow. These abnormal cells are arrested in the lymphoblast stage of the normal maturation pathway. Abberations in proliferation and differentiation of these cell are common, and normal hematopoiesis is suppressed. Symptoms result from varying degrees of anemia, neutropenia and thrombocytopenia or from infiltration of ALL cells into tissues (1).

Although ALL is the most curable form of leukemia, the price to be paid for this “therapeutic success” is enormous: growth problem, learning difficulties, sterility and higher risk of developing a secondary cancer. The impact of ALL on children and its burden on the health care system need to be reduced. Even though impressive biologic advances have increased understanding of leukemogenesis, we still largely unknown about cause of the childhood leukemia (2,3). Here, this thesis particularly reviews the genetic susceptibility to childhood acute lymphoblastic leukemia.

3.1.1 The biologic features and classification

Childhood acute lymphoblastic leukemia is a disease in which too many underdeveloped infection-fighting white blood cells, called lymphocytes, are found in a child’s blood and bone marrow. The cytomorphology characteristics of lymphoblasts are varied but rare usually sufficient to suggest a blastic or neoplastic

process for which phenotyping can confirm and further characterize the process. The most typical lymphoblast is the small to intermediate sized cell with round or oval nucleus that has smudgy nuclear chromatin, absent or small nucleoli, and scanty cytoplasm. Comparison to normal-appearing 'mature' lymphocyte in the blood and bone marrow aspirate is useful for the assessment of size and degree of chromatin condensation. The cells are larger than small lymphocytes (usually twice the size), and the chromatin is more homogeneous and less condensed and block-like. The scanty cytoplasm is quite dramatic in many cells as the nucleus has an appearance of bulging out of the cell cytoplasm. The cytoplasm is pale blue and not intensely stained. Lymphoblast with these typical features have been considered 'L1' lymphoblasts according to the French-American-British (FAB) classification scheme and are particularly common in paediatric cases, approximately 80% to 85%. In some cases, lymphoblasts exhibit significant morphology variation. Approximately 15% to 20% of childhood ALL. Such lymphoblasts are larger than the typical 'L1' lymphoblast and have oval or irregular nuclear outlines and less homogeneous chromatin. Nuclei are variable but frequently prominent, and sometimes multiple. The cytoplasm is more abundant but still pale blue. Cases with these more variable lymphoblasts usually contain at least some typical lymphoblasts, which are helpful to note, as they are less likely to be confused with myeloblasts. Cases with the morphologically varied lymphoblasts were referred to as 'L2' ALL by the FAB, but this classification is now believed to have little significance and the terminology is used here only for descriptive purposes. Other than being more common in children and adults, respectively, 'L1' and 'L2' ALL do not define specific disease entities, show no consistent correlation with phenotypic or cytogenetic features, and have not been adopted in the WHO classification of ALL, which is based on immunophenotype and genotype.

Compared to the blasts described above, blasts in cases of Burkitt lymphoma/leukemia ('L3' blast by the FAB scheme, referred to as Burkitt leukemia) are usually quite distinctive. The blasts are large and homogeneous and have distinctive deep blue cytoplasm. Which commonly contains sharply defined vacuoles. The nuclei of Burkitt cells are large and round or oval. They have finely stippled chromatin, and variable nucleoli, which sometimes are quite prominent. The larger

size and intense cytoplasmic basophilia with vacuolization are decidedly the most distinctive features but are not entirely specific. Vacuoles can be seen in other cases of ALL as well as in some cases of AML. Conversely, some cases of Burkitt leukemia with the associated chromosomal translocations lack the usual 'L3' morphology.

A number of additional cytological variants of lymphoblasts deserve mention. They can be seen in case as predominating cell type, or more commonly, as a less prominent component admixed with the 'L1' and 'L2' blasts described above. Although there are no particular clinical, phenotypic or genetic correlates with these variant blasts, their recognition will help to avoid exclusion of ALL from diagnostic consideration in cases where they are seen.

Small lymphoblasts can be seen in rare cases of ALL. These blasts are closer in size to small 'mature' lymphocytes, making them difficult to distinguish from the small lymphoid cells of chronic lymphocytic leukemia (CLL). The small lymphoblasts also have more condensed chromatin making the distinction further difficult. Lymphoblast with cytoplasmic granulation can be seen in small percentage of ALL cases. The granules are usually present in the larger blasts rather than in small 'L1' type. They are azurophilic granules seen in myeloblasts but are not distinguishable from the granules seen in natural killer cells. Nuclear clefts can be seen in lymphoblasts. They are usually present as deep nuclear grooves and are unlike the clefts seen in circulating follicular lymphoma cells. The so called hand-mirror cell is probably not a defining characteristic for certain entity.

Phenotypic evaluation begins with cytochemical studies, and specifically with a myeloperoxidase or Sudan black B reaction, as well as non-specific esterase reactions (ANA, ANB) to quickly exclude most cases of AML. AML with minimal differentiation (FAB: M0). Erythroleukemia and megakaryoblastic leukemia are the exceptions as these require additional studies for exclusion. Additional cytochemistry, such as PAS and oil red O, are being used less commonly due to the reliance on surface and cytoplasmic markers evaluated by flow cytometry and immunohistochemistry.

Table 3.1 FAB classification of ALL

L1	L2	L3
Small	Larger	Large
Almost no cytoplasm	About 20% cytoplasm	Basophilic cytoplasm, cytoplasmic vacuolation
Round to cleaved nuclei, perinuclear chromatin	More prominent nucleoli	

Immunophenotypic analysis is critical to confirm a morphological diagnosis of ALL, to resolve a difficult differential diagnosis and to further subclassify cases into precursor-B and precursor-T lineage types. However, a specific immunophenotype identified at diagnosis might also be useful for evaluating residual disease by flow cytometry. Most immunophenotyping studies are performed from blood or marrow aspirates with surface and cytoplasmic markers by flow cytometry, but a growing number of markers are now available for immunophenotyping on tissue sections by immunohistochemical techniques. This is of importance especially in cases which have low peripheral blast counts and in which bone marrow material is insufficient for flow analysis or in which a diagnosis is being made from an extramedullary site. ALL blasts are derived from either B-cell or T-cell lineages, as determined by cell surface and other markers (**Figure 3.1**).

The vast majority of cases of ALL (~85%) are of B lineage. These have been grouped into further subtypes, which may correspond to different levels of maturation in normal B cell development. However, such differentiation schemes are not universally agreed upon and the terminology for different subtypes is also not uniform. In fact, due to the lack of conformity, and questionable significance of the further sub-classification, the WHO classification scheme simply classifies cases as “precursor-B” and “precursor-T” ALL without additional categorization. The most common B lineage ALL is the precursor-B phenotype with B cell markers (CD19, CD22), TdT, cytoplasmic CD79A, CD34, CD10 (CALLA), and lack of cytoplasmic μ

and of surface immunoglobulin (sIg) expression. This type have variably been called “common precursor B ALL” or “early precursor-B ALL”. A less common type lacks CALLA and may be at an earlier level of maturation that has been termed “pre-B ALL”. This type has a worse prognosis. A type with more maturation than common precursor-B ALL. Reports are conflicting, but this may be more commonly associated with t(1;19)(q23;p13). Burkitt leukemia has the immunophenotype of mature B cells with sIg expression. Whether rare cases of non-Burkitt ALL also exhibit a mature B phenotype (sIg⁺) is questionable, although such cases have been reported on.

T lineage ALL accounts for only 15-20% of cases and can also be separated into phenotypic groups which may correspond to different stages of thymic T cell development. As in B-lineage ALL, a type with intermediate differentiation is the most common. This “common thymocyte” type shows expression of the pan T cell markers, CD2, cytoplasmic CD3 (cCD3), CD7, CD5 and distinctively shows coexpression of CD4 and CD8, and expression of CD1a. A more primitive type called “prothymocyte” or “immature thymocyte” type has TdT, cCD3 and variable expression of CD5, CD2 and CD7, but lacks CD4, CD8 and CD1a. A phenotype more mature than the ‘common thymocyte’ type has variable TdT, the pan T cell markers, CD4 or CD8 but lack of CD1a. Again, because of lack of conformity and variability of the marker expression, the WHO classification recognizes only the “precursor-T” group without further immunophenotypic categorization. B-lineage and T-lineage ALL phenotype are listed in **Table 3.2** (1, 4).

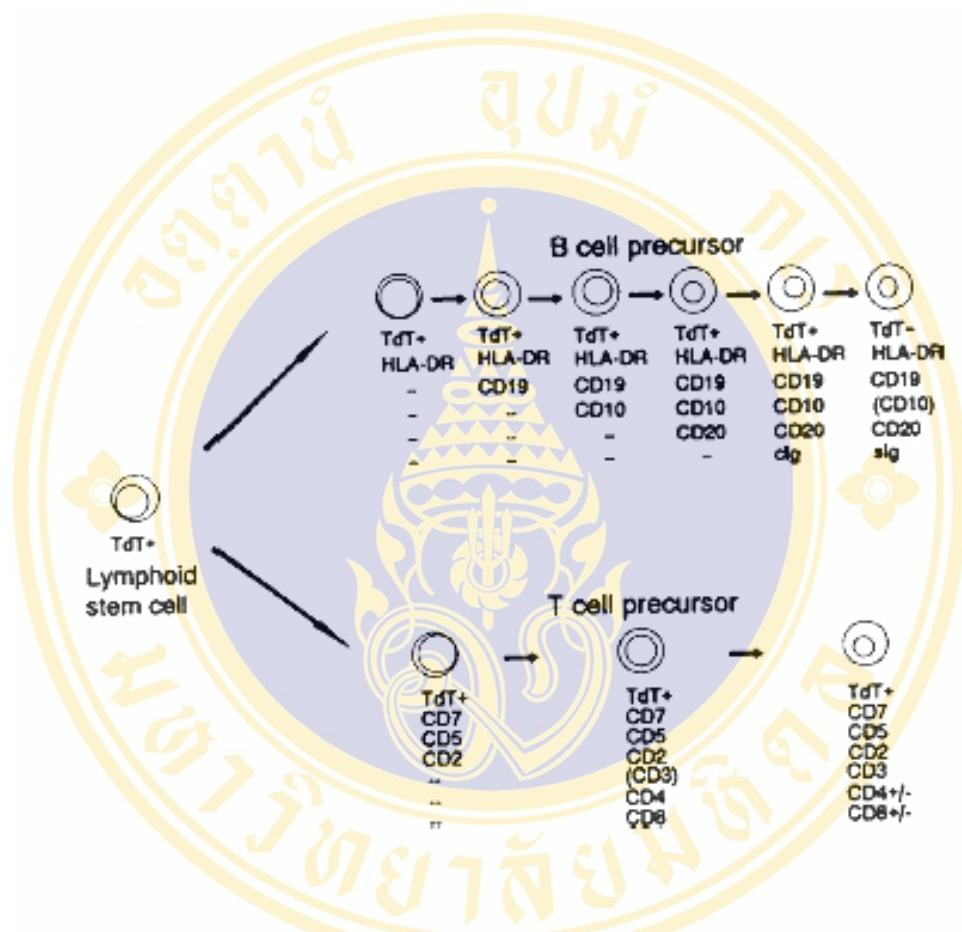


Figure 3.1 Human lymphoid differentiation: immunologic characteristics.

Table 3.2 Immunophenotype of subtype ALL with biologic characterization.

Lineage	ALL Subtype	Immunophenotype	Comment
B	Early pre-B ALL	HLA-DR, TdT, CD34, CD10, CD19/22/79A	70% of childhood ALL. Associated with favorable prognosis.
	Pre-B ALL	HLA-DR, TdT, CD34, CD10, CD19/22/79A, CD20, cytoIgH	20% of childhood ALL. Almost are classified as FAB 'L1'. Associated with great risk of marrow and CNS relapse, and shorter survival than early pre-B ALL.
	Mature B ALL	HLA-DR, CD10, CD19/22/79A, CD20, surIgH	Approximately 1-2% of childhood ALL with age older than those with other B-lineage subtype. Approximately 75% of cases share morphologic (FAB 'L3') and cytogenetic similarity with Burkitt's lymphoma. A higher incidence of CNS involvement with poor prognosis.
T	T ALL	CD2, CD3, CD5, CD7, CD4, CD8	Occurs more often in male and is associated with a high WBC count at diagnosis, and a poor prognosis. 50-60% of patients present with mediastinal mass with higher incidence of CNS involvement than other subtypes. FAB 'L2' morphology.

CD: Cluster of differentiation, HLA-DR: Human leukocyte antigen subclass DR, TdT: Terminal deoxynucleotidyl transferase, cyto: Cytoplasmic, sur: Surface, IgH: Immunoglobulin heavy chain.

3.1.2 Epidemiology and pathogenesis

Childhood ALL is the heterogenous groups of leukemias, all sharing a common characteristic of lymphoid markers and morphology. This disease account for approximately 75% of newly diagnosed leukemias and 25% of cancers in childhood. The peak incidence occurs between the ages of 3 to 5 years of age. Studies done by the Surveillance Epidemiology and End Results (SEER) Program indicate a 20% cumulative increase in the incidence of childhood ALL from 1971 to 1993. These findings may be attributed to increased exposure to environmental factors and possibly some “carcinogenic” factors.

After its early peak, the incidence of this disease declines and subsequently begins to rise again early in the third decade of life. The etiologic importance of this early peak is unclear, but may be related to the initial development of the immune system.

Acute lymphocytic leukemia occurs more often in males than females (1.2:1.0). The exact figures are variable. Sex is also an important prognostic factor, which females having a better survival and disease-free survival. The socioeconomic status and race are also important predisposing factors. The occurrence of ALL in Caucasians is greater than in African-American. Seasonal variations influencing the incidence of ALL have been the subject of several investigations, but geographic variations seems to have more influence on the incidence and frequency of this disease. Numerous other constitutional, genetic congenital or neurologic defect and environmental factors are associated with the development of ALL (4).

In ALL, a lymphoid progenitor cell becomes genetically altered and subsequently undergoes dysregulated proliferation and clonal expansion of hematopoietic progenitors blocked in differentiation at discrete stages of development. Recent data challenge this theory and suggest that leukemia arises from the stem cell that acquires features of differentiated cells. The malignant transformation of a single abnormal progenitor cell leading to an infinite self-renewal is sufficient to induce a leukemia. Responsible of the malignant transformation of lymphoid cell reflects the altered expression of genes whose products contribute to normal development of B cells and T cells. Indeed, during the normal lymphoid evolution, along with the gene

rearrangement processes, the lymphocytes precursors have a high risk of spontaneous mutations because of their high proliferation rate with intrinsic mutagenic activity.

3.2 DNA repair and childhood acute lymphoblastic leukemia

Although no one really knows causes of ALL in humans, leukemic transformation is unlikely to be the result of a single event but rather the culmination of multiple processes involving complex interactions (1), many certain risk factors make individuals more prone to developing leukemia. Many factors, such as age and genetics, are probably beyond our control. Other factors, such as environmental or lifestyle-related variables, may be more correctable. It is now known that all cancer, including leukemia, begin with a mutation in the genetic material, the DNA (deoxyribonucleic acid), within certain cells. The external or internal causes of such change probably add up over a lifetime. Leukemia begins when one or more white blood cell experience DNA loss or damage. Those errors are copied and passed on the subsequent generations of cells. The abnormally leukemic cells remain in an immature blast form that never matures properly. They do not die off like normal cells, but tend to multiply and accumulate within the body.

DNA in most cells is regularly damaged by endogenous and exogenous mutagens. Unrepaired damage can result in apoptosis or may lead to unregulated cell growth and cancer. If DNA damage is recognized by cell machinery, several responses may occur to prevent replication in the presence of genetic errors. At the cellular level, checkpoints can be activated to arrest the cell cycle, transcription can be up regulated to compensate for the damage, or the cell can apoptose. Because of the importance of maintaining genomic integrity in the general and specialized functions of cells as well as in the prevention of carcinogenesis, genes coding for DNA repair molecules have been proposed as candidate cancer-susceptibility genes (1).

DNA repair pathways are usually specific for a class of given damage: mismatched structural abnormalities at the replication forks are repair by mismatch repair pathway; double-stranded breaks are repair by homologous or illegitimate recombination process; damaged bases are usually by base excision repair (BER); and

bulky DNA lesions are exclusively repair by the nucleotide excision repair (NER) pathway (13).

3.3 Base excision repair (BER)

BER is a major DNA repair pathway protecting mammalian cells against single base DNA damage caused by methylating and oxidizing agents, other genotoxicants, and a large number (about 10,000 per cell per day) of spontaneous depurinations. BER is mediated through at least two subpathways, one involving single nucleotides. BER can be initiated through removed base by an DNA glycosylase, which bind the altered deoxynucleoside in an extrahelical position and catalyze cleavage of the base-sugar bond. This generates an apurinic/apyrimidic site (AP site). BER can also occur at a site of spontaneous depurination. An AP endonuclease, then makes a 5' nick in the DNA backbone. This is followed by poly (ADP-ribose)polymerase-1 (PARP-1) acting as a nick surveillance protein, binding to the nicked DNA. PARP-1 binds even more strongly if there is a stalled single nucleotide BER block at the excision step. Subpathway, repair patch synthesis and DNA ligation complete the process (**Figure 3.2**) (51).

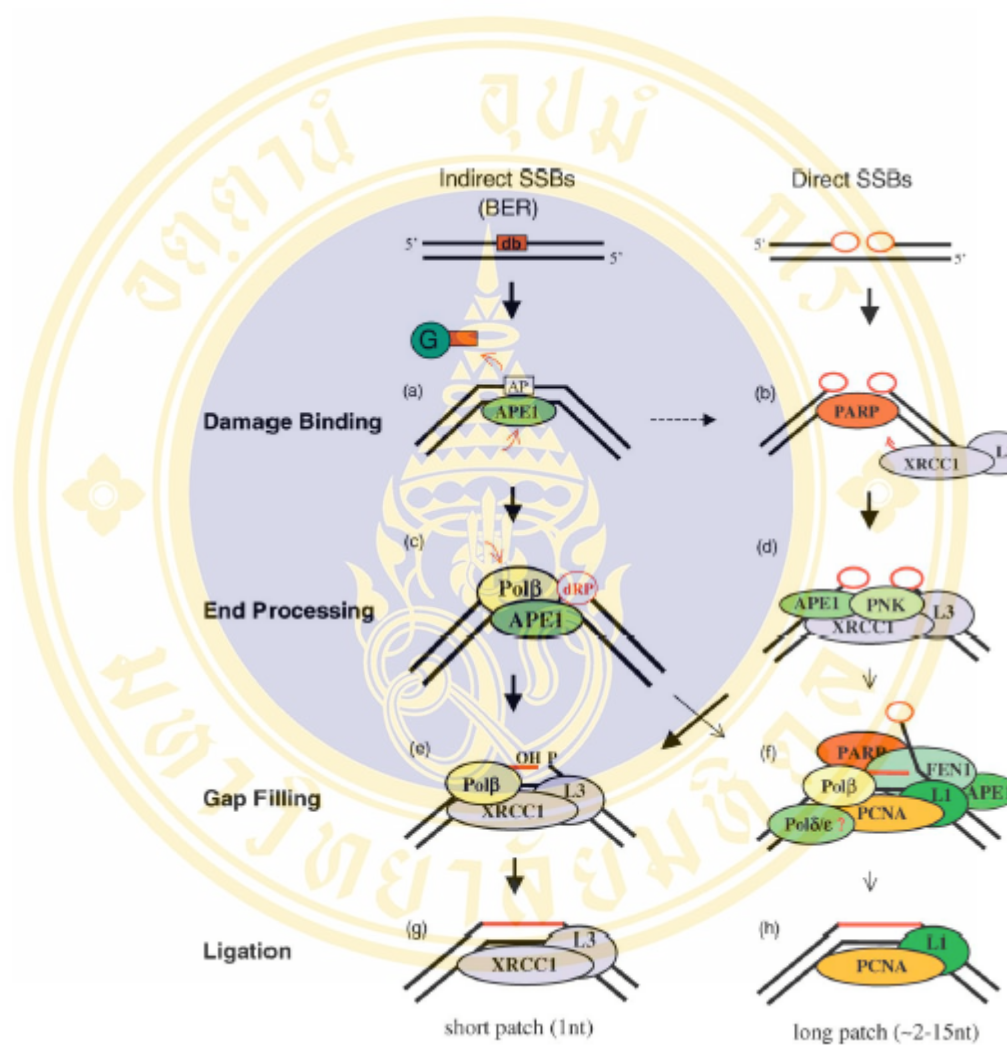


Figure 3.2 Base excision repair (BER) (modified from Caldecott KW. (66))

3.4 Nucleotide excision repair (NER)

NER repairs DNA with helix-distorting damages, including the damages of cyclobutane pyrimidine dimers and 6-4 photoproducts produced by UV light, and adducts produced by the chemotherapeutic agents cisplatin and 4-nitroquinoline oxide (52,53). About 30 polypeptides are involved in NER, and the NER process has been reconstituted with purified components (54). Key steps of NER include: recognition of DNA defect; recruitment of a repair complex; preparation of the DNA for repair through action of helicase; incision of the damaged strand on each side of the damage, with release of the damage in a single-stranded fragment about 24-32 nucleotides long; filling in of the gap by repair synthesis; ligation to form the final phosphodiester bond (53, 55). Two subpathways of NER are global genomic repair (GGR)(**Figure 3.3, 3.4**) and transcription coupled repair (TCR)(**Figure 3.5, 3.6**). These pathways are initiated somewhat differently, with GGR acting on damages in non-transcribed regions of DNA and TCR acting on damages in actively transcribed DNA. However, after initiation, most enzymatic steps utilize the same enzymes and enzyme complexes (53, 55).

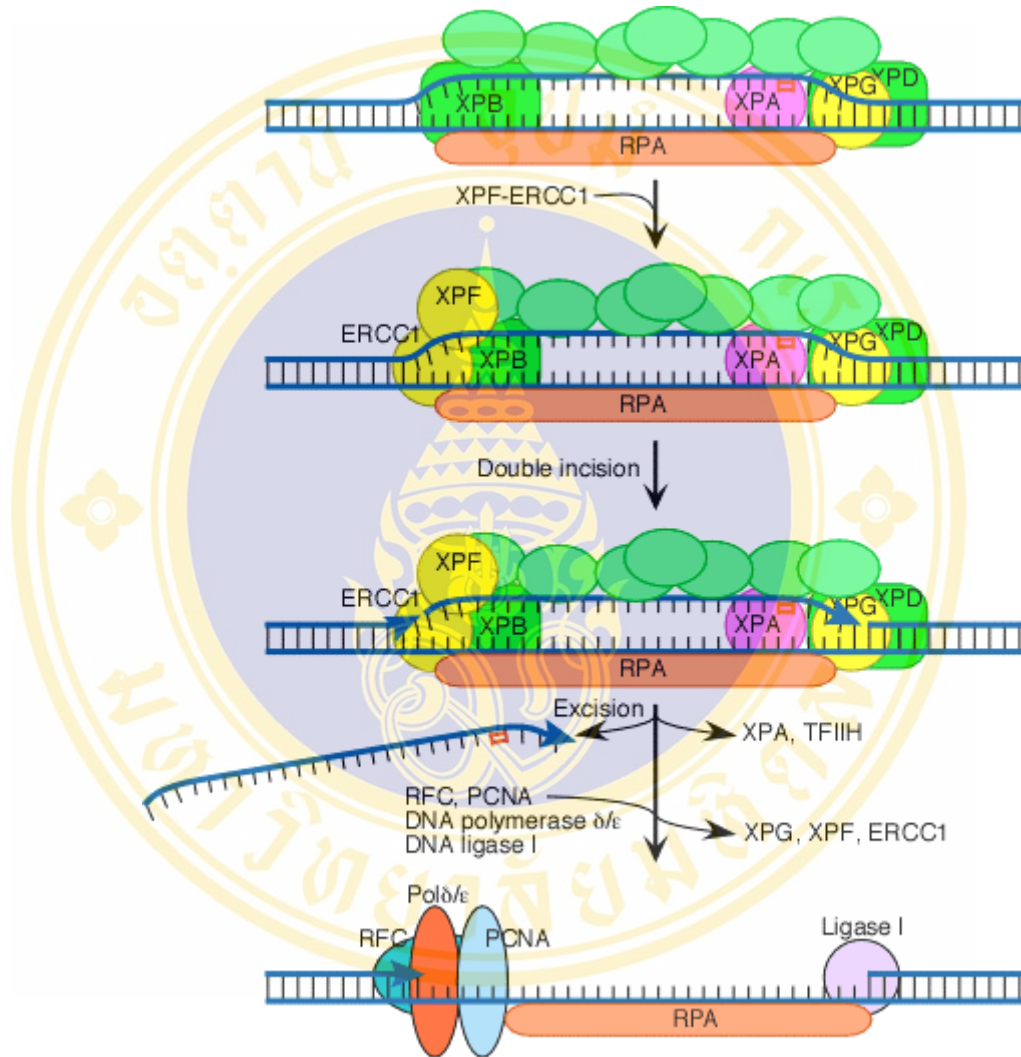


Figure 3.4 Nucleotide excision repair in global genomic repair (GGR) (continue).

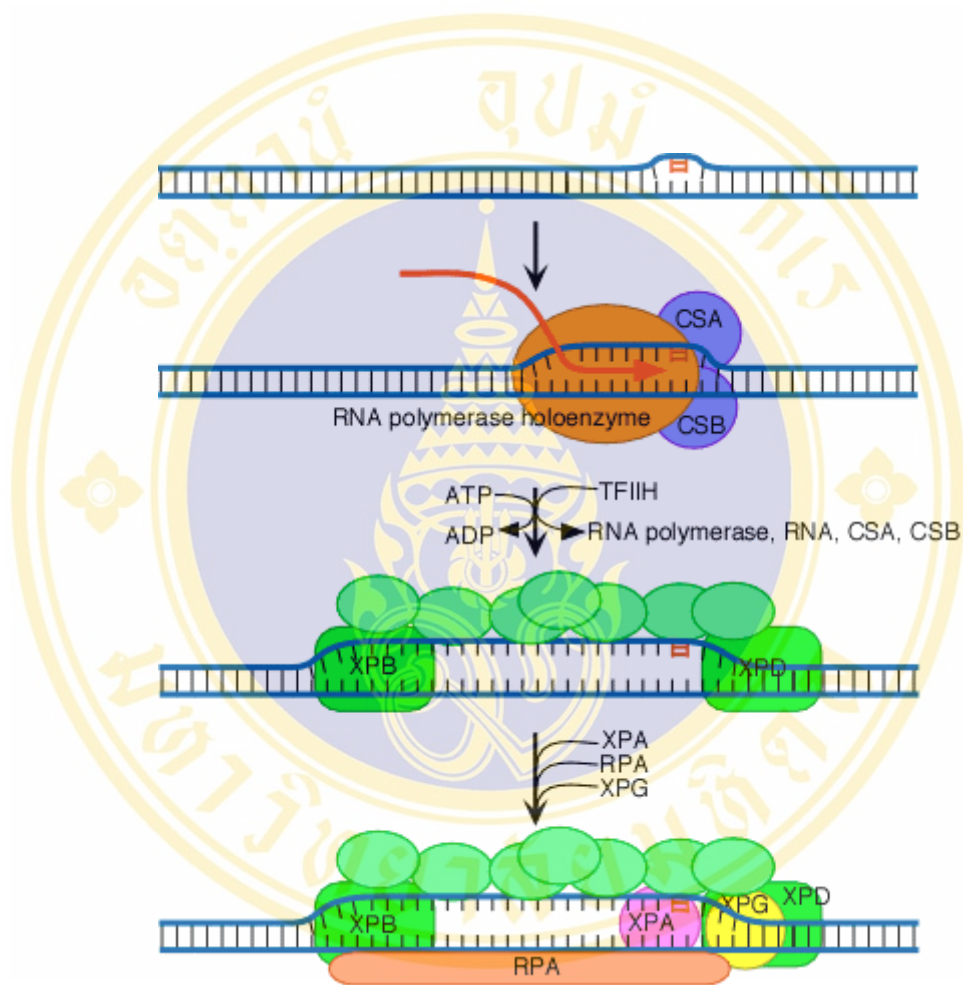


Figure 3.5 Nucleotide excision repair in transcription coupled repair (TCR)

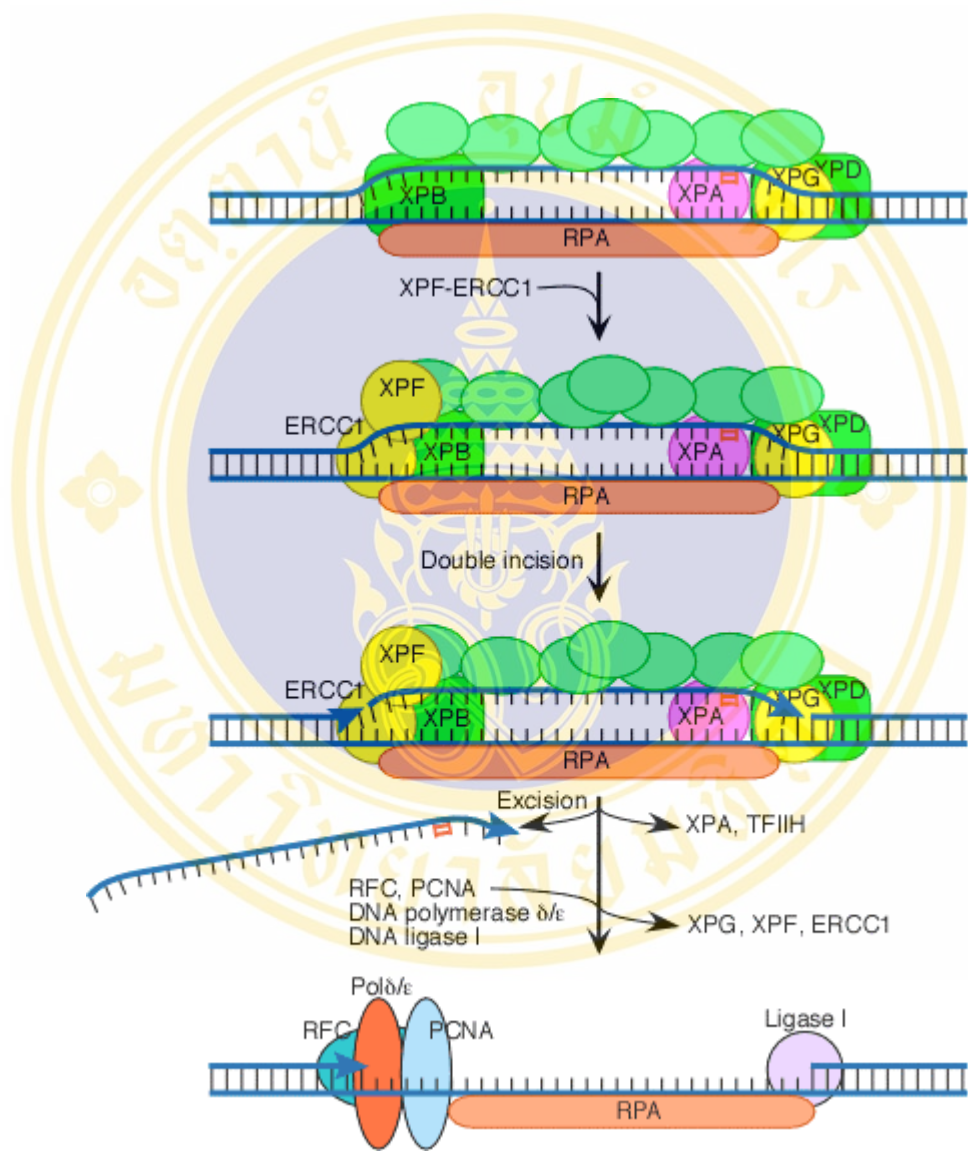


Figure 3.6 Nucleotide excision repair in transcription coupled repair (TCR) (continue).

3.5 Genetic polymorphisms in DNA repair and cancer risk

Everyone has a unique combination of polymorphic traits that modify susceptibility and respond to drugs, chemical and carcinogenic exposures. Genotypic and phenotypic variation in DNA repair may affect risk of somatic mutation and cancer (56). More than 130 human DNA repair genes have been described (57) and a number of single-nucleotide polymorphisms (SNPs) in DNA repair genes have been identified (21, 58-59). These variants of DNA repair enzymes either increase the activation of chemical carcinogens or decrease the cell's ability to repair mutagenic damages. Although on an individual basis these variant alleles may only slightly change catalytic activity and increase cancer risk. Identifying these genotypic alterations in DNA repair enzymes and their association with cancer may help to elucidate the mechanisms of cancer etiology and to predict both disease risk and response to cancer therapy, since most antineoplastic treatments mediate their effects through DNA damage (60).

DNA damage in a cell is complex, even when the exposure is relatively simple (61, 62). Mammalian cells have developed four, generally nonredundant pathways to repair this complex array of damages, include: BER involves 25 genes that process damaged bases, abasic sites, and other damage, mostly resulting from the action of free radicals (63); NER pathway include over 35 genes and removes UV-induced damage (e.g., pyrimidine dimers) and bulky DNA adducts associated with chemical exposures (64, 65).

3.5.1 XRCC1 genetic polymorphisms

XRCC1 protein plays an important role in BER; after excision of a damaged base, it stimulates endonuclease action and acts as a scaffold in the subsequent restoration of the site (1). Human XRCC1 was cloned more than 10 years ago, yet experimental analysis of the XRCC1 gene product is still unveiling new insights into the DNA damage response. The most striking feature of XRCC1 to have emerged during the past 10 years is its ability to interact with other DNA repair proteins. Indeed, although lacking any known enzymatic activity itself, XRCC1

interacts with enzymatic components of each stage of DNA strand break repair. The interactions known to date are summarised in Figure 3-5 (66). XRCC1 was identified by its ability to restore DNA repair activity in Chinese hamster ovary cell lines, EM-9 and EM-1, which are hypersensitive to ionizing radiation and alkylating agent (3-5). These cells have increased spontaneous and mutagen-induced sister chromatid exchange and have defects in rejoining single-strand breaks after exposure to X-ray (5,6). Both EM-9 and EM-11 cells contain a mutated XRCC1 gene and lack XRCC1 protein (7). The absence of XRCC1 activity in the mouse is an embryo-lethal condition (8). The XRCC1 protein complexes with DNA ligase III via a BRCT domain in its COOH terminus and with DNA polymerase β via the NH₂ terminus domain to repair gaps left during BER (9). PARP detects DNA strand breaks induced by ionizing radiation and is believed to participate in BER (10). XRCC1 negatively regulates PARP by binding to it via the XRCC1 central domain (amino acids 301-402 (10)). This central region also includes a BRCT domain and shares homology to the yeast *rad4/cut5* DNA repair gene (11, 12). Functional importance of this region is also suggested by the determination that the DNA repair-deficient EM-11 cell line contains a cysteine to tyrosine mutation at codon 399 (7).

Shen et al. (21) identified three coding polymorphisms in the XRCC1 gene at codons 194 (Arg to Trp), 280 (Arg to His) and 399 (Arg to Gln), occurring at conserved sequences (**Figure 3.7**). Two of the variants (Arg194Trp and Arg280Gln) reside in the linker regions separating the DNA polymerase β domain from the PARP-interaction domain (24, 25). The Arg194Trp change is a nonconservative substitution occurring within a hydrophobic core. The Arg399Gln change resides at the COOH-terminal side of the PARP-interacting domain and within an identified BRCT domain. The Arg399Gln substitution is within a relatively nonconserved region between conserved residues of the BRCT domain. The Arg280His variant is another nonconservative substitution. Single amino acid substitutions in both the BRCT domain and in the DNA polymerase β -interacting regions in the hamster XRCC1 have been shown completely disrupt the functionality of the XRCC1 protein (7). Arg194Trp studies reported a reduced risk of cancer associated with Trp/Trp allele (26-31). Only one small study of SCCHN(98 cases, 161 controls) estimated an increase in risk associated with Trp/Trp allele (32). No associations with Arg194Trp were seen in

small studies (≤ 125 cases) of esophageal cancer (33), non-small cell lung cancer (34), or melanoma (35). A second XRCC1 polymorphism (Arg399Gln) has also been well studied: however, the results suggested associations in different directions for different cancers: decreased risk for nonmelanoma skin carcinoma (36), esophageal cancer (33), and bladder cancer (26, 37); increased risk for breast cancer (27), and stomach cell cancer (31). There were inconsistent results for SCCHN (30, 32) and lung cancer (28, 29, 34, 38, 39), and no association was seen with melanoma (35).

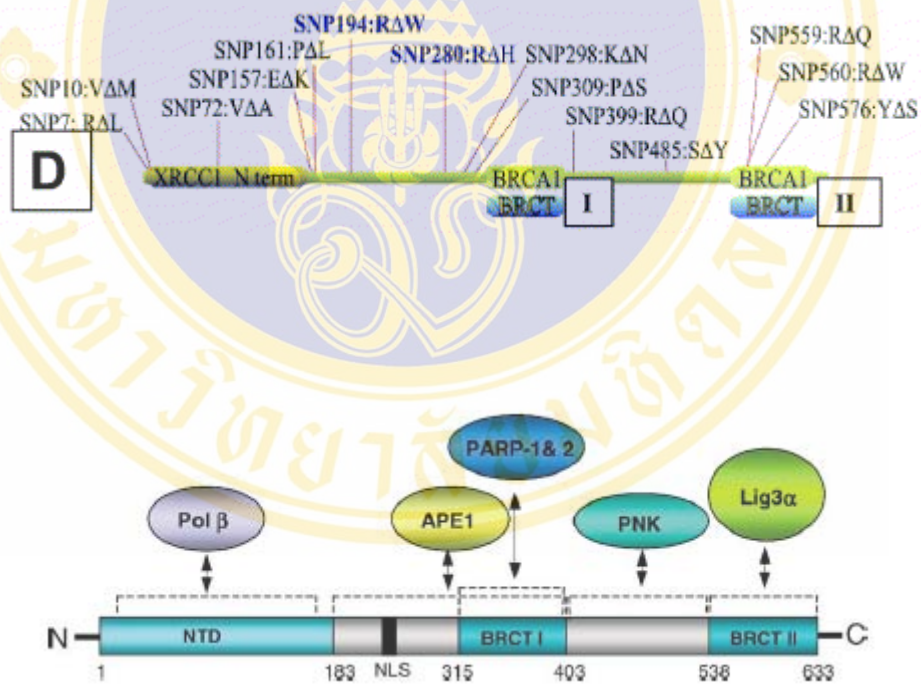


Figure 3.7 Polymorphisms of XRCC1 gene and binding domains of XRCC1 protein (modified from Caldecott KW. (66))

3.5.2 XPD genetic polymorphisms

Alterations at specific sites within the XPD protein have been identified as affecting one of two different primary functions of XPD: stabilization of the transcription factor complex TFIIH, and a 5' to 3' helicase function most strongly expressed when XPD is part of TFIIH complex (20). The helicase function of XPD is essential for NER. When XPD helicase function is defective due to an alteration in its ATP hydrolysis region, neither 5' nor 3' incision in defined positions around a DNA adduct can be detected (67). XPD function is also required for p53-mediated apoptosis (18, 19).

Several polymorphisms in the XPD gene (**Figure 3.8**) including the most common Lys751Gln and Asp312Asn have been reported (21). The 312 variant has the acidic moiety of aspartic acid removed and the 751 variant completely changes the electronic configuration of the amino acid. This is a major change, located in the important domain of interaction between XPD protein and its helicase activator, p44 protein inside the TFIIH complex (40). An Asn/Asn at codon 312 increases protection from lung cancer (34). In addition, an alteration at amino acid 751 give some protection against basal cell carcinoma (41), but possibly increases risk among smokers and drinkers for squamous cell carcinoma of the head and neck (42). The XPD polymorphic variant at amino acid 312 did not have an altered binding efficiency for p53. However, when present in lymphoblastoid cell lines, this XPD variant produced a 2.5-fold higher apoptotic response to UV- induced damage than that shown by the majority type of XPD (43). This higher apoptotic response many account for the protective effect of this variant against lung cancer.

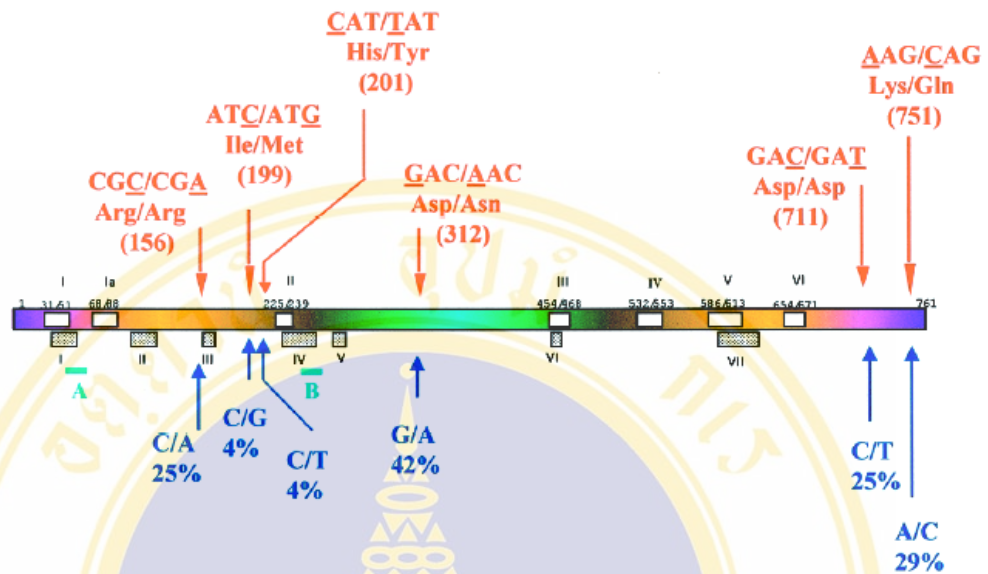


Figure 3.8 Polymorphisms of XPD gene

3.6 Genotyping methods

The most common type of human genetic variant is the single nucleotide polymorphisms (SNPs), a position at which two alternative bases occur an appreciable frequency (>1%) in the human population. Understanding the relationship between genetic variation and biological function on a genomic scale is suspected to provide fundamental new insights into the biology, evolution and pathophysiology of human and other species. The hope that single nucleotide polymorphisms (SNPs) will allow genes that underline complex disease to be identified, together with progress in identifying large set of SNPs, are the driving forces behind intense efforts to establish the technology for large-scale analysis of SNPs.

SNPs are usually used in high-throughput projects, so detection methods need to be appreciated to facilitate analysis of large numbers of polymorphisms and patient samples. Most methods require target sequence amplification followed by determination of distinct sequence variants by short hybridization probes, restriction endonucleases, discrimination of mismatched DNA substrates by polymerases or

ligases, or by observing templated-dependent choice of nucleotide incorporated by a polymerase.

3.6.1 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

Commonly used methods include gel electrophoresis-based techniques such as PCR coupled with restriction fragment length polymorphisms analysis. This method involves digestion of a PCR product with a restriction enzyme that distinguishes between the wild-type and mutant sequence. The method is most valuable when the polymorphisms introduce a direct alteration in a restriction site so that a restriction site is lost or created, but even if there is no change in restriction enzyme sites as a site of polymorphism, it may also be possible to engineer one or more base changes using a mismatched primer so that a new site seen only for either the mutant or wild-type sequence, is created. Restriction patterns are analyzed by gel electrophoresis on either agarose gels when the difference in fragment sizes is greater than approximately 40 bp or polyacrylamide gels where the difference is smaller such as in the case of engineered restriction site (68). Restriction site cleavage is shown in **Figure 3.9**. Restriction endonuclease of the DNA when the SNP alter the specific-recognition site for the restriction enzyme. Target molecules with intact recognition site will be cleaved, where as target molecules with altered site remain uncleaved.

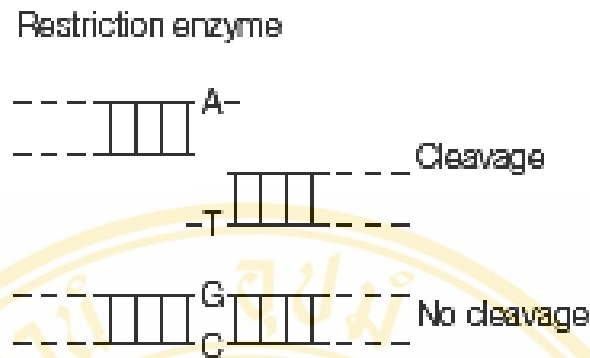


Figure 3.9 Principle for SNP genotyping by restriction enzyme method

3.6.2 Hybridization methods

With the hybridization approach, two allele-specific probes are designed to hybridize to the target sequence only when they match perfectly (**Figure 3.10**). Under optimized assay conditions, the one-base mismatch sufficiently destabilizes the hybridization to prevent the allelic probe from annealing to the target sequence. The challenge to ensure robust allelic discrimination lies in the design of the probe.

When the allele-specific probes are immobilized on a solid support, labeled target DNA samples are captured, and the hybridization event is visualized by detecting the label after the unbound targets are washed away. Knowing the location of the probe sequences on the solid support allows one to infer the genotype of the target DNA sample (69).

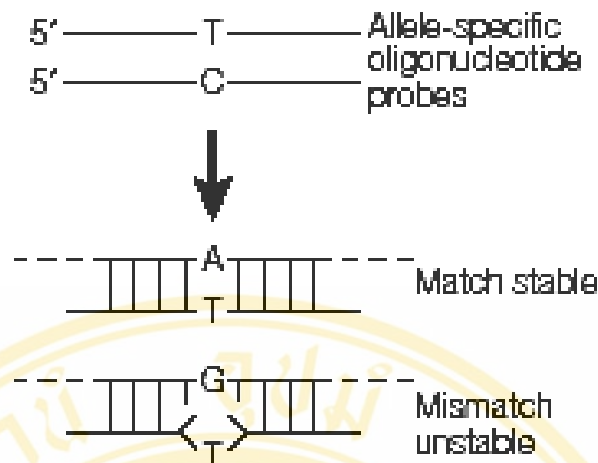


Figure 3.10 Allele-specific hybridization.

3.6.3 Primer extension

Primer extension is a very robust allelic discrimination mechanism. It is highly flexible and requires the smallest number of primers/probes. Probe design and optimization of the assay are usually very straightforward. There are numerous variations in the primer extension approach that are based on the ability of DNA polymerase to incorporate specific deoxyribonucleosides complementary to the sequence of the template DNA. However, they can be grouped into two categories. First is a sequencing (allele-specific nucleotide incorporation) approach where the identity of the polymorphic base in the target DNA is determined. Second is an allele-specific PCR approach where the DNA polymerase is used to amplify the target DNA only if the PCR primers are perfectly complementary to the target DNA sequence.

In the sequencing approach, one can either determine the sequence of amplified target DNA directly by mass spectrometry or perform primer extension reactions with amplified target DNA as a template and analyze the products to determine the identity of the bases incorporated at the polymorphic site (allele-specific nucleotide incorporation; see **Figure 3.11**). A number of ingenious ways have been devised for primer extension product analysis in homogeneous assays. Most of these approaches combine novel nucleic acid analogs and monitoring of interesting differences in physical properties between starting reagents and primer extension products.

In the allele-specific PCR approach, one relies on the DNA polymerase to extend a primer only when its 3' end is perfectly complementary to the template (**Figure 3.12**). When this condition is met, a PCR products is produced. By determining whether a PCR product is produced or not, one can infer the allele found on the target DNA. Several innovative approaches have been utilized to detect the formation of specific PCR products in homogeneous assays. Some are bases on melting curve analysis, and some are based on hybridization of target specific probes. A variation of this approach is the allele-specific primer extension. Here, the PCR product containing the polymorphic site serves as template, and the 3' end of the primer extension probe consists of the allelic base. The primer is extended only if 3' base complements the allele present in the target DNA. Monitoring the primer extension event, therefore, allows one to infer the alleles found in the DNA sample (69).

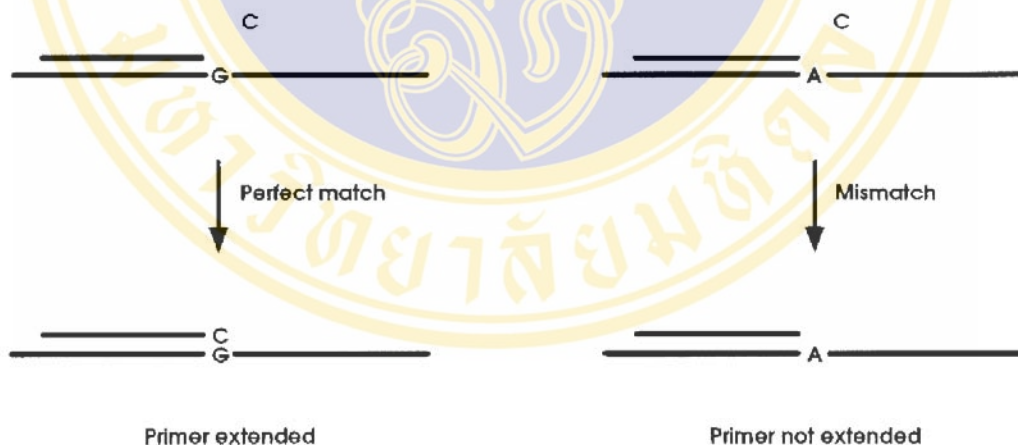


Figure 3.11 Allele-specific nucleotide incorporation

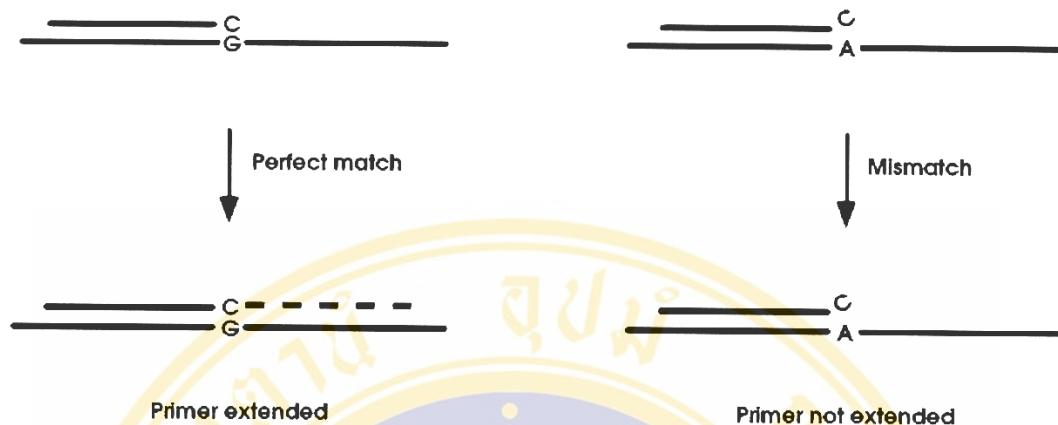


Figure 3.12 Allele-specific primer extension

3.6.4 Homogeneous hybridization assays and homogeneous allele-specific PCR

Allele-specific hybridization is also the basis of several elegant homogeneous genotyping assays. These assays differ in the way they report the hybridization event (69). At present, the most widely used ASO hybridization methods distinguish between the SNP alleles in real time during PCR in homogeneous, solution-phase hybridization reactions with fluorescence detection. The TaqManTM or Molecular Beacon probes, which were originally designed for quantitative PCR analysis, can also be applied to SNP genotyping.

The TaqManTM and Molecular Beacon assays are both based on a principle of energy transfer in which fluorescence is detected as a result of a change in physical distance between a reporter fluorophore and a quencher molecule on hybridization of ASO probe to its perfectly matched target sequence (**Figure 3.13**) (70).

The example illustrates the detection of an A-to-G transition. Panel a shows the 5'-exonuclease (TaqManTM) assay: allele-specific oligonucleotide probes are labelled with different fluorophores (F_A(blue) or F_G(red)) at their 3'-ends and with a quenching their fluorescence. The allele-specific probes are included in the PCR reaction mixture. During the annealing phase of PCR, the probes hybridize to the

strands of PCR products and during the extension phase of PCR, the 5'-3' exonuclease activity of the DNA polymerase degrades perfectly matched, annealed probes. The fragmented probes are released into the solution, separating the fluorophore from the quencher, which leads to an increase in fluorescence. Mismatched probes are displaced from the target without degradation. Molecular Beacon probes (shown in panel b) consist of a sequence that is complementary to the target sequence and a short stretch of self-complementary 5'- and 3'-nucleotides with a fluorophore (F_A or F_G) at the 5'-end and a DABCYL moiety as quencher (D) at the 3'-end. When free in solution, the Molecular Beacon probes adopt a stem-loop structure that brings the fluorophore and quencher into close proximity. When a Molecular Beacon probe hybridizes to the perfectly matched target during the primer annealing phase of PCR, the stem-loop structure opens, and the distance between quencher and fluorescent molecule increases, which restores the fluorescence. Mismatched probes readily adopt the stem-loop structure. It should be noted that the physical energy transfer mechanism differs between the TaqMan™ probes, with the fluorophore TAMRA as quencher, and the Molecular Beacon probes, with DABCYL as a quencher (70).

The Molecular Beacon probes described above have been adapted to allele-specific PCR primers for homogeneous SNP-genotyping assays. The incorporation of the primer into the PCR product releases the fluorescent label from the action of the quencher molecule.

Allele-specific PCR has been rationalized by using primary allele-specific PCR primers that contain a universal 5'-tail sequence that becomes part of the PCR product on amplification. A universal pair of secondary-energy-transfer-labelled, hairpin-structured primers can therefore be used for all SNPs (**Figure 3.13**).

Universal allele-specific energy transfer primers are shown in panel c, primary allele-specific PCR primers carry different 5'-tail sequences. The secondary primers consist of a 3'-sequence complementary to the tail sequences and a sequence that is held in a hairpin loop conformation by complementary stem sequences similar to the Molecular Beacon probes. During the primary allele-specific PCR, the 5'-tail sequence of the primer becomes incorporated into the PCR product. The energy transfer primer is used to initiate a secondary PCR. On synthesis of reverse strand of the PCR product, the stem-loop structure of the secondary primers opens, separating

the quencher from the fluorophore, and therefore restoring the fluorescence.

Fluorescence measurement is shown in panel d. The increase in the fluorescence at the emission wavelengths for the fluorophores F_A or F_G is monitored in real time during PCR. At the threshold cycle, the fluorescence rises to a detectable level and increases as more PCR products accumulate. Alternatively, the result can be interpreted by measurement of fluorescence intensity at the end point of PCR. (DABCYL, 4-dimethylaminoazobenzene-4'-sulphonyl; ET, energy transfer; TAMRA, 6-carboxytetra-methylrhodamine.) (70).

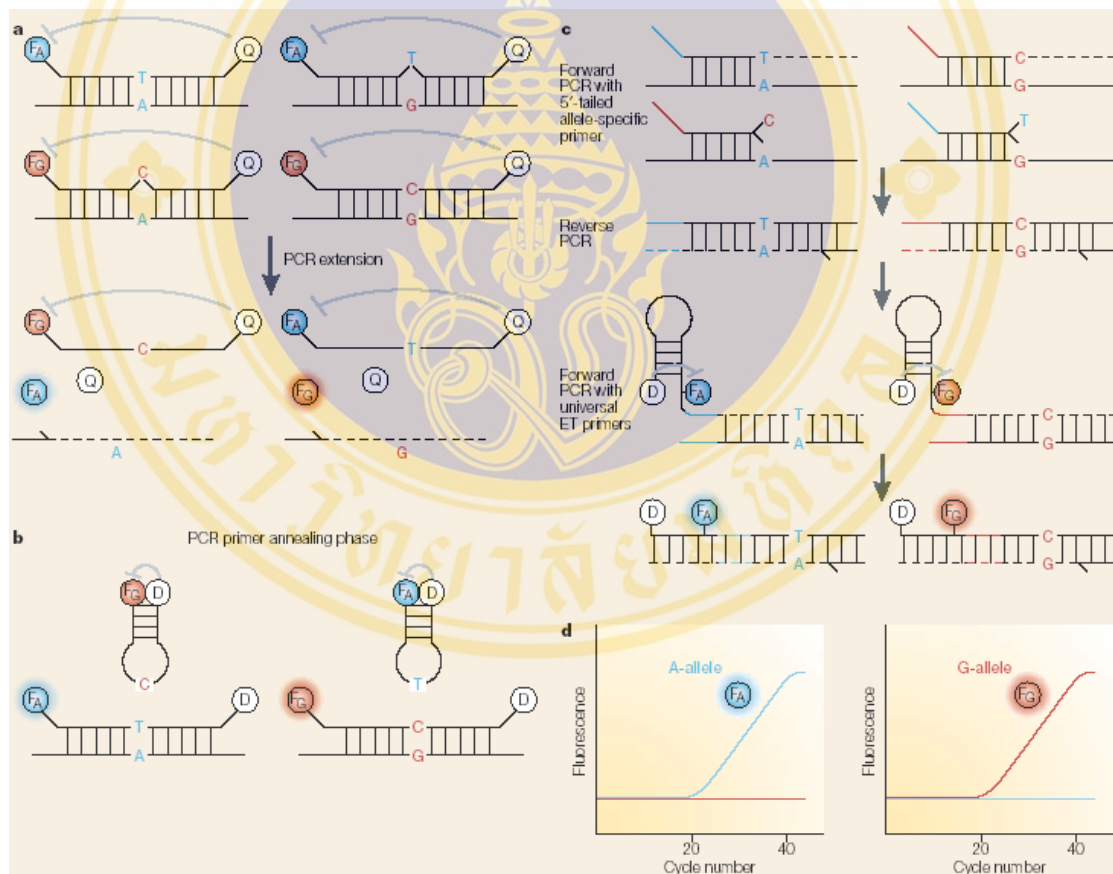


Figure 3.13 Principle for genotyping by homogeneous hybridization assays and homogeneous allele-specific PCR

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

4.1.1 Subjects

The case group of the study consisted of 108 patients with childhood ALL. They were diagnosed at the Division of Pediatric Hematology-Oncology, Ramathibodi Hospital, Department of Pediatrics Faculty of Medicine, Mahidol University. The criteria for inclusion in this group were: (1) complete clinical history and (2) availability of biological material.

A general population control group composed of 317 samples was collected from healthy volunteer. The criteria for inclusion in control group were: (1) Unrelated with malignancy and (2) availability of blood sample. Informed consent was obtained from all subjects.

4.1.2 Instruments

1. Automated thermal cycler (Mastercycler gradient, Eppendorf, Germany)
2. Autoclave (HA-3D, Hiroyama, Japan)
1. Automatic pipette; size 10 μ l, 100 μ l and 1000 μ l (Eppendorf, Germany)
4. Balance (1507MP8, Sartorius, Germany)
5. Centrifuge (Hermle Z283K, Berthold HermelenGmbH, Germany)

6. Freezer-70 °C (Frost free, White Westing House, USA.) Hot air oven (TV406, Memmert, Germany)
7. Hot plate with magnetic stirrer (Thermolyne Type 1000, Syborn/Thermolyne, USA.)
8. Hood
9. Mixer vortex (Vortex-2 Genie, Scientific Industries, USA.)
10. Microwave oven (Sanyo, Japan)
11. Power supply for electrophoresis system (MJ-105 short minihorizontal gel electrophoresis system, Basic Science Inc., Canada)
12. pH meter
13. Refrigerator 4-8 °C (GE, USA.)
14. UV transilluminator (Bio Imaging Systems, Syngene, UK.)
15. Water bath (Model 83, Precision Scientific, USA.)

4.1.3 Chemical and reagent

Chemical and reagent for DNA isolation

1. Absolute ethanol (BDH Laboratory, UK.)
2. Chromosome (BDH Laboratory, UK.)
3. EDTA (Merk, Germany)
4. Gracial acetic acid (Merk, Germany)
5. Hydrochloric acid (conc.)(Merk, Germany)
6. 8-Hydroxyquinolone (Sigma, USA.)
7. Isoamyl alcohol (Merk, Germany)
8. Nonidet (NP40)(Sigma, USA.)
9. Phenol (Merk, Germany)
10. Protinase K (Gibco, BRL)
11. Sodium dodecylsulfate (Sigma, USA.)
12. Sodium acetate anhydrous (Merk, Germany)
13. Sodium hydroxide (Merk, Germany)

14. Sodium chloride (Merk, Germany)
15. Sucrose (Ultra pure, Bio Basic Inc., USA.)
16. Sterile distilled water
17. Trizma base (Ultra pure molecular biology grade, Research Organics Inc., USA.)
18. Triton X-100 (Research Organics Inc., USA.)
19. Tris-HCl (Sigma, USA.)

Chemical and reagent for PCR-RFLP

1. Sterile distilled water
2. dNTP Mixture (Bio Basic Inc., Canada)
3. MgCl₂ (Promega, USA.)
4. Oligonucleotide primer (Bio Basic Inc., Canada)
5. PCR 10X reaction buffer (Promega, USA.)
6. *Taq* DNA polymerase (Promega, USA.)
7. *Pst*I Restriction enzymes (New England Bio Lab Inc., USA.)
8. *Sty*I Restriction enzymes (New England Bio Lab Inc., USA.)
9. *Msp*I Restriction enzymes (New England Bio Lab Inc., USA.)
10. *Rsa*I Restriction enzymes (New England Bio Lab Inc., USA.)
11. Restriction enzyme reaction buffer (New England Bio Lab Inc., USA.)

Chemical and reagent for gel electrophoresis and staining

1. Agarose (Molecular biology grade Reseach Organics Inc., USA.)
2. DNA molecular weight markers 100 bp (SibEnzyme, USA.)

3. Ethidium bromide
4. Loading dye
5. 1X TBE buffer

4.1.4 Supplies

1. Aluminium foil
2. Autoclave tape
3. Cylinder, size 100 ml
4. Duran bottle
5. Erlenmeyer flask, size 250 ml
6. Falcon tube, size 20 ml
7. Gloves
8. Microcentrifuge tube, size 1.5 ml
9. Magnetic stirrer
10. Separating funnel
11. Gel casting platform
12. Thin-wall PCR tube, size 0.2 ml
13. Pipette tips, size 10, 100 and 1000 μ l

4.2 Methods

4.2.1 DNA isolation

Genomic DNA was isolated from peripheral leukocyte using phenol-chloroform methods(71, 72). This method produces very pure DNA, which can be stored for long periods of time. The DNA isolation is first extracted with a phenol-chloroform mixture to remove protein contaminants, then precipitated with 100% ethanol. The DNA is pelleted after precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in buffer at a concentration suitable for further experimentation.

4.2.1.1 Cell lysis and protein digestion

DNA isolation from whole blood need to prepare white blood cells from anticoagulated whole blood by red blood cell lysis. In general, it is necessary to perform 2 cycles of red blood cell lysis in order to remove the majority of the heme contamination. Described, collect 3 ml of EDTA blood and place in 20 ml falcon tube. Then add 3-5 ml lysis buffer and rotate gently for 10-15 min. After that centrifuge for 10 min at 4,500 rpm to remove the supernatant without touching to the leukocyte. Add 5 ml of 0.1% nondidet to completely lysis and rotate gently for 10-15 min. Centrifuge for 10 min at 4,500 rpm and dicard the supernatant. Finally, add 1,000 µl of distilled water, 25 µl of proteinase K, 25 µl of 10% SDS and 50 µl of 10X STE and mix well, incubate overnight at 56°C.

4.2.1.2 Phenol-Chloroform extraction and ethanol precipitation of DNA

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Briefly, after the step for cell lysis and protein digestion add an equal volume (1 ml) of phenol to the nucleic acid sample in falcon tube and mix by rotating the tube slowly (20 rpm) on a wheel. Centrifuge for 10 min at 4,500 rpm, remove upper aqueous layer with a Pasteur pipette and place into the new microcentrifuge tube. Then add equal volume of chloroform and mix by rotating (20 rpm) on a wheel. Add 50 µl of 5M sodium acetate, 1,000 µl of freezer cold absolute ethanol and invert gently. DNA precipitate should appear. Wait as long as 10 min for all samples to develop precipitate. Centrifuge for 10 min at 6,000 rpm and carefully decant liquid so not dislodge pellet, then fill each tubes with 70% ethanol. After that centrifuge for 10 min at 14,000 rpm and then carefully decant off liquid. Don't touch the pellet and air dry pellet. Finally, add 100 µl of 1X TE buffer to each samples, dissolve DNA and freeze at -20°C until need.

4.2.2 Genotyping

4.2.2.1 Genotyping for detect XRCC1 codon 194 and 399 polymorphisms

Genotypic analysis of the XRCC1 gene was determined from the participants by a multiplex PCR assay. PCR primers for codon 194 and 399 previously described by Lunn et al.(2) were used to generate a 491-bp product and a 615-bp product containing the polymorphic sites. The reaction mixture (50 µl) for multiplex method consisted of isolated DNA (50 ng), 0.075 µM each of codon 194 primers and 0.15 µM each of codon 399 primers, 200 µM dNTPs, 5 µl of 10X PCR buffer, 1.5 mM MgCl₂, and 1 U Taq DNA polymerase.

The PCR conditions consisted of an initial denaturation of 94°C (5 min) followed by 30 cycles of denaturation (94°C, 30 s.), annealing (60°C, 1 min), and extension (72°C, 45 s.). A final extension step (72°C, 5 min) terminated the reaction.

Restriction enzyme digestion was performed on the PCR fragment in 5 µl PCR product, 1 µl 10X Restriction buffer, 10 U *MspI* in total volume of 10 µl and incubated at 37°C overnight, which recognizes the wild-type Arg allele at codon 194 and the wild-type Arg allele at codon 399. The digestion product was then resolved on 3% agarose gel and it was staining with ethidium bromide. Bands were then visualized directly by UV trans-illumination.

As show in **Figure 4.1**, the wild-type Arg allele for codon 194 is identified by the presence of a band at 292-bp, while the mutant Trp allele is identified by the presence of a band at 313-bp (indicative of the absence of *MspI* cutting site). The wild-type Arg allele for codon 399 is identified by the presence of the two bands at 374 and 221-bp, while the mutant Gln allele is identified by the presence of the uncut 615-bp band (indicative of the absence of the *MspI* cutting site). In addition of these bands, a 174-bp band, resulting from an additional invariant cutting site for *MspI* in the 491-bp amplified fragment (codon 194), is always present and serves as an internal control for complete *MspI* digestion.



Figure 4.1 XRCC1-194 and XRCC1-399 genotyping from PCR-RFLP

4.2.2.2 Genotyping for detect XRCC1 codon 280 polymorphism

The method for genotyping codon 280 polymorphism was modified from Lunn et al.(2). A 861-bp PCR product was amplified using two primers were shown in **Table 4.1**. PCR condition for this method consisted of 50 ng of genomic DNA, 200 μ l dNTPs, 3 mM $MgCl_2$, 0.2 μ M of each primers and 0.25 U Taq DNA polymerase in 25 μ l PCR reaction volume.

After initial denaturation step (94°C, 4 min), followed by 30 cycles of denaturation (94°C, 30 s.), annealing (68°C, 90 s.), and extension (72°C, 45 s.), with the final extension by 7 min. Five μ l of PCR product was then digested with 1 unit *RsaI* at 37°C overnight, in 10 μ l total volume.

The digestion fragment (60-bp, 221-bp, 580-bp, and 640-bp) were separated on 3% agarose gel. The Arg allele creates a *RsaI* site and results in the 580-bp and 60-bp products that are not recognized by the His allele but is contained in the 640-bp fragment. The 221-bp fragment is a result of an invariant *RsaI* site present in all of the samples (**Figure 4.2**).

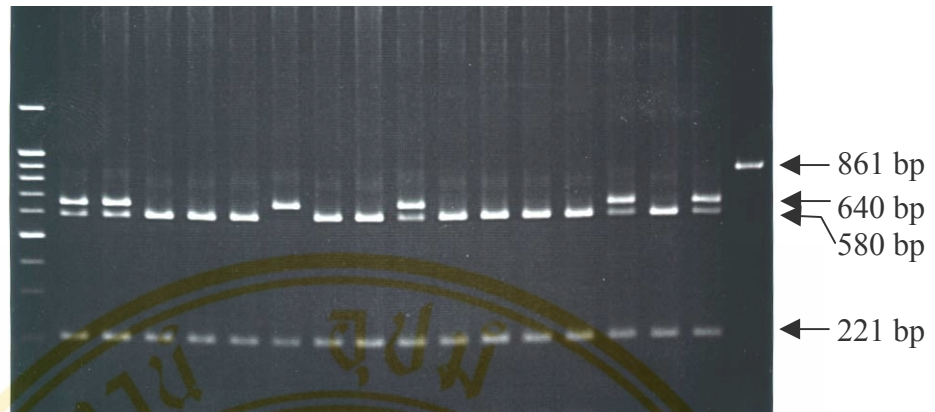


Figure 4-2. XRCC1-280 genotyping from PCR-RFLP

4.2.2.3 Genotyping for detect XPD codon 312 polymorphism

For amplification of the exon 10 region of XPD (Asp312Asn), which contains the polymorphic *StyI* restriction site. Olinucleotide primers were shown in **Table 4.1**. PCR was performed in 25 μ l reaction mixtures containing 1.5 mM MgCl₂, 200 μ M dNTPs, 3% DMSO, 0.05 μ M primers, 1 μ g of template DNA, and 1 U Taq DNA polymerase in PCR buffer. After the an initial denaturation at 94°C for 4 min, the DNA was amplified by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C, and then by a final extension step of 5 min at 72°C.

Five μ l of PCR product was digested with 5 U *StyI* at 37°C overnight, in total volume of 10 μ l. The digestion products were then separated by gel electrophoresis on 3% agarose gel and it was staining with ethidium bromide. The homozygous wild-type Asp allele was identified by two DNA bands (507 and 244), the homozygous mutant-type Asn allele produced three DNA bands (474, 244, and 33-bp); and heterozygotes (Asp/Asn) displayed all four bands (507, 474, 244, and 33-bp) (**Figure 4.3**).

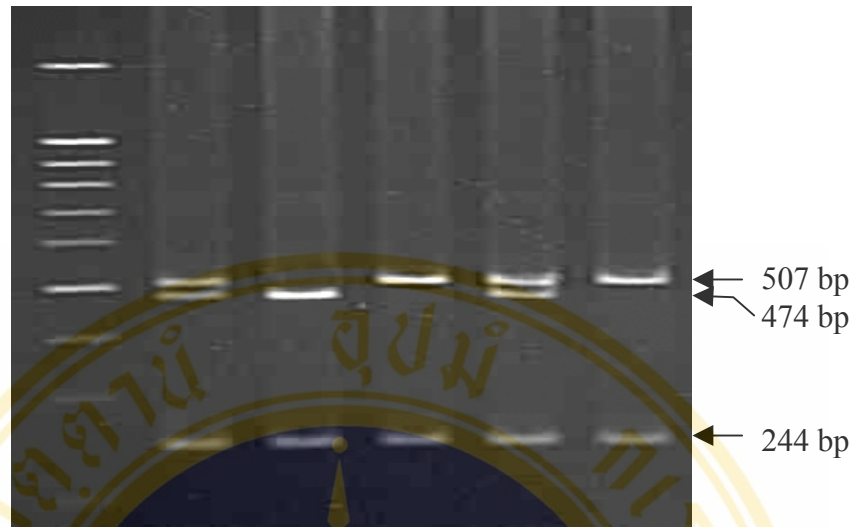


Figure 4.3 XPD-312 genotyping from PCR-RFLP

4.2.2.4 Genotyping for detect XPD codon 751 polymorphism

The method for genotyping exon 23 polymorphism (Lys751Gln) was modified from Hemminki et al.(46). A 322-bp PCR product was amplified using two intronic primers flanking exon 23 (primers were shown in **Table 4.1**). Approximately 50 ng DNA template was used in a 25 μ l PCR reaction containing 2.5 μ l 10X PCR buffer, 250 μ M dNTPs, 1.5 mM MgCl₂, 0.1 μ M each primers, and 1 U Taq DNA polymerase. The DNA was then amplified after 5 min at 94°C for initial denaturation, followed by 30 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 45s) with the final extension by 7 min.

Two μ l of PCR product was digested by 5 U *Pst*I at 37°C overnight, in total volume of 10 μ l, followed by electrophoresis in a 3% agarose gel and it was staining with ethidium bromide. The wild-type Lys allele was cut into two fragments (104 and 218-bp) while the mutant-type Gln allele was cut into three fragments (104, 155 and 63-bp)(**Figure 4.4**).

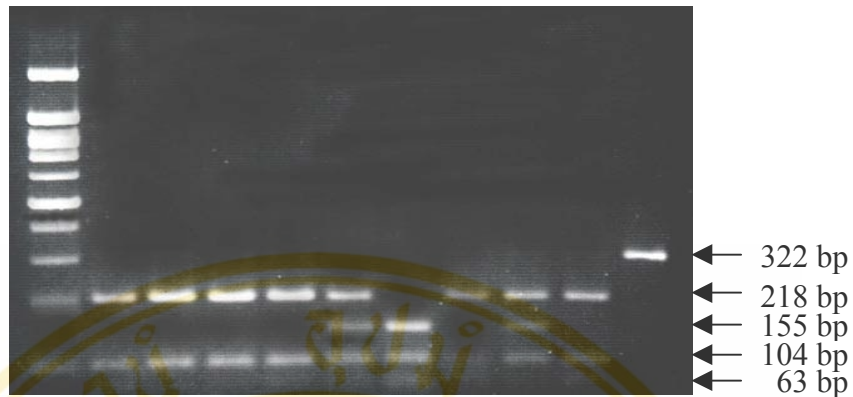


Figure 4.4 XPD-751 genotyping from PCR-RFLP

4.2.3 Agarose gel electrophoresis and ethidium bromide staining

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying DNA fragment. DNA is negative charged due to the phosphate backbone and moves to a positive pole in the electric field. This movement of charged particles in various media due to an applied electric field is called electrophoresis. The rate of migration is inversely proportional to size. The larger fragments move slower than smaller ones. Therefore, DNA fragment of different size can be separated and identified with this method. The migrated DNA fragment were detected by using ethidium bromide which is a fluorescent dye that intercalates double strand DNA. DNA bands can be made visible upon exposure to ultraviolet light.

In brief, prepared adequate volumes of electrophoresis buffer (1X TBE) to fill the electrophoresis tank and prepare the gel. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. The 3% agarose gel was melted in a microwave oven. Pour in the melted agarose and insert the gel comb, make sure that no air bubbles are trapped underneath the combs and all bubbles on the surface the agarose are removed before the gel sets. After the gel has hardened withdraw the gel comb, place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm. DNA sample should be prepared in a volume (10 μ l) that will not overflow addition of the appropriate amount of 10X

loading buffer (2 μ l). Molecular weight marker (M) (1 μ l) was used to differ the size of base pair of PCR products. The gel was eletrophoresed in 1X TBE at 100 volt. Then the gel was stained with ethidium bromide. The PCR products and digested PCR products were visualized by UV transillumination.

4.3 Statistical analysis

Chi-squared test was used to examine differences in frequencies between DNA repair (XRCC1 and XPD) genotypes in cases and controls. The level of significance was calculated at P -value < 0.05 . Crude odds ratios (OR) are given with 95% confidence intervals (CI). All statistical tests were performed with SPSS 12.0 software.

Table 4.1 PCR-RFLP primers, size of PCR products, restriction enzyme used: XRCC1-194, 399 and 280; XPD-312 and 751.

Primers	Nucleotide substitution		Oligonucleotide sequence 5' to 3'	PCR product size (bp)	Restriction enzymes
	Position	Change ^a			
XRCC1 codon 194	26304	TCAGCC/ <u>T</u> GGATC	F: GCCCCCGTCCCAGGTA R: GCCCCAAGACCCCTTCATC	491	<i>MspI</i>
XRCC1 codon 280	27466	AACTCG/ <u>A</u> TACCC	F: TTGACCCCCCAGTGGTGCTAA R: CGCTGGGACCCACCTGTGT	861	<i>RsaI</i>
XRCC1 codon 399	28152	CTCCCG/ <u>A</u> GAGGT	F: TTGTGCTTTCTCTGTGTCCA R: TCCTCCAGCCTTTCTGATA	615	<i>MspI</i>
XPD codon 312	23591	TGCCG/ <u>A</u> ACGAA	F: GCCCGCTCTGGATTATACG R: CTATCATCTCCTGGCCCCC	751	<i>StyI</i>
XPD codon 751	35931	CGCTGA/ <u>C</u> AGAGG	F: ATCCTGTCCCTACTGGCCATTC R: TGGACGTGACAGTGAGAAAT	322	<i>PstI</i>

F: Forward primer, R: Reverse primer

The GenBank accession numbers for XRCC1 and XPD are L34079 and L47234, respectively.

^aThe variant residues are underlined, with the common nucleotide followed by the variant.

CHAPTER 5

RESULTS

5.1 Genotyping result

5.1.1 Genotype and allele frequencies of XRCC1 and XPD genes in ALL cases and controls

The distribution of XRCC1-194, XRCC1-280, XRCC1-399, XPD-312 and XPD-751 genotypes in ALL cases and controls were shown in **Table 5.1** and **Table 5.2**, respectively. The frequencies of each polymorphisms in XRCC1 and XPD genes were given in **Table 5.3** and **Table 5.4**, respectively. The frequency of XRCC1-194, CC genotype was observed in 57.4% of cases compared to 47.3% among controls. The frequency of XRCC1-194, TT genotype was 1.9% in the ALL patients and 6.9% in controls. Both frequencies were statistically different (OR= 0.220, 95%CI, 0.50-0.964). But the frequency of CC genotype was not statistically different compared to the frequency of CT genotype between cases and controls (OR= 0.734, 95%CI, 0.469-1.150).

The genotyping of XRCC1-280 for ALL group demonstrated that frequencies of GG and GA were 87.0% and 13.0%, respectively. The AA genotype of XRCC1-280 was not found in this group. In control group, the frequencies of genotype for GG, GA and AA genotypes were 85.8%, 13.2% and 0.9%, respectively. Comparing between both groups, there were no statistical difference in the frequencies of each genotypes.

The XRCC1-399 genotype, the genotype frequencies for GG, GA and AA in the ALL patients were 36.1%, 55.6% and 8.3%, respectively. In control group, the genotype frequencies for GG, GA and AA were 55.2%, 39.1% and 5.7%, respectively.

But GA and GA+AA genotype frequencies were statistically different (OR= 2.171, 95%CI, 1.365-3.454 and OR= 2.180, 95%CI, 1.389-3.422, respectively).

The genotyping in both polymorphisms of XPD gene (codon 312 and 751) for ALL and controls groups were shown in **Table 5.4**. Comparing between both groups, there was no statistically difference in the frequencies of each genotypes in both polymorphisms.

Further analysis of the allele frequencies of XRCC1 and XPD polymorphisms have shown in **Table 5.5**. The study did not observed significant different in XRCC1-194 and XRCC1-399 allele frequencies between the two groups. For XRCC1-194, T allele showed a protective effect to this disease (OR=0.67, 95% CI, 0.47-0.97) while A allele of XRCC1-399 demonstrated increased risk to childhood ALL with an OR of 1.67 (95% CI, 1.2-2.3).

Table 5.1 Genotyping result in ALL patients

ALL code	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
ALL001	GA	CC	CC	GG	GA
ALL002	GG	AA	CT	GG	GG
ALL003	GG	AA	CC	GG	GA
ALL004	GG	AC	CT	GG	GA
ALL005	GG	AA	CC	GG	GA
ALL006	GG	AA	CC	GG	AA
ALL007	GA	CC	CC	GG	GA
ALL008	GG	AA	CT	GA	GG
ALL009	GG	AA	CC	GA	GA
ALL010	GG	AA	CT	GG	GG
ALL011	GG	AC	CC	GG	GA
ALL012	GG	AC	CC	GA	GA
ALL013	GG	AA	CC	GA	GA
ALL014	GG	AA	CT	GG	GG
ALL015	GG	AA	CC	GG	GA
ALL016	GG	AA	CT	GA	GG
ALL017	GG	AA	CT	GG	GA
ALL018	GG	AA	CC	GG	GA
ALL019	GG	AA	CC	GG	GA
ALL020	GG	AA	CT	GG	GA
ALL022	GG	AC	CT	GA	GG
ALL023	GG	AA	CT	GG	GG
ALL024	GG	AA	CC	GG	GA
ALL025	GG	AA	CC	GG	GA
ALL026	GG	AA	CC	GG	GA
ALL026	GG	AA	CC	GG	GA
ALL027	GG	AA	CC	GA	GG
ALL028	GG	AA	CT	GG	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.1 Genotyping result in ALL patients (continue)

ALL code	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
ALL029	GG	AA	CT	GG	GG
ALL030	GG	AA	CT	GG	GG
ALL031	GA	AC	CT	GG	GA
ALL032	GG	AA	CC	GG	GA
ALL033	GG	AA	CC	GG	GA
ALL034	GG	AA	CC	GG	GA
ALL035	GG	AA	CC	GG	GG
ALL036	GG	AA	CT	GA	GG
ALL037	GG	AA	CT	GG	GG
ALL038	GG	AA	CC	GG	GA
ALL039	GG	AA	CT	GG	GA
ALL040	GA	AC	CC	GG	GG
ALL041	GG	AA	CT	GG	AA
ALL042	GG	AA	CC	GG	GA
ALL043	GG	AC	CC	GG	GA
ALL044	GG	AA	CT	GG	GG
ALL045	GG	AA	CT	GA	GG
ALL046	GG	AA	CT	GA	GG
ALL047	GG	AA	TT	GG	GG
ALL048	GG	AA	CC	GG	GA
ALL049	GG	AA	CC	GG	GA
ALL050	GG	AA	CC	GG	GA
ALL051	GG	AA	CC	GA	GA
ALL052	GG	AA	CC	GG	GA
ALL053	GG	AA	CC	GG	GA
ALL055	GG	AA	CT	GG	GA
ALL056	GG	AA	CT	GG	GG
ALL057	GA	AC	CC	GG	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.1 Genotyping result in ALL patients (continue)

ALL code	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
ALL058	GG	AA	CT	GA	GA
ALL059	GG	AA	CT	GG	GA
ALL060	GG	AA	CT	GG	GA
ALL061	GG	AA	CC	GG	GA
ALL062	GG	AA	CT	GG	GA
ALL063	GG	AA	CT	GG	GA
ALL064	GG	AA	CC	GA	GG
ALL065	GG	AA	CC	GG	GG
ALL066	GG	AC	CC	GG	GA
ALL067	GG	AA	CC	GG	GA
ALL068	GG	AA	CC	GG	GA
ALL069	GG	AA	CC	GG	GA
ALL070	GA	AC	CC	GG	GA
ALL071	GA	AC	CC	GG	GA
ALL072	GG	AA	CC	GG	GA
ALL073	GG	AA	CC	GG	GA
ALL074	GG	AA	CC	GG	GA
ALL075	GG	AA	CC	GG	GA
ALL076	GG	AA	CC	GG	AA
ALL077	GG	AC	CC	GG	AA
ALL078	GG	AA	CC	GG	AA
ALL079	GG	AA	TT	GG	GG
ALL080	GG	AA	CC	GG	GA
ALL081	GG	AA	CT	GG	AA
ALL082	GG	AA	CT	GG	GG
ALL083	GG	AA	CC	GG	GG
ALL084	GA	AC	CT	GG	GG
ALL085	GG	AA	CT	GG	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.1 Genotyping result in ALL patients (continue)

ALL code	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
ALL086	GG	AA	CC	GG	GG
ALL087	GG	AA	CT	GG	GA
ALL088	GG	AA	CC	GG	GA
ALL089	GG	AA	CC	GG	GG
ALL090	GG	AA	CT	GG	GG
ALL091	GG	AA	CC	GG	GG
ALL092	GG	AC	CT	GG	GA
ALL093	GG	AA	CT	GG	GG
ALL094	GG	AA	CT	GG	GA
ALL095	GA	AC	CT	GG	GG
ALL096	GG	AA	CC	GG	AA
ALL097	GG	AA	CT	GG	GG
ALL098	GG	AC	CT	GG	GG
ALL099	GG	AC	CC	GG	GA
ALL100	GG	AA	CT	GG	GA
ALL101	GG	AA	CC	GG	GA
ALL102	GG	AA	CT	GG	GG
ALL103	GG	AA	CC	GG	GA
ALL104	GA	AC	CC	GA	GG
ALL105	GG	AA	CC	GG	GA
ALL106	GG	AA	CT	GG	GA
ALL107	GG	AA	CT	GG	GG
ALL108	GG	AA	CC	GG	AA
ALL109	GA	AC	CC	GG	GG
ALL110	GG	AA	CC	GG	AA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C001	GG	AC	CT	GG	GA
C002	GG	AC	CT	GG	GG
C003	GA	AA	CT	GG	GG
C004	GG	AA	CC	GG	GA
C005	GA	AC	CC	GG	GA
C006	GG	AA	CT	GG	GG
C007	GA	AA	TT	GG	GG
C008	GG	AA	CT	GG	GA
C009	GG	AA	CT	GG	GA
C010	GG	AA	CT	GG	GG
C011	GG	AA	CC	GG	AA
C012	GG	AA	CT	GG	GG
C013	GG	AA	CT	GG	GA
C014	GG	AA	CC	GA	GA
C015	GG	AC	CC	GG	GA
C016	GG	AA	TT	GG	GG
C017	GG	AA	CT	GG	GA
C018	GA	AC	CT	GG	GG
C019	GG	AA	CT	GG	GG
C020	GG	AA	CT	GG	GA
C021	GG	AA	CC	GG	GA
C022	GG	AA	CC	GG	GA
C023	GA	AC	CT	GG	GA
C024	GG	AA	CT	GG	GG
C025	GG	AA	CT	GG	GA
C026	GG	AC	CT	GG	GA
C027	GG	AA	CC	GA	GA
C028	GG	AA	CC	GG	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C029	GG	AA	CC	GA	GA
C030	GG	AA	CT	GG	GA
C031	GA	AA	CT	GG	GA
C032	GG	AA	CT	GG	GG
C033	GG	AA	CC	GG	GG
C034	GG	AA	CT	GG	GA
C035	GG	AA	CC	GG	AA
C036	GG	AA	CT	GG	GG
C037	GA	AC	TT	GG	GG
C038	GG	AA	CT	GG	GA
C039	GG	AA	CC	GG	GA
C040	GG	AA	CT	GG	GG
C041	GG	AA	CC	GA	GG
C042	GG	AA	CT	GG	GA
C043	GG	AA	CC	GG	AA
C044	GG	AA	CC	GG	AA
C045	GG	AA	CT	GG	GG
C046	GA	AC	CT	GG	GG
C047	GG	AA	CC	GG	GA
C048	GG	AA	CC	GG	GA
C049	GG	AA	CC	GG	GG
C050	GG	AA	CT	GG	GA
C051	GA	AA	CT	GG	GG
C052	GG	AA	CC	GG	AA
C053	GG	AA	TT	GG	GG
C054	GG	AA	CT	GG	GG
C055	GG	AA	CT	GG	GG
C056	GG	AA	CC	GG	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C057	GA	AC	CC	GG	GA
C058	GG	AA	CC	GG	GG
C059	GG	AA	CT	GG	GA
C060	GG	AA	CT	GA	GA
C061	GG	AA	CT	GG	GA
C062	GG	AA	CT	GG	GG
C063	GG	AA	CT	GG	GG
C064	GG	AA	CT	GA	GG
C065	GG	AA	CC	GA	GA
C066	GG	AA	CT	GG	GA
C067	GG	AA	CC	GG	GG
C068	GG	AA	CC	GG	GG
C069	GA	AC	CT	GA	GG
C070	GG	AA	TT	GG	GG
C071	GA	AC	CC	GG	AA
C072	GG	AA	CC	GG	GG
C073	GG	AC	CT	GG	GA
C074	GG	AA	CC	GG	GA
C075	GA	AC	CC	GG	GA
C076	GA	AC	CC	GG	GA
C077	GG	AA	CT	GG	GG
C078	GG	AA	CT	GG	GG
C079	GG	AA	TT	GG	GG
C080	GA	AC	CT	GG	GG
C081	GG	AA	CT	GG	GA
C082	GG	AA	CC	GA	GG
C083	GG	AA	CT	GA	GG
C084	GG	AA	CT	GG	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C085	GG	AA	CT	GG	GA
C086	GG	AA	CC	GA	GA
C087	GG	AA	CC	GG	GA
C088	GG	AC	CC	GG	GA
C089	GG	AA	TT	GG	GG
C090	GG	AA	CT	GG	GG
C091	GA	AA	CT	GG	GG
C092	GG	AA	CT	GG	GG
C093	GG	AA	CC	AA	GG
C094	GG	AA	CC	GG	AA
C095	GA	AC	CC	GG	GG
C096	GG	AA	CT	GG	GA
C097	GG	AA	CC	GG	GA
C098	GG	AA	CC	GG	GA
C099	GG	AA	CT	GG	GG
C100	GG	AA	CT	GG	GG
C101	GG	AA	CC	GG	GA
C102	GG	AA	CC	GG	GG
C103	GG	AA	CT	GG	GG
C104	GG	AA	CC	GG	GA
C105	GG	AA	CC	GG	GG
C106	GG	AA	CT	GG	GA
C107	GG	AA	CC	GG	GG
C108	GG	AA	CC	GG	GA
C109	GG	AA	CC	GG	GG
C110	GG	AA	CC	GG	GA
C111	GG	AA	CC	GG	GG
C112	GG	AC	CC	GA	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C113	AA	AA	CT	GG	GG
C114	GG	AA	CT	GA	GG
C115	GG	AA	CT	GG	GA
C116	GG	AA	CT	GG	GA
C117	GG	AA	CT	GG	GG
C118	GG	AA	CC	GG	GA
C119	GG	AA	CC	GG	GG
C120	GG	AA	TT	GG	GG
C121	GG	AA	CT	GG	GG
C122	GG	AC	CC	GA	GG
C123	GG	AA	CC	GG	AA
C124	GG	AA	CT	GG	GA
C125	GG	AA	CC	GG	GA
C126	GG	AA	CC	GG	GG
C127	GG	AA	CC	GG	GA
C128	GG	AA	CC	GA	GA
C129	GG	AA	CT	GG	GG
C130	GG	AA	CT	GG	GG
C131	GG	AA	CC	GG	GA
C132	GG	AA	CT	GG	GA
C133	GG	AA	CT	GG	GG
C134	GG	AA	CC	GA	GG
C135	GG	AA	CT	GA	GG
C136	GG	AA	TT	GG	GG
C137	GG	AA	CC	GG	AA
C138	GG	AA	CT	GG	GG
C139	GG	AA	CC	GA	GA
C140	GG	AA	CT	GG	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C141	GG	AA	CT	GG	GG
C143	GG	AA	CT	GA	GG
C144	GG	AC	CC	GG	GG
C145	GG	AA	CC	GG	GG
C146	GG	AA	CC	GG	GG
C147	GG	AA	CC	GG	GA
C148	GG	AC	CC	GG	GA
C149	GG	AA	CC	GG	GG
C150	GG	AA	CC	GA	GG
C151	GA	AC	CC	GA	GG
C152	GG	AA	CT	GG	GG
C153	GG	AA	CT	GG	GA
C154	GG	AA	CT	GG	GG
C155	GG	AA	CC	AA	GG
C156	GG	AA	CT	GG	GA
C157	GG	AC	CT	GG	GG
C158	GG	AA	CC	GA	GG
C159	GG	AA	TT	GG	GG
C160	GG	AA	CC	GG	GG
C161	GA	AA	CT	GG	GG
C162	GA	AC	CT	GG	GG
C163	GG	AC	CC	GA	GA
C164	GG	AC	CC	GG	GA
C165	GG	AA	CT	GA	GG
C166	GG	AA	CC	GA	GG
C167	GG	AA	CT	GG	GG
C168	GG	AA	CT	GG	GG
C169	GG	AA	CT	GA	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C170	GG	AA	CT	GG	GG
C171	GG	AA	CT	GG	GG
C172	GG	AA	CC	GG	GG
C173	GG	AA	CC	GG	AA
C174	GG	AA	CC	GG	GA
C175	GG	AA	CC	GG	GA
C176	GA	AC	CC	GG	GG
C177	GG	AA	CT	GG	GG
C178	GG	AA	TT	GG	GG
C179	GG	AA	CT	GA	GG
C180	GG	AA	TT	GG	GG
C181	GG	AA	CC	GG	GA
C182	GG	AA	CT	GG	GA
C183	GG	AA	CC	GG	GA
C184	GG	AA	CT	GG	GG
C185	GA	AC	TT	GG	GG
C186	GG	AA	CC	GA	GG
C187	GA	AC	CC	GG	GA
C188	GG	AC	CT	GG	GG
C189	GG	AA	CT	GA	GG
C190	GG	AA	TT	GG	GG
C191	GG	AA	CC	GG	GG
C192	GA	AC	CC	GG	GG
C193	GG	AA	CT	GG	GG
C194	GG	AA	CC	GG	GG
C195	GG	AA	CC	GG	GA
C196	GG	AA	CT	GG	GG
C197	GG	AA	CT	GG	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C198	GG	AA	CC	GG	GA
C199	GG	AA	CC	GG	GA
C200	GG	AA	CC	GG	GA
C201	GG	AA	CC	GG	GG
C202	GG	AA	TT	GG	GG
C203	GA	AC	CC	GG	GG
C204	GG	AA	CT	GG	GG
C205	GG	AC	CC	GG	GA
C206	GG	AA	CT	GG	GA
C207	GG	AA	CC	GG	AA
C208	GG	AA	CC	GG	AA
C209	GG	AA	CT	GG	GG
C210	GG	AA	CT	GG	GA
C211	GA	AC	CC	GA	GG
C212	GG	AA	TT	GG	GG
C213	GG	AA	CT	GG	GG
C214	GG	AC	CT	GA	GG
C215	GA	AC	CT	GG	GG
C216	GG	AA	CT	GG	GA
C217	GG	AA	CT	GG	GA
C218	GG	AA	CT	GG	GA
C219	GA	AC	CT	GG	GA
C220	GG	AC	CC	GG	GG
C221	GG	AC	CT	GG	GG
C222	GG	AA	CC	GG	GG
C223	GG	AA	CT	GG	GG
C224	GA	AC	CT	GA	GG
C225	GG	AA	CT	GG	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C226	GG	AA	CT	GG	GA
C227	GG	AA	CT	GG	GG
C228	GG	AA	CC	GG	AA
C229	GG	AA	CC	GG	GA
C230	GG	AA	CC	GG	GA
C231	GG	AA	CC	GG	GA
C232	GG	AA	CT	GG	GG
C233	GG	AA	CT	GG	GG
C234	GG	AA	TT	GG	GG
C235	GG	AA	CC	GG	GG
C236	GA	AC	CC	GA	GA
C237	GA	AC	CT	GG	GG
C238	GG	AA	CT	GG	GA
C239	GG	AC	TT	GG	GG
C240	GA	AC	CT	GG	GA
C241	GG	AC	CC	GG	GG
C242	GG	AA	CT	GG	GA
C243	GG	AA	CT	GG	GG
C244	GG	AA	CT	GG	GA
C245	GG	AA	CT	GG	GG
C246	GA	AC	CT	GG	GG
C247	GG	AA	TT	GG	GG
C248	GG	AA	CC	GG	AA
C249	AA	CC	CC	GG	GA
C250	GG	AA	CC	GG	GG
C251	GG	AA	CC	GG	GA
C252	GG	AA	CC	GG	GG
C253	GG	AA	CC	GA	GA

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C254	GA	AC	CC	GA	GG
C255	GA	AA	CT	GG	GA
C256	GG	AA	CT	GG	GG
C257	GG	AA	CT	GA	GG
C258	GG	AA	CT	GG	GG
C259	GG	AA	CC	GG	GA
C260	GG	AA	CC	GG	GG
C261	GG	AA	CC	GG	GA
C262	GG	AA	CT	GG	GA
C263	GG	AA	CT	GG	GG
C264	GG	AA	CT	GG	GG
C265	GG	AA	CC	GG	GA
C266	GG	AA	CT	GG	GA
C267	GG	AC	CT	GG	GA
C268	GG	AA	CT	GG	GG
C269	GG	AA	CT	GG	GG
C270	GG	AA	CC	GA	GG
C271	GG	AA	CC	GG	GG
C272	GG	AA	CC	GG	GG
C273	GG	AA	CC	GA	GG
C274	GG	AA	CC	GG	GA
C275	GG	AA	CT	GG	GG
C276	GG	AA	CT	GG	GA
C277	GG	AA	CC	GG	GA
C278	GA	AC	CC	GG	GA
C279	GG	AA	CC	GG	AA
C280	GG	AA	CT	GG	GG
C281	GA	AC	CC	AA	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C282	GG	AA	CC	GG	AA
C283	GG	AA	CC	GG	GA
C284	GG	AC	CC	GG	GG
C285	GG	AA	CC	GG	GA
C286	GG	AC	CC	GG	GA
C287	GA	AA	CC	GA	GA
C288	GG	AA	CC	GG	GG
C289	GG	AA	CT	GG	GG
C290	GG	AA	CC	GG	GG
C291	GG	AA	CC	GG	GA
C292	GG	AA	CT	GG	GG
C293	GG	AA	CC	GG	GG
C294	GA	AC	CC	GG	GG
C295	GG	AA	CC	GG	GG
C296	GG	AA	CC	GG	AA
C297	GG	AA	CT	GG	GG
C298	GG	AA	CC	GG	GA
C299	GG	AA	CC	GG	GA
C300	GG	AA	CT	GG	GG
C301	GG	AA	TT	GG	GG
C302	GG	AA	CT	GG	GG
C303	GA	AC	CC	GG	GA
C304	GG	AA	CC	GG	GA
C305	GG	AA	CT	GG	GA
C306	GA	AA	CT	GA	GG
C307	GG	AA	CC	GG	GA
C308	GA	AA	CC	GA	GG
C309	GG	AA	CC	GA	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.2 Genotyping result in controls (continue)

Control sample	XPD-312 G23591A, Asp-Asn	XPD-751 A35931C, Lys-Gln	XRCC1-194 C26304T, Arg-Trp	XRCC1-280 G27466A, Arg-His	XRCC1-399 G28152A, Arg-Gln
C310	GG	AA	TT	GG	GG
C311	GG	AA	TT	GG	GG
C312	GG	AA	CC	GG	GG
C313	GG	AA	CC	GG	GA
C314	GG	AA	CT	GG	AA
C315	GG	AA	CT	GG	GA
C316	GG	AA	CC	GG	GG
C319	GA	AA	CT	GG	GA
C320	GG	AC	CT	GG	GG
C310	GG	AA	TT	GG	GG
C311	GG	AA	TT	GG	GG
C312	GG	AA	CC	GG	GG
C313	GG	AA	CC	GG	GA
C314	GG	AA	CT	GG	AA
C315	GG	AA	CT	GG	GA
C316	GG	AA	CC	GG	GG
C319	GA	AA	CT	GG	GA
C320	GG	AC	CT	GG	GG

A; Adenine allele, G; Guanine allele, C; Cytosine allele, T; Thymine allele

Table 5.3 XRCC1 genotypes and ALL risk

Genotype	ALL (n = 108)	Controls (n = 317)	OR (95%CI)	P-value
XRCC1 codon 194 (C26304T, Arg-Trp)				
CC	62	150	1.0	-
CT	44	145	0.734 (0.469-1.150)	0.176
TT	2	22	0.220 (0.500-0.964)	0.029
CT+TT	46	167	0.666 (0.429-1.035)	0.070
XRCC1 codon 280, (G27466A, Arg-His)				
GG	94	272	1.0	-
GA	14	42	0.965 (0.504-1.845)	0.913
AA	0	3	-	-
GA+AA	14	45	0.900 (0.473-1.714)	0.749
XRCC1 codon 399 (G28152A, Arg-Gln)				
GG	39	175	1.0	-
GA	60	124	2.171 (1.365-3.454)	0.001
AA	9	18	2.244 (0.938-5.367)	0.064
GA+AA	69	142	2.180 (1.389-3.422)	0.001

Table 5.4 XPD genotypes and ALL risk

Genotype	ALL (n = 108)	Controls (n = 317)	OR (95%CI)	P-value
XPD codon 312 (G23591A, Asp-Asn)				
GG	97	272	1.0	-
GA	11	43	0.717 (0.356-1.447)	0.352
AA	0	2	-	-
GA+AA	11	45	0.685 (0.341-1.379)	0.287
XPD codon 751 (A35931C, Lys-Gln)				
AA	87	260	1.0	-
AC	19	56	1.014 (0.571-1.800)	0.962
CC	2	1	5.977 (0.535-66.728)	0.099
AC+CC	21	57	1.101 (0.631-1.920)	0.734

Table 5.5 XRCC1, XPD allele frequencies and ALL risk

Allele	ALL (n = 108)	Controls (n = 317)	OR (95%CI)	P-value
XRCC1-194 (C26304T)				
C (Arg)	168	445	1.0	-
T (Trp)	48	189	0.673 (0.468-0.967)	0.032
XRCC1-280 (G27466A)				
A (Arg)	202	586	1.0	-
T (His)	14	48	0.846 (0.457-1.567)	0.595
XRCC1-399 (G28152A)				
G (Arg)	138	474	1.0	-
A (Gln)	78	160	1.674 (1.203-2.330)	0.002
XPD-312 (G23591A)				
G (Asp)	205	587	1.0	-
A (Asn)	11	47	0.670 (0.341-1.317)	0.243
XPD-751 (A35931C)				
A (Lys)	193	576	1.0	-
C (Gln)	23	58	1.183 (0.711-1.970)	0.517

5.1.2 Comparing of XRCC1 and XPD genotypes with other ethnicity

For the XRCC1-194 and XRCC1-399, allele frequencies in previously of Caucasians, African-American, Egyptian, Chinese and Taiwanese population have been previously described (**Table 5.6**). The allele frequency of XRCC1-194 was found 25.0% in Chinese, 27.0% in Taiwanese, 6.0% in Caucasians, 5.0% in African-American and 5.0% in Egyptian. The XRCC1-194 allele frequency was not significantly different between Thai and Chinese or Taiwanese (p -value > 0.05). The XRCC1-399, allele frequency was found 27.0% in Chinese, 26.0% in Taiwanese, 37.0% in Caucasians, 17.0% in African-American and 14.0% in Egyptian. The XRCC1-399 allele frequency was significantly between Thai and Egyptian (p -value < 0.05), but was not significantly different between Thai and Chinese, Taiwanese, Caucasians or African-American (p -value > 0.05).

For the XRCC1-280, allele frequencies in previously published reports of Chinese, Taiwanese and Caucasians were also included in **Table 5.6**. This allele frequency was not statistically different from other ethnic populations which described in above.

The allele frequencies of XPD-312 and XPD-751 polymorphisms in previously reports of Chinese, Caucasians and Danish were shown in Table 5-6. The allele frequencies of XPD-312 and XPD-751 were statistically different between Thai and Chinese, Caucasians or Danish. The present data showed lower frequencies of both XPD genetic polymorphisms (7% and 9% of XPD-312 and XPD-751, respectively) in a Thai population compared with other ethnicities.

Table 5.6 Allele frequencies of XRCC1 and XPD genes in Thai and other ethnic populations

Gene	Allele	Allele frequency previous reports	Present study	
			ALL cases (n = 108)	Healthy controls (n = 317)
XRCC1	194 Trp	0.25 ^a , 0.06 ^b , 0.05 ^c , 0.25 ^d , 0.05 ^f , 0.27 ^g	0.22	0.30
	280 His	0.08 ^a , 0.03 ^b , 0.02 ^f , 0.11 ^g	0.07	0.08
	399 Gln	0.25 ^a , 0.37 ^b , 0.14 ^c , 0.27 ^d , 0.17 ^f , 0.26 ^g	0.36	0.25
XPD	312 Asn	0.42 ^a	0.05	0.07
	751 Gln	0.29 ^a , 0.40 ^d , 0.35 ^e	0.11	0.09

^a Shen et al. (21): 12 unidentified individuals

^b Lunn et al. (2): 169 healthy American Caucasians

^c Abdel-Rahman et al. (44): 48 healthy Egyptian

^d Chen et al. (73): 109 healthy Chinese

^e Dybdahl et al. (41): 20 healthy Danishes

^f Lunn et al. (2): 98 healthy African-Americans

^g Lunn et al. (2): 120 healthy Taiwanese

CHAPTER 6

DISCUSSION

Variants of the following genes were examined in epidemiological studies: BER gene XRCC1 and NER gene XPD. Shen *et al.*(21) identified three coding polymorphisms in XRCC1 gene at codon 194(Arg to Trp), 280(Arg to His) and 399 (Arg to Gln), and XPD gene at codon 312(Arg to Asn) and 751(Lys to Gln). DNA repair pathway protects cells against instability and oxidative stress. DNA repair pathway plays a vital role in maintaining genetic integrity, and it is becoming clear that defects in repair pathways are connected to many different types of disease including leukemia and other cancer (74). However, no consistent conclusion has yet been established especially on the role of these polymorphisms in developments of acute lymphoblastic leukemia.

This research determined the frequencies of XRCC1 genetic polymorphisms (XRCC1-194, XRCC1-280, XRCC1-399) and XPD genetic polymorphisms (XPD-312 and XPD-751) in patients of childhood ALL and in a control group. This study found a reduced risk of childhood ALL associated with the T allele (OR,0.673, 95%CI, 0.468-0.967) and TT genotype (OR, 0.220, 95%CI, 0.50-0.964). The obtained result was quite similar to the previous publication. Most of the published XRCC1-194 studies reported a reduced risk of cancer associated with the T allele (26, 27, 28, 29, 30, 31). The largest study was a breast-cancer study of African-Americans (n = 253 cases) and Caucasians (n = 386 cases) that showed age-adjusted ORs of 0.7 (CT/TT versus CC; Caucasians: 95%CI, 0.3-1.5; African-American: 95%CI, 0.4-1.3) and no evidence interactions with smoking, menopausal status or occupational exposure (27). Two lung-cancer studies and a bladder-cancer study also observed inverse associations with the T allele; adjusted ORs and 95% CIs (CT/TT versus CC) were 0.7 (0.4-1.2), 0.4 (0.2-0.9), and 0.6 (0.3-1.0), respectively (26, 28, 29). Possible interaction with smoking and drinking status were seen in these studies (26, 28, 29); such stratification by relevant exposures may provide information regarding the underlying biological

mechanisms. Studies of SCCHN and stomach cancer also suggested decreased risks of individuals with CT or TT genotype are compared to those with CC genotype (30, 31); in each of these studies, this inverse association was statistically significant only when restricted to subtype of disease (oral/pharyngeal cancers: OR, 0.4; 95% CI, 0.2-0.8; gastric cardia: OR, 0.5; 95% CI, 0.3-0.9) (30, 31). Because different subsets of a cancer may result from different molecular pathways, this strategy of refining the phenotype may improve power to detect genetic associations, although subset analyses can be misleading in the absence of a clear biological rationale. Only one small study of SCCHN (98 cases, 161 controls) estimated an increase in risk associated with the T allele, but the 95% CI was not inconsistent with a reduced risk of the magnitude discussed above (32). No associations with codon 194 were seen in small studies (\leq 125 cases) of esophageal cancer (33), non-small cell lung cancer (34), or melanoma (35).

A second XRCC1 polymorphism (Arg399Gln) has also been well studied. In this study, we found an increased risk of childhood ALL associated with the A allele, GA and GA or AA genotype (OR, 1.674, 95% CI, 1.203-2.303; OR, 2.171, 95% CI, 1.365-3.454, OR, 2.186, 95% CI, 1.389-3.422, respectively). However, the results suggested associations in different direction for different cancers: decreased risk for nonmelanoma skin carcinoma (36), esophageal cancer (33), and bladder-cancer (26, 37); increased risk of breast-cancer (27) and stomach cancer(31). There were consistent results for SCCHN (30, 32) and lung cancer (28, 29, 34, 38, 39) and no association was seen with melanoma (35), although the melanoma study was plagued by the use of cadaver controls. A relatively large population-based study of the nonmelanoma skin cancer revealed inverse associations with the AA genotype (AA versus GG: basal cell carcinoma, adjusted OR, 0.7; 95% CI, 0.4-1.0; squamous cell carcinoma, adjusted OR, 0.6; 95% CI, 0.3-0.9)(36). Putative interactions with the number of lifetime sunburns were seen. The inverse association was limited to those with fewer than three sunburns, and an increased risk with AA genotype was seen among those three or more sunburns (36). Inverse associations with 399A allele were also suggested in studies of esophageal cancer (among drinkers only)(33) and bladder cancer (particularly among former or light-smokers)(26, 37), similar to the codon 194 results in these studies. An increased risk of breast cancer was seen among African-

American carriers of the 399A allele in one study (GA/AA versus GG; age adjusted OR, 1.7; 95% CI, 1.1-2.4). Data were also consistent with interactions with smoking and occupational exposure to ionizing radiation (27). A stomach cancer study found an increased risk associated with the 399A allele (GA/AA versus GG adjusted OR, 1.5; 95% CI, 1.0-2.4)(31), and combined risk genotypes at codon 194 and codon 399 and the association with risk was stronger (194CC +399GA /399AA versus 194TT/194CT +399GG adjusted OR, 1.7; 95% CI, 1.1-2.7)(31), however, it is not clear that combined associations were greater than expected given the individual associations. Two hospital-based SCCHN studies yielded inconsistent results at XRCC1-399 polymorphism (30, 32). The larger study observed an increase in risk associated with the AA genotype (AA versus GG/AA adjusted OR, 1.6; 95% CI, 1.0-2.6)(30), and the smaller study observed a decrease in risk (AA versus GG adjusted OR, 0.1; 95% CI, 0.04-0.6)(32). Lung-cancer studies of XRCC1-399 have also shown inconsistent results. One population-based analysis suggested increased risk for lung cancer among individuals with AA genotype (OR, 2.5; 95% CI, 1.1-5.8)(38), as did a second hospital-based study that found a further increase in risk among squamous cell cases only (OR, 3.3; 95% CI, 1.2-9.2)(39). However, another larger lung-cancer study suggested decreased risk (African-American: OR, 0.6; 95% CI, 0.2-2.3; Caucasians: OR, 0.6; 95% CI, 0.3-1.3 (29)), and two lung-cancer studies showed no differences (28, 34). Inconsistent XRCC1-399 results in these lung-cancer studies may be attributable to the differences of population sample differences or other study-design issues; the relationship between XRCC1 and lung-cancer risk is yet to be clearly elucidated.

From the results, two ALL patients had XRCC1-194 TT genotype that reduced risk of childhood ALL and XRCC1-399 GG genotype that was not significantly different between childhood ALL and controls. This demonstrated that both polymorphisms are independent on childhood ALL risk.

For XRCC1-280 polymorphism, the result demonstrated that no differences of A allele frequencies between childhood ALL patients and a control group (OR, 0.846; 95% CI, 0.457-1.567). The AA genotype was not found in childhood ALL group. Comparing between both groups, there was no statistical difference in the frequencies of each genotype. Previous studies assessed the less common XRCC1-280

polymorphism (26, 28, 33, 34). One small nested case-control study of lung cancer reported an OR of 1.8 (95% CI, 1.0-3.4) for carriers of one or two A alleles and a statistically significant interaction with alcohol consumption ($P = 0.02$)(28). Three small studies of bladder, esophageal, and non-small cell lung cancer did not suggest any association, although small sample size and low frequency of the A allele limited power (26, 33, 34).

The XPD gene product is a subunit of TFIIH and is necessary for NER and transcription. Whereas XPD mutations are clearly deleterious (75), effects of common polymorphisms (codon 751, 312) on risk of carcinoma remain unclear. The most commonly studied polymorphism was the XPD-312 and 751 polymorphisms, although no statistically significant findings have been reported, suggestive results were observed in some studies. In this study, found essentially no statistical difference in frequencies of each genotypes and alleles in both of childhood ALL and controls. The largest study of codon 751 was a United States hospital-based lung cancer study (1092 cases, 1240 controls) that found essentially no increase in risk in the data set overall but did observe evidence of an interaction with smoking status ($P = 0.01$) with an increased risk among nonsmokers (CC versus AA adjusted OR, 2.0; 95% CI, 1.1-3.4) (76). A similar relationship between codon 751 and smoking status was seen in a smaller Swedish study of lung cancer (77), although two other lung cancer studies found no suggestions of association or interactions with smoking (78, 79). One study of SCHN among United States Caucasians reported a multivariate-adjusted OR of 1.7 (CC versus AA; 95% CI, 1.0-2.8); ORs in this study were higher among older individuals and among those who smoked or drank at the time of the study (42). Small, possibly under-powered studies of bladder-cancer (37), basal-cell-carcinoma (80), non-small cell lung cancer (34), and melanoma (35) did not observe any associations.

Five of the above mentioned XPD studies also examined associations with the XPD-312 polymorphism. A large lung-cancer study (1092 cases, 1240 controls) reported an elevated risk (AA versus GG adjusted OR, 1.5; 95% CI, 1.1-2.0) and an interaction with smoking ($P < 0.01$), again with the increased risk limited to nonsmokers (AA versus GG adjusted OR, 3.4; 95% CI, 1.9-6.0)(76). This interaction was also seen in a smaller lung cancer study (77). An inverse association was seen

between the rare allele and risk of non-small cell lung cancer (GA/AA versus AA; OR, 0.5; 95% CI, 0.3-1.0)(34). A further decreased risk was observed among light smokers but not among non-and heavy-smokers; the small number of subjects in this study precludes interpretation of smoking interaction (34). A small basal-cell carcinoma study found no association with codon 312 except when restricted to individuals with a family history of nonmelanoma skin cancer (GA/AA versus GG OR, 5.3; 95% CI, 1.2-23.9)(80); however, reasons for this stratification are unclear. No association with this polymorphism was seen in a British study of melanoma (35).

Our allele frequency distribution data indicate that the frequencies of the 194Trp and the 399Gln alleles in our controls population were 0.30 and 0.25, respectively, consistent with those reported by Shen *et al.*(21)(0.25 for 194Trp and 0.25 for 399Gln) from sequence analysis of XRCC1 gene in 12 presumably healthy individuals. The 194Trp allele frequency observed in this study is consistent with that observed by Chen *et al.*(73), for Chinese (0.25), and by Lunn *et al.*(2), for Taiwanese (0.27), but not with the frequency reported for Caucasians (0.06) and African-Americans (0.05) residing in North Carolina, and for Egyptians (0.05) by Abdel-Rahman *et al.*(44). The frequency of the 399Gln allele (0.25) was comparable with that reported for Taiwanese (0.26), and Caucasians (0.37), but much higher than that reported for African-Americans (0.17) by Lunn *et al.*(2), and Egyptians (0.14) by Abdel-Rahman *et al.*(44).

Both XPD polymorphisms in this study were not associated with childhood ALL risk. But these allele frequencies distribution data showed low frequencies of both XPD gene polymorphisms (0.07 for 312Asn and 0.09 for 751Gln), much lower than those reported by Shen *et al.*(21)(0.42 for 312Asn and 0.35 for 751Gln), Chen *et al.*(73), for Chinese (0.40) and 0.35 for Taiwanese by Lunn *et al.*(2). These differences suggest a possible ethnic variability in the allelic distribution of both DNA repair genes and point out the need for additional studies of polymorphisms frequencies in different populations. Therefore, this present data of DNA repair genetic polymorphisms could be studied further in pharmacogenetics and outcome of childhood ALL in Thai population.

XRCC1 is involved in the repair of direct DNA single strand breaks (SSBs) and during BER, the most common alteration produced by DNA damage. The XRCC1

gene codes for a multidomain protein that interacts with PARP, DNA polymerase β , DNA ligase III, polynucleotide kinase(PNK), and AP-site endonuclease1(APE1)(66). Three XRCC1 polymorphisms occur at residues that these amino acid are evolutionary conserved (34). Although, the functional significance of these polymorphisms in XRCC1 have not been proposed. The XRCC1-194 is located within a APE1 binding domain, it is revealed that APE1 binds to XRCC1 by interacting with the central section of the XRCC1 protein. XRCC1 binds to the amino terminal of APE1, and weakly stimulates (~3 fold) APE1 endonuclease activity if the XRCC1 is present in limiting amounts, albeit when added in several thousand fold molar excess. This raises the possibility that XRCC1 may influence the creation of a SSB during BER, in addition to the subsequent steps of removing the SSB. However, XRCC1 also weakly stimulated the 3'-end processing activity of APE1, which would be consistent with its identified role in processing 3'- and 5'-ends with human polynucleotide kinase. Whether or not XRCC1 stimulates this activity remains to be determined. In this study, the data suggest the amino acid substitution in XRCC1-194Trp that tend to increase APE1 stimulation to reduce DNA damage. The XRCC1-399 polymorphism is located at BRCT I domain, which is located in the central region of XRCC1 protein. The central BRCT I domain binds to and down regulates the SSB recognition protein PARP1, and is required for efficient SSB repair during both G1 and S/G2 phases of the cell cycle. Amino acid substitutions in the BRCT domain have been reported to disrupt the functionality of XRCC1 (28). Seedhouse *et al.* (74), the presence of at least one XRCC1-399Gln allele indicated a protective effect in therapy-related AML (OR, 0.44; 95% CI, 0.20-0.93). In contrast, in this study was found an increased risk of childhood ALL of at least one Gln allele. Amino acid change in XRCC1-399 polymorphism may be altered the protein structure that tend to decrease the ability of the PARP1 suppression. Interpretation of this data is limited by the lack of knowledge about the functional significance of the polymorphisms on the XRCC1 and XPD gene. The results of the other polymorphisms (XRCC1-280, XPD-312, and XPD-751) in this study may suggest these polymorphisms were not significantly associated with childhood ALL. Very low allele frequencies of both XPD polymorphisms in this population, and was not significantly associated with childhood ALL, may suggest that there are no potential roles for these

polymorphisms in genetic predisposition to the childhood ALL among Thai population. However, additional studies with larger sample sizes are required to detect the small effect observed. Future studies of XPD and XRCC1 polymorphisms and characterization of the functional significance of these variants will help to an understanding the roles of XRCC1 and XPD gene in carcinogenesis.

In this study, genomic DNA was extracted by using a phenol-chloroform standard method (71, 72). This method produces very pure DNA, which can be stored for long periods of time. However, the method used phenol, which is hazardous and is more time consuming. A wide range of methods for the analysis of pharmacogenetic polymorphisms is now available. Most genotyping assays currently used to screen for known pharmacogenetic polymorphisms involve use of the PCR followed by a specific detection step. Here, this study used PCR-RFLP to determined specific genotype. PCR-RFLP is relatively simple and not required expensive equipment, but slow and low throughput method, while real-time PCR, DNA microarray, and direct DNA sequencing analysis are more specialized higher-throughput (70). However, the high-throughput methods are required expensive equipment. In addition to the normal controls needed in carrying out any amplification by PCR, it is important that control DNA samples of known genotype are analyzed in parallel with test samples regardless of the method being used for genotyping. Although, this study did not have DNA samples of known genotypes used analyzed in parallel with test samples, but internal control cutting site of each restriction enzymes are check specific restriction site in each PCR products, this study was found all genotyping patterns (wild-type, heterozygous, variant-type) to genotyping for individuals in test samples of all polymorphisms, that indicate corrected of each different genotypes.

The case and control groups of this study were the same ethnic and culture (Thai population). However, the distribution of age was different between childhood ALL (median 6.3 yrs.) and controls (20-40 yrs.) This study was examining in host germ line polymorphisms or DNA sequence variation among individuals that is inherited and transmitted from one generation to the next (81). Additionally, volunteers in the controls group were older than the onset of childhood ALL, and had never been diagnosed with any malignancies. Therefore, this group should contain an appropriate individuals to be normal controls in their study.

In summary, we report an association between decreased childhood ALL risk and coding polymorphisms in the XRCC1 gene: a C to T substitution at position 26304 (codon 194, exon 6), and increased childhood ALL risk and polymorphism in the XRCC1 gene: a G to A substitution at position 28152 (codon 399, exon 10). Other DNA repair genetic polymorphisms in this study (XRCC1-280, XPD-312 and XPD-751) had no significant associations between the studied genotypes and risk of development of childhood ALL. However, childhood ALL is a wide diversity disease, which may differ in etiology, pathology, and behaviors. As we know that carcinogenesis is a multi-factorial process, it is unlikely that any simple genetic polymorphisms accounts completely for the individual variability in host susceptibility of childhood ALL. Consequently, the combination effect of various genetic polymorphisms and the interracial differences of the DNA repair genes and other genes in childhood ALL are recommended for further study.

CHAPTER 7

CONCLUSION

The purpose of this study was to evaluate the effect of XRCC1 and XPD genetic polymorphisms on the risk of childhood ALL. The relevance of genotyping in 108 childhood ALL patients and 317 healthy controls was investigated. Interestingly, the XRCC1-194 T allele showed a statistically significant difference between cases and controls (OR = 0.673; 95% CI, 0.468-0.967). In contrast, the XRCC1-399 A allele, GA and GA or AA genotypes were significantly increased in ALL group (OR = 1.674; 95% CI, 1.203-2.330; OR = 2.171; 95% CI, 1.365-3.454; OR = 2.180; 95% CI, 1.383-3.422, respectively). This data suggested that XRCC1-194 T allele play a role of protective on to this disease, and increased risk of childhood ALL with XRCC1-399 A allele. Additionally, the result presented simple DNA based test that can be used to investigate interindividual differences in DNA repair gene expression. This will greatly facilitate studies on the relevance of pharmacogenetics for DNA repair genes with respect to disease risk and to the pharmacokinetics and pharmacodynamics of many therapeutic drugs. In conclusion, this study demonstrated for the first time that polymorphisms in DNA repair genes may influence childhood ALL risk. Our data indicate an association between two XRCC1 polymorphisms and childhood leukemogenesis.

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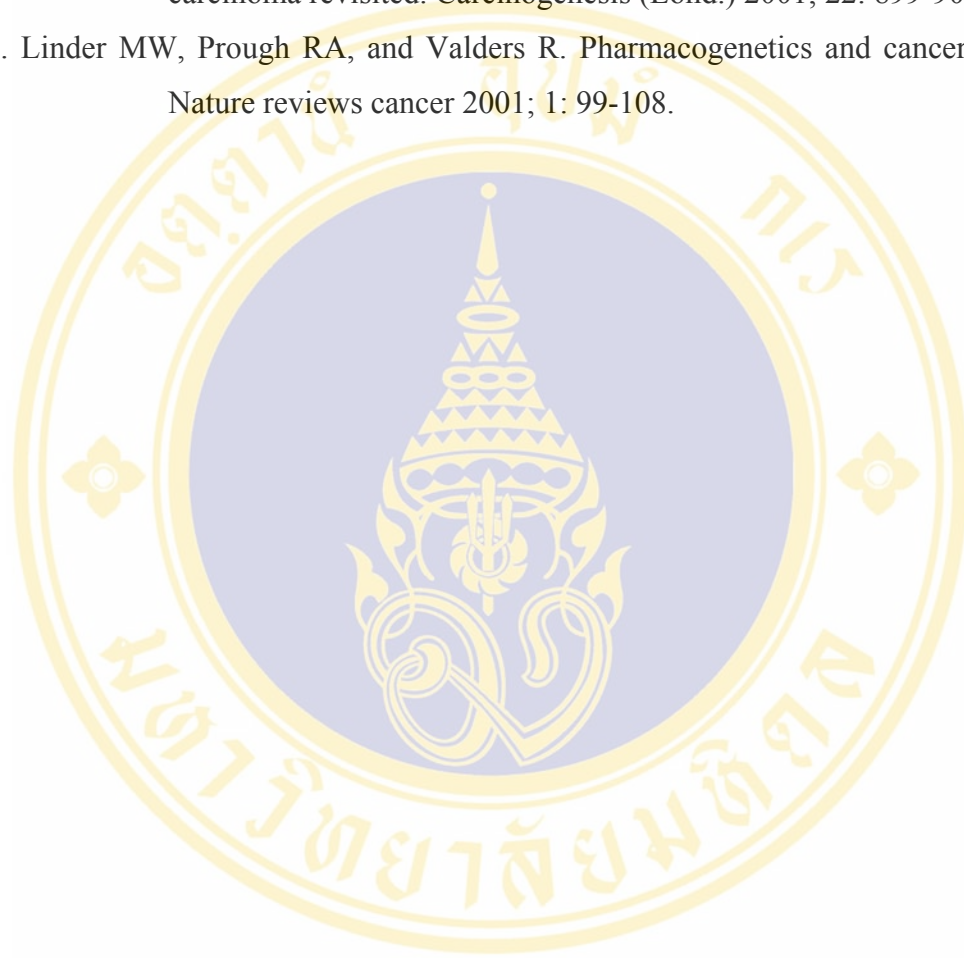
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APPENDIX

Preparation of buffer and solution

1. DNA isolation solution

1.1	10% SDS	50	ml
	Sodium dodecylsulfate (SDS)	5	g
	DW	50	ml
	Keep in room temperature		
1.2	Proteinase K (5 mg/ml)	20	ml
	Proteinase K	100	mg
	DW	20	ml
	Aliquot the solution 1.0 ml per vial and keep in freezer below -20°C		
1.3	0.5 M Tris-HCl pH 8.0	400	ml
	Trisma base	24.22	g
	DW	320	ml
	Adjust pH to 8.0 with HCl and adjust the volume to 400 ml with DW, keep in room temperature.		
	(Note: use 250 ml at step 1.5, to make saturated phenol pH 8.0 then dilute the rest with 1:4 pf water to make 0.1 Tris-HCl pH 8.0 adjust enough for step 1.6 and 1.7		
1.4	Saturated Phenol pH 8.0	250	ml
	Phenol (melt at 60°C)	250	g
	8-Hydroxyquinolone	0.25	g
	Add while Phenol still at 60°C		

1.5	0.5 M Tris-HCl pH 8.0	250 ml
	Approximately used and pore buffer in phenol bottle, continuously shake for 15 min. Let solution separate into 2 layers, the top should be transparence within a few min, otherwise shake again. Discard the top layer by suction gently, leave the least possible.	
1.6	0.1 M Tris-HCl pH 8.0	250 ml
	Mix with phenol, shake for 15 min then follow the rest of step 1.5	
1.7	0.1 M Tris-HCl pH 8.0	250 ml
	As step 1.6, pH should be 8.0 and keep in the brown bottle or clean glass bottle wrapped in aluminium foil at 4-10°C	
1.8	Chloroform-Isoamyl alcohol (24:1 v/v)	1000 ml
	Chloroform	960 ml
	Isoamyl alcohol	40 ml
1.9	3 M Sodium acetate pH 5.6	100 ml
	Sodium acetate	24.61 g
	DW	80 ml
	Adjust pH to 5.6 with glacial acetate and to make a final volume to 100 ml with DW, sterile by autoclave and keep in 4-10°C.	
1.10	70% Ethanol	250 ml
	Absolute ethanol	175 ml
	DW	75 ml
	Keep in freezer	
1.11	Lysis buffer	1,000 ml
	Tris base	12.1 g

Sucrose 109.5 g

DW 800 ml

Adjust pH to 7.5 with conc. HCl

Triton X 100 10 ml

To make a final volume to 1,000 ml with DW, then sterile by autoclave and keep at 4-10°C

1.12 10X STE buffer 100 ml

Tris base 6.05 g

EDTA 0.37 g

NaCl 5.84 g

DW 80 ml

Adjust pH to 7.5 with conc. HCl, and to make a final volume 100 ml with DW, then sterile by autoclave and keep at 4-10°C

1.13 TE buffer pH 7.4 500 ml

Tris-HCl 6.06 g

EDTA 0.19 g

Adjust pH to 7.4 by conc. HCl, to make a final volume 500 ml with DW, then sterile by autoclave and keep at room temperature.

1.14 0.1% Nonidet 1,000 ml

Nonidet (conc.) 1 ml

DW 1,000 ml

Keep in sterile bottle at 4-10°C

2. Electrophoretic analysis solution and Ethidium bromide staining

2.1	10X TBE buffer (stock solution)	1,000	ml
	Tris base	108	g
	Boric acid	55	g
	EDTA	7.4	g

Dissolved the Tris base and Boric acid in 700 ml DW, then add the EDTA and adjusted the volume to 1,000 ml with DW, keep at room temperature. Working solution (1X TBE) was prepared by adding 100 ml of 10X TBE with 900 ml of DW.

2.2	50 mg% Ethidium bromide solution	100	ml
	Ethidium bromide	0.5	g

Dissolved in 100 ml of DW (5 mg/ml) and sterile on magnetic stirrer for several hours to ensure that the dye was dissolved. Stored in dark bottle in aluminium foil and stored at 4°C

BIOGRAPHY

NAME	Mr. Tanasan Sirirat
DATE OF BIRTH	July 29, 1980
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTION ATTENDED	Huachiew Chalermprakiet University, 1998-2001: Bachelor of Science (Medical Technology) Mahidol University, 2002-2004: Mater of Science (Clinical Pathology)
POSITION & OFFICE	Division of Clinical Microscopy and parasitogy, Faculty of Medical Technology, Huachiew Chalermprakiet University, Samut Prakarn, Thailand, 10540 Position: Lecturer Tel. 0-23126300 E-mail: tansno@hotmail.com
HOME ADDRESS	55/59 Moo.5 Chalermprakiet Rama IX Road, Pravet District, Bangkok, 10250, Tel. 0-23615850