ABSTRACT

Title of Document:	GENETIC POLYMORPHISMS IN DNA EXCISION REPAIR GENES IN RELATION TO CANCER AND CANCER THERAPIES
	Rui Gao, Doctor of Philosophy, 2011
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DNA excision repair pathways, including the nucleotide excision repair (NER) and the base excision repair (BER) pathways, play significant roles in maintaining genomic stability. However, these pathways are found to be responsible for therapeutic resistance to cancer therapies causing DNA damage. Platinum-containing drugs are important therapies for various solid tumors. Genetic polymorphisms in NER and BER genes have been identified, and some have been correlated to altered clinical outcome to platinum-based chemotherapies. Here I studied the genetic polymorphisms in the NER genes, ERCC1 and XPD, and the BER

genes, XRCC1 and PARP1, and found that the polymorphic variants had significantly higher frequencies in European Americans (EAs) for ERCC1 N118N ($p<0.000001$), XPD K751Q ($p=0.006675$), XRCC1 R399Q ($p<0.000001$) and PARP1 V762A ($p=0.000001$), compared to those in African Americans (AAs), which may reflect a mild reduction in DNA excision repair function in EA population. However, these polymorphisms were not associated with risk of prostate cancer or the clinical outcome of radiation therapy in prostate cancer in EAs. I also investigated the functional consequences of the most well studied NER polymorphism ERCC1 N118N (500C>T) by introducing the ERCC1 cDNA clones containing either the C or T allele into an ERCC1 deficient cell line UV20. However, neither the ERCC1 expression levels nor the cellular sensitivity to platinum drugs were affected by this silent mutation. These data suggests that the N118N itself does not contribute to the phenotypic differences in *ERCC1*, but rather this polymorphism may be linked to other causative variants or haplotypes. Therefore, I examined 4 polymorphisms in ERCC1, including rs3212948 (G>C), rs3212950 (C>G) in intron 3, and rs3212929 (T>G) in the 5' UTR, in addition to N118N (500C>T), and found that the haplotypes of these polymorphisms were associated with risk of skin melanoma, indicating the potential functional significance of other ERCC1 polymorphisms. Understanding the functional significance the genetic polymorphisms in DNA excision repair genes may facilitate the administration of personalized medicine.

GENETIC POLYMORPHISMS IN DNA EXCISION REPAIR GENES IN RELATION TO CANCER AND CANCER THERAPIES

By

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Dedication

To my family and extended family --- this is for you!

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From the deepest of my heart, I want to say something to Grace and Rita, and many others. I couldn't make it through without you. Thank you for opening your

hearts and families to me and including me in your lives. Knowing you is probably the best thing that has happened in the past 3 years.

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List of Abbreviations

6-4PP: pyrimidine 6-4 pyrimidone photoproducts
AA: African American
APE1: Apurinic/apyrimidinic endonuclease
BER: base excision repair
BRCT1: BRCA1 C-terminal 1
CPD: cyclobutane pyrimidine dimmers
CS: Cockayne syndrome
CSA: Cockayne syndrome group A
CSB: Cockayne syndrome group B
DNA Pol: DNA polymerase
DNA-PK: DNA-activated protein kinase
DSB: double-strand break
EA: European American
EJ: end joining
ERCC1: Excision repair cross-complementing group 1
FEN1: Flap endonuclease 1
GG-NER: global genome NER
GSH: Glutathione
hOGG1: human 8-Oxoguanine DNA Glycosylase 1
hHR23B: human homolog of yeast Rad23 protein B
HR: homologous recombination
ICL: inter-strand cross-link
IMRT: intensity modulated radiation therapy
MMR: MisMatch repair
NER: nucleotide excision repair
NSCLC: non-small cell lung cancer
PARP1: Poly(ADP-ribose) polymerase 1
PCNA: proliferating cell nuclear antigen
PNK: polynucleotide kinase
PSA: prostate-specific antigen
POLH: Polymerase eta
RFC: replication factor C
RNA Pol II: RNA polymerase II
ROS: reactive oxygen species
RPA: replication A protein
TC-NER: transcription-coupled repair
TFIIH: Transcription factor II H
TTD: trichothiodystrophy
XP: xeroderma pigmentosum
XPA-XPG: xeroderma pigmentosum, complementation group A-G
XRCC1: X-ray repair cross-complementing protein 1

1. Introduction

1.1. Platinum containing drugs

Platinum containing drugs are currently used for treating cancer, and are actually the largest class of drugs used in cancer treatment. Their therapeutic use was discovered accidentally in the 1960s when Rosenberg and his coworkers (1) were studying how electric or magnetic dipole fields might be involved in cell division. The Escherichia coli cells in the chamber containing a set of platinum electrodes, which were considered to be inert, appeared as very long filaments rather than as the normal short rods. A chemical analysis followed and revealed that the electrolysis products arising from the platinum electrodes were causing the biological effect and the active product was subsequently named cisplatin. In 1968, cisplatin was given to mice bearing a standard murine transplantable tumor, and caused marked tumor regression (2). Since then, platinum-based drugs have been widely used against various solid tumors. Over the years cisplatin has significantly improved the disease free survival in patients with testicular cancer when discovered early, hence becoming an essential component of the chemotherapy regimen. The major platinum containing drugs are cisplatin, carboplatin, oxaliplatin and satraplatin. They destroy cancerous cells primarily by interfering with and causing cross-links in DNA, thereby triggering apoptosis.

Cisplatin was approved by the Food and Drug Administration (FDA) for the treatment of metastatic testicular and ovarian cancers in 1978 and transitional bladder cancer in 1993 (3). Though important in cancer treatment, cisplatin has some

drawbacks such as severe cytotoxicity, drug resistance and poor oral bioavailability. Cisplatin has cis-diammine as its carrier ligand and chloride as its leaving group. Carboplatin, approved in 1989 for ovarian cancer, shares the same carrier ligand with cisplatin but has a bidentate dicarboxylate ligand as its leaving group (refer to **Figure 1-1**). It is less toxic than cisplatin and can thus be given at a much higher dose than cisplatin, although it is only active in the same range of tumors as cisplatin (4). Oxaliplatin, approved for colorectal cancer in 2002, has a 1,2-diaminocyclohexane (DACH) carrier ligand, and has shown efficacy in cisplatin- and carboplatin-resistant tumor cell lines, indicating the important role that carrier ligands play in the specificity of tumor cytotoxicity. Satraplatin is the first orally available platinum-containing drug and has been under consideration for approval by the FDA for hormone-refractory prostate cancer (HRPC). It has two chlorine atoms attached to the platinum as in cisplatin, but differs from it with two acetate groups attached, and a cyclohexyl moiety substituted on one of the amino groups. Satraplatin shows similar anti-tumor activity to that of cisplatin and carboplatin but has an improved cytotoxicity profile and less cross-resistance to cisplatin (5). Nedaplatin is a second-generation platinum analog and is closely related to cisplatin in its chemical structure. It contains a ring structure in which glycolate is attached to the platinum by a bidentate ligand and has shown reduced cytotoxicity (6). It has been exclusively used in Japan to treat several types of cancers, such as head and neck, ovarian and lung cancer (3). Picoplatin is rationally designed to provide a bulkier carrier ligand and has subsequently shown less cross-resistance with cisplatin and carboplatin in vitro (7). It has shown clinical efficacy in cisplatin resistant small cell lung cancer (8). **Figure 1-1**

shows the chemical structures of the major platinum-containing drugs mentioned above.

One major challenge in applying and designing the platinum compounds has been overcoming the intrinsic resistance, mostly seen in colorectal cancer, prostate cancer, lung and breast cancer, or the resistance that is acquired during treatment cycles, as seen in ovarian cancer.

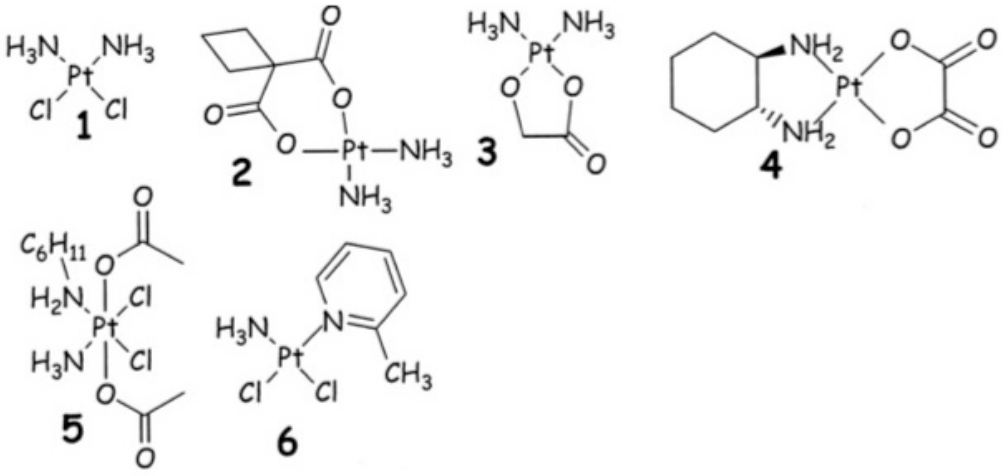


Figure 1-1: Chemical structures of major platinum compounds: cisplatin (1), carboplatin (2), nedaplatin (3), oxaliplatin (4), satraplatin (5) and picoplatin (6).

1.2.Mechanisms of action

1.2.1. Drug accumulation

Cisplatin is the first member of the class of platinum containing anti-cancer drugs and provides the structural basis for the rational design of other platinum analogs. It has two chloride ion ligands situated adjacent to one another and two relatively inert ammonia ligands in a square planar structure. Once the drug is in the bloodstream, it remains intact due to high concentrations of chloride ions (~100mM).

Cisplatin primarily enters cells by passive diffusion (9), but specialized transporters such as copper transporters (CTR1) and copper-transporting P-type ATPases (ATP7A and ATP7B) can also facilitate its uptake and efflux (10). Once cisplatin has entered the cells, aquation and reactions with the cellular components take place. Specifically, inside the cell, the two chloride ions are substituted with water due to the relatively low intracellular concentrations of chloride ions (~3-20 mM), whereas the two ammonia ligands remain intact and are known to carry anti-tumor activity. The aquated species then goes on to react with nucleophilic groups such as DNA, RNA, proteins, membrane phospholipids, and microfilaments (11).

1.2.2. Binding to DNA

Cisplatin primarily causes cytotoxicity by binding to the DNA and producing DNA-distorting lesions. The first clues of DNA being the primary target for cisplatin were the filamentous growth of the bacteria induced by cisplatin (1) and lysis of *Escherichia coli* cells containing bacteriophage λ (12), characteristics shared by DNA damaging agents. Cisplatin binds preferentially to the N7 positions of two adjacent guanines or a guanine and an adenine located in the major grooves of the double helix (13). The major platinum-DNA adducts are 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpXpG) intrastrand cross-links, which account for 60−65% of adducts formed by cisplatin (**Figure 1-2**). A small portion of interstrand cross-links and monofunctional adducts are also present (14). There is still debate on which types of cisplatin-DNA adducts are the most important in mediating cytotoxicity of the drug. Since transplatin, the inactive trans isomer of cisplatin, mainly forms 1,3-intrastrand and interstrand cross-links, it is believed that the 1,2-intrastrand adducts formed by

cisplatin play the most significant role in inducing tumor cell death (15, 16). Moreover, it has also been found that the 1,2-intrastrand adducts are more resistant to DNA repair by human cell extracts (17). However, the cytotoxic effects caused by the minor types of DNA cross-links should not be overlooked. For example, the interstrand cross-links formed by cisplatin can cause the extrusions of cytosines of the cross-linked sites, the bending of the helix towards the minor groove, and a large DNA bending, although the interstrand cross-links are unstable under physiological conditions and are easily cleaved and rearranged into intrastrand cross-links (18).

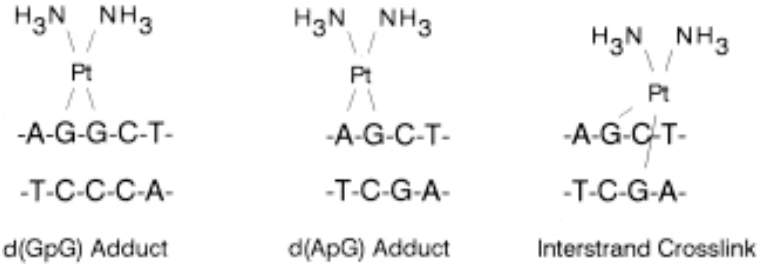


Figure 1-2: Platinum-DNA adducts (19).

1.2.3. Binding to non-DNA targets

In addition to platinum-DNA adducts, cisplatin also binds to non-DNA cellular components containing the nucleophilic groups, though the contribution of these targets to the cytotoxic effects induced by cisplatin has been poorly studied. DNA-protein cross-links are also formed by cisplatin and other platinum compounds (20). Less than 10% of covalently bound cisplatin is found in the DNA fraction. The vast majority of the drug binds to proteins (21). Cisplatin binds to phosphatidylserine in model membranes in a pH and chloride concentration dependent manner; this binding is suggested to be of biological importance in the cellular processes in which phosphatidylserine are involved (22). Due to cisplatin's strong reactivity against S-

donor molecules, the most important non-DNA cellular targets of cisplatin may be glutathione (GSH), which presents at high cellular concentrations (0.5−10 mM). The binding of cisplatin and GSH is proposed to serve as a drug reservoir modulating the kinetics of DNA platination. On the other hand, the binding of cisplatin to sulfur atoms of cysteine and/or methionine residues and to nitrogen atoms of histidine residues in proteins may affect their functions and thus cause cytotoxicity (23). A study showed that cisplatin binds to ubiquitin in vitro and forms at least three different adducts, while transplatin only forms one major adduct and does not bind as efficiently as cisplatin does, suggesting that the cytotoxic effects are caused by the binding of cisplatin to cellular proteins (24).

1.3. Repair of platinum-DNA adducts

The cytotoxic effect of platinum-containing drugs is a complex process and involves the initial stages of the drug entry into cells, interaction with cellular components, and the final stages of cell death. Reduced uptake and increased efflux may result in lower intracellular concentrations of the drug. Increased concentrations of thiol-containing molecules upon chronic platinum exposure can lead to decreased levels of platinum available for interaction with the target DNA. The formation of DNA adducts is an essential step for inducing the anticancer activity by these compounds. Platinum-DNA adducts interrupt major cellular processes such as DNA replication, transcription, cell cycle checkpoint arrest and apoptosis, and are recognized and processed by several DNA repair mechanisms. In addition, down regulation of the apoptotic signal by various mechanisms or increased DNA damage tolerance are also considered to contribute to platinum resistance (25).

However, the most important mechanism by which platinum resistance occurs is the increased DNA damage removal by various DNA repair pathways. The inhibition of DNA synthesis, especially in the actively transcribed strand, by platinum-DNA adducts is thought to be the main contributor to the cytotoxicity of platinum compounds (26). Following the formation of platinum-DNA adducts, the cellular repair systems respond to recognize the lesion and continually act until the fate of the cell is decided. Multiple pathways and proteins are involved in this process.

1.3.1. Mismatch repair (MMR) pathway

MMR facilitates the repair of post-DNA replication base pairing errors and functions in an ATP-dependent manner. Recognition of these errors and recruitment of repair machinery is carried out by the MutSα complex (composed of the MMR proteins MSH2 and MSH6) or MutSβ complex (containing MSH2 and MSH3) (27). MMR proteins have been indicated to bind to cisplatin-DNA adducts and initiate MMR protein-dependent cell death, specifically, by caspase-dependent apoptotic signaling pathways. Deficient MMR function increases replicative bypass of cisplatin-DNA adducts and compromises cisplatin induced cell death but not oxaliplatin-DNA adducts (28).

1.3.2. High-mobility-group box (HMGB) proteins

The HMGB proteins, such as HMGB1 and HMGB2, are highly conserved, non-histone architectural chromosomal proteins. They have been shown to have high binding affinity to the intrastrand cross-links caused by cisplatin (29) but play less

important roles in recognizing carboplatin- and oxaliplatin-DNA adducts (30-32). When the HMGB proteins bind to platinum induced DNA cross-links, they are deprived from their natural binding sites on the genome and protect the platinum-DNA lesions from cellular repair machineries, a process termed repair shielding. Over-expression of HMGB proteins has been shown to sensitize cancer cells to platinum containing drugs (33), possibly by reducing the recognition and removal of platinum-DNA adducts by other mechanisms.

1.3.3. DNA-activated protein kinase (DNA-PK)

DNA-PK is a nuclear serine/threonine protein kinase that requires DNA for activity. It mainly recognizes DNA double-strand breaks (DSB) (34) induced by ionizing radiation or small gaps and nicks in DNA (35). Ku is the DNA binding subunit of DNA-PK. It has been reported to also bind to the cisplatin-DNA adducts, however, the binding does not activate DNA-PK catalytic subunit (36). Nevertheless, Cisplatin-DNA adducts can inhibit translocation of Ku subunit along DNA, resulting in decreased association of DNA-PK to the Ku−DNA complex and therefore compromised protein kinase activity (37).

1.3.4. Nucleotide Excision Repair (NER) pathway

The NER pathway is the primary mechanism for removing platinum-DNA adducts. It has been found to excise cisplatin, oxaliplatin and satraplatin adducts with equal efficiency, indicating that it does not contribute to the carrier ligand specificity of platinum resistance (28). The molecular mechanism by which the platinum-DNA adducts are removed by NER has been well studied.

NER is a multi-step process that requires the coordination of multiple proteins. Based on the substrate specificity, the NER pathway can be divided into two sub-pathways: global genome NER (GG-NER) and transcription-coupled repair (TC-NER), as illustrated in **Figure 1-3** (38). GG-NER surveys the entire genome for helix-distorting injuries, while TC-NER focuses on the damage that is in the actively transcribed strand of a gene. The TC-NER is believed to be more important in repairing platinum-DNA adducts as TC-NER deficient cells are hypersensitive to cisplatin irrespective to their GG-NER status (39).

1.3.4.1.Lesion detection

The XPC-HR23B complex is the earliest damage detector to initiate GG-NER specifically (40). In comparison, in the TC-NER sub-pathway, RNA polymerase II (Pol II), and probably CSA and CSB, take the role of XPC. Elongating RNA Pol II is blocked by many lesions in the actively transcribed strand. The transcription bubble presented at the lesion can serve as an efficient damage sensor (41, 42). Once lesions have been detected, an open DNA complex is formed by a reaction intermediate composed of XPA, RPA, XPC-hHR23B plus TFIIH (Transcription factor II H). In this step, the DNA is unwound on either side of the lesion symmetrically and an open complex containing no more than 20 nucleotides is formed around the lesion. The addition of XPA, RPA, as well as XPG, is needed to obtain the full opening that is required for the following dual-incision step. XPA plays a key role in recognition of DNA damage and has a preference for binding to the single-stranded damaged DNA (43, 44). RPA binds to the undamaged DNA strand (45) and fully opens the repair intermediate to about 30 nucleotides in length. Independent of its 3’ incision activity,

XPG has a structural function in the stabilization of the open complex and the assembly of the NER DNA-protein complex (46).

1.3.4.2.Dual incision

Upon lesion demarcation, the actual incisions are carried out by the structure-specific endonucleases ERCC1-XPF (47, 48) and XPG (49). Though in principle, ERCC1-XPF and XPG can cut either strand of DNA, the nucleases are directed to the damaged strand only during repair. The binding polarity of RPA is crucial for the proper positioning of ERCC1-XPF (45). XPA's interaction with both RPA and ERCC1-XPF may facilitate and stabilize their binding to the DNA (50-54). RPA is not necessary but contributes to the proper positioning of XPG. TFIIH associates with XPG at physiological ionic strengths (55). The two proteins form a stable complex that is active in transcription and NER, suggesting that XPG and TFIIH play a role in stabilizing each other (56). Both TFIIH and XPG are essential for coupling transcription to different repair pathways, such as end joining and homologous recombination repair. Incisions are made asymmetrically around the lesion and the exact incision positions seem to depend on the type of the lesion (57). The presence, but not the catalytic activity, of XPG is required for the 5' incision by ERCC1-XPF whereas efficient 3' incision by XPG requires the catalytic activity of ERCC1-XPF (58). This indicates the importance of ERCC1-XPF in the NER pathway. The 5' incision by ERCC1-XPF leaves a free 3' hydroxyl (-OH)-group on the damaged strand which serves as the primer for repair synthesis.

1.3.4.3.Repair synthesis

After the incision, most NER proteins leave the repair complex before repair synthesis. However, RPA remains bound to the undamaged strand to protect the template strand against nucleases, and may facilitate replication. The DNA repair synthesis that follows requires either DNA Pol δ or ε with their cofactors, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). Briefly, RFC preferentially binds to the 3' end of DNA primers and facilitates the loading of PCNA, which forms a trimeric ring-shaped clamp that can travel along the duplex DNA. This complex serves as a docking platform for both Pol δ and Pol ε, which carry out single-stranded DNA synthesis using the undamaged strand as the template. An illustration of the process is shown in **Figure 1-4**. Finally, the NER is completed by the ligation of the newly synthesized patch to the original sequence, which is executed by DNA ligase I.

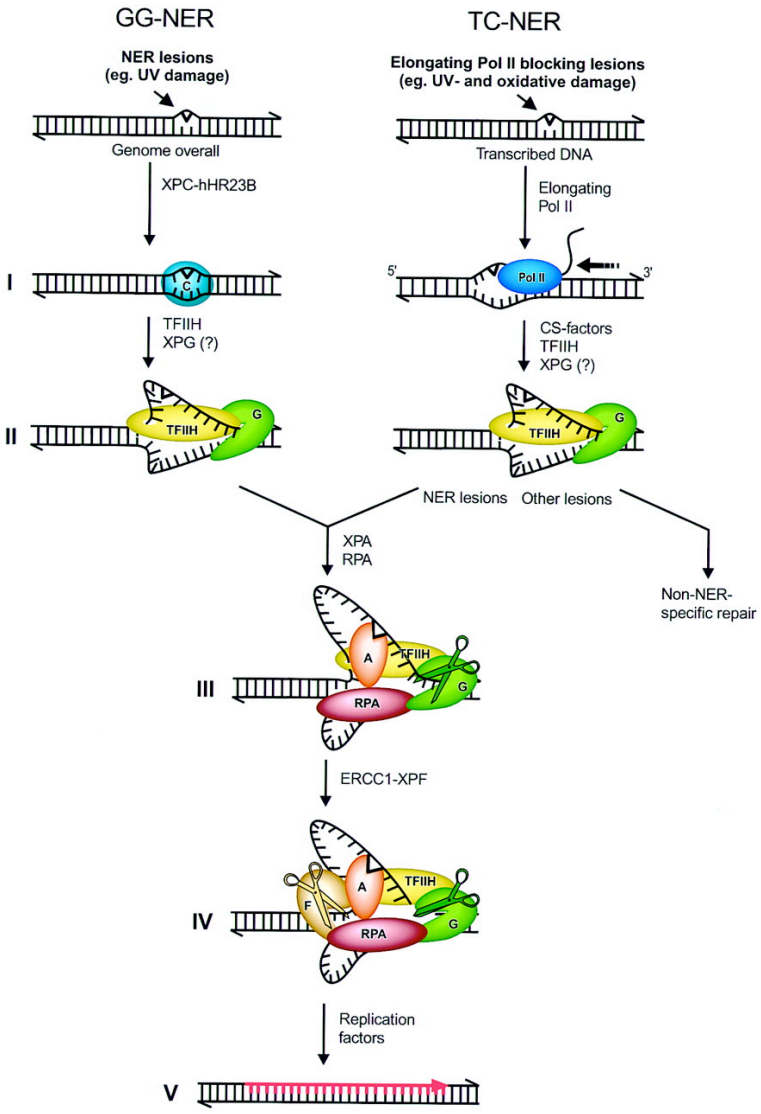
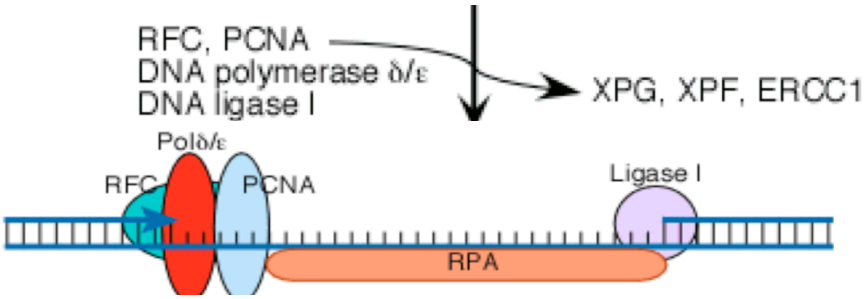


Figure 1-3: Molecular mechanisms for the Nucleotide excision repair (38). In global genome NER (GG–NER) (Left), the hetero-dimeric protein complex XPC–hHR23B (represented by the letter C on the double helix) senses DNA helix-distorting lesions, leading to conformational alterations of the DNA. In transcription-coupled repair (TC–NER) (Right), lesions are detected by elongating RNA polymerase II (Pol II) blocked by DNA lesions. In GG-NER, XPC–hHR23B at lesion recruits TFIIH, and possibly XPG (represented by the letter G) to the distorted DNA. TFIIH creates a 10- to 20-nucleotide opened DNA complex around the lesion by virtue of its helicase subunits XPB and XPD; this step requires ATP. XPC–hHR23B

hetero-dimer may be released at this or one of the subsequent steps. In TC-NER, CSA, CSB, TFIIH, XPG, and possibly other cofactors displace the stalled Pol II from the lesion, which now becomes accessible for further repair processing; depending on the type of lesion, repair is completed by NER or by other repair pathways. The single strand-binding proteins XPA (represented by the letter A in the complex) and RPA stabilize the opening caused by TFIIH and recruit and position other proteins. XPG stabilizes the fully opened complex. XPG, positioned by TFIIH and RPA, makes the 3′ incision. ERCC1–XPF (represented by the letter F in the complex), positioned by RPA and XPA, makes the second incision 5′ of the lesion. This is the critical step of the NER process. Dual incision is followed by gap-filling DNA synthesis, using the undamaged strand as the template, and ligation.



Modified from http://asajj.roswellpark.org/huberman/dna_repair/ner.html

Figure 1-4: Repair synthesis after the dual incision in NER. Before repair synthesis, most proteins in the repair complex have left except RPA, which remains bound to the undamaged DNA strand as a protection from its being attacked by nucleases and facilitation of following DNA replication. Then RFC is recruited to bind to the 3′ termini of DNA primers and facilitate the loading of PCNA. The complex formed by RFC and PCNA serves as a docking platform for both Pol δ and Pol ε, which accomplishes the synthesis of the single-stranded DNA using the undamaged strand as the template. DNA ligase I fixes the nick between the newly synthesized patch and the original sequence.

Reduced expression of NER proteins, such as XPA, ERCC1 and XPF, is found in testis tumor cell lines (59, 60) and is suggested to be responsible for the exceptional sensitivity of testicular tumors to cisplatin. Greater levels of ERCC1 and XPC mRNA are found in tissues from patients with ovarian cancer that are clinically proven to be resistant to platinum-based chemotherapy (61). The suppression of the XPF-ERCC1 complex significantly decreases cellular viability which correlates with the decrease in DNA repair capacity (62). In vitro repair assays have shown that inhibition of NER results in failure to remove a site-specific d(GpG) cisplatin adduct by strongly inhibiting DNA repair synthesis and less strongly inhibiting incision and ligation (63). Common genetic polymorphisms in NER genes have been discovered and their associations with the clinical outcome of platinum-based chemotherapies have been widely investigated and reported, as discussed in following sections.

1.3.5. Base excision repair (BER) pathway

In addition to the NER pathway, other DNA repair mechanisms have been found to contribute in removing platinum-DNA adducts, depending upon the type of damage it causes. The BER pathway protects cells from endogenous DNA damage and lesions caused by ionizing radiation and strong alkylating agents. It is important in cancer and chemotherapies that cause DNA damage, such as platinum-containing drugs and radiation, which will be discussed in detail in chapter 3.

BER begins with an abasic site that is generated by glycosylases or by spontaneous hydrolysis. The core BER reaction is initiated by strand incision at the abasic site by the APE1 endonuclease (64). Poly(ADP-ribose) polymerase 1 (PARP1) binds to and is activated by DNA strand breaks (65). If BER is initiated from a single

strand break, the polynucleotide kinase (PNK) is important to protect and trim the ends for repair synthesis (66). This is followed by the one-nucleotide gap-filling by DNA Pol ß and the removal of the 5'-terminal baseless sugar residue via its lyase activity (67). The remaining nick is sealed by the XRCC1-ligase 3 complex (68). The XRCC1 scaffold protein interacts with most of the above BER core components and may therefore be instrumental in protein exchange. This is the so-called short-patch repair and is the dominant mode in mammals. The long-patch repair mode requires DNA Pol ß, Pol δ/ε and PCNA to synthesize the 2–10 bases of nucleotides using the other strand as the template, as well as the FEN1 endonuclease to remove the displaced DNA flap, and DNA ligase 1 to seal the end (69). In some cases, the BER lesions block transcription. The problem is resolved by coupling with the TC-NER pathway described above. Thus, the BER pathway is joined with the NER pathway. An illustration of the BER pathway is shown in **Figure 1-5**.

BER is suggested to have an additional role in processing DNA damage caused by anti-tumor agents, and may share the similar mechanism of the MMR pathway in responding to DNA damage (70). Though little evidence suggests that the BER pathway plays direct roles in repairing platinum-caused DNA damage, the genetic polymorphisms in several BER genes have been found to be associated with clinical outcomes of platinum-based chemotherapies, which will be discussed in more details in later sections in this chapter.

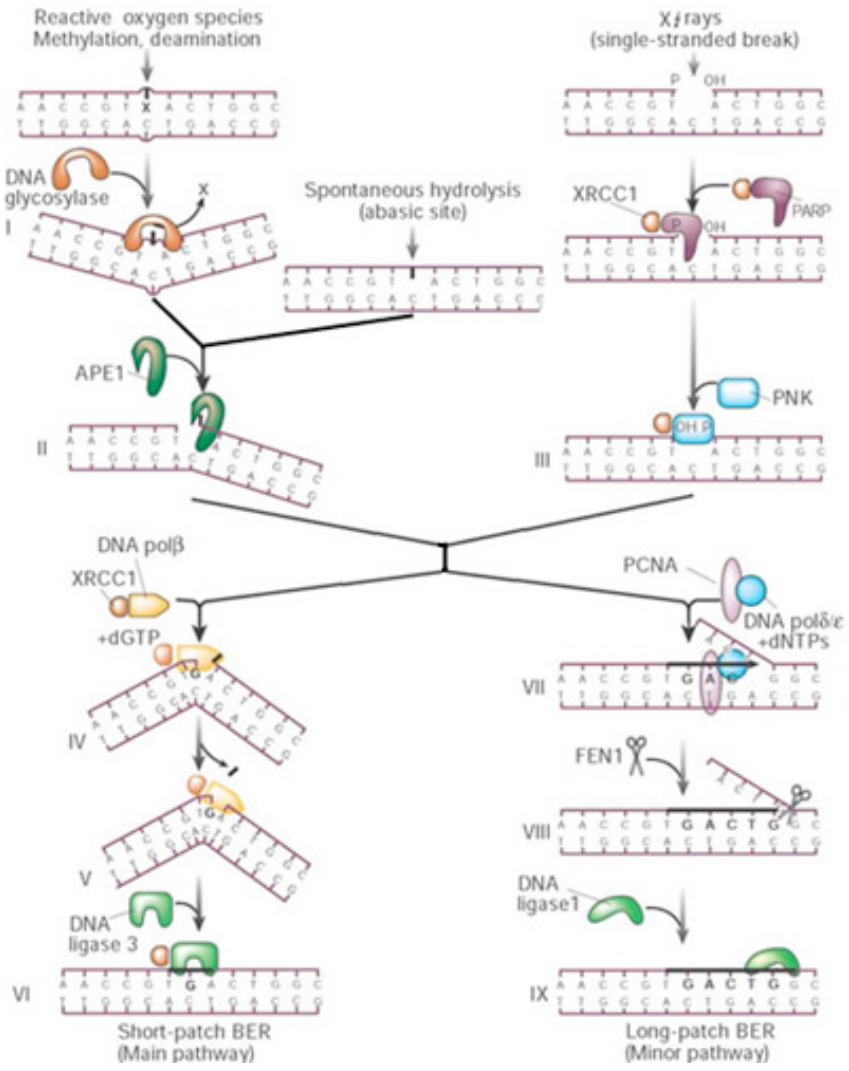


Figure 1-5: The molecular mechanism of the BER pathway (71).

1.4. Pharmacogenetics in DNA excision repair genes

Unpredictable efficacy and toxicity are major hurdles in drug development, especially in cancer treatment as most of the chemotherapy agents are extremely toxic. Many drugs that show therapeutic potential never get approved because of their adverse effect in some individuals, while others show effectiveness only in some of the patients that have received the treatment. Thus pharmacogenetics is an important

research field with both biomedical and commercial significance by identifying inherited DNA polymorphisms that could influence drug disposition and effects.

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genomic sequence is altered. SNPs make up about 90% of all human genetic variation and occur every 100 to 300 bases along the 3-billion-base human genome. Many SNPs have no effect on cellular function, but some predispose people to disease or influence their response to a drug. Common SNPs are found in most DNA excision repair genes. There is cumulative evidence that altered DNA repair capacity, implied by the common variations in DNA repair genes, might affect cancer disposition and response to DNA damaging chemotherapy regimens.

1.4.1. ERCC1

ERCC1 was the first gene cloned in the NER pathway (72). It is located on chromosome 19q13.2-q13.3 with 10 exons spread over 15-kb on the chromosome, and encodes a protein approximately 33 kDa in size. The gene product of ERCC1 forms a tight complex with XPF, its catalytic partner. The formation of the hetero-dimeric protein complex is important for both the stability of the two proteins in vivo and in vitro, and their proper functionality in DNA lesion incisions. The structure-specific dimeric endonuclease ERCC1-XPF cleaves strands containing DNA distorting lesions from the 5'-end. During NER, this activity is required to create a nick 5' to the lesion, releasing a 3' single-stranded DNA for further repair synthesis. However, the function in DNA repair of the ERCC1-XPF hetero-dimer is not limited to NER only. The ERCC1-XPF complex also plays an important role in homologous

recombination repair by removing long non-homologous tails from invading homologous strands (73), and is critical in removing interstrand cross-links (ICL) (74).

Though the inter-individual variations in response to platinum-based chemotherapy are widely observed, the genetic basis is not fully understood. A large body of evidence suggests that ERCC1 mRNA and/or protein expression levels may correlate with platinum resistance. Suppression of ERCC1 either enhances platinum sensitivity in human cancer cells or restores the platinum sensitivity in resistant cells (75-80). Greater levels of ERCC1 mRNA are found in ovarian cancer tissues resistant to platinum-based chemotherapy (61). In patients with non-small-cell lung cancer (NSCLC), ERCC1-negative tumors are found to benefit from cisplatin-base adjuvant chemotherapy (81-83). Patients with advanced head and neck squamous cell carcinoma that are characterized by low ERCC1 expression have a 4-fold greater odds of benefiting from an objective response to chemotherapy compared with patients with high ERCC1 expression (84). The ERCC1 promoter is located in a region of ±170 bps upstream of the transcriptional start site of this gene (76). A computer analysis of the transcriptional factors binding sites in the 5'-flanking region of ERCC1 (−415 to +32 bp) revealed 7 transcription activators and 1 transcription repressor binding sequences (85). However, the regulation of ERCC1 expression is less studied with only AP1 (86) and MZF1 (85) having been investigated.

The ERCC1 N118N (500C>T) polymorphism was first reported by Reed and colleagues (87). It has been the focus of many genetic association studies, and has shown the potential as a predictive marker in patients treated with platinum-based

chemotherapy in several cancers, as summarized in **Table 1-1**. However, the results vary in different types of malignancies. For example, in NSCLC, some studies suggest that the C allele signifies a better response to platinum-based chemotherapy (88-91), while others reported no association (92, 93). Alternatively, in metastatic colorectal cancer, both C and T alleles were found to be associated with either an improved or impaired outcome.

Table 1-1: Previous pharmacogenetics studies on *ERCC1* N118N polymorphism in multiple malignancies. mCRC: metastatic colorectal cancer, NPC: Nasopharyngeal Carcinoma, EOC: Epithelial Ovarian Cancer, NSCLC: Non-Small Cell Lung Cancer, PFS: Progression free Survival, OS: Overall Survival, HR: Hazard Ratio.

Disease	Therapy	# of pts	Association	HR (95% CI)	P value	Ref.
mCRC	FOLFOX	118	T allele: worse progression-free survival	2.62 (1.14-6.0)	0.02	(94)
	FOLFOX	168	T allele: increased ERCC1 expression, lower response, and shorter OS and PFS	N/A	≤ 0.01	(95)
	Fluorouracil/oxaliplatin	49	C allele correlated with a shorter PFS	1.96 (0.99–3.92)	0.050	(96)
	Platinum-based chemotherapy	106	CC genotype had longer median survival	N/A	N/A	(97)
	Oxaliplatin/5-fluorouracil	91	TT genotype had higher response rate	N/A	0.018	(98)
	Oxaliplatin/5-fluorouracil	106	CC genotype showed the most favorable survival	2.29 (1.19, 4.41) for CT and 1.86 (0.91, 3.83) for TT	0.021	(99)
NSCLC	Paclitaxel plus carboplatin	153	No association	1.2 (0.74-1.96)	0.45	(92)
	Platinum-based chemotherapy	119	C allele associated with better response	0.10 (0.013-0.828)	0.033	(88)
	Cisplatin combination	245	CC had longer OS in patients having over 50 packs per year	N/A	0.03	(89)
	Docetaxel-cisplatin	62	CC genotype had longer OS		0.01	(90)
	Platinum-based chemotherapy	128	No association	N/A	0.41	(93)
	Cisplatin combination chemotherapy	109	CC had better survival		0.0058	(91)

Pancreatic cancer	Cisplatin-based chemotherapy	67	T allele: longer PFS and OS		0.006 for PFS and 0.03 for OS	(100)
EOC	Platinum-based chemotherapy	159	TT signalized a better response		0.026	(101)
Gastric cancer	Oxaliplatin-based adjuvant chemotherapy	102	No association	N/A	> 0.05	(102)
Esophageal cancer	Cisplatin	262 with or 108 without cisplatin	No association	N/A	0.1 for cisplatin-treated and 0.49 for no-cisplatin	(103)
NPC	Gemcitabine and oxaliplatin	29	No association	N/A	0.76	(104)

Another SNP in ERCC1, C8092A, is found in the 3'-UTR of the gene. It has been suggested to be an indicator of altered chemo-sensitivity. The homozygous wild type CC is found to be associated with shorter median survival time compared to CA and AA genotypes in patients with advanced NSCLC who received platinum-based chemotherapy (88). However, other studies found that individuals with the CC genotype benefit from platinum-based chemotherapy (93, 105) or no association (106).

1.4.2. XPD

XPD (also called ERCC2), one of the two subunits of TFIIH, is a 5'-3' helicase that participates in DNA strand separation prior to the 5' incision step performed by the ERCC1-XPF heterodimer (107, 108). Although several polymorphisms have been reported in this gene, the most frequent is the K751Q (A>C) polymorphism. The interethnic variance of XPD polymorphisms has been previously reported among European, African and Asian populations (109). The lowest variant allele frequency occurs in Asians and the highest in Europeans, with Africans having a median variance rate. The homozygous wild type (AA) is correlated to suboptimal DNA repair of X-ray-induced DNA damage (110). Also, the AA genotype is found to be beneficial in patients with colorectal cancer treated with oxaliplatin-based chemotherapy (111, 112), patients with advanced NSCLC treated with platinum-based chemotherapy (113), and patients with acute myeloid leukemia treated with radiotherapy and chemotherapy (114). However, the CC variant is associated with reduced DNA repair capacity in patients with lung cancer (115) and is overrepresented in patients with lung cancer (116). ERCC1, as well as XPD, are

considered to be clinically useful molecular predictors for overall NER activity, and have been studied in bladder, lung, ovarian, colorectal, and other cancers where platinum compounds are used (97, 110, 111, 113, 117-122).

1.4.3. XRCC1

XRCC1 was the first human gene cloned in the BER pathway; it encodes a 633 amino acid protein. XRCC1 works as a stimulator and scaffold protein for other enzymes involved in the BER pathway. It interacts with DNA ligase-III, DNA Pol β and poly(ADPribose) polymerase (PARP1), and assists in sealing the nick in DNA after DNA Pol β has filled the nucleotide gap. Cells lacking this gene product are hypersensitive to ionizing radiation. Approximately half of the more than 60 validated polymorphisms in XRCC1 are located in exons or promoter regions (123). The most extensively studied SNPs are R399Q in exon 10 and R194W in exon 6. The R399Q polymorphism is in the BRCT1 domain, which provides a binding site for PARP1 polymerase (124). The wild type GG appears to be associated with increased sensitivity to platinum-based chemotherapy in cervical cancer in Asian populations (125) and in one study of 112 patients with non-small cell lung cancer (126), while the variant allele showed improved survival in one Spanish population (127) and in bladder cancer (122). The variant AA genotype is associated with smoke-induced pancreatic cancer (128), and is associated with breast cancer risk in African Americans (129).

Another prevalent polymorphism in XRCC1 is at codon 194, which is in a possible binding site for several protein partners in BER, and the positively charged arginine is changed to a hydrophobic tryptophan. This polymorphism affects binding

and DNA repair efficiency (130), and the variant allele was associated with a better response to platinum-based chemotherapy in patients with advanced NSCLC (131).

1.4.4. PARP1

Poly(ADP-ribose) polymerase 1 (PARP1) is a DNA repair enzyme that catalyzes the poly(ADP-ribosyl)ation of proteins using NAD^+ as the substrate. PARP1 is activated 100-fold by DNA damage and has a major role in the BER pathway (132, 133). A common PARP1 polymorphism at codon 762 results in the substitution of valine by alanine in the catalytic domain. This change was proven to dramatically reduce the enzymatic activity (134). The variant genotype contributes to prostate cancer susceptibility and altered DNA repair function to oxidative damage (135), association with risk of esophageal squamous cell carcinoma in a Chinese population (136) and increased risk of smoking-related lung cancer (137).

1.5.DNA repair and cancer

Genomic stability is important for preventing oncogenesis. All cancers are thought to be caused by gene mutations. The lesions in DNA can arise from endogenous and environmental causes. The endogenous damage includes mismatches that arise during DNA replication, (by)products of normal cellular processes, such as reactive oxidative species (ROS) derived from oxidative respiration and products from lipid oxidation, and spontaneous nucleotide hydrolysis which creates abasic sites. Spontaneous or induced deamination of cytosine, adenine, guanine or 5-methylcytosine converts these bases to the miscoding uracil, hypoxanthine, xanthine and thymine, respectively (138). The environmental damage can be caused by the

ultraviolet (UV) component of sunlight that produces cyclobutane pyrimidine dimers and other photoproducts, ionizing radiation that generates base damage and strand breaks, alkylating agents that alkylate bases, cross-linking agents that create intrastrand and/or interstrand covalent links and double-stranded DNA breaks, and other genotoxic chemicals such as cigarette smoke.

The outcome of DNA damage is generally adverse. All eukaryotic cells have evolved multifaceted mechanisms to counteract the potential deleterious effects of DNA damage. Acute effects trigger cell cycle checkpoint arrest to allow time for repair or cell death. Chronic effects arise from irreversible or unrepaired mutations that lead to oncogenesis. With the various types of DNA damage, no single repair pathway can cope with all kinds of damage. Instead, human cells have evolved sophisticated, interwoven DNA repair systems, including MMR, BER, recombinational repair (Homologous Recombination and End Joining), and NER. If, despite these repair mechanisms, DNA damage persists, cells can make use of a mechanism called translesion synthesis, which uses special DNA polymerases to bypass these specific types of DNA lesions.

In general, multiple mutations are needed to transform a normal cell into a tumor cell. However, normal human cells replicate their DNA with exceptional accuracy. The average frequency of spontaneous mutations in normal tissues is less than 1×10^{-8} per base pair, whereas the frequency of mutations increases at least two orders of magnitude in tumors from the same individuals (139). Therefore, tumor cells must exhibit a "mutator" phenotype arising from inherited or acquired faulty genome surveillance and repair machinery (140). Deficiency in DNA repair

mechanisms can lead to more than 1000-fold increased in cancer risk (141), thus underscoring the importance of DNA repair mechanisms in carcinogenesis.

1.6.Summary and research goal

DNA repair is a double-edged sword. It guards our genome, protecting us from endogenous and exogenous DNA damage, while causes resistance to cancer therapies that take effects by causing DNA damage. Although many studies have investigated the genetic polymorphisms in DNA excision genes in the context of cancer risk and clinical outcome of platinum-based chemotherapy and radiation therapies, there is still a lack in our understanding of the biological consequences of these genetic polymorphisms and how they can contribute to improved delivery of cancer therapies. We are particularly interested in how the genetic polymorphisms in DNA excision genes are associated with inter-individual and inter-populational differences in cancer incidence, how they may affect the respective gene expression and biological function, and what the functionally important polymorphisms are in genes involved in the DNA excision repair pathways. The aim of this dissertation research is to increase our understanding in these areas, and we focused our study on the NER and BER gene polymorphisms, especially ERCC1 which is of significant clinical importance.

2. Ethnic disparity in DNA excision repair gene polymorphisms

2.1. Abstract

NER and BER pathways are DNA repair pathways that are important in carcinogenesis and in response to DNA damaging chemotherapy. ERCC1 and XPD are important molecular markers for NER; XRCC1 and PARP1 are important molecular markers for BER. Functional polymorphisms have been described that are associated with altered expression levels of these genes, and with altered DNA repair capability. We assayed genomic DNA from 156 Americans of European descent (EAs) and 164 Americans of African descent (AAs), for the allelic frequencies of specific polymorphisms of ERCC1 N118N (500C>T), ERCC1 C8092A, XPD K751Q (2282A>C), XRCC1 R399Q (1301G>A), XRCC1 R194W (685C>T) and PARP1 V762A (2446T>C). Differences were observed between EAs and AAs in the allelic frequencies of the ERCC1 N118N polymorphism ($p<0.000001$), XPD K751Q ($p=0.006675$), XRCC1 R399Q ($p<0.000001$) and PARP1 V762A ($p=0.000001$). The polymorphic variants of DNA excision repair genes that are seen most commonly in EAs may reflect a mild reduction in NER and BER function, thus the repair of cisplatin-DNA lesions.

2.2.Introduction

Nucleotide excision repair (NER) is the DNA repair pathway that repairs bulky lesions that are covalently bound to DNA bases. This includes DNA damage

from ultraviolet light, polycyclic aromatic hydrocarbons, and selected anticancer pharmaceuticals such as the platinum compounds, e.g., cisplatin, carboplatin, and oxaliplatin (142, 143). ERCC1 and XPD are two of the multiple proteins that participate in NER to excise the bulky lesion from the DNA strand (144, 145). ERCC1 performs a number of functions, and, along with XPF, is essential for the 5' incision into the DNA strand that releases bulky DNA lesions (146, 147). XPD is a 5'-3' helicase that participates in DNA strand separation, prior to the 5' incision step performed by the ERCC1-XPF heterodimer (107, 108). The functions of ERCC1 and XPD are discussed in chapter 1. They are important clinical predictors for NER activity and have been extensively studied in many types of cancer, especially those where platinum-containing drugs are widely used.

Spontaneous DNA hydrolysis, oxidative damage to DNA, as well as simple alkylations to DNA bases are repaired by the BER pathway. BER has not been as well studied as NER in the setting of anti-cancer chemotherapy. However, data suggests that BER may play a role in clinical and cellular resistance to simple alkylating agents (148). The important enzymes involved in BER include XRCC1 and PARP1. XRCC1 stimulates endonuclease activities following the excision of a damaged nucleotide, and acts as both a scaffold and a regulator for other BER proteins (130). PARP1 is a zinc-finger DNA-binding enzyme that is activated by DNA breaks and converts DNA damage into intra-cellular signals that either trigger DNA repair by the BER pathway or cell death. PARP1 is required for XRCC1 function at sites of oxidative DNA damage (149). Inhibition of PARP1 is useful in

sensitizing chemotherapy and radiation therapy that damage DNA. Thus, PARP1 inhibitors are being actively investigated to facilitate cancer therapy.

There are several common diseases where there is a long history of ethnic differences in treatment outcomes and mortality. These include breast cancer, prostate cancer, lung cancer, and colorectal cancer as summarized in **Table 2-1**. Breast cancer is treated with adriamycin and cyclophosphamide, drugs which cause oxidative damage to DNA (150) and alkylation of DNA, respectively (151). In these circumstances, BER may be of major importance. Lung cancer is commonly treated with cisplatin or carboplatin based regimens (152), while colorectal cancer is commonly treated with an oxaliplatin-based combination (153). In lung cancer and colorectal cancer, NER appears to be the most important DNA repair mechanism (142). Bladder cancer, cervical cancer, stomach cancer, and head and neck malignancies are also treated with DNA damaging therapies and have had ethnic differences in disease outcome observed (154).

Ethnic disparity in treatment outcomes is a problem that is receiving increased recognition in clinical oncology, but has been poorly studied. It is not completely clear whether differences noted between EAs and AAs in treatment outcomes are due to matters of patient access to care, differences in medical care delivery, differences in clinical response to the same therapies, or most likely, a combination of all of these. The molecular causes of clinical resistance to chemotherapy have been elucidated for some of the commonly used anticancer agents. For example with cisplatin, carboplatin, and oxaliplatin, the NER DNA repair pathway appears to be of great importance (142), while agents that generate oxidative DNA damage within

cells, or produce simple alkylations to DNA, are more influenced by the BER pathway (71, 155). This information led us to investigate the following: if ethnic differences exist in the clinical treatment outcomes of patients who are treated with drugs that may be impacted by NER and/or by BER, are these outcomes associated with ethnic differences in polymorphism frequency in genes that are involved in these DNA repair pathways?

ERCC1, XPD, XRCC1 and PARP1, each have been reported to have polymorphic variants that appear to impact the functioning of the respective gene. Also, for ERCC1, XPD and XRCC1, the polymorphic variants have been associated with clinically important endpoints. The aim of this study is to assess the allelic frequency of the noted polymorphisms in the genes ERCC1, XPD, XRCC1 and PARP1.

Table 2-1: summary of previous studies on racial disparity of mortality and treatment outcome in cancer.

Cancer type	Treatment	Ethnic groups (# of samples)	Major findings	OR (95% CI)/P value	Ref.
Breast cancer	Not defined.	AA*(185) and EA (10,937)	Overall survival significantly favored EAs	2.27 (1.82,2.84)	(156)
	Surgery, radiation and adjuvant chemotherapy	AA (771) and EA (5651)	Increased risk of death in AA patients	1.57 (1.18,2.10)*	(157)
	Surgery, cyclophosphamide, methotrexate, and 5-fluorouracil	AA (543) and EA (7582)	Increased death in AA patients	1.21 (1.01,1.46) † 1.18 (1.03,1.34) ‡	(158)
Prostate cancer	Orchiectomy or LHRH analogue therapy	AA (55) and EA (90)	No difference		(159)
	Surgery, radiation, hormone therapy and others	AA (14,307) and EA (108,067)	Increased risk of death in AA patients	1.61 (1.50,1.72)†† 0.99 (0.92,1.06)‡‡	(160)
Lung cancer	Not defined.	AA and EA, numbers not defined	Lower 5-year survival rate in AA patients.	P ≤0.0001	(161)
Colorectal cancer	Surgery	AA (199) and EA (292)	Lower 5- and 10-year survival rate in AA	1.67 (1.21,2.33) ††† 1.52 (1.12,2.07) ‡‡‡	(154)
Rectal cancer	Methyl-lomustine, vincristine, fluorouracil, leucovorin and/or radiation therapy after surgery	AA (104) and EA (1,070)	Higher mortality in AA patients	1.45 (1.09,1.93)	(162)
Colon cancer	Not defined.	AA (454) and EA (521)	Higher risk of death among AA patients after adjusted for stage	1.2 (1.1,1.5)	(163)

* EA: Americans of European descent, AA: Americans of African descent, *Breast-cancer-specific survival, adjusted for tumor characteristics and major treatments, †Lymph node-negative disease, ‡Lymph node-positive disease, ††Adjusted only for age, ‡‡Adjusted for stage, treatment, grade, socioeconomic status and year of diagnosis, †††Within 5 years of surgery, ‡‡‡Within 10 years of surgery.

2.3.Materials and Methods

2.3.1. Sample collection & DNA isolation

320 whole blood samples, 156 EAs and 164 AAs, from healthy male volunteers (Valley Biomedical Inc., Winchester VA) were analyzed. All volunteers gave informed consent to allow their samples to be used for genotyping, and none had a diagnosis of cancer. Plasma was the source used to isolate genomic DNA using the UltraSens Virus Kit following the manufacturer's instructions (Qiagen, Valencia, CA).

2.3.2. Genotyping methods

2.3.2.1.Polymerase Chain Reaction (PCR)

PCR was performed using the Platinum Taq PCR Kit from Invitrogen (Carlsbad, CA) with gene-specific primers. PCR reactions were denatured at 94°C for 5 min, followed by denaturation at 94°C for 30 sec; annealing at optimal temperature for each pair of primers for 30 sec and synthesis for 30 sec at 72°C for 40 cycles; the final extension was carried out at 72°C for 7 min. PCR efficiency was examined by electrophoresis on 2% agrose gels. 50 to 150 ng of template DNA were used for each PCR, depending on the quality of the DNA samples.

2.3.2.2.SAP purification of the PCR product

After PCR, the product was purified by adding 1 U of shrimp alkaline phosphatase (SAP) and 3 U of exonuclease I (Exo I) to 15 ul of PCR product and heating for 90 minutes at 37°C and 20 minutes at 70°C to deactivate the enzymes.

2.3.2.3.Sequencing reaction

Direct nucleotide sequencing PCR was conducted using the Big Dye Terminator Cycle Sequencing Ready Reaction kit V3.1 (Applied Biosystems, Foster City, CA). The sequencing reaction was carried out by mixing 3 ul from the SAP and Exo I treated PCR product above to 1ul of BigDye™ terminator mix, 1.6 pmole of sequencing primer, and sterile H2O to bring the final volume to 15 ul. The PCR reaction was denatured at 94°C for 5 min, followed by denaturation at 96°C for 10 sec; annealing at 50°C for 5 sec and amplification for 4 min at 60°C for 25 cycles; no final extension was needed for the sequencing PCR. Primers and PCR conditions are listed in **Table 2-2**.

2.3.2.4.Direct sequencing

Before loading the sequencer, the DNA samples were processed using the PERFORMA® DTR V3 96-Well Short Plates (Edge BioSystems, Gaithersburg, MD) to remove extra dye terminators, along with dNTPs, salts and other low molecular weight materials from the sequencing reactions. An ABI Prism 3130 Genetic Analyzer was used for direct sequencing following the manufacturer's instructions.

Table 2-2: Primers and PCR conditions.

SNPs	Primers	Size (bp)	Annealing Temp (°C)
ERCC1 N118N	FW 5'-TGG ATC AGA GGA TCA GGG AC-3' RE 5'-TTC CTG AGA CCC AGG AGT TC-3' Seq 5'-AGG GAG GAG GTG CAA GAA GAG-3'	542	64 °C
ERCC1 C8092A	FW 5'- TCC TGC ACG AGC CCT TCT TG -3' RE 5'- TCA GGC AGC TCC CAC ATC CAC -3' Seq 5'- TCC CAG GCC AGG CTC CTG C -3'	497	66 °C
ERCC2 K751Q	FW 5'- CCT TCT CCT GCG ATT AAA GGC TGT -3' RE 5'- TCA GCC CCA TCT TAT GTT GAC AGG -3' Seq 5'-ACC AGG GCC AGG CAA GAC TC-3'	415	66 °C
XRCC1 R399Q	FW 5'-TGC ATC TCT CCC TTG GTC TCC-3' RE 5'-TGC ACA AAC TGC TCC TCC AGC-3' Seq 5'-CAC CAG CTG TGC CTT TGC CA-3'	522	66 °C
XRCC1 R194W	FW 5'–AGA CAA AGA TGA GGC AGA GG-3' RE 5' – TCA ACC CTC AGG ACA CAA GAG-3' Seq 5'- AAT ACT GAC CTT GCG GGA CC -3'	419	60 °C
PARP1 V762A	FW 5'- TC CCA AAT GTC AGC ATG TAC GA -3' RE 5'- TCC AGG AGA TCC TAA CAC ACA TGG -3' Seq 5'- AGG TAA CAG GCT GGC CCT GAC -3'	479	62 °C

2.3.3. Statistical analysis

For each SNP, the genotyping results were categorized into wild type, heterozygotes and variant genotypes. Hardy-Weinberg Equilibrium (HWE) tests were conducted using the HelixTree software (Golden Helix, Bozeman, MT). P values were calculated by the Chi-square test using Number Cruncher Statistical Software (NCSS, Kaysville, Utah).

2.4. Results

A summary of the six polymorphisms studied is provided in **Table 2-3**. The genotype distribution of each SNP is in Hardy-Weinberg equilibrium ($p>0.05$). No genotype frequency differences were observed between EAs and AAs for the ERCC1 C8092A polymorphism. However, significant differences in genotype frequency were noted for the ERCC1 N118N polymorphism ($p<0.000001$). The CC genotype occurred more frequently in AAs (76%) as compared to EAs (21%), while the TT genotype is seen in only 3% of AAs and in 30% of EAs. The CT genotype is seen in 21% and 49% of the respective groups. Reed and colleagues have shown that the TT genotype is associated with reduced expression of ERCC1, reduced cisplatin-DNA adduct repair, and increased sensitivity to cisplatin (87, 164).

For the XPD K751Q (2282A>C) polymorphism, differences between EAs and AAs in the distribution of the AA, AC, and CC genotypes were noted ($p=0.006675$). The AA genotype was seen more frequently in AAs (56% versus 42%), but the other two genotypes were observed more frequently in EAs: AC (47% versus 40%) and CC (11% versus 4%)

Table 2-3**:** Genotype distribution. P value is calculated by Chi-square test based on allelic frequency.

[‡]Indicates: gene name, amino acid change and position, nucleotide change and the position. [#] EA: Americans of European descent, AA: Americans of African descent. [*]Indicates: Count (frequency).

Gene	Genotype	EA[#]	AA	Allele	EA	AA	P value
ERCC1[‡]	CC	23(0.21)[*]	96(0.76)	C	99(0.46)	219(0.86)	
N118N	CT	53(0.49)	27(0.21)	T	117(0.54)	35(0.14)	
(500C>T)	TT	32(0.30)	4(0.03)				
	TOTAL	108	127	TOTAL	216	254	<0.000001
ERCC1	CC	77(0.53)	74(0.52)	C	213(0.73)	204(0.72)	
C8092A	AC	59(0.40)	56(0.40)	A	79(0.27)	78(0.28)	
	AA	10(0.07)	11(0.08)				
	TOTAL	146	141	TOTAL	292	282	0.870913
XPD	AA	49(0.42)	81(0.56)	A	154(0.65)	219(0.76)	
K751Q	AC	56(0.47)	57(0.40)	C	82(0.35)	69(0.24)	
(2282A>C)	CC	13(0.11)	6(0.04)				
	TOTAL	118	144	TOTAL	236	288	0.006675
XRCC1	GG	49(0.46)	113(0.80)	G	145(0.68)	252(0.89)	
R399Q	AG	47(0.44)	26(0.19)	A	67(0.32)	30(0.11)	
(1301G>A)	AA	10(0.10)	2(0.01)				
	TOTAL	106	141		212	282	<0.000001
XRCC1	CC	120(0.87)	133(0.90)	C	257(0.93)	280(0.95)	
R194W	CT	17(0.12)	14(0.09)	T	19(0.07)	16(0.05)	
(685C>T)	TT	1(0.01)	1(0.01)				
	TOTAL	138	148	TOTAL	276	296	0.460941
PARP1	TT	80(0.67)	108(0.91)	T	192(0.81)	227(0.95)	
V762A	CT	32(0.27)	11(0.09)	C	46(0.19)	11(0.05)	
(2446T>C)	CC	7(0.06)	0(0)				
	TOTAL	119	119	TOTAL	238	238	0.000001

For the XRCC1 R399Q (1301G>A) polymorphism, substantial differences between ethnic groups were also noted ($p<0.000001$). The GG genotype occurred in 80% of AAs, but only in 46% of EAs. The other two genotypes occurred more frequently in EAs: AG (44% versus 19%), and AA (10% versus 1%). The XRCC1 R194W (685C>T) polymorphism did not differ in genotype frequency between ethnic groups.

The TT genotype of PARP1 V762A (2446T>C) occurred more frequently in AAs than EAs (91% versus 67%; $p=0.000001$), and the other genotypes occurred more frequently in EAs: CT (27% versus 9%), and CC (6% versus 0%).

2.5. Discussion

We assessed genomic DNA from 156 EA individuals, and 164 AA individuals, for allelic frequency of the noted polymorphisms in the genes ERCC1, XPD, XRCC1 and PARP1. Our data suggest a profound difference between these two ethnic groups in three genes: ERCC1, XRCC1 and PARP1.

Of the differences demonstrated between ethnic groups, one of the most interesting is the difference observed for the N118N polymorphism of ERCC1. The polymorphism of AAC to AAT at codon 118 of ERCC1 was first reported by Reed and colleagues. This codon change results in the same amino acid, but the C>T transition decreases the translation rate from mRNA to protein by 50% (87). This polymorphism was noted to be associated with reduced mRNA expression of ERCC1, reduced repair of platinum-DNA adducts, and greater sensitivity to platinum compounds (87, 98). Codon 118 of ERCC1 has also been studied in several

malignancies, such as lung cancer, ovarian cancer, colorectal cancer, and other malignancies (97, 110, 113, 118-122, 142, 165). Our data suggests the possibility that reduced NER capacity may occur more commonly in EAs that carry the variant T allele more frequently, and this might result in greater sensitivity to platinum compounds in EAs. This would be consistent with the observed improved survival rates in EAs compared to AAs in malignancies where platinum compounds are important components of therapy, including lung, colorectal, head/neck and ovarian cancers.

Although several studies also suggested the C8092A mutation in the 3'-UTR of ERCC1 is an indicator of altered chemo-sensitivity (87) or cancer risk (166), we did not observe differences in genotype distribution of this polymorphism between EAs and AAs. Therefore, this polymorphism may be important to risk and clinical outcome in a similar fashion in both populations, but is likely not associated with health disparities between EAs and AAs.

XPD is a DNA helicase subunit of the transcription factor IIH (TFIIH), and catalyzes a local unwinding around a DNA lesion in a 5'->3' direction. The TFIIH-mediated opening generates junctions between duplex and single stranded DNA that in turn could be cleaved by a ERCC1-XPF heterodimer. XPD has a polymorphism at codon 751, K751Q (A>C), which is of particular interest. The codon 751 wild type of AA has been associated with suboptimal DNA repair (110). Also, the AA genotype has been seen with greater frequency in patients with colorectal cancer that respond to oxaliplatin based chemotherapy (111). These patients also show longer median survival time. However, the CC variant homozygote is associated with reduced DNA

repair capacity in patients with lung cancer (115), is significantly associated with risk and outcome in acute myeloid leukemia (114), and is overrepresented in patients with lung cancer of Chinese extraction (116). The mixed clinical picture for XPD makes it difficult to interpret the ethnic differences in allelic frequencies that we observe in this report. We report here that significantly low frequency of variant XPD K751Q was detected in AAs.

The BER pathway is essential in protecting cells from endogenous DNA damage, as well as lesions caused by ionizing radiation and alkylating agents. When these lesions cause replication blockage, BER is joined to TC-NER for more efficient DNA damage repair. A critical component of the BER is XRCC1, for which, one relevant polymorphism is at codon 399. This point mutation is in the BRCT1 domain, which is the docking site for PARP1 polymerase (130). Both the wild type GG genotype and the variant AA genotype are found to be associated with increased sensitivity to platinum based chemotherapy and cancer risk, but the results differ among populations and in different types of malignancies. The polymorphism of XRCC1 R194W (685C>T) is in a possible binding site for other proteins in the BER. This mutation causes the positively charged arginine to be changed to a hydrophobic tryptophan, and is susceptible to affect binding and DNA repair efficiency (130). The variant allele is beneficial to platinum-based chemotherapy in patients with advanced non-small cell lung cancer (131). Our data indicated significantly low frequency of XRCC1 R399Q, but not R194W, in AAs. Thus, the polymorphism of XRCC1 R399Q may contribute to the health disparity between the European American and African American populations.

Poly(ADP-ribose) polymerase 1 (PARP1) plays various roles in molecular processes including DNA damage detection and repair. PARP1 expression and activity are generally higher in cancer than in adjacent normal tissue, but cancer predisposition is reported to be greater in individuals with the polymorphism V762A (T>C) in the catalytic domain that reduces PARP1 activity (135-137, 167). The prevalence of variant genotype is extremely low in AAs (0% in our samples), which may indicate better protein function of PARP1 in this population.

Our data suggests the possibility that a comparatively modest reduction in base excision repair may occur more commonly in EAs. This would imply greater sensitivity to chemotherapy agents that alkylate DNA, such as cyclophosphamide, and/or to agents that generate free radicals that damage DNA, such as adriamycin. This would be consistent with observed differences in response to therapy in breast cancer in EA and AA patients.

The field of genetic epidemiology has much to offer in elucidating the effect of heritable variations and its contribution to the observed inter-patient variances in response to the same chemotherapy regimen. The ethnic disparity in mortality between the EA and AA patients is often seen in malignancies where DNA damaging chemo- and radiation therapies are frequently administered. Considering the important roles that DNA excision repair plays in response to these therapy regimens, the ethnic disparity in the genetic profiles of the DNA excision repair genes may serve as the molecular basis of the different responses to these cancer therapies between EAs and AAs, although other factors can not be ruled out by this study. Further understanding of the genetic composition of these DNA excision repair genes

among ethnic groups, and the functional impact of these genetic polymorphisms on the respective genes, will facilitate clinical decision-making. Studies investigating the correlation between these genetic polymorphisms in DNA excision repair genes and outcome of chemo- and/or radiation therapies would also be of great interest.

3. DNA repair polymorphisms and radiation therapy in prostate cancer

3.1. Abstract

Prostate cancer is the most frequent cancer among American men and is the second leading cause of cancer-related death in males. Radiation therapy is a potentially curative, important treatment option in localized prostate cancer. However, 8 years after radiation therapy, even in the low risk subset of patients, approximately 10% of patients will experience clinical disease recurrence. The identification of molecular markers of treatment success or failure may allow for the development of strategies to further improve treatment outcomes. Herein, we investigated the aforementioned molecular markers of DNA excision repair: ERCC1 N118N (500C>T), XPD K751Q (2282A>C), XRCC1 R194W (685C>T), XRCC1 R399Q (1301G>A) and PARP1 V762A (2446T>C), from 513 patients with castrate-resistant prostate cancer (CRPC), including 284 patients who received radiotherapy, 229 patients without radiotherapy, and 152 healthy individuals. The distribution of genetic polymorphisms in the patients with CRPC and in healthy controls was compared, and the association between the polymorphisms and overall survival was investigated. The polymorphisms evaluated did not show differences between the patient group and the healthy controls, nor did they show a trend toward an association with survival. We report here that the genetic polymorphisms in DNA

excision repair genes are not associated with risk of prostate cancer, nor are they predictive to the clinical outcome of radiotherapy for localized prostate cancer.

3.2. Introduction

Carcinoma of the prostate is the most frequent cancer among American males; it alone accounts for 28% of cancer cases in men (168). Prostate cancer is the second leading cause for cancer death in males, exceeded only by lung cancer. Although the specific molecular causes of prostate cancer still remain unknown, multiple etiologic factors, including African American ancestry, family history, increased age, genetic profile and environmental exposures, etc., are thought to be the risk factors for prostate cancer. Variations in exposure to these risk factors may account for inter-individual differences in risk of prostate cancer. Dietary habit may also be a modulator of prostate cancer risk, including phytoestrogen consumption (169), meat consumption, vitamins E and D, dairy and calcium (170), etc., although this has not been demonstrated consistently in clinical studies. Hormone levels, including higher serum levels of testosterone and lower levels of estrogen, may influence prostate carcinogenesis. Therefore, it is suggested that genetic variation in androgen biosynthesis and metabolism may play a role in prostate cancer risk (171).

3.2.1. DNA excision repair and prostate cancer

DNA repair mechanisms are paramount in maintaining genomic stability and play an important role in carcinogenesis. A robust DNA repair capacity may lessen any risks conferred by mutations from environmental or endogenous risk factors.

Numerous SNPs in DNA excision repair genes have been reported, and studies of these SNPs and prostate cancer risk will provide additional understanding of the etiology of prostate cancer. For example, the genetic variation in the BER gene APE1 is found to be associated with prostate cancer risk in Brazilian men (172). XRCC1 encodes a stimulator and scaffold protein for the BER pathway. It interacts with PARP1, DNA ligase III and DNA Pol ß, etc., as aforementioned. Polymorphisms have been previously identified in XRCC1 that correlate with phenotypic changes (130). Although many SNPs have been discovered in XRCC1, only three have been investigated regarding prostate cancer risk, R194W, R280H, and R399Q. The XRCC1 R194W is indicated to have no association with prostate cancer risk in two small studies (173, 174), or to be protective in a Chinese population (175). The studies evaluating the XRCC1 R280H polymorphism have not produced any conclusive findings on its association with prostate cancer risk (174, 175). The XRCC1 R399Q polymorphism has been the most frequently investigated among the BER gene polymorphisms. It is significantly associated with prostate cancer risk in European Americans (176) and Asians (173, 175, 177), but not in African Americans (176), while other studies do not replicate the positive associations (177, 178). Low intake of antioxidants may modify the risk (174, 179). The human 8-Oxoguanine DNA Glycosylase 1 (hOGG1) catalyzes the excision and removal of single base adducts. The hOGG1 S326C polymorphism is reported to be associated with prostate cancer risk in Caucasian patients (180). Genetic polymorphisms in XRCC7 and XRCC3, genes in the non-homologous end-joining (NHEJ) and homologous recombination (HR) repair pathways, are associated with prostate cancer risk and

high Gleason scores in North Indian populations (172). PARP1 V762A polymorphism is observed to have an increased risk of prostate cancer and a decrease in enzyme function in response to oxidative damage (135).

The NER pathway is associated with the repair of bulky adducts induced by several suspected environmental prostate cancer carcinogens, such as polycyclic aromatic hydrocarbons, heterocyclic aromatic amines from well-done meats, and pesticides (181). Deficient NER capacity is found in lymphocytes from patients with prostate cancer (182). XPD D312N and K751Q are reported to have no association with prostate cancer in Caucasian and Chinese populations, but XPF R415Q is moderately associated with an increased risk of prostate cancer though not statistically significant (183-185). Additional studies on other NER genes, such as XPG, XPC and hR23B, do not observe positive association in the polymorphisms in these gene with prostate cancer risk (183).

Efforts to identify prostate cancer susceptibility genes will be useful in understanding the etiology and molecular genetics of prostate cancer and hopefully in early detection of prostate cancer.

3.2.2. Radiation therapy in Prostate Cancer

Localized prostate cancer is treated by active monitoring, radical prostatectomy, or radiation therapy, depending on the tumor characteristics and the patient's life expectancy. Radiation therapy is an important treatment option for patients with localized, early stage prostate cancer. In patients with T1 to T3 lesions, without nodal or distant metastases, similar clinical results are obtained through

surgery (radical prostatectomy) or radiation therapy. Radiation therapy can be delivered by any of several approaches: external beam, brachytherapy, and intensity modulated radiation therapy (IMRT). However, with surgery or with radiation therapy, a percentage of patients with well-documented localized disease will experience the return of their malignancy.

In patients with low risk localized prostate cancer, treated with modern IMRT, actuarial PSA relapse-free survival is 85% to 89%. In unfavorable risk localized prostate cancer, the actuarial PSA relapse-free survival is 59% to 72% (186). Therefore, even in the group of patients with the best clinical features and the most favorable prognosis, 11% to 15% of these patients have intra-tumor characteristics that lead to relapse of disease.

Though considerable inter-patient differences in response to radiotherapy occur, the mechanisms behind these different responses are not well understood. A variety of patient, tumor, treatment, and molecular factors contribute to the various outcome of radiotherapy. The understanding of this mechanism may increase the predictability of outcome and selection of the optimal treatment. The work published by the Radiation Therapy Oncology Group (RTOG) investigated a total of 11 potential prognostic markers, and only p53 and DNA ploidy showed association with overall survival (187). One question is whether there are intra-tumor considerations, such as DNA repair pathways, that may make some prostate cancer cells more resistant to radiation therapy, and therefore make those tumors more likely to clinically recur.

3.2.3. Radiation and DNA excision repair

Ionizing radiation acts through the creation of various types of DNA damage, the inter-individual DNA repair capacity may influence the patient's radiosensitivity. The types of DNA damage induced by radiation include DNA base damage and both single- and double-strand DNA breaks (188). Such lesions, if inadequately repaired, can lead to cell death by lethal chromosomal aberrations or apoptosis, the desired outcome of radiation therapy. Multiple DNA repair pathways are involved to maintain the genomic integrity, and the homologous repair (HR) and non-homologous end-joining (NHEJ), NER and BER pathways are thought to contribute heavily to remove the damage caused by ionizing radiation (71, 188).

The genetic polymorphisms in DNA repair genes are believed to contribute to such inter-individual differences. One important polymorphism in XRCC1 is R194W, located in the linker region separating the NH_2-terminal domain (NTD) from the central BRCT1 (BRCA1 C-terminus) domain, as illustrated in **Figure 3-1**. The linker region was also suggested to be a potential binding domain of several interactive proteins, and is rich in basic amino acids. The substitution of arginine to hydrophobic tryptophan may affect the protein binding efficiency. According to a review by Goode et al. (189), the R194W polymorphism was related to reduced risk of cancer, and this was confirmed by two later association studies (190, 191). However, another study showed a highly significant association ($p=0.0005$) of R194W with the increased risk of head and neck cancer in a Korean population (192). The possible reasons for these confounding results include that the epidemiological studies could

be misleading and this polymorphism might not be directly associated, but linked to another relevant polymorphism in a haplotype (130). The XRCC1 R194W polymorphism is not associated with chromosome aberration frequencies in retired radiation workers and is not thought to influence the response to occupational exposure to radiation (193). The second XRCC1 polymorphism, R399Q, is a well-studied SNP located in the BRCT1 domain, which is essential for PARP1 binding. Cells carrying this mutation have been shown to be defective in responding to both X-ray radiation and UV light (194). Studies correlated the polymorphisms in XRCC1 with either adverse effects (195) or protective effects resulting from radiotherapy (196, 197), or favorable response to therapeutic radiation (198-200) in several cancers.

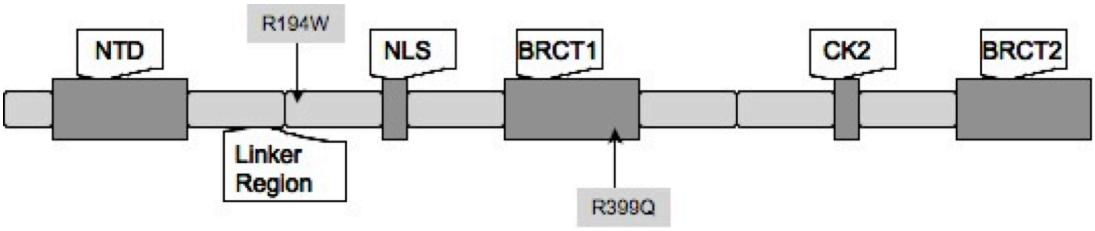


Figure 3-1**:** Structure of XRCC1 domains and locations of the single nucleotide polymorphisms (SNPs) genotyped in this study.

NTD: N-terminal domain, NLS: nuclear localization signal domain, BRCT: BRCA C-terminus domain, CK2: Ck2 phosphorylation sites, modified from (130).

PARP1, another important gene in DNA repair, assists by recruiting XRCC1 after sensing DNA damage. The variation, V762A in PARP1, causes the loss of two

methyl groups that in turn increases the distance between 762 and its closest neighbor in the active site. This steric change looses the binding of NAD^+ and reduces the enzymatic activity nearly two fold (134). As a consequence, the variant enzyme may be less able to sense the damage in DNA and reduce the recruitment of XRCC1 and other proteins involved in the repair process. Since PARP1 also plays an important role in repairing radiation inflicted lesions, several PARP1 inhibitors have been tested in clinical trials to try to increase the effectiveness of ionizing radiation in the treatment of cancer (201-203).

In addition to the BER, the NER pathway also plays a role in removing multiple types of DNA damage, including those caused by UV light and platinum-containing chemotherapy agents. Important genes in the NER, ERCC1, and XPF, are essential for the 5' incision into the DNA strand that releases bulky DNA lesions (146, 147). XPD is a 5'- 3' helicase that participates in DNA strand separation prior to the 5' incision step performed by the ERCC1-XPF heterodimer (108). Important genetic polymorphisms in these genes have also been described and studied extensively, as discussed before.

Our previous study shows that the variant alleles of the polymorphic DNA excision repair genes have significantly higher frequencies in EAs compared to AAs, suggesting a mild reduction in DNA excision repair function in the EA population (204). The aim of this study is to investigate whether the DNA excision repair gene polymorphisms are related to prostate cancer risk and the outcome of radiation therapy in prostate cancer in EA populations.

3.3. Materials and Methods

3.3.1. Sample collection & DNA isolation

Five hundred and thirteen patients with castrate-resistant prostate cancer (CRPC) were analyzed in this study. These include 284 patients who received external beam radiotherapy (XRT) and/or brachytherapy and 229 patients with the same disease that did not receive radiotherapy. All patients were Caucasians and were enrolled in an institutional review board–approved clinical trial within the intramural program of the National Cancer Institute, and were arbitrarily assigned a number in our database to protect confidentiality. Informed consent was obtained from all subjects before trial participation. The 152 male Caucasian control samples were the same as those used in chapter 2. All volunteers had signed informed consent to allow their samples to be used for genotyping, and none had a diagnosis of cancer. Genomic DNA was extracted from serum or white blood cell buffy coat layers of whole blood of patients using the UltraSens Virus Kit according to the manufacturer's instructions (Qiagen, Valencia, CA).

3.3.2. Genotyping methods

The genotyping methods are the same as those used in the study in Chapter 2.

3.3.3. Statistical analysis

Confidence intervals for the odds ratios of the distributions of individual polymorphisms relative to the wild type between controls and patients with cancer

were determined using the exact method. The probability of survival as a function of time since diagnosis was determined by the Kaplan-Meier method. The statistical significance of the differences in survival among the genotypes was determined by the log-rank test. All p-values are two-tailed.

3.4. Results

3.4.1. Polymorphisms in DNA excision repair genes are not associated with prostate cancer

Five hundred and thirteen patients with CRPC were assayed for 5 polymorphisms: ERCC1 N118N (500C>T), XPD K751Q (2282A>C), XRCC1 R399Q (1301G>A), XRCC1 R194W (685C>T), and PARP1 V762A (2446T>C). The distribution of these SNPs among the 513 patients studied was compared to the 152 healthy volunteer controls. Statistical analyses of the genotype prevalence for all five polymorphisms revealed no evidence of any differences between the two groups (**Table 3-1**). All of the genotype distributions were in Hardy-Weinberg equilibrium in both cases and controls.

Table 3-1: Distribution of polymorphisms among healthy controls and patients with CRPC.

SNP	Genotype	Control*	Patients	OR	95% CI	*P* Value
ERCC1	CC	23 (0.21)	91 (0.21)	Referent	-	-
N118N	CT	53 (0.49)	197 (0.46)	0.940	0.5426 to 1.627	0.8899
(500C>T)	TT	32 (0.30)	143 (0.33)	1.129	0.6218 to 2.052	0.7595
XPD	AA	49 (0.42)	186 (0.43)	Referent	-	-
K751Q	AC	56 (0.47)	178 (0.42)	0.837	0.5419 to 1.294	0.4399
(2282A>C)	CC	13 (0.11)	64 (0.15)	1.297	0.6608 to 2.546	0.5129
XRCC1	CC	120 (0.87)	402 (0.89)	Referent	-	-
R194W	CT	17 (0.12)	43 (0.09)	0.755	0.4154 to 1.372	0.3399
(685C>T)	TT	1 (0.01)	7 (0.02)	2.090	0.2544 to 17.16	0.6893
XRCC1	GG	49 (0.46)	145 (0.41)	Referent	-	-
R399Q	AG	47 (0.44)	151 (0.43)	1.086	0.6850 to 1.721	0.8144
(1301G>A)	AA	10 (0.10)	56 (0.16)	1.892	0.8967 to 3.994	0.1248
PARP1	TT	80 (0.67)	315 (0.70)	Referent	-	-
V762A	CT	32 (0.27)	123 (0.27)	0.976	0.6163 to 1.546	0.9068
(2446T>C)	CC	7 (0.06)	15 (0.03)	0.544	0.2147 to 1.380	0.1873

OR = odds ratio, CI = exact confidence interval.

*Values are number (percentage).

3.4.2. Polymorphisms in DNA excision repair genes are not associated with the median survival time in prostate cancer

We determined whether the polymorphisms in DNA excision repair genes were associated with the median survival time in prostate cancer using the univariate method. None of the polymorphisms evaluated showed a trend toward an association with the median survival time individually. The results are shown in **Table 3-2**. Patients displayed similar median survival time regardless of what ERCC1 and XPD genotypes they had. While in XRCC1 genotype groups, patients having the variant

genotype of XRCC1 R399Q had the longest survival time of 11.12 years. Patients having either the heterozygous genotype of XRCC1 R194W or the variant genotype of PARP1 V762A had relatively short median survival time (6.52 and 5.88 years, respectively), though not statistically significant.

Table 3-2**:** Median survival time comparison in all patients with CRPC according to their genotypes of the DNA excision repair genes. * Two-tailed log-rank test p-values.

SNP	Genotype	Median survival time (years)
ERCC1 N118N (500C>T)	CC	8.21
	CT	7.84
	TT	8.33
	P Value*	0.7622
XPD K751Q (2282A>C)	AA	8.13
	AC	8.21
	CC	7.155
	P Value	0.9925
XRCC1 R399Q (1301G>A)	GG	8.17
	AG	7.77
	AA	11.12
	P Value	0.5256
XRCC1 R194W (685C>T)	CC	8.06
	CT	6.52
	TT	9.22
	P Value	0.5493
PARP1 V762A (2446T>C)	TT	8.17
	CT	7.69
	CC	5.88
	P Value	0.8469

3.4.3. Polymorphisms in DNA excision repair genes are not associated with the median survival in radiation therapy in prostate cancer

To determine whether the polymorphisms in DNA excision repair genes are associated with the outcome of radiation therapy for prostate cancer, we categorized patients into two groups according to whether they have received radiation therapy for their prostate cancer, i.e. a radiation group and a non-radiation group, and compared the survival times for each genotype group. However, we did not find any of the polymorphisms showing a trend toward an association with the median survival time in either of the two groups. The results are presented in **Table 3-3**.

In general, the median survival time in the radiation group was longer than those of the non-radiation group. In both groups, individuals carrying the XRCC1 R194W CT genotype only had shorter median survival time compared to the homozygous genotypes of CC or TT. While in patients with the AG genotype of XRCC1 R399Q had similar median survival time as that of the variant genotype AA in the radiation group, but in the non-radiation group, patients with the AG genotype had a much shorter median survival time than those with the AA genotype (5.41 and 8.305 years, respectively). Patients carrying the variant genotype of PARP1 V762A polymorphism had a median survival time as long as 11.675 years compared to 3.9 years in the non-radiation group (**Table 3-3**). However, none of the comparisons reached statistical significance.

Table 3-3: Median survival time comparisons in patients who received radiation therapy for their prostate cancer and patients who did not according to their genotypes of the DNA excision repair genes. * Two-tailed log-rank test p-values.

SNP	Genotype	Median survival time_radiation group (years)	Median survival time_non-radiation group (years)
ERCC1 N118N (500C>T)	CC	9.72	6.915
	CT	10.35	4.781
	TT	8.86	6.381
	P Value*	0.9649	0.4028
XPD K751Q (2282A>C)	AA	8.86	6.7
	AC	10.33	5.32
	CC	9.22	4.15
	P Value	0.9325	0.6019
XRCC1 R399Q (1301G>A)	GG	9.22	5.88
	AG	10.41	5.41
	AA	11.75	8.305
	P Value	0.8456	0.6261
XRCC1 R194W (685C>T)	CC	9.66	5.88
	CT	6.81	4.24
	TT	9.22	10.595
	P Value	0.3361	0.8515
PARP1 V762A (2446T>C)	TT	9.55	5.9
	CT	8.82	4.985
	CC	11.675	3.9
	P Value	0.6805	0.0949

3.5. Discussion

The study presented here investigated the possible association between polymorphisms in the NER and BER DNA repair genes and clinical outcome of radiation therapy in patients with prostate cancer. First, all five SNPs assessed in this study were not associated with prostate cancer as compared to healthy volunteers.

Second, none of the polymorphisms investigated here showed a significant trend toward an association with the median survival time in patients with prostate cancer, nor did they show any association with the median survival time in patients who received radiation therapy for their localized prostate cancer. Regarding the BER genes, neither the XRCC1 R399Q nor the XRCC1 R194W was associated with the median survival time individually.

DNA excision repair is an important mechanism for maintaining genomic stability and to prevent accumulation of DNA damage. Epidemiologic studies of genetic polymorphisms in DNA excision repair genes may inform individual susceptibility and provide further understanding on molecular mechanisms of carcinogenesis. The XRCC1 polymorphisms are indicated to have no association with prostate cancer risk in several studies (173, 174, 179, 185) or to be associated with increased risk of prostate cancer among heavy smokers in Chinese population (175). The same confounding results are seen for the NER genes as well. Our study of 5 polymorphisms in 4 genes involved in both the NER and the BER pathways did not reveal any associations between the polymorphic DNA excision repair genes and the risk of prostate cancer in a European American population ($p>0.05$). The current challenge is the validation of the functional impact of the genetic polymorphisms in these DNA excision repair genes that have been identified by epidemiological studies.

Laboratory studies indicate that the variant genotype of XRCC1 R399Q is more sensitive to X-ray and UV-light than the other two genotypes within this codon (194). XRCC1 R399Q is located in the BRCT1 domain (**Figure 3-1**), a critical region that is required for PARP1 mediated recruitment of XRCC1 upon DNA damage. This

site is involved in survival after methylation damage (205). It is suggested that the substitution of an arginine to glutamine may cause the loss of a secondary structure feature such as an alpha helix that is important for correct protein-protein interactions in the BRCT1 domain, thus compromising the DNA repair capability (206). A study showed that the number of variant alleles in APE1 D148Q and XRCC1 R399Q genotypes was significantly correlated with prolonged cell-cycle delay following ionizing radiation (IR), which resulted in IR hypersensitivity in breast cancer cases (207). Theoretically, the variant allele of the XRCC1 R399Q may impair the interaction between XRCC1 and other proteins, resulting in inefficient removal of radiation induced DNA damage and prolonged cell cycle arrest, which delivers favorable response to radiotherapy. However, the results from our study did not show any correlation between the XRCC1 R399Q genotypes and the survival time in the patients who received radiation therapy for their prostate cancer.

The R194W polymorphism is located in a linker region (residues 158–310) between the NTD and the central BRCT domain of XRCC1 (**Figure 3-1**), enriched in basic amino acids. The high pI and overall positive charge of this region was suggested to have an important role in proper secondary structure formation (208). This domain is also the potential protein-binding domain for several interactive protein partners (PCNA, APE1, etc.) of the XRCC1 protein. The transition from the positively charged arginine to a hydrophobic tryptophan could affect binding and DNA repair efficiency. An *in vitro* study suggested that the presence of the variant allele of R194W might result in a damaging effect and an intolerant protein (130). We found a low frequency of the variant genotype TT of this SNP in our study population

(1% in the healthy volunteers and 2% in the patient group). A previous study showed that the variant allele of R194W had higher frequency in radiation-sensitive breast cancer cases (OR 1.98, 95% CI 0.92–4.17) (209). But our study showed no association between the R194W genotypes and the survival time of radiation therapy. Though some epidemiological studies did suggest the variant allele of XRCC1 R194W confers reduced cancer risk (189), others suggested vice versa (192). As suggested by another study (210), possessing more than 4 SNPs in DNA repair genes resulted in hypersensitivity to radiation in cells obtained from patients with cancer ($p<0.001$). Another study shows that the wild type allele G of R399Q along with the variant allele T of R194W, and the wild type allele of XRCC1 R280H had shorter overall survival than other haplotypes in patients with lung cancer that received radiotherapy ($p=0.04$) (200). Taken together, these results suggest that a complex intergenic interaction between the alleles of XRCC1 polymorphisms may exist.

Due to the important role that PARP1 plays in DNA damage detecting and responding, PARP inhibitors have been actively investigated to enhance the cytotoxic effects of ionizing radiation and DNA-damaging chemotherapy regimens. However, our study did not find positive association between the PARP1 V762A genotypes and the outcome of radiation therapy in patients with prostate cancer.

One shortcoming of this retrospective study is that the patients in the non-radiation group usually had more severe malignancies and received therapies other than radiation. That may account for the shorter median survival time in this group in general. Therefore, the comparisons of the median survival time between each genotype groups were made within the radiation and non-radiation groups.

DNA excision repair pathways help to maintain genetic stability and prevent the development of cancer. However, they also represent a potential mechanism of resistance to DNA damaging chemotherapy and radiation therapy. The polymorphisms in DNA excision repair genes provide the genetic basis for various DNA excision repair capability. To identify radiosensitive cancer patients before treatment may allow tailored radiation therapy and improve effectiveness and reduce unnecessary toxicity of ionizing radiation in clinical practice.

4. The ERCC1 N118N polymorphism does not change cellular ERCC1 protein expression or platinum sensitivity

4.1. Abstract

Genetic polymorphisms in ERCC1 are thought to contribute to altered sensitivity to platinum-based chemotherapy in the clinic. Although ERCC1 N118N (500 C>T, rs11615) is the most studied polymorphism, the impact of this polymorphism on platinum-based chemotherapy remains unclear. This is the first study where the functional impact of ERCC1 N118N on gene expression and platinum sensitivity was explored. An ERCC1 deficient cell line, UV20, was transfected with the ERCC1 cDNA clone with either C or T allele and examined for the changes in ERCC1 transcription, translation and platinum sensitivity. Both ERCC1 mRNA and protein expression levels increased upon cisplatin treatment, and peaked at 4 hours post-treatment, however, there were no differences between the two alleles ($p>0.05$). Cells complemented with ERCC1 showed significantly higher survival proportion than the parental cell line following platinum exposure ($P<0.0001$), although no differences were observed between the cells transfected with the wild type or the polymorphic allele. These data suggest that N118N itself is not related to the phenotypic differences in ERCC1 expression or function, rather the polymorphism may be linked to other causative variants or haplotypes.

4.2. Introduction

4.2.1. ERCC1 in NER and Platinum-based chemotherapy

ERCC1 has been highly conserved through evolution and is constitutively expressed in all tissues. Mutational analysis revealed that the N-terminus of ERCC1 is poorly conserved and is dispensable for repair functions, whereas the C-terminus possesses a strongly conserved motif sharing sequence homology with many DNA repair proteins. Most missense mutations in the central region of the protein produce an unstable protein or complex (47). An ERCC1 deficient Chinese Hamster Ovary (CHO) cell line 43-3B has the full length ERCC1 mRNA but a mutation (V98E) in the coding region of XPA-binding domain so that the produced protein is unable to bind XPA (211), indicating the importance of the integrity of the central region in the normal functioning of ERCC1. Whether ERCC1 has functions other than DNA repair has yet to be clarified, but it is thought that ERCC1 is essential for life. Deletion or disruption of mouse ERCC1 gives rise to the accumulation of endogenously generated DNA interstrand cross-links and severe symptoms including postnatal growth failure, nuclear abnormalities, life-limiting liver and kidney disease and senescence (212-214). The CHO cell lines defective in ERCC1 display unique, extreme sensitivity to ultraviolet radiation and cross-linking agents (215-217). The patient described with human inherited ERCC1 deficiency has severe developmental failure and morphological features very similar to cerebro-oculo-facio-skeletal (COFS). Patient cells exhibit moderate hypersensitivity to UV irradiation, indicating impaired DNA repair capability (218).

Platinum-based chemotherapies have been widely used in the treatment of several solid malignancies since its discovery. The response of tumor cells to platinum-based drugs involves DNA repair mechanisms. DNA lesions caused by platinum compounds are mainly repaired by the NER pathway. It recognizes the DNA damage and excise the platinum-DNA adducts from the injured DNA strand. ERCC1 is one of the proteins that make up the NER complex and several studies have linked ERCC1 to platinum resistance in cell lines and in human cancers. It forms a heterodimeric protein complex with XPF to carry out the 5' incision in the presence of a DNA lesion. Reduction of ERCC1 function may predispose people to cancer due to the inefficiency of DNA damage removal. However, improved response to DNA-damaging chemotherapy in those individuals is anticipated. Inter-individual differences are observed for ERCC1 protein expression levels and have been correlated to the outcome of platinum-based chemotherapies. In a study conducted in patients with non-small cell lung cancer, cisplatin-based adjuvant chemotherapy was beneficial to those with ERCC1-negative tumors. While in the patients who did not receive adjuvant chemotherapy, individuals with ERCC1-postive tumors had prolonged survival time compared to those have ERCC1-negative tumors (81). However, the genetic contribution behind the mechanism of variation in ERCC1 expression has yet to be clarified. To date, more than 190 single nucleotide polymorphisms (SNPs) have been reported for ERCC1, most of which are located in intronic or untranslated regions. Missense mutations in ERCC1 are rare and not well studied; the only reported human case with ERCC1 missense mutations shows severe phenotypic deficiency (218). Of all the SNPs that have been discovered in ERCC1, a

silent polymorphism in exon 4 (500 C>T in mRNA, N118N, rs11615) has been extensively studied and associated with altered outcome in platinum-based chemotherapy in multiple malignancies, as summarized in **Table 1-1**. However, these studies were performed in different tumor types, different therapeutic regimens, using different criteria to measure response or outcome, and most of them had small groups of patients. Therefore, the results generated a mixed clinic picture, which poses the challenge to the understanding of the functional consequences of this silent mutation.

4.2.2. Implications of silent mutations

Silent mutations do not alter amino acid sequence, and therefore they are not expected to change the function of the protein. However, increasing evidence supports that codons encode more information than merely amino acid sequences, contrary to the widely accepted concept that the information necessary to define the three-dimensional structure of a protein largely resides in its amino acid sequence. For example, a study shows that a naturally occurring rare silent mutation in the MDR1 gene affects the timing of co-translational folding and insertion of P-glycoprotein into the membrane, thereby altering the structural and functional properties of the gene product (219). In addition, the moving of ribosome on mRNA is not uniform. The rate of the traffic is primarily determined by the genetic code and the relative abundance of tRNA molecules surrounding the translational machinery. The translational speed slows down at the transition into secondary structure, and the alteration in tRNA concentration and the introducing of synonymous codons in the

slow translating regions can affect folding efficiency (220, 221). Finally, alternative mRNA splicing may also give rise to more than one protein from one mRNA.

The residues from 96-214 form a deep V-shaped cleft on ERCC1 that is the binding site for XPA. The N118 residue is within the region 107-156 that makes critical contacts with XPA though N118 itself does not directly interact with XPA. Therefore, a perturbation in translation could affect the effective folding of the encoded protein, and consequently the proper function of the protein product. The two codons, AAC and AAT, which encode asparagine, have different usage frequencies (21.30 for AAC and 16.43 for AAT) according to "The human codon usage and codon preference table" (http://genome.crg.es/courses/genefinding/T4/main/).

Hence, to test if the silent mutation of ERCC1 N118N has phenotypic impact on gene expression and protein function, we introduced a ERCC1 cDNA clone with either C or T at the specific position into an ERCC1 deficient cell line UV20, denoted by $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$. We then assayed for ERCC1 mRNA and protein expression levels upon cisplatin treatment and the cellular sensitivity to platinum-containing drugs.

4.3. Materials and Methods

4.3.1. Cell culture

The ERCC1 deficient cell line UV20 was obtained from the American Type Culture Collection (Manassas, VA). UV-20 was isolated as an UV sensitive mutant

by mutagenizing the parental cell line AA-8 with EMS (222, 223). The ERCC1 gene in this cell line encodes a truncated protein that is barely detectable by Western blot (224). It is an ideal model for studying ERCC1 mutations. The cells were maintained in a humidified incubator at 37°C equilibrated with 5% CO_2 and 95% air in Alpha minimum essential medium containing 10% fetal bovine serum and 1% Penicillin-Streptomycin. To determine the minimum concentration of Geneticin®, 5 X 10^4 cells were plated in 6-well plates, and a range of Geneticin concentrations were tested. 750 ug/ml of Geneticin was used to select the stable transfected cell line. The plasmids containing the reference or variant allele of ERCC1 N118N, as well as the vector control, were transfected into UV-20 cells using the Lipofectamine™ LTX Reagent according to the manufacurer's instructions (Invitrogen, Carlsbad, CA). The stable transfected cell lines were designated as $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$, respectively.

4.3.2. LR recombination reaction

The ERCC1 Ultimate™ ORF clone (IOH5754, NM_001983) was purchased from Invitrogen. The plasmid was grown in competent E. coli and purified. Then the target gene was introduced into the expression vector pcDNA™3.2/V5-DEST by the LR recombination reaction (225): mix 150 ng of the entry clone and 150 ng of the destination vector (pcDNA™3.2/V5-DEST) with 4µl of 5X LR Clonase Reaction Buffer and TE buffer, bring to the final volume of 8 µl, then add 2 µl LR Clonase; incubate reactions at 25°C for 60 minutes; add 1 µl of the Proteinase K solution to each sample to terminate the reaction, and incubate the samples at 37°C for 10 minutes.

Then, the LR reaction product was transformed into DH5α™ competent E. coli cells by the heat-shock method to amplify the plasmids. The transformed cells were selected by ampicillin. pUC19 DNA was used as the control plasmid for transfection efficiency. The plasmid DNA was purified using the QIAGEN® Plasmid Purification kit. The purified plasmid DNA containing the ERCC1 ORF clone then served as the template for the mutagenesis PCR.

4.3.3. Site-directed mutagenesis

The mutant was generated using the ERCC1 ORF clone in the pcDNA™/V5-DEST vector as the template using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following primers were used in the mutagenesis PCR: 5'-ACT GAA GTT CGT GCG CAA TGT GCC CTG GG-3' and Primer2: 5'-CCC AGG GCA CAT TGC GCA CGA ACT TCA GT-3'. The introduced allele is in italics. 50 ng of starting DNA template, PfuUltra HF (high fidelity) DNA polymerase and low number of thermal cycles (18 cycles) were used in the mutagenesis PCR to eliminate the unwanted second-site errors and obtain high mutation efficiencies. The thermal cycles included one heating step at 95°C for 2 minutes in the beginning, 18 cycles of 95°C for 20 seconds, 60°C for 10 seconds, and 68°C for 1.5 minutes/kb of plasmid length, followed by a final extension at 68°C for 5 minutes. After the PCR, 1 µl Dpn I restriction enzyme was applied to the PCR product to digest the parental methylated or semimethylated DNA. The reaction was carried out by heating at 37°C for 10 minutes. Then the Dpn I-treated DNA was transferred into the XL10-Gold ultracompetent cells by heat shocking according to

the protocol. The nicked vector DNA containing the desired mutations was fixed in the cells. The pWhitescript™ 4.5-kb control plasmid and the control primers were used to monitor the efficiency of mutant plasmid generation.

The proper construction of both the original ORF clone and the synthesized mutant strand were verified by direct sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit V3.1 (Applied Biosystems, Foster City, CA) and an ABI Prism 3130 Genetic Analyzer using the manufacturers instructions.

4.3.4. Real-time RT PCR

Expression of ERCC1 mRNA was measured by quantitative reverse transcription-PCR (RT-PCR) using the Stratagene Mx3005P™ Real-Time PCR System. Briefly, cell lysates were collected at the appointed time following treatment of cisplatin (3.75×10^{-2} mg/ml) using AllPrep RNA/Protein Kit (Qiagen, Valencia, CA). Total RNA extracted was reverse-transcribed using the RT² First Strand Kits (SABiosciences, Frederick, MD). Each sample was analyzed in duplicate and the results are an average of four analyses. Analysis of mRNA expression was conducted using the RT² qPCR Primer Assay (PPH01539A, SABiosciences, Frederick, MD) for ERCC1 and normalized to the expression of CHO ACTB (226).

4.3.5. Western blotting

Expression of ERCC1 protein was assessed by Western blot analysis. Cells were washed in cold PBS and lysed in 100 µl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 10

µl/ml of Halt™ Protease Inhibitor Cocktail (Pierce) at 4°C. Samples were then sonicated on ice and collected by centrifuging at 14,000 × g for 15 minutes. Protein concentration was determined with BCA Protein Assay Kit (Pierce, Rockford, IL) and 20 µg of protein was subjected to electrophoresis on a NuPage® 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Proteins were transferred electrophoretically to a PVDF membrane using the iBlot® Dry Blotting System (Invitrogen, Carlsbad, CA). The membrane was blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE) followed by an overnight incubation with a 1: 200 dilution of ERCC1 primary antibody (FL-297, Santa Cruz Biotechnology, Santa Cruz, CA). For detection with the Odyssey imaging system, a 1: 5,000 dilution of the infrared fluorophore conjugated antibody, IRDye™ 800-conjugated goat anti-rabbit IgG was used. Proteins were visualized using the Odyssey Infrared Imaging System and Odyssey software (LI-COR, Lincoln, NE). Quantification of western blots was performed using ImageJ (http://rsbweb.nih.gov/ij/) according to the developer's instructions.

4.3.6. Cell cytotoxicity assays

The night before treatment, cells were plated in 96-well plates at a density of 5000 cells/well. A range of cisplatin (2.56×10^{-6} to 0.2 µg/µl), carboplatin and oxaliplatin concentrations (3.2×10^{-4} to 1 µg/µl) was tested in triplicate. Cells were exposed to these concentrations of toxicants for 1 hour and then incubated with fresh medium without toxicants for another 72 hours. Cell viability was tested using CCK-8 (Dojindo, Rockville, MD) and CellTiter-Blue® (Promega Corporation, Madison, WI) assays according to the technical manuals.

4.3.7. Statistical analysis

Data are presented as the mean ± SD. To assess statistical significance of differences, Student's t test or one-way ANOVA was conducted. P values <0.05 were considered statistically significant. All statistical analyses were conducted using GraphPad Software (GraphPad Software, Inc., La Jolla, CA).

4.4. Results

4.4.1. ERCC1 expression in UV20 cell lines

In order to compare the effects of the genetic polymorphism of ERCC1 N118N (500 C>T) on ERCC1 expression and platinum sensitivity in the same genetic background, the ERCC1 deficient cell line UV20, a derivative from CHO cell line, was employed. The UV20 cell lines stably expressing ERCC1 with C or T allele, denoted by $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$, were established. ERCC1 protein expression levels in each cell were measured by western blot. As shown in **Figure 4-1**, the parental UV20 cell line and the empty vector pcDNA™/V5-DEST transfected cells did not show detectable ERCC1 protein expression, while cells transfected with ERCC1 cDNA containing either genotype of 500 C>T showed comparable ERCC1 protein expression levels.

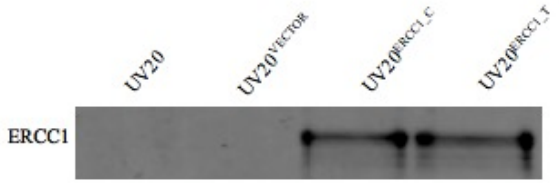


Figure 4-1: ERCC1 protein expression levels in transfected UV20 cell lines are comparable.

The UV20 cell lines stably expressing ERCC1 with C or T allele were established. ERCC1 protein expression levels in each cell were measured by western blot. The parental UV20 cell line and the empty vector pcDNA™/V5-DEST transfected cells ($UV20^{VECTOR}$) did not show detectable ERCC1 protein expression, while cells transfected with ERCC1 cDNA containing either genotype of 500 C>T, $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$, showed comparable ERCC1 protein expression levels.

4.4.2. Real-time quantitative PCR analysis reveals no difference in levels of ERCC1 transcripts in $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines

To study how exogenous stress induces ERCC1 transcription in the transfected UV20 cell lines, we assayed for changes in ERCC1 mRNA expression levels upon cisplatin treatment in UV20 cells transfected C or T allele by quantitative RT-PCR. The basal levels of ERCC1 transcripts in $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines were similar, as normalized to beta-actin expression in each cell line (**Figure 4-2**). Upon cisplatin treatment, the expression levels of ERCC1 transcripts increased in both cell lines immediately. This increase peaked at 4 hours following cisplatin induction. However, there were no differences in ERCC1 transcript levels between the $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines ($p=0.1737$). At 8 hours after

cisplatin treatment, the ERCC1 transcription level started to decrease. The two cell lines showed comparable levels of ERCC1 transcripts (p= 0.6376).

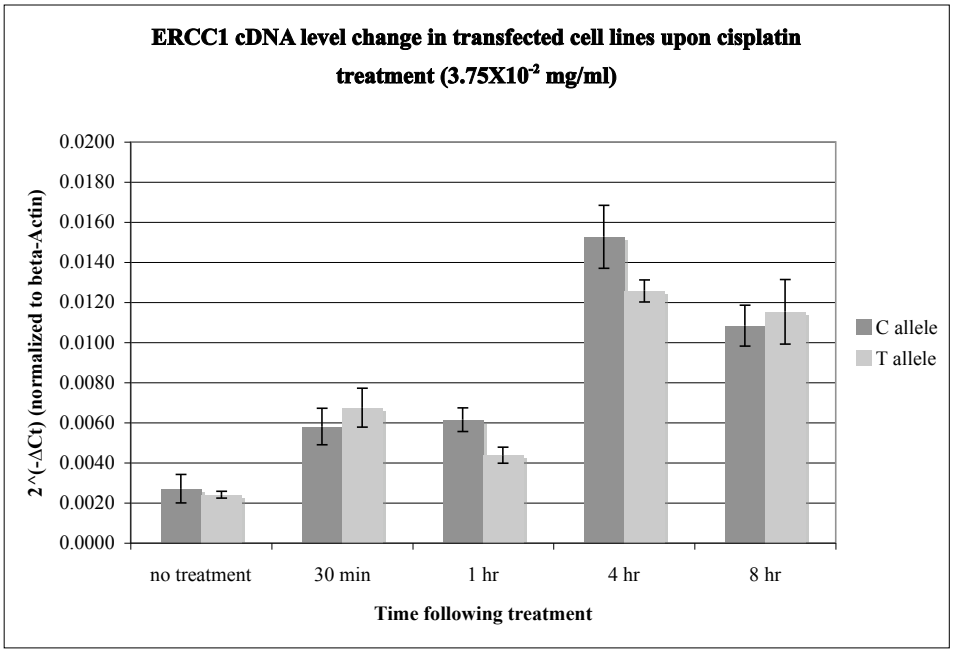
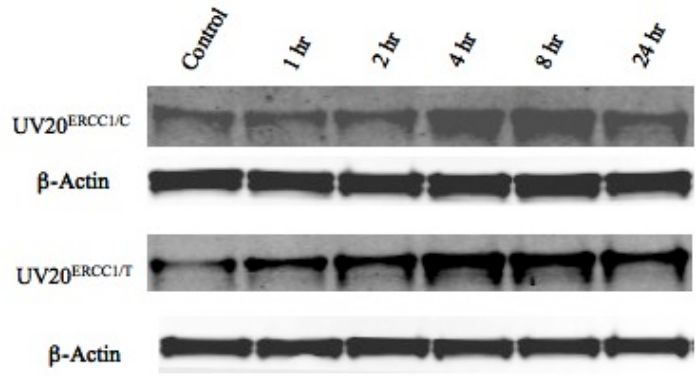


Figure 4-2: The levels of ERCC1 transcripts in $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines remained the same upon cisplatin treatment.

There was no difference in ERCC1 transcripts in the cells transfected with either C or T allele before treatment (p=0.495). ERCC1 transcription increased in both cell lines 30 minutes after cisplatin treatment. This increase in transcription peaked at 4 hours following cisplatin induction. However, there was no significant difference in ERCC1 gene expression levels between the two transfected cell lines, $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ (p=0.1737). Results are obtained from 4 independent experiments with duplicates in each experiment.

4.4.3. ERCC1 protein expression levels in UV-20 cells with different ERCC1 N118N (500 C>T) genotypes remained the same

To investigate how the 500 C>T allele affects ERCC1 translation upon cisplatin induction, we used western blot analysis to examine ERCC1 protein levels in the $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines. **Figure 4-3** shows that in both cell lines, ERCC1 protein expression levels increase upon cisplatin treatment, and peak at 4 hours post-treatment, reaching a 2.28 fold increase in $UV20^{ERCC1\_C}$ cell line and 1.71 fold increase in $UV20^{ERCC1\_T}$ cell line ($p=0.5061$). This increase in protein production dropped after 24 hours. However, there was no significant difference in ERCC1 expression upon cisplatin challenge, although $UV20^{ERCC1\_C}$ cell line did show slightly more ERCC1 expression compared to that of the $UV20^{ERCC1\_T}$ cell line.



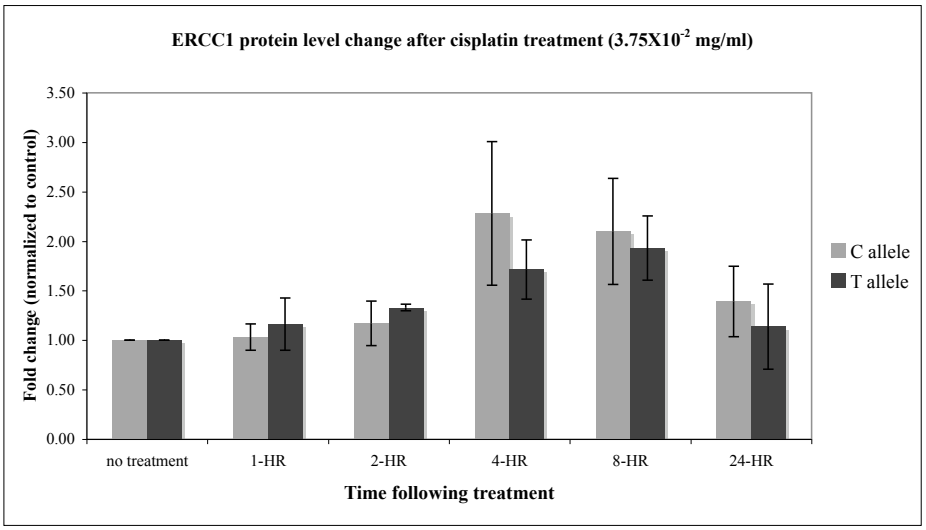


Figure 4-3: ERCC1 expression levels in UV20^{ERCC1_C} and UV20^{ERCC1_T} cell lines following cisplatin treatment did not show difference.

Upper panel: Western blots for ERCC1. Lower panel: Quantification of ERCC1 expression change following cisplatin treatment. Data are mean ± SD obtained from three independent experiments. The expression levels of ERCC1 in the control cells pre-treatment were arbitrarily assigned 1. The change in ERCC1 level is expressed as the fold change compared to untreated cells. Data were normalized for equal loading.

4.4.4. Cellular sensitivity to platinum compounds did not exhibit difference in UV20^{ERCC1_C} and UV20^{ERCC1_T} cell lines

Lastly, cell viability assays were performed to determine if the cells transfected with different alleles of ERCC1 N118N would respond to platinum drugs differently. Both the UV20^{ERCC1_C} and UV20^{ERCC1_T} cells showed significantly increased viability upon cisplatin treatment compared to the parental cell line and vector transfected control cell line, as confirmed by CCK-8 and CellTiter-Blue assays independently (**Figure 4-4**, upper panel). The survival proportion of UV20^{ERCC1_C}

and UV20^{ERCC1_T} cells were not affected by cisplatin at the concentration of 3.2×10^{-4} mg/ml. However, only 32% of the parental cell line and 47% of cells transfected with the empty vector survived after treated by 3.2×10^{-4} mg/ml of cisplatin (P<0.0001). When the cisplatin concentration was increased to 0.0016 mg/ml, 13% and 27% cells survived for the parental and control cell lines while 87% of UV20^{ERCC1_C} cells and 88% of UV20^{ERCC1_T} cells survived, respectively (P<0.0001). Nonetheless, the UV20^{ERCC1_C} and UV20^{ERCC1_T} cell lines did not show differences in terms of cisplatin sensitivity. Similar results were obtained for carboplatin and oxaliplatin (**Figure 4-4**, lower panel).

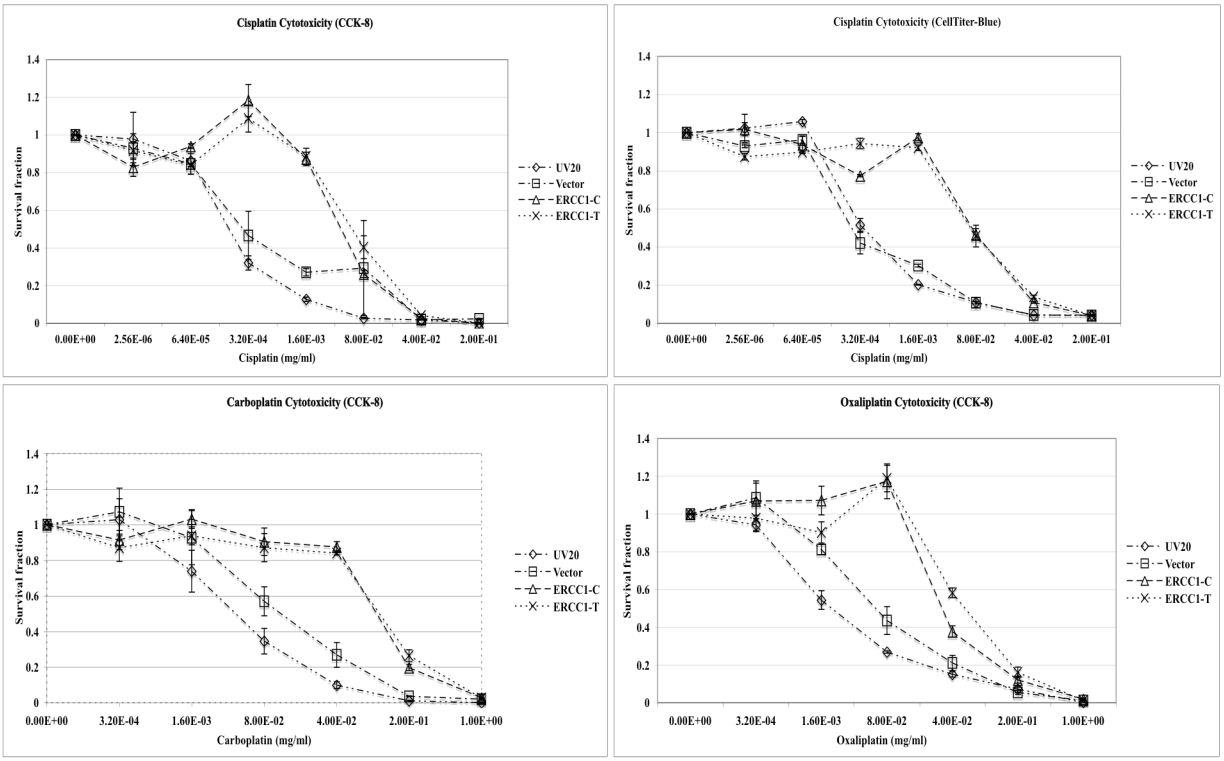


Figure 4-4: Cell viability did not change in the $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines.

The parental cell line UV20, empty vector transfected control cell line, and ERCC1 cDNA with either allele of 500 C>T transfected cell line $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ were treated with cisplatin (0, 2.56×10^{-6}, 6.40×10^{-5}, 3.2×10^{-4}, 1.6×10^{-3}, 8×10^{-2}, 4×10^{-2}, 0.2 μg/μl), carboplatin (0, 3.2×10^{-4}, 1.6×10^{-3}, 8×10^{-2}, 4×10^{-2}, 0.2 and 1.00 μg/μl) and oxaliplatin (0, 3.2×10^{-4}, 1.6×10^{-3}, 8×10^{-2}, 4×10^{-2}, 0.2 and 1.00 μg/μl). The cell cytotoxicity was tested by CCK-8 and CellTiter-Blue assayes. Only the results from CCK-8 assay for carboplatin and oxaliplatin are shown. Data are mean ± SD obtained from three independent experiments with triplicates in each experiment.

4.5. Discussion

Personalized medicine is the use of both a patient's genotypic and phenotypic data to choose a treatment or therapy that will best help the patient by maximizing benefit and minimizing harm (227). Therefore, it is important to understand how the inter-individual variations in the DNA sequence of specific genes affect drug responses. By introducing the cDNA clone of ERCC1 containing either C or T allele of N118N into an ERCC1 deficient cell line UV20, we examined the functional consequences of the genetic polymorphisms on ERCC1 expression and function. We found that this polymorphism did not contribute to altered ERCC1 expression upon cisplatin treatment or cellular sensitivity to platinum-containing drugs in vitro.

The ERCC1 N118N silent mutation was first described by genotyping a series of human cell lines and ovarian cancer tumor tissue specimens, and it was suggested that the conversion of the common codon AAC to an infrequently used codon AAT could affect protein translation rate and response to cisplatin (87). A subsequent study

suggested that this polymorphism could affect the DNA repair capacity in human ovarian cancer cell lines through a reduction in peak ERCC1 mRNA production and a consequent reduction in the translation of ERCC1 mRNA into protein (164). A growing body of literature shows that ERCC1 expression level correlates with response to platinum containing reagents (81, 228-231) while the results of genetic association studies are not always consistent (**Table 1-1**). The codon usage frequency for the SNP at position 500 with AAC changed to AAT (both encode Asn) changes moderately from 21.30 to 16.43 per thousand. This codon usage preference is conserved among species.

To test whether the synonymous codons of AAC and AAT in exon 4 of ERCC1 affects gene expression and function in response to platinum drugs, we established UV20 cell lines constitutively expressing ERCC1 with C or T allele at the 500 position in the mRNA, named $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$. Our results showed that the two cell lines had close basal ERCC1 transcription levels and performed similarly in ERCC1 transcription upon cisplatin induction. The $UV20^{ERCC1\_C}$ cells showed slightly faster production of ERCC1 protein than the $UV20^{ERCC1\_T}$ cells that corresponds to the moderate codon usage bias toward the AAC codon. However, it did not reach statistical significance. Nonetheless, the $UV20^{ERCC1\_C}$ and $UV20^{ERCC1\_T}$ cell lines exhibited the same level of sensitivity to platinum-containing drugs, including cisplatin, carboplatin and oxaliplatin.

The amino acid asparagine at this position is conserved among species, however, the wild type allele at the third position of this codon is not conserved, according to the analysis performed using the UCSC Genome Browser

(http://genome.ucsc.edu). Some species use AAC, such as rhesus, dog and opossum, etc., while others use AAT, such as human, mouse, elephant and zebrafish, etc. In addition, this SNP ERCC1 N118N (rs11615) is not under selective pressure in European, African or Chinese decent as indicated by low positive Tajima's D values (232, 233). These results confirm that this SNP has little to no phenotypic effects although there is a clear preference for asparagine at the 118th amino acid. Furthermore, the LD (linkage disequilibrium) plot shows that this SNP is linked in a haplotype block of 18 KB within ERCC1 and the adjacent genomic region in European population. But this is not true for African or Asian populations (234). Although another NER gene, ERCC2 (also called XPD), is located in the same chromosome, the haplotype block does not extend to ERCC2 in any of the three populations, suggesting linkage disequilibrium with causative SNPs within ERCC1 might account for previous clinical associations with ERCC1 N118N. Mutations in ERCC1 that cause protein sequence change are not common and the only reported case is an infant possessing two point mutations that has severe developmental abnormality and does not thrive (218). Therefore, instead of focusing on the ERCC1 N118N polymorphism, future genetic association studies should be expanded to include other polymorphisms linked to ERCC1 N118N, such as those in the regulatory regions of this gene.

5. ERCC1 polymorphisms are associated with melanoma

5.1. Abstract

Reduced DNA repair capacity has been proposed as a risk factor for skin melanoma. The incidence of skin melanoma is 20 times higher in European Americans (EAs) than in African Americans (AAs). And in fact, increased frequencies of DNA repair gene polymorphisms have also been found in EAs compared to AAs. Here we evaluated 6 genetic polymorphisms in 4 DNA excision repair genes in relation to skin melanoma risk. 165 patients with malignant skin melanoma were screened, as well as 156 EA and 164 AA healthy controls. The polymorphism N118N in ERCC1 that showed a significant association with melanoma ($p=0.0047$) was further investigated and our data revealed that it was linked to two other polymorphisms in a nearby intron; and the haplotypes of these ERCC1 polymorphisms are associated with skin melanoma by significant margins. Carrying the variant haplotype allele or not carrying the wild type haplotype allele significantly increased risk of melanoma ($p=0.0034$ and $p=0.0011$, respectively). The variant haplotype allele of ERCC1 confers melanoma risk in an additive and recessive way. AAs have significantly fewer melanoma risky alleles ($p<0.0001$). EAs have a higher genetic risk of getting skin melanoma than AAs based on our study of ERCC1 polymorphisms. However, when the fusion genes containing either the wild type or the variant haplotype allele of ERCC1 were transiently expressed in a human skin

melanoma cell line, they did not exhibit any differences in the transcriptional levels, suggesting that these alleles may not be causal.

5.2. Introduction

5.2.1. NER deficiency and skin cancer

Cancer development requires the accumulation of numerous genetic alterations that are usually believed to occur through the presence of compromised DNA repair machinery. The NER pathway is the most versatile and best-studied DNA repair pathway in humans. NER can repair a variety of bulky DNA damage, including UV-light induced DNA photoproducts. It is believed that at least 11 NER genes are involved in more than 8 clinical syndromes (235). However, the relationship between the genetic defects in the NER genes and the clinical diseases is complex. NER deficiencies result in profound photosensitivity and at least three autosomal recessive disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). NER-defective XP can be classified into 7 complementation groups, XPA to XPG. XP is known to be associated with mutations in the XP genes, ERCC1 and POLH (Polymerase eta), while mutations in XPA and XPC account for approximately 50% of XP (235). XP is characterized by serious UV sensitivity, a strong disposition to skin cancers and, in severe cases, neurodegeneration. TC-NER is defective in Cockayne syndrome, which is characterized by UV sensitivity, short stature, neurodisability, dysmorphic features, etc., (236). Mutations in XPB, XPD and TTDA genes have been found in TTD,

which is also characterized by UV sensitivity, in addition to brittle hair and scaling of skin (237, 238). However, TTD and CS patients have normal skin cancer risk (239). In addition, COFS syndrome is a recessively inherited rapidly progressive neurologic disorder which shares clinical similarities with CS. Deficiencies in NER genes have also been proposed as causes for COFS (240).

The estimated incidence of skin cancer is elevated more than 1000-fold in individuals with XP. Cells from XP patients exhibit inability to repair UV damage to their DNA (141). This results in the accumulation of mutations in the DNA of skin cells after exposure to sunlight, which eventually leads to enhanced mutational burden in cells and neoplastic transformation.

5.2.2. ERCC1 and skin melanoma

Basal cell carcinoma and squamous cell carcinoma are the two most common forms of skin cancer and are together referred to as the nonmelanoma skin cancer, whereas melanoma is the most lethal form of skin cancer, and is characterized by therapeutic resistance and predisposition for metastasis. Substantial epidemiological evidence suggests that hair-color and freckling, as indirect measurements of the skin's reaction to sun, family history, age and the number of raised nevi are the risk factors of malignant melanoma. Overexposure to sun is generally thought to increase the risk of melanoma. Solar UV light, the major environmental carcinogen, is absorbed in DNA and results in the formation of pyrimidine dimers. Nucleotide excision repair is the mammalian DNA repair mechanism that removes these UV-caused DNA lesions

(119). Inefficient repair of DNA damage can result in cancerous transformation of the cells, as aforementioned.

Cumulative evidence has suggested the importance of DNA excision repair mechanism in the etiology of melanoma. Previous case-control studies indicated that variations in NER genes, including ERCC1, XPF and XPD, are associated with an increased risk of melanoma (241-243). Using host cell reactivation assay to evaluate the repair of UV-induced DNA damages, Wei et al. showed that cells from patients with melanoma had significantly lower DNA repair capacity than control cells (244). Another study has shown that mice with skin-specific ERCC1 deficiency are hypersensitive to UV irradiation (245).

The incidence of melanoma is 20 times higher in European Americans than in African Americans (http://www.cdc.gov/cancer/npcr/uscs/pdf/2002_USCS.pdf). The variant genotypes in DNA repair genes, including ERCC1 and XPD in the NER pathway and XRCC1 and PARP1 in the BER pathway, were found to be significantly more prevalent in European Americans, compared to African Americans (**Table 2-3**). This is thought to be associated with a reduction in DNA excision repair capability and thus a possibly better response to DNA damaging chemotherapy in European American patients (204). Therefore, we hypothesize that the polymorphisms in DNA excision repair genes that have different allelic distributions between African and European populations may be responsible for the increased risk to melanoma. In this study, we investigated the association between the DNA excision repair genes and melanoma. Furthermore, we focused on 4 polymorphisms in ERCC1 that have different distributions in European and African populations, according to the NCBI

SNP database, in both melanoma cases and healthy controls. The haplotypes identified by the association analysis were then fused with reporter genes and transfected to a skin melanoma cell line, and their effects on transcription were examined.

5.3. Materials and Methods

5.3.1. Genotyping methods

320 whole blood samples from healthy male volunteers were the same as those used in the previous study in chapter 2.

165 patients with malignant melanoma were analyzed. All patients were EAs and were enrolled in an institutional review board–approved clinical trial within the intramural program of the National Cancer Institute, and were arbitrarily assigned a number in our database to protect confidentiality. Informed consent was obtained from all subjects before trial participation.

The genotyping methods are described in details in Chapter 2. The genotyping method for ERCC1 N118N is the same as used in Chapter 2 and 3. The PCR primers and conditions used for ERCC1 rs3212948, rs3212950 and rs3212929 are listed in **Table 5-1**. The forward primers are used as the sequencing primers for each polymorphism.

Table 5-1**:** Primers and PCR conditions for ERCC1 polymorphisms.

SNPs	Primers	Size (bp)	Annealing Temp (°C)
rs3212948 and rs3212950	FW 5'- AGG AGA GAC GCC CAA CCA GG-3' RE 5'- TGG CAC CAG GCC TTT CCT AAA G-3'	576	66 °C
rs3212929 T>G	FW 5'- TCA GAG AGC TGC AAG TTA GAA CAG TG-3' RE 5'- TTG ACT TGG CTT CAG TTT CCT C -3'	381	64 °C

5.3.2. Statistical analysis

Haploview (234) was used to calculate allele frequencies for the investigated ERCC1 polymorphisms from the data. Haploview software was also used to perform linkage disequilibrium assessment and to define the haplotype blocks by the incorporated confidence interval method (246). The standard chi-squared statistics was also used for association test and Hardy-Weinberg Equilibrium test.

5.3.3. Cell culture

SK-Mel-5 cell line was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in a humidified incubator at 37°C equilibrated with 5% CO_2 and 95% air in RPMI-1640 medium containing 10% fetal bovine serum and 1% Penicillin-Streptomycin.

5.3.4. Plasmid constructs and transfection

The gene fragments containing ERCC1 intron 3 and exon 4 regions were amplified from patients with melanoma that have either homozygous wild type or

homozygous variant haplotypes as defined in the association analysis. The PCR products were cloned in-frame between the EcoRI and KpnI sites of the pCMV-HA and pCMV-Myc vector set. The vector pair was co-transfected into SK-Mel-5 cells using Lipofectamine™ 2000 (Invitrogen, CA) when cells were 70 % confluent per manufacturer's instructions.

5.3.5. RNA isolation and RT–PCR

Expression of HA or Myc-tagged ERCC1 fragments were measured by quantitative reverse transcription-PCR (RT-PCR) using the Stratagene Mx3005P™ Real-Time PCR System (La Jolla, CA). Briefly, cells were lysed and the total RNA was collected 48 hours after transfection using the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA extracted was reverse-transcribed using RT² First Strand Kits (SABiosciences, Frederick, MD). Each sample was analyzed in duplicate and the results are an average of four analyses. Analysis of mRNA expression was conducted using primers specific to the tag and the ERCC1 sequence, and normalized to a human housekeeping gene GAPDH by RT² qPCR Primer Assay (SABiosciences, Frederick, MD).

5.4. Results

5.4.1. DNA excision repair genes and melanoma

First we genotyped the patients with melanoma for the DNA excision repair gene polymorphisms as investigated in the previous study in Chapter 2, and compared the allele frequencies with those in the EA healthy controls. Among all of these

polymorphisms, only ERCC1 N118N (rs11615) was found to be associated with melanoma (**Table 5-2**). Therefore, we focused our future studies on polymorphisms within the ERCC1 gene.

Table 5-2: Allele distribution of DNA excision repair genes in patients with melanoma and EA controls.

SNP	ALLELE	EA controls (Counts (freq.))	Patients with melanoma (Counts (freq.))	OD (95% CI)	P
ERCC1 N118N (500C>T)	C	99 (0.46)	104 (0.33)	1.692 (1.185 to 2.417)	0.0047
	T	117 (0.54)	208 (0.67)		
	TOTAL	216	312		
ERCC1 C8092A	C	213 (0.73)	73 (0.79)	0.701751 (0.398 to 1.237)	0.2193
	A	79 (0.27)	19 (0.21)		
	TOTAL	292	92		
XPD K751Q (2282A>C)	A	154 (0.65)	36 (0.67)	0.939024 (0.502 to 1.756)	0.8438
	C	82 (0.35)	18 (0.33)		
	TOTAL	236	54		
XRCC1 R399Q (1301G>A)	G	145 (0.68)	60 (0.64)	1.22637 (0.736 to 2.044)	0.4332
	A	67 (0.32)	34 (0.36)		
	TOTAL	212	94		
XRCC1 R194W (685C>T)	C	257 (0.93)	86 (0.96)	0.629131 (0.2084 to 1.901)	0.4076
	T	19 (0.07)	4 (0.04)		
	TOTAL	276	90		
PARP1 V762A (2446T>C)	T	192 (0.81)	78 (0.85)	0.749164 (0.390 to 1.440)	0.3854
	C	46 (0.19)	14 (0.15)		
	TOTAL	238	92		

5.4.2. Allele distribution of ERCC1 polymorphisms

In this study, the DNA samples from 165 European American patients with skin melanoma, 156 healthy individuals of European American descent, and 164 healthy individuals of African American descent were genotyped for 4 SNPs in the DNA excision repair gene ERCC1, including rs11615 (N118N, 500C>T) in exon 4,

rs3212950 (C>G) and rs3212948 (G>C) in intron 3, and rs3212929 (T>G) in the 5' utr. All the SNPs analyzed were in Hardy-Weinberg Equilibrium, except rs3212929, which showed Hardy-Weinberg disequilibrium in AA population (p=0.0021) with a lower observed heterozygosity (0.33) than expected (0.483).

The allele frequencies in patients with melanoma and EA and AA healthy individuals are summarized below. Patients with melanoma carry more variant alleles T, G, C, G of rs11615, rs3212950, rs3212948 and rs3212929 than controls (**Table 5-3**), and African Americans carry fewer melanoma risk alleles than European Americans by a strongly significant margin (p<0.0001, **Table 5-4**).

Table 5-3: Distribution of ERCC1 alleles in patients with melanoma and European American (EA) healthy controls.

Allele Name	Assoc Allele	Patients with melanoma (Counts (freq.))	EA (Counts (freq.))	OR (95%CI)	P value
rs11615	T	196(0.681)	105(0.553)	1.73 (1.18 - 2.52)	0.0051
	C	92(0.319)	85(0.447)		
rs3212950	G	201(0.698)	107(0.563)	1.79 (1.22 - 2.62)	0.0033
	C	87(0.302)	83(0.437)		
rs3212948	C	202(0.701)	104(0.547)	1.94 (1.33 - 2.84)	0.0007
	G	86(0.299)	86(0.453)		
rs3212929	G	287(0.997)	183(0.963)	10.98 (1.34 - 90.01)	0.0077
	T	1(0.003)	7(0.037)		

Table 5-4: Distribution of ERCC1 alleles in African American (AA) and European American (EA) populations.

Allele Name	Assoc Allele	AA (Counts (freq.))	EA (Counts (freq.))	OR (95%CI)	P value
rs11615	C	180(0.874)	85(0.447)	8.55 (5.18 - 14.11)	P<0.0001
	T	26(0.126)	105(0.553)		
rs3212950	C	168(0.816)	83(0.437)	5.70 (3.62 - 8.98)	P<0.0001
	G	38(0.184)	107(0.563)		
rs3212948	G	168(0.816)	86(0.453)	5.35 (3.40 - 8.41)	P<0.0001
	C	38(0.184)	104(0.547)		
rs3212929	T	84(0.408)	7(0.037)	18.00 (8.05 - 40.24)	P<0.0001
	G	122(0.592)	183(0.963)		

5.4.3. Haplotype and diplotype reconstruction and association analysis

To determine whether specific ERCC1 alleles might be associated with melanoma, and therefore overrepresented in the patient group, haplotypes were derived using Haploview. The pairwise r^2 values in patients with melanoma, EA and AA healthy volunteers are shown in **Table 5-5**. Three SNPs, including rs11615, rs3212950 and rs3212948 were defined in one haplotype block. Three common haplotypes were identified: homozygous variant TGC, homozygous wild type CCG and heterozygous CGC. The diplotypes were estimated based on the haplotype frequencies, as summarized in **Table 5-6**.

Table 5-5: Pairwise r^2 in patients with melanoma, EA and AA healthy volunteers.

L1	L2	r^2		
		Patients with melanoma	EA controls	AA controls
rs11615	rs3212950	0.86	0.916	0.515
rs11615	rs3212948	0.876	0.895	0.515
rs11615	rs3212929	0.007	0.021	0.099
rs3212950	rs3212948	0.984	0.938	1
rs3212950	rs3212929	0.008	0.022	0.083
rs3212948	rs3212929	0.008	0.02	0.083

Table 5-6: Haplotypes (upper) and diplotype reconstruction (lower).

ERCC1 haplotypes	rs11615 (C>T)	rs3212950 (C>G)	rs3212948 (G>C)	Haplotype Counts (freq)	
				Patients with melanoma	EA controls
TGC	T	G	C	194 (0.674)	102 (0.536)
CCG	C	C	G	85 (0.295)	82 (0.431)
CGC	C	G	C	7 (0.024)	2 (0.012)

	Patients with melanoma		EA controls	
DIPLOTYPE	Counts	Freq.	Counts	Freq.
TGC-TGC	69	0.486	27	0.294
TGC-CCG	50	0.352	45	0.489
CCG-CCG	16	0.113	18	0.196
TGC-CGC	5	0.035	1	0.011
CCG-CGC	2	0.014	1	0.011

By comparing the haplotype allele frequencies, we observed that patients with melanoma carried the variant haplotype allele TGC at a much higher frequency than controls (68% vs. 54%, p=0.0034), and also carried the reference haplotype allele

CCG at a significantly lower frequency than controls (30% vs. 45%, p=0.0011), shown in **Table 5-7**. The comparison between AAs and EAs showed that the AAs carried TGC at a significantly lower frequency than EAs (12% vs. 54%, p<0.0001), and carried the CCG haplotype at a much higher frequency than EAs (83% vs. 45%, p<0.0001). The individuals with missing genotypes or rare haplotypes were excluded in further analysis.

Table 5-7: Association analysis of ERCC1 haplotype allele frequencies in patients with melanoma and EA healthy controls.

Haplotype Alleles	Patients with melanoma (Counts (freq.))	EA controls (Counts (freq.))	OR (95%CI)	P Value
Carries TGC	193 (0.68)	100 (0.54)	1.782 (1.215 - 2.612)	0.0034
Does not carry TGC	91 (0.32)	84 (0.46)		
Does not carry CCG	200 (0.70)	102 (0.55)	1.914 (1.300 - 2.818)	0.0011
Carries CCG	84 (0.30)	82 (0.45)		
Carries CGC	7 (0.02)	2 (0.01)	2.3 (0.4723 - 11.20)	0.493
Does not carry CGC	277 (0.98)	182 (0.99)		

Diplotype analysis showed that 52% of patients with melanoma had a diplotype without the wild type allele CCG, while only 30% of healthy controls had a diplotype without the wild type allele (OR=2.487, p=0.0024). However, whether the individuals had a diplotype with or without the variant allele TGC did not show association toward melanoma (p=0.2828). Data are shown in **Table 5-8**.

Table 5-8: Diplotype association analysis.

DIPLOTYPE	Patients with melanoma (Counts (freq.))	EA controls (Counts (freq.))	OR (95%CI)	P Value (adjusted for MCs)
Does not carry CCG	74 (0.52)	28 (0.30)	2.487 (1.431 - 4.324)	0.0024
Carries CCG	68 (0.48)	64 (0.70)		
Carries TGC	124 (0.87)	73 (0.79)	1.793 (0.8844 - 3.635)	0.2828
Does not carry TGC	18 (0.13)	19 (0.21)		

To examine which genotype has a greater effect on melanoma risk, not carrying the wild type CCG allele or carrying the variant TGC allele, we then compared the frequencies of individuals having two copies CCG, TGC-CCG, and two copies TGC in the patient groups and the control group. 11% of patients with melanoma had the homozygous wild type diplotype CCG-CCG, while its frequency is doubled (20%) in the healthy controls. The heterozygous diplotype TGC-CCG did not show a significant association with melanoma risk, however, carrying two copies of the variant TGC allele significantly increased the individual's risk of getting melanoma (OR=2.875, p=0.0119, **Table 5-9**).

Table 5-9: Association of variant allele with risk of melanoma.

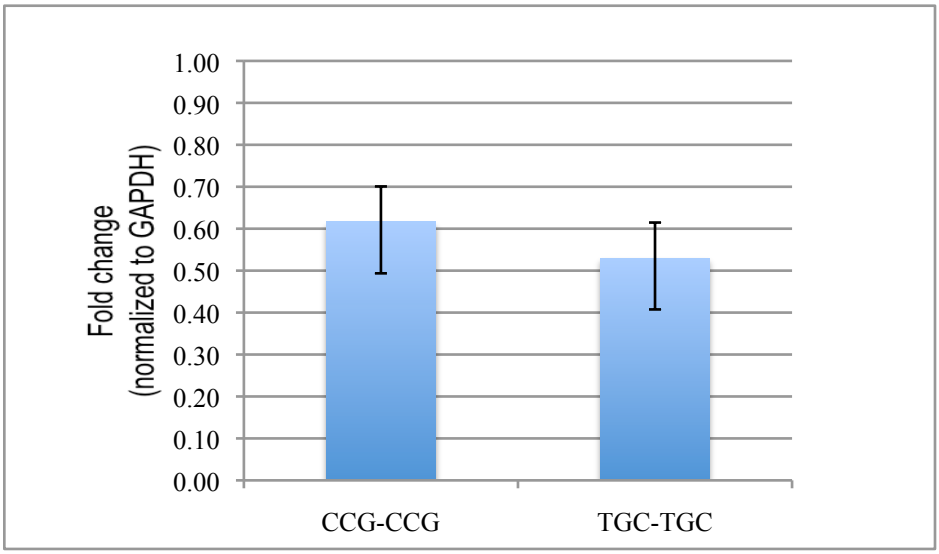
DIPLOTYPE	Patients with melanoma (Counts (freq.))	EA controls (Counts (freq.))	OR (95%CI)	P Value
CCG-CCG	16 (0.11)	18 (0.20)	1.0 (N/A)	N/A
TGC-CCG	50 (0.35)	45 (0.49)	1.25 (0.5702 - 2.740)	0.6899
TGC-TGC	69 (0.49)	27 (0.29)	2.875 (1.282 - 6.445)	0.0119

The same comparison was made between the EA and the AA populations, and the results showed that the AA individuals carried the TGC-TGC diplotype at a frequency as low as 1%, while 66% of them had the homozygous wild type CCG-CCG ($p<0.001$).

5.4.4. Effect of ERCC1 haplotypes on transcripts abundance

In order to determine if the haplotypes identified in the association study are causal, the homozygous wild type and variant haplotypes CCG-CCG and TGC-TGC of ERCC1 intron 3 and exon 4 were cloned in-frame between the EcoRI and KpnI sites of the pCMV-HA and pCMV-Myc vector set. The plasmids were transiently transfected into a skin melanoma cell line, SK-Mel-5, and the relative transcripts levels of the fusion genes were measured the quantitative RT-PCR. The level of transcripts of the wild type fusion gene was 1.16 fold higher than the variant genotype ($p=0.6217$), shown in **Figure 5-1**.

Figure 5-1: Relative abundance of transcripts of the two fusion genes with wild type or variant genotypes.



5.5. Discussion

Melanoma is the most dangerous type of skin cancer. According to American Academy of Dermatology, it is the leading cause of death from skin cancer, accounting for 75% of all skin cancer deaths, and one American dies from melanoma almost every hour. Overexposure to the UV component of sunlight is an established risk factor for developing skin cancer. The incidence of skin melanoma is surprisingly higher in the European American population than in African Americans. Pigmentation is generally thought to be a protective factor for African Americans. However, a recent study found that the variant polymorphisms of DNA excision repair genes are significantly more prevalent in European Americans, which may indicate a relatively lower DNA excision repair capacity in the respective repair pathways, NER and BER (204). DNA repair mechanisms play fundamental roles in

maintaining genomic integrity. Reduction in DNA repair capacity is thought to be responsible for increased risk for several types of cancer (71). Despite the high penetrance of NER deficiency in XP patients, little is known about the correlations of the common genetic polymorphisms in DNA excision repair genes and risk of skin melanoma. Many DNA repair gene polymorphisms have been investigated in terms of genetic predisposition to skin melanoma, and positive associations were found for XPF (243) and XPD (241, 242, 249). In addition, the wild type ERCC1 polymorphism was found to be associated with melanoma in patients under 50 (243).

In this study, we investigated genetic polymorphisms in DNA excision repair genes in relation to skin melanoma. Only the ERCC1 N118N polymorphism showed significant association with melanoma among the 6 DNA excision repair gene polymorphisms. ERCC1 is a gene essential to life and plays multiple roles in addition to its function in the NER pathway, such as in recombination repair and some unknown processes (218). When extended to more SNPs in ERCC1 that have different allelic distributions in African and European populations, a haplotype was identified and showed a strong correlation with risk of skin melanoma. Two SNPs in intron 3 are in linkage with the N118N polymorphism in exon 4. The variant haplotype has a significantly higher frequency in patients with melanoma, while the variant haplotype has a protective effect toward the risk of skin melanoma. The homozygous variant diplotype of ERCC1 associates with increased risk of skin melanoma but not the heterozygous diplotype, suggesting that the ERCC1 polymorphisms behave additively and recessively. African Americans carry significantly fewer melanoma risky alleles. However, the fusion genes with the wild

type or variant diplotype of ERCC1 did not show differences in transcriptional levels when transiently expressed in a skin melanoma cell line.

Based on our study, European Americans have an increased genetic risk for skin melanoma than African Americans. The melanoma risky ERCC1 alleles identified in this study may not be causal. This association could be due to the relatively high frequencies of the investigated polymorphisms in population or their linkage with causal alleles. It should be kept in mind that haplotype analysis may increase power to detect rare casual alleles but may decrease power to detect common causal alleles (250). The haplotype block extends to an 18 KB region including part of ERCC1 and the neighboring genome. The linkage patterns of ERCC1 and the surrounding genomic regions are different in African and Asian populations. Further work on fine mapping and on functional characterization of the polymorphisms in ERCC1 and linked regions is required.

In addition, two recent genome-wide association studies identified several melanoma risk chromosomal loci, including 16q24 encompassing melanocortin-1 receptor, 11q14-q21 encompassing tyrosinase, which are associated with well-recognized melanoma risk factors such as pigmentation, freckling and cutaneous sun sensitivity, 9p21 (247) and 20q11 (248), which may contain common polymorphisms associated with melanoma. These studies were both done in melanoma high-risk populations, i.e., Europeans and Australians. These melanoma risk regions may have different implications in melanoma etiology.

6. Summaries and future directions

The human DNA excision repair mechanisms are complex and interweaving, and act as double-edged swords in cancer and cancer therapy. The goal of our study was directed towards increasing our understanding of the translational aspects of the DNA excision repair genes. Cancer therapies that cause cytotoxicity by damaging DNA, such as platinum-based drugs and radiation therapy, are useful in clinical oncology. However, these therapeutic regimens also have severe side effects and can be carcinogenic as well. Therefore, being able to assess who would benefit from such therapies would be useful and would allow the avoidance of unnecessary toxicity to those who would be predicted to have no benefit.

Pharmacogenetics is a fast-evolving field in oncology. Ideally, it will allow physicians to use a patient's genotypic and phenotypic data to choose a treatment that will best help the patient by maximizing benefit and minimizing harm (227). To achieve this goal, a thorough and extensive understanding of the genetic composition and the biological consequences is necessary. In this study, we assessed the genetic polymorphisms in the DNA excision repair pathways in the context of cancer and cancer therapies causing DNA damaging.

In addition to inter-individual variation in drug response, there are also observed inter-population differences in the outcome of certain chemotherapies that have been widely used, such as platinum-based chemotherapies. In general, the mortality rate is higher in African American patients in malignancies where these therapeutic regimens are widely used, as summaried in chapter 2. Of course this can

be due to a variety of reasons, such as differences in patient access to medical care and/or medical care delivery. It is also likely that these differences are the results of patients' response to the same therapies. In our investigation of the genetic polymorphisms of DNA excision repair genes involved in the molecular mechanisms of DNA-damaging chemotherapies, we found that a population difference in the genetic makeup generally exists in these genes. The genetic polymorphisms that we assessed in this study include ERCC1 N118N (500C>T), ERCC1 C8092A, XPD K751Q (2282A>C) from the NER pathway, and XRCC1 R399Q (1301G>A), XRCC1 R194W (685C>T) and PARP1 V762A (2446T>C) from the BER pathway. Significantly higher frequencies of the genetic variants of the assessed genes were found to occur in European Americans, as compared to African Americans. This may imply a moderate reduction in the repair capabilities of the respective pathways in European Americans, thereby leading to a better outcome once DNA-damaging chemotherapies are used this population.

DNA damage and repair is far broader and deeper than we used to think, especially in our understanding and successful treatment of cancer. The most widely used class of anticancer compounds is the platinum-containing agents. Radiation is also critically important in treating various early stage diseases. Although both therapeutic methods are useful, they are also toxic. Therefore, being able to stratify patients by genetic or molecular markers for efficacy and/or toxicity will be extremely helpful. In order to understand if these genetic polymorphisms also contribute to the outcome of radiation therapy, we investigated the aforementioned polymorphisms in patients with castrate-resistant prostate cancer. Our data is

summarized in chapter 3. Firstly, we compared the distributions of these DNA excision repair gene polymorphisms between patients with prostate cancer and the healthy controls that were assessed in chapter 2. However, we found that the genetic polymorphisms in DNA excision repair genes are not associated with risk of prostate cancer. Secondly, we assessed whether the above-mentioned DNA excision repair gene polymorphisms were correlated to the outcome of radiation therapy in patients with prostate cancer. Our results indicated that none of these polymorphisms showed an association toward the overall survival of radiation therapy in prostate cancer.

As reviewed in previous chapters, genetic association studies on the DNA excision repair genes and cancer and DNA-damaging cancer therapies have generated confusing results. This is partly due to the limitations of such studies including limited sample sizes, mixed cancer types and treatment regimens, accuracy and consistency of diagnostic criteria, as well as the tendency of both the investigators and the journals to report positive results. Consequently, the literature has become weighted toward unconfirmed associations. This is especially true for the ERCC1 N118N polymorphism, one of the most important genetic markers for platinum-based chemotherapies, as summarized in chapter 1. Therefore, in the following study presented in chapter 4, we investigated the biological consequences of this synonymous mutation N118N in ERCC1 in a well-controlled in vitro system. By introducing the human ERCC1 cDNA clones with either the wild type or the variant genotype of the N118N polymorphism into an ERCC1 deficient CHO cell line, we were able to test the two alleles' effects on ERCC1 gene transcription, translation and cellular sensitivity to platinum drugs. However, the cells that differ at the

polymorphism did not differ in the biological consequences that we assayed. Therefore, we believe that any observed association between this polymorphism and the outcome of platinum-based chemotherapies is most likely due to its linkage with other polymorphisms in ERCC1. This hypothesis led to our next investigation, which was aimed at looking for the possible causal genetic polymorphisms for ERCC1 function.

DNA damage and repair is also important in the etiology of skin melanoma, which is the most lethal form of skin cancer. DNA damage induced by UV irradiation is mainly removed through DNA excision repair mechanisms. The incidence of melanoma is 20 times higher in European Americans than in African Americans. Skin tone is usually thought to be a protective factor for African Americans. However, we found that the genetic variants of DNA excision repair gene rarely occur in this population. Rather, the frequencies of these variant DNA excision repair gene polymorphisms are significantly higher in European Americans, as shown in chapter 2. Hence, the causal genetic polymorphisms for reduced DNA excision repair functions might also show an association toward skin melanoma. Subsequently, we found that haplotypes in ERCC1 that are associated with skin melanoma, as reported in chapter 5. The wild type alleles of ERCC1 polymorphisms had protective effects toward skin melanoma; the variant alleles work in an additive and recessive fashion. In addition, European Americans have a higher genetic risk of getting melanoma than African Americans, based on the results of our study. However, the functional analysis did not reveal any differences in transcriptional levels of the fusion genes with either the wild type or variant haplotypes of ERCC1, indicating that the

identified haplotypes in ERCC1 might be associated with skin melanoma but not causal for ERCC1 functional changes.

Intrinsic or acquired resistance to DNA-damaging chemotherapy is widely observed, and the mechanisms underlying this have been extensively investigated but not fully defined. Varied levels of ERCC1 protein expression were found in the tumor specimens from patients with NSCLC, and were associated with the outcome of platinum-based adjuvant chemotherapy (81). However, it would be helpful to be able to predict ERCC1 protein expression levels without taking tissues samples from a patient before administering platinum-based chemotherapies. This causes a thorough understanding of the genetic polymorphisms of this gene. As discussed in chapters 4 and 5, the LD plot shows a haplotype block of 18 KB within ERCC1 and the adjacent genomic region in European. Therefore, the causal SNPs in ERCC1 may be located in the linked region, and are worth investigating. However, using the HapMap data may result in missing the uncommon polymorphisms since only 90 individuals were sequenced in each population by the HapMap project. Hence, sequencing and comparisons of the entire ERCC1 gene in individuals with a known response to platinum-based chemotherapies would greatly advance our knowledge about this gene in the context of platinum sensitivity. Additionally, the biological consequences of such polymorphisms should also be assessed before too many association studies are conducted. On top of that, genome wide association studies could also be performed, taking advantage of the high-throughput genotyping methods, to locate the possible genes and genomic regions that might be responsible for differences to platinum sensitivity.

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