DNA REPAIR GENES, XRCC3 AND RAD51, POLYMORPHISMS AND RISK OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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DNA REPAIR GENES, *XRCC3* AND *RAD51*, POLYMORPHISMS AND RISK OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

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In this study, the role of two DNA repair genes, X-ray repair cross complementing group 3 (*XRCC3*) Thr241Met and *Rad51* G135C polymorphisms were investigated in the risk of development of childhood ALL in Turkish population among 193 healthy controls and 184 ALL patients, by using PCR-RFLP technique. For *XRCC3* Thr241Met polymorphism, the frequencies of both heterozygous and homozygous mutant genotypes were found to be higher in the controls compared to ALL patients (OR: 0.59, p = 0.02; OR: 0.48, p = 0.02, respectively). In addition, either heterozygous (Thr/Met) or homozygous mutant (Met/Met) genotypes were significantly more common in the controls than the ALL patients (OR: 0.55, p =0.005). In case of *Rad51* G135C polymorphism, no significant associations have been found with the risk of childhood ALL. Combination of *XRCC3* heterozygote and *Rad51* heterozygote genotypes increased the protective effect for risk of childhood ALL. (OR=0.35 ; p

=0.02). Combination of homozygote mutant genotype of *XRCC3* with homozygote wild type genotype of *Rad51* gave a highly statistically proved protective effect for the development of disease (OR= 0.36; *p*= 0.004). To our knowledge, this is the first study showing the protective role of *XRCC3* Thr241Met polymorphism either alone or in combination with *Rad51* G135C variant on the risk of development of childhood ALL.

In addition, interactions of these polymorphisms with non-genetic risk factors were investigated. Only in terms of paternal exposure, the heterozygote (Thr/Met) genotype for *XRCC3* gene in children whose father exposed to cigarette smoke demonstrated a significant risk of 3.0 fold (*p*=0.05). Moreover, the frequency of *Rad51* 135C allele was determined for the first time in Turkish population. The frequency of the mutant allele was found to be very similar to that observed in other Caucasian populations.

Keywords: *XRCC3*, *Rad51*, genetic polymorphism, risk of childhood ALL,

ÖZ

DNA TAMİR GENLERİNİN POLİMORFİZMLERİ VE ÇOCUKLUK ÇAĞI AKUT LENFOBLASTİK LÖSEMİ RİSKİ

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Bu çalışmada, DNA tamir genlerinden olan X-ray repair cross complementing group 3 (*XRCC3*) geni Thr241Met ve *Rad51* geni G135C polimorfizmlerinin çocukluk dönemi akut lenfoblastik lösemi (ALL) hastalığı riskini modifiye edici rolleri 184 ALL hastası çocuk ve 193 sağlıklı yetişkinden oluşan Türk popülasyonunda PCR-RFLP tekniği kullanarak araştırılmıştır. *XRCC3* Thr241Met polimorfizmi için, hem heterozigot hem de homozigot mutant genotip frekansı ALL hastalarına kıyasla kontrollerde daha yüksek bulunmuştur (OR: 0.59, p = 0.02; OR: 0.48, p = 0.02, respectively). Ayrıca, heterozigot veya homozigot mutant genotip önemli bir şekilde kontrollerde ALL hastalarına kıyasla daha yaygın olmuştur (OR: 0.55, p =0.005). *Rad51* G135C polimorfizmi ile çocukluk çağı ALL riski arasında herhangi belirgin bir ilişki bulunamamıştır. *XRCC3* heterozigot genotipi ile *Rad51* heterozigot genotiplerinin kombinasyonu, çocukluk çağı

ALL riskinde koruyucu etkisini artırmıştır (OR=0.35; p =0.02). *XRCC3* homozigot genotipi ile *Rad51* homozigot genotiplerinin kombinasyonu, hastalığın gelişiminde istatistiksel bakımdan oldukça anlamlı bir koruyucu etki sağlamıştır (OR= 0.36; *p*= 0.004). Bilindiği kadarıyla, bu çalışma *XRCC3* Thr241Met polimorfizminin tek başına veya *Rad51* G135C polimorfizmi kombinasyonu ile birlikte çocukluk çağı ALL hastalığı oluşması riskinde koruyucu bir role sahip olduğunu gösteren ilk çalışmadır.

İlaveten, adı geçen genetik polimorfizmlerin diğer genetik olmayan risk faktörleriyle ilişkisi de incelenmiştir. Sadece, *XRCC3* heterozigot genotipine sahip olan çocukların babalarının sigaraya maruziyeti durumununun önemli bir risk faktörü oluşturduğu görülmüştür (OR= 3.0 fold, *p*=0.05). Ayrıca bu çalışma ile, ilk defa *Rad51* 135C alel frekansı Türk popülasyonu için bulunmuştur. Mutant alelin frekansı diğer Kafkas popülasyonlarına yakın bulunmuştur.

Anahtar Kelimeler: *XRCC3*, *Rad51*, genetic polimorfizm, çoçukluk çağı ALL riski.

To my family...

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χ^2	Chi-Square
ALL	Acute Lymphoblastic Leukemia
XRCC3	X-ray Cross Complementing Group 3
DSBR	Double Strand break Repair
HR	Homologous Recombination
DSBs	Double Strand Breaks
OR	Odds Ratio
COR	Case-only Odds Ratio
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
CYP	Cytochrome P450
BaP	Benzo (a) pyrene
SULTs	Sulfotransferases
TMPT	Thiopurine methyl-transferase
NAD	Nicotinamide adenine dinucleotide

CHAPTER 1

INTRODUCTION

During the lifespan of the cell, DNA is regularly damaged by endogenous and exogenous carcinogens. In order to protect genome stability, cells have evolved DNA repair mechanisms. When DNA is exposed to damage, cells activate several pathways such as cell cycle arrest, transcriptional and post-transcriptional activation of DNA damage response genes including DNA repair genes, and in some cases apoptosis. An inability to repair the damage properly due to genetic polymorphisms impairing the DNA repair capacity, can lead to genetic instability and potentially modulate individual's susceptibility to various cancers.

1.1 Genetic Polymorphism

The coexistence of multiple alleles at a locus is called genetic polymorphism (Lewin, 2004). An allele is usually defined as polymorphic if it is present at a frequency of more than 1% in the population. Polymorphism caused by the presence of more than one allele for the same gene which result in more than one phenotype in the organisms. The differences between mutations and polymorphisms are that polymorphisms generally do not cause any sickness or other problems affecting the reproductive efficiency so that their frequencies in a population are

relatively high compared to mutations(more than 1%), whereas a mutation causing a serious disease would affect the reproductive efficiency (Gonzalez, 1999). That is the reason why mutations are found at extremely low frequency (less than 1%) in populations. The other difference between mutation and polymorphism is that, mutations occur at random sites within the DNA, whereas polymorphims are found at spesific alleles.

1.1.1 Mechanism of Genetic Polymorphism

Genetic polymorphisms can be categorized into functional and nonfunctional polymorphism depending on their effect on protein profile that the gene is translated. A functional polymorphism is a change in the DNA sequence of a gene that results in different levels of expression of protein or enzyme, or in alteration of the activity, while a nonfunctional polymorphism results in neither of them (Gonzalez, 1999).

The functional polymorphisms can be either in the coding or in the noncoding regions of the gene. Variations in the coding region of a gene have the potential to alter enzyme activity or protein function by changing the primary sequence of the protein. The non-coding regions of a gene are comprised of introns –which are spliced of posttranscriptionally; and regulatory regions, which are not transcribed but regulate the level of expression of the protein. However, if the polymorphism occurs at the splice junction, it can affect the structure of the protein or enzyme. Genetic variability in these noncoding regions is associated with altered levels of protein rather than changes in the protein itself (McKinnon and Evans, 2000).

1.1.2 Types of Genetic Polymorphisms

Structurally, polymorphisms could occur as deletions/insertions (InDel), varying number of tandem repeats (VNTR), and single nucleotide substitutions (SNP).

Insertion and deletion polymorphism is the insertion or deletion of DNA sequences that are 1 to 1000 nucleotides long.

The name microsatellite (VNTR) is usually used when the length of the repeating unit is shorter than 10 bp, and the name minisatellite (VNTR) is used when the length of the repeating unit is between 10 to 100 bp. The high variability of minisatellites makes them useful for genomic mapping.

The most widely observed polymorphism in human genome is single nucleotide substitutions. (SNP)

1.1.3 Single Nucleotide Polymorphism (SNP)

SNP is the substitution of a single nucleotide with a frequency of more than 1% in at least one population (Evans and Johnson, 2001). SNPs are distributed throughout the human genome at an estimated overall frequency of one in every 1330 bases in the human genome (Lewin, 2004). In human, more than 130 genes have been identified which codes for proteins of the various DNA repair pathways, and within 80 genes over 400 SNPS characterized (Mohrenweiser *et al.*, 2003). Because of the importance of maintaining genomic integrity in order to prevent development of carcinogenesis, DNA repair genes have strong relevance in determining susceptibility to cancer (Cairns, 1982; Knudson, 1989, and Shields, 1981).

For that reason, SNPs in DNA repair genes are an important area of investigation for epidemiology of carcinogenesis.

1.2 Childhood Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is the most common form of leukemia in childhood. It is the uncontrolled growth of immature white blood cells (lymphocytes) in the blood. Domination of lymphoblasts or immature hematopoietic cell precursors with malignant cells which express various phenotypes and variable response to therapy is characteristics of ALL. Death from anemia, infection or bleeding is the possible outcome of the disease if not treated, in which outcome of the disease depends on the type of the leukemic clone and on progression of disease when recognition of pathological symptoms, diagnosis and treatment were performed. It accounts for about 30-50 new cases per million children (Bonet, 1995) and represents 25-30% of all childhood malignancies (Ross, 1994; Pui, 2000; and Greaves, 2000). "Within this population, acute lymphocytic leukemia (ALL) occurs approximately five times more frequently than acute myelogenous leukemia (AML) and accounts for approximately 78% of all childhood leukemia diagnoses" (Belson, 2007). According to the study of Kaatsch (2010) incidence rates of Childhood leukemia in Europe has shown an annual increase of 0.6% during the period of 1978-1997. Throughout the world each year 2000-2500 new cases of childhood ALL are diagnosed. According to LÖSEV webpage, in Turkey every year, approximately 1200-1500 children are diagnosed with ALL.

As a treatment, multiple chemotherapeutics has led to a significant improvement in disease treatment. The probability of survival has increased by 8% when comparing the 2000-2002 period to the 1995-1999 period, and considering the years between 1995 and 2002, overall 5-year survival probability was found to be 81% for Europe and USA (Kaatsch, 2010). However, still 20-40% of the patients developed resistance to chemotherapic agents (Pui, 2000; Chessells, 1995; and Schorin, 1994). Usage of chemotherapeutics is being the only effective treatment for the disease at this moment, in which intensive treatment has also long term drawbacks, such as causing secondary malignancies and cognitive impairments (Waber, 1995; and Garre, 1994). In order to improve the efficiency of childhood leukemia treatment, further investigations should be studied at least to decrease the drawbacks of the treatment.

As an example for efficiency of ALL treatment, genetic polymorphism of thiopurine methyltransferase (TPMT) can be given. 6-Mercaptopurine (6MP) is an anticancer drug which is used in the treatment of childhood ALL. It is a prodrug, which is activated via cellular metabolisms to form active cytotoxic thioguanine nucleotides (TGNs) which binds to DNA and trigger death of cell (Lennard *et al.,* 1983). On the other hand, 6MP is inactivated by conjugation of S-methylation reaction catalyzed by TPMT enzyme. So, the concentration of active drug within plasma is dependent on the activity of TPMT enzyme. Studies on TPMT revealed that, TPMT gene is polymorphic and resulted in deficient enzyme activity, and individuals bearing low activity alleles suffered from toxicity when administired with standard doses of 6MP (McLeod *et al.,* 1999; Tumer *et al.,* 2007). So that, it is important to know the genetic polymorphism of TPMT gene for the patients

who were taken 6MP treatment in order to determine the safe dose of the drug.

Even though childhood ALL is the highly death causing disease among children, there is little known about the molecular etiology of it, since cancer is a multistage disorder. It was shown that over 80% of infant B-cell ALL patients have 11q23 chromosomal translocations which resulted in breakage and recombination of the mixed lineage leukemia (*MLL*) gene, that is responsible for regulation of gene expression and maintanence of chromation stability (Yu *et al.*, 1998), with additional several potentional genes (Heerema *et al.,*1994). Experiments conducting on mice in which *MLL, TEL,* and *AML1* were knocked out, were shown the necessity of these genes for normal haemopoiesis (Enver and Greaves, 1998; Speck and Gilliland, 2002; Tenen, 2003]. Identification of the translocated fusion gene sequence in neonatal blood spots or Guthrie cards suggested that chromosomal translocations can occur in utero which might initiate leukemia progression prenatally (Gale *et al.*, 1997; Wiemels *et al.*, 1999; Hjalgrim *et al.*, 2002). An interesting finding was that around 1% of newborn cord blood sample had the *TEL-AML1* fusion gene, and within that the percentage of ALL developed children under 15 years old constitutes 1% of that population. In other words, within 100 children having fusion gene, there is only 1 child with ALL (Mori *et al.,* 2002*).* This implies that conversion of preleukemic clone to overt disease is not highly possible and the progression from preleukemic clone to progressed disease requires at least one additional postnatal event (Graves, 1999). The expression of the fusion gene is a common event and alone it is not sufficient to induce ALL, whereas the postnatal event is rare and acting as a rate determining step in the disease progression (Andreasson *et al.*, 2001). Indeed, the preleukemic stem cell can remain

silent in most cases. In a minority of children this preleukaemic stem cell progress to leukemia after receiving further postnatal genetic mutations such as deletions of a part of fusion gene (Maia *et al.*, 2001).

Although there are numerous studies support the multistage model of leukemia progression, possible exposures or molecular events that cause these chromosome breakages which are repaired inefficiently or incorrectly, are needed to be identified. There are some known exposure dependent factors which influencing the disease risk and likely to be associated with the ALL inducing events such as environmental agents (e.g., carcinogens, mutagens) and genetic factors (e.g., genetic polymorphisms of DSB repair genes) (Kim *et al.*, 2006).

1.3 Environmental Risk Factors for ALL

In the previous section, it was stated that genetic susceptibility is not solely effective in disease progression. Addition to genetic susceptibility, some external or internal factors may accompany with genetics factors to result in disease development. In addition to genetic susceptibility to ALL disease, environmental agents have been found to be a risk factor for ALL disease progression. Exposure to environmental agent can affect the development of the disease in three critical periods: preconceptional-before pregnancy, prenatal-during pregnancy and postnatal periods (Kim *et al.*, 2006).

Exposure to cigarette smoking, alcohol usage, dietary habits, and exposure to ionizing radiation are environmental exogenous agents that

parents and infant might be exposed during all three periods. So these exogenous agents will be given below in detail for all three periods.

As a preconceptional risk factor, the ages of mother and father before the conception can be important factors. Actually in terms of preconceptional transmission, “exposures occurring during an individual's father's life may be more important than exposures occurring during their mother's life. This is because spermatogenesis continues from puberty to old age and hence there is more opportunity for preconceptional mutant gene accumulation in men than women (Anderson, 2000).” The mother’s ability to metabolize and detoxify carcinogens is also an important risk factor for disease progression. Exposure of mother to agents during preconceptional period, in which mother has a slow metabolic clearance might result in the deposition of embryotoxic agents into the early stages of pregnancy.

The age of the mother and father during the pregnancy is an important risk factor for ALL. “The risk for childhood ALL has been shown to be significantly higher among children who were born when their parents were older; significant trends in ALL incidence have been related to increasing mother’s (> 35 years, $p < 0.001$) and father’s (> 40 years, $p < 0.002$) ages (Dockerty, 2001).” “For children born to mothers and fathers ≥ 40 years of age, the odds ratio for offspring developing childhood leukemia was 1.97 and 1.45, respectively (Dockerty, 2001).”

The prenatal exposure mostly related with mother exposure since fetus expose to the environmental agents via placental transmission. Risk factor associated with breastfeeding mothers and children after birth are potentially linked to the postnatal events (Kim, 2006).

Environmental agents that are exposed during preconceptional period, during pregnancy or postnatal period are ionizing radiation, chemicals such as hydrocarbons and pesticides; and daily habits such as alcohol usage, cigarette smoke.

Ionizing radiation is one of the exposure types for which the association with childhood leukemia has been confirmed (Mahoney *et al.* 2004; Ron 1998; Sali *et al.* 1996; United Nations Scientific Committee on the Effects of Atomic Radiation 1994). The magnitude of the risk is dependent on some factors such as the radiation level, the exposure time, and the age of the person at the time of exposure. As an example, survivors living within the 1 km of the atomic bomb explosions at Hiroshima and Nagasaki showed 20 fold higher rates for leukemia disease compared to general population (Mahoney MC, *et al.,*2004)

Hydrocarbons are organic compounds that are present in many household and industrial products such as paint removers, thinners and solvents. The most widely known hydrocarbon is benzene, which is used heavily in paint and plastics production and as a component in motor fuels and hobby glues. Strong correlation between exposure to benzene and ALL leukemia was established at a range of exposures not so higher than recommended for workers (Rinsky RA, 1981). Prof. Dr. Muzaffer Aksoy established a link between leukemia and exposure to benzene in a first large-scale epidemiological study in 1974 (Aksoy *et al*., 1976). According to that study, "a large group of leukemic patients were among the shoemakers, or leather manufacturing workers who were exposed to high level of benzene since benzene is used as a solvent in leather manufacturing and also being the major component of glue used in shoe making process." In the study of Aksoy *et al.*, (1976), "from 1967 to 1974, in Istanbul, Turkey, 31

patients with leukemia were diagnosed among 28,500 shoe, slipper, and handbag workers who are chronically exposed to benzene." Although the incidence of leukemia in general population of Istanbul in that time was 4.00/100,000 (Yarış *et al.,* 2004), it was found to be 13.59/100,000 by study of Aksoy. In other words, incidence of leukemia was more than 3 times higher among shoe workers compared to general population of Istanbul (Aksoy *et al*., 1976). Freedman *et al.* (2001) studied the relationship between parental hobbies and incidence of childhood leukemia. "According to that study ALL had a statistically significant association with prenatal exposure to painted homes (> 4 rooms) (OR = 1.7; 95% CI, 1.1–2.7) and to artwork with solvents (OR = 4.1; 95% CI, 1.1–15.1)."

According to study of Shu *et al.* (1996) "The risk for AML with maternal alcohol consumption (OR = 2.6; 95% CI, 1.4–5.1) was almost twice that of ALL. Paternal alcohol consumption before conception did not appear to increase risk."

Cigarette smoke contains various well-known carcinogens, and both active and passive smoking are associated with the development of various cancers during adulthood (Boffetta *et al.*, 2002). Chemicals in cigarette smoke can cross the placental barrier, and have been linked to an increased frequency of chromosomal abnormalities (Pluth *et al.*, 2000) oxidative damage (Fraga *et al.*, 1996), and aneuploidy of sperm (Shi *et al.*, 2001). However, there are contradictory results for whether maternal or paternal cigarette smoking before and during pregnancy is a risk factor for the developing childhood leukemia. A study reported a positive association between paternal preconceptional cigarette use and risk for childhood leukemia (Shu *et al.*, 1996). Whereas, another study demonstrated that "the risk of ALL was not associated with the father's ever having smoked (odds

ratio [OR], 1.04, 95% confidence interval [CI] 0.90 –1.20) or the mother's ever having smoked (OR, 1.0, 95% CI 0.9–1.2)" (Brondum *et al.*, 1999).

1.4 DNA Repair Mechanisms

DNA Repair can be defined as a range of cellular responses in order to restore the genetic information provided by the normal primary DNA sequence. Searching for the causative effects in human disease resulted in discovery of human DNA repair gene. The first human repair deficiency disease due to an inherited defect in the Nucleotide Excision Repair (NER) pathway, identified was *xeroderma pigmentosum* (XP) (Jung and Bantle, 1971; Stich, 1975). "The other human disorders related to defective DNA repair or defective cellular responses to DNA damage are *trichothiodystrophy* [Rebora and Crovato, 1987], *Cockayne syndrome*, *Fanconi's anemia* [Poon *et al.*, 1975], *ataxia telangiectasia* [Vincent *et al.*, 1975], *Bloom's syndrome* [Inoue *et al.*, 1977], and *hereditary nonpolypopsis colorectal cancer* (HNPCC) [Fishel *et al.*, 1993; Bronner *et al.*, 1994]." Association between DNA repair mechanisms deficiencies and related cancers or syndromes resulted in chromosomal mapping, cloning, and sequencing of DNA repair genes, and also biochemical studies of specific pathways and responses.

DNA repair mechanisms divided into 5 groups, named as; Direct Repair, Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR), and Double Strand Break Repair.

1.4.1 Direct Repair Mechanism

This is the simplest repair mechanism compared to other repair system regarding number of molecules involved. In that repair mechanisms, the lesion is removed or reversed by a single step reaction restoring the local sequence to its original state. There are several direct repair enzymes each having a different substrate. As an example, photolyases reverse the DNA damage resulting from UV or cisplatin treatment (Sancar, 1996). Secondly, O^6- methyl guanine DNA methyltransferase (MGMT) repairs the alkylation damage. It transfers the alkyl group from the damaged site to a cysteine residue found in the active site of MGMT (Hazra *et al.*, 1997). 20% of human tumor cell lines have decreased MGMT activity and increased sensitivity to alkylating agent (Sancar, 1995), but there are few direct data suggesting that mutations in the *mgmt* gene contribute to cancer.

1.4.2 Base Excision Repair

Nonbulky base adducts such as methylated, oxidized, reduced bases and also fragmented bases by ionizing radiation or oxidative damage are repaired by Base Excision Repair (BER) system. The repair system involves three steps; removal of damaged bases from DNA by DNA glycosylases, than formed abasic site is removed and finally gap is filled by DNA polymerase and DNA ligase seals the nick. Schematic representation of BER is given in Figure 1.1 A. X-ray repair cross-complementing group 1 (XRCC1) is a critical enzyme for this repair pathway, which enables the assembly and activity of DNA ligase III, DNA polymerase β, human AP endonuclease, polynucleotide kinase and, poly(ADP-Ribose) polymerase (PARP) at the site of DNA damage (Caldecot, 1996; Masson, 1998; Whitehouse, 2001; and Vidal, 2001). The human XRCC1 gene is located on chromosome 19q13.2 which contains 17

exons and it encodes a protein of consisting 633 amino acids (Lindahl T *et al.,* 1999). Shen *et al.* (1998) have identified three polymorphisms of XRCC1 gene at codon 194(Arg to Trp), 280 (Arg to His), and 399 (Arg to Gln). All these 3 polymorphisms are studied with association to different types of cancer. It is reported that XRCC1 Arg399Gln polymorphism located within the BRCT1 domain which interacts with PARP and may result in deficient DNA repair (Lunn RM *et al.* 1999; Zhang X *et al.,* 1998).

1.4.3 Nucleotide Excision Repair

The NER pathway is responsible for repair of bulky lesions such as larger chemical adducts, pyrimidine dimers, other photo-products;, and cross-links (Squire, 1998). NER pathway involves 4 steps; damage recognition, incision, gap filling, and ligation.

The enzymes of that pathway are multifunctional and involved in other cellular processes, like cell cycle regulation (Hwang, 1996). Schematic representation of NER is given in Figure 1.1 B

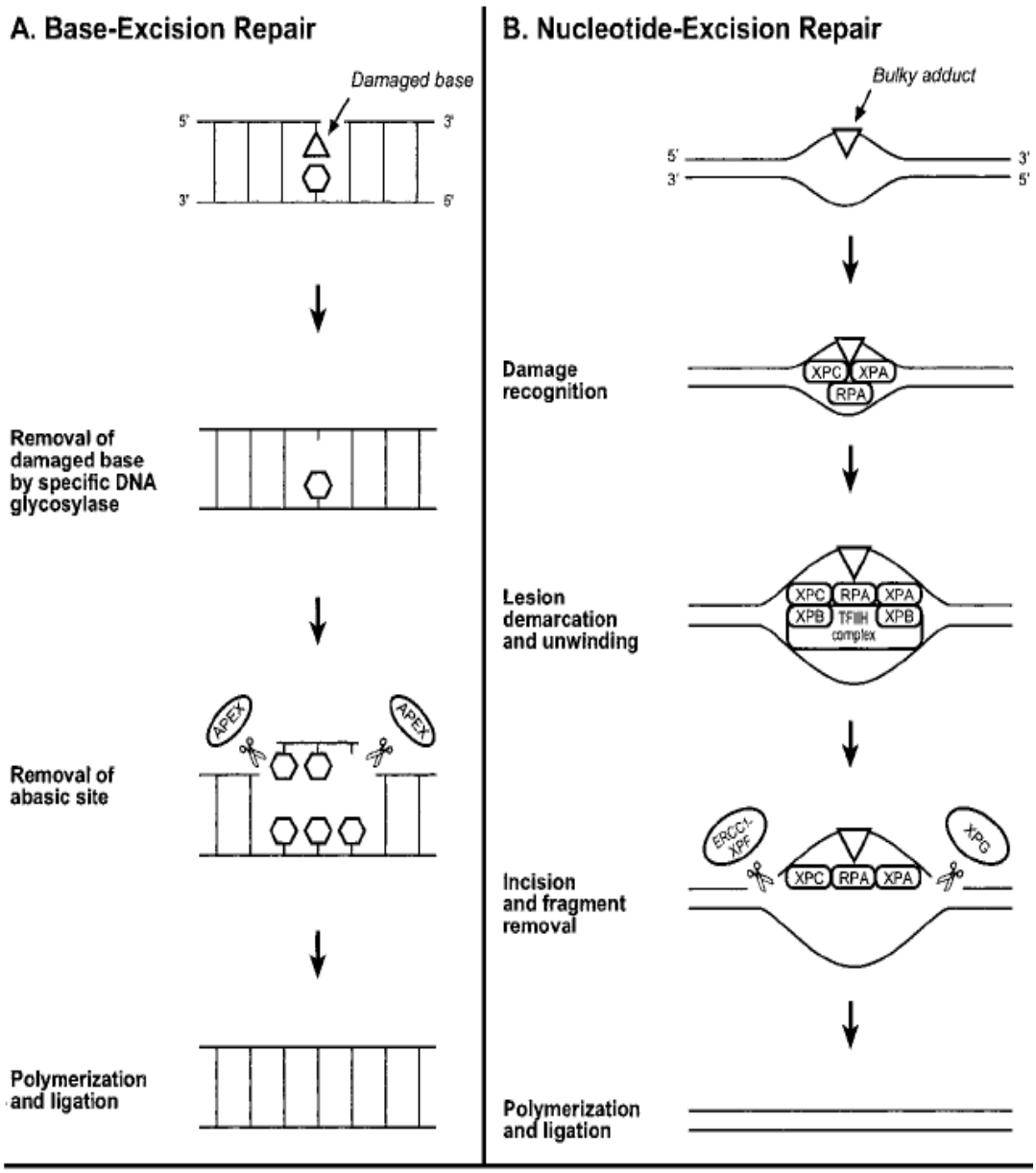

Figure 1.1 A: Base excision repair; **B**, Nucleotide Excision Repair (Figure is taken from Ellen *et al.*, 2002)

1.4.4 Mismatch Repair

Mismatch Repair (MMR) is responsible for repair of mismatched basepairs which occurs through processes including misincorporations during DNA replication, formation of heteroduplexes, and secondary structure such as imperfect palindromes (Bishop, 1985). Also, deamination of 5-methylcytosine to uracil, following by removal of uracil by uracil N-glycosylase results in a G: T mismatch. The repair mechanism is similar to that of excision repair; a patch of nucleotides is removed from one strand, and followed by resynthesis and ligation processes.

There are two types of mismatch repair systems, long-patch and short-patch. In short-patch repair system there are 3 enzymes possessing nicking activities specific for mismatch repair; T/G specific (Wiebauer, 1989), A/G specific, and all-type mismatch nicking enzymes (Yeh, 1991). That three enzymes have different mode of action, but interestingly in either an A/G mismatch or a T/G mismatch, it is usually the guanine that remains untouched by mismatch specific glycosylases (Wiebauer, 1990; Yeh, 1991).

1.4.5 Double Strand Break Repair

Double strand break repair is responsible for the repair of double strand DNA breaks. Double strand breaks (DSBs) can be produced due to exogenous agents such as ionizing radiation (IR) (Dizdaroglu, 1992), some chemotherapeutic drugs, endogenously formed reactive oxygen species, mechanical stress on the chromosomes. When DNA replication forks encounter DNA single strand breaks or other types of lesion, it might result in formation of DSBs. In addition, DSBs are generated to initiate

recombination between homologues chromosomes during meiosis, and also during the immunoglobulin class-switch recombination.

DSBs are the most dangerous type of DNA damage, because unrepaired DSBs block replication, and transcription of involved sequences. Moreover, the breaks are prone to nuclease attack with subsequent destruction (Rufer, 1992). Furthermore, DSBs can result in death of the cell, if it occurs within an essential gene. Repair of DSBs is intrinsically more difficult than other type of DNA damage because there is no undamaged template available (Khanna, 2001)

There are two pathways of double strand break repair; homologous end joining (HR) and non-homologous end joining (NHEJ).

In HR pathway, DNA ends are resected in the 5' to 3' direction by nucleases, newly exposed 3' single stranded tails then invade the double helix of the homologous, undamaged partner molecule, and strands are extended by action of DNA polymerase, the cross-overs yield two intact DNA molecules (Khanna, 2001). The key molecules of HR are *Rad51* and X-ray cross-complementing group 3 (*XRCC3*) genes (Richardson, 2005).

The *Rad51* recombinase interacts directly with breast cancer-associated tumor suppressor BRCA2 (Tarsounas, 2004; Davies, 2001), and this interaction is required for the control of *RAD51* function (Davies, 2001), necessary for normal recombination proficiency, radiation resistance and genome stability (Tarsounas, 2003). Homozygous loss of *Rad51* in mice results in early embryonic lethality and cells recovered from mutant embryos do not proliferate (Lim, 1996; Tsuzuki, 1996)

The *XRCC3* gene product is a member of the *RAD51*- related gene family, and directly interacts with *Rad51* (Liu, 1998; Brenneman, 2000; and Schild, 2000). *XRCC3* protein associates directly with DSBs and recruit *Rad51* (Forget, 2004), and *XRCC3*-*Rad51* complex cooperatively modulate the progression of the replication forks on damaged chromosomes (Henry-Mowat, 2003). Deficiency of *XRCC3* gene resulted in increased hypersensitivity to ionizing and UV radiations as well as mono- and bifunctional alkylating agents (Xu, 2005; Alsbeih, 2007).

In NHEJ, there is no need to undamaged partner, two DNA ends are degraded limitedly at the termini and the DNA ends are ligated together (Khanna, 2001). Since there is no template for the repair of damaged DNA, it is often prone to error, and small sequence deletions are usually introduced to the DNA. Schematic representation of double strand break repair is given in Figure 1.2

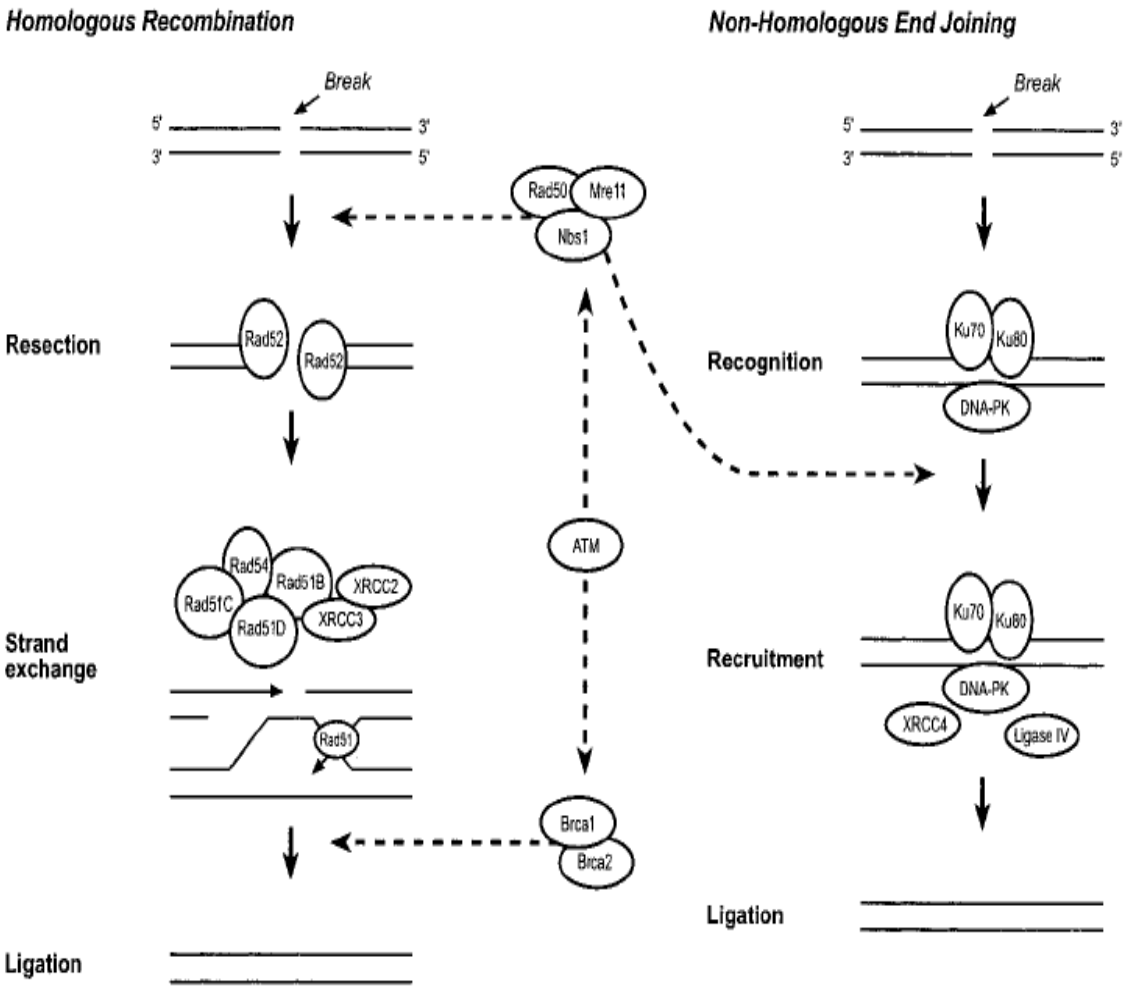

Figure 1.2 Schematic representation of Homologous Recombination end joining and Non-homologous recombination end joining (Figure is taken from Ellen *et al.*, 2002).

1.5 Genetic Polymorphisms and Risk of Childhood ALL

1.5.1 Genetic Polymorphisms in the Xenobiotic Metabolizing Enzymes

It is known that individuals show different resistance and sensitivity to environmental carcinogens. Actually, the reason for that individuals have different metabolic rates for metabolizing chemicals, due to polymorphic form of metabolizing enzymes. Metabolism of external chemicals are performed by various numbers of enzymes, and nearly each enzyme has various types of polymorphic forms, and when considering the genetic profile of each individual, it will explain the different responses of an each individual to same exposure. Most of the chemical carcinogens are metabolized by Xenobiotic metabolizing enzymes. Xenobiotic metabolism is divided into two parts; Phase I and Phase II reactions. The exogenous chemicals first converted to a more soluble form by either of oxidation, reduction and hydrolysis reaction by Phase I reactions. Then, by phase II reactions, more soluble product formed by Phase I reactions are conjugated with chemicals to facilitate the elimination of the chemical from the body. Almost all enzymes in Phase I and Phase II are polymorphic, which might affect the individual's response to exposure. Phase I enzymes are mainly responsible for forming metabolic activation reactions which mainly causes DNA adducts compounds, and also reactive oxygen species. So, any polymorphic forms resulting in higher activity of Phase I enzymes (there are some exceptions) causes increased metabolic carcinogen activation. Whereas, Phase II enzymes have role in detoxification and elimination of these activated metabolites of phase I reactions as well as protection against oxidative stress caused by carcinogen exposure of Phase I reactions.

So that, polymorphisms resulting in higher phase II detoxification leads to more efficient detoxification of activated carcinogenes and also for oxidative stress (Not all Phase II reactions). So exposure to environmental chemicals in a "high phase I, low phase II" individual may lead to more toxicity than that in a "low phase I, high phase II" individual (Nebert and Roe, 2001).

The major enzymes of Phase I are cytochrome P450 dependent monooxygenases (P450). P450 enzyme superfamily, comprise about 80% of Phase I enzymes, and their main function is to introduction of a moiety to chemicals, such as one or more hydroxy groups (Schenkman and Johnson, 1975; Arınç and Philpot, 1976; and Black and Coon, 1986, Arinç, 2010). CYP1A1, CYP1A2 and CYP2E1 are isoforms of P450 and have particular interest in carcinogenesis, since these enyzmes have roles in bioactivation of environmental chemicals into carcinogenic forms.

Phase II enzymes have the role of conjugating either the parent compound or the metabolite from Phase I by glucuronidation (by UDP-Glucuronosyltransferases-UGTs), sulfation (by sulfotransferases- STs), acetylation (by N-acly transferases- NATs), methylation (by thiopurine methyltransferase- TPMT), or glutathione conjugation by glutathione transferases- GSTs) to facilitate elimination (Gunaratna, 2000)

Polymorphism of CYP1A1 and exposure to chemicals such as polyaromatic hydrocarbons (PAHs) in relation to cancer may be given as an example for xenobiotic metabolizing enyzmes polymorphisms, chemical exposure and cancer susceptibility. Studies were carried out to demonstrate the association between CYP1A1 polymorphisms and smoking related cancers. CYP1A1 polymorphisms were found to be associated with lung (Coles and Ketterer, 1990; McClellan, 1996), head and neck (Doll,

1998), prostate (Murata *et al.,* 2001) and colorectal (Sivaraman *et al.,* 1994) cancers. Benzo(a)pyrene (BaP) found in the tobacco smoke, is one of the substrate of CYP1A1. Within body, BaP is converted to BaP 7, 8 diydrodiol-9, 10-epoxide (BPDE) by several reactions of CYP family. BPDE is one of the most carcinogenic compound ever known by CYP1A1, which can bind to DNA and forms DNA-adducts (Parke *et al.,* 1991). Interestingly, CYP1A1 is induced by BaP itself, which causes higher rate of CYP1A1 expression and so higher activity of CYP1A1 enzyme and increased rate of accumulation of carcinogenic compound (Stegeman, 1995; Arınç *et al.,* 2000). The polymorphism of CYP1A1 and its association with childhood ALL were studied by several groups. An association between *2A allele and Childhood ALL (Krajinovic *et al.,* 1999; Joseph *et al.*, 2004), between *2B allele and childhood ALL (Infante-Rivard *et al.,* 2000) were found.

Another important enzyme of CYP family is CYP2E1. A number of different chemicals of diverse structures have been found to be metabolized by CYP2E1, such as ethanol, endogenous compounds, exogenous compounds including industrial solvents, procarcinogens, and a few pharmaceutical agents. CYP2E1 activates its subtrates to more toxic and carcinogenic forms. The most known substrate of CYP2E1 is benzene. When body is exposed to benzene, it is first converted to benzene oxide by CYP2E1 enzyme. From that point, benzene oxide can be detoxified by GSTs via glutathione conjugation, however, rearrangement of benzene oxide to phenol occurs spontaneously. Phenol can be further oxidized to hydroquinone by CYP2E1. Phenol and hydroquinone can be detoxified by Phase II enzymes such as sulfotransferases and glucuronyltransferases (Seaton *et al.,*1995). Alternatively, hydroquinone or phenol can diffuse to the blood and distributed to other tissues, including bone marrow, where it can further metabolized to 1, 4 benzoquinone by meyeloperoxidases (MPO)

(Snyder and Hedli, 1996). These benzoquinone metabolites can be detoxified back to hydroquinone by NAD(P)H: quinone oxidoreductase (NQO1), so kept in a reduced state where they can more readily be conjugated and excreted (Bauer *et al.,* 2003). Another fate of benzene oxide is the conversion of it to trans-trans muconaldehyde (MA), which is a direct acting alklating agent and proposed to be the most toxic species resulting from benzene metabolism.

CYP2E1 plays a critical role in activation of benzene metabolism (and also other procarcinogenic compounds). NQO1, which detoxifies the quinone metabolites of benzene in bone marrow, is of crucial importance for the protecetion against CYP2E1 activated benzene toxicity. GSTs also have an important role as they conjugate benzene oxide. Myeloperoxidase (MPO) is another important enzyme in benzene metabolism, since it converts catechol into carcinogenic metabolite 1, 2- benzoquinone.

Due to its importance in bioactivation of several procarcinogenic compounds, CYP2E1 is highly studied in relation to risk of various cancer types, including childhood ALL. For example, CYP2E1 catalyzes the activation of numerous low molecular weight compounds including ethanol, acetone, drugs like acetaminophen, isoniazid and many procarcinogens such as vinyl chloride and vinyl bromide, dimethylnitrosamine and diethylnitrosamine, acrylonitrile, urethane, styrene, acrylamide, carbon tetrachloride, and chloroform. CYP2E1 is also induced by its substrates like ethanol, benzene, pyridine and acrylamide (Koop and Casazza 1985; Johansson and Ingelman-Sundberg 1988; Arınç *et al*, 2000a; Arinç *et al.,* 1991; Arınç *et al.*, 2000b; Nuyan, 2008), as well as some patophysiological conditions like obesity, diabetes and starvation

(Koop and Casazza 1985; Hong *et al.*, 1987; Arınç *et al.*, 2005; Arınç *et al.*, 2007).

There are several studies which examined the relationship between CYP2E1 polymorphisms and risk of childhood ALL. Ulusoy *et al.,* (2007) investigated the possible association of *CYP2E1*5B, *6* and **7* alleles, alone or in combination, with the risk of incidence of childhood ALL in Turkish population. Accordingly, when both CYP2E1 *5B and *6 alleles were considered together, the risk of childhood ALL have been found to be significantly increase to 2.9 fold (95% CI 1.0–8.5) (Ulusoy et al., 2006, 2007).

Recently, Tumer et al. (2010) investigated the possible association of DNA repair gene XRCC1 Arg399Gln polymorphism alone, and in combination with CYP2E1 polymorphism with the risk of incidence of childhood Acute Lymphoblastic Leukemia (ALL) in Turkish population. In that study, Gln399Gln genotype significantly increased the risk of disease up to 2.0 fold (p=0.04). When CYP2E1 *5B, *6B and XRCC1 codon 399 were considered together, the risk factor significantly increased to 3.7 fold (p=0.049).

Among Phase II enzymes, SULT1A1 and EPHX1 are highly studied in relation to various cancer types. As mentioned above, EPHX1 converts benzene oxide to benzene dihydrodiol which is then converted to catechol and 1, 2 benzoquinones by further reactions. The latter compound can bind to DNA and proteins and causes carcinogenic effects. SULT1A1 is responsible for the detoxification of phenol and hydroquinones. Besides benzene metabolism, both SULT1A1 and EPHX1 participate in the metabolism of various chemical compounds including environmental and tobacco carcinogens. In this aspect, genetic polymorphisms altering the

activity of SULT1A1 and mEH may modify the individual's susceptibility for the childhood ALL. Recently Tumer *et al.* (2010) investigated the interaction between EPHX1 gene Tyr113His polymorphism (exon3) with childhood ALL development. According to that study, homozygote mutant allele were shown to be significantly higher in ALL patients than controls, and represent a risk factor for the childhood ALL (OR= 2.3, p=0.01).

1.5.2 *Rad51* Polymorphism and Childhood ALL Risk

Rad51 is a member of homologous DNA repair systems, and it plays a crucial role in maintaining the genetic stability of the cell. It catalyzes strand transfer between a broken sequence and its undamaged homologue to allow re-synthesis of the damaged region (West, 2003). *Rad51* has a function of strand invasion. It polymerizes onto 3' DNA end and mediates the transfer and annealing of resulting nucleoprotein filament to a complementary homologous strand on the intact chromatid (Thacker, 2005). *Rad51* has to overcome internal DNA bonding forces to unwind and separate DNA strands, and also competing with other DNA binding proteins (Thacker, 2005). These problems are overcome by recruitment of *Rad51* like proteins like *XRCC3*, which all have sequence similarity. These all *Rad51* like proteins have DNA-stimulated ATPase activity and preferentially bind single stranded DNA (Braybrooke, 2000; Sigurdsson, 2001; Masson, 2001a; Masson, 2001b; Lio, 2003).

The human *Rad51* Gene is located on chromosome 15q15.1 and consists of 36998 bases (Gene Cards Database). The protein product of *Rad51* gene comprised of 339 amino acids and it is 36966 Da (Gene Cards Database). It has 6 domains, one for ATPase activity, one for DNA binding (Helix hairpin helix motif), and the other domains are specific to action of

Rad51. According to NCBI SNP database, *Rad51* gene has 296 SNPs. The most important polymorphism identified for *Rad51* is G135C SNP in 5' untranslated region (5' UTR). The *Rad51* G135C polymorphism is associated with *Rad51* protein over-expression and to increased DNA repair (Vispe, 1998; Kim, 2001; and Richardson, 2004).

Regarding the role of *Rad51* in the Homologous DNA repair mechanism, several studies have examined the relationship between *Rad51* G135C polymorphism and risk of certain cancers. However the results from these previous studies are conflicting. For this reason, additional studies to address the role of *Rad51* G135C polymorphism in human carcinogenesis are needed. In case of childhod ALL, so far, there has been no study evaluating the role of *Rad51* G135C polymorphism as risk modifier. In this study, the role of *Rad51* G135C polymorphism in the development of childhood ALL was investigated.

1.5.3 *XRCC3* Polymorphism and Childhood ALL Risk

X-ray cross-complementing group 3 (*XRCC3*) is a member of homologous DNA repair mechanisms and play a key role in that repair system. *XRCC3* contains ATPase motifs composed of the Walker A, and B boxes, and the integrity of the Walker A motif of *XRCC3* is required for biological activity and governs *Rad51*C-*XRCC3* complex formation (Yamada, 2004). Moreover *XRCC3* protein interacts with *Rad51* protein, enabling *Rad51* protein multimers to assemble at the site of damage (Bishop, 1998)

The human *XRCC3* gene is located on chromosome 14q32.3 and consists of 17870 bases (Gene Cards Database). The protein product of *XRCC3* gene comprised of 346 amino acids and it is 37850 Da (Gene Cards

Database). According to NCBI SNP database, *XRCC3* gene has 111 SNPs. The most important polymorphism identified for *XRCC3* is Thr241Met SNP in 7th exon region. Some studies demonstrated that *XRCC3* Thr241Met variant allele is associated with relatively high DNA adduct levels in lymphocyte DNA, indicating relatively low DNA repair capacity (Matullo, 2001; Shen, 1998)

Regarding the role of *XRCC3* in the Homologous DNA repair mechanism, several studies have examined the relationship between *XRCC3* Thr241Met polymorphism and risk of certain cancers. However the results from these previous studies are conflicting. For this reason, additional studies to address the role of *XRCC3* Thr241Met polymorphism in human carcinogenesis are needed. In case of childhod ALL, so far, there has been no study evaluating the role of *XRCC3* Thr241Met polymorphism as risk modifier. In this study, the role of *XRCC3* Thr241Met polymorphism in the development of childhood ALL was investigated.

1.6 The Aim of This Study

Acute lymphoblastic leukemia is the most common type of childhood cancer, accounting for 30% of all cancers diagnosed in children younger than 15 years (Linet, 1999). However, there are limited and little information about risk factors for childhood leukemia progression. Observation of association between some cancer types and DNA repair defects suggested the DNA repair genes as candidate cancer susceptibility genes. Homologous recombination repair is the most important mechanism for the sake of the cell. It is well established that individuals having a modified ability to repair double strand breaks are at increased susceptibility to cancer. Therefore, polymorphisms in genes encoding DSB

repair molecules have strong relevance in determining susceptibility to cancer.

As described in more detail previously, *Rad51* and *XRCC3* are two susceptible enzymes due to their dual roles in repair of double strand DNA breaks. Genetic polymorphisms altering the activity or the structure of *Rad51* and *XRCC3* may modify the individual's susceptibility for the childhood ALL. Polymorphisms of both *Rad51* and *XRCC3* have been widely studied in relation to various cancer types as risk modifiers. However, so far, there have been no reports evaluating the clinical significance of *Rad51* and *XRCC3* genetic polymorphisms for the risk of developing childhood ALL. Therefore, this study focused on the effects of *Rad51* and *XRCC3* genetic polymorphisms, alone or in combination, as risk modifier for the development of childhood ALL and aims related with this part of the present study included;

- Investigation of genetic polymorphism of *Rad51* G135C on the pediatric patients with ALL and healthy adult controls and comparison of the frequencies in case and control groups to elucidate the possible risk modifier effects of polymorphisms on the development of childhood ALL. So far, there has been no study evaluating the role of ***Rad51* G135C** polymorphism as risk modifier. Therefore this study, investigated the association of *Rad51* G135C polymorphisms with the risk of childhood ALL for the first time.

- Investigation of *XRCC3* Thr241Met polymorphism in control and case group, and determination of the effects of this polymorphisms on the risk of development of childhood ALL. So far, *XRCC3* Thr241Met polymorphism has not been studied in relation to childhood ALL.

Therefore this study, investigated the association of *XRCC3* Thr241Met polymorphism with the risk of childhood ALL for the first time.

- The multi-locus analysis of two *Rad51* polymorphisms together with *XRCC3* polymorphism, thereby this study aimed to investigate the effects of combined polymorphisms in the risk of childhood ALL development, which would efficiently describe the risk factors for the disease.

- Investigation of the interaction between *Rad51* and *XRCC3* genetic polymorphisms with non-genetic factors such as parental age at conception, smoking status of the parents, postnatal exposure of the children to cigarette smoke, in the risk of development of childhood ALL, based on case-only model.

- Lastly, investigation of the frequencies of *Rad51* polymorphisms and *XRCC3* polymorphism in control samples, which would reflect the Turkish population frequencies for these polymorphisms, and compare the frequencies of these polymorphisms in Turkish population with those in other population of different ethnic origins. By the current work, the frequency of *Rad51* G135C polymorphism has been identified for Turkish population for the first time.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Subjects and Blood Sample Collection

Control groups comprised of 193 subjects and blood samples were obtained from healthy volunteers with the collaboration of METU Health Center, Biochemistry Laboratory. Written informed consents were taken from each participant. The consent form also included questions regarding their age, birth place of volunteer and hid/her parents, and any diseases they had had. Subjects having disease- like any type of cancer, diabetes etc.- were excluded from the study.

A total of 184 subjects were included in the ALL-patient group. The blood samples for the patient group were obtained by the collaboration of Sami Ulus Children's Hospital and Ankara University, Faculty of Medicine, Department of Pediatric Hematology between June 2005 and November 2007. Ethical approval was obtained from research ethics committee of Medical Faculty, Ankara University. The information on demographic data, like age, birth place of child, mother and father-,clinical diagnosis, treatmen protocol, stage of therapy, familial relationship between mother and father

and patient's risk group were obtained with a questionnarie. Information on subtype of ALL, age at diagnosis, white blood cell count at diagnosis were obtained from the medical patient's log. Written informed consents were taken from the parents together with questionnaire.

Questinnaire on the smoking status of mother and father was also obtained for 108 of the patients. Smoking status of mother and father, and the duration of exposure and the number of cigarettes smoked per day for active smokers were obtained. The questionnaire also included information on the smoking status of mother during pregnancy, and postnatal exposure of child to cigarette smoke.

4-5 ml of blood samples from control and ALL-patient group subjects were taken in EDTA-containing vacuumed tubes and stored at – 20 ^{0}C till use for DNA isolation.

2.1.2 Enzymes and Chemicals Used in the Genotyping Studies

Agarose (A-9539), bromophenol blue (B-5525), ethidium Bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), sodium chloride (NaCl; S-3014), sodium dodecyl sulfate (SDS; L-4390), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T-5941) were purchased from Sigma Chemical Company, Saint Louis, USA.

Borate (11607), and absolute ethanol (32221) were the products of Riedel de Haën, Seelze. Magnesium Chloride (A4425) and potassium chloride (A2939) were purchased from AppliChem, Ottoweg, Darmstadt. Sucrose (7653) and Triton-X-100 (11869) were the products of Merck & Co., Inc., Whitehouse Station, NJ, USA.

Taq DNA Polymerase -supplied together with $MgCl_2$ ad amplification buffer- (#EP0407), dNTP mix (#R0191), Gene Ruler™ 50 bp DNA Ladder (#SM0371) and restriction enzyme *Nla III* (#ER1831), *Mva I* (#ER0551) which were supplied with their buffers Tango™ were purchased from Fermentas, Int, Inc., Ontario, Canada.

All chemicals used in this study were of molecular grade and were obtained from commercial sources at the highest grade of purity.

2.1.3 Primers

Primers used throughout the study were selected by literature search and were derived from known sequences of human. The primer pairs were purchased from MWG (MWG Biotech, Ebersberg, Germany) or Metabion (Metabion InternationalAG, Martinsried, Deutschland) and were all purified by HPLC. Primers stocks were brought to 100 pmol/µl concentration and stored at -20 ^{0}C. Aliquots of 10 pmol/µl concentration were prepared and used for PCR.

2.2 Methods

2.2.1 Isolation of Genomic DNA from Human Whole Blood Samples

2.2.1.1 Manual Isolation of Genomic DNA

Genomic DNA from human whole blood samples was isolated according to the method of Lahiri and Schnabel (Lahiri and Schnabel,1993), with some modifications. 500 µl of whole blood which was taken into EDTA-

containing vacuumed tubes is treated with an equal volume of low-salt buffer containing 10 mM Tris-HCl pH(A) 7.6, 10 mM KCl, 2mM EDTA, 4mM $MgCl_2$ (TKME buffer) and 2.5% Triton X-100. The cells were lysed by inversions and suspension is centrifuged at 1000 g for 10 minutes at room temperatures using Sigma 1-15 bench top microfuge(Sigma, Postfach 1713-D-37507, Osterode). The pellet was washed three more times with TKME buffer and centrifuged using same conditins. The final pellet was suspended in 0.2 ml of TKME buffer and 20 µl of 10% SDS was added. The suspension was mixed vigorously and incubated at 55 ^{0}C for 20 minutes. Then was added to precipitate the proteins, the tube was mixed well and centrifuged at 14000 g for 7 minutes at 4 ^{0}C. The supernatant, which contained the DNA, was precipitated using absolute ethanol, kept at -20 ^{0}C for 30 min-1 hour to increase the efficiency of precipitation, and pellet was obtained by centrifugation at 10000 g for 10 min at 4 ^{0}C. Then DNA pellet was washed once with 70% ethanol, air dried to final DNA pellet is resuspended in 0.2 ml of 10 mM tris-HCl pH 8.0, 1 mM EDTA pH 8.0. (TE) and incubated overnight at 37 ^{0}C. The DNA samples were stored at 4 ^{0}C while they were in active use and kept at – 20 ^{0}C for long term storage.

2.2.1.2 Genomic DNA Isolatin by Nucleospin Blood Kit

Nucleosping Blood Kit (Macherey-Nagel, Germany) was used for isolatin of DNA from old blood samples or from the blood samples of ALL patients that have reduced number of white blood cells, which manual isolation did not give effective PCR results.

After complete thawing, 200 µl of whole blood was mixed with 25 µl of proteinase-K in a 1.5 ml eppendorf tube, and vortexed immediately. Then 200 µl of lysis buffer “B3” was added, vortexed vigorously for 10-20 seconds

and the tube was incubated at 70 ^{0}C in a heater block for 30 minutes. In every 10 minutes of incubation, tube was vortexed for 10-20 seconds. The lysate turns into a greenish-brownish color at the end of the incubation period.

At the end of the incubation period, 210 µl of absolute ethanol was added to the mixture, vortexed and the sample in the tube was transferred to a spin column. The sample in the column was centrifuged at 14000 g for 2 minutes at room temperature using Thermo Microlite RF (refrigerated centrifuge, Waltham, MA, USA) micro centrifuge. The collecting tube with flow-through was discarded. The spin column was placed in a new collecting tube and 500 µl of byffer "BW"was added to the spin column. The sample in the spin column was centrifuged at 14000 g for 2 minutes at room temperature and the flow through was discarded. 600 µl of buffer "B5" was added to the column, centrifuged again as described above, and the flow through was discarded. Then the spin column was centrifuged once more as descrived above, without adding anything to the column, in order to get rid of the ethanol that was present in buffer "B5" completely.

In order to elute the DNA in the column, the spin column was placed in a new 1.5 ml eppendorf tube, 100µl of preheated (to 70 ^{0}C) buffer"BE" was dispended directly onto the membrane, the tube was incubated at room temperature for 5 minutes and then centrifuged at 14000 g for 2 minutes at room temperature. The sample eluted from the spin column was loaded onto the same spin column again, incubated for 5 minutes and centrifuged as described above, to increase the yield of DNA.

2.2.2 Spectrophotometric Quantification of Genomic DNA

The concentrations of each genomic DNA that was isolated were determined by measuring the absorbance values at 260 nm and 280 nm in quartz cuvettes using Schimadzu UV-1201 Spectrophotometer (Schimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). As the DNA molecules gave maximum absorption at 260 nm, reading at this wavelength was used to calculate the concentration of nucleic acid in the sample. Based on the knowledge that an optical density of 1.0 corresponded to approximately 50 µg/ml for double stranded DNA,the concentration of DNA in the sample was calculated according to the formula:

"Concentration (µg/ml) = OD_{260nm} × 50 (µg/ml) × Dilution Factor" (2.1)

The ratio between OD values at 260 nm and 280 nm (OD_{260}/OD_{280} ratio)was used to estimate the purity of the nucleic acid. Pure DNA preparations gave the ratio of 1.8 while the higher or lower values indicated either RNA or protein contaminations, respectively. However the ratio between 1.6 and 2.0 can be acceptable.

2.2.3 Qualification of Genomic DNA by Agorose Gel Electrophoresis

The intactness of DNA samples were determined by horizontal agorose gel electrophoresis. Agarose gel was prepared by dissolving agarose powder in 0.5% concentration by 0.5x TBE buffer (450 mM Tris, 450 mM Boate, 10 mM EDTA, pH 8.3) using a microwave oven. When the gel solution was cooled enough (approximately 60 ^{0}C), ethidium bromide was added

from a stock solution of 10 mg/mL so as to obtain a final concentration of 0.5 µg/ml and the solution was mixed throughly.

The warm agarose solution was poured into the pre-settled mold and any air bubles-if present- especially under or between the teeth of the comb were removed by the help of a pipette tip. The gel was allowed to solidfy completely which approximately takes 20-40 minutes at room temperature. After the gel is solidified, it was mounted in the electrophoresis tank which was filled with 0.5 x TBE Buffer so that the slots of the gel faced the negative pole-cathode. 5 µl of DNA sample was mixed with 1 µl of gel loading dye by the use of a micropipette, and the mixture was slowly added to the wells of the gel. After loading of the DNA samples were completed, the lid of the tank was closed and the electrical leads were attached to the power supply. The power supply was set to a constant voltage so that not more than a voltage of 5V/cm (measured as the distance between electrodes) was applied (corresponds to maximum of 150 volts for Scie-Plas HU13W horizontal gel electrophoresis unit).

The gel was run until the bromophenol blue dye reached to the bottom of the gel, and visualized under UV light and the photograph was taken by Vilber Lourmant Gel Imaging System (Marne La Vallee, Cedex, France) and InfinityCapt (Version 12.9) computer software. Pure DNA preparations give a single band in agarose gel electrophoresis. Presence of more than one band indicates breaking in DNA, presence of RNA or contamination of DNA during isolation.

2.3 Genotyping of Single Nucleotide Polymorphisms

In this study the genes of two DNA repair enzymes: *XRCC3* and *Rad51* were genotyped for their most common single nucleotide polymorphisms. ***XRCC3* C18067T and *Rad51* G135C** polymorphisms were identified by PCR amplification of SNP regions followed by appropriate restriction enzyme digestions. The details of these methods were described below. Techne Progene (Cambridge, UK) and Eppendorf Mastercycler (Hamburg, Germany) thermocyclers were used for PCR.

2.3.1 Genotyping of *XRCC3* C18067T Polymorphism

In this study, *XRCC3* gene was genotyped by PCR-RFLP method for the detection of *XRCC3* **C18067T** (Thr241Met) polymorphism. The sequence of the amplified fragment, location of the SNP site and sequence of the recognition site for the restriction enzyme were given in Figure 2.1

Figure 2-1 Sequence of amplified fragment in exon 7 region of *XRCC3* gene that includes C18067T SNP. The yellow highlighted sequences are forward and reverse primers. The red highlighted sequence shows the recognition site for the restriction enzyme *Nla III* which is an internal cut site. The blue highlighted sequence shows the recognition site for the restriction enzyme *Nla III*. The Red marked nucleotide shows the location of C18067T SNP. (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov, last accessed on 13/01/2011)

2.3.1.1 Polymerase Chain Reaction

C18067T SNP is located on exon 7 region of the gene. A 456 bp fragment covering C18067T point mutation was amplified with forward primer XrF: 5'-GGTCGAGTGACAGTC CAAAC and reverse primer XrR:5'-TGCAACGGCTGAGGGTCTT (primer sequences were taken from Yen *et al.*, **2008**). In order to obtain a single band devoid of nonspecific bands, different parameters were tested in PCR. These parameters included the change in concentration of $MgCl_2$ as 0.5, 1.0, 1.25, 1.5 and 2 mM; change in concentration of primer pairs as 10, 20 and 40 pmol; and change in annealing temperature as 56, 58 and 60 ^{0}C. The optimized PCR conditions were given in Table 2.1

Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.0 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM (20 pmol in 50 µl reaction mixture) of forward and reverse primers, approximately 200 ng of genomic DNA, and 2.5 U of Taq polymerase.

Table 2.1 Components of PCR mixture for *XRCC3* Thr241Met

Constituents	Stock Concentration	Volume added (µl)	Final Concentration in 50 µl Reaction mixture
Taq buffer (KCl)	10x	5	1x
$MgCl_2$	25 mM	2	1.0 mM
dNTPmixture*	10 mM	1	200 µM
Forward Primer	10 pmol/µl	2	20 pmol
Reverse Primer	10 pmol/µl	2	20 pmol
Template DNA	Varies	Varies	200 ng
Taq DNA Polymerase*	5 U/µl	0.5	2.5 U
dH_2O		To 50 µl	

* See Appendix B for these reagents

The PCR program used for the amplification of exon 7 region consist of an initial denaturation step at 95 ^{0}C for 3 min followed by 40 cycles of denaturating at 95 ^{0}C for 30 sec, annealing at 58 ^{0}C for 30 sec, extension at 72 ^{0}C for 30 second. When cycles were completed, a final extension step at 72 ^{0}C for 7 minute applied (Yen *et al.*, 2008).

Amplification programme used was as follows:

Initial Denaturation	95 °C	3 min	
Denaturation	95 °C	30 sec	40 cycles
Annelaing	58 °C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	

The bands were analyzed on 2% agarose gel as described in **section 2.2.3.** 10 µl of PCR product was mixed with 4 µl of gel loading dye and applied to the wells of the gel. 5 µl of DNA ladder (50-1000bp, see Appendix B) was applied to one well. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed.

2.3.1.2 Restriction Endonuclease Digestion of PCR Products for *XRCC3* C18067T Polymorphism

Genotyping for *XRCC3* C18067T point mutation was carried out by digestion 10 µl of PCR product with 4 U of *Nla III* (*Hin 1II*) enzyme in a final volume of 30 µl reaction mixture. The components of the reaction mixture were given in Table 2.2.

Table 2.2 Components of digestion mixture for *XRCC3* Thr241Met

Constituents	Stock Concentration	Volume added (µl)	Final Concentration in 30 µl Reaction mixture
Buffer*	10x	3 µl	1x
PCR Product		10 µl	
Restriction Enzyme *(Nla III)*	5 U/µl	0.8 µl	4 U
		16.2 µl	
Sterile apyrogen H_2O			

* Tango™ Buffer (See Appendix B)

The reaction mixture was incubated overnight at 37 ^{0}C for 18 hours for complete digestion. The mutant type allele (Met allele) that contain T base at position 18067 bear a recognition sites around that nucleotide for *Nla III* restriction enzyme thus providing *Nla III* to cut the product from that position. And there is a one more recognition site within the amplified fragment which is an invariant restriction site around **17963**, which serves as an internal control for complete enzyme digestion. Therefore, *Nla III* digestion results in 3 bands of 105, 141 and 210 bp for the mutant type. However in wild type alleles with C in position **18067,** there is not a recognition site around the SNP, thus *Nla III* can not cut the amplified fragment around the SNP but it still cuts the invariant cut site. Therefore, wild type allele results in 2 bands of length 315 and 141 bp. Figure 2.2

shows the basis of *Nla III* digestion and represents the resulting fragments upon digestion in case of different genotypes.

Accordingly, the expected banding patterns upon digestion of amplified region in exon 7 of *XRCC3* gene with *Nla III* restriction enzyme were as follows: In homozygous wild types, absence of recognition sequences around SNP but presence of invariant cut site would yield 2 bands of 315 and 141 bp, while in homozygous mutants, as recognition sequence around SNP is presented due to base substitution (C at 18067 was substituted by T in mutant allele), 3 bands would be yielded. The heterozygotes would contain in total 4 bands in lengths of 315, 210, 141, and 105 bp.

Restriction products were analyzed on 2.5% agarose gel. 30 µl of digestion product was mixed with 6 µl of gel loading dye and applied to the wells of the gel. 6 µl of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run until the bromophenol blue reached to the bottom of the gel, visualized under UV and photographed.

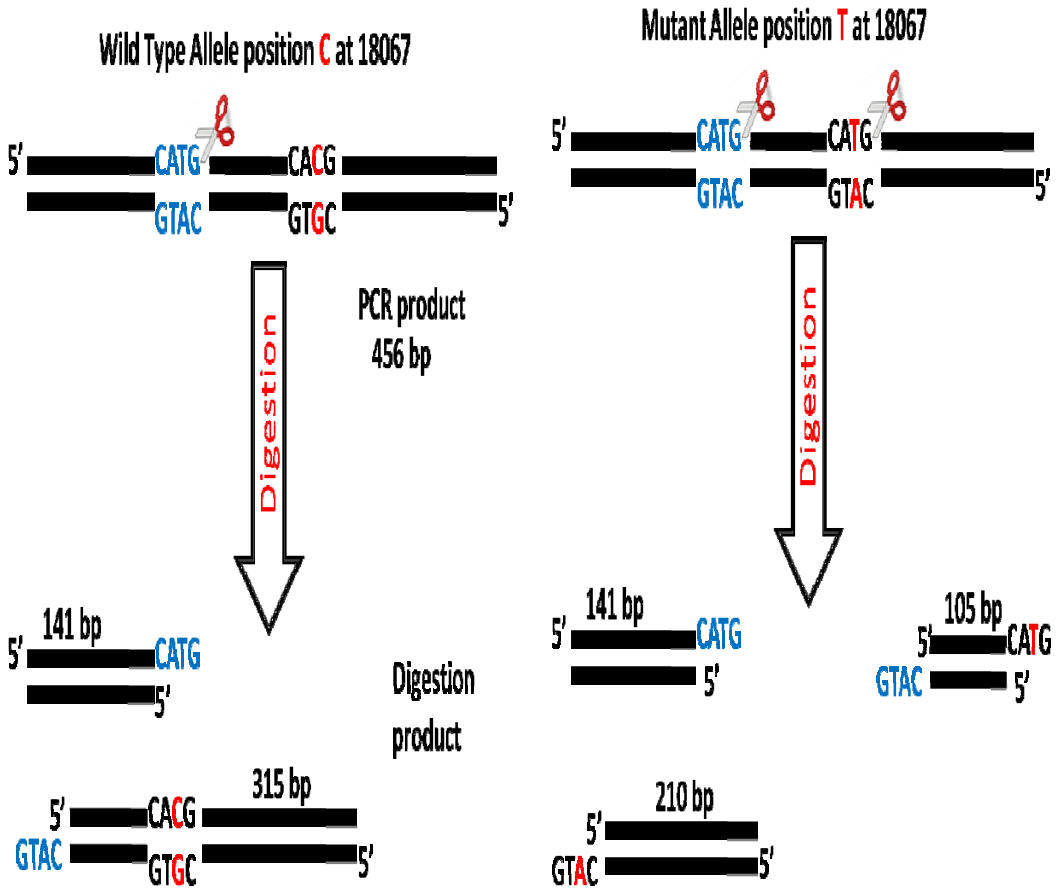

Figure 2-2 Schematic representation of the banding patterns of the amplified exon 7 region of *XRCC3* gene upon digestion with *Nla III* restriction enzyme.

2.3.2 Genotyping of *Rad51* G135C Polymorphism

In this study, *Rad51* gene was genotyped by PCR-RFLP method for the detection of *Rad51* G135C polymorphism located at position -135 in the 5' UTR region. The sequence of the amplified fragment, location of the SNP site and sequence of the recognition site for the restriction enzyme were given in Figure 2.3

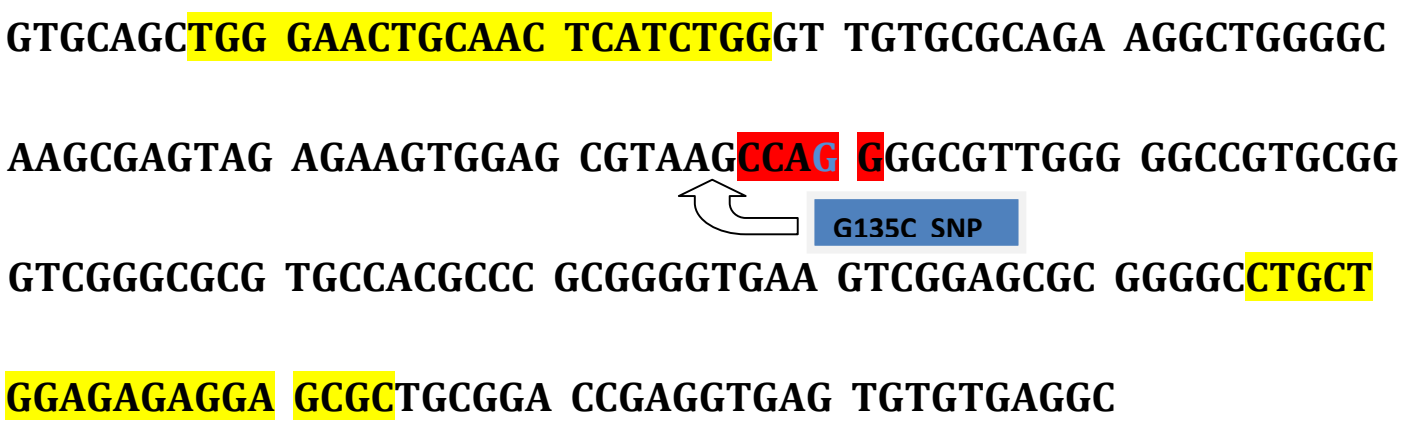

Figure 2-3 Sequence of amplified fragment around -135 base in 5' UTR region of *Rad51* gene that includes G135C SNP. The yellow highlighted sequences are forward and reverse primers. The red highlighted sequence shows the recognition site for the restriction enzyme *Mva I*. The blue marked nucleotide shows the location of G135C SNP. (The nucleotide sequence was taken from http://www.ncbi.nlm.nih.gov, last accesed on 13/01/2011)

2.3.2.1Polymerase Chain Reaction

G135C SNP is located at position -135 in the 5' UTR region. A 157 bp fragment covering G135C point mutation was amplified with forward primer Rd51F: 5'-TGGGAACTGCAACTCATCTGG and reverse primer XrR:5'-GCGCTCCTCTCTCCAGCAG (primer sequences were taken from Lahad *et al.*, 2001). In order to obtain a single band devoid of nonspecific bands, different parameters were tested in PCR. These parameters included the change in concentration of $MgCl_2$ as 0.5, 1.0, 1.25, 1.5 and 2 mM; change in concentration of primer pairs as 10, 25 and 40 pmol; and change in annealing temperature as 60, 61, 62, 63 and 64 ^{0}C. The optimized PCR conditions were given in Table 2.3.

Briefly, PCR reaction was carried out in 50 µl of a solution containing amplification buffer (supplied commercially with the Taq polymerase enzyme: 20 mM Tris HCl, 50 mM KCl; pH 8.5), 1.0 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM (10 pmol in 50 µl reaction mixture) of forward and reverse primers, approximately 200 ng of genomic DNA, 5% DMSO and 2.5 U of Taq polymerase.

Table 2.3 Components of PCR mixture for *Rad51* G135C

Constituents	Stock Concentration	Volume added (µl)	Final Concentration in 50 µl Reaction mixture
Taq buffer (KCl)	10x	5	1x
$MgCl_2$	25 mM	2	1.0 mM
dNTPmixture*	10 mM	1	200 µM
Forward Primer	10 pmol/µl	4	20 pmol
Reverse Primer	10 pmol/µl	4	20 pmol
Template DNA	Varies	Varies	200 ng
DMSO	100%	2.5	5%
Taq DNA Polymerase*	5 U/µl	0.5	2.5 U
dH_2O		To 50 µl	

* See Appendix B for these reagents

The PCR program used for the amplification of 5' upstream region consist of an initial denaturation step at 95 ^{0}C for 5 min followed by 35 cycles of denaturating at 95 ^{0}C for 30 sec, annealing at 62 ^{0}C for 30 sec, extension at 72 ^{0}C for 35 second. When cycles were completed, a final extension step at 72 ^{0}C for 7 minute applied.

Amplification programme used was as follows:

Initial Denaturation	95°C	3 min	
Denaturation	95°C	30 sec	35 cycles
Annelaing	62°C	30 sec	
Extension	72°C	35 sec	
Final Extension	72°C	7 min	

The bands were visualized as described in **section 2.3.1.1**. Instead of using bromophenol blue, xylene cyanol was used as a gel loading dye since bromophenol blue runs as the same speed as pcr products run which causes covering of pcr product and preventing clear observation of bands.

2.3.2.2 Restriction Endonuclease Digestion of PCR Products for *Rad51* G135C Polymorphism

Genotyping for *Rad51* G135C point mutation was carried out by digestion of 10 µl of PCR product with 10 U of *Mva I* (*BstNI*) enzyme in a final volume of 30 µl reaction mixture. The components of the reaction mixture were given in Table 2.4.

Table 2.4 Components of digestion mixture for polymorphism of *Rad51* G135C

Constituents	Stock Concentration	Volume added (µl)	Final Concentration in 30 µl Reaction mixture
Buffer*	10x	3 µl	1x
PCR Product		10 µl	
Restriction Enzyme *(Mva I)*	10 U/µl	1 µl	10 U
		16 µl	
Sterile apyrogen H_2O			

* Buffer R™ Buffer (See Appendix B)

The reaction mixture was incubated overnight at 37 ^{0}C for 18 hours for complete digestion. The wild type allele that contain G base at position -135 in 5' UTR region bear a recognition sites around that nucleotide for *Mva I* restriction enzyme thus providing *Mva I* to cut the PCR product from that position. Therefore, wild type allele results in 2 bands of lengths 86 and 71 bp. However in mutant type allele with C in position -135 in 5' UTR region**,** there is not a recognition site around the SNP, thus *Mva I* can not cut the amplified fragment around the SNP. Figure 2.4 shows the basis of *Mva I* digestion and represents the resulting fragments upon digestion in case of different genotypes.

Accordingly, the expected banding patterns upon digestion of amplified region around -135 of *Rad51* gene with *Mva I* restriction enzyme were as follows: In homozygous wild types, presence of recognition sequences around SNP would yield 2 bands of 71 and 86 bp, while in homozygous mutants, as recognition sequence around SNP is lost due to base substitution (G/C), single band (157 bp) is expected. Nucleotide position was substituted by T in mutant allele), 3 bands would be yielded. The heterozygotes would contain in total 3 bands in lengths of 157, 86, and 71 bp.

Restriction products were analyzed on 2.5% agarose gel. 30 µl of digestion product was mixed with 6 µl of gel loading dye and applied to the wells of the gel. 6 µl of DNA ladder (50-1000 bp) was applied to the first well of the gel. The gel was run for 1 hour, and visualized under UV and photographed.

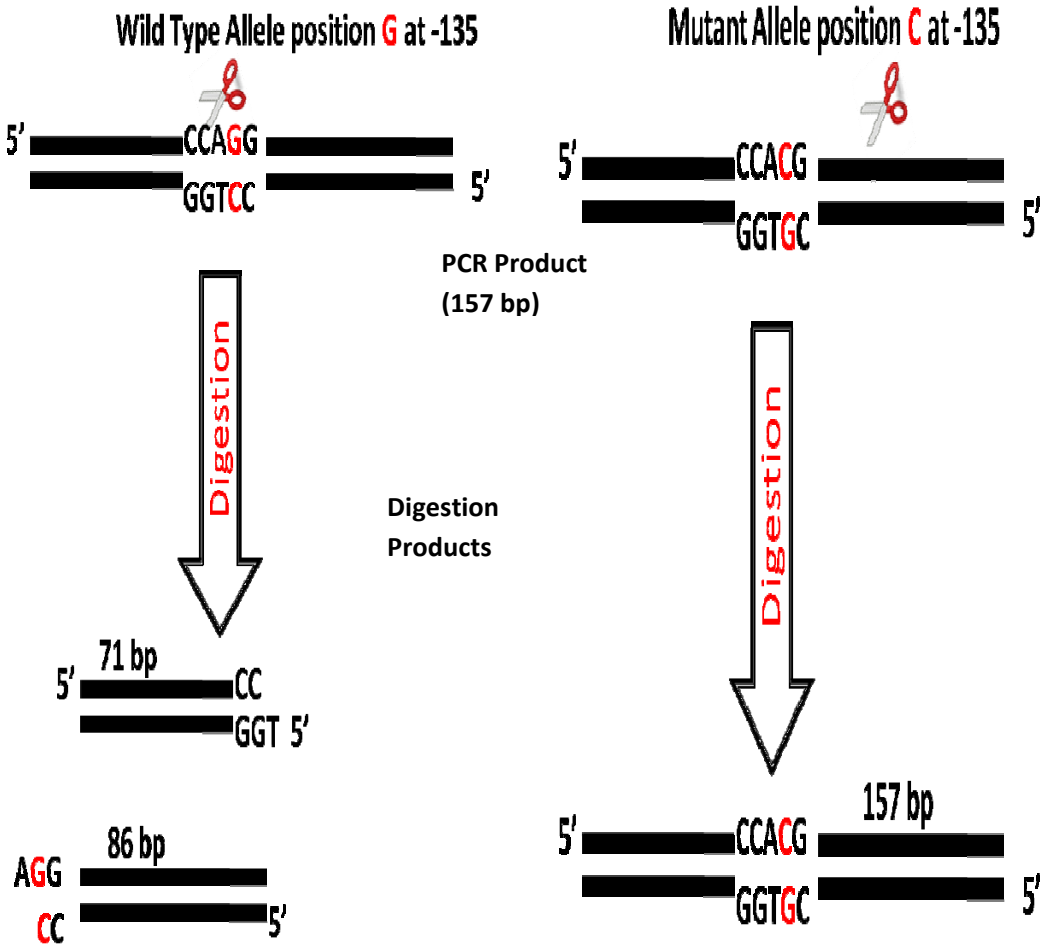

Figure 2-4 Schematic representation of the banding patterns of the amplified 5' UTR region of *Rad51* gene upon digestion with *Mva I* restriction enzyme.

2.4 Statistical Analysis

The allele frequency for each allele was determined by gene counting. The allele and genotype frequencies of patients were given together with the 95% confidence interval (CI). Statistical analysis were conducted using SPSS software. The genotype distributions of polymorphisms were compared by

χ^2 – test (Pearson), Yate's correction factor was used if necessary. A p value< 0.05 was considered to be statistically significant throughout the population comparision. Comparisions of genotypes in control and patient groups was done by calculation odds ratios (OR). The formula of Chi-Square test is given below:

$$X^2 = \sum \frac{(|\ \text{Observed frequency} - \text{Expected frequency}\ | - 0.5)^2}{\text{Expected frequency}} \qquad (2.2)$$

CHAPTER 3

RESULTS

3.1 Study Populations

3.1.1 Control Population

Control population comprised of 193 healthy Turkish volunteers between the ages of 12 and 65 and mean age of 31.3 ±12.0. Among the control groups, 77 were male (mean age: 32.3 ± 12.3; range: 14-65 years) and 116 were female (mean age: 30.7 ± 11.9; range: 12-63 years). The number of controls involved in this study and corresponding means for ages is represented in Table 3.1.

Table 3.1 Number of control samples involved in the study and their corresponding mean age, and range of their ages

Control Sample	Number	Mean Age (years)	Range (years)
Whole Controls	193	31.3 ±12.0	12-65
Females	116	30.7 ± 11.9	12-63
Males	77	32.3 ± 12.3	14-65

Subjects having dieases- like any type of cancer, diabetes, etc.- were excluded from the study. The blood samples were collected from Middle East Technical University (METU) Health Center, Biochemistry Laboratory. As obtained from the birth place information of volunteer and his/her parents, all the regions of Turkey were equally represented by the control population. So it could be concluded that control population is a sample of Turkish population. The distribution of control subjects according to their birth place was given in Figure 3.1.

(A) **(B)**

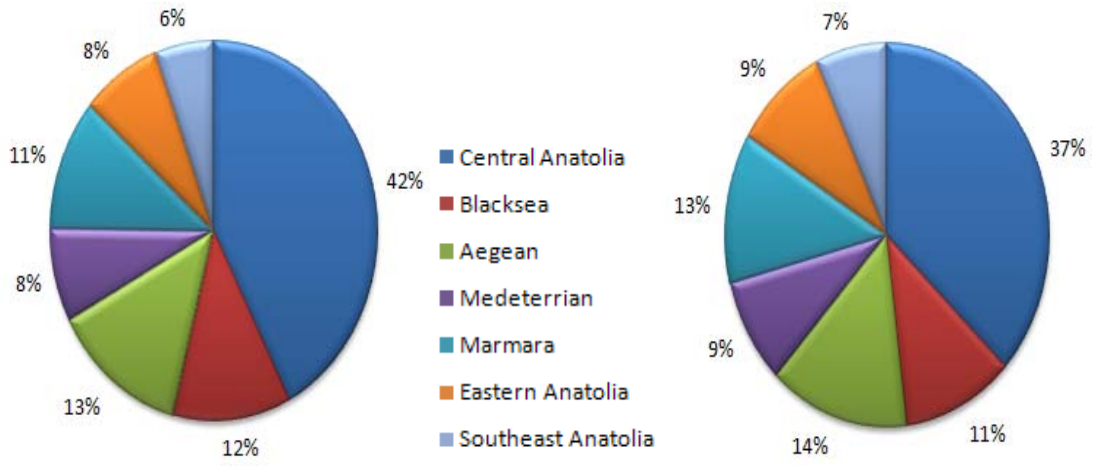

Figure 3-1 Distribution of control subjects to seven regions of Turkey, according to **A)** The birth place of subject; **B)** The birth place of parents of the subjects.

3.1.2 Patient Population

The diagnosis of ALL was made by using French-American-British (FAB) criteria after conventional cytochemical and surfacemarker analysis (Tumer *et al.*, 2010). The FAB classification categorizes leukemia into 8 subtypes based on the type of cell and its degree of maturity. Diagnosis is made by examining the appearance of the malignant cells under light microscopy and also by using cytogenetics to characterize chromosomal abnormalities. For ALL diagnosis, lymphocytes are counted and examined. Differentiation of blood cells and their types were indicated in Figure 3.2. Patient population was comprised of 75 female (mean age 6.9 ± 3.9 years; range: 0.6-16 years) and 109 male (mean age 7.0 ± 3.9 years; range: 2-16 years), in total 184 children (mean age: 7.0 ± 3.8 years; range: 0.6-16 years). Treament of these patients were applied in Ankara University, Faculty of

Medicine, Department of Pediatric Hematology and Sami Ulus Children's Hospital which are one of the most crowded health centers of the Turkey and these hospitals receive patients from all over the country. So it could be said that patient sample is a representative of the Turkish population, which was proved by the demographic data of the samples. Information about birth place of patients and their parents was given in Figure 3.3

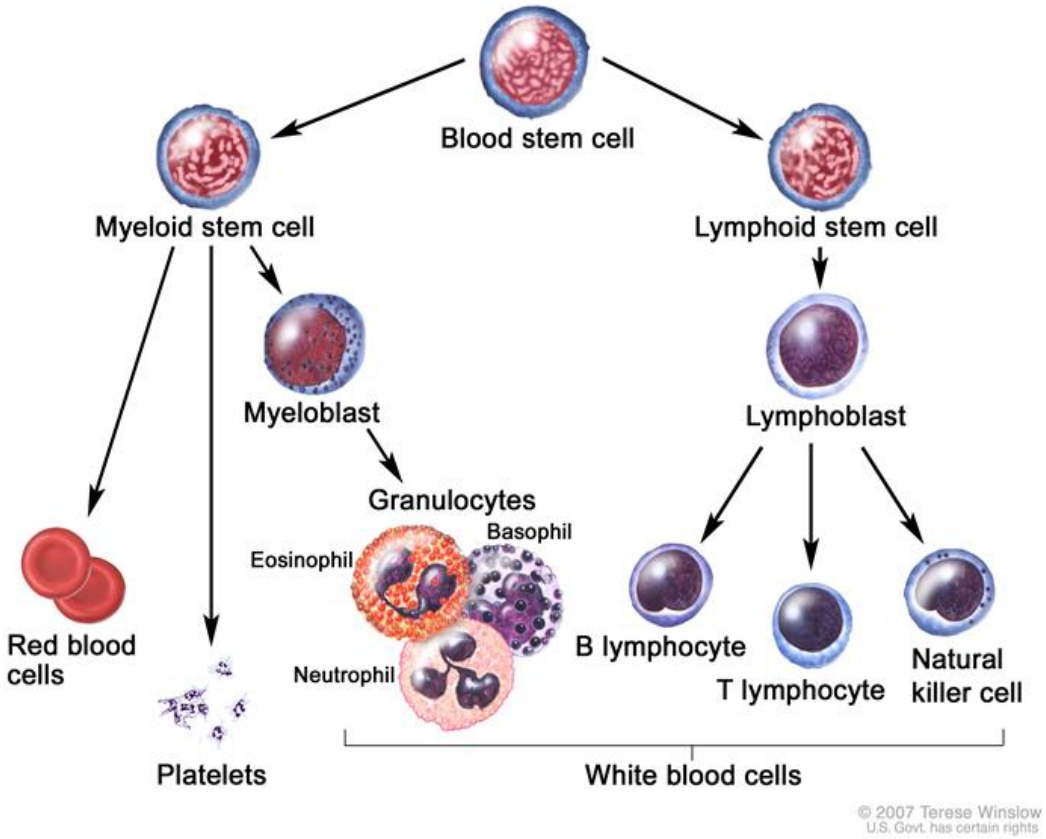

Figure 3-2 Blood stem cell differentiation and blood cells types (Retrieved from http://www.cancer.gov/cancertopics/pdq/treatment/childALL/Patient last accesed on 10/01/2011).

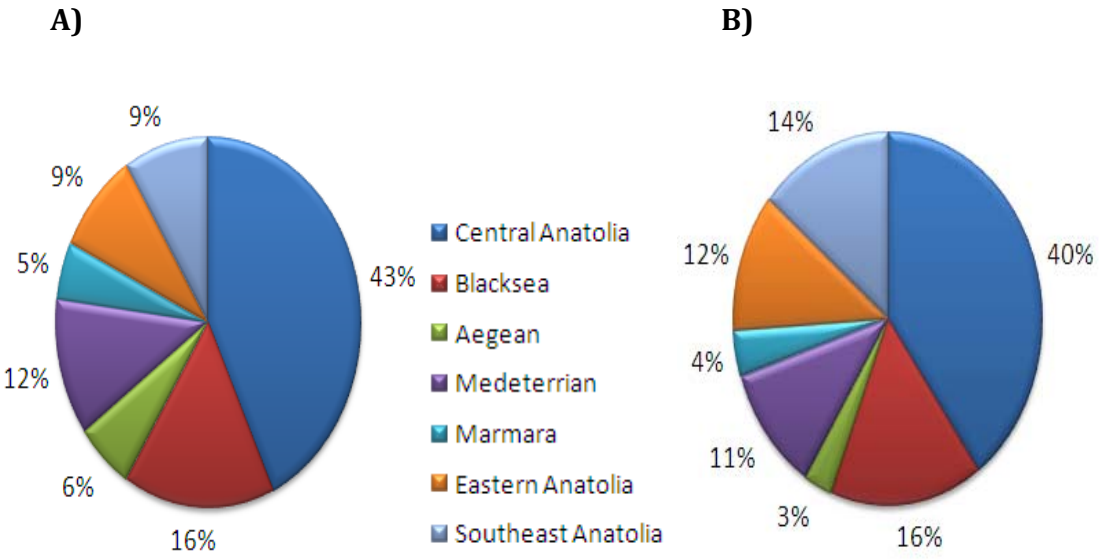

Figure 3-3 Distribution of patients to seven regions of Turkey, according to **A)** The birth place of patient; **B)** The birth place of parents of the patients**.**

The questionnaires about parents' smoking status, and medical logs of the patients presented some information about the characteristic of the patient population. This information was summarized in Table 3.2 and below within the text. However, data was not available for all patients due to some unanswered questions in questionnaires by patients' parents.

Table 3.2 (A) Characteristics of the patient population and **(B)** Information gathered from cigarette smoking questionnaire. (Data is collected by Gürses Şahin and modified from Ulusoy Ph.D. Thesis, 2009)

(A)

Characteristic	**Total Patient**	**Male Patients**	**Female Patients**
N	184	109	75
Age at diagnosis	7.0 ± 3.8 years; range: 0.6-16 years	7.0 ± 3.9 years; range: 2-16 years	6.9 ± 3.9 years; range: 0.6-16 years
Risk Group	171 (13 missing)	104 (5 missing)	67 (8 missing)
Low Risk	17 (9.94%)	9 (8.7%)	8 (12.0%)
Standard Risk	63 (36.8%)	39 (37.5%)	24 (35.8%)
High Risk	91 (53.2%)	56 (53.8%)	35 (52.2%)
Familial relationship between parents	163 (21 missing)	97 (12 missing)	66 (9 missing)
Present	43 (26.4%)	27 (27.8%)	16 (24.2%)
Absent	120 (73.6%)	70 (72.2%)	50 (75.8%)

Table 3.3 Cont'd

(B)

Information obtained from cigarette smoking questionnaire		
Maternal age at conception	**Total Patients**	
	109 (75 missing)	
Mean age	26.4 ± 6.9 years; range 16-54 years	
<20 years of age	13 (11.9%)	
20-35 years of age	80 (73.4%)	
>35 years of age	16 (14.7%)	
Paternal age at conception	**Total Patients**	
	N=106 (78 missing)	
Mean age	30.2 ± 9.1 years; range 16-68 years	
<40 years of age	92 (86.8%)	
>40 years of age	14 (13.2%)	
Parental Smoking Status	**Maternal**	**Paternal**
N	109 (75 missing)	108 (76 missing)
Non-smoker	30 (27.5%)	22 (20.4%)
Passive smoker	59 (54.1%)	5 (4.6%)
Active smoker	20 (18.4%)	81 (75.0%)
Maternal smoking during pregnancy	**Total Patients**	
	109 (75 missing)	
Smoked	12 (11.0%)	
Not smoked	97 (89.0%)	
Postnatal exposure of child to cigarette smoke	**Total Patients**	
	107 (77 missing)	
Exposed	71 (66.4%)	
Not exposed	36 (33.6%)	

For an efficient treatment of patients, they are grouped into risk groups at the time of diagnosis which are low risk group, standard risk group and high risk groups. The risk group information assigned to determine the intensity of the therapy and was available for 171 patients. (Information on 13 patients was missing). In total, 17 (9.94%) patients were assigned to low-risk group, 63 (36.8%) patients were assigned to standard risk group and 91 (53.2%) were assigned to high risk group. There was no statistically significant a difference between each risk groups and gender of the patients ($\chi 2$ =0.45; df=2; and p=0.8).

From the "smoking status questionnaires" information about maternal age and paternal age at conception, parental smoking status, maternal smoking status during pregnancy and postnatal exposure of child to cigarette smoke were obtained and were summarized at below paragraphs.

Answers for question about maternal age at conception were available for 109 patients' mother and 106 patients' father. The mean age of mothers at conception was 26.4 $\pm$ 6.9 years, range 16-54 years old and fathers' mean age was 30.2 $\pm$ 9.1 years, range 16-68 years old. Mothers' age was divided into three age range- below 20, between 20 and 35 and older than 35. 13 (11.9%) of them were younger than 20 years of age, 80 (73.4%) were at the ages between 20 and 35 and 16 (14.7%) of them were older than 35 years of age at the time of conception. The father of ages are grouped into two categories, ages older than 40 and ages younger than 40. Among fathers 92 (86.8%) of them were younger than 40 years of age and

14 (13.2%) of them were older than 40 years of age at the time of conception.

Answers for question about smoking status of parents were available for 109 mothers and 108 fathers. Among 109 mothers, 30 (27.5) of them were non-smokers, 59 (54.1%) of them were passive smokers and 20 (18.4%) of them were active smokers. Among these 20 active smokers, 12 (11.0%) mothers smoked also during the pregnancy period. Remaining 97 (89.0%) mothers did not smoke during pregnancy. Among 108 fathers, 22 (20.4%) were non-smokers, 5 (4.6%) were passive smokers and 81 (75.0%) were active smokers. The question about postnatal exposure of child to cigarette smoke were replied by 107 parents, and 71(66.4%) of them indicated that their children were exposed to cigarette smoke, whereas 36 (33.6%) of them indicated that their children were not exposed to cigarette smoke during postnatal period.

The information gathered from questionnaire was only available for patient population, not for control population, so that these non-genetic factors as indicated above were investigated to evaluate their interaction with genetic polymorphisms of *XRCC3* and *Rad51* genes by using case-only approach. This investigation was done to observe any effects of these non-genetic factors on having risky allele.

This study were performed to investigate the role of genetic polymorphisms of DNA repair genes-*XRCC3* and *Rad51*- on the development of childhood ALL. The genotyping of single nucleotide polymorphisms of these two genes were done for all 193 control and 184 patient sample.

3.2 Case Control Analyses

In order to investigate the interaction between genetic polymorphisms of these genes and childhood ALL, genotypes of patients (or cases) group were compared to genotypes of control group. The statistical value used for comparison between the groups is the "odds ratio (OR)". OR is calculated by proportioning the ratio of numbers of patients having risk elevating allele (heterozygote mutant or homozygote mutant) to numbers of patient having not risky allele (homozygote wild type) and ratio of numbers of controls having risk elevating allele (heterozygote mutant or homozygote mutant) to numbers of controls having not risky allele (homozygote wild type). Simply, formula of calculation for OR was given below:

$$\mathbf{OR} = (Case_{Risk} / Case_{No\ Risk}) \,/\, (Control_{Risk} / Control_{NoRisk})$$

Odds of Case Odds of Control

From the formula, OR is the proportion of case's odds to control's odds. An Odds ratio significantly greater than 1 indicates that odds of case is higher than odds of control, in other words number of subjects having risky allele in patient group is higher than number of subjects having risky allele in control group. So this denotes the association of the risk elevating genotype with the disease. Odds ratio significantly less than 1 indicates the protective role of the genotype for the disease, since risky allele present in

higher values in control group. If odds ratio value is close to 1, there is no association between the risky genotype and disease. In other words, having homozygote mutant or heterozygote genotype is not a risk factor for the development of ALL. Odds ratio is an essential term for genetic epidemiologic studies since it verifies either the presence or absence of association and if present, it shows the magnitude of it, in terms of times-fold of risk. (Green *et al.*, 2000)

Investigation of genetic risk factors for development of a disease state can be studied on a single gene polymorphism and also with combinations of several gene polymorphisms. When association was studied based on one gene polymorphism, than homozygote wild type genotype was stated as "no-risk" group, whereas heterozygote and homozygote mutant genotypes were stated as "risk" group as it is stated above. On the other hand, if association of several genes were studied, any combination of genes having heterozygote or homozygote mutant within were stated as "risk" group, whereas in "no-risk" group all genotypes were in homozygote wild type. Risk groups were analyzed against no-risk groups. In that study, single nucleotide polymorphism of DNA repair genes were studied separately and then their combined effects were tested.

3.2.1 Genetic Risk Factors for the Development of Childhood ALL

In this study, single nucleotide polymorphism of two homologous recombination DNA repair genes-*XRCC3* and *Rad51*- were investigated as risk factors for the development of childhood ALL. *XRCC3* Thr241Met and *Rad51* G135C polymorphisms were determined in 193 control sample and 184 patient samples.

3.2.1.1*XRCC3* Thr241Met (C18067T SNP) as Genetic Risk Factor

C18067T single nucleotide polymorphism is located on exon 7 region of the homologous recombination DNA repair gene *XRCC3*. Substitution of wild type C base with T base at that point results in replacement of Threonine aminoacid with Methionine aminoacid at position of 241st of encoded protein. Wild type allele is indicated as Thr, and mutant allele is indicated as Met.

Genotyping of *XRCC3* Thr241Met polymorphism were performed by using PCR-RFLP method. A 456 bp fragment, obtained by PCR, covering C18067T point mutation were digested with *Nla III* (*Hin 1II*) enzyme. The mutant type allele (Met allele) that contain T base at position 18067 bear a recognition sites (recognition sequence 5'-CATG↓-3') around that nucleotide for *Nla III* restriction enzyme thus providing *Nla III* to cut the product from that position. And there is a one more recognition site within the amplified fragment which is an invariant restriction site around base at 17963 position, which serves as an internal control for complete enzyme digestion. Therefore, Nla III digestion resulted in 3 bands of 105, 141 and 210 bp for the mutant type (Figure 3.4, lanes 5, 10). However in wild type alleles with C in position **18067,** there is not a recognition site around the SNP, thus *Nla III* can not cut the amplified fragment around the SNP but it still cuts the invariant cut site. Therefore, wild type allele resulted in 2 bands of length 315 and 141 bp (Figure 3.3, lanes 3, 4, 8, and 9). The heterozygotes resulted in total 4 bands in lengths of 315, 210, 141, and 105 bp. (Figure 3.3, lanes 1, 2, 6, and 7).

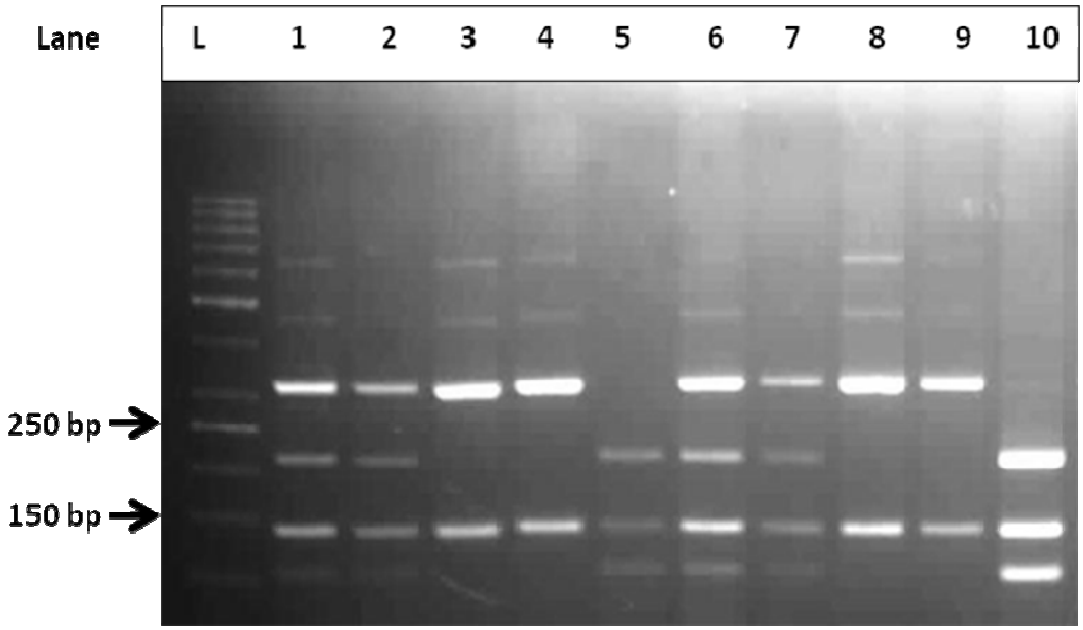

Figure 3-4 Agorose Gel photo for *Nla III* digestion of amplified exon 7 region of *XRCC3* gene. L stands for DNA Ladder (50-1000 bp). Lanes **3, 4, 8** and **9** represent the band patterns of individuals having homozygote wild type genotype of *XRCC3* with bands of length 315 and 141 bp. Lanes **1, 2, 6** and **7** represent the band patterns of individuals having heterozygote genotype of *XRCC3* with bands of length 315, 210, 141 and 105 bp. Lanes **5** and **10** represent the band patterns of individuals having homozygote mutant genotype of *XRCC3* with bands of length 210, 141, and 105 bp.

The genotype distributions and allele frequencies of *XRCC3* Thr241Met polymorphism were given in Table 3.3. In total of 184 patient sample, 89 individuals (48.4%) were homozygote wild type. In total of 193 control sample, 66 individuals (34.2%) were homozygote wild type. The frequency is higher in patient group. For heterozygosity, 71 individuals (38.6%) out of 184 patient sample were heterozygote, whereas in 193 control sample, 90 individuals (46.6%) were heterozygote. The frequency

difference is statistically higher in control sample compared to patient sample (OR=0.59; 95% CI=0.38-0.91; *p*=0.018). Since odds ratio is less than 1, having heterozygote genotype has a role of protective factor for development of ALL with an 0.59 fold. For homozygote mutant case, 24 individuals (13.0%) out of 184 patients were homozygote mutant, whereas 37 individuals(19.2%) out of 193 control sample were found to be homozygote mutant genotype. The allele frequency distribution is statistically different in patient group compared to control sample (OR=0.48; 95% CI=0.26-0.88; *p*=0.017). So, possesing a homozygote mutant genotype is a protective factor for development of ALL with an factor of 0.48 fold. In other words, having homozygote mutant genotype results in 0.48 fold less risk for development of ALL disease. When heterozygote and homozygote mutant individuals were combined, the frequency distribution was again found as statistically diferent between control and patient group. (OR=0.55; 95% CI=0.37-0.84; *p*=0.005). When allele frequency distrubition was calculated, 67.7% of ALL patients had Thr241 (wild type) allele (N=184), whereas 57.5% of control population had Thr241 allele (N=193). The distribution of allele frequencies between patient group and control sample is statistically different. (OR=0.65; 95% CI=0.48-0.87; *p*=0.004). Therefore, considering frequencies of allele and genotypes both in control and patient group, it can be concluded that mutant allele has a protective role for childhood ALL development.

Upto now, there is not a study about genotype and allele frequencies of *XRCC3* Thr241Met polymorphisim for Turkish population. This study provided genotype distribution and allele frequency for *XRCC3* Thr241Met polymorphism. From that study, genotype of homozygote wild type was 34.2%, genotype of heterozygote mutant type was 46.6% and genotype of

homozygote mutant type was 19.2% for Turkish population regarding control sample. The frequency of wild type allele was 0.58%, and frequency of mutant allele was 0.42 for Turkish population.

Table 3.4 Frequencies of *XRCC3* Thr241Met genotypes and alleles in ALL patients (n=184) and controls (n=193) and Odd Ratios.

Genotype	Patients (n=184)	Controls (n=193)	OR (95% CI)	P
XRCC3 Genotypes, n (%)				
Wild types (Thr241/ Thr241)	89 (48.4%)	66 (34.2%)	1.0 (Referent)	
Wt/Mut (Thr241/Met241)	71 (38.6%)	90 (46.6%)	0.59 (0.38-0.91)	**0.018***
Mut/Mut (Met241/ Met241)	24 (13.0%)	37 (19.2%)	0.48 (0.26-0.88)	**0.017***
Thr241/Met241plus Met241/ Met241	95	127	0.55 (0.37-0.84)	**0.005***
Allele frequency				
Total	368	386		
Thr241	249 (67.7%)	222 (57.5%)	1.0 (Referent)	
Met241	119 (32.3%)	164 (42.5%)	0.65 (0.48-0.87)	**0.004***

*Statistically significant difference p<0.05

In Table 3.4, comparision of allele frequencies of *XRCC3* Thr241Met polymorphism obtained from control sample of that study for Turkish population with different control populations were given. In all

comparisions, allele frequencies of Turkish population were significantly different than other populations except for Turkish population studied by Atar *et al.,* (2010). Wild type allele frequencies were significantly higher in all populations except for Atar *et al.* (2010) studies compared to Turkish population ($p < 0.05$). The allele frequencies were found to be very similar to Turkish population studied by Atar with 101 control samples.

Table 3.5 The *XRCC3 Thr241Met* allele frequencies in different ethnic populations

Populations	**Allele Frequency**		**N of alleles**	**References**
	Thr	***Met***		
Turkish	**0.575**	**0.425**	**386**	**This Study**
Turkish	0.564	0.436	202	Atar *et al.,* 2010
Brazil	0.73	0.27	236	Dufloth *et al.*,2005
Spanish	0.64	0.36	868	Cima *et al.*, 2007
Chinese	0.82	0.18	1724	Long MD *et al.*, 2008
South American	0.77	0.23	1000	Jara *et al.*, 2009
Taiwan	0.96	0.04	196	Yen *et al.*, 2007
Thai	0.93	0.07	1014	Sangrajrang *et al.,* 2007

3.2.1.2*Rad51* G135C SNP as Genetic Risk Factor

G135C single nucleotide polymorphism is located at position -135 in the 5' UTR region of the homologous recombination DNA repair gene *Rad51*. Substitution of wild type G base with C base at that point does not result in replacement of any aminoacids since this base at non coding region of the

gene. G135 stands for wild type allele, whereas 135C stands for mutant allele.

Genotyping of *Rad51* G135C polymorphism were performed by using PCR-RFLP method. A 157 bp fragment, obtained by PCR, covering G135C point mutation were digested with *Mva I* enzyme. The wild type allele containing G base at position -135 bear a recognition sites (recognition sequence 5'-CCA↓*G*G- 3') around that nucleotide for *Mva I* restriction enzyme thus providing *Mva I* to cut the product from that position. Therefore, wild type allele results in 2 bands of lengths 86 and 71 bp (Figure 3.3, lanes , 2, 3, 4, 5, 6, 7, 8 and 9). However in mutant type allele with C in position -135 in 5' UTR region**,** there is not a recognition site around the SNP, thus *Mva I* can not cut the amplified fragment around the SNP. Therefore, mutant type allele resulted in 1 band of length 157 bp (Figure 3.5, lane 10). The heterozygotes resulted in total 3 bands in lengths of 157, 86, and 71 bp. (Figure 3.3, lane 11).

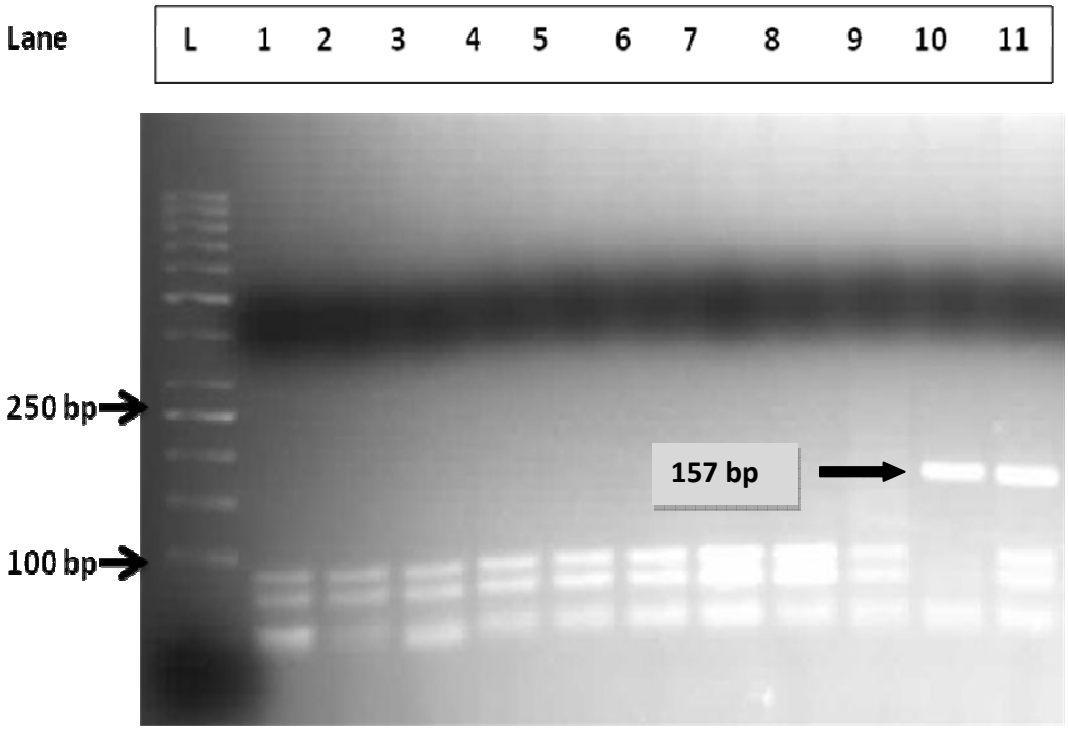

Figure 3-5 Agorose Gel photo for *Mva I* digestion of amplified 5' upstream -135 region of *Rad51* gene. L stands for DNA Ladder (50-1000 bp). Lanes **1, 2, 3, 4, 5, 6, 7, 8** and **9** represent the band patterns of individuals having homozygote wild type genotype of *Rad51* with bands of length 86 and 71 bp. Lane **11** represent the band patterns of individuals having heterozygote genotype of *Rad51* with bands of length of 157, 86, and 71 bp. Lane **10** represent the band patterns of individuals having homozygote mutant genotype of *Rad51* with band of length 157 bp.

The genotype distributions and allele frequencies of *Rad51* G135C polymorphism were given in Table 3.5. In total of 184 patient sample, 152 individuals (82.6%) were homozygote wild type. In total of 193 control sample, 154 individuals (79.8%) were homozygote wild type. The frequencies are very similar to each other. For heterozygosity, 30 individuals (16.3%) out of 184 patient sample were heterozygote, whereas in 193 control sample, 37 individuals (19.2%) were heterozygote. The frequency difference was not statistically important (OR=0.82; 95%

CI=0.48-1.40; *p*=0.47). For homozygote mutant case, 2 individuals (1.10%) out of 184 patients were homozygote mutant, and 2 individuals (1.00%) out of 193 control sample were found to be homozygote mutant genotype. The frequency distribution was not statistically valuable (OR=1.01; 95% CI=0.14-7.29; *p*=0.99). So, possesing a homozygote mutant genotype or heterozygote mutant genotype is not a risk factor for development of ALL. When heterozygote and homozygote mutant individuals were combined, the frequency distribution was not again found as statistically diferent between control and patient group. (OR=0.83; 95% CI=0.50-1.40; *p*=0.48). When allele frequency distribution was calculated, 90.8% of ALL patients had G135 (wild type) allele (N=184), and 89.4% of control population had 135C allele (N=193). The distribution of allele frequencies between patient group and control sample was not statistically different. (OR=0.86; 95% CI=0.53-1.38; *p*=0.53). Therefore, considering frequencies of allele and genotypes both in control and patient group, it can be concluded that mutant allele was not a risk factor for development of childhood ALL.

Up to now, there is not a study about genotype and allele frequencies of *Rad51* G135C polymorphisim for Turkish population. This study provided genotype distribution and allele frequency for *Rad51* G135C polymorphism. From that study, genotype of homozygote wild type was 79.8%, genotype of heterozygote mutant type was 19.2% and genotype of homozygote mutant type was 1.0% for Turkish population regarding control sample. The frequency of wild type allele was 0.89%, and frequency of mutant allele was found to be 0.11 for Turkish population considering control sample.

Table 3.6 Frequencies of *Rad51* G135C genotypes and alleles in ALL patients (n=184) and controls (n=193) and Odd Ratios**.**

Genotype	ALL Patients (n=184)	Controls (n=193)	OR (95% CI)	P
Rad51 Genotypes, n (%)				
Wild types (G135/G135)	152 (82.6%)	154 (79.8%)	1.0 (Referent)	
Wt/Mut (G135/135C)	30 (16.3%)	37 (19.2%)	0.82 (0.48-1.40)	0.47
Mut/Mut (135C/135C)	2 (1.1%)	2 (1.0%)	1.01 (0.14-7.29)	0.99
G135/135C plus 135C/135C	32	39	0.83(0.5-1.40)	0.48
Allele frequency				
Total	368	386		
G135	334 (90.8%)	345 (89.4%)	1.0 (Referent)	
135C	34 (9.2%)	41 (10.6%)	0.86 (0.53-1.38)	0.53

In Table 3.6, comparision of allele frequencies of obtained from control sample of that study for Turkish population with different control populations were given. In all comparisions, allele frequencies of Turkish population were significantly different than other populations except for Werbrouck *et al.*, studies with Caucasian population and USA population. Wild type allele frequencies were significantly higher in all populations compared to Turkish population ($p < 0.05$).

Table 3.7 The *Rad51 G135C* allele frequencies in different ethnic populations

Populations	Allele Frequency		N	References
	G135	***135C***		
Turkish	**0.89**	**0.11**	**386**	**This Study**
South American	0.94	0.06	1000	Jara *et al.*, 2009
Portuguese	0.94	0.06	870	Costa *et al.*, 2006
British	0.93	0.07	1872	Rollinson *et al.*, 2006
Caucasian	0.93	0.07	314	Werbrouck *et al.*, 2008
South Australia	0.95	0.05	264	Yeoh *et al.*, 2009
European Caucasian	0.94	0.06	1694	Auranen *et al.*, 2005
USA	0.93	0.07	838	Auranen *et al.*, 2005

3.2.1.3 Combination of *RAD51* G135C SNP and *XRCC3* Thr241Met Polymorphisms as Genetic Risk Factor for Childhood ALL

Mutant allele of *XRCC3* Thr241Met gene (Met) was found to have a significant protective role for childhood ALL development, Whereas mutant allele of *Rad51* G135C (C allele) was found not to have any effect on disease development or occurence. *XRCC3* Thr241Met was alone have a protective role in disease progression, but with combined effect of any type of polymorphism of *Rad51* gene, it might have higher protective role, or *Rad51* could have a risk factor with combined effect of a type of polymorphic form of *XRCC3*. All possible combination of two genes genotypes (9 possibilities), and their distribution was presented in Table 3.7. Homozygote wild type genotype of *XRCC3* and homozygote genotype of *Rad51* assigned as no-risk group, and other combinations were analyzed against that group. Whatever

genotype is *Rad51*, when genotype of *XRCC3* is homozygote is wild type, there was not a statistically differences between patient and control sample. Combination of heterozygote *XRCC3* Thr/Met genotype with homozygote or heterozygote *Rad51* genotype was found as significantly different between patient and control sample. Combination of *XRCC3* heterozygote genotype and *Rad51* heterozygote genotype increased the protective factor for risk of childhood ALL with an odds ratio of 0.35. (95% CI= 0.14-0.90; p =0.02). Heterozygote genotype of *XRCC3* alone had an protective factor of 0.59 fold, together with heterozygote *Rad51* it increased to 0.35 fold. Combination of Thr/Met genotype with homozygote mutant genotype of *Rad51* were not associated with the risk of development of childhood ALL (OR= 1.3; 95% CI=0.12- 15.0; *p*=0.82), but the number of individuals in that genotypes were very low due to low frequency of homozygote mutant genotype of *Rad51* (1.1% for patient sample, 1.0% for control sample). Combination of homozygote mutant genotype of *XRCC3* with homozygote wild type genotype of *Rad51* gave a highly statistically proved protective factor for development of disease (OR= 0.36; 95% CI = 0.18- 0.73; *p*= 0.004). Combination of homozygote mutant genotype of *XRCC3* with heterozygote mutant genotype of *Rad51* was not associated with the risk of development of childhood ALL (OR= 0.77; 95% CI= 0.25-2.43; *p*= 0.66). Homozygote mutant allele of *XRCC3* gene alone had a protective factor for disease development, but not with combination of it with heterozygote mutant genotype of *Rad51*, since number of individuals having heterozygote mutant allele for *Rad51* were too low both for patient and control sample (16.3%, and 19.2%, respectively) There was no any individual having homozygote mutant allele for both gene.

Table 3.8 Combination analyses for *XRCC3 Thr241Met* polymorphism and *Rad51* G135C gene polymorphism as risk factors for the development of childhood

Genotype of *XRCC3* Thr241Met	**Genotype of *Rad51* G135C**	**Control (N=193)**	**ALL (N= 184)**	**OR (95% CI)**	**P**
Thr/Thr	GG	49	74	1.0 (Referent)	-
Thr/Thr	GC	16	15	0.62 (0.28-1.37)	0.24
Thr/Thr	CC	1	0	-	-
Thr/Met	GG	74	61	0.55 (0.33-0.90)	**0.016***
Thr/Met	GC	15	8	0.35 (0.14-0.90)	**0.02***
Thr/Met	CC	1	2	1.3 (0.12-15.0)	0.82
Met/Met	GG	31	17	0.36 (0.18-0.73)	**0.004***
Met/Met	GC	6	7	0.77 (0.25-2.43)	0.66
Met/Met	CC	0	0	-	-

*Statistically significant difference p<0.05

3.3 Effect of Non-Genetic Factors on the Risk of Genetic Factors for the Development of Childhood ALL : Case- Only Analyses

In previous chapters, association between genotypes of two genes with a disease state was investigated seperately and combined by using case-control analyses with help of odds ratio value. Odds ratio is a value which gives the ratio of proportion of risky genotype present in case group to risky genotype present in control group. When information about control group was not present, and effects of non-genetical factors were investigated, "Case-Only Odd Ratio (COR)" was used. Like odds ratio, COR is used to show the interaction between two variables, and the magnitude of it. The logic is to assess the magnitude of the interaction between the exposure of interest (like patient and control group for OR) and susceptibility genotype among case subjects. This value was calculated by a new 2 by 2 table, and model for that calculation was represented below in Table 3.8.

Table 3.9 Model for gene-environment interaction analyses in the context of a case-only study (Khoury *et al.*, 1996)

Susceptibility / Genotype — Environmental Factor	Negative	Positive
Not Risky allele	a	b
Risky allele	c	d

Case-only Odds Ratio (COR) can be calculated as:

$$COR = (d/b)/(c/a) \quad (3.1)$$

COR values calculated from only case population were shown to be similar to OR as a numerical value, for both the case population were the same (Khoury *et al.*, 1996). And like Odds ratio, COR value represents the interaction between independent effects of the exposure and the genotype, not the evaluation of factors alone (Khoury *et al.,* 1996; goodman and Flanders, 2007)

In this study, there are non-genetic factors which could be examined for their interaction with the genetic factors in the risk of development of childhood ALL, like parental age at conception, prenatal or postnatal exposure of child to cigarette smoke. These information were not available for control sample, so case- only approach was applied to examine the interaction of these factors on development of childhood ALL.

3.3.1 Interaction of Age of Parents at Conception with the Genetic Polymorphisms in Risk of Childhood ALL

It has been shown that young (<20) and old (>35) maternal ages; and ages older than 40 years were risk factor for the development of childhood ALL (Kaye *et al.,* 1991; Dockerty *et al.,* 2001)

Age at conception information was available for 109 mothers and age of mothers were grouped into 3 subgroups according to risk factor. Age group between 20 and 35 was taken as reference group and compared with ages older than 35 and ages younger than 20 at conception. COR values were calculated and presented in Table 3.9. Among 109 mothers, 80 of them were

in between ages of 20 and 35, 13 of them were younger than 20 and 16 of them were older than 35 at the time of conception. There were no significant associations between ages of mother at the time of conception and risk for childhood ALL development. In other words, neither ages younger than 20 nor ages older than 35 at conception for the mother, together with genetic polymorphisms was a risk factor for childhood ALL.

Table 3.10 Analyses of interaction of single genetic polymorphisms and maternal age at conception as risk factors for development of childhood ALL.

Analyses (Total N=109)	**Age 20-35 N= 80 Referent**	**Age<20 N=13**	**Age>35 N=16**	**COR for Age<20 95%CI**	**COR for Age>35 95%CI**
XRCC3 Thr241Met					
Thr/Thr	41	4	7	1.0 (Referent)	1.0 (Referent)
Thr/Met	28	8	8	2.9 (0.8-10.7)	1.7 (0.5-5.1)
Met/Met	11	1	1	0.9 (0.1-9.2)	0.5 (0.1-4.8)
Thr/Met plus Met/Met	39	9	9	2.4 (0.7-8.3)	1.4 (0.5-4.0)
Rad51 G135C					
GG	69	10	12	1.0 (Referent)	1.0 (Referent)
GC	10	3	4	2.1 (0.5-8.8)	2.3 (0.6-8.5)
CC	1	0	0	-	-
GG plus CC	11	3	4	1.9 (0.4-7.9)	2.1 (0.6-7.7)

Age of parents at conception was available for 106 fathers. 14 of them were older than 40 years of age, and 92 of them were younger than 40 years of age at the time of conception. Ages younger than 40 years old was taken as reference group. There were no significant associations between ages of fathers at the time of conception and risk for childhood ALL development as represented in Table 3.10. In other words, neither ages older than 40 nor ages younger than 40 at conception for the father, together with genetic polymorphisms was a risk factor for childhood ALL.

Table 3.11 Analyses of interaction of single genetic polymorphisms and paternal age at conception as risk factors for development of childhood ALL.

Analyses (Total N=106)	**Age <40 N= 92**	**Age>40 N=14**	**COR 95%CI**	**p**
XRCC3 Thr241Met				
Thr/Thr	44	7	1.0 (Referent)	
Thr/Met	37	6	1.0 (0.3-3.3)	1.0
Met/Met	11	1	0.6 (0.1-5.1)	0.6
Thr/Met plus Met/Met	48	7	0.9 (0.3-2.8)	0.9
Rad51 G135C				
GG	76	12	1.0 (Referent)	
GC	15	2	0.8 (0.2-4.2)	0.84
CC	1	0	-	-
GG plus CC	16	2	0.8 (0.2-3.9)	0.8

3.3.2 Interaction of Cigarette Smoking Status of Parents with the Genetic Polymorphisms in Risk of Childhood ALL

Cigarette smoking questionnaires were answered by 109 mothers and 108 fathers. Smoking status of fathers and mothers were given previously in Table 3.2. While investigating effect of parental smoking status on development of childhood ALL, passive and active smokers were grouped into together as exposed group, non-smokers were grouped into expose group. There were no significant associations between maternal cigarette smoking status and risk for childhood ALL development as represented in Table 3.11.

Table 3.12 Analyses of interaction of single genetic polymorphisms and maternal smoking status as risk factors for development of childhood ALL.

Analyses (Total N=109)	**Not Exposed N= 30**	**Exposed N=79**	**COR 95%CI**	**p**
XRCC3 Thr241Met				
Thr/Thr	17	35	1.0(Referent)	
Thr/Met	10	34	1.7 (0.7-4.1)	0.3
Met/Met	3	10	1.6 (0.4-6.7)	0.5
Thr/Met plus Met/Met	13	44	1.6 (0.7-3.8)	0.2
Rad51 G135C				
GG	25	66	1.0 (Referent)	
GC	4	13	1.2 (0.4-4.1)	0.7
CC	1	0	-	-
GG plus CC	5	13	1.0 (0.3-3.0)	1.0

Cigarette smoking questionnaires were answered by 108 fathers. Smoking status of fathers were given previously in Table 3.2. While investigating effect of parental smoking status on development of childhood ALL, passive and active smokers were grouped into together as exposed group, non-smokers were grouped into not exposed group. Exposure of father either passively or by active smoking together with heterozygote mutant *XRCC3* genotype gene increased the risk of ALL 3.0 fold. (95%CI: 1.0-9.0, p=0.05). There were no other significant associations between paternal cigarette smoking status and risk for childhood ALL development as represented in Table 3.12.

Table 3.13 Analyses of interaction of single genetic polymorphisms and paternal smoking status as risk factors for development of childhood ALL.

Analyses (Total N=108)	**Not Exposed N= 22**	**Exposed N=86**	**COR 95%CI**	**p**
XRCC3 Thr241Met				
Thr/Thr	14	37	1.0 (Referent)	
Thr/Met	5	39	3.0 (1.0-9.0)	**0.05***
Met/Met	3	10	1.3 (0.3-5.3)	0.8
Thr/Met plus Met/Met	8	49	2.3 (0.9-6.1)	0.08
Rad51 G135C				
GG	16	74	1.0 (Referent)	
GC	5	12	0.5 (0.2-1.7)	0.3
CC	1	0	-	-
GG plus CC	6	12	0.4 (0.1-1.3)	0.1

The cigarette smoke questionnaire also includes the information of whether mothers smoked during pregnancy or not. Cigarette smoking questionnaires were answered by 109 mothers. 12 of them smoked during pregnancy, and 97 of them did not smoke during pregnancy. There were no significant associations between maternal smoking during pregnancy and risk for childhood ALL development as represented in Table 3.13.

Table 3.14 Analyses of interaction of single genetic polymorphisms and maternal smoking during pregnancy as risk factors for development of childhood ALL.

Analyses (Total N=109)	**Not Smoked N= 97**	**Smoked N=12**	**COR 95%CI**	**p**
XRCC3 Thr241Met				
Thr/Thr	44	8	1.0 (Referent)	
Thr/Met	40	4	0.6 (0.2-2.0)	0.4
Met/Met	13	0	-	-
Thr/Met plus Met/Met	53	4	0.4 (0.1-1.5)	0.2
Rad51 G135C				
GG	80	11	1.0 (Referent)	
GC	16	1	0.5 (0.06-3.8)	0.5
CC	1	0	-	-
GG plus CC	17	1	0.4 (0.05-3.5)	0.4

The cigarette smoke questionnaire also includes the information of whether children were exposed to cigarette smoke or not postnatally. Information related with that question were available for 107 patients. 36 of them were exposed to cigarette smoke postnatally, and 71 of them were not exposed to cigarette smoke postnatally. There were no statistically significant associations between postnatal exposure to cigarete smoke and risk for childhood ALL development as represented in Table 3.14.

Table 3.15 Analyses of interaction of single genetic polymorphisms and postnatal exposure to cigarette smoke as risk factors for development of childhood ALL.

Analyses (Total N=107)	**Not Exposed N= 36**	**Exposed N=71**	**COR 95%CI**	**p**
XRCC3 Thr241Met				
Thr/Thr	20	32	1.0 (Referent)	
Thr/Met	13	30	1.4 (0.6-3.4)	0.4
Met/Met	3	9	1.9 (0.5-7.8)	0.4
Thr/Met plus Met/Met	16	39	1.5 (0.7-3.4)	0.3
Rad51 G135C				
GG	29	61	1.0 (Referent)	
GC	6	10	0.8 (0.3-2.4)	0.7
CC	1	0	-	-
GG plus CC	7	10	0.7 (0.2-2.0)	0.5

CHAPTER 4

DISCUSSION

This study was performed to investigate the association between polymorphisms of two DNA double strand break repair genes, *XRCC3* and *Rad51*, and their role in risk of development of childhood ALL. These two genes' products have a central role in DNA double strand break repair. Genetic polymorphisms of these two genes might cause change in DNA repair capacity, which will be a risk factor for development of childhood ALL.

As previously mentioned in "Introduction part", acute lymphoblastic leukemia is the most common form of leukemia in childhood. It accounts for 30-50 new cancer cases per million children (Bonet, 1995) and represents 25-30% of all childhood malignancies (Rose, 199; Pui, 2000; and Greaves, 2000). The molecular etiology of the disease has not been known yet, but there are some established risk factors for childhood leukemia (such as, sex, age, race and certain congenital diseases such as Down syndrome, and neurofibromatosis), however these risk factors account for only 10% of the childhood leukemia cases (Chang, 2009).

“Genetic polymorphisms in the DNA repair mechanisms result in interindividual variation in DNA repair capacity and may account, in part, for the susceptibility of a cell to genotoxic agents and to malignancy. The DNA repair systems correct the damage inflicted by carcinogens and anticancer agents and the defective DNA repair has been reported to be a risk factor for various malignancies” (Krajinovic, 2002). Therefore, polymorphisms in DNA repair genes affecting the DNA repair capacity have strong relevance in determining susceptibility to cancer.

As previously stated in detail, *XRCC3* and *Rad51* are two susceptible gene products due to their role in double strand break repair which is the most important repair mechanisms within the cell. Genetic polymorphisms altering the kinetics of either *XRCC3* and *Rad51* may modify the individual’s susceptibility for the Childhood ALL. Polymorphisms of both *XRCC3* and *Rad51* have been widely studied in relation to various cancer types as risk modifiers, however, to our knowledge, there have been no reports evaluating the clinical significance of *Rad51* and *XRCC3* genetic polymorphisms for the risk of developing childhood ALL. Besides, genetic polymorphisms of *Rad51* gene have not been studied in Turkish population, so this study also provided allele frequencies of *Rad51* gene in Turkish population.

The study population comprised of 193 healthy adult control and 183 pediatric patients with ALL. Case-contol studies were carried out for total of two polymorphisms, *XRCC3* Thr241Met polymorphism and *Rad51* G135C polymorphism, either alone or in combination. The results were represented in Tables 3.3 through 3.14. Here in Table 4.1, the results found to be important for discussion part were summarized.

Table 4.1 Analysis of genetic polymorphisms, alone, or in combination, as risk factors for the development of childhood ALL. The data was drawn from Tables 3.3 to 3.7

Genotypes	OR (95% CI)	P value
XRCC3		
Thr/Met	**0.59 (0.38-0.91)**	**0.018**
Met/Met	**0.48 (0.26-0.88)**	**0.017**
Thr/Met plus Met/Met	**0.55 (0.37-0.84)**	**0.005**
Rad51		
G/C	0.82 (0.48-1.40)	0.47
C/C	1.01 (0.14-7.29)	0.99
G/C plus C/C	0.83 (0.5-1.40)	0.48
Combined Genotypes		
Thr/Met plus GG	**0.55 (0.33-0.90)**	**0.016**
Thr/Met plus GC	**0.35 (0.14-0.90)**	**0.02**
Met/Met plus GG	**0.36 (0.18-0.73)**	**0.004**

Considering single locus polymorphisms as risk factors for the development of childhood ALL, only *XRCC3* Thr241Met polymorphism was found to be a protective factor for development of childhood ALL. *Rad51* G135C polymorphism was not found to be a risk factor for the development of childhood ALL, statistically. For *XRCC3* Thr241Met polymorphism, presence of either heterozygote or homozygote mutant was significantly decreased the risk of disease development.

The heterozygote presence of *XRCC3* Thr241Met gene (Thr/Met) and homozygote presence of *XRCC3* (Met/Met) were significantly found to be a protective factor for childhood ALL (OR: 0.59, ***p*=0.018** and OR= 0.48, ***p*= 0.017**, respectively, see Table 4.1). In literature, there have been some studies investigated the interaction between *XRCC3* Thr241Met poymorphism and various cancer types, but the results are contradictory. As an example, the study of Juan Jin *et al.,* 2005 comprised of 140 patients with colorectal cancers and 280 controls demonstrated that heterozygote genotype and homozygote mutant genotypes increased the risk of developing colerectal cancers as 3.25 and 3.13 times respectively (OR= 3.25 *p*= 0.005, and OR= 3.13, *p*= 0.005, respectively) (Jin, 2005). The study of Voso *et al.,* (2007) with 161 controls and 160 patients with acute myeloid leukemia (AML) demonstrated that *XRCC3* Met/Met genotype was increased the risk of developing AML 2.27 fold when compared to sum of homozygote wild type and heterozygote mutant genotypes (OR=2.27, *p*= 0.009) (Voso, 2007). In the study of Sangrajrang *et al.*, (2007)the *XRCC3* Thr241Met polymorphism was investigated for the risk of breast cancer in 424 healthy Thai women and 507 Thai patients with breast cancer. Accordingly, presence of either heterozygote or homozygote mutant genotype (presence of at least 1 mutant allele) was associated with risk of having breast cancer (OR= 1.58, *p*= 0.04) (Sangrajrang *et al.*, 2007).There is a recent publication investigated the interaction of *XRCC3* Thr241Met polymorphism with Endometriosis in Turkish population. In that study (Attar, 2010), *XRCC3* Thr241Met genetic polymorphism was investigated among 101 controls and 52 endometriosis patients, and Thr/Thr genotype was found significantly greater in endometriosis patients compared to controls (*p*=0.005). In other words, *XRCC3* Thr/Met genotypes (*P*= 0.022) and the Met allele (*P*=0.005) was found to have a protective role against endometriosis (Atar *et al.,*2010).

This is the first study investigated the polymorphism of *XRCC3* Thr241Met gene in Turkish population, and demonstrated the protective role of Met allele in endometriosis. However, our study was the first demonstrating the protective role of Met allele in Childhood ALL in Turkish population.

Moreover there are numerous studies in some of which *XRCC3* Thr241Met polymorphism have been a risk factor, in some of which it has been a protective factor and in some it does not have any association with the disease development. Studies about *XRCC3* polymorphisms and their interaction with the related diseases were listed in Table 4.2, below.

As it is demonstrated in the table, there are so many studies regarding the association of polymorphisms of *XRCC3* with various cancer types, although results remain inconsistent.

Table 4.2 Genetic polymorphism of *XRCC3* Thr241Met gene and its interaction with various types of cancers

Cancer types	Association	N of control	Reference
Lung Cancer	Increased risk	190	*Wang et al., 2003*
Bladder Cancer	Increased Risk	209	*Stern et al.,2002*
Acute Myeloid Leukemia	Increased Risk	186	*Seedhouse et al.,2004*
Colorectal cancer	Increased Risk	100	*Krupa et al.,2004*
Breast Cancer	Increased Risk	1826 268	*Kuschel et al., 2002;* *Smith et al., 2003*
Acute Lymphoblastic Leukemia	Protective Role	193	***This Study***
Bladder Cancer	Protective Role	214	*Shen et al., 2003*
Colerectal Cancer	Protective Role	128	*Mort et al., 2003*
Skin Cancer	Protective Role	873	*Han et al., 2004*
Supraglottic cancer	Protective Role	172	*Benhamou et al., 2004*
Lung Cancer	No association	272	*Jacobsen et al., 2004*
Gastric Cancer	No association	166	*Shen et al., 2004*
Cutaneous Malignant Melanoma	No association	319	*Duan et al., 2002*
Colerectal Adenoma	No association	725	*Tranah et al., 2004*

The frequency of Thr241Met polymorphism of *XRCC3* gene was found to be 0.42 (Met allele) in Turkish population. The comparison of *XRCC3* 241Met allele frequency in Turkish population (obtained from control population) with various control population was previously represented in Table 3.4. That polymorphism has been studied in Turkish population by Attar *et al.,* 2010, recently. In the study of Attar *et al.*, allele frequency of Met allele was found to be 0.44 which is highly similar to our result. When compared to other Caucasian populations, the frequency of Met allele in Turkish population was found to be similar. *XRCC3* 241Met allele frequency was found to be highly different compared to Asian populations. There were significant differences in terms of mutant allele frequency between the major ethnicities, Caucasian and Asian populations in terms of *XRCC3* Thr241Met polymorphism. Inconsistent results about association of *XRCC3* Thr241Met polymorphism with various cancer types may be resulted from different allelic frequencies among ethnicities. Actually, it should be not uncommon for the same polymorphism to play a different role in cancer susceptibility across different populations.

How polymorphism of *XRCC3* Thr241Met may be a protective factor for Childhood ALL? Information about function of *XRCC3* within the cell is limited. It plays a central role in homologous recombination repair, and for its function to take place, it has to be interacted with *Rad51* protein molecule. According to the study of Yamada *et al.*(2004), *XRCC3* presents as a heterodimer with *Rad51*C. When *XRCC3* binds ATP molecule, due to steric hinderence of ATP molecule, *Rad51*C dissociates from *XRCC3* and binds to damaged site. Any change in the ATP binding domain of *XRCC3* gene or in a site of *XRCC3* interacting with other molecules will disrupt the function of the *XRCC3* protein. (Kuschel, 2002). However, this variation does not reside

in the ATP binding domain, the only functional domain identified in the protein (Manuguerra, 2006). But *XRCC3* 241Met allele has been associated with less efficient DNA repair (Matullo, 2001), as well as an increased number of centrosomes and binucleated cells (Lindh, 2006). "However, it has also been shown that the wild type and mutant alleles are functionally equivalent in the double strand break repair pathway (Araujo, 2002)". So that this polymorphism might change the function of *XRCC3* product in a way that it does not affect the interaction of *XRCC3* with the molecules of double strand break repair, but it might change the function of it in other cellular pathways.

To sum up for the polymorphism of *XRCC3* Thr241Met, the present work is the first study that demonstrated the mutant allele of *XRCC3* as the protective factor for the development of childhood ALL, not only in Turkish population, but also in the literature.

In the present study, *Rad51* G135C polymorphism did not confer a risk factor for the development of childhood ALL (see Table 4.1). *Rad51* protein catalyses strand transfer between a broken sequence and its undamaged homologue to allow resynthesis of the damaged region (West *et al.,* 2003). The function of *Rad51* protein is controlled by breast cancer-associated tumour suppressor BRCA2 (Tarsounas *et al.,* 2004; Davies *et al.*, 2001). Due to the interaction of *Rad51* protein with BRCA2 (and also with BRCA1), polymorphism of this gene is highly studied in breast cancer cases. Although there are many studies in the literature examining the role of polymorphisms in the development of certain cancers, especially breast cancer(Sun *et al*, 2010; Krupa *et al.,* 2009; Jara *et al.*, 2009), the results are contradictory. Some studies demonstrated significant association of *Rad51* 135C allele with breast cancer, but some studies did not. So far, no studies

have been found in the literature for the investigation of this polymorphism as risk factor for the childhood ALL. Accordingly, this study demonstrated that polymorphism of *Rad51* G135C does not play a risk factor for the development of childhood ALL. There might be several possibilities explaining this situation. Since this polymorphism presents at 5' upstream of the gene, it does not change the structure and function of the *Rad51* protein. It is demonstrated that *Rad51* G135C polymorphism is associated to *Rad51* protein over-expression and to increased DNA repair (Vispe, 1998; Kim, 2001; and Richardson, 2004). Presence of polymorphic variant of *Rad51*, alone might not be enough to cause childhood ALL. The occurrence and progression of Childhood ALL might be caused by polygenic factors. Therefore, further investigations are needed to elucidate these possibilities.

As it is stated above, occurrence and progression of a cancer might not be caused only due to one gene polymorphism. Combinations of two or more risk genotypes can significantly increase the risk of disease. In that study, when genotypes of *XRCC3* and *Rad51* are combined, it was observed that the co-presence of *XRCC3* polymorphism and *Rad51* G135C polymorphisms had a combined effect on the risk of development of childhood ALL. The co-presence of homozygote wild type genotype of *Rad51* gene with both heterozygote genotype and homozygote mutant genotype of *XRCC3* gene resulted in a protective factor for development of childhood ALL (OR= 0.55**, *p*= 0.016**, and OR = 0.36**, *p*= 0.004**, respectively) (See table 4.1). When analysis was carried out for the combination of heterozygote genotype of *Rad51* with heterozygote genotype of *XRCC3* gene, this combination gave the highest protective factor for the development of childhood ALL (OR= 0.35, *p*= 0.02, even higher than *XRCC3* alone). Combination analysis of *Rad51* and *XRCC3* makes sense, since these two

proteins are interacted within the cell and they are the central molecules for the homologous repair of double strand breaks. As previously stated in Discussion part, *XRCC3* presents as a heterodimer with *Rad51*. Upon *XRCC3* interacted with ATP, ATP binding results in dissociation of *Rad51* from the *XRCC3-Rad51* complex. So *XRCC3* functions in a way that it holds the *Rad51* until it is needed for its function. Therefore, the combined effect of *XRCC3* Thr241Met with *Rad51* G135C polymorphism on the risk of development of childhood ALL supported the fact that genetic polymorphism of one single gene on it is own may not be enough to significantly increase or decrease the risk for the disease. However, the accumulation of multiple high risk genotypes within a DNA repair pathway can have dominating effects on disease occurrence and progression.

It is well known that genetic background on its own may not be enough all the time to result in occurrence and progression of cancer. For that reason, interaction of some non-genetic factors –parental age at conception and cigarette smoking exposure of parents and children- with genetic polymorphisms of *XRCC3* and *Rad51* genes were investigated, too. These analyses were carried out by using case-only approach as the information related with non-genetic factors was only available for patient group. Non-genetic factor categories and related patient numbers are previously represented in Table 3.2 (B).

One of the non-genetic risk factor for the development of childhood ALL that was analyzed for the interaction with genetic polymorphism in this study was parental age at conception. It is well demonstrated that maternal age younger than 20 and older than 35, and, paternal age older than 40 at the conception are risk factors for the development of childhood ALL (Kaye *et al.*, 1991; Dockerty *et al.*, 2001). In the study of Kaye *et al.* (1991), it was

demonstrated that risk of childhood ALL was significantly increased 2.1 fold among the children of mothers older than 35 years of age and 1.6 fold among the children of fathers older than 40 (Kaye *et al.,* 1991). For that analysis, mothers of age younger than 20 and older than 35 are grouped into risky groups, whereas ages of mother between 20 and 35 are grouped into the non-risky groups. Analysis was done by comparing mother age groups against different genotypes of both *XRCC3* and *Rad51* genes. However, neither ages younger than 20 nor ages older than 35 for the mother together with genetic polymorphisms represents a risk factor for the development of childhood ALL. Besides, same analysis was done for father age older than 40, but again, no significant association has been found.

The second non-genetic risk factors for the development of childhood ALL that was analyzed for the interaction with genetic polymorphisms of *XRCC3* and *Rad51* were smoking exposure of mother, father and postnatal exposure of child. It has been known that cigarette smoke contains at least 60 known carcinogens which can disrupt the integrity of genomic material when the cell is exposed to these chemicals. If the cell has a reduced DNA repair capacity due to polymorphisms in *XRCC3* or *Rad51*, that can result in leukemia development. It is well proved that tobacco smoke is a risk factor for the development of adult acute myeloid leukemia (Chang, 2009). However, studies that investigate the association between parental smoking and childhood leukemia have produced inconsistent results (Shu *et al.*, 1996; Brondum *et al.*, 1999; Chang *et al.*, 2006).

Detailed information about analysis and categories of smokers were previously explained in **Section 3.3.1.** Briefly, active and passive smokers were categorized as exposed groups, and case-only odd ratio was calculated for exposed individuals against non-exposed individuals. Among the

analyses only paternal exposure demonstrated a significant association. Maternal exposure to cigarette smoke, smoking of mother during pregnancy and postnatal exposure of child did not demonstrate a significant effect with the genetic polymorphisms on the development of childhood ALL. In terms of paternal exposure, only the heterozygote (Thr/Met) genotype for *XRCC3* gene in children whose father exposed to cigarette smoke demonstrated a significant risk of 3.0 fold (p=0.05). Interaction of paternal exposure to cigarette smoke with development of childhood ALL has been reported in other studies, too (Chang *et al.,* 2006; Chang, 2009). Chang *et al.,* (2006); Chang, (2009) studies also did not find any correlation between maternal smoking status with childhood leukemia risk. Since the spermatogenesis continues from puberty to old age, there is more opportunity for mutant gene accumulation in men than women (Anderson *et al.,* 2000). These genetic mutations accumulated in the father's sperm may transmit cancer susceptibility to the child. The biological mechanism for more positive association of paternal cigarette smoking with childhood ALL risk could be explained by the accumulation of cigarette smoke-induced oxidative DNA damage in human semen, which may cause chromosome breaks that ultimately lead to translocations in uterus and childhood leukemia development.

It is worth to mention that no single gene polymorphism might cause the development of childhood ALL. When several polymorphisms of the genes involving in the various cellular pathways combined with the environmental risk factors; the risk of development of childhood ALL increases. Cells are exposed to various carcinogens present in the environment and the interindividual differences in the activity of xenobiotic metabolizing enzymes due to genetic polymorphisms results in increased

damage in DNA, directly or indirectly. Besides, variable DNA repair capacity due to genetic polymorphism within those genes may further modulate induced DNA damages. Up to date the genetic polymorphisms of various drug metabolizing enzymes like CYP1A1, CYP2E1, GSTs, NQO1, EPHX1, SULT1A1 etc., were studied in association with childhood ALL. CYP2E1 is the most studied enzyme in relation to childhood ALL due to its role in the metabolism of various carcinogens. In the study of Ulusoy et al., (2007), it was demonstrated that co-presence of CYP2E1 *5B and *6 alleles significantly increased the risk of childhood ALL to 2.9 fold (Ulusoy et al., 2006, 2007). In another study of Ulusoy (2009-PhD Thesis), GSTT1 null polymorphism, alone was shown to be risk factor for childhood ALL (OR: 1.8, p=0.01). The co-presence of GSTT1 null with either CYP2E1*6 or CYP2E1*7B considerably increased the risk of childhood ALL 4.2 (p=0.02) and 4.1 (p=0.03) fold, respectively. Moreover, co-presence of GSTT1 null, CYP2E1*5B and *6 together (the combinations of three polymorphisms) increased the risk of childhood ALL strikingly, 7.6 fold (p=0.04) (Ulusoy, 2009). In the study of Tumer, (2009 PhD Thesis) it was demonstrated that EPHX1 exon 3 homozygote mutant polymorphism increased the risk of childhood ALL development to 2.0 fold (p=0.01) (Tumer, 2009). Recently, Tumer et al., (2010) demonstrated that Gln399Gln genotype of XRCC1 gene significantly increased the risk of disease up to 2.0 fold (p=0.04). When this genotype were combined with CYP2E1 *5B, *6B, the risk factor significantly increased to 3.7 fold (p=0.049). All these studies indicated that, etiology of Childhood ALL does not rely on one gene polymorphism.

To sum up, by this study, information about frequencies of *XRCC3* and *Rad51* gene polymorphisms obtained. To our knowledge, this is the first study that provided the allele frequency of *Rad51* G135C polymorphism for

Turkish population. Risk assessment part of current study provided a strong association for *XRCC3* 241Met allele as a protective role for childhood ALL disease development in Turkish population, and also in literature. Interaction of *Rad51* G135C polymorphism with childhood ALL was studied for the first time in Turkish population and in literature, and no interaction was found between *Rad51* polymorphism and childhood ALL. Case-only analysis revealed that heterozygote genotype of *XRCC3* gene together with paternal smoking exposure has considerably positive interactive effect on the development childhood ALL.

CHAPTER 5

CONCLUSION

In that study, the polymorphisms of two DNA repair genes were investigated as risk modifiers in the development of childhood ALL. In this respect, *XRCC3* and *Rad51* genes were selected due to their key role in double strand break repair. To the best of our knowledge, in the scope of this study, the polymorphisms of *XRCC3* and *Rad51* have been studied for the first time in relation to development of childhood ALL. *XRCC3* Thr241Met and *Rad51* G135C polymorphisms were investigated in 193 healthy adult controls and 184 pediatric patients with ALL.

In case-control analyses, the risk modifier role of *XRCC3* Thr241Met and *Rad51* G135C polymorphisms were investigated either alone or in combination in the development of childhood ALL. When investigated alone, only *XRCC3* Thr241Met polymorphism was found to be associated with development of disease. Both heterozygote and homozygote mutant genotype of *XRCC3* gene has a protective role for development of disease (OR=0.48, p=0.017 for heterozygote; OR=0.55, p=0.005 for homozygote mutant allele). Although, *Rad51* G135C polymorphism did not confer any association with disease development, but when combined with *XRCC3* gene polymorphism, homozygote wild type of *Rad51* gene with either heterozygote or homozygote mutant genotype of *XRCC3* and heterozygote genotype of *Rad51* gene with heterozygote genotype of *XRCC3* demonstrated

an association with the childhood ALL. The homozygous presence of *Rad51* gene with either heterozygous or homozygous mutant genotype of *XRCC3* significantly had a protective role for the risk of childhood ALL (OR=0.55, *p*=0.016; OR=0.36, *p*=0.004, respectively). Similarly, heterozygote presence of *Rad51* gene with heterozygous genotype of *XRCC3* significantly had a protective role for the risk of childhood ALL (OR=0.35, *p*=0.02).

In case only analysis, some non genetic risk factors -parental age at conception and cigarette smoking exposure of parents and children- were investigated to evaluate their interactions with genetic polymorphisms of *XRCC3* and *Rad51* genes. Accordingly, we could not find an interactive association with the paternal age at conception and studied genetic polymorphism on the risk of childhood ALL. In terms of cigarette smoking exposure, maternal exposure to cigarette smoke, smoking of mother during pregnancy and postnatal exposure of child did not demonstrate an interactive effect with the genetic polymorphisms in combination on the development of childhood ALL. In terms of paternal exposure, only the heterozygote (Thr/Met) genotype for *XRCC3* gene in children whose father exposed to cigarette smoke demonstrated a significant risk of 3.0 fold (*p*=0.05).

The genotyping for control samples of this study also served to determine the population frequencies of these polymorphisms, and to make comparison with other population frequencies. In the present study, the frequency of *Rad51* 135C allele was determined for the first time in Turkish population. The frequency of the mutant allele was found to be very similar to that observed in other Spanish Population (See Table 3.4)

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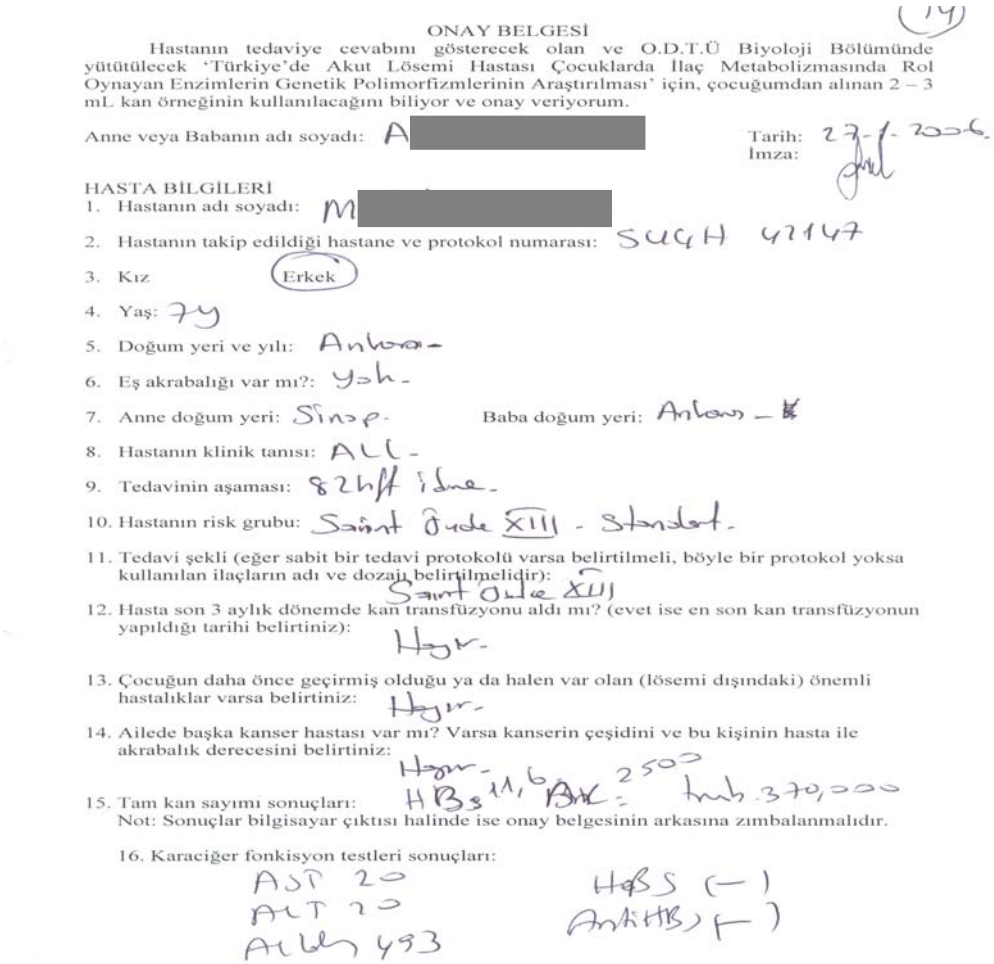
APPENDIX A

WRITTEN INFORMED CONSENT FORM FOR ALL-PATIENT GROUP



ONAY BELGESİ

Hastanın tedaviye cevabını gösterecek olan ve O.D.T.Ü Biyoloji Bölümünde yütütülecek 'Türkiye'de Akut Lösemi Hastası Çocuklarda İlaç Metabolizmasında Rol Oynayan Enzimlerin Genetik Polimorfizmlerinin Araştırılması' için, çocuğumdan alınan 2 – 3 mL kan örneğinin kullanılacağını biliyor ve onay veriyorum.

Anne veya Babanın adı soyadı: A[redacted] Tarih: 27-1-2006
İmza:

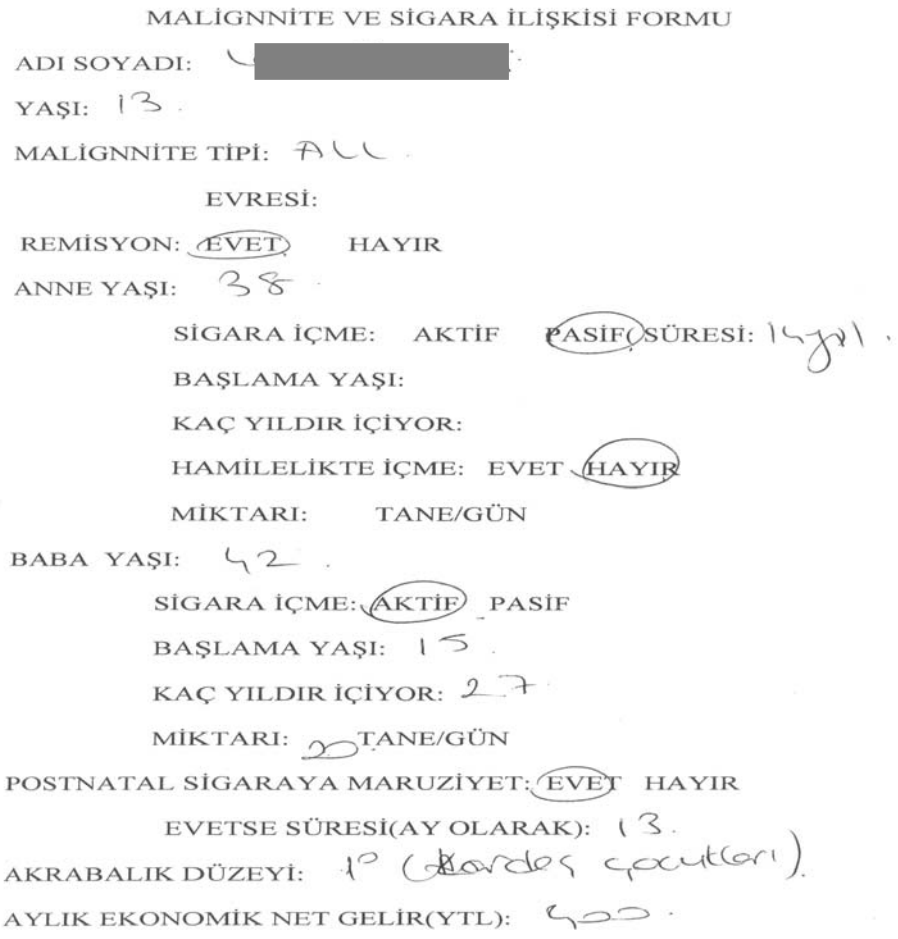
HASTA BİLGİLERİ

1. Hastanın adı soyadı: M[redacted]
2. Hastanın takip edildiği hastane ve protokol numarası: SUGH 47147
3. Kız (Erkek)
4. Yaş: 7y
5. Doğum yeri ve yılı: Ankara-
6. Eş akrabalığı var mı?: Yok-
7. Anne doğum yeri: Sinop- Baba doğum yeri: Ankara
8. Hastanın klinik tanısı: ALL-
9. Tedavinin aşaması: 82hft idame-
10. Hastanın risk grubu: Saint Jude XIII - standart-
11. Tedavi şekli (eğer sabit bir tedavi protokolü varsa belirtilmeli, böyle bir protokol yoksa kullanılan ilaçların adı ve dozajı belirtilmelidir): Saint Jude XIII
12. Hasta son 3 aylık dönemde kan transfüzyonu aldı mı? (evet ise en son kan transfüzyonun yapıldığı tarihi belirtiniz): Hayır-
13. Çocuğun daha önce geçirmiş olduğu ya da halen var olan (lösemi dışındaki) önemli hastalıklar varsa belirtiniz: Hayır-
14. Ailede başka kanser hastası var mı? Varsa kanserin çeşidini ve bu kişinin hasta ile akrabalık derecesini belirtiniz: Hayır-
15. Tam kan sayımı sonuçları: HB: 11,6 BK: 2500 Trb: 370,000
Not: Sonuçlar bilgisayar çıktısı halinde ise onay belgesinin arkasına zımbalanmalıdır.
16. Karaciğer fonkisyon testleri sonuçları:
AST 20
ALT 20
Alb 4,93
HBS (-)
Anti HBS (-)

Figure A 2 Written informed consent form for all-patient group

APPENDIX B

CIGARETTE SMOKING STATUS QUESTIONNAIRE



MALİGNNİTE VE SİGARA İLİŞKİSİ FORMU

ADI SOYADI: [redacted]

YAŞI: 13

MALİGNNİTE TİPİ: ALL

EVRESİ:

REMİSYON: (EVET) HAYIR

ANNE YAŞI: 38

SİGARA İÇME: AKTİF (PASİF) SÜRESİ: 14 yıl

BAŞLAMA YAŞI:

KAÇ YILDIR İÇİYOR:

HAMİLELİKTE İÇME: EVET (HAYIR)

MİKTARI: TANE/GÜN

BABA YAŞI: 42

SİGARA İÇME: (AKTİF) PASİF

BAŞLAMA YAŞI: 15

KAÇ YILDIR İÇİYOR: 27

MİKTARI: 20 TANE/GÜN

POSTNATAL SİGARAYA MARUZİYET: (EVET) HAYIR

EVETSE SÜRESİ(AY OLARAK): 13

AKRABALIK DÜZEYİ: 1° (kardeş çocukları)

AYLIK EKONOMİK NET GELİR(YTL): 400

Figure B 1 Cigarette smoking status questionnaire

APPENDIX C

BUFFERS AND SOLUTIONS

All the glassware used for DNA isolation, genotyping and lymphocyte isolation procedures were sterilized by autoclaving and sterilized distilled water was used for the preparation of solutions.

Tris-HCl, pH 8.0 (100 mM);

12.1 g Tris was weighed and dissolved in 700 mL of dH2O. pH was adjusted to 8.0 with concentrated HCl and volume was completed to 1 L. Solution was autoclaved for sterilization and stored at 4°C.

EDTA, pH 8.0 (500 mM);

186.1 g Na2EDTA.2H2O was weighed and dissolved in 700 mL dH2O. Dissolution of EDTA was achieved by adjusting the pH to 8.0 with NaOH. Volume was completed to 1 L. Solution was autoclaved for sterilization and stored at 4°C.

TKME (Tris-KCl-MgCl2-EDTA) Buffer, pH 7.6;

10mM Tris-HCl (pH 7.6), 10 mM KCl, 4 mM MgCl2, 2 mM EDTA.
Solution was autoclaved for sterilization and stored at 4°C.

Saturated NaCl (6M)

3.5064 g NaCl was weighed and dissolved in 10 mL of sterilized dH2O.

Solution was autoclaved for sterilization and stored at 4°C.

TE (Tris-EDTA) Buffer, pH 8.0;

10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0).

Solution was autoclaved for sterilization and stored at 4°C.

TBE (Tris-Borate-EDTA) Buffer, pH 8.3;

5x stock solution: 54 g Trizma-base and 27.5 g boric acid were weighed and dissolved in appropriate amount of water. 20 mL of 500 mM EDTA (pH 8.0) was added. pH was set to 8.3. Volume was completed to 1 L. Solution was autoclaved for sterilization and stored at room temperature to prevent precipitation.

0.5x solution: The stock solution was diluted 10 times with sterilized dH2O prior to use to achieve 45 mM Tris-borate, 1 mM EDTA.

Ethidium Bromide (10 mg/mL);

0.1 g ethidium bromide was dissolved in 10 mL dH2O. Solution was stirred on magnetic stirrer for several hours to ensure that dye had completely dissolved. As this solution is light sensitive, the bottle was covered with aluminum foil and stored at room temperature.

Gel loading dye

0.25% bromophenol blue, 40% sucrose in sterilized dH2O.

Solution is stored at 4°C.

PCR Amplification Buffer (10x) (Fermentas);

100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% Nonidet P40.

This buffer and 25 mM MgCl2 solution were supplied together with Taq DNA Polymerase. Taq Polymerase, amplification buffer and MgCl2 solutions were stored at -20°C.

dNTP Mixture (Fermentas);

10 mM of each dATP, dCTP, dGTP and dTTP in aqueous solution. The solution was stored at -20°C.

TANGOTM Buffer (digestion buffers) (Fermentas);

33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Buffer B+ (digestion buffer) (Fermentas);

10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.1 mg/mL BSA.

Restriction enzymes and buffers were stored at -20°C.

Gene Ruler 50 bp DNA Ladder (0.5 mg DNA/mL) (Fermentas);

This commercial DNA ladder was prepared from a specially designed plasmid pEJ3 DNA, containing pUC, λ phage and yeast genome sequences. pEJ3 DNA was completely digested with Eco147I and PvuI. The ladder was dissolved in storage buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA).

6x Loading dye solution: 0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA.

The ladder was prepared by mixing DNA ladder: 6x loading dye solution: dH2O in 1:1:4 ratio, mixed well and applied to the gel.

The DNA ladder contained the following discrete fragments (in base pairs): 1031 900 800 700 600 500 400 300 250 200 150 100 50.