I, Jiadi Xu, hereby submit this original work as part of the requirements for the degree of Master of Science in Molecular & Developmental Biology.

It is entitled:
DNA repair defects as a mechanism contributing to the development of lupus.

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This work and its defense approved by:

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Committee member: Kenneth M. Kaufman, Ph.D.
DNA repair defects as a mechanism contributing to the development of lupus

A thesis submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Master of Science in the Department of Developmental Biology of the College of Medicine

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by

Jiadi Xu

B.S. China Agricultural University 2010

Committee Chair: John Harley, M.D., Ph.D
Abstract

Lupus system erythematosus (SLE) is chronic autoimmune that is characterized by chronic inflammation and the production of anti-nuclear auto-antibodies together with a strong environmental and genetic components. The pathogenesis of SLE is not completely understood. Genetic, epigenetic and environmental factors are components. For clues to how genetic components contribute to SLE development, we use the exome sequencing and genome wide association study (GWAS) designs to reveal the genetics of SLE. There is evidence that defective DNA repair is one of the causative factors that associated with SLE. SLE patients are usually photosensitive to sunlight and frequently develop malar and vasculitic rashes. Studies of lymphocytes and fibroblasts in SLE patients have shown increased sensitivity to ionizing radiation and ultraviolet light. Ionizing radiation is known to cause both single and double stranded DNA breaks. Recently, there are studies showing that there are both single and double strand DNA break repair defects in pediatric SLE patients. Also, studies have shown that DNA damage in cells caused by etoposide response to interferon (IFN) signaling. IFN is well known to the initiating pathogenic factor of human SLE. Hence, we hypothesized that DNA repair defect after radiation caused by specific variants found in SLE patients is a potential cause of disease pathogenesis through the IFN induced SLE. From GWAS study of SLE, over 50 genetic variants have been associated with SLE, some of the genes are important in DNA repair pathway such as TREX1, BRCC1, BRCC3 and BRCC4. Currently, we found RAD51B as a new SLE susceptibility gene in our African American GWAS data. In addition, from our exome sequencing project, we found one family that has two potentially dysfunctional variants of RAD51B. The coincidence that we found
variants in same DNA repair gene by using two different genetic methods suggests that DNA repair pathway is playing an important role in SLE. In addition, from another pediatric lupus trio, we found a de novo variant in RAD54B in the child. Child shows DNA repair defect after ionizing radiation by clonogenic survival assay and neutral comet assay. We developed the lymphoblastoid cell lines (LCLs) of this particular trio as the standard model. We transduced a wild type vector of RAD54B cDNA into the mutant cells and show that this procedure complements the DNA repair deficiency. Our data are consistent with the phenotype that this de novo variant in a DNA repair gene affects DNA repair efficiency in SLE patients, which also leads us to hypothesize that de novo variants contribute to an important proportion of pediatric SLE. Collectively, our work uncovers the previously unappreciated mechanism how genetic components in DNA repair pathways are associated with SLE. We not only demonstrate the genetic variants by both exome sequencing and GWAS studies, but also study the biological function of variants by developing a model system in LCLs, identifying a new potential mechanism of pathogenesis and opening a window for a new approach to clinical treatment.
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>DSB</td>
<td>Double strand DNA break</td>
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<tr>
<td>SSB</td>
<td>Single strand DNA break</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ANA</td>
<td>Anti nuclear antibody</td>
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<tr>
<td>IR</td>
<td>Ionizing radiation</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>pSLE</td>
<td>Pediatric systemic lupus erythematosus</td>
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<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
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<tr>
<td>HRR</td>
<td>Homologous recombinational repair (HRR)</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
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<tr>
<td>CSA</td>
<td>Clonogenic survival assay</td>
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<td>NCA</td>
<td>Neutral comet assay</td>
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<td>WB</td>
<td>Western blot</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>QC</td>
<td>Quality Control</td>
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<tr>
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<td>Murine stem cell virus</td>
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<tr>
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<td>Transducin beta-like protein 3</td>
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<tr>
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<td>Ultraviolet</td>
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<td>DNA</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>SIFT</td>
<td>Scale-invariant feature transform</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>LFRR</td>
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Chapter 1: General Introduction

Overview of Systemic Lupus Erythematosus

General Introduction of SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that involves organ system and can affect any part of the body. SLE is often harmful for the heart, joints, skin, lungs, blood vessels, liver, kidneys, and nervous system. It is usually caused by the inflammation and cytotoxicity resulted from the antibody-immune complex formation. As other autoimmune diseases, cells and tissue in the immune system are attacked, resulting in inflammation and tissue damage which may lead to significant morbidity and mortality [1, 2]. The course of the disease is unpredictable. The disease occurs nine times more often in females than in males, this is particularly true in women of childbearing age. Lupus is also more common in those of non-European ancestry. Childhood systemic lupus erythematosus generally occurs between the ages of 3 and 15 years, with girls outnumbering boys 4:1, butterfly eruption on the face and photosensitivity are the typical skin manifestations [3]. SLE is usually treated with immunosuppression, mainly with mycophenolate mofetil, cyclophosphamide and other immunosuppressants. SLE can be fatal, but fatalities have decreased recent years. Survival for SLE patients in the United States, Canada, and Europe has risen to approximately 95% at five years, 90% at ten years, and 78% at twenty years [4].
Genetic Susceptibility of SLE

SLE is nine times more frequent in female than male and three to seven times more frequent in non-European populations [5]. The heritability is about 66% overall with a sibling risk between 8 and 29. The concordance is about 30% in monozygotic twins and only 3% in dizygotic twins [6]. There are over 50 genetic variants established by GWAS which have association with SLE [7].

SLE and SLE-like disease in both man and mouse have single gene defects that many occur in around 40-50 genes in one species or the other, which means that genetics variants in single gene is sufficient enough to contribute to SLE [7-12]. Recently, studies which bolster this perspective show that disruptive de novo mutations in coding region may cause lupus, such as TREX1 [13-15].

Pathogenesis of SLE

The pathogenesis of SLE is partially understood, many aspects remain unknown. Genetic (antigens and complement alleles), epigenetic, environmental (sun exposures, ionizing radiation), hormonal and immunoregulatory all appear to have a role. SLE patients have features of autoantibody formation. There is an association between pathogenesis and autoantibody production, such as anti-double-stranded DNA antibodies and antinuclear antibodies (ANAs) [16]. Autoantibodies appear to mediate many clinical manifestations [17]. Autoantibodies against cell surface antigen or phospholipid can lead to autoimmunity, organ dysfunction and damage [18]. Hence, B cells have a very important role in SLE since autoantibodies are important factors of SLE pathogenesis. The clinical
heterogeneity of the disease led to the establishment of 11 criteria, with 4 needed for the classification as systemic lupus erythematosus. The organs and tissues involved include brain, blood, and the kidney in many patients. In the United States, people of Hispanic, African, or Asian ancestry tend to have an increased prevalence of SLE and greater involvement of vital organs as compared with those of other racial or ethnic groups such as the European ancestry [2, 3].

**Pediatric SLE**

15%-20% of all the SLE patients are diagnosed during childhood with onset prior to 16 years old. Most pediatric patients with SLE have no family history of autoimmune disease [19]. SLE is more severe in children than adulthood with more major organ system involvement [20-22]. The GWAS study can only explain a small portion of the genetic heritability of SLE [23]. Genetic components including *de novo* mutation, recessive mutation and compound heterozygous mutations are studied by exome sequencing. These mutations explain some recessive models of genetic inheritability of SLE. Patients without family history and with severe course of disease are selected for whole exome sequencing.

**Genetic approach to SLE**

**Exome sequencing**

Exome sequencing is a comprehensive way to explore the protein coding regions of the whole genome (exons) [24]. There are about 180,000 exons in human genome, which constitute about 1-2% of the whole genome and translate to about 30-50 megabases in total length. It is estimated that the protein coding regions of the human genome
constitute about 85% of the disease-causing mutations [25]. The method generates sequencing data from millions of short DNA fragments in parallel [26]. A random input library of DNA fragments is generated and sequenced (Figure 2). DNA from Pediatric lupus patients that have no family history and with severe form of disease is selected for next generation sequencing. After getting the next generation sequencing data back in VCF file format, the data are analyzed by Golden Helix software. Compared to the standard reference sequence, there are about 40,000-50,000 variants individually and 80,000-120,000 variants in each trio. The Mendel error frequency of each trio is around 3,000 or about 35 of the variants. However, the concordance of replicate sample is around 90%, which means that there must be sequencing artifacts in the next generation sequencing data. We have developed a filtering system which reduces sequencing artifacts and flawed data. For Quality Control (QC), we use criteria of read depth, Quality Score and alternate ratio. After passing the QC, less than 50 instances of Mendel errors remain and the concordance of replicates sample is more than 99%. The next step is to remove variants that are not predicted to protein function. Minor Allele Frequency of < 1% will then be used to filter different types of mutations, including de novo mutations, recessive mutations and compound heterozygous mutations. Sanger sequencing is then used to confirm the variants by a second method.
Genomic DNA
Shear DNA
Break genomic DNA into short fragments of about 300bp
Add adaptors
Ligate adaptors to allow genomic DNA be sequenced
Enrich Library for exon
Hybridise the library to baits and select only the exome regions
Wash non-exome regions
Eluting selected exome fragments to create a library with protein coding sequences
Sequencing
Next generation sequencing generates base pairs of data
Data Processing
Alignment, quality control, and variant calling
Filter based on criteria
Candidates variants

Figure 1. Schematic of the current method applied to exome sequencing.

The genomic DNA used for exome sequencing is sheared into double strand DNA fragments. The fragments are added with adaptors which contain universal priming sequences. The fragments are captured by RNA oligonucleotides on the streptavidin-labeled magnetic beads of the microarray [25, 27]. The oligonucleotides on the microarray are designed to cover 62 mb of the exon region with 50% overlap. The microarray is incubated with selected DNA library together with streptavidin-labeled magnetic beads. PCR will be used to amplify the target DNA, which is then sequenced [27].

*De novo mutation*

A *de novo* mutation is a mutation that first occurs in the individual where it is first found not transmitted from nor possessed by parents. It is occurs for the first time in a gene in a
child caused by a mutation in the germ cell of one of the parents or in the fertilized egg itself [28, 29]. Disruptive *de novo* variants have the potential to cause many rare genetic disorders [30-33]. Other investigators have used exome sequencing to evaluate trios and have found *de novo* variants in coding region that cause disruptive gene product and cause diseases, such as autism and mental retardation [34-37].

**Compound Heterozygous Mutations**

Compound heterozygosity is the genetic condition that having two different mutant variants at a gene locus, one in each of the two alleles [38, 39].

**GWAS**

The Genome wide association study is a statistical and genetic method to identify any variant or single nucleotide polymorphisms (SNP). Typically a case - control study design is used where subjects with the phenotype of interest are collected as cases. GWAS has been shown to be a very important genetics approach to study the genetics of SLE. There are almost 50 SLE susceptibility genes have been identified by GWAS in recent years, including genes with unknown pathogenesis of SLE [40, 41].

**Genetic Association**

Genetic association evaluates the relation between phenotypes and genotypes. Genetic association is present when there is non-random association of the SNP on the same region of a gene or a chromosome. The genetic association study is used to test whether a SNP or a genotype frequency is different between two groups of individuals, usually between cases and controls or in family based studies [42].
DNA repair in Lupus

DNA repair response and mechanism

DNA damage is caused by many sources. Cellular DNA is challenged by different exposures, including chemicals and radiation [43]. Damage to DNA can lead to different responses. DNA repair proteins can sense and repair DNA damage. The cellular responses to damage involve recruitment of repair enzymes, activation of signal transducers that regulate cell cycle checkpoints, initiation of transcriptional program, DNA repair, cell survival, apoptosis and response to IFN signaling pathways [44] [45].

The key signal transducers and regulators in the DNA damage response are the nuclear kinase ataxia-telangiectasia mutated (ATM, ataxia telangiectasia, mutated) and ataxia telangiectasia and RAD3 related (ATR, ATM and RAD3-related) protein kinases [46]. Cell cycle checkpoints are then activated after sensing DNA damage and control the order and time point of cell cycle transitions and completion. Subsequently, DNA damage prevents the cell cycle from progressing and saves time for DNA repair [47]. In addition to the activation of cell cycle checkpoints, DNA damage response also initiates transcriptional programs, enhancement of DNA repair pathways, and apoptosis [48]. Both of the two proteins belong to a family of serine-threonine kinases which contain a phosphatidylinositol 3-kinase domain. Both ATM and ATR are able to phosphorylate many downstream substrates [49]. These two proteins can sense different types of DNA damage. ATM usually responds to DNA double strand breaks (DSBs) that are caused by exposure to ionizing radiation (IR). Cells that have mutations in ATM fail to repair DSBs and have chromosome instability, such as AT cell lines (ataxia telangiectasia mutant cell
line). ATR responds to UV damage and stalls DNA replication. It serves the response to DSB as a back-up of ATM [50, 51].

**The DNA repair pathways in lupus**

There is evidence that defective DNA repair is one of the causative factors associated with systemic lupus erythematosus. SLE patients are usually photosensitive to sunlight and frequently develop malar and vasculitic rashes, which is consistent with the possibility that damaged DNA is accumulated in cells [16] [52]. Studies of lymphocytes and fibroblasts from SLE patients have shown increased sensitivity to ionizing radiation and ultraviolet light [53]. In some SLE patients, fibroblasts with decreased levels of DNA repair induced by UV light may present DNA damage as antigenic denatured DNA. Under abnormal immune responsiveness, these antigens will form immune complexes, which presumably could cause photosensitivity in SLE patients [54]. Ionizing radiation is known to cause both single and double stranded DNA breaks. Studies have shown that there are DNA damage and DNA repair defects in cells from SLE patients after ionizing radiation [55]. Recently, there are studies showing there are both single and double strand DNA break repair defects in pediatric systemic lupus erythematosus patients. Our collaborator Dr. Deborah McCurdy at UCLA and her group has developed nine assays to assess and recognize DSBs, signaling and the DNA repair mechanisms in EBV transformed B lymphoblastoid cell lines from pediatric SLE patients [56]. Recently, there are studies showing that DNA damage in human cells caused by DSBs led to IFN production and induction of IFN-stimulated genes and IFN-α and IFN-λ genes [45]. Since increased IFN signature and IFN-inducible genes expression are highly associated with
SLE. DNA damage responses that are caused by DNA repair defects and lead to an IFN signature may have a mechanism in the pathogenesis of the SLE [57, 58].

**Genome-Wide association studies have identified genetic associations in genes in DNA repair pathways**

There are over 50 genetic variants that have been associated with SLE according to the GWAS study [7]. Several of them are involved in DNA repair pathway, such as TREX1, XRCC1, XRCC3 and XRCC4 [15, 59]. *De novo* mutations in the coding region of Trexl cause SLE [15, 60]. Trexl is a DNA exonuclease in human cells that can degrade DNA in both the cytoplasm and nucleus and prevents innate immune activation. Trexl can degrade abnormal replication intermediates to attenuate DNA damage checkpoint signaling and prevent pathological immune activation. The accumulation of undegraded DNA cause by mutant Trexl dysfunction can active type1 IFN signaling which leads to SLE autoimmunity [61, 62]. XRCC1, XRCC3 and XRCC4 are associated with SLE. XRCC genes play very important roles in DNA repair processes, including DSB repair. Most of the XRCC genes have the ability to prevent DNA damage hypersensitivity in different DNA repair pathways such as base-excision repair, non-homologous end joining, and homologous recombination. Mutations in XRCC genes lead to their protein dysfunction and cause disease disorders such as cancer [63].

**RAD51B**

RAD51B belongs to the RAD51 family and is essential for DNA repair by homologous recombination. It is a human protein encoded by RAD51L1. RAD51B usually forms a heterodimer with RAD51C and interacts with other DNA repair genes such as RAD51,
XRCC2 and XRCC3. It plays a role in sensing DNA damage and over expression can cause cell apoptosis [64]. Deficiency for RAD51B can lead to dysfunction of homologous recombinational repair (HRR). Cells with defects in RAD51B are sensitive to gamma radiation and other agents such as cisplatin and mitomycin C, which suggests that RAD51B is essential for DNA repair and chromosome integrity [65].

**RAD54B**

RAD54B is a gene that belongs to the DEAD-like helices superfamily which plays major roles in homologous recombination. RAD54B has the SWI2/SNF2 domain which displays DNA-stimulated ATPase activity. It can destabilize histone DNA-interactions and remodel the nucleosome. It also has functional interaction with RAD51[66]. RAD54B knock out mice ES cells and mice have hypersensitivity to ionizing radiation. It is highly expressed in testis and spleen, which suggests important roles in meiotic and mitotic recombination. Homozygous mutations of this gene were found in both primary lymphoma and colon cancer. It is localized in both of the nucleus and cytoplasm [67].
Reference


Chapter 2: DNA repair defects identified by exome sequencing in pSLE trios

Abstract

We use exome sequencing to identify de novo variants in pediatric lupus patients that have no family history. We developed our filter system to obtain reliable variants results from next generation sequencing data. From the variants obtained, we predict protein function. We use Sanger sequencing to confirm the candidate variants. From our current study, we found a de novo mutation in RAD54B from one lupus trio. RAD54B is a DNA repair gene. Much research and clinical evidence suggest that DNA repair is associated with lupus. The RAD54B variant identified is predicted to be damaging to protein function. We used real time PCR and Western blot to assess the expression level, which is not affected by the variant. Since RAD54B is a DNA repair gene, we used clonogenic survival assay and neutral comet assay to detect DNA repair defects in the transformed patient B cell line. The patient with the specific de novo mutation showed DNA repair defects after radiation. We complemented the mutant cell line with WT-RAD54B and tested the radiation sensitivity by neutral comet assay. The DNA repair defects in the mutant cell line had been partially recovered by the complement experiment. We also found two compound heterozygous variants in another lupus trio. The variants are in RAD51B which is also a DNA repair gene.
Introduction
Systemic lupus erythematosus (SLE) is a chronic, multisystem autoimmune disease. 10–20% of all the SLE cases present in children. Pediatric-onset SLE (pSLE) has more severe clinical course than adult-onset SLE (aSLE), which includes more extensive organ damage [1]. Most pediatric patients with SLE have no family history of autoimmune disease. SLE and other similar diseases may be associated with single gene defects in both humans and mice. Usually, human SLE is considered to be a complex genetic disorders, consistent with the GWAS results that show more than 50 associated genes making small contributions toward disease risk [2].

De novo mutation is a genetic mutation that arises in progeny and is neither possessed nor transmitted from parents [3]. Disruptive de novo variants can cause many rare genetic disorders, such as mental retardation [4-7]. De novo mutations found in TREX1 prove that de novo mutations could explain some cases of pSLE [8, 9]. In our study, we use family-based whole-exome sequencing approaches to identify de novo, non-synonymous variants in trios that include a pediatric lupus patient without family history of autoimmune disease. This mechanism of genetic inheritance has never been studied or explored before in pSLE. In our first lupus trio, a 12 year old girl who was diagnosed with pSLE and had no family history of autoimmunity, we identified two de novo mutations are each in RAD54B and DOCK8. In another lupus trio, a boy was diagnosed with pSLE and had no family history of autoimmunity as well. We found two compound heterozygous variants in RAD51B. All the variants have been confirmed by Sanger sequencing. The available in silico algorithms, including SIFT, PolyPhen 2 and
MutationTaster score variably predict damaging of the amino acid changes to the protein function. RAD54B is a DNA repair gene belongs to the DEAD-like helicase superfamily and is a double-stranded DNA-dependent ATPase [10, 11]. RAD51B is another a DNA repair gene encoded by the RAD51L1 gene and essential for DNA repair by homologous recombination and plays a role in sensing DNA damage [12, 13]. We performed the Clonogenic Survival Assay and Neutral Comet Assay using the EBV transformed B cell lines of the first lupus trio, which show that and the patient that has de novo variant in RAD54B also has DNA repair defects after radiation. We did complementary experiments by transducing wide type RAD54B vector into the mutant patient cell line and used neutral comet assay as the read out. DNA repair defects in the mutant patient cell line had been partially rescued. We concluded that the de novo mutation in RAD54B might be one of the reasons that cause DNA repair defects in this particular patient.

Results
Exome sequencing identifies a de novo mutation in DNA repair gene RAD54B and two compound heterozygous mutations in DNA repair gene RAD51B.

We have DNA from 67 complete pSLE trios selected from 3265 SLE cases and 2519 pedigrees available in the Lupus Family Registry and Repository (LFRR). We conduct the exome sequencing on these trios and identified variants in DNA repair genes in two of the pSLE cases from our collection. We used Illumina TruSeq to capture the exonic DNA and used next generation sequencing. After getting the next generation sequencing data back in VCF file, the data is analyzed by Golden Helix software (Table 1). Compared to the standard reference sequence, there are about 40,000-50,000 variants individually and 80,000-120,000 variants in each trio. The Mendel error frequency of
each trio is around 3,000 or about 35 of the variants. However, the concordance of replicate sample is around 90%, which means that there must be 10%-15% sequencing artifacts in the next generation sequencing data. We have developed a filtering system to identify variants that alter the amino acid sequence of a protein, including non-synonymous, a stop codon gain or loss, alter the open-reading frame, or alter the exon-intron splice site. By performing the filter system, we reduce the huge number of variants from next generation sequencing data down to a couple of candidates. For Quality Control (QC), we use criteria of read depth, Quality Score and alternate ratio. (Figure1). After passing the QC, we get rid of 20% variants as artifacts. This has left less than 50 Mendel errors in one trio and a high concordance of 99% in replicates sample. Variants that alter protein function will then be selected as functional polymorphism and minor allele frequency will be used to filter different types of mutations. We use 1% for de novo mutations and recessive mutations, 1% and 5% for compound heterozygous mutations. We found eight candidates for de novo mutations, 18 candidates for rare recessive homozygous variants and 23 candidates for compound heterozygous variants in our first lupus trio. As our filter system is being developed and modified, we usually find around an average about 3 candidates for de novo mutation, 5 for recessive mutation and 10 for compound heterozygous mutation. Two of the de novo mutations were confirmed by Sanger sequencing. One of the de novo mutations is in DNA repair gene RAD54B and the other de novo mutation is in DOCK8, which is a component in cytokinesis. We also found two compound heterozygous variants in DNA repair gene RAD51B in another lupus trio (Note 1).

**De novo mutation in RAD54B and compound heterozygous variants in RAD51B identified by Sanger Sequencing**
Candidate variants are validated by Sanger sequencing. We developed a semi-automated system to design PCR primers for multiple SNP at the same time. PCR products then are cleaned up with shrimp alkaline phosphatase and sent for Sanger sequencing. DNA sequence is analyzed by the DNASTAR Lasergene 8 SeqMan software (Figure 2).

**Gene disruption is predicted for de novo mutation and compound heterozygous variants**

We used SIFT, PhlyPhen2 and MutationTester to predict whether the genetics variants altering amio acid could affect the gene function. All the three methods predict that *de novo* mutation in RAD54B would be damaging to protein function (Table 2). The *de novo* mutation is in the N-terminal SWI/SNF domain of RAD54B with the DNA–stimulated ATPase activity, which alters the position of nucleosomes (Figure 3). The mutation changes an R (Arginine) to a Q (Glutamine) in RAD54B. We considered SNF2 domains in 290 sequenced eukaryotes proteins, aligned them, and tallied the amino acid frequencies at this position. The non-risk R is presented at this position in half of the 2313 SNF2 domains in the 96 metazoan genomes. By contrast, only 10 SNF2 domain-containing proteins have the risk Q at this position (Table 2). Q is not a preferred amino acid across the eukaryotic genome while R is. We are more confident to believe that the R amino acid is likely to have preserve function.

The predictions showed *de novo* mutation in DOCK8 is tolerated. However, DOCK8 is a gene plays an important role in inflammatory pathways [14]. We, of course, can not determinate that DOCK8 is related to lupus risk.

**DNA repair gene RAD51B has suggested association with SLE in the African American GWAS data.**
We have African American GWAS data shows that RAD51B has a suggested association with SLE. There are multiple SNP associated in this gene and the strongest associations are flanking one of the exon (Figure 4).

**The de novo mutation and compound heterozygous mutations do not result in the detection of less RAD54B expression.**

We performed Real Time PCR to detect RAD54B RNA expression level in the EBV transformed B cell lines from the first lupus trio. The patient showed similar level of RNA to her parents and other five non-lupus controls (Figure 5A). We also used Western blot to detect RAD54B protein expression in the EBV transformed B cell lines, the patient also shows similar protein expression with her parents (Figure 5B). Similar real time PCR has been done on RAD51B RNA expression, the gene did not show less expression neither (Figure 5C).

**Patient cells with de novo mutation in RAD54B show the most severe sensitivity to ionizing radiation at different doses.**

Clonogenic survival assay (CSA) and neutral comet assay (NCA) were used to assess double stranded DNA breaks (DSBs). The two assays are performed on EBV transformed B cell lines from AT cell lines, the three cell lines from the first lupus trio and eight non-lupus control human cell lines. The CSA is the gold standard method to assess relative sensitivity of cell lines to ionizing radiation. We used the AT cell line as the positive control and the patient with *de novo* mutation shows reduced clonogenic survival and cellular radiosensitive which is similar to the AT cell line. Both parental cell lines have a response similar to non-lupus human cell lines (Figure 6A).
Same experiment was done with cells from the second trio, which has the compound heterozygous mutations in the patient. In this second trio, the patient cell line showed similar clonogenic survival to the parents (Figure 6B).

The NCA was performed to assess DNA DSBs damage recovery. Cell lines were irradiated and then incubated for either 30 minutes or 5 hours before assay. Radiation caused DNA damage in the cell and resulted in a comet tail in DNA fragment after 30 minutes incubation. DNA damage in properly functioning cells over the 5 hours incubation after radiation as expected to demonstrate religation of broken DNA fragments in normal cells. The activity of this DNA repair machinery results in a shorter tail in the NCA (Figure 7A). DNA repair defects lead to an inability to repair and thus a long tail moment after 5 hours. Tail moment was quantified to show the average DNA damage in a certain cell at different incubation times. Tail moment increased sharply after 30 minutes incubation after radiation (Figure 7B). The tail moment repair ratio was quantified. The patient cell line with de novo mutation shows a similar repair ratio compared to the AT cell, the mother cell line shows a similar repair ratio compared to non-lupus human cells, the father cell line shows a repair ratio that is between the mutant and wide type cells (Figure 7C).

Same experiment had been done to our second lupus trio. This is patient showed a similar repair ratio with parents (Figure7 E F).

**Retrovirus transduction overexpresses RAD54B in cells with de novo mutation in RAD54B**

EBV transformed B cell lines from patient with de novo mutation in RAD54B were transduced with either the control MSCV virus or MSCV retrovirus expressing RAD54B.
Retrovirus alone and the retrovirus construct expressing RAD54B were produced in 293 T cells and transduced into the EBV transformed B cell lines from patient with de novo mutation in RAD54B. Flow cytometry was performed to detect the GFP percentage in transduced cell lines. Transduction efficiency was around 60% for MSCV retrovirus and 30% for RAD54B-MSCV retrovirus (Figure 8A). The GFP positive transduced cells were sorted. Expression of the RAD54B protein in 293 cells was confirmed by Western blot, with the MSCV empty vector transduced 293 cells as a control (Figure 8B).

**Repair of DNA damage in patient cells with de novo mutation in RAD54B increased after complemented with RAD54B**

Neutral Comet assay (NCA) was performed with cells either transduced with RAD54B-MSCV plasmid or MSCV as a negative control. Tail moment increased sharply after 30 minutes incubation after radiation (Figure 9A). Tail moment repair ratio was quantified. The patient cell with de novo mutation in RAD54B transduced with RAD54B-MSCV showed higher repair ratio than the cells transduced with MSCV control vector (Figure 9B).

**Discussion**

Lupus system erythematous is a chronic autoimmune disease that is characterized by chronic inflammation and the production of anti-nuclear auto-antibodies. SLE has a strong genetic component [15]. The pathogenesis of SLE is not completely understood yet. GWAS studies have revealed many genetic associations with SLE. From GWAS study of SLE, over 50 genetic variants have been associated with SLE [2]. Some of the genes are involved in DNA repair pathways such as TREX1, BRCC1, BRCC3 and
BRCC4 [16, 17]. In this project, we found RAD51B as a new SLE susceptibility gene in our African American GWAS data.

There is other evidence suggesting that defective DNA repair is associated with SLE. SLE patients are usually photosensitive to sunlight and frequently develop malar and vasculitic rashes [15], which is consistent with the idea that SLE patients have DNA repair deficiencies. Studies of lymphocytes and fibroblasts in SLE patients have shown increased sensitivity to ionizing radiation and ultraviolet light. Ionizing radiation is known to cause both single and double stranded DNA breaks. Recently, there are studies showing that there are both single and double strand DNA break repair defects in pediatric SLE patients[18]. Also, studies have shown that DNA damage in cells response to the interferon (IFN) signaling. IFN is well known to be important to human SLE. Hence, we hypothesized that a DNA repair defect after radiation is caused by specific variants found in SLE patients as a potential cause of disease pathogenesis through the IFN induced SLE [19, 20]. However, the genetic variants in DNA repair pathway in SLE patients have not been studied and the entire mechanism of DNA repair pathway in SLE is not clear yet.

We used exome sequencing and genome wide association study (GWAS) to study the genetics of SLE. We used trios with pediatric SLE patient without a family history in autoimmune disease to study the genetic variants. We looked for de novo mutation, compound heterozygous mutation and recessive mutation. From our exome sequencing project, we found one family that has two potentially dysfunctional variants of RAD51B, the case child inherit one variant from mother and the other from father which suggests that the child has compound heterozygous variants in RAD51B. In addition, from another
pediatric lupus trio, we found a *de novo* variant in RAD54B in the child. The *de novo* variant has been verified by Sanger sequencing. We generated the Epstein-Barr virus (EBV) transformed B cell lines from this particular trio as the standard model. The child showed DNA repair defects after ionizing radiation by clonogenic survival assay and neutral comet assay. We next asked whether the DNA repair defects in the patient cell line is due to *de novo* mutation in RAD54B. We transduced a wide type vector of RAD54B cDNA into the mutant cells and complemented part of the DNA repair deficiency.

Collectively, our data strongly suggest that the *de novo* variant in RAD54B DNA repair gene affects DNA repair efficiency in this particular pediatric SLE patient. Since the *de novo* mutation is heterozygous and DNA repair defect had not been 100 percent complemented by RAD54B cDNA, we consider that there might be other genetic variants could affect the DNA repair ability of this child as well. In the neutral comet assay, the father showed a repair ratio between the child and mother together with other non-lupus control cell lines, maybe there are some genetic factors in the father which also lead to damaged DNA repair and the child inherited these variants which work together with the *de novo* mutation.

In addition, there is another *de novo* mutation evaluated in DOCK8 in this patient. DOCK8 is stands for dedicator of cytokinesis 8 and involved in intracellular signaling networks [14]. Mutations in DOCK8 usually cause autosomal recessive hyper-IgE syndrome, combined immunodeficiency, allergic disease and also autoimmunity [21]. *De novo* mutation in DOCK8 may associate with SLE in this particular patient through the intracellular pathways. There are also 18 rare recessive homolozygous mutation
candidates and 23 compound heterozygous mutations candidates in this particular patient. Most of the variants are in genes with unknown functions in SLE pathways. All of these are candidate genes potentially contributing to this patient developing lupus.

In summary, our work uncovers the previously unknown mechanism of how genetic variants in DNA repair pathways are associated with SLE. We not only demonstrate the genetic variants by exome sequencing and GWAS, but also study the function of variants by developing a biological model in EBV transformed B cells, identifying both the genotype and phenotype. The study reveals DNA repair as a newly pathway associated with SLE, with that does not only prove that the \textit{de novo} mutation is responsible for the ionizing radiation sensitivity in cells, but also is responsible for the development of SLE in this child. In order to have a global assessment of the role of RAD54B gene, we have shown that the level of RAD54B protein produced is normal from the EBV transformed B cell line from the child with RAD54B \textit{de novo} mutation (Figure 5). Real time PCR of this gene produced the same result. Both results are expected since it is not the production of the protein that is predicted to be affected. Rather, its activity once normally produced is reduced in the child with SLE and the \textit{de novo} mutation in RAD54B. The transduction of RAD54B into the child’s cell line returns DNA repair toward normal is contented.

\textbf{Materials and Methods}

\textbf{SIFT.} SIFT (Sorting Intolerant From Tolerant) is a tool used to predict whether an observed sequence variant will be tolerable or damaging to the protein product of a gene. SIFT prediction scores are available for every possible SNP variant that may occur within an exon sequence. SIFT gives a score for each variant, and scores less than 0.05 indicate that a variant is expected to be damaging. Since we are using exome sequence data, SIFT
is a promising tool to identify which of the variants in our data are likely to be harmful [17, 22, 23].

**Polyphen2 (Polymorphism Phenotyping version 2).** PolyPhen-2 uses predictive features based on sequence and structure. The features were selected by a Bayesian analysis approach automatically. Most of these features contain comparison of the reference allele and the alternate allele, which can define the replacement of amino acid. Prediction of the functional significance of an allele replacement is based on its individual features. PolyPhen-2 score calculates the probability that whether a particular mutation is damaging and estimates false positive and true positive rates. A mutation is also appraised, as benign, possibly damaging, or probably damaging [23, 24].

**Mutationtaster.** Mutationtaster is an analysis method which integrates information from different biomedical databases and predicts disease-causing potential of DNA sequence alterations. Mutationtaster evaluation includes splice-site changes, evolutionary conservation, loss of protein features and alteration that affect mRNA expression level. The final results predict the potential of getting a certain disease [25].

**Neutral Comet Assay** (NCA, single –cell gel electrophoresis) NCA was performed according to the protocol of the manufacturer from Trevigen [26]. Cells were radiated at 15 Gy. Each slide was electrophoresed at 33V for 15 minutes. Slides were washed and dried and stained with SYBR green for 30 minutes and DNA comet tail were observed using a fluorescence microscope equipped with camera and acquisition software. The images were analyzed by Comet Score IV software.
**Clonogenic Survival Assay** (CSA). CSA was performed in the EBV transformed B cell lines [27]. Cells were plated in 96 well plates at a concentration of 200 cells/well and radiated at 0.5, 1, 1.5, 2.0 Gy. After 10-13 days incubation, cells were stained with MTT and colonies are counted. Colony survival fraction was calculated and used to the result.

**Retroviral vector and construct**

The MSCV vector was kindly provided by Dr. James Mulloy. The RAD54B cDNA was cloned with from RAD54B cDNA clone vector from Origene at restriction enzymes sites BamHI and XhoI. The PCR product was subcloned into MSCV between the sites BglII and XhoI. The RAD54B-MSCV ligation product was transformed into TBL3 competent cells and positive clone was cultured and Maxiprep was performed to extract high quantify RAD54B-MSCV construct. Sanger sequencing was used to establish that the construct has the expected sequence.

**Retroviral production**

RAD54B-MSCV, pEQ-PAM3 (-E) was transiently transfected into 293 T cells. The precipitate was washed away after 12 hours and viral supernatants were collected at 24, 36, 48 hours. The virus was subsequently used for transduction. We transduced 4 million EBV transformed human B cell lines with retrovirus using retronectin-coated dishes. The cells were expanded and cultured for 48 hours and subsequently analyzed on a FACScan and sort for GFP positive cells.
Figures

Table 1

<table>
<thead>
<tr>
<th>Filters Applied: GATK Best practices during alignment and variant calls Human Build 37.1.</th>
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<tr>
<td>Genotype Filters:</td>
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<td>Variant Read Depth</td>
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<tr>
<td>Variant Genotype Quality Score</td>
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<td>Software</td>
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Overview of Filter Criteria

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<th>Variants in trio</th>
<th>80,000-120,000</th>
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<td>Pass QC</td>
<td>Depth of reads &gt;15</td>
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<tr>
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<td>Genotype quality score &gt;20</td>
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<td></td>
<td>Alt ratio</td>
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<tr>
<td></td>
<td>Homozygous Ref &lt;0.15</td>
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<td>Homozygous Alt &gt;0.85</td>
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<td></td>
<td>Heterozygous between 0.3 and 0.7</td>
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<tr>
<td>Protein Altering</td>
<td>Functional Polymorphism</td>
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<td>Minor Allele Frequency</td>
<td>De novo mutation 1%</td>
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<tr>
<td></td>
<td>3 Candidate</td>
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<td>Recessive mutation 1%</td>
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<td>5 Candidate</td>
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<tr>
<td></td>
<td>Compound heterozygous mutation 5%</td>
</tr>
<tr>
<td></td>
<td>10 Candidate</td>
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</table>

Figure 1 Overview of Filter System Performance of QC.
**Note 1**

**Trio 1**

The female patient was diagnosed with SLE at the age of 12 years and had ACR classification criteria of photosensitive, malar rash, oral ulcerations, hematologic (leukopenia, lymphopenia, thrombocytopenia with a history of idiopathic thrombocytopenic purpura), immunologic (dsDNA and anti-Sm) and ANA (+). She has had recurrent infections of different types: many upper and lower respiratory tract infections, sinusitis, ear infections (all of these were bacterial and/or viral); shingles (VZV) and fungal infections (repetitive episodes of Candidiasis).

**Trio 2**

The male patient was diagnosed with SLE at the age of 12.5 years and had ACR classification criteria of photosensitive, malar rash, oral ulcerations, immunologic ANA (+) and anti-La. He has Anti phospholipid syndrome, a few seizures as a child.
Figure 2. A. Sanger sequencing validation of candidate variants

Sanger sequencing confirmes *de novo* mutations in the child, one in RAD54B and one in DOCK8. Child is heterozygous and parents are homozygous.

RAD54B: Child AG Father GG Mother GG (complementary strand)

DOCK8:  Child CG 301415 Father CC Mother CC

B . Sanger sequencing confirms compound heterozygous variants in RAD51B in the child.

Variant A : Child TG Father TT Mother TG

Variant B: Child TG Father TG Mother GG
Figure 3 The Structure of RAD54B showing the position of the De novo mutation. The de novo mutation we identified is in the N-terminal domain of RAD54B which is the region that interacts with RAD51. It occurs two amino acids before the SWI/SNF domain which has the DNA-stimulated ATPase activity and is capable of altering the position of nucleosomes along DNA [11].
Table 2

A. Summary of the *de novo* mutations.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Gene</th>
<th>Classification</th>
<th>Exon</th>
<th>Amino Acid Change</th>
<th>Genotype Child</th>
<th>Genotype Father</th>
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<td>RAD54B</td>
<td>Nonsyn SNV</td>
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<td>Arg113Gln</td>
<td>C_T</td>
<td>C_C C_C</td>
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<td>DOCK8</td>
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<td>C_C C_C</td>
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<table>
<thead>
<tr>
<th>Marker</th>
<th>SIFT Score</th>
<th>SIFT Pred</th>
<th>PolyPhen2 Score</th>
<th>PolyPhen2 Pred</th>
<th>MutationTaster Score</th>
<th>MutationTaster Pred</th>
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B. Summary of the compound heterozygous mutations.

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<th>Exon</th>
<th>Amino Acid Change</th>
<th>Genotype</th>
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<th>PolyPhen2 Pred</th>
<th>MutationTaster Score</th>
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Figure 4

Association Test of RADR51B from African American GWAS data. P values for differences in allele frequencies were calculated by chi-square test. The –Log P value for SNP at different positions on RAD51B gene on Chromosome 14 are shown.
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Table 3  The frequency risk and non-risk amino acid showing up in SNF-containing proteins at the particular position of the *de novo* mutation.
Figure 5  Real Time PCR and Western blot detecting RAD54B and RAD51B expression. A. Real Time PCR of RAD54B using EBV transformed B cell lines. B. Western Blot of RAD54B. C Real Time PCR of RAD51B using EBV transformed B cell lines.
Figure 6. Clonogenic Survival Assay. A. Survival Fraction of different cell lines from our first lupus trio which has the *de novo* mutation in RAD54B in patient at different radiation doses. B. Survival Fraction of different cell lines from our second lupus trio which has the compound heterozygous mutations in RAD51B in patient at different radiation doses.
A - 30min  5Hrs

Child

WT

B

Tail Moment

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<th></th>
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<th>5 Hr</th>
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<tbody>
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<tr>
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Figure 7 Neutral Comet Assay. A. Representative photomicrographs show tail lengths of patient cell line and a wide type cell line from non-lupus human cell. Original magnification 20X. B. Tail moment at different time point. C. Tail moment repair ratio at 5 hours post-irradiation. E Tail moment at different time point. F. Tail moment repair ratio at 5 hours post-irradiation.
Figure 8. Transduction of human EBV transformed B cell lines and overexpression of human RAD54B. A. Flow cytometry analysis of transduced cells. Negative cells with transduction showed are used as control. Cells transduced with MSCV vector showed around 60% GFP percentage and cells transduced with RAD54B-MSCV showed around 30% GFP percentage.

B. Western Blot analysis demonstrating expression of the RAD54B protein in transfected 293 cells which were used to produce virus.
Figure 9. Neutral Comet Assay showing RAD54B transduced cell line with increased repair of DNA damage. A. Tail moment at different time point. B, Tail moment repair ratio at 5 hours post-irradiation.