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Genomic Analysis of inherited Melanoma in a Palestinian family: a Next Generation Sequencing study.

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Declaration

I certify that this thesis submitted for the degree of master in the result of my own research, except where otherwise acknowledged. This thesis has not submitted for a higher degree to any other university or institution.

Eman Fares Mohammad

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Abstract

Cutaneous melanoma is a cancer type that arises from melanocytes, the pigment-producing cells in our skin. It is the most aggressive skin cancer and is responsible for about 75% of skin cancer deaths. Familial predisposition is among the strongest risk factors for cutaneous melanoma and accounts for around 10% of the cases. About half of these melanoma familial cases can't be explained by mutations in known moderate-to-high penetrance melanoma genes, thus impairing genetic testing and counseling in families with a predisposition to melanoma. The present study concerns a Palestinian family with melanoma, of which the patient had no pathogenic mutations identified in a previous targeted gene panel analysis of 94 genes. Therefore, the primary objective of this thesis is to identify germline mutations predisposing to melanoma in the family via the application of WES. The secondary objective is to determine family members at high risk of developing the disease. We hypothesize that the underlying genetic cause of melanoma is a rare germline mutation segregating in the family. We used whole exome sequencing approaches to identify candidate mutations from DNA samples of the patient, his brother with a brain tumor, and his unaffected brother. We then analyzed WES data using bioinformatics pipelines to generate variant call format for variant filtration. Afterwards, Sanger sequencing of the candidate variant was applied on DNA samples from the patient and family members for validation and segregation analysis. This is followed by *In silico* analysis of the candidate variants and genes. WES analysis identified three heterozygous rare missense variants, *WRN* (p.L383F and p.A995T) and *TYRP1* (p.T262M). Importantly, we identified a pathogenic homozygous missense mutation in *ERCC2* (p.R683Q), associated with the rare autosomal recessive xeroderma pigmentosum, which increases the risk of developing melanoma. The pathogenic

mutation cosegregated with the clinical phenotypes presented in the two affected brothers and was confirmed by sanger sequencing analysis. Segregation analysis of this mutation showed its absence in the homozygous state in the unaffected family members. These findings confirm that the homozygous *ERCC2* (p.R683Q) mutation is the causative mutation for melanoma in the Palestinian family. In conclusion, this study expands the knowledge of the mutational background of familial melanoma in the Palestinian population, which is valuable to guide the diagnosis, prevention, and treatment of affected patients and their families.

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List of Abbreviations

Abbreviation	Definition
ACMG	American College of Medical genetics and genomics
ATM	Ataxia-Telangiesctasia Mutated
BRIP1	BRCA1 Interacting Protein 1
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
EBF3	EBF Transcription Factor 3
GOLM1	Golgi membrane protein 1
MITF	Inducing Transcription Factor
MLH1	MutL homolog 1
µL	Microliter
MTAP	Methylthioadenosine Phosphorylase
NEK2	NIMA Related Kinase 2
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
OCA2	Oculocutaneous Albinism type 2
pM	Picomole
POLE	DNA polymerase epsilon
PolyPhen2	Polymorphism Phenotyping v2
PTEN	Phosphatase And Tensin Homolog
RB1	Retinoblastoma gene
SIFT	Sorting Intolerant from tolerant
SLC45A2	Solute Carrier Family 45 Member 2
UVR	Ultraviolet radiation

Chapter 1

Introduction

Among the strongest risk factors for cutaneous melanoma (CM) is the presence of familial predisposition factors. In the current work, we searched for the genetic risk factors predisposing a Palestinian family to CM by using WES. This chapter provides an overview of the subject by discussing incidence, genomics, therapies, treatment, risk factors, and the genetics of hereditary melanoma. We discuss the contribution of known high, moderate, and low penetrance melanoma predisposition genes in melanoma prone families. Then, we reviewed familial cancer syndromes and their risk to melanoma. The final section of this chapter focuses on exploring novel melanoma genes identified in recent studies before closing with a short summary leading into the hypotheses and specific aims of this thesis.

1.1 Cancer

1.1.2 Cancer mortality and situation in Palestine

Cancer is a major health problem, ranked as the second leading cause of death worldwide, by being responsible for a yearly estimated 9.6 million deaths. [2] In Palestine, cancer is also the second most common cause of death. It causes 14.0% of deaths, exceeded only by cardiovascular disease at 30.6% of total deaths in 2016.[3] It is estimated that the total number of reported cancer-related deaths was 10,940.[3] The high mortality rate of cancer in Palestine is due to the lack of access to cancer prevention and control strategies, late-stage diagnoses, low availability of cancer screening programs, and inadequate treatment

facilities. For example, there is no organized program or plan for preventable cancers such as cervical cancer and tobacco-related cancers, including lung, head, and neck .[4,5]

1.1.3 Cancer Genetics

Most cancer cases are sporadic and caused by acquired mutations occurring in somatic cells. Only around 10% of all cancers are hereditary. [6] Hereditary cancer develops due to an inherited germline mutation that can pass to through generations. [7] Early age of onset and the occurrence of multiple tumors or different cancers in the same family strongly suggest hereditary cancer.[8] The majority of somatic mutations in tumors don't confer a growth advantage to cancer cells, and these are known as passenger mutations. In contrast, Driver mutations are found in a subset of genes that are causally involved in cancer development by conferring selective growth advantage to the cell.[9] These driver mutations fall into two broad categories of genes, oncogenes, and tumor suppressor genes. Oncogenes have an activating effect in which a single allele is enough to cause growth advantage to the cancer cell by expressing the gain-of-function phenotype.[10,11] Loss-of-function is another event that also can lead to tumorigenesis through mutations resulting in the inactivation of tumor suppressor genes (TSGs). This is associated with the second event in a phenomenon known as loss of heterozygosity (LOH), which involves the loss of a wild-type allele at the tumoral level. In some cases, however, the loss of one gene copy of a TSG has a tumorigenic effect, and this refers to haploinsufficiency in which the reduced levels of the encoded protein cause the disease phenotype.[12] TSGs can be gatekeepers that directly control tumor growth (e.g., *RB1, CDKN2A*, and *APC*) or caretakers that maintain genomic stability, including genes that repair DNA (e.g., *BRCA1, BRCA2*, *MLH1*, and *ATM*).[13] Caretakers are commonly mutated in cancer, and most often increase the rate of mutations in other genes. Genome stability is ensured

through triggering DNA repair pathways, which include the mismatch-repair (MMR), base excision repair (BER) pathways, and nucleotide-excision repair (NER) pathway. Each of these has a different repair mechanism and is involved in repairing a specific type of damage. [10,13] Mutations can affect both the structure and function of the proteins. The former mainly affect the stability and/or folding of its protein product, while the latter affects specific functional sites . Both effects can be compatible with damaging non-synonymous mutations (nsSNPs) which are nucleotide substitution that changes the amino acid in the protein at conserved regions needed for protein stability, folding, and function.[14] Around 95% of mutations are single-base substitutions (such as C>G), while the remaining are deletions or insertions of one or a few bases (such as CTT>CT). Of the base substitutions, 90.7% result in missense changes, 7.6% result in nonsense changes, and 1.7% result in alterations of splice sites or untranslated regions immediately adjacent to the start and stop codons.[9]

1.2 Melanoma

1.2.1 Incidence

Melanoma is a cancer type that forms as a result of unregulated growth of pigment-producing cells known as melanocytes. The incidence of melanoma has been continuously increasing worldwide, especially in the Caucasian population.[15] The great majority of the diagnosed cases are cutaneous melanoma (CM), which is responsible for about 75% of skin cancer deaths.[16] Other rare types of melanomas are mucosal and uvea. According to the global cancer statistics that estimated the incidence and mortality of cancer in 2018, cutaneous melanoma had about 300,000 new cases.[2] The incidence rates of CM differ across countries. It is predominant in fair-skinned populations of Europe

and North America with high sun exposure [17]. Worldwide, incidences were the highest in Australia (36/ 100 000 person-years) and New Zealand (31.6/ 100 000 person-years) as estimated by the age-standardized world standard population 2020.[18] Unfortunately, the increasing incidence of CM has been accompanied by increased rates of mortality. [19] According to the Annual Health Report of Palestine of the year 2016, only 7 cases of melanoma were reported.[3] It is worth noting, however, that cancer incidence in Palestine is affected by incomplete reporting and surveillance issues.[4]

1.2.2 Genomics of Melanoma

The mutational landscape of melanoma is complex, since melanoma has the highest mutation burden among cancer genomes.[20–22]Next-generation sequencing (NGS) techniques are invaluable tools and have been used by some recent studies to identify somatic mutations underlying melanoma genesis.[21] In 2015, The Cancer Genome Atlas Network (TCGA) published a landmark study of melanoma genomics, which characterized alterations using DNA, RNA, and protein-based analyses of 333 cutaneous melanomas, providing the largest integrative analysis to date (Figure 1).[23] The great majority of these mutations, which are known as drivers, affect the MAPK signaling pathway. Four genomic subtypes of melanomas were classified according to the identified significantly mutated genes (SMGs) and their distribution in the cohort. *BRAF* hot-spot mutations form the largest genomic subtype and account for approximately 50% of all melanoma cases. The most common of these mutations is V600E substitution. The second subtype is characterized by the presence of RAS mutations, most frequently *NRAS*, followed by *KRAS* and *HRAS* mutations. *NF1* is the third most commonly seen SMG in the MAPK pathway and accounts for 14% of the samples. The last subtype is the

Triple-wild-type melanoma, which represents around 6% of melanoma and is defined by a lack of hot-spot *BRAF*, *NRAS,* or *NF1* mutations.[23]

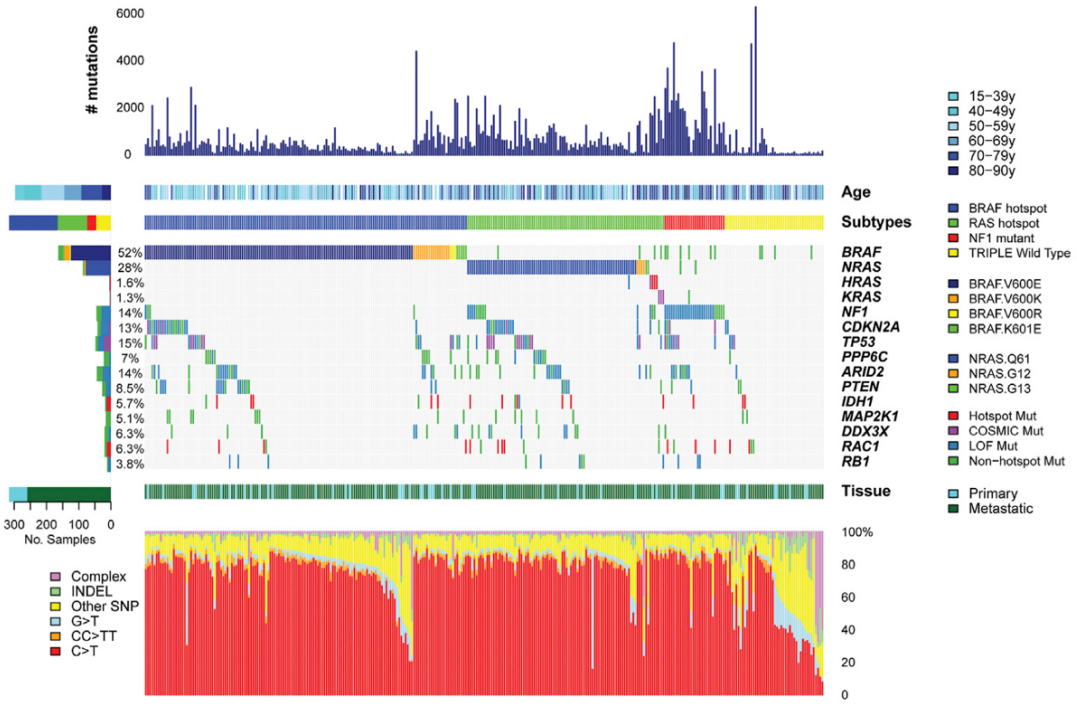


Figure 1. Landscape of Driver Mutations in Melanoma, adapted from [23] Total number of mutations, age at melanoma accession, and mutation subtype (BRAF, RAS [N/H/K], NF1, and Triple-WT) are indicated for each sample (top). (Not shown are one hyper-mutated and one co-occurring NRAS BRAF hot-spot mutant). Color-coded matrix of individual mutations (specific BRAF and NRAS mutations indicated) (middle), type of melanoma specimen (primary or metastasis), and mutation spectra for all samples (bottom) are indicated. For the two samples with both a matched primary and metastatic sample, only the mutation information from the metastasis was included.

1.2.3 Therapies for Melanoma Treatment

Most cases of melanoma are diagnosed at an early stage with a 5-year survival rate of 95%.[24] However, poor survival has been reported for melanoma diagnosed at a metastatic stage, where it is very aggressive and resistant to most of the standard treatments.[25,26] The high tendency of CM to spread and to resist treatments is related to the extensive mutational burden of CM, causing increased genetic heterogeneity. Due to this large heterogeneity, a huge effort is directed toward identifying targets for therapeutic

intervention and more importantly for early detection and diagnosis.[27] In recent years, effective targeted molecular therapies and immunotherapy regimens have emerged for the treatment of melanoma. Targeted therapies have been developed for melanoma with the hot spot mutation in proto-oncogene BRAF codon V600, which is present in around 50% of the cases.[28] Ipilimumab and vemurafenib are among the FDA-approved *BRAF* drug inhibitors that have been shown to attack advanced melanoma.[27] Although monotherapy with *BRAF* inhibitors shows great success in *BRAF*-mutant melanomas, patients can acquire resistance through upregulation of RTK or *NRAS*.[29] Nevertheless, *BRAF* inhibitors combined with MEK inhibitors such as trametinib and cobimetinib provide complete blockade of melanoma growth pathways and have shown improved overall survival (OS) in phase 3 clinical trials. [29] However, tumor resistance develops within 4-9 months following treatment through reactivating MAPK pathway.[30,31] Melanoma is widely considered as a highly immunogenic tumor.[32] Therefore, Immunotherapy has been intensively studied and has become one of the most powerful approaches for the treatment of patients with advanced and metastatic melanoma.[33] One such approach was the development of antibodies targeting immune checkpoints, mainly CTLA-4 and PD-1. These antibodies known as immune checkpoint inhibitors, act by removing the inhibition of T- cell activation, hence, allowing the immune system to recognize and attack the tumor.[34] The emergence of these immune checkpoint inhibitors has shown more durable responses and prolonged survival.[34] Unfortunately, a significant number of melanoma patients do not respond to immunotherapies.[35] An association between mutational tumor load and the clinical benefit from immunotherapy has been suggested. A recent study has shown that patients with *CDKN2A* mutations had an improved response rate to immunotherapy regimens, probably due to increased tumor

mutational load that leads to more neoantigens and stronger antitumoral immune responses. [35] An additional immunotherapy option for the treatment of melanoma is adoptive cell therapy (ACT) using Tumor-infiltrating lymphocytes (TILs)[36]. Treatment with TILs consists of the outgrowth of the patient's autologous T cells from tumor material, their expansion ex *vivo*, and then re-infusion into the patient to mediate anti-tumor response. [36]TILs have been a focus of interest in the past few decades due to their potential to serve as an independent prognostic factor in melanoma.[32] The presence of dense TILs has been associated with a good prognosis in patients with melanoma.[32] Multiple clinical trials have consistently observed objective responses ranging between 40 and 70%.[37–39]

1.3 Risk Factors for CM

The pathogenesis of cutaneous melanoma arises from the interplay between genetic, epigenetic, and environmental factors contributing to its development. A significant environmental risk factor for CM is exposure to UVR and history of sunburns. The carcinogenic effect of UVR causes DNA damage, specifically cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (64PP), which may induce mutations in the epidermal cells resulting in the development of skin cancer**.**[19] Studies have supported that exposure to UVR during childhood can result in frequent BRAF V600E mutations involving thymine to adenine (T>A) transversion, identified by an early age of diagnosis. [17] Other risk factors are related to individuals' phenotype, including fair skin, blue or green eye color, blond or red hair color, and many freckles.[40] Moreover, the presence of an increased number of nevi is considered to be among the most important risk factors for developing melanoma. Nevi are benign

melanocytic tumors, and although the majority are stable, some can progress and become melanoma. In one meta-analysis, it has been found that those with more than 100 common nevi had a 7- fold higher melanoma risk compared with those that have less than 15, and the risk of an individual with five dysplastic nevi is 6-fold higher than that of an individual with no dysplastic nevi.[41] Beyond environmental and phenotypic factors, genetic factors are significant for the pathogenesis of melanoma, with most of the melanomas, around 90%, are sporadic and the remaining being familial.[34] Melanoma pathogenesis can be triggered by activating proto-oncogenes or inactivating tumor-suppressor genes through molecular mechanisms, such as mutations, deletions, and translocations. Moreover, epigenetic alternation such as microRNA expression or DNA methylation is an additional mechanism involved in melanoma pathogenesis.[34] Finally, positive family history has an increased risk for developing melanoma, which indicates the presence of a shared germline mutation among family members. [42,43]

1.4 Genetics of Hereditary CM

Around 10% of reported melanoma cases occur in individuals with a strong family history of melanoma. It is known as familial or hereditary melanoma, which is characterized by two or more affected first-degree relatives.[42,44] Genetic analysis has suggested an autosomal dominant pattern of inheritance with incomplete penetrance. [45] Melanoma predisposition genes can be classified based on their contribution to melanoma risk into, high, moderate, and low penetrance genes (Figure 2). High-penetrance genes are rare, but they have an extremely high risk of developing melanoma when mutated in the germline cells, and they cause Mendelian-like disease in families. These genes include *CDKN2A, CDK4, BAP1, POT1, ACD, TERF2IP*, and *TERT* .[16] Melanoma patients with such

mutations often present with melanoma of an early onset and develop multiple primary tumors. [46] On the other hand, medium and low penetrance genes are found more frequently in the population but with a much-reduced effect size. They might combine with environmental risk factors, thus activate melanoma growth pathways. [44] Medium-penetrance genes include MITF and MC1R, while the low-penetrance genes include *ASIP, TYRP1, PLA2G6*, and maney others, all of which were identified through genome-wide association studies (GWAS). Collectively, melanoma-known genes are responsible for only 50% of familial melanoma cases [42], which means there are novel, high-penetrance genes that are very likely to exist. Therefore, some candidate novel genes have been identified by recent studies. [47–50] Melanoma patients and their relatives who can experience different types of cancer are within the context of mixed cancer syndromes. These syndromes are caused by mutations in cancer genes, including *PTEN, TP53*, and *BRCA1/2* genes (Table 1).[51] Cancers associated with melanoma have been studied, providing useful information regarding the shared genetic and environmental risk factors.[52,53] A recent study based on Swedish Family-Cancer Database has assessed the relative risk (RR) for any cancer in families with a high number of first-degree relatives who have been diagnosed with melanoma. In at least two independent analyses, an association has been found between melanoma and other cancers, including breast, prostate, colorectal, skin, and nervous system cancers.[53]

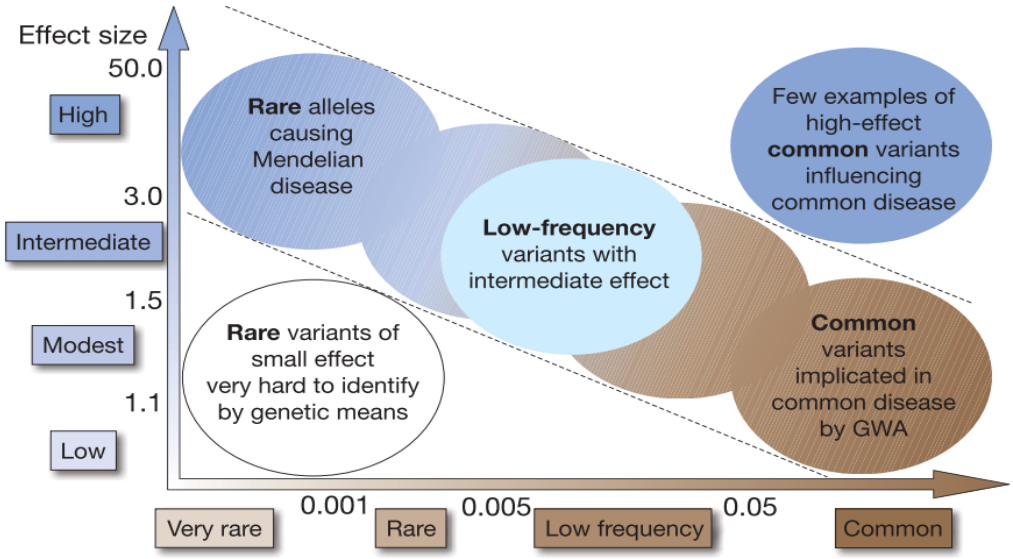


Figure 2. Effect sizes and allele frequencies of genetic variants associated with melanoma risk, adapted from[1]. The dotted line shows where the most emphasis and interest is when searching for predisposition loci. GWA = genome wide association.

1.5 High-Penetrance Genes

Several high-penetrance familial melanoma genes have been identified, and account for about 50% of all melanoma families. The identified genes are involved in cell cycle regulation, *CDKN2A, CDK4,* and *BAP1* or genomic stability by telomere maintenance, *TERT, POT1, ACD*, and *TERF2IP*.[54–58]

1.5.1 *CDKN2A*

Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*) is the most frequently mutated high-penetrance gene leading to hereditary melanoma. The *CDKN2A* tumor suppressor gene is located on chromosome 9p21.3. It encodes two proteins involved in cell cycle regulation, p16INK4A, and p14ARF.[59] p16INK4A binds to cyclinD1/CDK4 complex and inhibits their function in phosphorylating retinoblastoma protein (pRB), therefore preventing the cell cycle from entering the S-phase. Engaging the p16Ink4A/RB pathway

leads to senescence and regulation of cell damage response. However, mutation of *CDKN2A* prevent the binding of p16Ink4A to cyclinD1/CDK4, thus evading senescence. [60,61] p14ARF is a positive regulator of *TP53* that inhibits the *MDM2*- mediated *TP53* degradation, and thus its loss results in the accumulation of DNA damage. [25] Mutations in *CDKN2A* account for about 20–40% of melanoma-prone families, with variations in mutation frequencies between different geographic regions**.** [40,59] Geographically related founder mutations have been documented, with some occurring as a single dominant mutation according to common ancestry. Such founder mutations include p.Arg112dup, which explains the majority of *CDKN2A* mutations in Sweden. [62,63]This mutation changes the peptide sequences of p16-INK4A, specifically by inserting (duplicates) of an arginine at codon 112 in one of the ankyrin repeats, disrupting its binding to *CDK4/6*.[63] Different variants were identified in different world populations including, a missense mutation, p.Asp108Tyr, in Sweden, which segregated with melanoma in three families, and p.Gly89Asp variant identified in the Icelandic population.[58,64] In Europe, G101W occurs as the most common recurrent mutation in France, Spain, and Italy, while the M53I, IVS2-105A/G, R24P, and L32P mutations are shared between Australia and the United Kingdom.[65] The penetrance of *CDKN2A* mutations, which is the proportion of people carrying the mutation and develop the disease, is incomplete because it reflects an association between environmental and genetic factors, including the geographic area, UV exposures, the melanoma incidence rate, genetic modifiers, and age. [66] Accordingly, penetrance for *CDKN2A* mutation carriers at the age of 50 is estimated to be 13% in Europe, 50% in the US, and 32% in Australia, while at the age of 80 the penetrance was 58% in Europe, 76% in the US and 91% in Australia. [40] Another variable that can affect *CDKN2A* mutation incidence is the presence of atypical moles such as in familial-atypical

mole and malignant melanoma syndrome (FAMMM). Relatives with this syndrome appear to have a three-fold risk to be a carrier for the *CDKN2A* germline mutation. [66] Moreover, families that carry *CDKN2A* germline mutations have an increased risk of developing other cancers. The most documented association is between *CDKN2A* and pancreatic cancer. [63,65] Besides familial melanoma, *CDKN2A* mutations have been found in sporadic melanoma but with a lower percent. In a study that investigated the incidence of somatic and germline mutations in Italian patients with multiple primary melanomas (MPM), It was shown that these patients with a known family history of melanoma have a higher frequency of *CDKN2A* mutation (37.5%) compared to those without a family history (3.5%).[67] Interestingly, families with *CDKN2A* germline mutations affecting P14ARF transcript, specifically exon 1β deletions, have been reported to develop neural system tumors in addition to melanoma in at least three families. [68] Screening for p16 mutations in 30 Israeli Jewish melanoma families has identified two p16 germline mutations, G101W, that have been previously described in melanoma families, and G122V, a novel missense mutation. Functional analysis has shown that G122V novel mutation significantly interfered in its ability to cause a G1 cell cycle arrest in human fibroblasts, besides having partial interaction with cyclin-dependent kinases (CDKs) *in vitro*, suggesting that G122V substitution affects the 3-dimensional structure of the p16 protein.[69]

1.5.2 *CDK4*

CDK4 is the second high-risk melanoma susceptibility gene after *CDKN2A*. [40] A small number of melanoma families with mutations in *CDK4* have been reported worldwide. Germline mutations in *CDK4* have oncogenic effects on the control of the cell cycle by

the same pathway as p16INK4A. Only two distinct mutations in *CDK4* have been identified and both occur in codon 24 of exon 2, p.R24C and p.R24H. Functional studies have shown that these mutations with arginine substitution at codon 24 result in binding loss of *CDK4* to p16INK4A. [68] However, a recent study has reported a novel germline *CDK4*, c.71G>T,p.Arg24Leu, in a melanoma family that segregated into three family members and predicted to be damaging by *In silico* analyses.[70] Screening of 17 familial melanoma pedigrees with *CDK4* mutation has revealed that the median age at first melanoma diagnosis was 39 years and based on estimates from available data, the lifetime mutation penetrance was 74%.[57] Moreover, melanoma families with *CDK4* mutations were described to be phenotypically similar to those with *CDKN2A* families regarding the age of diagnosis, predisposing to early-onset MPM, and increased atypical nevi. [57] Although mutations in this gene are rare, screening of exon2 at the *CDK4* locus is recommended whenever melanoma families have a negative test for *CDKN2A*.[57]

1.5.3 *BAP1*

Germline mutations in the BRCA1-associated protein (*BAP1*) gene have been associated with a cancer syndrome of various tumor types including, cutaneous melanoma, uveal melanoma, mesothelioma, renal cell carcinoma (RCC), and others. [59] Due to this diversity of cancers, *BAP1* mutation exhibits a variable penetrance that possibly depends on unidentified genetic modifiers or environmental factors.[61] *BAP1* is a deubiquitinating enzyme that functions as a tumor suppressor and is associated with the regulations of critical pathways including, cell proliferation, differentiation, and DNA damage response. The prevalence of *BAP1* mutations has been reported to be present in around 1% of melanoma families worldwide.[60]A recent population-based analysis in the united

kingdom has detected 22 *BAP1* variants among 1977 CM cases; 5 variants among controls and 3 of the detected variants had a missense mutation (S98R), that was confirmed to affect the *BAP1* activity and was associated with a family history of other *BAP1*-related cancers. The study concluded that deleterious *BAP1* germline mutations harbored by CM patients are rare.[71] In a recent study published in 2020, six variants, five frameshifts, and one non-sense were identified in the *BAP1* gene, which was included in a targeted gene panel, testing for 273 probands lacking *CDKN2A/ARF* and *CDK4* mutations. Of the identified variants, two novel frameshift variants, c.1337delA and c.1777dupC.[72]

1.6 Telomere-Associated Mutations

The length of a telomere is variable among chromosomes and even between individuals. It ranges from 0.5 kb to 15 kb in humans depending on age and type of tissue.[73] Progressive shortening of telomeres at each cell division is associated with the normal aging of cells. Environmental risk factors such as UVR may hasten the aging process. However, several mechanisms for telomere length maintenance exist to overcome this gradual telomere shortening, primarily through two protein complexes the telomerase complex and the shelterin complex [16] Telomerase is an enzyme that contains reverse transcriptase (*TERT*), which functions in elongating telomeres by adding new telomeric repeats. Telomeric DNA is bound to the shelterin complex, which it is composed of six subunits; *TRF1, TRF2, RAP1, TIN2, TPP1*, and *POT1*.[74] These proteins protect chromosome ends against degradation and chromosomal fusion, thus maintain genomic stability and integrity. [55]Melanoma susceptibility involves various genes that play a role in telomere maintenance and lengthening. Germline mutations in *TERT, POT1, ACD,* and

TERF2IP have been reported to cause familial melanoma, and they explain around 1% of melanoma cases.[55,75,76]

1.6.1 *POT1*

Protection of telomere 1 (*POT1*) gene is located on chromosome 7q31.33 and composed of 19 exons spanning around 108,000 base pairs. *POT1* encodes a protein that affects telomerase activity and DNA replication. Mutations in this gene can result in telomere elongation. One study has identified 9 germline rare variants of *POT1* gene, most of them affect the highly conserved oligonucleotide/oligosaccharide-binding (OB) fold domains, which are necessary for the specific binding of *POT* to the single-stranded telomeric repeats. [77] Whole-exome sequencing (WES) analysis on melanoma families from UK, Netherlands, and Australia have identified loss of function mutations in the *POT1* gene. It has been shown that all nine carriers of *POT1* mutations developed melanoma in addition to developing breast and small cell lung cancer in two of the individuals. Mutations of *POT1* that were detected include a p.Tyr89Cys variant in the N-terminal OB domain in a five-case pedigree and a splice-acceptor variant found between exons 17 and 18 carried by a six-case pedigree. Another two variants of the OB domain, p.Gln94Glu, and p.Arg273Leu, each of which were observed in a case from distinct families. [78] Similarly, in an Italian cohort, a rare variant in the *POT1* gene (Ser270Asn) has been identified in five unrelated families. This variant was found at a highly conserved residue in vertebrates and predicted to be deleterious by most *In silico* tools evaluated. Additionally, two more rare *POT1* variants were detected in all cases sequenced in two distinct Italian families, yielding a frequency of *POT1* variants comparable with the *CDKN2A* mutations among melanoma families in this population.[77]

In a Spanish study of *CDKN2A*/*CDK4* wild-type melanoma families, four pedigrees out of 228 (1.75%) were identified with additional distinct *POT1* mutations (p.Ile78Thr, p.Glu344*, c.255G > A and p.Asp598Serfs*22).[79] In another study, Wong et al. (2019) have identified the POT1 p.I78T variant in 3 melanoma families of Jewish ancestry, that disrupted the *POT1*-telomere binding. The authors suggested that this variant represents a candidate susceptibility allele for melanoma development. [80]

1.6.2 *ACD/TERF2IP*

High penetrant mutations in other shelterin complex genes, adrenocortical dysplasia homolog (*ACD*), and telomeric repeat binding factor 2 interacting protein (*TER2IP*) have been described in melanoma-prone families. In a cohort of 510 melanoma families that were negative for mutations in known predisposition genes, six families had mutations in *ACD*, and four families carried *TER2IP* mutations, including segregating nonsense mutations for the two genes. A p.Q320X nonsense mutation in *ACD* identified in an Australian family, segregated in all four melanoma cases, and was linked with early-onset at diagnosis. Additionally, a p.N249S mutation was identified in two separate families from Australia and Denmark, with a founder haplotype shared across the *ACD* locus. The mutation segregated in all seven cases of the Australian family, with eight confirmed and four unconfirmed CM cases. Both p.Q320X and p.N249S mutations clustered in the POT1 binding domain of *ACD*, which reflects the important role of the *ACD-POT1* heterodimer in mediating the interaction between shelterin and *TERT*, thus the telomerase activity. *TERF2IP* is a negative regulator of telomere length that acts by repressing homology-directed repair (HDR). An identified non-nonsense mutation, p.R364X, resulted in a C-terminal truncated protein, leading to disruption of the TERF2IP binding

site, and therefore loss of binding to the shelterin complex. In addition, a novel missense variant, p.Q191R was observed in a two-case melanoma family and was associated with an age of melanoma at 15 and 24 years.[55] More recently, Potjer et al. identified a very rare missense variant *TERF2IP* (c.398G>A, p.R133Q) in a proband of a two case Dutch family. The variant is located in the MyB DNA binding domain and was predicted to be deleterious by at least two *in-silico* tools. [43]

1.6.3 *TERT*

Telomere length maintenance is a function of telomerase, and altered regulation of telomerase lead to the unlimited replicative potential of cancer cells. Mutations occurring in the *TERT* promoter were found to be associated with familial CM. [40,56,81] Two studies have identified a single nucleotide germline mutation in the *TERT* promoter (c.-57T>G) in two independent large families of Northern-European ancestry. One of them has included four affected and four unaffected members of a 14-case melanoma family using a High-throughput sequencing. The identified mutation was detected in all four affected individuals in addition to one non-affected individual. This family was described by the development of melanoma at early-onset, at age 20 and age 30, as well as the development of various types of cancer, suggesting that this mutation is highly penetrant. Functionally, the mutation resulted in the creation of a new binding motif for Ets transcription factors which lead to a two-fold increase in the transcriptional activity of *TERT*.[76] In the other study, targeted sequencing involving 675 melanoma families have identified the same mutation in two affected individuals in a one 7-case melanoma family. Multiple primaries and early age of onset were characteristics of this family.[56] These results provide evidence confirming that germline *TERT* promoter mutation is extremely rare in familial melanoma

and accounts for less than 1%. In addition to germline mutations, somatic mutations within the *TERT* promotor from sporadic melanoma cases have also been described. These mutations are all found in the C-to-T transitions, revealing a recurrent UVR-signature.[76]

1.6 Medium-Penetrance Genes

1.6.1 *MC1R*

Pigmentation characteristics are one of the phenotypes associated with melanoma risk. The Melanocortin-1 receptor (*MC1R*) regulates melanin pigment production, which protects the skin against UVR damage. It encodes the G-protein coupled receptor MC1R, which binds its ligand -melanocyte-stimulating hormone (α-MSH). This binding triggers cyclic AMP (cAMP) production through the activation of adenylate cyclase (AC). Increased levels of cAMP activate signaling cascade via downstream *MITF* and tyrosinase and stimulate the production of eumelanin pigment. The increased amount of photoprotective black/brown eumelanin pigments decreases the relative amount of red/yellow pheomelanin, which are poorly protective against UVR. *MC1R* is a highly polymorphic gene responsible for variable skin phenotypes. Certain *MC1R* polymorphisms cause a switch in pigment synthesis from eumelanin to pheomelanin and are associated with the red hair color (RHC) phenotype. An association has also been shown between such variant and the risk of developing both sporadic and hereditary melanoma. The most common variants of *MC1R* have been classed as (r) variants when they are weakly associated with RHC (p.V60L, p.V92M, p.R163Q) and (R) variants, when there is a strong association with RHC (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H). [40]A pooled analysis from 17 case-control studies has found that

carriers of a single *MC1R* variant had around 40% increased risk of melanoma compared with wild-type controls, and the risk attributed to *MC1R* variants was 28%. For individuals who carry more than one variant allele, the risk increases to more than double the risk found for wild-type controls.[82] Moreover, studies on melanoma families have shown that common *MC1R* variants serve as a modifier gene for *CDKN2A* by increasing the penetrance of *CDKN2A* mutations from 50% to 84% and decreasing the age of melanoma onset, particularly in MPM patients. [57,83]

1.6.2 *MITF*

MITF is a transcription factor encoding gene, and the master regulator for the functions of melanocytes, including development and differentiation. [16] MITF regulates various biological processes in melanocytes through the expression of genes involved in pigmentation (*TYR, TYRP1,* and *MC1R*), proliferation, and regulation of cell cycle (p16, p21, CDK2), and apoptosis (BCL2). [84] A gain-of-function mutation in *MITF*, p.E318K has been reported to increase the risk to CM by 5-fold.[51,74] This mutation disrupts a conserved small-ubiquitin-like modifier (SUMO) site, which consequently decreases the SUMOylation of *MITF*.[16,85] SUMOylation of *MITF* suppresses its transcriptional function, p.E318K increases the *MITF* transcriptional regulatory activity and can result in the up-regulation of distinct sets of target genes[40] Two reports on the germline *MITF* mutation, have shown that p.E318K co-segregated with melanoma in multiple families. One of the reports has identified 31 melanoma-prone families that were carriers for the p.E318K variant and segregated in some but not all family members indicating that it is a medium penetrance melanoma variant same as *MC1R*. From the analysis of the variant allele in an Australian case-control study that has also been replicated in a UK case-

control sample, the p.E318K variant has been shown to be associated with an increased nevus number, non-blue eye-color, and multiple primary melanomas.[86,87]

1.7 Low-Penetrance Genes

In addition to the known medium penetrance genes *MC1R* and *MITF,* 18 other low-penetrance genes with high allele frequencies were revealed by GWAS.[88] These genes are implicated in various biological processes. One group is involved in naevi count and pigmentation. Agouti signaling protein (*ASIP*) encodes an antagonist of *MC1R*, and ASIP SNPs have been associated with red hair and freckling. Tyrosinase (*TYR*) encodes a protein involved in eye color and tanning response, where its enzyme activity determines the ratio of eumelanin to pheomelanin, and hence *TYR* mutations can contribute to a fair skin phenotype. Tyrosinase-related protein 1 (*TYRP1*) SNPs have also been significantly associated with melanoma. It stabilizes the protein encoded by *TYR*, and thus alternations in this gene can also influence tanning response. Other phenotypic associations with melanoma have been reported in pigment-associated SNPs in the *HERC2/OCA2* at chromosome 15q13.1. The major SNPs that were found to be associated with melanoma risk are rs1129038 and rs12913832. The latter being a determinant factor of blue-brown human eye color. Phospholi- pase A2, group VI (PLA2G6) is associated with both pigmentation and naevi, while *CASP8, TERT, IRF4, AGR3, MTAP/CDKN2A*, and *FTO* are associated with variation in naevi count. [16,40] Other groups of genes are those encoding proteins involved in cell growth and differentiation (*BRAF, EGF, VDR*, and *EDNRB*), DNA repair (*XPC, XPD*, *XRCC3, PARP1 and ATM*), and metabolism and detoxification (*GSTM1* and *GSTT1*).[45,89]

1.8 Familial Caner Syndromes and Risk to Melanoma

Many rare autosomal familial syndromes have been described, with the occurrence of multiple cancers, including melanoma. These syndromes include Li-Fraumeni syndrome, Cowden syndrome, Werner syndrome, xeroderma pigmentosum, and familial breast cancer. Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer disorder linked to germline mutations in TP53. It is associated with a wide spectrum of cancers including, bone and soft tissue sarcomas, breast cancer, brain tumors, and adrenocortical carcinomas. Although the inclusion of melanoma in the syndrome is controversial, some melanoma cases were described, including a single patient harboring a germline mutation in *TP53* who presented with multiple primary cutaneous melanomas. Cowden syndrome is also autosomal dominant caused by germline mutations in the tumor suppressor *PTEN*. *PTEN* mutation carriers have elevated risk of breast, thyroid, and endometrial cancers, as well as melanoma. By contrast, xeroderma pigmentosum (XP) is an autosomal recessive disorder caused by mutations in one of eight genes involved in the nucleotide excision repair pathway (NER) that repairs UVR-induced DNA damage. 600- to 8000-fold increased risk of malignant melanoma has been reported in individuals with XP.[42] Another example of autosomal-recessive inheritance of cancer predisposition syndromes is Werner syndrome, caused by loss of function mutations in the *WRN* gene results in premature aging, with a wide range of cancers including thyroid cancer, meningioma, melanoma, sarcomas, and leukemia. Other melanoma cancer syndromes include, hereditary breast and ovarian syndrome, which is caused by mutations in the *BRCA1* or *BRCA2* genes. Carriers of *BRCA1* or *BRCA2* mutations have an increased risk of developing breast, ovarian, prostate, and pancreatic cancer. [42] *BRCA1* and *BRCA2* are genes involved in the DNA repair machinery, and mutations in these genes affect

the ability to repair DNA damage, particularly the double-stranded breaks.[42] Analysis has shown that *BRCA2* mutations are associated with a 2.6-fold increased risk of CM, while there's no increased risk associated with *BRCA1* mutation**.** [90]

1.9 Novel Genes

Several rare genes have been recently proposed as candidate melanoma genes, with some might be highly penetrant. A recent study has performed WES targeting 42 candidate melanoma susceptibly genes among 144 melanoma cases in 76 American families. [91]The analysis has revealed a high frequency of rare variants in *OCA2* and other albinism genes such as *TYR* and *TYRP1*. Interestingly, a nonsense variant in *TYR* was reported in a six-case melanoma family and showed near-to-complete co-segregation. It is suggested that albinism genes are good candidates for further investigation. Additional findings were a potentially deleterious missense variant in *ATM* and a start gain variant in the 5'UTR region of *PLA2G6*, both occurred in a single-family, and co-segregated with the disease in three affected members.[91] A study published in 2020 has searched for germline mutations in melanoma patients to characterize it's prevalence through NGS analysis targeting 217 genes. The study found that 3.4% had mutations in high-to-moderate melanoma risk genes (*CDKN2A, POT1,* and *ACD*), and 8.3% of patients carried mutations in other cancer syndrome genes (*NBN, BRCA1/2, CHEK2, ATM, WRN,* and *RB1*). The most frequent germline mutation in melanoma patients was observed in the *NBN* gene (2.7%), coding for nibrin, a protein part of an MRN complex, functioning in DNA repair, followed by *BRCA2*.[92] The role of mutations in *BRCA1/2, CHEK2*, and *PALB2* genes in the development of familial melanoma is still a matter of debate. [93–95] *POLE* is a polymerase involved in DNA repair and replication and is mainly associated

with colorectal cancer. A study by Lauren G has reported a novel germline mutation in the exonuclease domain of *POLE*, p.(Trp347Cys), in a seven-case melanoma family that showed a near-complete co-segregation. Furthermore, multiple cancer types were observed in the melanoma families that performed targeted sequencing of *POLE*, including colorectal cancer, prostate cancer, and acute myeloid leukemia.[48] An analysis of 30 candidate melanoma susceptibility genes in a large cohort of Dutch melanoma-prone families lacking a known *CDKN2A* or *CDK4* mutation has identified a novel truncating variant in the *BRIP1* gene, two missense variants in the exonuclease domain of the *POLE*, and nine potentially deleterious variants in the *OCA2* gene. Along with other research groups, additional candidate genes were detected and occurred in only one proband with some lack of co-segregation data. These include four truncating variants in *ERCC3, NEK2, RASE*, and *POLH*, two canonical splice site variants in *NEK2*, and *NEK4*, and several potentially deleterious missense variants in *CBLB, ERCC3, MLLT6*, *NEK2, NEK4*, *NEK10, NEK11, PARP1, POLH*, and *RASEF*.[96]A recent study that used NGS to investigate germline mutations in families negative for the known melanoma sociability genes has identified several heterozygous variants of interest in *OCA* genes, *TYR, OCA2, TYRP1*, and *SLC45A2*. Of particular interest, *SLC45A2* was identified as a likely pathogenic frameshift variant and showed complete segregation in a 4-case CM family. *SLC45A2* functions as a membrane-associated transport protein (MATP) and compound heterozygous variants in *SLC45A2* have been strongly linked with distinct changes in pigmentation. *SLC45A2* has been proposed as a low-penetrance melanoma susceptibility gene in sporadic CM. However, the authors concluded that this is the first study to report a segregating deleterious *SLC45A2* variant in a familial context.[97] Other rare variants identified by WES were reported in GOLM1, EBF3, and *TP53AIP1* genes, even though

their effect size and functional effect still need future studies to clarify.[89,98,99] In a rare variant germline genome-wide association study, the EBF3 showed statistical significance and was identified as a novel risk candidate for CM. Loss of EBF3 expression correlated with aggressiveness, poorer outcome, and high *MITF* in tumors. [89]A recent study has assessed 525 cancer or DNA repair genes in a cohort of patients with CM and a history of at least two independent additional primary cancers. The study has identified variants in *JAK1, JAK2, SF3B1, SRSF2, TET2*, and *TYK2* that haven't previously been reported in the germline. All patients with these variants had a history of hematological malignancy and CM in addition to colorectal cancer and/or prostate cancer. Other potentially pathogenic germline variants were found in *BUB1B, POLE2, ROS1*, and *DNMT3A*.[100] Recently, a Dutch family with 4 cases of cutaneous melanoma was subjected to whole-exome sequencing. Interestingly, all members were negative for the known melanoma predisposition genes (*CDKN2A, BAP1, POT1, TERT, TERF2IP, ACD, MITF, GOLM1, EBF3*). However, a nonsense protein-truncating variant (p.Arg374Ter) was identified in *NEK11*, a family of kinases that function in cell cycle regulation. Previously, somatic mutations in *NEK11* have been reported in various cancer types, including prostate cancer and melanoma. Accordingly, *NEK11* was defined as a potential novel high-penetrance melanoma susceptibility gene. [101] In another recent study, three novel melanoma Susceptibility genes were identified, following a high-throughput sequencing of eight patients with MPM, a high number of nevi, and negative for germline mutations in high and intermediate-risk genes. The novel genes were *CDH23*, *ARHGEF40*, and *BRD9*, the most promising susceptibility genes among 13 new potentially pathogenic identified variants. *In silico* analysis has suggested that *CDH23* and *ARHGEF40* variants may alter the protein structure and function. *BRD9* is involved

in oncogenic and DNA repair mechanisms, make it the most promising gene in hereditary melanoma to be further investigated. [50] In another WES study, ten rare, co-segregating, predicted deleterious missense variants, were identified in two affected members from a family of 4 melanoma cases without known mutations in high penetrance melanoma-susceptibility genes. Of the ten rare variants, only two, *DOT1L* (R409H) and *SLCO4C1* (P597A), co-segregated with melanoma in all four affected cases of the family. *DOT1L* is a unique histone methyltransferase that methylate histone the nucleosome core on H3K79. It has been previously reported that mutations in *DOT1L* compromise the removal of UV photoproducts in UV-irradiated melanocytes, thus enhance the malignant transformation. Although the functional analysis of the recently identified variant didn't reveal the expected role of the gene, the role of *DOT1L* as a novel melanoma susceptibility gene deserves further examination.[49] More recently, a study aiming to identify rare variants from five Brazilian melanoma-prone families had reported seven novel putative candidate genes affected by loss of function (LOF) variants which are *ADGRG7, FAM221A, SERPINB4, UNC93A, HRNR, OR51M1*, and *SLC5A11*. Variants in these genes have been detected in three families and co-segregated in affected relatives of each family. Additionally, 281 rare missense variants with co-segregation in genes such as *MYO7A* and WRN have been reported.[102]A Genome-wide linkage analysis study has identified a novel melanoma susceptibility loci, with significant linkage at 11q14.1-q14.3 in Spanish melanom prone families. The genes located in this region were *DLG2, PRSS23*, *FZD4*, and *TMEM135*. There is limited knowledge about their biological role, but they are likely candidates for cancer susceptibility, and future sequencing studies are necessary to explain their possible role in melanoma susceptibility.[103]

Table 1. Genes associated with familial melanoma germline predisposition

Categories	Genes	Reference
High-to-Moderate penetrance genes	*CDKN2A, CDK4, BAP1, POT1, ACD, TERF2IP, TERT, MC1R, MITF.*	[46], [54–58]
Low penetrance genes	*ASIP, TYR, TYRP1, HERC2, OCA2, CASP8, IRF4, AGR3, MTAP, BRAF, EGF, VDR, EDNRB, XPC, XPD, XRCC3, RAP1, ATM, GSTM1, GSTT.*	[16,40], [45, 88], [89]
Syndromes associated genes	*TP53, PTEN, WRN, BRCA1/BRCA2, XPC, XPD.*	[42], [90]
Novel genes	*RB1, NBN, POLE, BRIP1, CBLB, ERCC3, NEK2, NEK4, NEK10, NEK11, PARP1, MLLT6 POLH, RASEF RASE, SLC45A2, GOLM1, EBF3, JAK1, JAK2, SF3B1, SRSF2, TET2, TYK2, BUB1B, POLE2, ROS1, DNMT3A, CDH23, ARHGEF40, BRD9, DOT1L, SLCO4C, ADGRG7, FAM221A, SERPINB4, UNC93A, HRNR, OR51M1, SLC5A11, MYO7A.*	[92] [48], [96], [97], [89], [98], [99], [100], [101], [50], [49], [102]

1.10 Overview of the ERCC2 Gene.

1.10.1 Molecular Biology of the ERCC2

Excision Repair Cross-Complementing Group 2 (*ERCC2*) encodes a protein called Xeroderma Pigmentosum D (XPD), an ATP- dependent DNA helicase. It is an essential subunit of the transcription factor complex TFIIH, which participates in two fundamental processes, basal transcription, and repair of damaged DNA by NER.[104] A wide variety of DNA damage, such as those caused by UV radiation produces significant bulky DNA lesions that must be removed by a repair pathway such as NER.[105] NER pathways cåan be broadly divided into two classes, transcription-coupled repair (TCR), targeting DNA lesions of expressed genes, where the progression of RNA polymerase is blocked, and global genome repair (GGR), removing DNA lesions in all regions of the genome.[106] The XPC and DDB2 proteins are required to recognize the UV-induced DNA damage and initiate the NER pathway. [107] The XPD protein binds to the DNA around the lesion and subsequently uses its 5'→3' helicase activity to unwind the two DNA strands, together with the other subunit of the TFIIH, XPB, which acts as a DNA-dependent ATPase. After unwinding, a dual incision around the lesion is performed by ERCC1-XPF complex and XPG endonuclease. The damaged nucleotide is removed, and the resulting gap is filled by DNA polymerase and then ligated by ligase I or III (Figure 3).[108,109]

1.10.2 Molecular Defects in the ERCC2

The *ERCC2* or *XPD* gene is located on chromosome 19q13.3 and is composed of 24 exons.[104] Mutations in this gene result in three clinically different autosomal recessive disorders including, Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD), and Cockayne Syndrome (CS). However, only XP patients suffer additionally from a very

high risk of developing sun-induced skin cancer.[109] They show extreme photosensitivity, often developing skin cancer by the age of 10, and have an increased risk of developing CM at a median age of 22 .[105] Moreover, freckling on sun-exposed skin is a typical sign for XP.[109] XP is caused by hereditary mutations in any of the NER genes, *XPA to XPG*, and *XPV* that codes for Translesion Synthesis (TLS) polymerase. [104] Over 100 mutations have been mapped to the *XPD* gene. Most of these mutations cluster at the C-terminus of the protein, which is the domain necessary for the interaction with p44, a component of the TFIIH complex, and a stimulator for the XPD helicase activity. Thus, such mutations interfere with the helicase activation function. Also, they interfere with the ability of XPD to interact with other TFIIH subunits, leading to the destabilization of the TFIIH complex, thereby affecting both the NER pathway and transcription. [108]

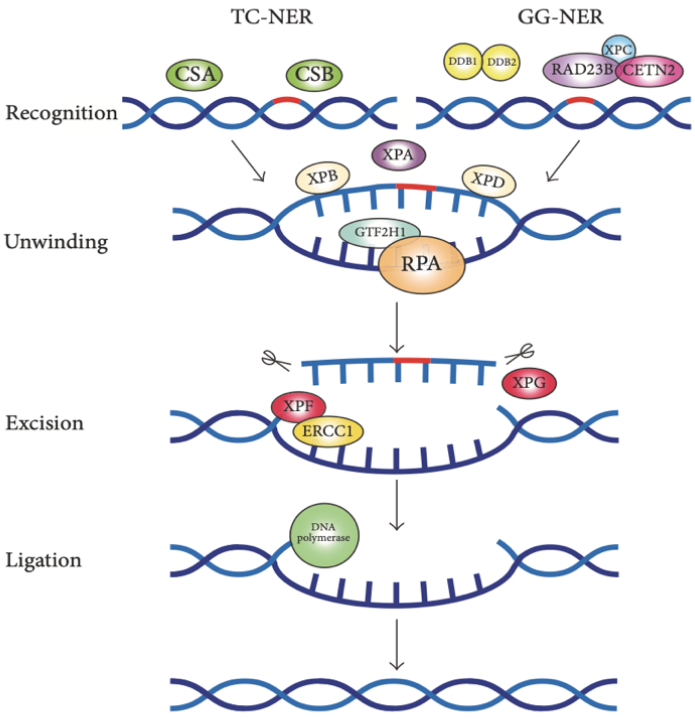


Figure 3. Graphical Overview of the Nucleotide Excision Repair (NER) Pathway, Adapted From [110]

1.11 Thesis Statement and Aims

The present study concerns a Palestinian family, of which the patient a 30 years old male, was diagnosed with left para nasal melanoma that metastasized to the lungs as well as to the brain. A family history of melanoma was present, with two of his brothers died at age of 30 due to metastatic melanoma. Furthermore, the patient was tested previously through targeted gene panel analysis of 94 genes in the coding and intronic sequences, but no pathogenic mutations were found in any of the genes, including the *CDKN2A, CDK4*, and *BAP1*. Reviewing the literature, with *CDKN2A* accounting for approximately 40% of melanoma families worldwide, and rare mutations in *CDK4, MITF, BAP1, TERT, POT1, TERF2IP*, *ACD, GOLM1, EBF3, POLE,* and *TP53* responsible for around 10% of the variation, still, there is about 50% of an unexplained genetic cause, resulting in impaired genetic testing and counseling in families with a predisposition to melanoma. WES has emerged as a powerful tool and an alternative to targeted gene panels since it permits the investigation of genetic mutations in the entire coding region of the genome. Therefore, the primary objective of this thesis is to identify germline mutations predisposing to melanoma in a Palestinian family via the application of WES. The secondary objective is to determine relatives at high risk of developing melanoma. We hypothesize that the underlying genetic cause of melanoma development is due to a rare germline mutation segregating in the family. We believe that identifying germline mutations associated with hereditary melanoma in the Palestinian population will be of great significance; for improving genetic counseling, cancer risk assessment, and testing in both patients and their relatives. In turn, this will decrease disease prevalence and morbidity. In addition, it will uncover genes that can be used as targets for cancer therapy.

Chapter 2

Methodology

2.1 Subjects and Samples

2.1.1 Clinical Phenotyping and Family Pedigree

This study included seven members of a Palestinian family, of which, one individual is affected with melanoma, two individuals with a brain tumor, and four un-affected Individuals. The pedigree (Figure 4) represents the family, 17 members, of which three were diagnosed with melanoma at the ages of 29, 17, and 30. The patients' father and two paternal uncles were all diagnosed with prostate cancer. The clinical phenotypes of the patient (9) and individual (6), who had a brain tumor with no melanoma, were an abnormality of skin pigmentation and hyperpigmentation of the skin. Individual (5) had normal skin pigmentation. No clinical data were available for the rest of the family members included in the study.

2.1.2 Ethical approvals

The study was ethically approved by the research center, Arab American University in Palestine.

2.1.3 Ethical Considerations

Consent forms were obtained from the patients and family members prior to participation in the study.

2.1.4 Sample collection

Blood samples were collected from the participant in EDTA tubes and stored at -80°C in the laboratory prior to DNA extraction.

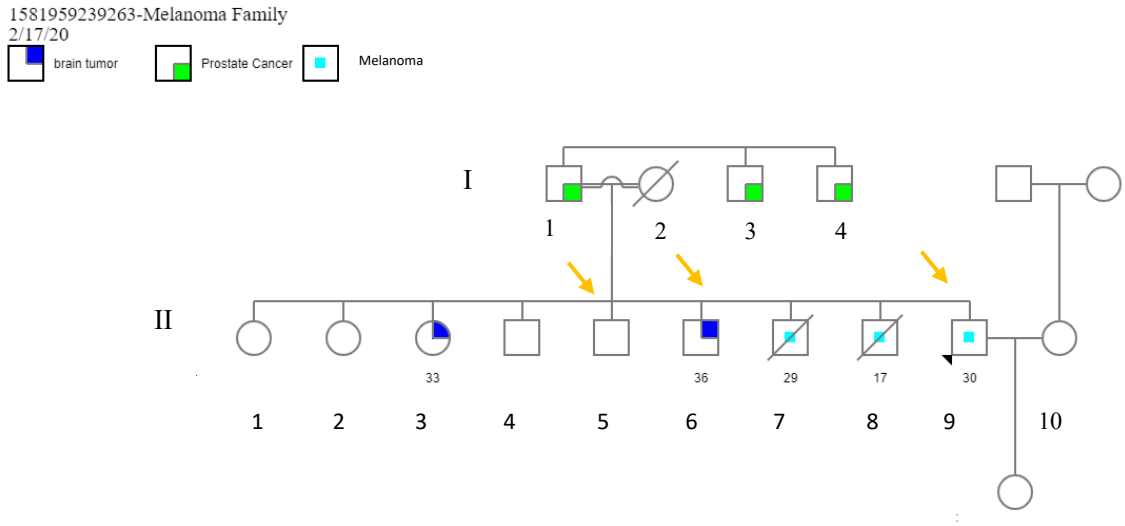


Figure 4. Family pedigree . Males are illustrated by squares, and females by circles. The patient is indicated by a black arrow head. The age is written under each individual symbol. The yellow arrow indicates the individuals whose samples were subjected to WES analysis.

2.3 DNA Extraction

Genomic DNA extraction was performed using the The Promega Blood kit, catalog #A1120 according to the manufacturer's instructions. Briefly, 300 µl from each blood sample is mixed with 900 µl of cell lysis solution, which lysis the red blood cells (RBCs). The samples are centrifuged at 13,000-16,000xg for 20 seconds, and the supernatant is removed, leaving only the pellet. Next, white blood cells (WBCs) are vortexed, followed by adding 300 µl nuclei lysis solution to lyse the WBCs. Then, 100 µl of protein precipitating solution is added to the nuclear lysate and vortexed for 10-20 seconds. The

solution is centrifuged, and a dark brown pellet is observed. The supernatant is transferred into a clean microcentrifuge containing 300 µl isopropanol and inverted several times. This step will precipitate the DNA. The DNA is washed by adding 70% ethanol to the sample, then vortexed for 5 seconds and centrifuged. The supernatant is removed, and the pellet is air-dried. Finally, 200 µl of TE buffer is added to the sample and vortexed for 10 seconds. The DNA concentrations and purity are examined using the Thermo scientific NanoDrop 2000c spectrophotometer, utilizing 1.5 µl of the undiluted DNA samples.

2.4 Whole-Exome Sequencing

2.4.1 Library Preparation

WES was performed on the DNA of the patient and two unaffected siblings, one of them with a brain tumor. DNA libraries were prepared from 500ng of genomic DNA using the Illumina DNA Prep with Enrichment - (S) Tagmentation, 16 Samples kit, catalog # 20025523 following the manufacturer's instructions. Briefly, DNA was fragmented and tagged to specific adapter sequences by adding a tagmentation master mix, consisting of Enrichment Bead-Linked Transposomes (eBL) and Tagementation Buffer 1 (TB1), into the DNA on a 96-well PCR plate. Following tagmentation, post tagmentation cleanup was performed by the addition of Stop Tagment Buffer 2 (ST2) to each reaction. Then, two rounds of washes with Tagment Wash Buffer (TWB) buffer were done to wash the adapter-tagged DNA. Next, tagmented DNA was amplified in a limited-cycle PCR reaction which included the addition of 10 µl Index 2 (i5) adaptors, Index 1 (i7) adapters, and sequences required for cluster generation. Thermal cycling conditions were as follows: 72°C for 3 min, 98°C for 3 min, and 9 cycles of 98°C for 20 sec, 60°C for 30 sec, and 72°C for 60 sec with a final extension of 72°C for 3 min. A cleanup of the

amplified libraries was carried out using a double-sided bead purification method in which the AMPure XP magnetic beads were used. At first, nuclease-free water was added to each sample well in a new midi plate containing supernatant, followed by adding 88 µl of AMPure XP Beads and subsequently mixing and incubating. During incubation, 20 µl of the AMPure XP Beads was added to a second new midi plate. Then, Beads were washed twice with 80% ethanol and allowed to Air-dry on the plate magnet to get rid of ethanol. The beads were resuspended with the addition of Resuspension buffer (RSB). Subsequently, single library concentrations were measured and then pooled by volume using 12-plex enrichment to prepare a single library. Following pooling, the single library was denatured, and biotinylated oligonucleotide probes were hybridized to the targeted regions of the library fragments, performed by adding Hyb Buffer 2 + IDT NXT Blockers (NHB2), Enrichment probe panel, and EHB2 sequentially to the pooled libraries. After hybridization, streptavidin Magnetic Beads (SMB3) were added to capture the targeted library fragments within the regions of interest. The captured DNA libraries were washed using preheated Enhanced Enrichment Wash (EEW) and then eluted from the beads by an elution mix containing Enrichment Elution Buffer 1 (EE1) and 2NaOH (HP3). A post capture PCR amplification was carried out by adding Enhanced PCR Mix (EPM) and then PCR Primer Cocktail (PPC) to each sample well**.** Thermal cycling conditions were as follows: 98°C for 60 sec, and 12 cycles of 98°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec with a final extension of 72**°**C for 5 min. The last step in library preparation required cleanup of the amplified enriched library, which was performed using the AMPure XP Beads followed by two washes with 80% ethanol (EtOH).

2.4.2 Purification and Quality Control

Library yield was determined using Qubit dsDNA HS Assay Kit, catalog # Q32850, and the mean fragment size was determined using an Agilent Technology 2100 Bioanalyzer with a High Sensitivity DNA kit, catalog #5067-4626 . Further, libraries were diluted to the required molarity for a starting concentration using RSP. Denaturation and final loading concentration of the libraries were performed according to the Denature and Dilute Libraries Guide for NextSeq 500 and NextSeq 550 Sequencing Systems. The library pool was denatured by the addition of 0.2 N NaOH followed by neutralization using 200 mM of Tris-HCl pH 7.0, at equal volumes of 5 µl. Then, the denatured libraries were diluted to 20 pM using HT1 before dilution to a final loading concentration of 1.5 pM with a total volume of 1.3 ml. PhiX library was prepared for use as a sequencing control starting with PhiX dilution to 4 nM with RSB followed by denaturation with 0.2 N NaOH and Tris-HCl. Next, the denatured PhiX was diluted to a final loading concentration of 1.5 pM with a total volume of 1.3 ml.

2.4.3 Sequencing

The diluted library and PhiX were combined and then loaded onto reagent cartilage for sequencing on a NextSeq550 platform.

2.5 NGS Data Analysis

2.5.1 Variant Filtering

Variant calls were generated from the WES data using a bioinformatic pipeline, so the variants can be filtered to obtain a final list of causal variants for segrigation analysis. The generated variant call format (VCF) file contained 36,521 variants that were filtered

based on several filtering criteria as follows. First, variants that were intronic, ncRNA_intronic, downstream, upstream, 3'UTR3, 5'UTR, and synonymous unknown, were filtered out. Then variants were assessed by their clinical significance from the Clinvar database. They were excluded if they have been reported as benign and likely_benign. Next, variants were filtered out if they had a general allele frequency (AF) and greater middle eastern allele frequency (GME_AF) above 0.1%, based on the gnomAD database. This filter ensures that the non-filtered variants have a level of evidence for pathogenicity and are not polymorphisms. In order to limit false positives, variants with mapping by quality (MQ) scores below 60, which are low-quality scores that have a higher probability of error were all removed. Also, variants having quality by depth (QD) scores below 3 were removed. Then variants were filtered out based on exomiser, which ranks candidate variants according to the patient phenotype similarity to known disease–gene phenotypes, using the human phenotype ontology terms (HPO). Variant scores were scaled from 0 to 1, and scores lower than 0.5 were filtered out. Following exomiser filtering, the genotype of the patient was evaluated, removing variants with wild-type alleles (WT), and considered either homozygous (HOM), heterozygous (HET), or low allelic balance (lowAB) genotypes. Next, all variants that are unrelated to cancer or melanoma according to OMIM and ClinVar databases were removed. After that, synonymous and ncRNA_exonic variants were filtered out. Finally, only variants that were present in both, the patient and his brother with a brain tumor not in the control were kept. After filtering, four variants in three genes remained for further analysis (Figure 5).

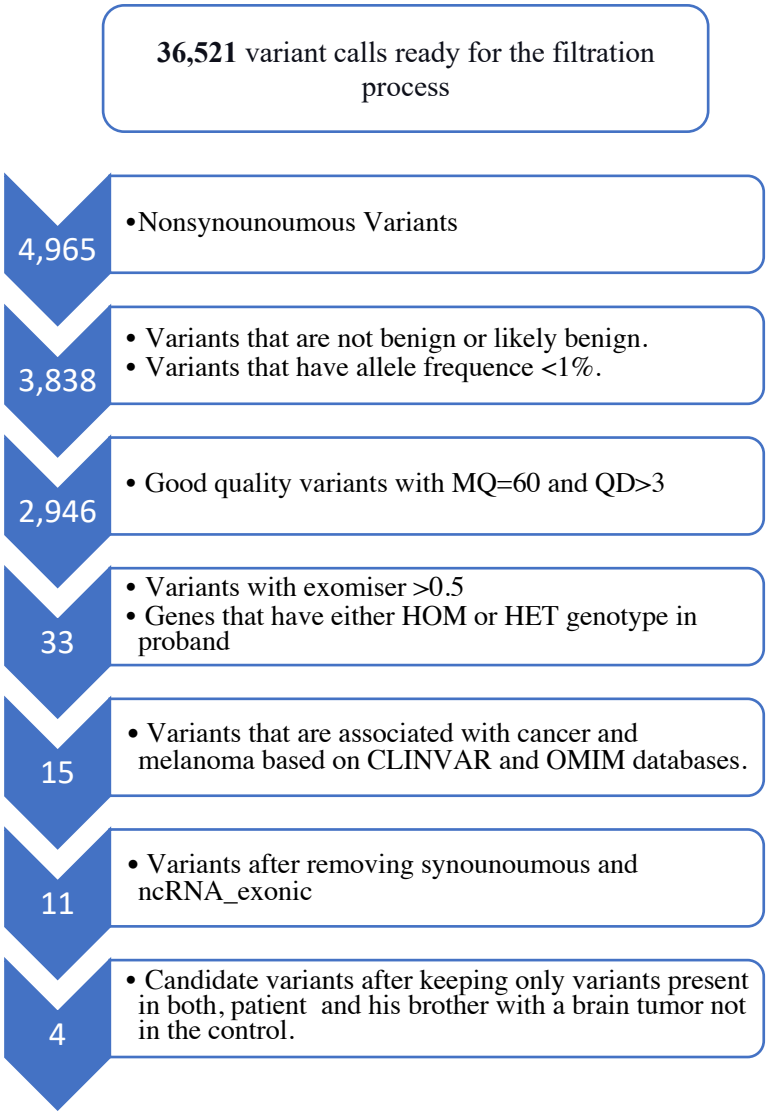


Figure 5. Flowchart of the variant filtration steps followed in the search for melanoma candidate genes.

2.7 Variant Selection for Sanger Sequencing

Candidate variants shared between the patient and his brother (6) with a brain tumor were further filtered for Sanger sequencing. Only variants that are reported as pathogenic or potentially pathogenic with a genotype-phenotype association based on ClinVar were selected.

2.8 Variant Validation and Segregation Analysis.

The identified homozygous mutation in ERCC2, p.R683Q, was confirmed by sanger sequencing and co-segregation analysis was performed on family members II 2, II 3, II 4, and II 10.

2.8.1 Primer Design

First, primers were designed for PCR and sanger sequencing based on the reference sequence for *ERCC2*: rs758439420, using the primer3 (https://primer3.ut.ee) and the UCSC *In Silico* PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr). The primer sequences were as follows: Forward: tcaggttgaggttggcatct, Reverse caagaaccaggctgtttccc. The total PCR product size was 191 bp.

2.8.2 PCR

PCR was performed in 20µL reaction volumes consisting of 10 µL of GoTaq PCR master mix 2x (Promega, Madison, WI, USA) 0.8-2µL of DNA (100 ng), 1 µL of forward, and reverse primers (19.6 nmol, and 29.1 nmol respectively), and nuclease free water to complete the volume to 20 µL . Thermal cycling conditions were as follows: Initial denaturation for 5 min at 95°C, and 31 cycles of 30 sec at 95°C for denaturation, 30 sec at 56°C for annealing, then 30 sec at 72°C with a final extension of 5 min at 72°C.

2.8.3 Agarose Gel Electrophoresis.

To confirm PCR amplification products, agarose gel electrophoresis was carried out using 2% (w/v) agarose gel in TBE 1X . 50 µLs of (0.5 µg/mL) ethidium bromide (Hy labs, Jerusalem) were added to aid DNA visualization. 4µls of PCR product were loaded into

each well of the gel. 50 bp DNA ladder (GeneDirex, hy.labs) was run alongside the samples at 120 mV for 30 minutes. PCR products were visualized by using UV transilluminator.

2.8.4 PCR Cleanup

Leftover primers and dNTPs were cleaned from PCR products using the EPPiC Fast kit (catalog #1021-500F, A&A Biotechnology).

2.8.5 Sanger Sequencing

5µl of cleaned PCR product was added to 1µl of the EPPiC Fast enzymatic solution. It is then run on a thermal cycler at 37°C for 10 minutes followed by 1 minute at 80°C. Sanger sequencing was run using the BigDye Terminator v3 kit (Applied biosystem) according to the manufacturer's instructions.

2.8.6 Sequence Analysis

The sequences of the PCR products were viewed and analyzed against reference sequences obtained from Varsome (https://varsome.com), using the accession number: NM_000400.

2.9 *In Silico* Analysis and Interpretation of the Melanoma Candidate Genes.

Candidate variants and the genes identified were investigated using various *In silico* analysis. The protein domains of the candidate genes were studied to locate these variants. SIFT PolyPhen2, PROVEAN, Mutation Taster, and Align GVGD, Predictive algorithms were used to predict the impact of the missense mutations. To check the

evolutionary conservation of the sites of the variants, the amino acid sequences of the candidate gene from Homo sapiens and other species were gathered from NCBI and aligned with COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi). Moreover, 3d structural modeling was performed to determine the site of the mutation using the Protein Data Bank (PDB) database. The candidate variants were interpreted and classified according to the ACMG guidelines. After that, the somatic mutation databases, Catalogue of Somatic Mutations in Cancer (COSMIC), and The Cancer Genome Atlas (TCGA) cBioPortal for cancer genomics were accessed to check whether the candidate variants are present in tumors of melanoma and other cancer types. To investigate whether or not the ERCC2 protein is connected with the TYRP1 and WRN proteins, we used the BioGRID protein interaction database (https://thebiogrid.org). Then, the MutPred *in silico* web-based tool (http://mutpred2.mutdb.org/index.html) was used to predict the pathogenicity and molecular alternations associated with the amino acid substitutions in the *TYRP1* and *WRN* genes. It is based on the impact of amino acid substitutions on conservation, structural and functional characteristics of the proteins. The *In-silico* tool, Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict the protein structure based on homology modeling technique. On the other hand, candidate genes were investigated for alternation frequency in melanoma and other cancer types using TCGA cBioPortal database. Additionally, THE HUMAN PROTEIN ATLAS database was used to obtain the Genotype-Tissue Expression (GTEx) dataset to examine the mRNA tissues expression of the genes in different tissues.

Chapter 3

Results

3.1 Demographic and Clinical Characteristics of the Palestinian Family

The study included seven family members, the patient II 9, his wife II 10, and his siblings from III 2 to III 6. The table 2 summarizes the main demographic and clinical features of the family members.

Table 2. Demographic data and clinical phenotypes of the melanoma Palestinian family. (-): Not Present

Family members (ID)	Relationship with patient (II 9)	Age (years)	Sex	Clinical phenotypes
II 9	-	30	Male	Skin melanoma, Abnormality of skin pigmentation, and Hyperpigmentation.
II 5	Brother	-	Male	Normal pigmentation
II 6	Brother	36	Male	Brain tumor, Abnormality of skin pigmentation, and Hyperpigmentation.
II 7	Brother	29	Male	Skin melanoma
II 8	Brother	17	Male	Skin melanoma
II 1	Sister	-	Female	-
II 2	Sister	-	Female	-

II 3	Sister	33	Female	Brain tumor
II 4	Brother	-		-
II 10	Wife	-	Female	-
I 1	Father	-	Male	Prostate cancer
I 3	Paternal uncle	-	Male	Prostate cancer
I 4	Paternal uncle	-	Male	Prostate cancer
III 1	Child	-	Female	-

3.2 WES Analysis Identified Rare Variants in Candidate Genes for Melanoma Germline Predisposition.

Here, we performed WES on DNA samples from a melanoma patient (II 9) and his two brothers, one with a brain tumor (II 6) and the other unaffected (II 5), to identify rare germline variants predisposing to melanoma in the Palestinian family. As per the filtering steps detailed in (Figure 5), 36,521 VCF WES dataset of the three siblings was filtered. 3,838 uncommon, protein-affecting variants predicted to be pathogenic, conflicting interpretation of pathogenicity, and uncertain significance by ClinSig were retrieved. Genes were further filtered based on run quality, and genotype-phenotype correlation. This filtering reduced the number of variants to 33. Of these variants, 15 were previously associated with cancer and melanoma based on OMIM and ClinVar databases. These 12

genes included *ERCC2; KLC3, WRN, SMARCA4, SDHB, TYRP1, MRE11, TERT, MLH1, RELCH; PIGN, SNORD132; BUB1, MEN1, CDKN1A; DINOL, CDKN1A,* and *COMT; MIR4761*. Synonymous mutations in *COMT; MIR4761, CDKN1A*; *DINOL, RELCH; PIGN* genes were excluded as they did not affect splicing. Additionally, the exonic ncRNA mutations in *SNORD132; BUB1* gene were excluded. The patient's brother, who had a brain tumor was treated as an affected family member. The top final variants and their corresponding genes most relevant to melanoma and are present in both the patient and the sibling with a brain tumor not in the control are in *WRN*, *TYRP1*, and *ERCC2*, shown in (Table 3) and discussed below.

Table 3. WES analysis and identification of germline candidate variants in the Palestinian family.

Gene	Mutation type	Amino acid change	ClinVar	Allele frequency (gnomAD)	II 9 genotype	II 5 genotype	II 6 genotype
TYRP1	missense	c.C785T: p.T262M	Uncertain significance	0.0011	HET	-	HET
ERCC2	missense	c.G2048A: p.R683Q	Pathogenic	0.0000159	HOM	HET	HOM
WRN	missense	c.G1149T: p.L383F	Conflicting interpretations of pathogenicity	0.0021	HET	-	HET
WRN	missense	c.G2983A:p.A995T	Conflicting interpretations of pathogenicity	0.0024	HET	-	HET

All of the variants identified by WES analysis were missense mutations. The *ERCC2* mutation (NM_000400, c.G2048A, p.R683Q), was present in the patient and his brother

with a brain tumor (6) in a homozygous state, while it is in the heterozygous state in the unaffected brother (5). This mutation is classified as pathogenic in ClinVar. Moreover, it is associated with the phenotype Xeroderma pigmentosum group D (XPD), characterized by cutaneous photosensitivity and predisposition to skin cancer on sun-exposed body sites, based on the OMIM description (OMIM #278730). The p.R683Q was observed in the gnomAD exome database with a low allele frequency of 0.0000159. Also, it has been seen four times as a heterozygous mutation and none as a homozygous mutation (ID 19-45855609-C-T). The other missenses mutations in *TYRP1* (c.C785T, p.T262M), and *WRN* (c.G1149T: p.L383F, and c.G2983A: p.A995T) were present in a heterozygous state in the patient and his brother with a brain tumor (6), while it was absent in the unaffected brother (5). The *TYRP1* mutation is classified as unknown significance by multiple submitters in ClinVar. Homozygous or compound heterozygous mutations in TYRP1 are associated with oculocutaneous albinism type 3 (OCA3) (OMIM #203290), a milder form of OCA. In WRN, both of the mutations are classified as Conflicting interpretations of pathogenicity based on ClinVar. They are present at an allele frequency of 0.0021, and 0.0024, respectively in gnomAD, and are associated with autosomal recessive disorder, Werner syndrome (WRN) (OMIM #277700).

3.3 The *ERCC2* Missense Mutation Segregate in A Palestinian Melanoma Family

The *ERCC2* missense mutation was selected for segregation analysis for the following reasons**:** 1) The p.R683Q mutation cosegregated with the disease phenotype in the patient and his affected brother (II 5) in a homozygous state. 2) Classified as pathogenic in ClinVar. The *ERCC2* missense mutation was validated by sanger sequencing and co-segregation analysis was performed on the unaffected members in the family. The

homozygous missense mutation, p.R683Q, was confirmed in the patient (II 9), and his affected brother (II 6). Moreover, it was detected in a homozygous wild type in the patient’s brother (II 4), one sister (II 2), and his wife (II 10). It was also detected in a heterozygous state in the patient's other sister with a brain tumor (II 3) (Figure 6).

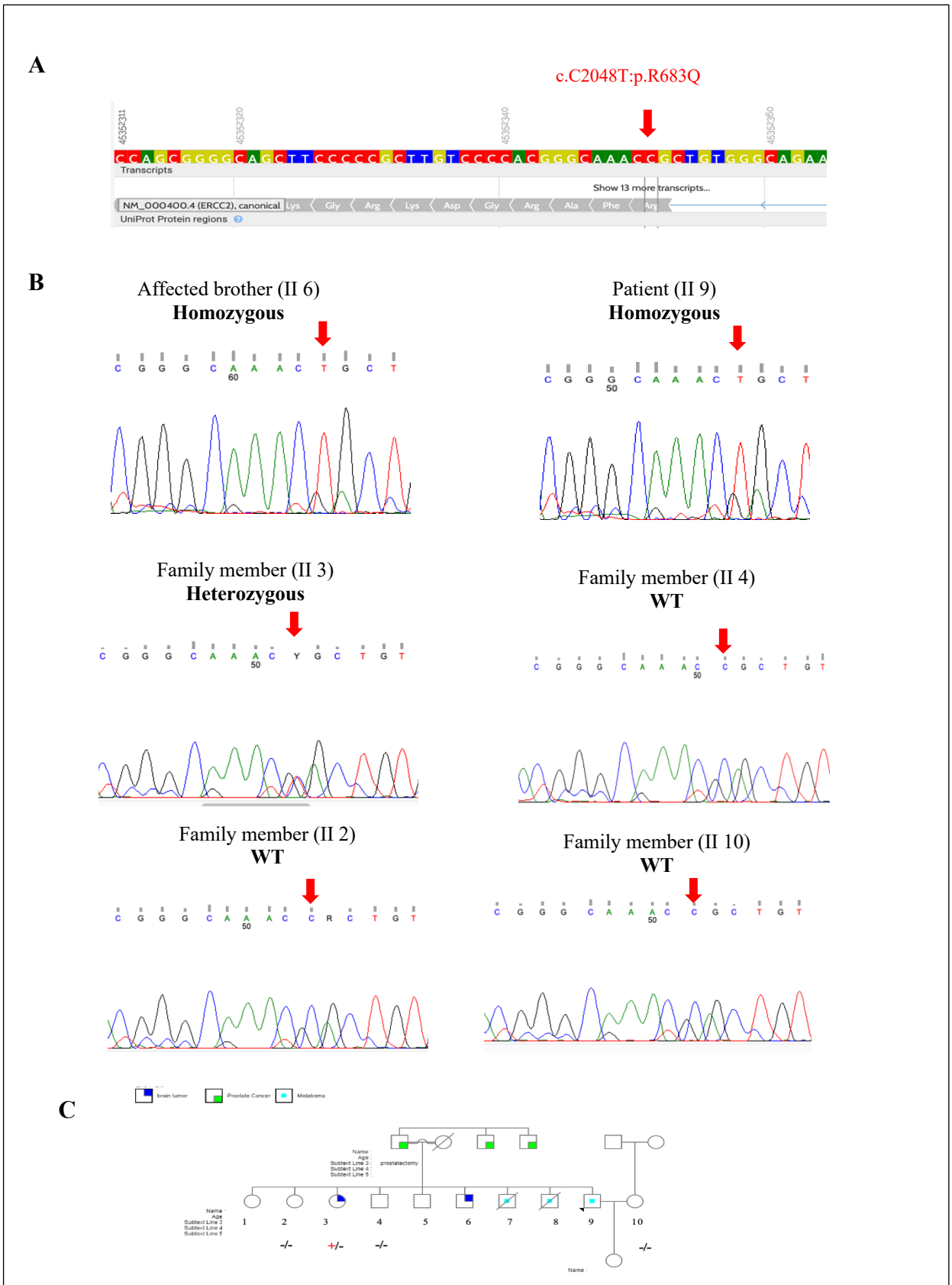


Figure 6. A) Wild-type nucleotide and amino acid sequences. The site and name of the mutation is indicated in a red. B) Sequence chromatogram of the patient (II 9), affected brother (II 6) and family members II 2, II 3, II 4, and II 10. The site of mutation is pointed in red. C) Pedigree of the Palestinian melanoma family, showing the genotype of each member after sanger sequencing analysis. The genotype +/+ (Homozygous), +/− (Heterozygous), or −/− (wild type) is represented below the symbol of each analyzed individual.

3.4 *In Silico* Analysis of the Candidate Mutations and Genes

Different *In silico* analysis are performed on the identified variants in order to predict their pathogenicity, and assess their functional and structural effect on the proteins. Moreover, the variants were checked for their presence in tumors of melanoma and other cancers using somatic mutation databases. The candidate genes were also investigated to determine their alternation frequencies and types of mutations in cutaneous melanoma tumors, as well as their mRNA expression in different human tissues.

3.4.1 *In Silico* Analysis of the *ERCC2* Mutation and Gene.

Schematic of the ERCC2 protein (Figure 7, A) shows that the p.R683Q is located in the ATP-dependent helicase region of the HD2 domain of the ERCC2 protein, required for the opening of the DNA. Multiple alignment of amino acid sequences of the HD2 domain showed that the residue 683 is highly conserved among different species (Figure 7, B). The p.R683Q mutation is predicted with high probability to be deleterious by all *In silico* algorithm tools (SIFT, PolyPhen2, Mutation taster, Align GVD, PROVEAN) (Table 4). Moreover, it is classified as pathogenic according to the ACMG. The XPD 3D structure: (General transcription and DNA repair factor IIH helicase subunit XPD - chain B) was obtained from the Protein Data Bank (PDB) ID: 6NMI. The interaction of XPD with other subunits of the TFIH protein complex is characterized in this 3D structure. Moreover, it revealed the site of the mutation in the XPD protein structure (Figure 7 C). To check whether or not the ERCC2 mutation is present in sequenced tumor samples of melanoma and other cancer types, it was carried out in COSMIC and cBioPortal databases. However, the mutation wasn’t reported

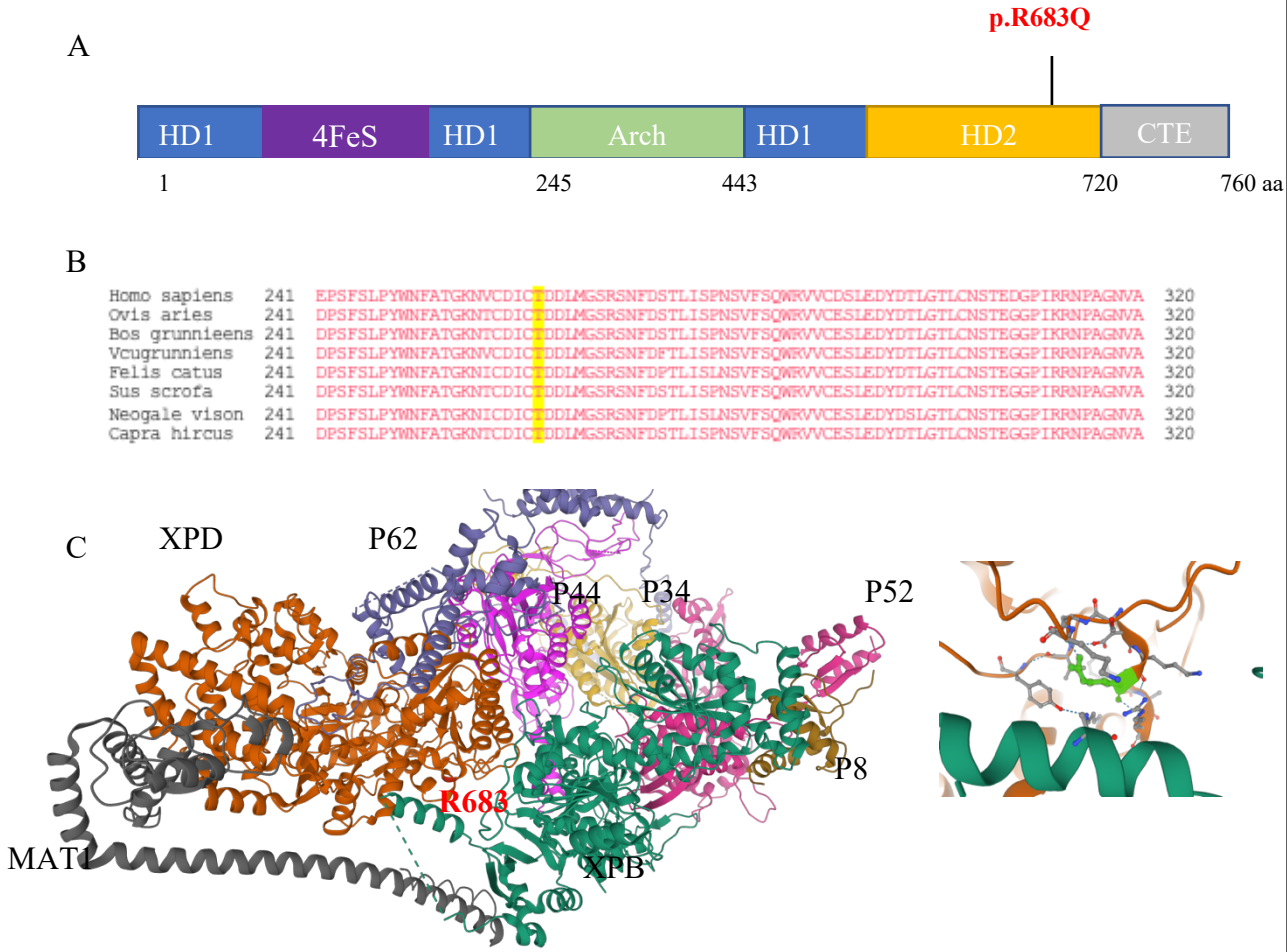


Figure 7. A) Schematic of the ERCC2 domains and location of the mutation. The position of the p.R683Q mutation is identified in the Helicase *domain* 2 (HD2) (indicated in red). B**)** Multiple sequence alignment of the XPD protein regions surrounding the R683 (highlighted in yellow) that is mutated to p.R683Q. C) Left: Crystal structure of the (General transcription and DNA repair factor IIH helicase subunit XPD - chain B): (6NMI, PDB), showing the XPD and other subunits of the TFIIH, and the site of the R683 residue affected by the mutation (red), Right: Zoom in to the site of the mutation on the XPD structure (green).

Analysis of cBioPortal, the TCGA, PanCancer Atlas database comprising 448 cutaneous melanoma tumors, found that ERCC2 is altered in 4% of the tumor samples (Figure 8, A, left). 3.8 % of the alternations were somatic mutations (n=17) (Figure 8, A, right). Of these mutations, two were truncated (drivers), and the rest were missense mutations of

uncertain significance forming the predominant type of the mutation. This demonstrate that melanoma genetics is complex, and that there are likely many mutations that need to be studied to find out the driver mutations causing the disease development and progression. We then used the Genotype-Tissue Expression (GTEx) to evaluate the mRNA expression of the ERCC2 in different tissues. The *ERCC2* was expressed in numerous tissues with similar values, including testis, prostate, skin, and endometrium (Figure 8, B). To check whether or not the ERCC2 protein interacted with the TYRP1 and WRN proteins, we used the BioGRID protein interaction database. BioGRIG analysis showed that the TYRP1 and WRN proteins are not among the protein network interaction of ERCC2 (Figure 9).

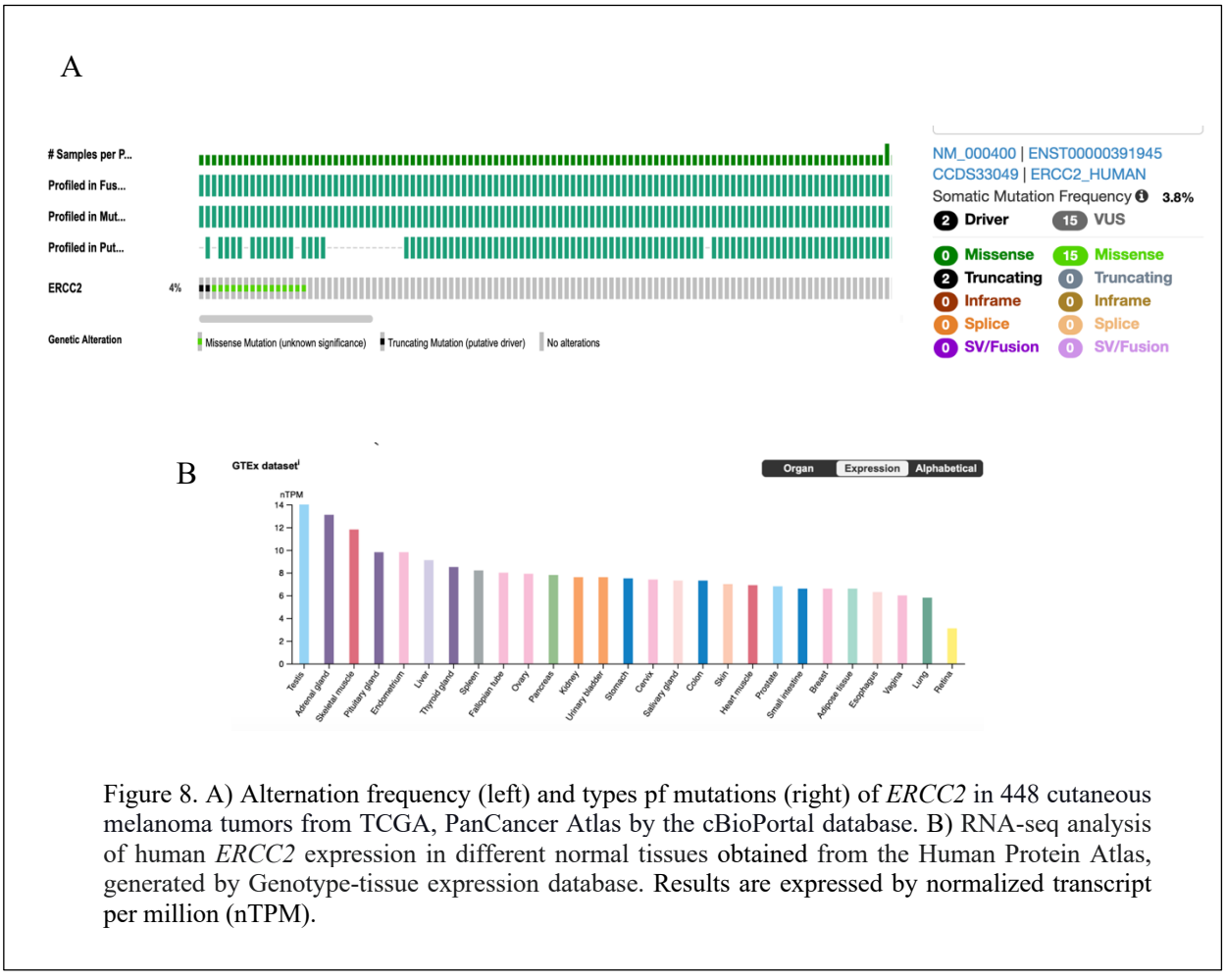


Figure 8. A) Alternation frequency (left) and types pf mutations (right) of *ERCC2* in 448 cutaneous melanoma tumors from TCGA, PanCancer Atlas by the cBioPortal database. B) RNA-seq analysis of human *ERCC2* expression in different normal tissues obtained from the Human Protein Atlas, generated by Genotype-tissue expression database. Results are expressed by normalized transcript per million (nTPM).

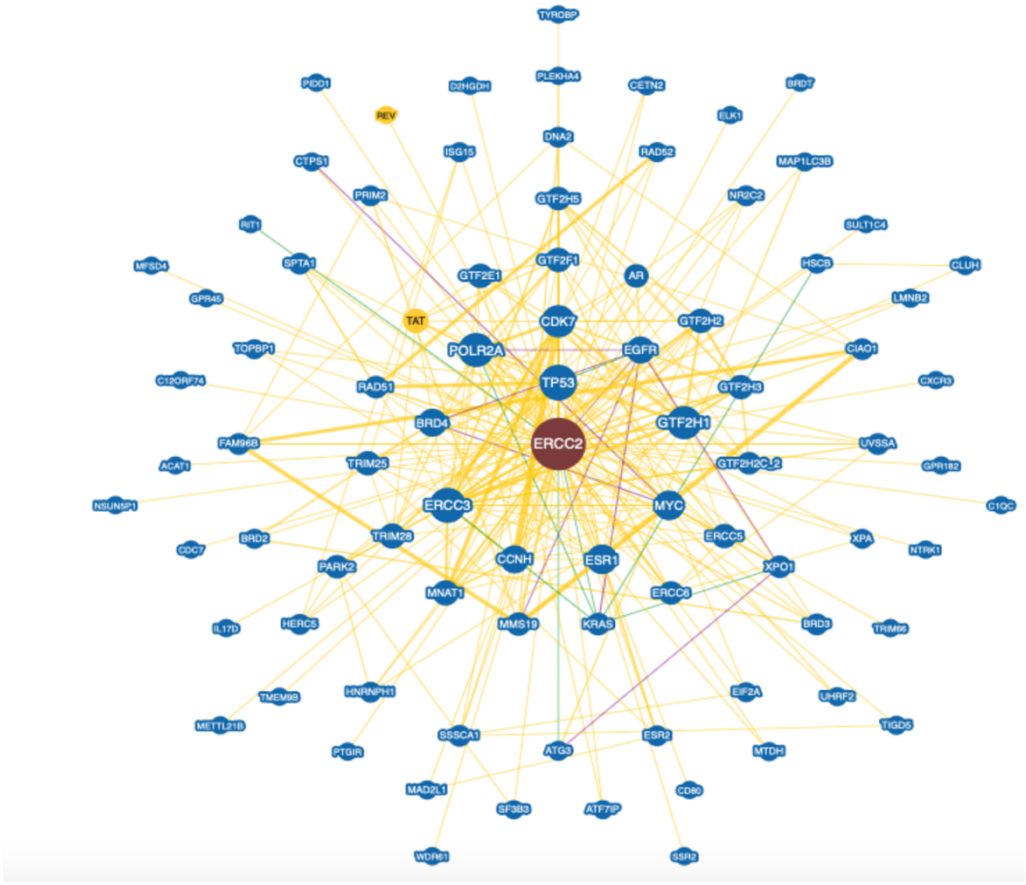


Figure 9. Protein network interaction of human ERCC2, determined by BioGRID database.

3.4.2 *In Silico* Analysis of the *TYRP1* Mutation and Gene.

Schematic of the TYRP1 protein shows that the T262M mutation is located within the tyrosine-like domain (Figure 10, A), and it occurs in a highly conserved site among different species (Figure 10, B). The T262M is predicted to be deleterious by all *In silico* algorithms (SIFT, PolyPhen2, Mutation taster, Align GVD, PROVEAN) (Table 4). However, it was classified as uncertain significance according to ACMG classification from Varsome, The crystal structure of the human tyrosinase related protein 1, (PDB) ID:

5m8l, revealed the site of the T262M mutation (Figure 10, C). Moreover, the T262M mutation was reported in the COSMIC database to occur somatically, three times in a stomach tissue (Adenoma), and two times in a skin tissue (CM) (Figure 10, D). MutPred2 analysis found that the T262M variant is deleterious with a score of 0.607 and predicted to cause some alternations in molecular mechanisms (Figure 11). The human TYRP1 protein was modeled using the structural prediction program, Phyre2. The topmost template (PDM ID: c5m8pA) has covered 83% of the TYRP1 mutant protein query sequence (Figure 12).

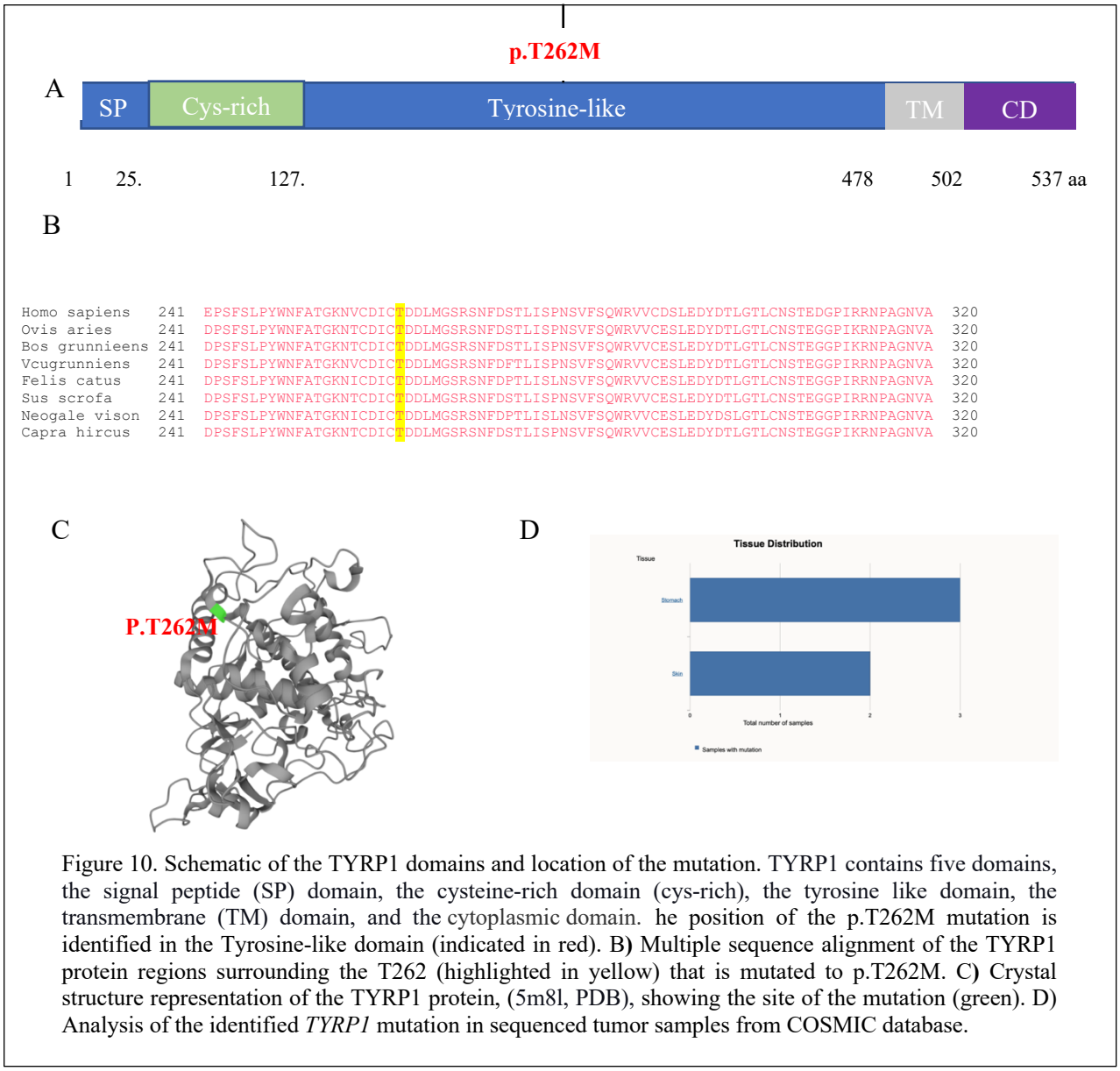


Figure 10. Schematic of the TYRP1 domains and location of the mutation. TYRP1 contains five domains, the signal peptide (SP) domain, the cysteine-rich domain (cys-rich), the tyrosine like domain, the transmembrane (TM) domain, and the cytoplasmic domain. he position of the p.T262M mutation is identified in the Tyrosine-like domain (indicated in red). B) Multiple sequence alignment of the TYRP1 protein regions surrounding the T262 (highlighted in yellow) that is mutated to p.T262M. C) Crystal structure representation of the TYRP1 protein, (5m8l, PDB), showing the site of the mutation (green). D) Analysis of the identified *TYRP1* mutation in sequenced tumor samples from COSMIC database.

ID	Substitution	MutPred2 score	Remarks	Affected PROSITE and ELM Motifs
sp|P17643	T262M	0.607	-	ELME000052
Molecular mechanisms with P-values <= 0.05			**Probability**	**P-value**
Altered Transmembrane protein			0.31	1.1e-04
Altered Metal binding			0.25	0.04
Loss of Relative solvent accessibility			0.24	0.04
Loss of Allosteric site at L265			0.22	0.03
Gain of Disulfide linkage at C258			0.20	0.02
Loss of Catalytic site at C261			0.14	0.02

Figure 11. MutPred2 and the molecular consequence of the T262M variant. MutPred2 score above 0.5 is considered deleterious, and a score above 0.75 is considered to have a high confidence deleterious prediction.

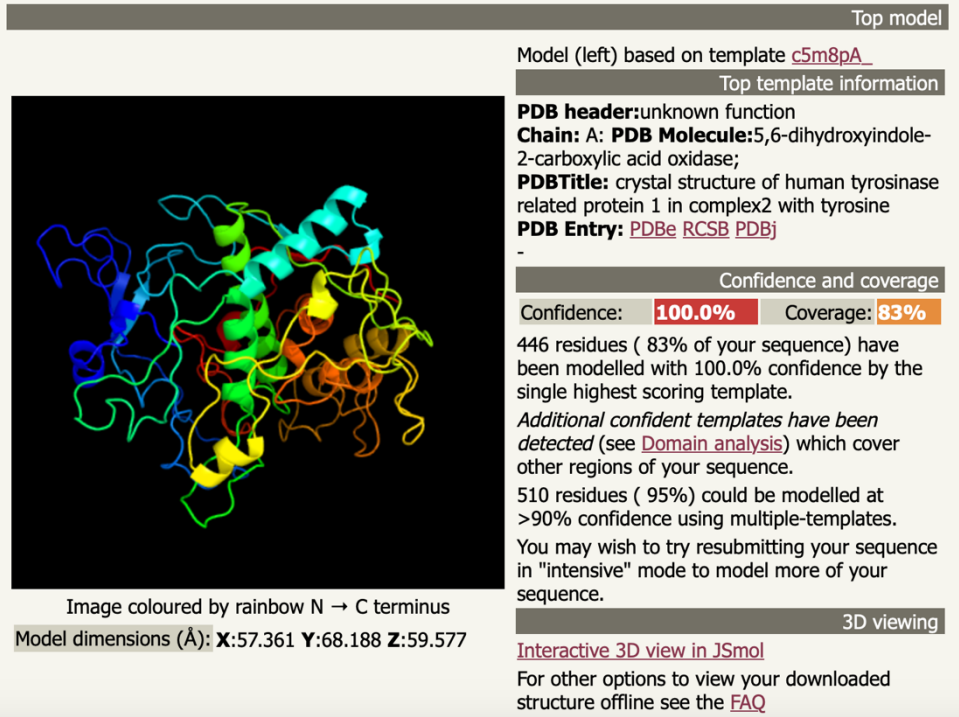


Figure 12. Predicted 3D structural model of the mutant *TYRP1*protein by Phyre2.

Analysis of cBioPortal, the TCGA, PanCancer Atlas database comprising 448 cutaneous melanoma tumors, found that *TYRP1* is altered in 6% of the tumor samples (Figure 13, A, lift). 3.6 % of the alternations were somatic mutations (n=17) (Figure 13, A, right). All of these mutations were missense mutations of uncertain significance, indicating the importance of studing these mutations. We then used the Genotype-Tissue Expression (GTEx) to evaluate the mRNA expression of the TYRP1 in different tissues. The *TYRP1* was mainly expressed in the skin, followed by the heart muscle (Figure 13, B).

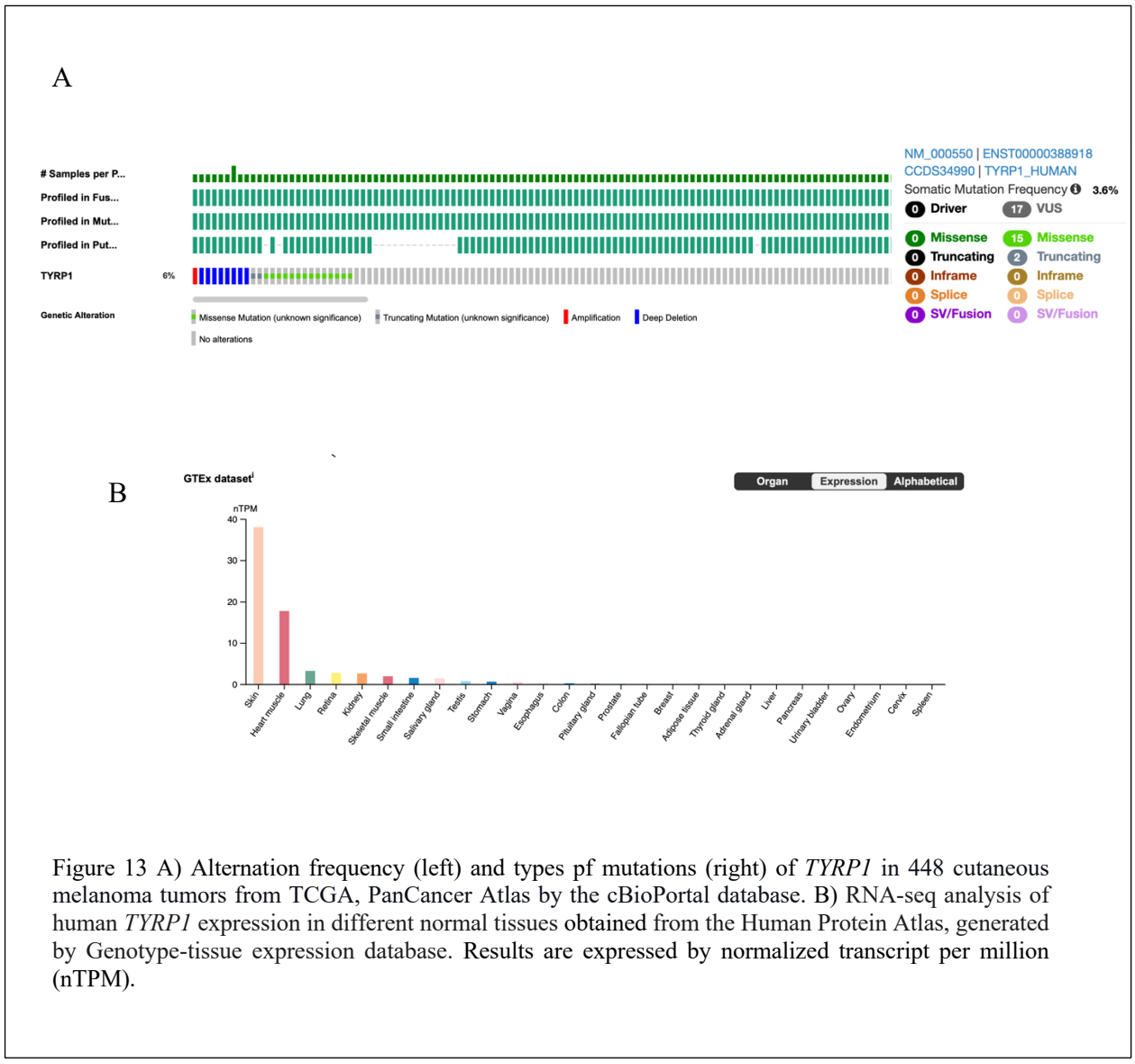


Figure 13 A) Alternation frequency (left) and types pf mutations (right) of *TYRP1* in 448 cutaneous melanoma tumors from TCGA, PanCancer Atlas by the cBioPortal database. B) RNA-seq analysis of human *TYRP1* expression in different normal tissues obtained from the Human Protein Atlas, generated by Genotype-tissue expression database. Results are expressed by normalized transcript per million (nTPM).

3.4.3 *In Silico* Analysis of the *WRN* Mutations and Gene.

Based on the WRN protein schematic (Figure 14, A), the p.L383F variant is located in the junction between the exonuclease and ATPase domains, while the p.A995T is located in the RQC functional domain of the protein. Analysis of the multiple sequence alignment of various species showed that both of the *WRN* variants occur at conserved regions (Figur 14, B). The two *WRN* variants, p.L383F, and p.A995T were predicted to be benign/tolerated respectively, by all *In silico* tools. (SIFT, PolyPhen2, Mutation taster, Align GVD, PROVEAN) (Table 4). Concerning ACMG, both variants were predicted as benign. The crystal structure of the human Werner syndrome helicase, (PDB) ID: 6yhr, revealed the site of the two mutations p.A995T, and p.L383F (Figure 14, C). The *WRN* variants were not reported in cBioPortal, but were reported in the Cosmic database. Both variants, p.L383F, and p.A995T were somatically mutated once in skin tissue of melanoma. Additionally, the p.A995T variant was mutated in prostate tissue two times (Figure 14, D). MutPred2 analysis showed that both *WRN* variants, p.L383F and p.A995T had a pathogenicity prediction score below 0.5, indicating no effect on the structural and functional properties of the protein (Figure 15, A). The human WRN protein was modeled using the structural prediction program, Phyre2. The topmost template (PDM ID: c6yhra) has covered 38% and 28% of the WRN, L383F, and A995T protein query sequence, respectively (Figure 15, B).

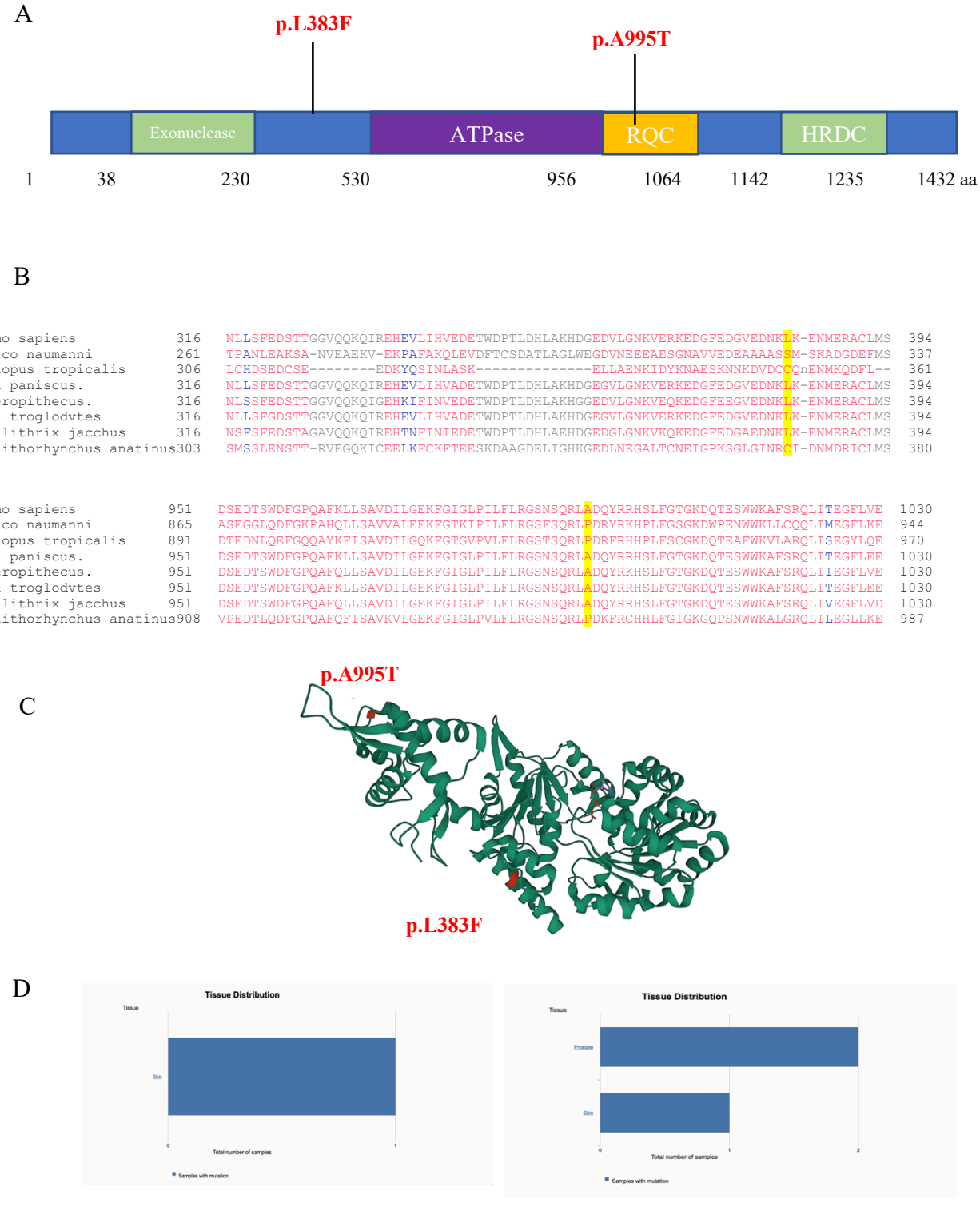


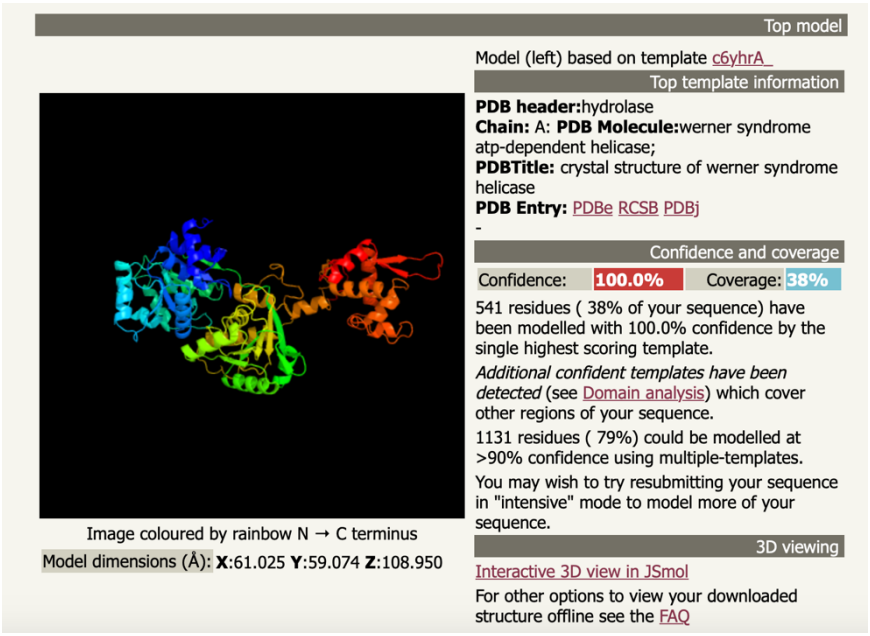
Figure 14. Schematic of the WRN domains and location of the mutation. The position of the p.L383F is identified in the junction between exonuclease domain and the ATPase domain, and the A995T mutation is identified in the RQC domain (indicated in red). B) Multiple sequence alignment of the WRN protein regions surrounding the two mutations (highlighted in yellow). C) Crystal structure representation of the WRN protein, (6YHR, PDB), showing the site of the two mutations (red). D) Analysis of the identified *WRN* mutations in sequenced tumor samples from COSMIC database.

A

ID	Substitution	MutPred2 score	Remarks	Affected PROSITE and ELM Motifs
sp|Q14191|	L383F	0.139	-	-

ID	Substitution	MutPred2 score	Remarks	Affected PROSITE and ELM Motifs
sp|Q14191|	A995T	0.126	-	-

B



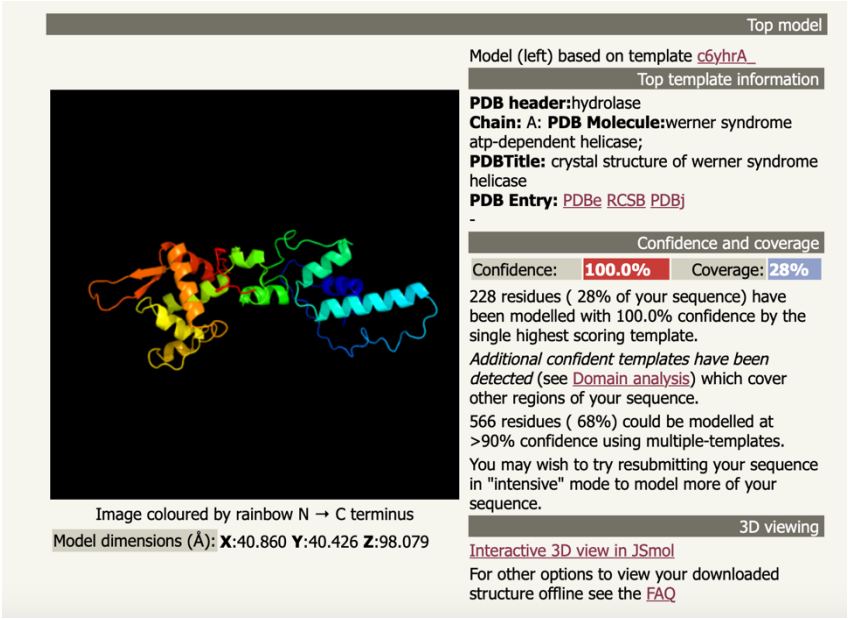


Figure 15. A) MutPred2 scores of the *WRN* variants, L383F (up), and A995T (down). B) Predicted 3D structural model of the mutant WRN protein, L383F (up), and A995T (down), by Phyre2.

Analysis of cBioPortal, the TCGA, PanCancer Atlas database comprising 448 cutaneous melanoma tumors, found that *WRN* is altered in 7% of the tumor samples (Figure 16, A, lift). 6.7 % of the alternations were somatic mutations (n=32) (Figure 16, A, right). All of these mutations were missense mutations of uncertain significance, indicating the importance of studying these mutations. We then used the Genotype-Tissue Expression (GTEx) to evaluate the mRNA expression of the WRN in different tissues. The *WRN* was expressed in numerous tissues with similar values, including retina, ovary, prostate, and skin (Figure 16, B).

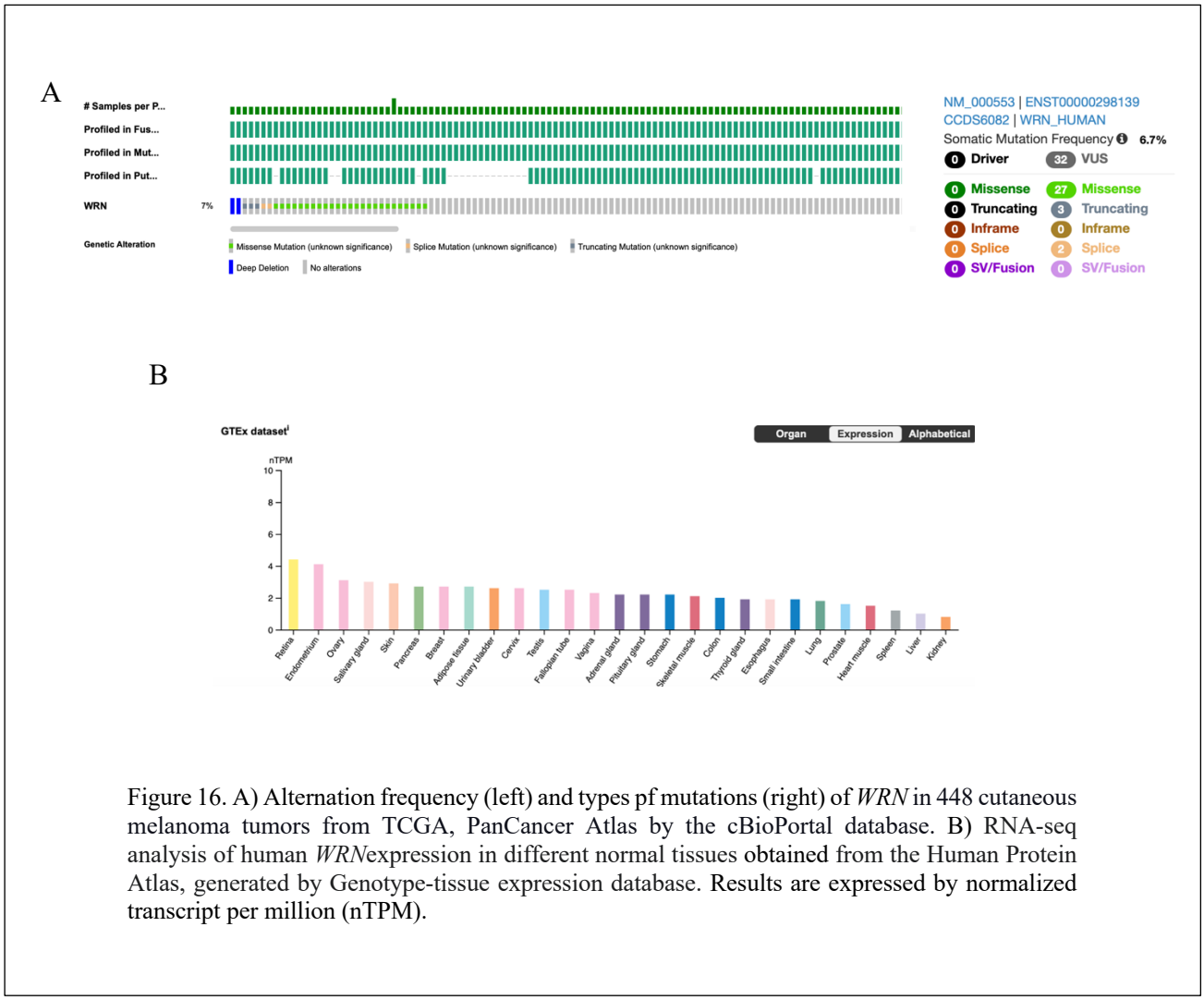


Figure 16. A) Alternation frequency (left) and types pf mutations (right) of *WRN* in 448 cutaneous melanoma tumors from TCGA, PanCancer Atlas by the cBioPortal database. B) RNA-seq analysis of human *WRN*expression in different normal tissues obtained from the Human Protein Atlas, generated by Genotype-tissue expression database. Results are expressed by normalized transcript per million (nTPM).

Table 4. Effect prediction of the melanoma candidate variants. The variants were predicted by the *In silico* algorithms: SIFT (Score under 0,05: “damaging”, score above 0.05; “tolerated”. Range 0-1), PolyPhen-2 (HumVar, "benign"-"possibly damaging”, "probably damaging", Range: 0–1), Provean (Scores equal to or below -2.5 are considered “deleterious”, while scores above -2.5 are considered “neutral”. Align-GVGD (Scores (C0, C15, C25, C35, C45, C55, C65) from C0 “likely benign” to C65 “likely pathogenic”.

Gene	Mutation	SIFT	PolyPhen2	Mutation taster	Align GVD	PROVEAN
ERCC2	p.R683Q	Damaging (0)	Probably damaging (1)	Deleterious (0.992)	C35	Deleterious (-3.662)
TYRP1	p.T262M	Damaging (0)	Probably damaging (1)	Deleterious (0.953)	C65	Deleterious (-5.102)
WRN	p.L383F	Benign (0.72)	Benign (0.032)	Neutral 0.345	C15	Neutral (-0.784)
WRN	p.A995T	Tolerated (0.2)	Tolerated (0.007)	Neutral 0.196	C55	Neutral (-0.246)

Chapter 4

Discussion

In this study, we used WES to identify rare germline variants predisposing to melanoma in a Palestinian family, of whom the patient had no pathogenic variants detected in a previously targeted gene panel test. Analysis of the WES data of the patient and his affected and unaffected brothers had identified four rare variants in *WRN, TYRP1*, *and ERCC2* candidate genes. *WRN* belongs to the RECQ DNA helicase family and is associated with multiple DNA transactions, including transcription, replication, recombination, and DNA repair.[111] The mRNA expression of the *WRN* gene has been observed in numerous tissues, including the ovary, breast, skin, colon, and prostate (GTEx dataset). Mutations in *WRN* cause the Werner syndrome, an autosomal recessive disorder characterized by premature aging and increased risk of cancers, including melanoma.[111,112] A previous study has identified two different *WRN* variants (rs4733225 and rs13251813), which were associated with increased risk of cutaneous melanoma-prone families.[113] Another study of 189 individuals with Werner syndrome has revealed a 53-fold elevated risk to melanoma. [114] In our study, the two *WRN* missense variants (c.G1149T:p.L383F, and c.G2983A:p.A995T) identified in the patient and his brother (5) had no typical presentation of Werner syndrome as these mutations were in a heterozygous state. In vitro analysis showed a degree of genetic instability for carriers of the heterozygous pathogenic variants in *WRN*. However, in our study, the two identified variants had conflicting reports of pathogenicity concerning their clinical significance.

Moreover, they were predicted to be benign/tolerated by all *In silico* tools. Heterozygous germline mutations in the *WRN* gene have been identified previously in a few studies performed on melanoma patients.[92,100] One of the studies has identified the *WRN* variant c.1105C>T (p.R369) and has been classified as an uncertain melanoma risk.[92] Based on cBioPortal, all the *WRN* missense mutations detected in melanoma are of uncertain significance, suggesting that pathogenic variants are mostly non-missense. The two variants haven't been seen associated with cancer in the literature. Also, they were absent in the TCGA data of cBioPortal, which also support that they have no role in any cancer type. In the COSMIC database, they were found somatically mutated in only one case of skin tissue, indicating that these variants are rare in melanomas. In addition to skin tissue, the p.A995T variant was mutated two times in prostate tissue, suggesting that this *WRN* variant might have a role in the predisposition to prostate cancer. In our study, the patient's father had prostate cancer, and since missense mutations in the *WRN* gene have been linked with the risk of prostate cancer [115,116], we think that the p.A995T variant can be potentially causative. In support, a previous study that used a hereditary cancer NGS panel has detected the two WRN variants that we identified (p.L383F, and p.A995T) in a proband with breast and endometrial cancers who had a history of prostate cancer in two first-degree relatives. This gives some evidence that these WRN mutations could play a role in prostate cancer predisposition. [117] Interestingly, a recently published study has identified the heterozygous WRN variants (p.L383F, and p.A995T) in one of five Brazilian melanoma-prone families and have co-segregated in affected relatives. Although the study didn't conclude a particular causative gene for melanoma, WRN variants could potentially play a role in its predisposition. [102] *TYRP1* encodes a regulatory protein, that is important to normal pigmentation and has a central role in the melanin

biosynthetic pathway. [91] Thus, it is highly expressed in melanocytes contained in the skin, but also expressed in other melanocytes containing tissues, such as the heart, eye, and brain (GTEx). Biallelic mutations in *TYRP1* are associated with the autosomal recessive disorder oculocutaneous albinism type 3 (OCA3). We have identified a rare missense variant (c.C785T, p.T262M) in the *TYRP1*. This variant was observed in a heterozygous state in both patient and his brother (6). *TYRP1* is one of the albinism genes that have been described previously as a low-risk melanoma gene in a meta-analysis GWAS.[88] p.T262M has been classified as a variant of unknown significance by the ClinVar database since there is insufficient evidence to determine its role in melanoma. Even though it was predicted to be deleterious by all *In silico* tools, these predictions have not been experimentally confirmed by functional studies, and their clinical significance is uncertain. Several rare heterozygous variants in *TYRP1* and other melanogenesis genes have been recently associated with familial melanoma, suggesting their partial effect in impairing pigmentation.[91,97] Three rare variants in *TYRP1*, two stop gain and one frameshift, have been detected in four melanoma cases of separate families among 144 melanoma cases from 76 American CM families. Similar to our study, these variants were predicted as deleterious by *In silico* tools.[91] Importantly, one study has identified the heterozygous missense variant p.T262M in familial CM.[97] It has been observed in a single family, present in 1 of 2 affected individuals, implying there is no clear evidence for the variant pathogenicity. Notably, several other missense variants of unknown significance have also been detected in the study. Among them, two variants (p.A24T and p.R153C) showed complete segregation in a four-case CM family, indicating a possible role of rare *TYRP1* variants in the risk of familial melanoma. [97] Nevertheless, further support for functional consequence and pathogenicity of VUS is required. The main finding in our

study is identifying a pathogenic missense mutation in *ERCC2* (rs758439420) on chromosome 19. This missense mutation (C to T) leads to a substitution of Arginine by a Glutamine at codon 683 (p.R683Q). *ERCC2* encodes an ATP-dependent DNA helicase called XPD that is important in the DNA damage repair by the NER mechanism. [104]According to GTEx, the mRNA of ERCC2 shows a high expression in numerous tissues, suggesting the essential function of *ERCC2* in maintaining genome integrity through the NER pathway. Thus, mutations in this gene are associated with the susceptibility to various cancer types including, bladder cancer, lung cancer, melanoma, and colon cancer.[118] However, *ERCC2* mutations seem to be uncommon in CM tumors since only 4% of cases from *TCGA* data harbored an alteration in *ERCC2*, specifically two truncating mutations were drivers. The p.R683Q mutation was absent from the COSMIC and cbBioPortal databases, indicating that this mutation in the *ERCC2* gene can't be a somatic mutation. Germline mutations in *ERCC2* have been rarely found associated with hereditary cancer. According to two previous studies, they have been identified in families with colorectal cancer and breast and ovarian cancer.[119,120] Biallelic germline mutations in *ERCC2* are associated with the rare autosomal recessive disorder, Xeroderma pigmentosum group D (XPD). XP Patients with mutations in *ERCC2* are characterized by photosensitivity, abnormality in skin pigmentations in UV exposed area of the body, neurodegeneration, and an increased risk of developing skin cancers, brain tumors, and tumors in other organs.[104,121] In the clinvar database (https://www.ncbi.nlm.nih.gov/clinvar), there have been 21 pathogenic non-synonymous mutations of *ERCC2* linked with xeroderma pigminitisoum, among which 8 are missense mutations. The p.R683Q missense mutation was present only four times at a heterozygous state and non at a homozygous state in the gnomAD database. This indicates

the rarity of the alleles and evidence for pathogenicity. In our study, *In silico* analysis of the 3D structure: General transcription and DNA repair factor IIH helicase subunit XPD, showed the different subunits of the TFIH protein complex, and identified the site of the mutation, p.R683Q, in XPD. The p.R683Q mutation is found toward the C-terminal end of the protein. Most mutations in XPD patients, including ours are within codon 683, among whom the Arg683Trp is the most predominant,[122] reported in more than 80% of the patients.[123] Functional studies has shown that mutations at the C-terminal end of the protein interfere with the p44 binding, thus preventing the stimulation of the helicase activity of XPD.[124] R683 is also involved in binding to double-strand/single-strand DNA junctions, and as expected, replacing R residue with Q at position 683 changes the positive charge, resulting in diminished DNA binding. Therefore, this explains the deficit in the NER system observed in XP patients. [125]A difference in clinical phenotypes has been reported, with the phenotype in patients with Arg683Trp, being more severe than the phenotype observed in patients with Arg683Gln.[104] Previous *In silico* analysis has found that Arg683Gln has more ATPase activity than the Arg683Trp. [126] The *ERCC2* pathogenic missense mutation identified by our study (c.G2048A, p.R683Q) was present in a homozygous state in the patient, who was diagnosed with Melanoma at the age of 30. In addition to melanoma (HP:0002861), the patient presented with an abnormality of skin pigmentation and hyperpigmentation of the skin (HP:0001000, HP:0000953). Notably, the homozygous variant was also identified in the patient's brother (6), who had a brain tumor besides the same clinical phenotypes found in the patient except for melanoma. These findings correlate with the phenotypic features of xeroderma pigmentosum group D, including the high risk of developing cutaneous melanoma, as a result of exposure to ultraviolet light from the sun. Furthermore, WES results showed that

the unaffected individual (5) is heterozygous for the variant. The patient's parents must also be considered heterozygote, implying that *ERCC2* has an autosomal recessive inheritance in familial cases. To confirm that p.R683Q mutation is the mutation that causes cancer in the family, we conducted Sanger sequencing of the area flanking the mutation site. Sanger sequencing analysis has confirmed the homozygous mutation and has shown that one of the unaffected family members is heterozygous while the three remaining members were wild-type (WT). The patient's daughter should be heterozygous since her mother was tested WT for the mutation. Individuals (5) and (3) who are known to be carriers have a high risk of transmitting the mutation to the next generation. These findings confirm that the homozygous *ERCC2* variant (p.R683Q) is absent in nonaffected family members. Moreover, it cosegregated with the phenotype in the investigated patient and his brother with a brain tumor. Therefore, it is the causative mutation of melanoma in the Palestinian family. The Arg683Gln mutation has been previously reported at a homozygous state in patients from various ethnic backgrounds, including Italian, German, Iraqi Jewish, Japanese, and Tunisia.[104,126–129] The study from Tunisian families has identified ten homozygous patients belonging to three families and has shown full co-segregation. These patients exhibited mild dermatological manifestations, late-onset of skin tumors, and an absence of neurological abnormalities.[104] A recent study in Vietnam has reported four siblings affected by XP with extreme sun sensitivity carried compound heterozygous mutations in *ERCC2*, p.R683Q in one allele of the gene and a novel p.Q452X nonsense mutation in the other allele. The study suggested that the latter mutation is responsible for the severe sun sensitivity experienced by the XP patients in contrast to the p.R683Q mutation that is associated with mild sun sensitivity.[125] This indicates that the different XPD mutations in

each patient strongly influence the range and severity of the phenotypes. The study has shown that two of the patients were diagnosed at the age of 38 and 35 and had developed melanoma. Additionally, they experienced phenotypes such as hyperpigmentation, irritation, and freckles.[125] In our study, we think that the homozygous *ERCC2* p.R683Q mutation could have contributed to the tumorigenesis in the patient's brother (36 years old), who has a brain tumor. Most probably, the brain tumor resulted from an increased rate of mutations due to the defective DNA repair caused by the *ERCC2* mutation. Still, he is at a high risk of developing melanoma, due to the strong history of melanoma in the family. Moreover, reports have suggested that the age of developing melanoma in patients with XP ranges between 2 and 47 years. [130] On the other hand, carriers of the *ERCC2* p.R683Q don't have a risk of melanoma development. Even though haploinsufficiency could play role in increasing the susceptibility to cancer without leading to a cancer syndrome, there have been no formal documents attributing cancer susceptibility to a heterozygous variant in a gene of an autosomal recessive cancer syndrome. Moreover, loss of heterozygosity (LOH) is not a common event in patients with *ERCC2* mutations. Only one study has reported the presence of LOH in tumors from patients with *ERCC2* germline mutations, and this has been found in bladder cancer patients carrying the p.Arg616Pro mutation in *ERCC2.*[131]A limitation of this study is that the patient's parents refused to participate. However, we were able to include siblings and perform a segregation analysis.

Conclusion

In summary, we have successfully applied exome sequencing for an unexplained molecular cause of melanoma to a diagnosis with xeroderma pigmentosum and identified a rare *ERCC2* missense mutation (c.G2048A, p.R683Q) responsible for melanoma in the family. This study expands the knowledge of the mutational background of familial melanoma in the Palestinian population, which is valuable to guide the diagnosis, prevention, and treatment of affected patients and their families.

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ملخص الرسالة

الورم الميلانيني الجلدي (الميلانوما) هو نوع سـرطاني ينشـأ من الخلايا الصـبغية، وهي الخلايا المنتجة لصبغة الميلانين في الجلد. إنه أكثر أنواع سرطانات الجلد خطورة وهو مسؤول عن حوالي 75٪ من وفيات ســرطان الجلد. وجود تاريخ عائلي للورم الميلانيني يعد من بين أقوى عوامل الخطر للإصــابة بالمرض ويمثل حوالي 10٪ من الحالات. حوالي نصـف هذه الحالات لا يمكن تفسيرها من خلال الطفرات في جينات الورم الميلانيني المعروفة بنفاذها المتوسط إلى المرتفع، مما يضــعف الاختبارات الجينية والاســتشــارة في العائلات التي لديها اســتعداد للإصــابة بســرطان الميلانوما. تتعلق الدراسة الحالية بعائلة فلسطينية مصـابة بسرطان الميلانوما، بحيث ان المريض لم يتم العثور لديه على طفرات جينية مسببة للمرض عن طريق فحص سابق لتحليل لوحة الجينات المستهدفة لـــ 94 جينًا . لذلك، فإن الهدف الأساسي من هذه الأطروحة هو تحديد الطفرات الجينية الوراثية المسببة لسرطان الميلانوما في الأسرة عن طريق تطبيق التسلس الكامل للاكسوم WES. الهدف الثانوي هو تحديد أفراد الأسـرة االذين لديهم عرضــة للإصــابة بالمرض. نحن نفترض أن السبب الجيني الكامن وراء الميلانوما في العائلة هو وجود طفرة جينية نادرة. استخدمنا نهج تسلسل الإكسـوم الكامل لتحديد الطفرات المتعلقة بالميلانوما من عينات الحمض النووي للمريض، شـقيقه المصاب بورم في المخ، وشقيقه غير المصاب. قمنا بعد ذلك بتحليل بيانات WES باستخدام خطوط الانابيب المعلوماتية الحيوية لإنشــاءVCF بهدف ترشـيح المتغيرات. بعد ذلك تم تطبيق تسـلسـل ســانجرSanger sequencing للمتغير المرشــح على عينات الحمض النووي للمريض وأفراد الأسـرة للتحقق من ان الطفرة المرشـحة هي المسـببة للمرض ومن اجل معرفة وجودها في افراد العائلة. تبع ذلك تحليل السيليكو للمتغيرات والجينات المرشحة. أظهرت نتائج تحليل ال WESوجود ثلاث طفرات نــادرة متبــاينــة الازدواج، WRN (p.L383F and p.A995T) و TYRP1 (p.T262M). الأكثر أهمية من ذلك، تم العثور على طفرة متماثلة الازدواج في جين ال ERCC2 (p.R683Q) ، متعلقة بالاضـطراب النادر، جفاف الجلد المصـطبغ او زيروديرما، و الذي يزيد من خطر الإصــابة بالميلانوما . ان هذه الطفرة المسـببة للميلانوما متناسـبة مع الأنماط الظاهرية السـريرية الموجودة في الأخوين المصـابين، وتم تأكيدها من خلال تحليل تسـلسـل سـانجر. أظهر تحليل سانجر لهذه الطفرة غيابها في الحالة المتماثلة المزدوجة في أفراد الأسرة غير المصابين.

تؤكد هذه النتائج أن طفرة ERCC2 متماثلة الازدواج (p.R683Q) هي الطفرة المسببة لسرطان الميلانوما في الأسرة الفلسطينية. في الختام، توسع هذه الدراسة المعرفة بالخلفية الجينية للطفرات المتعلقة بالميلانوما الوراثية لدى السكان الفلسطينيين، وهو أمر ذو قيمة لتوجيه التشخيص والوقاية والعلاج للمرضى المصابين وعائلاتهم.