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Research Article

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Analysis of Recurrent DNA Base Pair Mismatches in Eye Cancer Patient Genomes

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Abstract

This research investigates potential genetic drivers for uveal melanoma (UM), the most frequent primary cancer of the eye, through a frequency and ontology analysis of recurrent genetic mutations in UM patients. Genome data of 32 patients, 23 primary and 9 metastatic, was acquired from the U.S. National Institute of Health (NIH) and obtained from samples that were surgically collected from eye enucleations or resected from liver metastases. It was hypothesized that chromosomes 15 and 16 would predominantly exhibit genetic alterations linked to UM due to their role in the expression of eye color. During analysis, DNA from cancerous tumor cells was compared to a reference DNA sequence (extracted from normal tissue) to identify nucleotide base pair mismatches. The locus of each mutation noted to determine what genes mutated. Pareto analysis of cross-patient data performed to identify chromosomes with the most genetic mutations along with any recurrent genetic mutations across patients. A gene ontology (GO) analysis conducted to study the functions of mutated genes and investigate possible links to cancer, such as anomalies in genes with a known role in tumor suppression. A total of 130 genetic mutations were identified (7 recurrent and 123 non-recurrent), with most mutations occurring in chromosomes 3 and X. Recurrent mutations varied from 8.7% to 17.39% occurrence in the UM patient sample. The recurring mutations observed as missense mutations in seven genes. These findings suggest that UM is a recessive heterogeneous disease with selected homozygous mutations. Notably, this study has potential wider significance because the seven genes targeted by recurrent mutations affected in other cancers. It concluded that immunotherapy is a highly promising treatment for Uveal Melanoma due to the disease's heterogeneous nature.

1. Introduction

Melanoma is a life-threatening malignancy that affects melanocytes (pigment-producing cells) found throughout the body. Melanomas are of two types: cutaneous and non-cutaneous. Cutaneous melanomas, which account for approximately 95% of all melanomas, originate in the pigment-producing cells of the skin. On the other hand, non-cutaneous (non-skin) melanomas affect other regions of the body including the eyes and mucous membranes, such as those present in nasal passages and the oral cavity [1]. Although it is a rare disease, uveal melanoma is the most common form of non-cutaneous melanoma, and it is the most frequent primary cancer of the eye in the adult [2]. Uveal melanoma is known to affect ~7000 individuals worldwide annually, with incidence rates ranging from 0.2 to 0.3 per million individuals in African and Asian populations to up to 6 per million in white populations [3,4]. Diagnosis usually occurs at age 60, and this cancer is more prevalent among Caucasians. In most cases, UM forms in the choroid: the vascular layer of the eye lying between the sclera and the retina. Symptoms that exhibited by UM patients include but are not limited to variable and painless visual disturbances, discoloration of the iris, change in the shape of the pupil, or loss of peripheral vision [5].

One aspect that contributes to the lethal nature of melanomas, including uveal melanoma, is the risk of metastasis. Metastasis is the migration of cancerous cells through the bloodstream, which leads to the development of tumors elsewhere in the body. These tumors can cause tissue damage and other widespread effects, which accelerate the poor outcomes for cancer patients [6]. In the case of uveal melanoma, the liver is the most common organ affected by metastasis, which occurs in 80% of cases [7,8]. Although regular metastasis can be detected, undetectable micro metastases may occur, in which a small collection of cancerous cells spread to other parts of the body via the lymphovascular system. Micro metastasis poses a significant risk to all UM patients, which is why the patient should receive immediate treatment after diagnosis. Unfortunately, the causes for uveal melanoma are still somewhat unclear. Some studies have revealed that the DNA present within the cancerous cells showed alterations on chromosomes 1, 3, 6, and 8, but these conclusions can be researched more extensively. They have also found that genes BAP1, SF3B1, GNAQ, and GNA11

seem to play a role in the development of uveal melanoma [9,10]. Although these studies did uncover some genetic errors that may be responsible for UM, they had only analyzed chromosomal rearrangements rather than specific nucleotide alterations. Currently, there is a lack of effective therapies for the treatment of UM [11].

This study aimed to identify specific genetic mutations and base pair discrepancies that may contribute to the development of uveal melanoma using genome data, statistical analysis techniques, and gene ontology (GO) research of frequently mutated genes. This research was conducted with an NIH data set of 32 samples including 23 primary and 9 metastatic samples. Following the analysis, 130 unique genetic mutations were observed in all the UM patients, with 7 recurrences in the primary samples and no recurrences in the metastasis samples. The Seven recurrent mutations identified as ALG1L2, DMD, IL1RAPL2, KIA0825, LOC440040, NXF2, and PHYHD1.

2. Materials & Methods

All data for this experiment collected from Complete Genomics Inc., which hosts publicly available data published by the National Center for Biotechnology Information (NCBI). Sample genome data was publicly available and provided in the "Supplemental Data" section of a 2016 whole-genome sequencing study, which is accessible through the National Institute of Health (NIH) National Library of Medicine [10]. This data obtained from 32 UM patients, and the data package included 32 data files (23 Primary Uveal Melanoma (PUM) files, and 9 Metastases Uveal Melanoma (MUM) files). The UM patient data was in excel format, and each file included a sample reference DNA sequence (matched normal) and a mutated allele 2 sequence from each patient. Each file also contained specific data on each subject, including gender, date of data collection, and sample source. Microsoft Excel used in analysis of patient genome data to identify and locate genetic mutations. Minitab 2018 software then used to perform a Pareto analysis to analyze the frequency of all genetic mutations and chromosomes involved.

2.1. Analysis Methodology

Examine each SNV (Single-Nucleotide Variant) file in Microsoft Excel as follows:

1. Apply data filter on all column headings
2. Insert a new column labeled "Match" between the "reference" (matched normal sequence) column and "allele2Sequence" (mutated sequence) column.
a. Note that the "allele1Sequence" column can be ignored, as it only contains nitrogenous bases that are complimentary to the reference sequence.
3. Import an IF function into the "Match" column, and define the logical test with the following parameters:
a. Logical_test: value of 1st cell under reference column = value of 1st cell under allele2Sequence column
b. Value_if_true: " "
c. Value_if_false: "X"
4. Click on the "allele2Sequence" heading and uncheck "blanks" to filter out blanks because those cells are haploid, and only have 1 allele.
5. Click on the "allele2Sequence" heading and uncheck "?" to filter out unknown base pairs which were not recorded. These mismatches were disregarded due to uncertainty.
6. In all instances where there is a mismatch ("X" in the Match column), it indicates a mutation. For each observed mutation, note the corresponding zygosity, variation type, and gene name (labeled "gene_name" located in column AE)
a. The article supplementary data provides information on the zygosities and variation types
7. Repeat steps 1-6 to examine all patient files and consolidate results for genetic mutations observed across all primary and metastases UM patients.

Perform Pareto analysis for frequency of chromosomes with observed genetic mutations:

1. List all chromosomes with mutated genes sequentially from chromosome 1 to 23 (x), along with count of mutated genes on each chromosome
2. Count number of patients with one or more of the observed mutations (see Table 1)
3. Using Minitab software, perform pareto analysis of chromosomes with mutated genes by listing chromosomes in descending order by frequency distribution of counts of genetic mutations in all patients from the primary sample (non-metastatic). For example, chromosome 3 exhibited the highest number of mutations (45) and is the first bar in the Pareto chart (Figure 1)
4. Repeat the analysis performed in the previous step with data from the patients with Metastasis (Figure 2).
5. Perform Pareto analysis of mutated genes in all patients from the primary sample by listing mutated genes in descending order by frequency distribution of mutation count (Figure 3). For example, genetic mutation in ALG1L2 mutation was observed in 4 patients (17,39%).
6. Research gene functions of mutated genes to investigate link between gene function and anomalies that may result in cancer (Table 2).

Chromosomes Exhibiting Genetic Mutations	Number of Mutated Genes	Number of Patients	Percentage of Patients
3	45	11	47.83
X	30	6	26.09
1	7	3	13.05
9	12	3	13.05
11	2	3	13.05
5	1	2	8.70
6	5	2	8.70
8	3	2	8.70
12	2	2	8.70
17	2	2	8.70
16	1	1	4.35
18	1	1	4.35
19	1	1	4.35
20	2	1	4.35

Table 1: Frequency of Mutated Genes per Chromosome in Primary UM Samples (listed in Descending Order of Frequency per Patient)

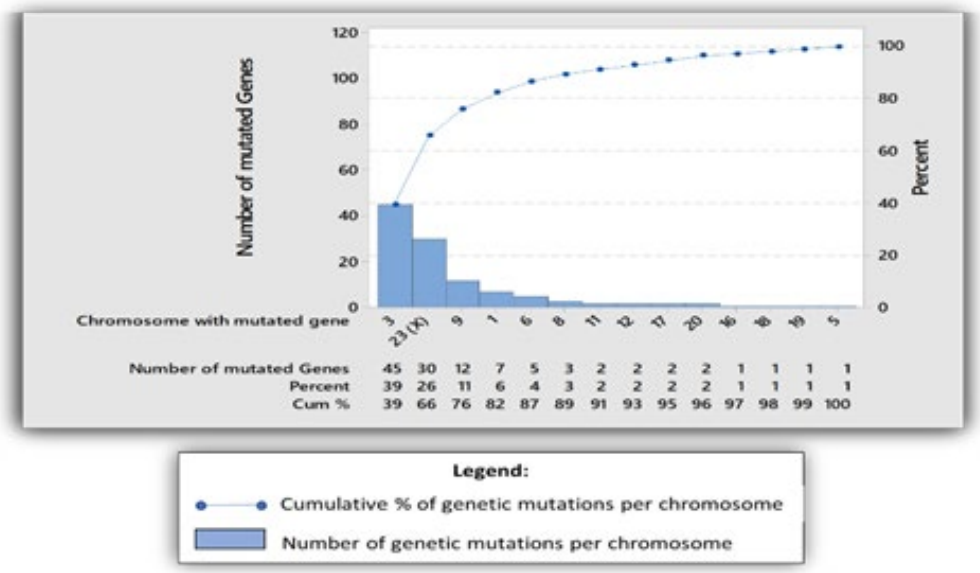

Figure 1: Pareto Chart of Frequency of Genetic Mutations per Chromosome in Primary UM Samples

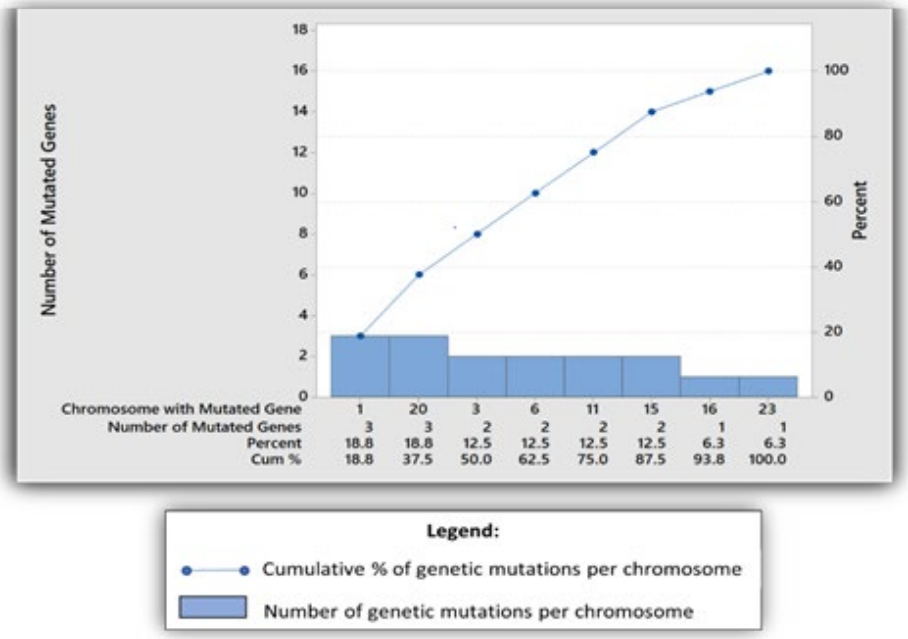

Figure 2: Pareto Chart of Frequency of Genetic Mutations per Chromosome in Metastasis UM Samples

Mutated Gene	Cytogenic band	Gene Function	Freq.
ALG1L2 (ALG1, Chitobiosyldiphospho-dolichol Beta-Mannosyltransferase Like 2)	3q22.1	Protein Coding gene. Gene Ontology (GO) annotations related to this gene include transferase activity, transferring glycosyl groups.	17.39%
DMD Dystrophin	Xp21.2-p21.1	Protein Coding gene. Diseases associated with DMD include Muscular Dystrophy, Duchenne Type and Muscular Dystrophy, Becker Type. Gene Ontology (GO) annotations related to this gene include calcium ion binding and structural constituent of cytoskeleton.	8.7%
IL1RAPL2 Interleukin 1 Receptor Accessory Protein Like 2	Xq22.3	Protein Coding gene. Diseases associated include Cold Urticaria and Anterior Scleritis. Gene Ontology (GO) annotations related to this gene include interleukin-1 receptor activity and interleukin-1, Type II, blocking receptor activity.	8.7%
KIAA0825	5q15	Protein coding gene. Possible risk factor in Type II Diabetes and associated with high levels of glucose in the blood.	8.7%
LOC440040 Glutamate Metabotropic Receptor 5 Pseudogene	11p11.12	Pseudogenes play essential roles in gene regulation of their parent genes, and many pseudogenes are transcribed into RNA. Pseudogenes regulate tumor suppressors and oncogenes [13].	8.7%
NXF2 Nuclear RNA Export Factor 2	Xq22.1	Protein Coding gene. Among its related pathways are RNA transport and Transport of Mature Transcript to Cytoplasm. Gene Ontology (GO) annotations related to this gene include RNA binding and nucleotide binding.	8.7%
PHYHD1 Phytanoyl-CoA Dioxygenase Domain Containing 1	9q34.11	Protein Coding gene. Gene Ontology (GO) annotations related to this gene include dioxygenase activity.	8.7%

Table 2: Information on Location, Function, and Frequency of Genes Targeted by Recurrent Mutations in Primary UM Samples

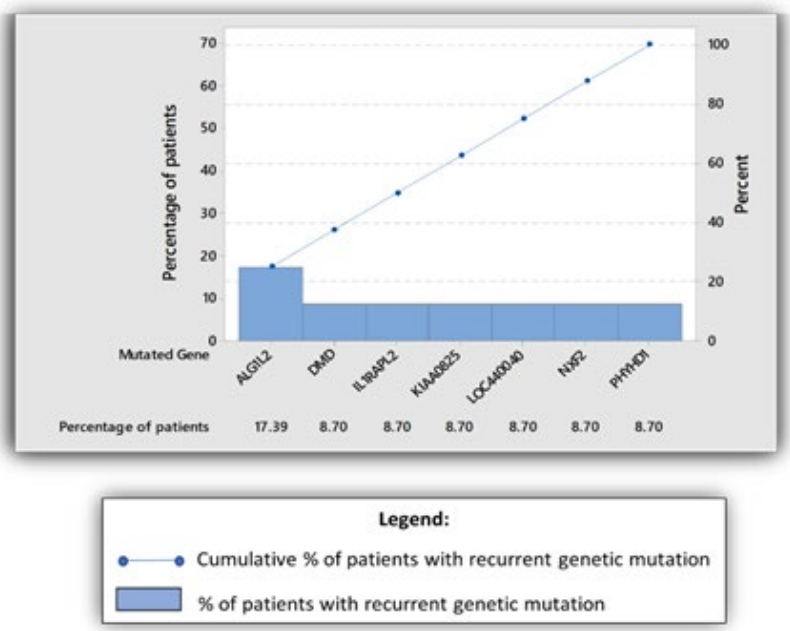

Figure 3: Pareto Analysis of the Frequency of Recurrent Genetic Mutations in Primary UM Samples

3. Results

Within this study, genetic alterations resulting from chromosomal rearrangements were disregarded, but base pair mismatches causing missense, nonsense, or frameshift mutations. Some patients had experienced metastasis of cancerous cells through the bloodstream; data from their sample genomes were also considered separately to identify any genetic mutations that could promote and accelerate metastasis. The basic procedures for this study involve the comparison of a mutated DNA sequence (found in cancerous somatic cells) with the matched normal sequence (found in healthy, unaffected somatic cells).

It was observed that most of the genetic mutations were present on chromosomes 1, 3, 5, 9, 11, and X, which reconfirms previous findings of chromosomes 1 and 3 being involved in UM, and identifies chromosomes 5, 9, 11, and X as potential new factors (Figure 4). Pareto analysis showed that chromosome 3 and X exhibited the most genetic mutations - 75 in total across all primary samples Figure 1.

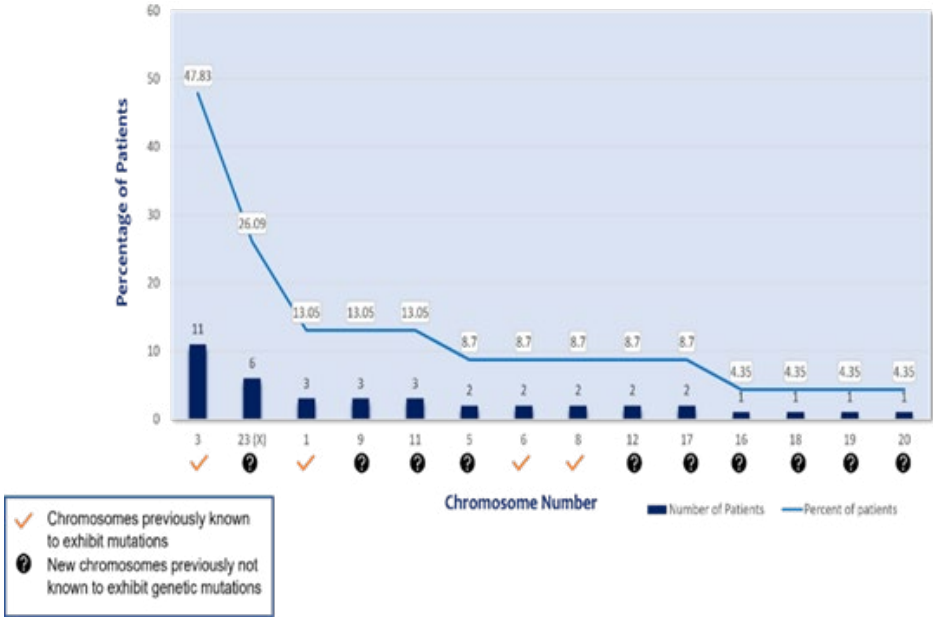

Figure 4: Bar Chart of Frequency of Chromosomes with Mutated Genes in Primary UM Samples

Somatic SNV (Single-Nucleotide Variant) files were examined from the data package. Each data file contained information on the patient's whole genome, including the locus, zygosity, and variation type of each DNA base pair, as well as the genes identified during sequencing [Table 3]. The two columns in Table 3 outlined in bold include the reference (normal) DNA sequence and the allele 2 (mutated) DNA sequence. The 'Match' column was not present within the data and was created with an automated IF function to compare each cell from the reference sequence with the corresponding cell from the allele 2 sequence. Whenever there was a mismatch, the function would automatically flag each mutation with an 'X' symbol in the corresponding cell within the 'Match' column. This IF function in the Match column was applied to the entire genome to search for any mutations. After the comparison was complete, the Match column was filtered to show the base pair mismatches (indicated by 'X' in the 'Match' column) [Table 3]. All '?' in the allele 2 sequence indicated that there was an unknown base present, therefore those mismatches were disregarded as mutations due to ambiguity. This analysis procedure was followed for both the primary (PUM) and metastases (MUM) files.

Locus	Ploidy	Chromosome	Begin	End	Zygosity	VarType	Match	Reference	Allele2Seq	Gene Name
656203	2	chr1	1E+08	1E+08	hom	snp	X	C	T	NA
5356442	2	chr6	7E+07	7E+07	half	snp	X	G	?	NA
5561068	2	chr6	1E+08	1E+08	hom	snp	X	G	T	FOXO3;
9276693	2	chr11	1E+08	1E+08	hom	snp	X	C	T	NA
9363079	2	chr11	1E+08	1E+08	hom	snp	X	G	T	PCSK7;
9379683	2	chr11	1E+08	1E+08	hom	snp	X	A	G	NA
9400430	2	chr11	1E+08	1E+08	hom	snp	X	A	G	DCPS;
12932227	2	chr20	2E+06	2E+06	hom	snp	X	C	T	SIRPB1;
12932231	2	chr20	2E+06	2E+06	hom	snp	X	C	A	SIRPB1;
12932233	2	chr20	2E+06	2E+06	hom	snp	X	C	A	SIRPB1;
12932279	2	chr20	2E+06	2E+06	half	snp	X	T	?	SIRPB1;
12932666	2	chr20	2E+06	2E+06	half	snp	X	T	?	SIRPB1;
12932778	2	chr20	2E+06	2E+06	hom	snp	X	A	G	SIRPB1;
12935032	2	chr20	2E+06	2E+06	hom	snp	X	A	G	NA
12970932	2	chr20	1E+07	1E+07	hom	snp	X	A	C	PAK7;
12984366	2	chr20	1E+07	1E+07	half	snp	X	T	?	NA
12996659	2	chr20	2E+07	2E+07	hom	snp	X	A	G	MACROD2;
13011746	2	chr20	2E+07	2E+07	hom	snp	X	C	T	NA
13641551	2	chrX	336920	3E+05	half	snp	X	C	?	PPP2R3B;
13653396	2	chrX	1E+06	1E+06	half	snp	X	C	?	NA
13653496	2	chrX	1E+06	1E+06	half	snp	X	ACACGCGTGA	?	NA
13653842	2	chrX	1E+06	1E+06	half	snp	X	C	?	NA
13663423	2	chrX	2E+06	2E+06	half	snp	X	C	?	NA
13663425	2	chrX	2E+06	2E+06	half	snp	X	GA	?	NA
13663427	2	chrX	2E+06	2E+06	half	snp	X	T	?	NA
13663433	2	chrX	2E+06	2E+06	half	snp	X	C	?	NA

Table 3: Snapshot of Uveal Melanoma Primary Sample Data

Legend:

SNV file contains reference and mutated (allele2seq) DNA sequences.

The "Match" column has been filtered to only show Xs whenever there is a mismatch between the reference and allele2seq columns, signifying a mutation.

All the mutations that occurred were noted along with their respective chromosome number, zygosity, and gene name. The gender of each patient was also considered to determine whether uveal melanoma is an autosomal or sex-linked disease. The variation type of each mutation including base substitutions, deletions, and insertions, was not assessed as that had already been investigated by other studies. However, the application of a data filter on the variation type (labeled "varType" in Table 3) to sort for chromosomal rearrangements coupled with the IF function from this procedure allowed for the reaffirmation of GNAQ, GNA11, and BAP1 as plausible genetic drivers for UM.

Pareto analysis was performed to visualize the frequency of the genetic mutations, as well as the chromosomes on which they were present. A Pareto chart is a bar chart in which the bars are ordered from highest frequency of occurrence to lowest frequency of occurrence [12]. This cause analysis tool can be used to measure the frequency of problems or causes in a process [13]. Although this technique is typically used in statistical decision-making, it helped visualize quantitative data on the frequency of genetic mutations across all samples in the study. Pareto analysis of chromosome involvement was initially conducted on data that was consolidated and sequentially ordered in Table 1. The analysis revealed that the highest frequency of patients exhibited genetic alterations to chro-

mosome 3, accounting for nearly 50 percent of all genomes analyzed. Chromosome X was also found to have a high frequency of mutated genes and exhibited alterations in more than a quarter of the patients analyzed. Chromosomes 1, 9, and 11 follow with the third-highest frequency of ~13 percent, and all other chromosomes were affected in 2 or fewer patients. Another Pareto analysis was conducted to determine the frequency of mutated genes in UM patients. As shown in Table 4, the analysis uncovered 7 genes that recurred in 2 or more UM patients. The gene ALG1L2 was found to have the highest recurrence, appearing to be mutated in 4 out of 23 (17.39%) patients analyzed. The Pareto analysis of mutated genes was only performed for the primary samples [Figure 3] because the results revealed that there were no recurring genetic mutations among the patients whose cells had undergone metastasis.

Mutated Gene	Number of Patients	Percentage of Patients
ALG1L2	4	17.39
KIAA0825	2	8.70
PHYHD1	2	8.70
LOC440040	2	8.70
NXF2	2	8.70
IL1RAPL2	2	8.70
DMD	2	8.70

Table 4: Frequency of Recurrent Genetic Mutations in Primary UM Samples (listed in Descending Order of Frequency per Patient)

4. Discussion

From this study, it can be concluded that uveal melanoma is a heterogeneous disease characterized by predominantly non-recurrent (130 total) mutations. This heterogeneous nature is a very common characteristic of most types of aggressive melanomas and serves as important information for predicting the appropriate form of treatment for UM patients. The Pareto analysis uncovered that the frequency of the recurrent genetic mutations in our UM cohort ranged from 2 to 4 out of 23 primary samples (8.7%-17.39%), which is significant considering the size of the human genome. Since hundreds of non-recurrent missense mutations were identified in this study, this relatively high rate of recurrence suggests a link for these genes to cancer. To confirm this inference, the functions of all the mutated genes were researched, and the recurrent ones were studied extensively [Table 2]. A gene ontology (GO) analysis of recurrent genetic mutations was conducted to explore the biochemical pathways, associated genes, and dysfunctional proteins that may contribute to the progression of UM.

ALG1L2, the most frequent genetic mutation, was observed in 4 patients out of the 23 primary samples. This frequency is quite high considering that there are an estimated ~25,000 genes in the human genome, and thus the recurrences were unlikely due to random chance [14]. ALG1L2 encodes a putative glycosyltransferase protein which is responsible for transferring glycosyl groups [15,16]. This activity plays a critical role in determining the structure, stability, and function of a protein, and could consequently affect tumor suppressor pathways if inhibited. DMD, which exhibited recurrent mutations in samples 19 and 21, codes for dystrophin. Although DMD deletions are commonly associated with muscular dystrophy, mutations in DMD could alter the structure of dystrophin, which has been found to suppress myogenic tumors and prevent metastasis of cancerous cells [17,18]. DMD mutations could therefore contribute to myogenic tumor growth in the ciliary body, a ring-shaped muscle located behind the iris, thus facilitating the development of ciliary body melanoma [19]. Additionally, research has shown that abnormal dystrophin levels are indicative of DMD involvement in the pathogenesis of several other cancers and melanomas [20].

Mutations on the protein-coding gene IL1RAPL2 were also found in two patients. IL1RAPL2 encodes an interleukin receptor accessory protein which is integral to immune response. The primary function of interleukins is to modulate growth, differentiation, and activation during inflammatory responses [21]. However, chronic inflammation can often damage DNA and promote carcinogenesis, and interleukin signaling in cancerous cells has been researched as a critical factor in cancer development, progression, and control [22]. KIAA0825 was also identified as a recurrently mutated gene, but little is known about its gene product. KIAA0825 shares interactions with several genes including PAX4, E2F1, and E2F4, which code for transcription factors and control the behavior of other tumor suppressor proteins [16,23]. Long noncoding RNA LOC440040 was mutated in samples 1 and 20, and it is a pseudogene of GRM5 on chromosome 11. GRM5 (Glutamate Metabotropic Receptor 5) is responsible for the regulation of neural network activity and synaptic plasticity [16,23]. According to the Gene Expression Database, GRM5 is expressed in the visual system and helps modulate synapse activity through glutamate signaling. Dysregulation of transporters and dysfunctional glutamate receptors can adversely affect glutamate signaling beyond the central nervous system, which could promote cancer development in visual pathways [24].

NXF2, which was mutated in samples 3 and 17, encodes a nuclear RNA export protein that is responsible for RNA and nucleotide binding [16,23]. Nuclear RNA export factor 2 is involved in the export of mRNA from the nucleus to the cytoplasm. Issues with mRNA transport can have a cascading effect on transcription and protein synthesis, which could potentially correlate with the devel-

opment of UM. Diseases associated with NXF2 include Progesterone-Receptor Negative Breast Cancer. Finally, PHYHD1, mutated in samples 19 and 22, codes for phytanoyl-CoA dioxygenase domain-containing protein 1 which has a role in the epigenetic regulation of gene transcription [25]. PHYHD1 also interacts with the FH (fumarate hydratase) and CAT (catalase) proteins, which act as a tumor suppressor and promote cell growth, respectively. It was also found that most of the non-recurrent genes that were mutated coded for transcription factors and tumor suppressors – proteins that, when defective, are known to cause other cancers.

The genetic mutations were homozygous, meaning that uveal melanoma is a recessive disorder, which explains the rarity of this cancer. Although two recurrent mutations target genes on the X chromosome, no pattern was observed between patient gender and the identified mutations, thus suggesting that uveal melanoma is an autosomal disease. All mutations were missense mutations, resulting from single base mismatches within the protein and non-protein-coding regions of the DNA. Across all samples, 15 genes were observed (8 pseudogenes and 7 RNA genes) to exhibit mutations in non-protein-coding regions. Only one of these genetic mutations, present on LOC44004, was recurrent. The other two types, frameshift and nonsense mutations, were not found within any of the samples that were analyzed. All of the recurrent genetic mutations that were found in this study have not been listed in any source of literature, suggesting that these are potentially new and yet undiscovered mutations responsible for uveal melanoma, and likely responsible for other cancers (the same genetic mutations are often responsible for multiple types of cancers, such as genetic mutations in BRAF V600E, TP53, and CDKN2A, which have a high recurrence in cutaneous melanomas).

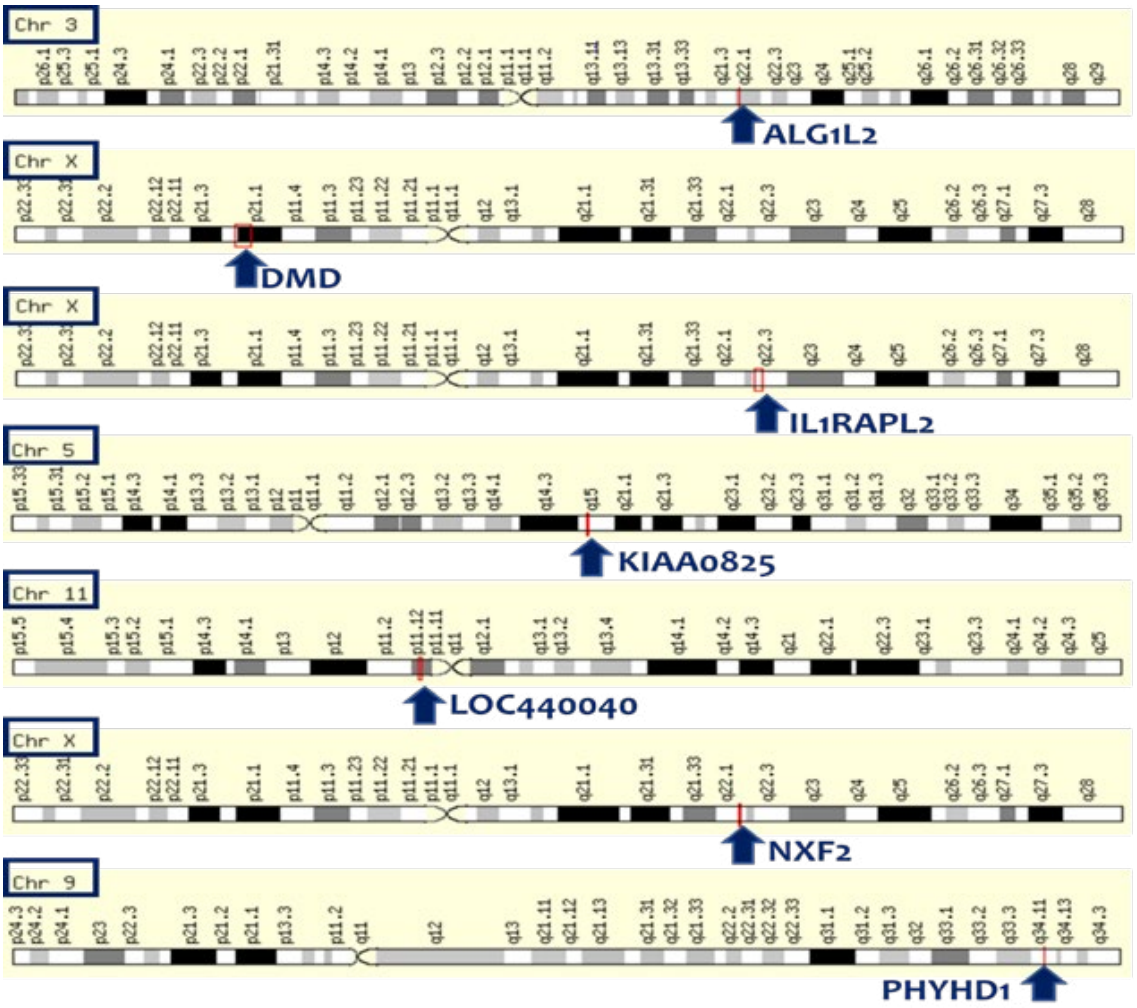

Figure 5: Genes Targeted by Recurrent Mutations and Respective Locations

5. Conclusion

One major implication of this study is that it suggests the best treatment approach for people suffering from UM. Since the results show that UM is caused by several unique genetic mutations, thus implying its heterogeneous nature, targeted therapy may not be the best approach. Targeted therapies are developed to target and inhibit the function of a specific gene or defective protein that results from genetic mutations. For example, Larotrectinib is a

medication for solid tumors that inhibits TRK (tyrosine kinase), a protein that promotes cancer [26]. TRK is produced because of the fusion of two genes due to an underlying genetic mutation in NTRK (neurotrophic receptor tyrosine kinase). The best treatment option predicted for UM would include immunotherapy, which prevents disease by stimulating and enhancing the immune system to fight against dysfunctional proteins caused by mutations. Immunotherapy would be effective in reducing the cumulative effects of all these harmful genes and proteins. In contrast, targeted therapy could be used to inactivate specific genes and pathways which are implicated in UM. Additionally, the recurrent genetic mutations identified in this study can be activated in mice models to assess the phenotypic consequences and potentially causational relationship with UM [27].

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