Investigating the genomic profile of inherited prostate cancer using whole exome sequencing data

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Abstract

Prostate cancer (PrCa) is the second most common cancer among men and a leading cause of cancer-related death. While hereditary factors contribute to approximately 20% of cases, the role of rare and low-frequency genetic variants in PrCa predisposition remains underexplored. This project aims to investigate the mutational landscape of rare (MAF $< 1\%$) variants in 96 PrCa patients from 45 families using whole exome sequencing (WES). The study will identify significantly altered genes and enriched pathways associated with PrCa susceptibility, focusing on frameshift, nonsense, splicing, and deleterious missense variants. This research aims to contribute to early detection, targeted treatment strategies and better genetic counseling for families with hereditary prostate cancer.

Keywords: hereditary prostate cancer; single nucleotide variations; whole exome sequencing.

1 Introduction

Repository location `https://github.com/Alexsf35/Projeto_Bioinf.git`

Context During their lifetime, men have a 40.1% chance of being diagnosed with any invasive cancer (slightly higher than women, 38.7%)[1, 2]. Among these diagnoses, approximately 14.2% are prostate cancers (PrCa). As of 2022, PrCa has been determined to be the second most prevalent cancer worldwide in terms of incidence and the fifth most prevalent in terms of mortality among the male population. It has been identified as the most common cancer among men in 118 different countries, including Portugal[3, 4].

A notable factor in PrCa is its hereditary component. The proportion of PrCa attributable to hereditary factors has been estimated to be 5 to 15%, with other estimates stating that up to 20% of men diagnosed with PrCa have a family history of the disease within their paternal or fraternal lineage[5]. In the case of any affected family member, the relative risk is doubled, with an additional increase observed in relation to the number of relatives and their age (under 60 years of age) at the time of diagnosis[6]. In addition, ethnicity has been shown to exert a significant influence on the outcomes of PrCa cases, with African ancestry being recognized as a well-established risk factor[1, 7, 8].

The studies done on hereditary cancer syndromes such as Hereditary Breast and Ovarian Cancer (HBOC) and Lynch Syndrome (LS), have contributed to the identification of many pathogenic and "likely/potentially pathogenic" germline mutations in homologous recombination genes (*BRCA1/2*, *ATM*, *PALB2*, *CHEK2*) and mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) respectively, that may also underlie hereditary prostate cancer (HPC)[5, 9, 10, 11]. It is estimated that these germline mutations may account for 7.4–21% of cases. However, it is noteworthy that 79–92% of cases are attributable to genes that have yet to be identified[5].

The etiology of PrCa remains to be fully explained; however, mounting evidence suggests that the aforementioned factors are significant indicators of genetic susceptibility to PrCa. This susceptibility is linked to the inheritance of a combination of rare germline variants (defined as having a minor allele frequency [MAF] $< 1\%$) in moderate to high-penetrance genes and common genetic alterations in low-risk genes[4, 11].

PrCa is thus characterized as a cancer with a multigenic etiology, that can be caused by single nucleotide variations (SNVs) (which can lead to truncating, missense or nonsense changes). While few such mutations may have low impact, the cumulative effects might be of particular interest in HPC studies[12].

Objectives Whilst the majority of studies on the genetic predisposition to PrCa have focused on common variants (MAF $> 5\%$) and very rare, high-risk variants (MAF $< 0.1\%$), the present project aims to bridge this gap by investigating the mutational landscape of rare (MAF $<1\%$) single nucleotide variants and small indels. The present study will analyse whole exome sequencing (WES) data from 96 PrCa patients across 45 families, this analysis would seek to characterize the low-frequency and rare variants that cause HPC. In addition, the study seeks to identify significantly impacted genes that play a role in predisposition to PrCa and identify enriched biological pathways that may be responsible for increased susceptibility. With all these objectives brought together, the project aims to provide a comprehensive picture of the enriched pathways implicated in PrCa susceptibility, which can eventually contribute to more effective early detection, targeted treatment strategies and better genetic counseling.

2 Epidemiology

PrCa is a major health issue worldwide with an average age of diagnosis being 66 years old[11]. The age-standardized incidence rate (ASR) in 2020 was of 31 per 100,000 males, with a lifetime risk of 3.9%. Still, these incidences vary tremendously by region.

For instance, incidences of as high as 83 per 100,000 are seen in Northern Europe, followed by Western Europe (78), the Caribbean (76), and Australia/New Zealand. Conversely, incidences as low as 6.3 per 100,000 have been observed in countries such as South-Central Asia, 14 for South-Eastern Asia, and 17 for Northern Africa. These variations are the reflection of the differences in screening efforts, access to healthcare and public awareness about the disease. As for mortality, in 2020 the global ASR was approximately 7.7 per 100,000 males. The less developed socioeconomic areas have much higher mortality rates, with the Caribbean at 28 per 100,000, Middle Africa at 25, and Southern Africa at 22. In contrast, Asian regions have significantly lower mortality rate, South-Central Asia at 3.1, Eastern Asia at 4.6, and South-Eastern Asia at 5.4 per 100,000[6].

In the future, projections are not too bright. It is estimated that new PrCa cases will nearly double, from 1.4 million in 2020 to approximately 2.9 million in 2040. Similarly, PrCa deaths are also estimated to rise by 85%, from 375,000 to nearly 700,000 in the same period[8]. This expected rise is partly due to world population aging and the varied effectiveness of screening and treatment initiatives around the world[1, 6].

PrCa incidence has been rising since the 1980s, largely as a result of widespread of prostate-specific antigen (PSA) testing and once again an increase in disease awareness. Which in turn results in early diagnosis and treatment, having reflected a decline in mortality rates, that have decreased by 52% from their peak in 1993, with recent patterns showing a moderate decline through 2020[1, 7, 8].

Lifestyle factors are also a significant in PrCa diagnosis. A meta-analysis in 2016 found that higher physical activity is correlated with a 38% reduction in PrCa-specific mortality, which suggests that an active lifestyle may be important in managing disease progression[13]. Other factors, such as diet and smoking habits, have also been shown to affect both incidence and mortality[6, 7, 8].

3 Genetic Etiology

To be diagnosed with HPC families must meet the Johns Hopkins criteria by either having three or more first-degree relatives diagnosed with PrCa; cases spanning three successive generations; or having at least two relatives diagnosed with early-onset PrCa (before the age of 56 years)[11].

Notably, any affected family member doubles PrCa risk and is increased by 2.5-fold if one first-degree relative is less than 60 years old at diagnosis (1.6 times if the first-degree relative is older than 60 years). If 2 or more relatives are diagnosed before 60 years old, the relative risk can escalate up to 5.7[6]. These elevated risks may be attributed to the existence of rare variants that are present in less than 1% of the population, which get pass down hereditarily and affect moderate- to high-penetrance genes. These genetic factors are frequently identified in families that also exhibit other hereditary cancer syndromes, which are among the most important risk factors compared to age, race, ethnicity and environmental factors for the development of PrCa and this risk is estimated at 40% to 50 %.[5] They are:

HBOC, is most commonly associated with inherited pathogenic mutations in the *BRCA1* and *BRCA2* genes, though other genes (such as *PALB2*, *ATM*, and *CHEK2*) can also play a role. *PALB2*, for instance, links *BRCA1* and *BRCA2* forming the "BRCA complex" that repairs DNA double-strand breaks via HR. Furthermore, it has been established that they also exert control over centrosome dynamics, chromosome segregation and cytokinesis, thereby contributing to the temporally and spatially stabilised genome within the cell cycle[9]. The other genes (*ATM* and *CHEK2*) are also related to the correction of damaged genome; *CHEK2* encodes a checkpoint kinase that interacts with cell cycle regulators and DNA repair proteins, like the aforementioned *ATM* serine threonine kinase that recognises double stranded DNA breaks and initiates multiple aspects of the damage response cascade[14].

LS, is caused by mutations in DNA mismatch repair (MMR) genes, most commonly *MLH1*, *MSH2*, *MSH6*, and *PMS2* . Individuals with LS have a higher lifetime risk of developing colorectal and endometrial cancer and a range of other cancers such as ovarian, gastric and urinary tract[15].Its influence in PrCa susceptibility is still controversial and the evidence is not consistent, but overall risk studies indicate a moderately increased risk in men who possess an MMR gene mutation[10, 11].

Distinct from the broader hereditary cancer syndromes, *HOXB13* is the only gene being specifically and consistently associated with an increased risk of HPC, especially the G84E mutation[11, 16]. Even though the mutations can be found in both affected and healthy man, the carrier rate was found to be considerably elevated among affected males (194 out of 382; 51% in some studies) in comparison to their counterparts within these families[16]. The protein expressed by *HOXB13* plays a crucial role in the embryonic development of the prostate gland and continues to be expressed in normal prostate tissue throughout adulthood. Moreover, it has been demonstrated to interact with the androgen receptor, thereby impacting the proliferation and differentiation of both normal and cancerous prostate cells, which underlines its significance as a PrCa susceptibility gene[17].

4 Methods

4.1 Dataset

Forty-nine of the 462 families provided DNA samples from two or three affected relatives (including the proband). Of those, forty-five families, specifically, six with three available DNA samples and 39 with two available DNA samples, were selected for WES according to the following prioritization: firstly, families with three available DNA samples; secondly, families with more than two family members diagnosed with PrCa; thirdly, families with probands diagnosed before the age of 61; and, lastly, families with the lowest average age at onset. The genetic screening with whole-exome sequencing therefore included 96 patients from 45 families of the 462 families recruited.

4.2 Capture and sequencing

Approximately 1μg of genomic DNA was enriched for exonic regions using the SureSelectXT2 Human All Exon v5 kit (Agilent Technologies, Santa Clara, CA, USA), according to the SureSelectXT2 Target Enrichment System for Illumina Multiplexed Sequencing protocol, at the Carvajal-Carmona Laboratory, Genome Center & Department of Biochemistry and Molecular Medicine, University of California, Davis, CA, USA. Sequencing of the pooled enriched libraries was an outsourced service, provided by the Beijing Genomics Institute sequencing facility at the UC Davis campus in Sacramento, CA, USA, and was performed with 100bp paired-end reads using the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA).

4.3 Data processing

Raw sequence data was received in paired-end FASTQ format and processed at the Carvajal-Carmona Laboratory. Data were demultiplexed and converted to Sanger encoding using seqtk (`https://github.com/lh3/seqtk`). Fastq quality was assessed and checked with FastqQC (`http://www.bioinformatics.babraham.ac.uk/projects/fastqc/`). Quality trimming was performed by SICKLE (`https://github.com/najoshi/sickle`). Sequence reads were trimmed and aligned to the Genome Analysis Toolkit (GATK) bundle human genome build GRCh37_decoy/hg19 using Burrows-Wheeler Alignment (BWA-mem, version 0.7.8-r455) [18, 19] and PCR duplicates were removed using Picard (1.118). GATK (v3.2-2) IndelRealigner and BaseRecalibrator were used for indel realignment and base quality score recalibration, and variants were called with five different callers: Freebayes [20], GATK HaplotypeCaller algorithm [21, 22], SAMtools [23], SNVer [24], and VarScan [25].

4.4 Pipeline and Filtering

Variant Processing

Variants for all callers were combined and filtered according to the following filters: coverage ≥10; variant counts ≥5; variant frequency ≥10%; average single nucleotide variants (SNV) base quality ≥22; and ≥10% of variant reads on both strands. For secondary variant filtering, intronic variants at more than 2-bp away from exon-intron boundaries, synonymous, UTR variants.

Sample Preparation

We began with a single multisample VCF containing 364,992 variants collated from all study participants. To ensure only heterozygous calls were analyzed, we split the master VCF into per-sample VCFs and filtered out homozygous reference and homozygous alternate genotypes. Each resulting VCF contained only heterozygous variants observed in that individual.

Conversion to ANNOVAR Input (AVINPUT)

The per-sample VCFs were converted to ANNOVAR's AVINPUT format. We compared three approaches: custom Python scripts for variant normalization (splitting multiallelics, adjusting coordinates, trimming), simpler Python reformatting scripts, and the default Perl script provided by ANNOVAR. Performance was evaluated based on the number of preserved variants and compliance with AVINPUT specifications. Ultimately, we selected the ANNOVAR Perl script for its superior retention of variant records and robust formatting.

Functional Annotation with ANNOVAR

ANNOVAR gathers information for MAF in non-Finnish European (NFE) and Iberic (IBS) populations, from the Genome Aggregation Database (gnomAD) and the 1000 Genomes Project [1000G, Phase 3 data], and for the pathogenicity predictors available at dbNSFP (v3.5), which compiles results from multiple prediction tools. Using the hg19 reference database, we annotated all AVINPUT files with ANNOVAR to generate a consolidated CSV containing 355,853 annotations. This file included standard gene, transcript, and consequence fields but lacked sample identifiers.

Restoring Sample Labels

To reintroduce sample information, we developed a Python utility that cross references the original per-sample VCFs with the annotated CSV. For each variant in the ANNOVAR output, the script identifies all samples in which it was present and appends a comma delimited Samples column, preserving the original sample count (355,853 entries).

Filtering by Minor Allele Frequency

We filtered variants to retain those with global MAF $\leq$ 1% (≤ 0.01) in the non-Finnish European cohort, as well as variants lacking frequency data (`.`).

Although the shell command underfiltered (which was corrected in later steps), this step reduced the dataset to 139,585 variants.

Loss of Function Truncating and Potentially pathogenic Subset

After MAF filtering, the pipeline diverged into two branches:

1. **Truncating variants:** We identified splice site changes and exonic truncating events, such as stopgain, stoploss, frameshift insertions and deletions, and startloss, while also retaining nonframeshift indels and variants of unknown consequence that could have functional impact. This curated subset ended with 2,885 unique truncating variant records.

2. **Potentially pathogenic missense variants:** A custom Python filter integrated multiple in silico pathogenicity and conservation scores to prioritize variants likely to affect protein function. From the filtered output, we further restricted to nonsynonymous single nucleotide variants (missense), yielding 1,002 high-confidence missense events.

Family Assignment and Master File Consolidation

Each subset was passed through another script, which annotates a Family only when a variant appears in two or more members of the same family. The two family annotated CSVs were then merged into a unified master file containing variant coordinates, gene symbols, comma delimited sample lists, and assigned family IDs.

Additional Post Import Filtering in R

Once the master CSV was loaded into `R`, we refined the variant set through a series of operations that refiltered variants to retain those with MAF $\leq 1\%$ (≤ 0.01), excluded certain classifications (e.g., `"Unknown"`), and removed ClinVar annotations with benign interpretations (e.g., `"Benign"` or `"Likely benign"`). After this comprehensive cleanup in `R`, the dataset was reduced to 552 high-confidence, family-associated variants, which were then subjected to further analysis, visualization, and statistical summarization.

Identification of significantly altered genes

Identification of significantly altered genes was performed using tools like OncodriveFML[26]. OncodriveFML accesses the functional bias of mutations, identifying genes enriched for functional alterations. This will help to identify genes whose mutational patterns are unlikely to occur by chance and may be indicative of involvement in PrCa susceptibility.

Gene set enrichment analysis

Following identification of significantly mutated genes, gene set enrichment analysis (GSEA) a method used to determine whether a group of genes (a *gene set*) has statistically significant differences, will be conducted to identify enriched or depleted biological processes and pathways potentially implicated in PrCa susceptibility. Enrichr[27], and g:Profiler[28] were used to assess the over-representation of gene sets within curated databases, including KEGG, and Gene Ontology (GO).

5 Results

Mutational Burden per Sample and per Family

First, we examined the overall mutation landscape, categorized by sample and family.

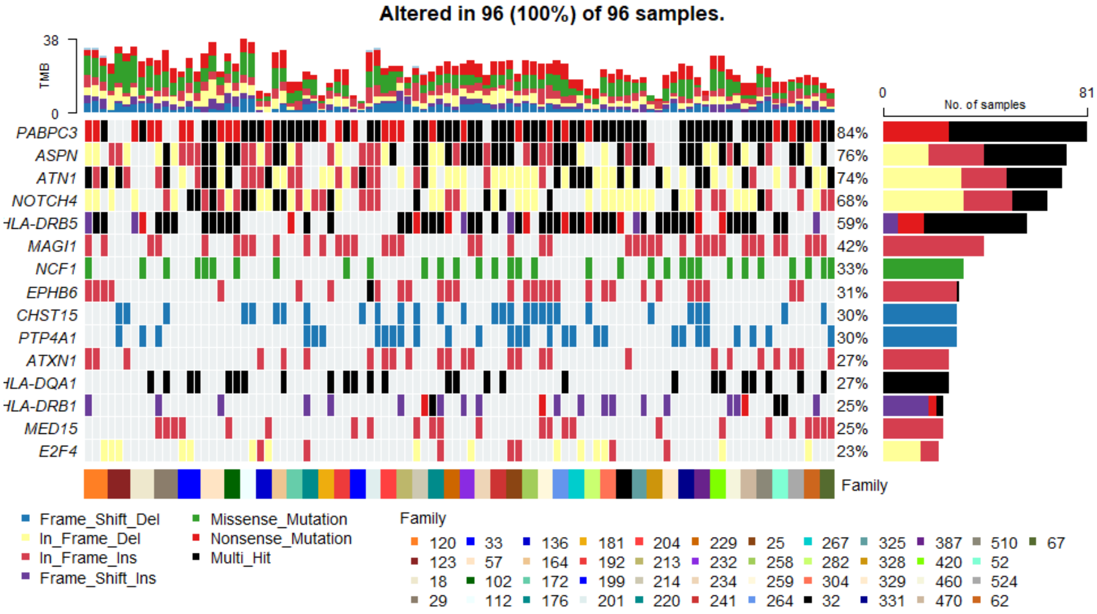


Figure 1: Oncoplot of the dataframe expanded by sample.

The oncoplot representing the 96 sequenced samples from 45 HPC families, shows recurrent mutations in several genes such as *PABPC3, ASPN, ATN1, NOTCH4, HLA-DRB5, MAGI1*, and *NCF1*, with potential implications for HPC predisposition and progression.

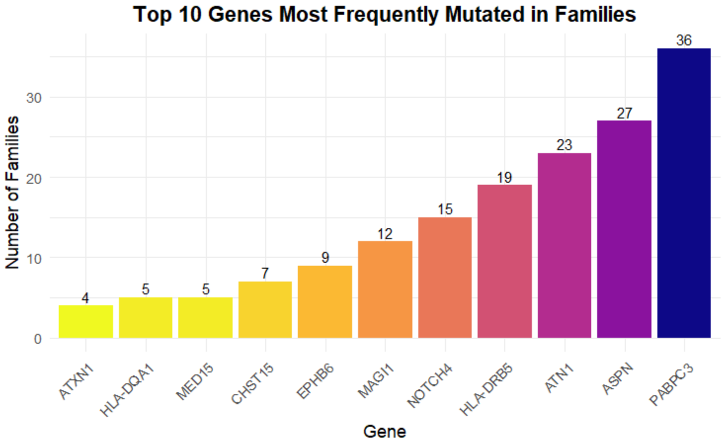
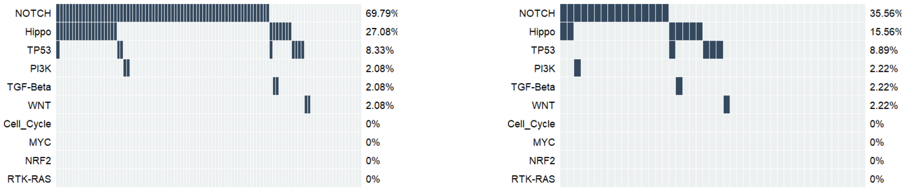


Figure 2: Top 10 genes most frequently mutated across families.

Each bar represents the number of families in which a given gene harbored a segregating variant (i.e., present in at least two affected members). Again, we see *PABPC3* as the most frequently mutated, with mutations in 36 out of the 45 families.

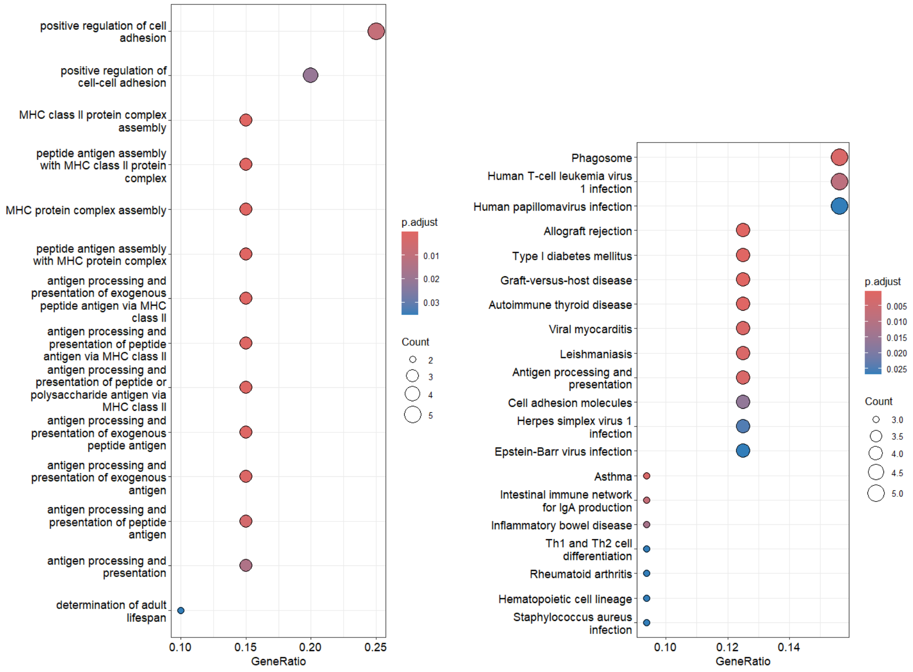


(a) Mutated pathways by sample

(b) Mutated pathways by family

Figure 3: Comparison of mutated pathways by sample (a) an by family (b).

In the comparison, we observe a shift in percentages between the sample-based and family-based analyses. This difference arises because, in the family-based context, a mutation is only considered segregating if it is present in at least two samples within the same family. For instance, the NOTCH pathway shows mutations in 69.79% of individual samples, but only in 35.56% of families, which suggests a lower level of familial segregation.



(a) GO enrichment

(b) KEGG enrichment

Figure 4: Dotplots of Gene Ontology (GO) and KEGG pathway enrichment analyses of mutated genes, highlighting overrepresented biological processes and signaling pathways.

The dotplots display the top 20 enriched Gene Ontology biological processes (GO:BP) and KEGG pathways based on the set of mutated genes. Each dot represents a significantly overrepresented term, where the size indicates the number of associated genes and the color reflects the adjusted p-value, with more significant terms appearing in red. The plots help identify biological functions and pathways potentially affected by the observed variants.

6 Discussion

Before continuing, it should be noted that some variants in this study either lack a dbSNP[29] entry or have alternative allele frequencies that do not match ANNOVAR annotations, this could be due to the fact that either ANNOVAR or the databases we used were outdated. These cases may have slipped through the filtering stage, which could help explain why genes such as *PABPC3* appear mutated across so many families. This gene encodes Poly(A) Binding Protein Cytoplasmic 3, involved in mRNA stability and translation initiation, with testis-predominant expression but emerging cancer relevance [30, 31].

The second most recurrently mutated gene, *ASPN*, encodes an extracellular matrix protein increasingly recognized as a key regulator in the tumor microenvironment. Its roles in tumor progression, metabolic adaptation, and cell migration [32] make it a strong prognostic biomarker candidate in prostate cancer [33]. It also as a roll in imune-related and cell adhesion processes, that are shown in figure 4 [34]. Similarly, *ATN1* encodes a transcriptional co-repressor expressed in prostate tissue that interacts with tumor suppressor pathways like PTEN and TGF-β signaling [35, 36], though its direct links to prostate cancer predisposition require further validation.

Other notable genes include *NOTCH4*, *HLA-DRB5/HLA-DRB1/HLA-DQA1*,*MED15*, *MAGI1* which show emerging roles in prostate cancer progression, immune regulation, and transcriptional control [37, 38, 39, 40]. These genes also participate in the pathways that are later discussed, for example *MAGI1* scaffolds Hippo components while stabilizing adherent junctions, connecting to adhesion terms[41]. The HLA class II genes can influence both autoimmune disease risk and cancer immune surveillance, connecting to immune terms [42].

Pathway analysis revealed significant alterations in key signaling cascades. The NOTCH pathway, mutated in 69.8% of samples but only 35.56% of families which suggests a lower familial segregation, is a fundamental cell-to-cell communication pathway that controls cell fate decisions, proliferation, apoptosis, and is clinically relevant since *NOTCH4* mutations correlate with higher Gleason scores and poor prognosis [38], they can also promote tumor growth, therapy resistance, and metastasis [43, 44]. The Hippo pathway, altered in 27.1% of samples and 15.56% of families suggesting a lower level of familial segregation as well, controls organ size through proliferation/apoptosis regulation, with disruption activating oncogenic YAP/TAZ programs in prostate tumorigenesis [45]. The TP53 pathway, though less frequently mutated (8.3%) but with a higher level of familial segregation then the others with alterations in 8.89% of families, remains critical as p53 inactivation drives genomic instability and therapy resistance in aggressive disease [46, 47].

Due to the fact that the previous pathways were obtained through the Maftools[48] function ”pathways” that only highlights enrichment in known driver pathways curated by Sanchez-Vega [49], gene set enrichment analysis (GSEA) was done for genes mutated in 3 or more families to understand the affected pathways, resulting in figure 4. The functional enrichment analyses of the most frequently mutated genes in our HPC cohort revealed a striking overrepresentation of immune-related processes, particularly those involving MHC class II-mediated antigen processing and presentation. This is consistent with the high mutation rate observed in *HLA-DRB5* and *HLA-DQA1*, underscoring a potential role for altered antigen presentation and immune surveillance in HPC [39]. Enrichment for cell adhesion pathways further suggests that changes in the tumor microenvironment and cell-cell interactions, possibly mediated by *ASPN* and *MAGI1*, may contribute to disease progression [32, 33]. Additionally, the prominence of autoimmune and inflammatory disease pathways in KEGG analysis points to a broader context of immune dysregulation, supporting emerging evidence that links inflammation and immune escape to PrCa risk and progression [50]. These findings are complemented by mutation-centric pathway analysis, which highlights recurrent alterations in NOTCH, Hippo, and TP53 signaling pathways known to modulate both immune responses and cellular plasticity in cancer [43, 44, 45, 46, 47]. Collectively, our results suggest that HPC in these families may be driven by a convergence of immune, adhesion, and classical cancer signaling pathway alterations.

7 Conclusion

Together, these results identify a set of biologically credible themes in hereditary prostate carcinogenesis. Mutational burden convergence on NOTCH, Hippo and p53 pathways aligns with known mechanisms of prostate cancer initiation and implicates new germline predictors of risk. From a translational perspective, our findings can inform genetic counseling by increasing the number of candidate predisposition genes and biologic pathways to consider in high-risk families. In particular, the pathways involved point to experimentally testable hypotheses: germline mutations that modulate Hippo or Notch signaling, for instance, can predispose progenitor cells to malignant transformation, or influence tumor–stromal dynamics. Follow-up experiments to confirm these candidate mutations in larger numbers and to characterize their functional impact will be essential. Finally, incorporation of such pathway-level information into risk assessment can potentially tailor individualized screening and prevention strategies for men from prostate cancer–prone families.

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